MTA DOKTORI ÉRTEKEZÉS

POLI(ADP-RIBÓZ) POLIMERÁZ-1 ÉS 2 ENZIMEK SZEREPE METABOLIKUS TRANSZKRIPCIÓS FAKTOROK SZABÁLYOZÁSÁBAN

BAY PÉTER

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A disszertációban bemutatott munkához nélkülözhetetlenek voltak a kollaborátorok. **Johan Auwerx** (EPFL, Lausanne, Svájc), **Gilbert de Murcia** és **Josiane Menissier de Murcia** (ESBS, Strasbourg, Franciaország)) indítottak el a PARP enzimek és a metabolizmus közti kapcsolat vizsgálata irányába, alakították, tágították a látókörömet, illetve megnyitották előttem az európai mozgásteret. **Carles Cantó** (Nestlé Institute of Health Sciences, Lausanne), **Anthony A. Sauve** (Weill Cornell Medical College, USA) és **Valérie Schreiber** (ESBS, Strasbourg, Franciaország) támogatása nélkülözhetetlen volt. **Tóth Attila** (Kardiológiai Intézet, Debreceni Egyetem) nemcsak önzetlen és odaadó barát, hanem kiváló kollaborátor, aki nélkül az érfunkció vizsgálatokat nem tudtuk volna végrehajtani. Köszönet illeti **Sander M. Houtent** (Icahn School of Medicine, Mount Sinai, USA), **Aline Hubert** (IBMC, Strasbourg, Franciaország), **Rutkai Ibolyát** és **Czikora Ágnest** (Kardiológiai Intézet, Debreceni Egyetem), illetve **Hughues Oudart**-t (CEPE, Strasbourg, Franciaország).

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Tartalomjegyzék

Rövidítések jegyzéke

ACC - acetil-KoA karboxiláz (acetyl-CoA carboxylase)
ACO – acil-KoA oxidáz
ADPR – ADP-ribóz
AM - automodifikáció
AMPK - AMP-aktivált kináz
AR – androgén receptor
ARH3 – ADP-ribozilhidroláz-3
ART – ADP-riboziltranszferáz (ADP-ribosyltransferase)
ARTD – Bakteriális diftériatoxin-típusú ADP-riboziltranszferáz (Bacterial diphtheria toxin-like ARTs)
BAT – barna zsírszövet (brown adipose tissue)
BRCT - BRCA1 C terminális domén
Chow diet – Normál zsírtartalmú (kontroll) táplálék
ChREBP – szénhidrát válaszadó elemhez kapcsolódó fehérje (carbohydrate response element binding protein)
COX – citokróm c oxidáz
CPT1 – karnitin palmitoil transzferáz-1 (carnitin palmitoyl transferase-1)
CREB – cAMP válaszadó elemhez kötődő fehérje (cAMP response element-binding protein)
CRTC2 – CREBP regulált transzkripció koaktivátor-2 (CREB regulated transcription coactivator-2)
CS – citrát szintáz
cyt c – citokróm c
DAB – diamino benzamid
DBD – DNS-kötő domén (DNA binding domain)
Dio2 – dejodináz 2
DOX - doxorubicin
ECL – felerősített kemilumineszcencia (enhanced chemiluminescence)
ER – ösztrogén receptor (estrogen receptor)
ERRα – ösztogén receptorhoz hasonló receptor α (estrogen-related receptor receptor α)
FCS – fötális borjú savó (fetal calf serum)
G6Pase – glükóz-6-foszfatáz (glucose-6-phosphatase)HA - hemagglutinin
GK - glükokináz
HE – hematoxillin-eozin
HFD – magas zsírtartalmú diéta (high-fat diet)
HIC1 – hypermethylated in cancer-1
Ins - inzulin
InsR – inzulin receptor
i.p. - intraperitoneális
ipGTT – intraperitoneális glükóz tolerancia teszt
ipITT - intraperitoneális inzulin tolerancia teszt
ipPTT - intraperitoneális piruvát tolerancia teszt
K19 – keratin 19
Kir6.2 – inward-rectifier potassium ion channel 6.2

LCAD – nagy lánchosszúságú acil-KoA dehidrogenáz (Long-chain acyl-CoA dehydrogenase)
LXR – máj X receptor (liver X receptor)
MCAD – közepes lánchosszúságú acil-KoA dehidrogenáz (Medium-chain acyl-CoA dehydrogenase)
mCPT-1 – karnitil palmitoil transzferáz-1 (carnitil parmitoil transzferáz-1)
MEF – egér embrionális fibroblaszt (murine embryonic fibroblast))
MMP9 - mátrix metalloproteináz-9
MHCI – I. típusú miozin nehéz lánc (myosin heavy chain type I)
MHCIIx – IIx. típusú miozin nehéz lánc(myosin heavy chain type IIx)
MHCIIa – IIa. típusú miozin nehéz lánc (myosin heavy chain type IIa)
MS – tömegspektrometria (mass spectrometry)
mtDNS – mitokondriális DNS
mTOR - mammalian target of rapamycin
mTORC1 – mammalian target of rapamycin complex 1
mTORC2 - mammalian target of rapamycin complex 2
NAM - nikotinamid
NCS – újszülött borjú savó (newborn calf serum)
NLS – nukleáris lokalizációs szignál
NoLS - nukleoláris lokalizációs szignál
NOR1 – Neuron-eredetű árva receptor-1 (Neuron-derived orphan receptor-1)
OGTT - orális glükóz tolerancia teszt
ORO – Oil Red O (Szudán-vörös)
pACC – foszfo-acetil-KoA karboxiláz (phospho-acetyl-CoA carboxylase)
PAR - poli(ADP-ribóz)
PARG – poli(ADP-ribóz) glikohidroláz
PARP - poli(ADP-ribóz) polimeráz
PARiláció – poli(ADP-ribozil)áció
PCAF - P300/CBP-associated factor
PDK4 – piruvát dehidrogenáz kináz 4. izoenzim (Pyruvate dehydrogenase kinase isozyme 4)
PDX1 - pankreatikus-duodenális homeobox-1 (pancreatic and duodenal homeobox 1)
PEPCK – foszfoenol-piruvát karboxikináz (phosphoenol pyruvate carboxykinase)
PGC1α – peroxisome activated receptor cofactor-1α (peroxiszóma proliferátor aktivált receptor kofaktor-1α)
PI – propídium jodid
PPAR – peroxiszóma proliferátor aktivált receptor
PR – progeszteron receptor
qPCR - kvantitatív polimeráz láncreakció
RT-qPCR – reverz transzkripcióval kapcsolt kvantitatív polimeráz láncreakció
RXR – retinoid X receptor
SIRT - sirtuin
SMA – simaizom specifikus aktin (smooth muscle actin)
SREBP - Sterol Regulatory Element-Binding Protein
SUMO - Small Ubiquitin-like Modifier

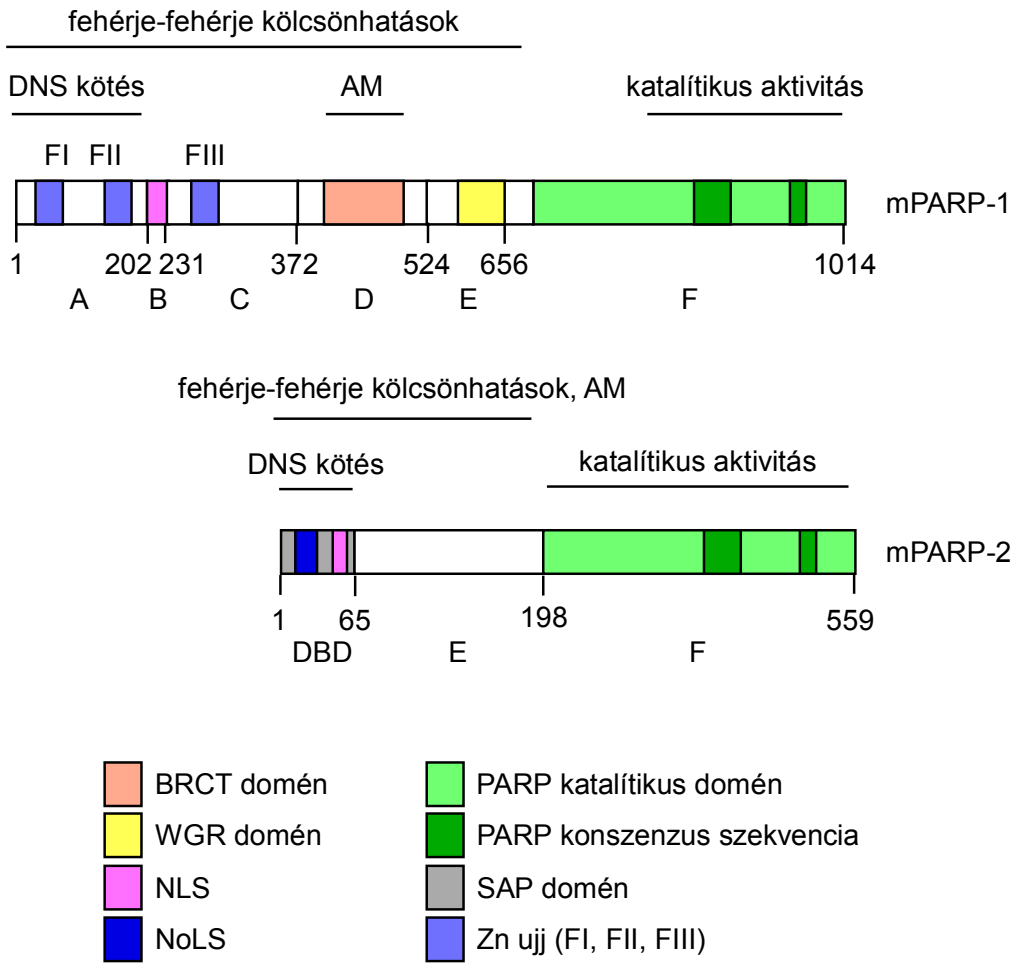
TBARS – Tiobarbitursav-reaktív anyagok
(Thiobarbituric acid reactive substances)
TFAM – mitokondriális transzkripciós faktor
A (mitochondrial transcription factor A)
TR – tiroid receptor
TropI – I. típusú troponin
TUNEL - Terminal deoxynucleotidyl
transferase dUTP nick end labeling
UCP – uncoupling protein

1. Bevezetés

1.1 A poli(ADP-ribóz) polimeráz enzimek biokémiai jellemzése

A poli(ADP-ribóz) polimeráz (PARP) aktivitást Pierre Chambon és munkatársai (Chambon és mtsai, 1963) írták le 1963-ban, majd a PARP-1 enzimet, amely az aktivitás jelentős részéért felelős, 1967-ben Shimizu és munkatársai jellemezték (Shimizu és mtsai, 1967). Homológia modellezéssel új PARP enzimeket azonosítottak a 2000-es évek elején (Ame és mtsai, 2004, Otto és mtsai, 2005), így jelenleg emberben 17, egérben 16 PARP enzimet ismerünk. 2010-ben Michael Hottiger és munkatársai (Hottiger és mtsai, 2010) – szerkezeti és biokémiai hasonlóságokra hivatkozva - azt javasolták, hogy minden ADP-ribozil csoport transzfert végző enzimet egy családba soroljanak, amelyeket ADP-ribozil transzferáz (ART) enzimeknek neveznek, melyek között a PARP enzimek az bakteriális diftéria toxin-szerű ART-k (ARTD) csoportba tartoznak. Az ART/ARTD elnevezés nem nyert egyelőre teret, ezért a jelen értekezésben a PARP elnevezést használom.

A PARP enzimek több doménből álló, multidomén fehérjék. Az értekezésben a PARP-1 és a PARP-2 enzimekkel foglalkozom, szerkezetüket az első ábra szemlélteti (1. ábra). Mindkét enzim N-terminálisán a DNS kötéséhez szükséges szerkezeti elemeket találunk: a PARP-1-ben két cink ujj (a harmadik cink ujj motívum a dimerizációhoz szükséges), míg a PARP-2-ben egy SAP domén segíti a DNS-sel történő kölcsönhatást (Huber és mtsai, 2004, Mazen és mtsai, 1989). Ezt mindkét enzim esetében fehérje–fehérje kölcsönhatások kialakítására alkalmas domének követik, mint a BRCT domén a PARP-1-ben (de Murcia és mtsai, 1994). PARP-2-ben az analóg szakaszon belül egyelőre nem írtak le ismert szerkezetű domén(eke)t. A PARP-1 és PARP-2 lokalizációját nukleáris és nukleoláris lokalizációs szignál biztosítja (Meder és mtsai, 2005, Schreiber és mtsai, 1992).



1. ábra. A PARP-1 és PARP-2 enzimek vázlatos doménszerkezete

Az ábrán a számozás az egér fehérjék aminosav szekvenciájának megfelelő domén határokat jelzi, illetve a nagybetűk a domének jelzésére szolgálnak. AM – automodifikáció, DBD – DNS-kötő motívum/domén, NLS – nukleáris lokalizációs szignál, NoLS – nukleoláris lokalizációs szignál, FI, FII, FIII – cink ujjak, BRCT – BRCA1 C-terminális domén.

A PARP-1 és a PARP-2 C-terminálisán található a PARP enzimekre jellemző katalitikus domén. Mindkét enzim NAD^+-ot bont nikotinamidra (NAM) és ADP-ribózra (ADPR), majd a két enzim az ADPR monomerekből elágazó polimereket épít különböző akceptor fehérjékre (Altmeyer és mtsai, 2009, Alvarez-Gonzalez és Mendoza-Alvarez, 1995, Burzio és mtsai, 1979, Ogata és mtsai, 1980). A PARP-1 és a PARP-2 által létrehozott polimerek hosszúak lehetnek, akár 200 ADPR egység is alkothatja (Hayashi és mtsai, 1983). A klasszikus elképzelés szerint a PARP-1 és -2, DNS törésekhez kötődve aktiválódik (Ame és mtsai, 1999, Benjamin és Gill, 1980, Kun és mtsai, 2002, Langelier és mtsai, 2012). A PARP aktivitás nagy részéért (a sejt

teljes alap és indukált PARP aktivitásának 85-90%-a) a PARP-1, míg a maradékért gyakorlatilag a PARP-2 felelős (Schreiber és mtsai, 2002). Ennek megfelelően az értekezésben amikor PARP aktivációról van szó, akkor sejtek össz-PARP aktivitásáról van szó (tulajdonképpen PARP-1 + PARP-2), míg a PARP-1, vagy PARP-2 gátlás az adott gén megszakítása során a PARP aktivitásban fellépő változásokra utal.

A PAR polimerek életciklusa rövid, a féléletidejét 1 perc körülinek fogadja el az irodalom (Goodwin és mtsai, 1978, Skidmore és mtsai, 1979). A PAR lebontásáért a poli(ADP-ribóz) glikohidroláz (PARG) és az ADP-ribozil liáz felelős (Kawaichi és mtsai, 1983, Ueda és mtsai, 1972). A fehérjéken maradó utolsó ADPR egységet az ún. makro domén (illetve az ilyen doménnel rendelkező) fehérjék hasítják le (Jankevicius és mtsai, 2013, Karras és mtsai, 2005, Posavec és mtsai, 2013) A PAR épülhet a PARP-1, vagy a PARP-2 enzimre (autoPARiláció), illetve más fehérjékre (transzPARiláció). Az autoPARiláció gátolja a PARP-1-et (Kawaichi és mtsai, 1981, Zahradka és Ebisuzaki, 1982) és valószínűleg a PARP-2-t is, így visszacsatolási kört alakít ki, ami megakadályozza a túlzott PARP aktivációt. A transzPARiláció a célfehérjék biológiai, biokémiai aktivitását befolyásolja.

A klasszikus DNS törés mellett poszttranszlációs módosítások és különböző jelátviteli útvonalak is befolyásolják a sejtek PARP aktivitását. A PARP-1 több helyen is foszforilálódik, ami befolyásolja az enzim aktivitását (Burkle és Virag, 2013, Cohen-Armon, 2007, Gagne és mtsai, 2009, Hegedus és mtsai, 2008). A később bemutatásra kerülő adatok értelmezéséhez nagyon fontos tudni, hogy a PARP-1 acetilált fehérje (a p300/CBP-association factor (PCAF) és a p300 acetilálja) és acetilált formában aktív (Hassa és mtsai, 2005, Rajamohan és mtsai, 2009). A PARP-1 enzimet a SIRT1 képes deacetilálni és ezáltal gátolni (Rajamohan és mtsai, 2009). A PARP-2 is acetilálódik a PCAF és a GCN5L enzimek által (Haenni és mtsai, 2008). Egy SUMO ligáz, a PIASy kölcsönhat a PARP-1-gyel és SUMOilálja hősokk esetén (Martin és mtsai, 2009), illetve a PARP-3 és a SIRT6 mono-ADP-ribozilálja és aktiválja a PARP-1-et (Loseva és mtsai, 2010, Mao és mtsai, 2011). A PARP-1 aktivitás összefüggést mutat a sejtek kalcium homeosztázisával (Bakondi és mtsai, 2003, Geistrikh és mtsai, 2011, Wyrsch és mtsai, 2012).

1.2 A PARP enzimek által befolyásolt biológiai folyamatok

Elsőként a PARP enzimek a PARP-1, majd később a PARP-2 és -3 DNS hibajavításban játszott szerepét ismerték fel (Boehler és mtsai, 2011, Durkacz és mtsai, 1980, Purnell és Whish, 1980, Schreiber és mtsai, 2002). Jelenlegi ismereteink szerint a PARP-1 és -2 enzimek bár nem eszenciálisak a DNS hibajavítás megindulásához (Allinson és mtsai, 2003, De Vos és mtsai, 2012) genotoxikus stressz (pl. gyökök jelenléte, ionizáló sugárzás, stb.) esetén

szükségesek a hatékony hibajavításhoz (Menissier-de Murcia és mtsai, 1997, Menissier-de Murcia és mtsai, 2003, Wang és mtsai, 1995). A PAR polimerek kijelölik a DNS károsodás helyét és a DNS repair fehérjék számára kapcsolódási felszínt biztosítanak (Karras és mtsai, 2005, Mortusewicz és mtsai, 2007, Tartier és mtsai, 2003). A PARP-1 és a PARP-2 enzim szerepét leírták az egyszálú és kétszálú törések javításában, illetve a báziskihasítással járó javítási folyamatokban (Dantzer és mtsai, 2000, Langelier és mtsai, 2012, Schreiber és mtsai, 2002, Yelamos és mtsai, 2008). Bár a PARP-1, vagy a PARP-2 hiányában valóban csökken a DNS hibajavítás hatékonysága ez önmagában úgy tűnik nem elég a tumoros transzformációhoz, ahhoz egyes tumorszupresszorok hiánya is szükséges (Huber és mtsai, 2004, Menissier-de Murcia és mtsai, 2003, Nicolas és mtsai, 2010, Tong és mtsai, 2001).

A PARP aktiváció mértéke befolyásolja a sejtek további sorsát. Amennyiben a DNS sérülése javítható a PARP aktiváció hozzájárul az hatékony DNS hibajavításhoz. Amennyiben a DNS sérülés nagymértékű a túlzott PARP aktiváció elhasználja a sejtek NAD^+ készletét és a NAD^+ újraszintézise pedig az ATP készletet meríti ki (Berger, 1985). A sejtek energetikai katasztrófájához hozzájárul, hogy lelassul a glikolízis (Ying és mtsai, 2002) és az alacsony ATP szint miatt a mitokondriális F1/F0 ATPáz az ATP bontásába kezd (Ha és Snyder, 1999). A sejtekben megnyílnak a mitokondriális tranzíciós pórusok, amik tovább erősítik az energetikai katasztrófát (Virag és mtsai, 1998). Mivel az apoptózis sikeres végrehajtásához szükséges a sejtek normális energiatöltöttsége, ezért az energetikai katasztrófa állapotában lévő sejtekben az apoptózis nem megy végbe, így ezek a sejtek nekrózissal pusztulnak el (Virag és Szabo, 2002). A PARP aktivitás gátlása megakadályozza az energetikai krízis kialakulását (Virag és Szabo, 2002).

A PARP-1 és a PARP-2 enzim több ponton is befolyásolja a génexpressziót (Frizzell és mtsai, 2009, Kraus és Hottiger, 2013). A PAR polimerek módosíthatják a kromatinszerkezetet és így a DNS hozzáférhetőségét (Aubin és mtsai, 1983, de Murcia és mtsai, 1986, de Murcia és mtsai, 1988). A PARP-1 és a PARP-2 transzkripciós kofaktorként enhanszer, promóter és inzulátor elemekhez kapcsolódhatnak és összetett génexpressziós változásokat okozhatnak (Kraus, 2008). A PARP enzimek és a transzkripció kapcsolatában több a megválaszolatlan kérdés. Nem ismert, hogy a PARP enzimek transzkripciós kofaktor funkciójához szükséges-e az enzimatikus aktivitás (Hassa és mtsai, 2001, Oliver és mtsai, 1999). Mindenesetre úgy tűnik, hogy a NAD^+ szint befolyásolja a PARP-1 működését a transzkripciós fókuszokban (Berger és mtsai, 2007, Kim és mtsai, 2004, Zhang és mtsai, 2012).

Az előbbiekben leírt folyamatok egyszerre jelennek meg különböző élettani és kórélettani folyamatokban (pl. gyulladásos folyamatok, vagy a gyök közvetítette események) (Virag és

Szabo, 2002). A PARP-1 delációja vagy PARP inhibitor kezelés védelmet nyújt az oxidatív stresszel jellemezhető betegségek ellen (Pacher és mtsai, 2007, Virag és Szabo, 2002). A PARP-2 delációja ezzel szemben csak részleges védelmet nyújt hasonló kórképekben ismeretlen hatásmechnizmuson keresztül (Kofler és mtsai, 2006, Popoff és mtsai, 2002). A PARP-mediált citotoxicitással jellemezhető betegségek száma jelentős. Etiológiájukat tekintve vegyesek, gyakoriak köztük a gyulladásos betegségek vagy egyes gyógyszerek (például citosztatikumok) mellékhatásaként jelenik meg a PARP aktiváció. Ilyen citosztatikum például a kísérleteinkben alkalmazott doxorubicin (DOX), amely egy antraciklin típusú citosztatikum és alkalmazása gyakran okoz kardiovaszkuláris károsodást (Singal és Iliskovic, 1998). A mitokondriális elektrontranszport lánc a DOX-ot részlegesen szemikinonná redukálja. A mitokondriumból kikerülő szemikinon kinonná oxidálódik, az elektront a környezetébe leadja és így szabad gyököket generál (Davies és Doroshow, 1986, Doroshow és Davies, 1986, Pacher és mtsai, 2003). A szabad gyökök DNS szálak töréséhez és a PARP-1 aktivációjához vezetnek (Pacher és mtsai, 2002), ami több útvonalon (pl. a mitokondriális funkció károsítása) keresztül a kardiomiociták, az erek simaizmai, illetve az endotél sejtek diszfunkcióját eredményezik (Bristow és mtsai, 1981, Danz és mtsai, 2009, Dawer és mtsai, 1988, Taga és mtsai, 1987).

1.3. A poli(ADP-ribóz) polimeráz enzimek metabolikus szerepe

A PARP-1 és a PARP-2 enzimek metabolizmust szabályzó szerepe, hasonlóan, mint a fent bemutatott folyamatokban, több elemi jelenségre vezethető vissza, szerepet játszik benne a NAD^+ szint, specifikus fehérjék PARilációja, jelátviteli utak, illetve a génexpresszió módosulása.

1.3.1 A PARP aktiváció akut metabolikus hatásai

Nagymértékű, akut genotoxikus stressz a DNS-függő PARP enzimek aktiválásán keresztül a sejtek NAD^+ szintjének drasztikus csökkenéséhez, és következményesen az ATP szint csökkenéséhez vezet (Berger, 1985). Ezen felül NAD^+ hiányában lelassul a glikolízis (Ying és mtsai, 2002). A fokozott PARP aktivációt követő glikolítikus fluxus csökkenést kimutatták asztrocitákban, májban, vesékben és a központi idegrendszerben (Devalaraja-Narashimha és Padanilam, 2009, Erdelyi és mtsai, 2009, Lin és Yang, 2008, Mongan és mtsai, 2002, Mongan és mtsai, 2003, Sharma és mtsai, 2005, Ying és mtsai, 2002, Ying és mtsai, 2003).

1.3.2 Mitokondriális poli(ADP-ribozil)áció

A PARP-1 aktiváció rontja a mitokondriális funkciót, bár jelenleg nem tudjuk pontosan meghatározni, hogy ez a NAD^+ szint csökkenéséből, a glikolízis lassulásából, vagy a PARP-1

valamilyen direkt hatásából adódik. Canuelo és munkatársai (Canuelo és mtsai, 2011) kimutatták, hogy PARP-1 hiányos fibroblasztokban a mitokondriális II, III és IV komplex aktivitása megnövekszik. Klaidman és munkatársainak (Klaidman és mtsai, 2003), illetve Zhou és munkatársainak (Zhou és mtsai, 2006) eredményei arra utalnak, hogy a PARP-1 aktiváció blokkolja az I. komplexet. Niere és munkatársai (Niere és mtsai, 2008) egy mesterséges, rövidített PARP fehérjeláncot és mitokondriális lokalizációs szignált tartalmazó konstrukt segítségével kimutatták, hogy a PARP aktivitás növelése a mitokondriumban a mitokondriális funkció zavarához vezetett, a glikolítikus fluxus változása nélkül. Több csoport is kimutatott PARilált mitokondriális fehérjéket, vagy a PARP-1 enzimmel kölcsönható proteineket (Du és mtsai, 2003, Lai és mtsai, 2008), bár többen vitatják ezeket az eredményeket. A PARG bizonyos izoformái és az ADP-ribozilhidroláz-3 (ARH3) is jelen vannak a mitokondriumban (Niere és mtsai, 2008, Oka és mtsai, 2006). Vagyis a teljes PARilációs ciklusból a PAR-t eltávolító enzimek megtalálhatóak a mitokondriumban, azonban a PAR-t szintetizáló enzim(ek)et nem tudtak minden kétséget kizáró módon kimutatni a mitokondriumban (jelenleg is több csoport dolgozik ezek azonosításán). Az akceptor fehérjékre vonatkozó eredményekkel kapcsolatban több kétely is felmerült (a preparálás során aktív, magi PARP-okkal kerülhettek kapcsolatba a mitokondriális fehérjék). A mesterségesen indukált mitokondriális PARiláció csökkenti a mitokondriális aktivitást, vagyis ha létezik mitokondriális PARiláció, annak mindenképpen jelentős hatása van a mitokondriális aktivitás szabályzására.

1.3.3. PARP enzimek és növekedési faktor receptor jelátviteli útvonalak kölcsönhatásai

A PI3 kináz-Akt útvonal kiemelt fontosságú az inzulin és a növekedési faktorok jelátviteli folyamataiban. A PI3 kináz a sejtmembránhoz asszociált és kapcsolatban van tirozin kináz receptorokkal. A tirozin kináz receptorok aktivációja esetén a jel továbbításában központi szerepe van a PI3 kináznak és tőle lefelé helyeződő Akt aktivációjának. Az Akt aktiváció befolyásolja a sejtek túlélését stresszben, a glükóz metabolizmust és a sejtek növekedését. Mint azt az előbbiekben bemutattam, hogy a PARP gátlás védelmet nyújt az oxidatív stressz által kiváltott NAD^+ és ATP depléció ellen. Sümegi Balázs kutatócsoportja kimutatta, hogy a PI3 kináz – Akt útvonal is részt vesz a PARP gátlás védő hatásának kialakításában (Palfi és mtsai, 2005, Radnai és mtsai, 2012, Tapodi és mtsai, 2005). Egyelőre nem ismert, hogy a PARP inhibitor kezelés során bekövetkező PI3 kináz – Akt útvonal aktivitálódása milyen metabolikus változásokat okoz.

1.3.4. PARP enzimek és energia stressz útvonalak kölcsönhatásai

A PARP-1 aktivitás összefüggést mutat az AMP-aktivált kináz (AMPK) és az mTOR (mammalian Target Of Rapamycin) útvonallal. Az mTOR két, alegységeiben különböző komplex formájában van jelen: mTORC1 és mTORC2 (Guertin és Sabatini, 2007). Az mTORC1 feladata a környezet tápanyaggal való ellátottságának (aminosav és glükóz) érzékelése. Az mTORC1 aktivációjához vezet, ha a környezetben magas az aminosavak és a glükóz mennyisége (Guertin és Sabatini, 2007). A PARP aktiváció gátolja az mTORC1 aktivitást (Ethier és mtsai, 2012, Huang és Shen, 2009, Huang és mtsai, 2009, Munoz-Gamez és mtsai, 2009). Az mTORC1 komplexet gátló rapamicin alkalmazása azonban gátolja a PARP aktivációt, bár nem közvetlenül kapcsolódik a PARP-1-hez - nem kizárt, hogy az mTORC1 aktivitásának változása áll a jelenség hátterében (Fahrer és mtsai, 2009).

Az AMPK egy több alegységből álló kináz, amely képes a sejtek energiatöltöttségének érzékelésére. Az ATP/AMP arány csökkenése, azaz növekvő AMP koncentráció az AMPK aktivációjához vezet, ami fokozza a mitokondriális biogenezist. A PARP-1 és az AMPK egyrészt fizikai kölcsönhatásban van egymással (Zhou és mtsai, 2012). Másrészt, a két enzim aktivitása egymást potencírozó hatást mutat - az AMPK aktiváció növeli a PARP-1 aktivitását és fordítva (Walker és mtsai, 2006, Zhou és mtsai, 2012). Itt kell megemlíteni, hogy az AMPK aktiváció gátolja az mTORC1 komplex aktivitását (Inoki és mtsai, 2012), vagyis a két útvonal működése összefügg, ami arra utal, hogy az energiaszenzor útvonalak és a PARP enzimek közötti összefüggés sokkal bonyolultabb lehet, mint arra jelenlegi ismereteink utalnak.

1.3.5 A poli(ADP-ribóz) polimeráz enzimekkel kölcsönható metabolikus regulátorok: a peroxiszóma proliferátor aktivált receptor-γ (PPARγ)

A magreceptorok központi szerepet játszanak a metabolikus és a hormonális szabályzásban. A magreceptor dimerek az egyes gének promótereiben található specifikus válaszadó szekvenciákhoz kapcsolódnak jellegzetes DNS-kötő doménjükön keresztül (Francis és mtsai, 2003). A magreceptorokhoz nagyszámú kofaktor kapcsolódik, amelyek alapvetően meghatározzák a magreceptorok aktivált vagy represszált állapotát (Feige és Auwerx, 2007, Francis és mtsai, 2003). A komplex tagjai a magreceptorok ligandkötésétől függően kicserélhetőek. Általánosságban elmondható, hogy a ligand hiányában a magreceptorokhoz represszorok kapcsolódnak, míg a ligand bekötődése a represszorok leválását és koaktivátor molekulák bekötődését okozza. Mind a koaktivátorok, mind a korepresszorok elsősorban a kromatinszerkezet modulálásával fejtik ki a hatásukat. A korepresszorok zártabb, nehezebben hozzáférhető kromatinszerkezetet alakítanak ki, míg a koaktivátorok nyíltabbá és

hozzáférhetőbbé teszik a kromatint - epigenetikai hatás (Feige és Auwerx, 2007). A magreceptorok aktivitásában bekövetkező változás a génexpressziós mintázat átrendeződéséhez vezet (Green és Chambon, 1988, Mangelsdorf és mtsai, 1995). A magreceptorok ligandjai gyakran hormonok, a metabolizmus közti termékei, vagy a táplálék bizonyos összetevői, vagyis a magreceptorok elősegítik a sejtek szövetek alkalmazkodását a metabolizmus, vagy a táplálékbevitel változásaihoz.

A PARP-1 több magreceptorral is kölcsönhat (1. táblázat). Munkánk során a peroxiszóma proliferátor aktivált receptor (PPAR)γ-val foglalkoztunk. A PPARγ receptoron kívül a PPARα és a PPARβ/δ tartozik a PPAR-ok közé. Mindhárom receptor dimert alkot a retinoid X-receptorral (RXR). Bár mindhárom receptor szerteágazó metabolikus szereppel bír, a PPARα elsősorban a máj, a PPARβ/δ (a disszertációban δ-ként fogom jelölni) pedig a harántcsíkolt izmok lipid háztartását (Francis és mtsai, 2003), míg a PPARγ (NR1C3) fehér zsírszövet működését és differenciálódását szabályozza (Auwerx és mtsai, 2003, Brun és Spiegelman, 1997, Fajas és mtsai, 1997). A PPARγ fehérjének két izoformája van, PPARγ1, amely ubikviter, illetve a PPARγ2, amely a zsírszövetre és a makrofágokra jellemző. A két izoforma alternatív splicing eredménye, a $PPAR\gamma_2$ 28 aminosavval hosszabb, mint a $PPAR\gamma_1$ (Fajas és mtsai, 1997). A PPARγ-t kisméretű lipofil ligandok aktiválják, például a táplálékból, vagy metabolikus útvonalakból származó zsírsavak, vagy az antidiabetikus tiazolidindionok (Auwerx és mtsai, 2003, Brun és Spiegelman, 1997, Fajas és mtsai, 1997). A PPARγ-RXR dimer az energia, a lipid és a glükóz homeosztázist befolyásoló gének expresszióját szabályozza (Auwerx és mtsai, 2003, Brun és Spiegelman, 1997, Fajas és mtsai, 1997).

1. táblázat. A PARP-1 enzimmel kölcsönható magreceptorok

Magreceptor	A PARP szerepe és/vagy a kölcsönhatás eredménye	Hivatkozás
Ösztrogén receptor (ER)	A PARP-1 aktivitása ahhoz szükséges, hogy az ER aktiváció során a topoizomeráz IIβ által kialakított DNS törések hatékonyan javíthatóak legyenek. Az ösztrogén gátolja a PARP-1 aktivációt. Az ösztrogén adása gátolja a PARP-1 aktivitást.	(Sripathy és mtsai, 2008) (Ju és mtsai, 2006) (Mabley és mtsai, 2005)
Progeszteron receptor (PR)	A PR kölcsönhat a PARP-1-gyel. A progeszteron aktiválja a PARP-1-et.	(Burzio és Koide, 1977) (Sartorius és mtsai, 2000) (Ghabreau és mtsai, 2004)
Reténsav receptor (RAR)	A PARP-1 az RAR pozitív kofaktora.	(Pavri és mtsai, 2005)
Retinoid X receptor (RXR)	A PARP-1 második cink ujja (FII) felelős az RXR-rel történő kölcsönhatásért.	(Miyamoto és mtsai, 1999)
Tiroid receptor (TR)	A PARP-1 a TR pozitív kofaktora. A PARP-1 aktivitása szükséges a TR/RXR dimer működéséhez, viszont a PARP-1 overexpressziója gátolja a dimer működését.	(Pavri és mtsai, 2005) (Miyamoto és mtsai, 1999)
RXR/PPARγ	A PARP-1 szükséges a normális PPARγ függő transzkripcióhoz. A PARP-1 túlaktivációja gátolja az adiponektin transzkripciót a PPARγ PARilációján keresztül.	(Erener és mtsai, 2012) (Huang és mtsai, 2009)
Neuron-derived orphan receptor 1 (NOR1)	A PARP-1 overexpresszió gátolja a NOR1 mediált transzkripciót.	(Ohkura és mtsai, 2008)
Androgén receptor (AR)	A PARP-1 az AR promoterének pozitív regulátora.	(Shi és mtsai, 2008)

A magreceptorokkal való kölcsönhatásban – az eddigi adatok szerint - a PARP-1 második cink-ujjának (Hossain és mtsai, 2009, Miyamoto és mtsai, 1999, Pavri és mtsai, 2005) és a BRCT (Ohkura és mtsai, 2008) doménnek van szerepe. A PARP-1 aktiváció diverz módon hat az egyes magreceptorokra. Míg a PPARγ (Huang és mtsai, 2009) és a NOR-1 (Ohkura és mtsai, 2008) esetében a PARP-1 aktivációja gátolja a magreceptorok aktivitását, ezzel ellentétben az ER-függő promóterek esetében a PARP-1 aktiváció szükséges az ER-függő transzkripciós aktivációhoz (Ju és mtsai, 2006). A PARP enzimek és a magreceptorok molekuláris kapcsolata és a kölcsönhatás fiziológiai jelentősége javarészt feltáratlan terület.

1.3.6 A poli(ADP-ribóz) polimeráz enzimekkel kölcsönható metabolikus regulátorok: a SIRT1

A sirtuin (SIRT) enzimek családjába 7 fehérje tartozik, közülük a legjobban a SIRT1 enzimet jellemezték. A SIRT1 egy NAD^+ függő, III. típusú deacetiláz enzim (Imai és mtsai, 2000), amely a magreceptorok kofaktoraként működik.

Az acetil csoport lehasításával egyidőben az enzim egy NAD^+ molekulát elhasít ADP-ribózra és nikotinamidra, majd a lehasított acetil csoportot az ADP-ribózhoz köti O-acetil-ADP-ribózt hozva létre (Imai és mtsai, 2000). SIRT1 NAD^+-ra vonatkoztatott Km értéke a sejtek fiziológiás NAD^+ szintjéhez közel esik, vagyis valószínű, hogy a sejtek NAD^+ szintjének változása szabályozza a SIRT1 enzim aktivitását (Chen és mtsai, 2008, Houtkooper és mtsai, 2010, Rodgers és mtsai, 2005). A NAD^+ szint emelkedése az éhezésre jellemző állapot. Ennek megfelelően a SIRT1 enzim aktivációja az éhezés, illetve az éhezésre hasonlító állapotokra jellemző (Guarente, 2000, Imai és mtsai, 2000). A NAD^+ szint központi szerepére utal a SIRT1 aktivitás szabályzásában az a megfigyelés is, hogy NAD^+ előanyagok adásával fokozható a SIRT1 aktivitása (Canto és Auwerx, 2012, Canto és mtsai, 2012).

A gyors szubsztrátfüggő szabályozáson kívül a SIRT1 expressziója is követi a tápanyag ellátottságot. A SIRT1 promóterének aktivitását több transzkripciós faktor befolyásolja. Éhezés során a cAMP response element binding protein (CREB), PPAR-ok, vagy a FOXO-k fokozzák a SIRT1 expressziót (Canto és Auwerx, 2012), míg a magas glükóz szint által (megfelelő tápláltság) aktivált faktorok, mint a carbohydrate response element binding protein (ChREBP) a SIRT1 expresszió negatív regulátorai (Noriega és mtsai, 2011).

A SIRT1 aktiváció a sejtek metabolikus adaptációját segíti elő stressz állapotokban a génexpresszió átalakításával. A SIRT1 számos transzkripciós faktor deacetilációjára képes (pl. peroxisome proliferator activated receptor γ coactivator (PGC)1α) PPAR-ok, sterol regulatory element binding protein (SREBP)-1, liver X receptor (LXR), FOXO-k, CREB, CREB regulated

transcription coactivator-2 (CRTC2) (Canto és Auwerx, 2012). Ezen transzkripciós faktorok működésének megváltozása az energiaigényes felépítő folyamatok helyett a lebontó, energiatermelő folyamatokat aktiválja. Az energiatermelés a zsírsavak és a szénhidrátok oxidációjához, illetve a mitokondriális biogenezis aktivációjához köthető (Canto és Auwerx, 2012). A SIRT1 aktiváció a mitokondriális biogenezis indukcióját okozza több szervben: a májban, a BAT-ban és a harántcsíkolt izomban (Feige és mtsai, 2008, Lagouge és mtsai, 2006). A harántcsíkolt izomban a mitokondriumok számának és aktivitásának a fokozódása együtt jár az ún. izomrost izotípusváltással. Az izotípusváltás során a kontraktilis rendszer elemei is megváltoznak (pl. miozin nehéz lánc), illetve megemelkedik az oxigén szöveti tárolásában fontos mioglobin expressziója is (Feige és mtsai, 2008, Lagouge és mtsai, 2006). A SIRT1 aktiváció az oxidatív I. típusú (lassú), illetve IIa típusú rostok megjelenésének kedvez (Feige és mtsai, 2008, Lagouge és mtsai, 2006). A pankreász béta-sejtjeiben a SIRT1 overexpresszió a mitokondriális funkció ugrásszerű javulásához és az inzulin szekréció növekedéséhez vezet (Moynihan és mtsai, 2005).

1.3.7. A PARP enzimek ismert metabolikus funkciói

A legelső megfigyeléseket, amelyek a PARP enzimeket metabolikus folyamatokhoz kötötték, a fehér zsírszövet vizsgálata során tették. Janssen és Hilz (Janssen és Hilz, 1989) kimutatta, hogy 3T3-L1 preadipociták differenciálódás során PAR keletkezik. Amikor Zhao-Qi Wang és munkatársai 1995-ben (Wang és mtsai, 1995) elkészítették az első PARP-1 knockout egértörzset, azt találták, hogy a PARP-1 deléciója a testsúly és a fehérzsír depók méretének növekedését okozta. Smulson és munkatársai (Smulson és mtsai, 1995) 3T3-L1 sejtek differenciációját vizsgálva kimutatta, hogy a PARP-1 aktivitása szükséges a sejtek megfelelő zsírsejt-irányú differenciációjához.

A PARP-1 túlaktiváció és a mitokondriális funkció romlása között az összefüggést Virág László és munkatársai tárták fel (Virag és mtsai, 1998), ez volt az első utalás arra, hogy a PARP-1 és a mitokondriális aktivitás között összefüggés van. Azonban sokáig a PARP-1 és a mitokondrium kapcsolatát egyirányúnak ismertük, ahol a PARP-1 aktiváció rontja a mitokondriális funkciót. A SIRT1 és a PARP-1 kapcsolatát - amelyet a disszertációban tárgyalni fogok - először Zhang vetette fel (Zhang, 2003), majd azt, hogy a PARP-1 – SIRT1 kapcsolatnak lehet metabolikus vetülete először Asher és munkatársai (Asher és mtsai, 2010) mutatták ki.

2. Célkitűzések

Mint azt az előző fejezetben áttekintettem, a PARP enzimek komplex módon kapcsolódnak a metabolikus regulátor hálózatokhoz. Megfigyelések utalnak arra, hogy a túlzott PARP aktiváció károsítja a mitokondriális funkciót (Virag és mtsai, 1998), illetve a PARP aktivitás szükséges egyes metabolikus szervek, vagy szövetek sejtjeinek működéséhez, vagy differenciációjához (pl. zsírsejtek (Janssen és Hilz, 1989, Smulson és mtsai, 1995)). Továbbá a PARP-1 több energiaszenzor útvonallal is kölcsönhat (Palfi és mtsai, 2005, Walker és mtsai, 2006).

A PARP enzimeket hagyományosan a DNS hibajavítás enzimeiként ismerik, transzkripciós szerepüket az elmúlt évtizedben kezdték részleteiben feltárni és megismerni, metabolikus hatásaikat illetően csak rész ismeretekkel rendelkezünk. Az eredmények egy része jelentős ellenmondásokat hordoz (például: a PARP-1 szükséges-e a zsírsejt differenciációhoz, vagy aktivációja inkább gátolja a folyamatot?). Célunk – ennek megfelelően - annak a mélyebb vizsgálata volt, hogy a PARP enzimek milyen módon vesznek részt a metabolikus regulációban, amelyhez az alábbi kérdéseket kívántuk megválaszolni.

1. A PARP-1 enzim hogyan befolyásolja a metabolikus szervek, szövetek működését?
2. A PARP-2 enzim hogyan befolyásolja a metabolikus szervek, szövetek működését?
3. Képes védelmet nyújtani a PARP-2 delációja oxidatív károsodás ellen?

3. Kísérleti módszerek áttekintése

3.1 *In vivo* kísérletek

3.1.1 Egerek

A kísérletekben használt PARP-$1^{+/+}$ és PARP-$1^{-/-}$ (Menissier-de Murcia és mtsai, 1997), illetve a PARP-$2^{+/+}$ és PARP-$2^{-/-}$ (Menissier-de Murcia és mtsai, 2003) egerek heterozigóta keresztezésekből származtak. Külön jelöltem, ahol C57Bl6 hím egereket használtam farmakológiai kísérletekben vagy sejtpreparáláshoz. Az állatházban a napszakok 12 óránként váltották egymást, az állatoknak ad libitum hozzáférése volt a táplálékoz (chow vagy magas zsírtartalmú táplálék, 60% hiperkalorikus diéta). Az egerek 87,5% C57/Bl6J 12,5% SV126 háttéren voltak.

3.1.2 Metabolikus mérések

Az állatok táplálékfogyasztását minden héten ugyanazon a napon mértük.

Az orális/intraperitoneális glükóztolerancia (ipGTT/OGTT), intraperitoneális inzulintolerancia (ipITT), az intraperitoneális piruvát tolerancia (ipPTT), illetve a hidegtolerancia kísérleteket Lagouge és mtsai szerint végeztük el (Lagouge és mtsai, 2006).

A PJ34 kezelés 12 óránként történt (7:00 és 19:00-kor) 10 mg/kg PJ34-et adtunk az állatoknak intraperitoneális injekció formájában öt egymást követő napon.

Az O_2 fogyasztást, CO_2 termelést és a spontán lokomotor aktivitást (aktimetria) indirekt kaloriméterben határoztuk meg (*Sabre systems*, Las Vegas, NV, USA) 48 órás megfigyelés során. Az energia felszabadulást az O_2 fogyásból egy konstans (20.1 J/ml O_2) segítségével számítottuk ki. Az aktimeria során az infravörös nyaláb megszakításokat 15 perces időintervallumokban összegeztük, ábrázoltuk az idő függvényében és AUC-t számoltunk.

Az euglikémiás-hiperinzulinémiás clamp kísérleteket Feige és mtsai (Feige és mtsai, 2008) szerint végeztük el.

Az egerek kifáradásig tartó futtatása (forced running) Cantó és mtsai (Canto és mtsai, 2009) eljárása alapján történt.

A metabolikus kísérletekben az állatokat CO_2 inhalációval vagy cervikális diszlokációval 14:00-kor öltük le 6 óra éhezés után.

3.1.3. Akut doxorubicin modell

A kísérletben négy kohortot alakítottunk ki *PARP-*$2^{+/+}$ és *PARP-*$2^{-/-}$ kontroll (CTL), illetve *PARP-*$2^{+/+}$ és *PARP-*$2^{-/-}$ doxorubicin (DOX) kezelt csoportokat. A DOX kezelt állatok i.p. 25 mg/kg DOX-

ot kaptak, majd az aortákat a kezelést követő 2. napon eltávolítottuk funkcionális és biokémiai vizsgálatok céljából.

Az aorta funkcionális vizsgálata során a kimetszett 4-5 mm-es aorta darabokat két fémdrót segítségével egy erőátviteli rendszerhez (Helpern-Mulvany arteriograph, DMT510A system, *Danish Myotechnology*, Aarhus, Dánia) kapcsoltuk, majd normalizáltuk (azonos mértékű feszülést hoztunk létre bennük) és megvizsgáltuk, hogy az aorták életképesek-e (KCl kezelés). A kontrakciót kiváltó anyagokat (szerotonin és norepinefrin) emelkedő hígításban adtuk a rendszerhez. A relaxáció vizsgálatához az ereket norepinefrinnel előfeszítettük, majd az endotél függő relaxációt acetilkolinnal, míg az endotélium független relaxációt nátrium-nitroprusszidal váltottuk ki.

3.2 Sejtes kísérletek

3.2.1. Sejtvonalak és azok kezelése

A *primer embrionális fibroblasztokat* (*MEF*-eket) a Menissier-de Murcia és mtsai (Menissier-de Murcia és mtsai, 2003) által leírt módon preparáltuk és 10% FCS DMEM (1 g/L glükóz) médiumban tartottuk fenn. A MEF sejtek zsírsejt irányú differenciációjához a MEF sejteket konfluenciáig növesztettük, majd differenciációs médiumot tettünk a sejtekre (DMEM (1 g/L glükóz) 10% NCS 5 µM troglitazone (TZD), 5 µM dexamethasone (Dex), 500 µM IBMX and 10 µg/ml inzulin), míg a kontroll sejtek 10% FCS DMEM (1 g/L glükóz) 0,21% DMSO médiumot kaptak. A médiumot két naponta cseréltük, a differenciáció nyolc napig tartott.

A *3T3-L1 preadipocitákat* DMEM (1 g/L glükóz) 10% NCS médiumban tartottuk fenn.

A *HEK293T* sejteket DMEM (1 g/L glükóz) 10% FCS médiumban tartottuk fenn.

SIRT1$^{-/-}$ és *SIRT1*$^{+/+}$ MEF sejteket Chua és mtsai (Chua és mtsai, 2005) írták le, 10% FCS DMEM (1 g/L glükóz)-ben tartottuk fenn.

A *C2C12 mioblasztokat* DMEM-ben (4,5 g/l glükóz, 10% FCS) tartottuk fenn, differenciációjukat a szérumszint csökkentésével indítottuk be (2% ló szérum). A sejteket két nappal a differenciáció megindítása után kezeltük farmakológiai szerekkel. Létrehoztunk PARP-2 csendesített C2C12 sejteket. C2C12 sejtekbe PARP-2 specifikus és egy nem specifikus szekvenciát vittünk be lentivirális vektorok felhasználásával, majd a sejteket 2,5 µg/ml puromicinnel szelektáltuk és a túlélő sejteket használtuk a kísérleteinkben (ezeket a sejteket fenntartottuk és differenciáltattuk kísérleteinkben). A depléció hatékonyságát RT-qPCR-ral, illetve Western blottal ellenőriztük.

MOVAS artéria simaizom sejteket az ATCC-től szereztük be és DMEM-ben tartottuk fenn (4,5 g/L glükóz, 10% FCS, 0,2 mg/ml G418). A PARP-2 depléciója a C2C12 sejtekhez hasonlóan történt. A sejteken a méréseket 7 órával a DOX kezelés után végeztük el.

3.2.2. Transzfekció

A transzfekciókat a BES pufferelt só módszerrel, vagy a JetPei reagenssel végeztük el (Schreiber és mtsai, 2002).

3.3 Molekuláris biológiai és biokémiai módszerek

3.3.1 Konstruktok

A *pGL3-(J_{wt})$_3$TKluc* riporter konstrukt, a *pSG5-PPARγ$_2$*, *pSG5-PPARα*, *pSG5-PPARδ*, *pCMX-ERβ*, a *vitellogeninA2-ERE-TKLuc (ER-luc)*, *pCMX-ERβ, pCMV-βGal,* a *SIRT1 promóter riporter konstruktok, a SIRT1 csendesítésére való adenovírus alapú konstrukt, a PARP-1 csendesítésére való konstrukt, a FLAG-HA-PGC1α* ismert konstruktok, leírásuk megtalálható a disszertáció alapját képező közleményekben.

A pSuper-siPARP-2 és a *pSuper-scrPARP2* konstruktok kétszálú oligonukleotidokat (melyek a PARP-2 specifikus szekvenciát (pSuper-siPARP-2 esetében) vagy az aspecifikus szekvenciát (a pSuper-scrPARP-2 esetében) tartalmazták kétszer egymás után egy hurokkal elválasztva).

A *pBabe-PARP2* vektor elkészítése során a PARP-2 cDNS-ét a pBABEpuro vektor SnaBI helyére klónoztuk.

3.3.2 Transzkripciós faktor transzaktiváció tanulmányozása

A transzkripciós faktorok transzaktivációjának meghatározására luciferáz riporter esszét alkalmaztunk. Az alábbiakban a kísérletek általános menetét ismertetem, a 2. táblázat tartalmazza az alkalmazott konstruktok koncentrációit, a sejtvonalakat és az inkubációs időket.

A sejteket hat lyukú lemezeken növesztettük. A HEK293T sejtekben a PARP-2-t vagy depletáltuk, vagy overexpresszáltuk, melyhez három napra volt szükség. MOVAS esetében PARP-2 csendesített és PARP-2 expresszáló (kontroll) vonalakkal dolgoztunk. A 2. táblázatban részletezett konstruktokkal a sejteket transzfektáltuk a mérés napján, majd a jelzett inkubációs idő letelte után a sejteket felkapartuk és meghatároztuk a β-galaktozidáz és a luciferáz aktivitást. A luciferáz aktivitást a β-galaktozidáz aktivitásra normalizáltuk.

A PPAR receptorok aktivitásnak a vizsgálata során a sejtek zsírmentes FCS-t tartalmazó médiumban voltak, az egyes PPAR receptorok aktiválására különböző ligandokat alkalmaztunk: PPARα – fenofibrát (FF, 50 μM); PPARδ - mono-etil-hexil-ftalát (MEHP, 100 μM) (Feige és

mtsai, 2007); PPARγ – troglitazon (TZD, 5 μM). Az ösztrogén receptor β (ERβ) aktiválására β-ösztradiolt (10 μM) használtunk.

2. táblázat. A luciferáz riporter esszékben alkalmazott eljárások részletei

Kísérlet	Sejtvonal	Konstruktok (név, mennyiség)	Inkubáció
PARP-2 expresszió hatása PPARα transzaktivációra	HEK293T	0,6 µg pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2 1 µg pSG-PPARα 1 µg pGL3-$(J_{wt})_3$TKluc 0,4 µg pCMV-βgal	6 óra
PARP-2 expresszió hatása PPARγ transzaktivációra	HEK293T	0,6 µg pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2 1 µg pSG-PPARγ 1 µg pGL3-$(J_{wt})_3$TKluc 0,4 µg pCMV-βgal	6 óra
PARP-2 expresszió hatása PPARδ transzaktivációra	HEK293T	0,6 µg pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2 1 µg pSG-PPARδ 1 µg pGL3-$(J_{wt})_3$TKluc 0,4 µg pCMV-βgal	6 óra
PARP-2 expresszió hatása ERβ transzaktivációra	HEK293T	0,6 µg pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2 1 µg pSG- ERβ 1 µg vitellogeninA2-ERE-TKLuc 0,4 µg pCMV-βgal	6 óra
PARP-2 expresszió hatása a SIRT1 promóterének működésére	HEK293T	1 µg of pSuper-shPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2 1.6 µg SIRT1 promóter riporter 0,4 µg pCMV-βgal	10 óra
PARP-2 expresszió hatása a SIRT1 promóterének működésére	MOVAS	8 µg SIRT1 -91 promóter riporter 4 µg pCMV-βgal	48 óra

3.3.3. Áramlási citometria technikák

Nílusvörös festés – A MEF-ek zsírsejt irányú differenciációjának a jellemzésére használt eljárás. A differenciált MEF-eket nílusvörös festékkel festettük (20 μg/ml, 5 perc), majd a sejteket mostuk, tripszineztük és áramlási citometriai vizsgálatnak vetettük alá. A differenciációt az összes mért sejt százalékában adtuk meg és levontuk a nem differenciáltatott kontroll sejtekben meghatározott differenciált sejtek százalékát.

Hidroetidin festés - hidroetidin festést Bai és munkatársai szerint végeztük el (Bai és mtsai, 2001).

3.3.4. Fehérje azonosításhoz használt technikák

SDS-PAGE, Western blot - A sejteket, vagy szöveteket lízis pufferben (50 mM Tris, 100 mM KCl, 1 mM EDTA, 1% NP40, 5 mM NAM, 1 mM Na-butirát, proteáz inhibitor koktél (Sigma, 100x hígítás) pH 7,4; illetve néhány esetben 50 mM Tris, 500 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM PMSF, proteáz inhibitor koktél, pH 8.0 puffert alkalmaztunk) feltártuk, majd a fehérjéket SDS-PAGE eljárással elválasztottuk és nitrocellulóz membránra blotoltuk. A membránok blokkolása után az elsődleges, majd a hozzá tartozó másodlagos antitestekkel kezeltük a membránokat és a jelet ECL technikával hívtuk elő. Az elsődleges antitestek gyártója, illetve az alkalmazott hígítása a vonatkozó közleményben található. A blotokat ImageJ szoftverrel is kiértékeltük.

Immunprecipitáció – A sejteket lízispufferben (50 mM Tris, 100 mM KCl, 1 mM EDTA, 1% NP40, 5 mM NAM, 1 mM Na-butirát, proteáz inhibitor koktél (Sigma, 100x hígítás) pH 7,4) feltártuk, majd a lizátumokból immunprecipitáltuk anti-PGC1α, anti-FOXO1, anti-tubulin és anti-Ndufa9 antitesttel. A precipitátumot két részre osztottuk, SDS-PAGE-et és Western blotot hajtottunk végre, az egyik membránt anti-acetil-lizin antitesttel, míg a másikat az immunprecipitációhoz használt antitesttel hívtuk elő (az immunprecipitált fehérjére normalizáláshoz). HEK293T sejtekben a HA-PGC1α-t overexpresszáltunk, ebben az esetben az immunprecipitációhoz anti-HA antitestet használtunk. C2C12 sejtekben FLAG-HA-PGC1α-t overexpresszáltunk (adenovírus konstruktról) és anti-FLAG antitesttel történt az immunprecipitáció két nappal a transzdukciót követően. A blotokat ImageJ szoftverrel is kiértékeltük.

Sejtmag izolálás izomrostokból – A preparálást Edelman és munkatársai (Edelman és mtsai, 1965) szerint végeztük el.

3.3.4. RT-qPCR és qPCR

Az értekezésben az RT-qPCR az RNS mennyiség meghatározását, míg a qPCR a DNS mennyiség meghatározását jelzi. A kvantitatív PCR reakciók SYBR green assay-k voltak, az alkalmazott primerek szekvenciáit nem részletezem, ezek a vonatkozó közleményekben megtalálhatóak.

Sejtekből és szövetekből teljes RNS-t Trizol reagenssel tisztítottunk, majd 2 µg RNS-t reverz transzkripcióval írtunk át cDNS-sé. A specifikus cDNS-ek mennyiségét hígítás után kvantitatív PCR-ral (qPCR) határoztuk meg.

Sejtekből és szövetekből teljes DNS-t (genomi és mitokondriális DNS-t is tartalmazó preparátum) proteináz K emésztést követő fenol-kloroformos extrakcióval tisztítottuk és qPCR-ral vizsgáltuk.

3.3.5. Kromatin immunprecipitáció

A kromatin immunprecipitációt Bálint és munkatársai (Balint és mtsai, 2005) szerint végeztük el. A kromatin fixálása és feltördelése után a kromatin egyes részeit antitestekkel gyűjtöttük össze. Minden mintából vettünk a teljes kromatin tartalmazó ún. „input"-ot A PARP-2 kötődésének kimutatására PARP-2 specifikus antitestet, a PPARγ kötődésének kimutatására PPARγ specifikus antitestet használtunk, míg a nem specifikus kötődés meghatározására egy nem specifikus antitestet (MMP9-re/MRE11-re/TNF-R1-re specifikus antitestet) és egy antitestet nem tartalmazó mintát alkalmaztunk. Az összegyűjtött kromatin darabokat qPCR-ral vizsgáltuk, olyan primerpárokkal, amelyek specifikusak:

- a PPARγ vezérelt gének promoterére,
- a PPARγ vezérelt gének kódoló régiójára,
- a SIRT1 promoterére,
- Az UCP3 promoterére,
- a PDK4 promoterére, illetve
- a keratin 19 (K19) promoterére.

Az egyes antitestekkel kapott c_t értékeket a input (totál) értékekre normalizáltuk.

3.3.6. A PARP aktivitás meghatározása

A PARP aktivitás jellemzésére több módszert használtunk. Egyrészt Western blot vagy immunhisztokémiai technikával mutattuk ki a PAR szinteket sejtekből, illetve szövetekből. Másrészt tríciált NAD^+ beépülését határoztuk meg és ezzel jellemeztük a PARP aktivitást. A módszer lényege, hogy a stimulált sejteket egy digitonint és ^3H-NAD^+-ot tartalmazó oldatba

helyezzük tíz percre, majd TCA-val kicsapjuk a fehérjéket és a hozzákapcsolódó PAR polimereket. A be nem épült ^{3}H-NAD^{+}-ot elmostuk, majd a csapadékot feloldva a beépült ^{3}H radioaktivitást szcintillációs számlálóval meghatároztuk (Bai és mtsai, 2001).

3.3.7. Sejtek oxigénfogyasztásának meghatározása

A sejtek oxigénfogyasztását a Seahorse Biosciences XF24, vagy XF96 készülékével (Seahrose Biosciences, North Billerica, MA, USA) mértük meg. A C2C12 sejteket a mérés előtt 48 órával transzdukáltuk FLAG-HA-PGC1α vagy SIRT1 shRNS-sel. A fúzionált C2C12 sejteket 1 μM PJ34-gyel kezeltük két napon át.

3.3.8. NAD^{+} meghatározás

A NAD^{+} meghatározására vagy kolorimetriás eljárást, vagy tömegspektrometriai (MS) módszert használtunk. Az utóbbit Anthony Sauve laboratóriumában végeztük (Sauve és mtsai, 2005).
Sejtfrakcionálást végeztünk a NAD^{+} sejten belüli eloszlásának vizsgálatára. A sejteket felkapartuk és két részre osztottuk. A sejtek egyik részéből meghatároztuk az össz-NAD^{+}-ot, míg a másik részét teflon-üveg homogenizátorral homogenizáltuk, majd a homogenizátumot 600 rpm-mel centrifugáltuk. A képződő csapadékot mag frakciónak, majd egy további centrifugálással (7000 rpm) képződő csapadékot a mitokondriális frakciónak tekintettük. A csapadékokat két részre osztottuk, az egyikből fehérjét, a másikból NAD^{+}-ot határoztunk meg.

3.3.7. Egyéb biokémiai eljárások

A szabad zsírsav és a triglicerid szintek meghatározására kolorimetriás kiteket használtunk.
Az inzulintartalmat ELISA módszerrel mértük (Mercodia).
A malondialdehid meghatározására (TBARS assay) során a sejteket, vagy szöveteket 1,15% KCl oldatban feltártuk és tiobarbitrusav jelenlétében melegítettük (90°C, 45 perc), majd a képződő rózsaszínű terméket 532 nm-en határoztuk meg fotometriásan. A lipid peroxidáció mértékét a kontroll százalékában adtuk meg.
A máj lipid tartalmának meghatározására Floch-extrakciót végeztünk el Lagouge és munkatársai szerint (Lagouge és mtsai, 2006).

3.3.8 Szekvencia összehasonlítás

Több gerinces faj SIRT1 promóterének a szekvenciáját a Pubmed-ről gyűjtöttük össze. Az első 300 bp-os szakaszt ($^{-}$1 - $^{-}$300) a ClustalW algoritmus segítségével hasonlítottuk össze.

3.4 Mikroszkópia

Hematoxilin-eozin (HE) festés – A HE festést 7 µm formalin fixált metszeteken végeztük el.

Immunhisztokémia – Az immunhisztokémiai protokollt Géhl és mtsai (Gehl és mtsai, 2012) szerint végeztük el 7 µm formalin fixált metszeteken anti-F4/80 antitest (Serotec, Raleigh, NC, USA, 1:100, DAB előhívás), anti-PAR (1:500, DAB előhívás), simaizom aktin (SMA, *Novocastra*, Newcastle upon Tyne, UK, 1:300, DAB), illetve inzulin DAKO primer antitestek felhasználásával.

Langerhans szigetek méretének meghatározása – Minden pankreászból 3-11 reprezentatív metszetet készítettünk, melyet inzulinra megfestettünk. Minden metszetet lefotóztunk és a szigetszervek méretét Image J szoftverrel megmértük.

Oil Red-O festés (ORO) – 1% ORO-val festettünk formalin fixált sejteket, majd PBS-sel kimostunk a többlet festéket.

TUNEL assay – A DNS töréseket terminális deoxiribonukleotidil transzferáz enzim segítségével digoxigenin kapcsolt dUTP-vel jelöltük, amit digoxigenin antitesttel mutattunk ki.

Szukcinát dehidrogenáz (SDH) festés – A lassú rostok kimutatására használt szövetkémiai módszer, amit Lagouge és mtsai szerint végeztünk el (Lagouge és mtsai, 2006).

Transzmissziós elektronmikroszkópia (TEM) – A vizsgálatok glutáraldehid fixált preparátumokon történtek az ICS-ben (Strasbourg, Franciaország).

3.5 Statisztikai feldolgozás

Két csoport összehasonlítására párosítatlan kétszélű t-tesztet alkalmaztunk. Több csoport összehasonlítására Anova tesztet használtuk, ahol a szignifikanciát post-hoc tesztekkel számítottuk ki. A szignifikancia jelzésére *-ot és #-et használtunk. A * a kontroll és transzgén/csendesített sejtek/állatok közti szignifikáns különbséget jelöli. A # azonos genotípusú, farmakológiai szerekkel (pl. PPAR ligandok) kezelt csoportok közötti szignifikáns különbséget jelzi. Az egyes jelek a következő szignifikancia szinteket jelzik: - *, vagy # $p<0,05$;
- **, vagy ## $p<0,01$; - ***, vagy ### $p<0,001$.

4. Eredmények

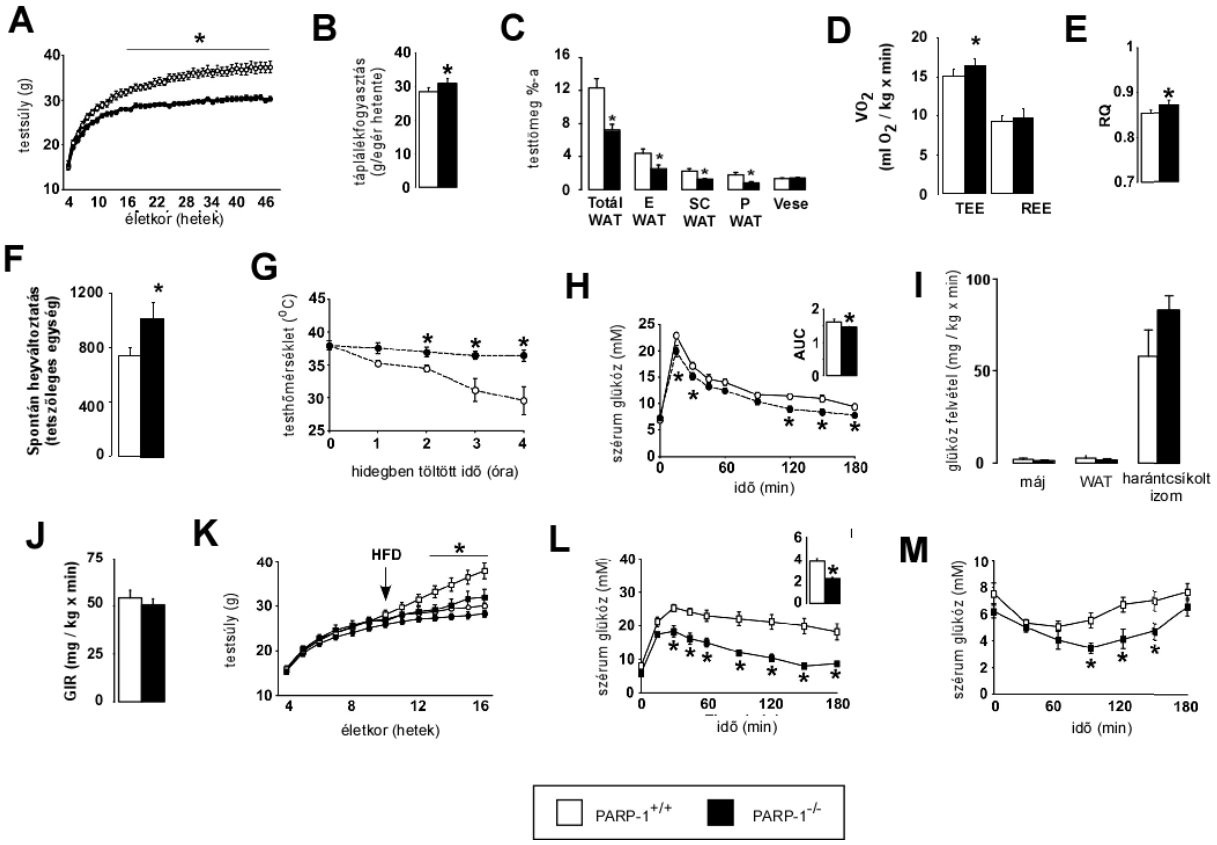
4.1. Hogyan befolyásolja a PARP-1 enzim a metabolikus szervek, szövetek működését?

4.1.1. A PARP-1$^{-/-}$ egerek metabolizmusának és energiaháztartásának jellemzése

Metabolikus vizsgálatainkat PARP-1 knockout egerek testtömeg változásának megfigyelésével kezdtük. A PARP-1$^{-/-}$ egerek a PARP-1$^{+/+}$ állatokhoz képest kevésbé gyarapodtak és a felnőtt testsúlyuk is kisebb volt (2A ábra), ami mögött metabolikus eltéréseket gyanítottunk. A PARP-1$^{-/-}$ egerek tápanyagfogyasztása magasabb volt, mint vad típusú társaiké (2B ábra). Ennek ellentmondó módon azonban a boncolás a zsírszövet csökkent mennyiségét mutatta ki (2C ábra), vagyis a magasabb tápanyagbevitel mellett alacsonyabb testtömege és tápanyagtartaléka alacsonyabb volt a PARP-1$^{-/-}$ állatoknak, ami az energiaegyensúly zavarára utalt.

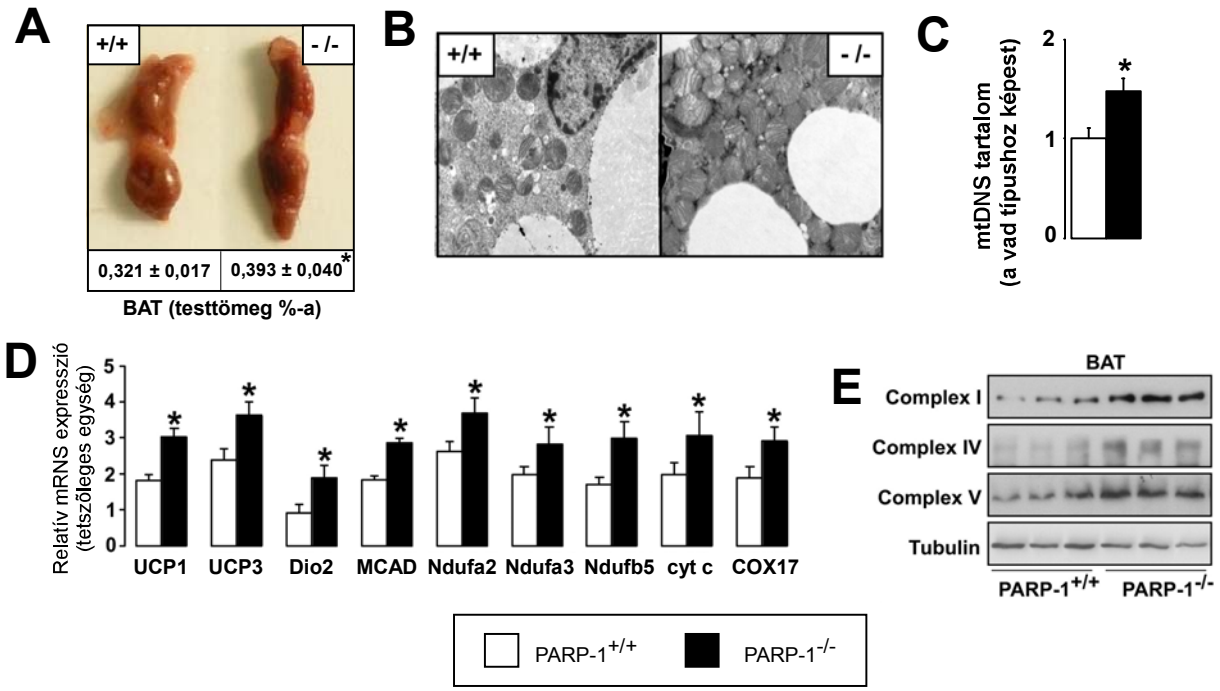
Valóban, indirekt kalorimetriával kimutattuk, hogy a PARP-1$^{-/-}$ állatok több oxigént fogyasztanak az aktív periódusokban a PARP-1$^{+/+}$ egerekhez képest (2D ábra), illetve az RQ értékük magasabb, mint vad típusú társaiké (2E ábra), ami együtt a biológiai oxidáció és a glükóz felhasználás megemelkedésére utalt. Bár a PARP-1$^{-/-}$ egerek spontán helyváltoztatása magasabb, mint vad típusú társaiké (2F ábra) több más metabolikus eltérés is a mitokondriális biogenezis javulására utalt, amelyet a továbbiakban részletezek. A PARP-1$^{-/-}$ egerek hosszabb ideig tudták fenntartani a testhőmérsékletüket, mikor táplálék nélkül 4 ^{o}C-on tartottuk az állatokat (hideg shock, 2G ábra). A PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egerek glükóz toleranciája javult (2H ábra). A glükóz eltávolításában elsősorban a harántcsíkolt izmok voltak felelősek (2I ábra).

Az inzulinérzékenységet hiperinzulinémiás-euglikémiás clamp-ben vizsgálatuk, ahol az euglikémiához szükséges glükóz infúzió sebessége bár kisebb volt a PARP-1$^{-/-}$ egerekben ez nem volt szignifikáns (2J ábra). Amikor az állatokat 60%-os hiperkalorikus magas zsírtartalmú diétát (HFD-t) kaptak a PARP-1$^{-/-}$ egerek kisebb mértékben híztak el (2K ábra) és a glükóz toleranciájuk (2L ábra), illetve inzulin szenzitivitásuk (2M ábra) is kevésbé károsodott a HFD során, mint a PARP-1$^{+/+}$ egereknek. Ezen kívül a PARP-1$^{-/-}$ egerekben a HFD diéta végén alacsonyabb volt a szérum szabad zsírsav szint (0,66 ± 0.05 mEq/l versus 0.53 ± 0.03 mEq/l; p = 0.026). Mindezek a változások köthetőek a mitokondriális aktivitás növekedéséhez is, ezért két fontos, az energia leadásban szerepet játszó szervben (izom és barna zsírszövet (BAT)) megvizsgáltuk a mitokondriális aktivitás paramétereit.



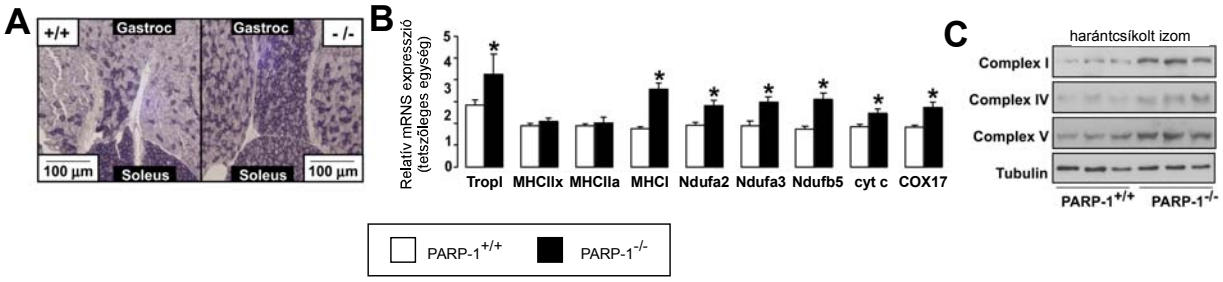
2. ábra. A PARP-1 egerek metabolikus fenotipizálása
PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egerek (n=8/9) (A) testsúlyváltozását és (B) táplálékfogyasztását vizsgáltuk. Ugyanezekben az egerekben a mérés végén meghatároztuk (C) a különböző zsírdepók arányát, az (D) oxigénfogyasztást, az (E) RQ-t és a (F) spontán helyváltoztatást. (G) PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egereken (n=6/5) hideg shock kísérletet végeztünk el. PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egereket (n=9/9) (H) OGTT, illetve (I-J) hiperinzulinémiás-euglikémiás clamp vizsgálatnak vetettük alá. Ennek során meghatároztuk (I) a glükóz felvételt és az (J) inzulin érzékenységet (glükóz infúzió sebessége – GIR). (K-M) PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egereket (n=10/10) HFD diétán tartottuk 6 héten át, eközben (K) vizsgálatuk a testsúly változását, majd a diéta után meghatároztuk az egerek (L) glükóz (OGTT) és (M) inzulin (ipITT) érzékenységét.

A PARP-1$^{-/-}$ egerekben a BAT élénk piros színe és tömegének növekedése (3A ábra) utalt a mitokondriális aktivitás emelkedésére, amelyet elektronmikroszkópos eljárással (3B ábra) és a mitokondriális DNS mennyiségének (3C ábra), illetve a mitokondriális funkcióhoz kapcsolódó gének és fehérjék expressziójának (3D és 3E ábra) meghatározásával bizonyítottuk.



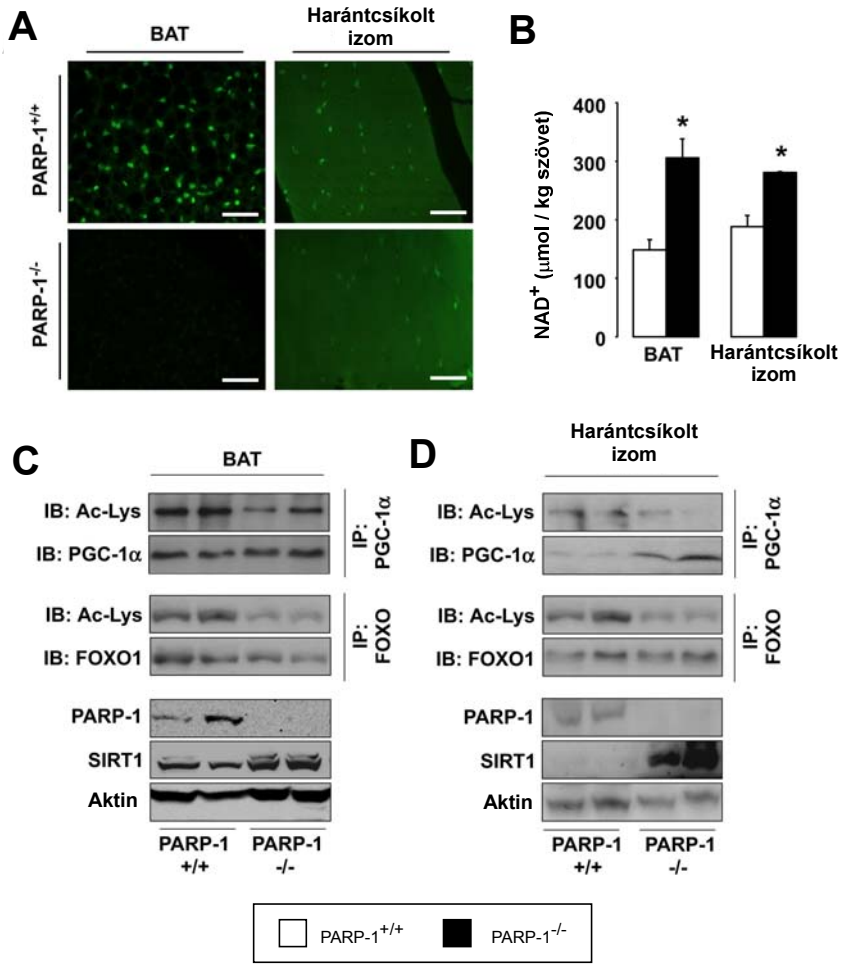
3. ábra. Az egerek barna zsírszövetében (BAT-ban) lezajló változások jellemzése
PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egerek (n=8/9) BAT-ját vizsgáltuk. (A) A boncolás során megmértük és kiszámítottuk a BAT relatív tömegét és lefotóztuk a szervet. A szövetmintákon (B) elektronmikroszkópiai vizsgálatot végeztünk, majd megmértük (C) a mitokondriális DNS mennyiségét, illetve meghatároztuk több, a mitokondriális funkcióhoz szükséges (D) mRNS és (E) fehérje mennyiségét.

A harántcsíkolt izomban (*m. gastrocnemiusban*) szukcinát dehidrogenáz hisztokémiai eljárással (4A ábra), illetve a mitokondriális funkcióhoz kapcsolódó gének és fehérjék expressziójának (4B és 4C ábra) meghatározásával mutattuk ki a mitokondriális aktivitás emelkedését. Az indukálódó gének közé tartozik a BAT-ban az uncoupling protein (UCP)1 és 3, a dejodináz-2 (Dio2) a közepes hosszúságú acil-KoA dehidrogenáz (MCAD), Ndufa2, Ndufb3, Ndufb5, citokróm c (cyt c), citokróm c oxidáz (COX)17, míg a *m. gastrocnemiusban* a I. típusú troponin (TropI), I. típusú miozin nehéz lánc I (MHCI), Ndufa2, Ndufb3, Ndufb5, cyt c, COX17. A *m. gastrocnemiusban* leírt változások izomrost izotípus váltásra utalnak.



4. ábra. A *m. gastrocnemiusban* lezajló változások jellemzése
PARP-1$^{+/+}$ és PARP-1$^{-/-}$ egerek (n=8/9) *m. gastrocnemiusát* vizsgáltuk. A szövetmintákon (A) SDH hisztokémiai vizsgálatot végeztünk, majd meghatároztuk több, a mitokondriális funkcióhoz szükséges (B) mRNS kifejeződését és (C) fehérjetermékének mennyiségét.

A NAD$^+$-függő sirtuin fehérjék kiemelten fontosak a mitokondriális aktivitás szabályzásában és ezek közül a SIRT1 és a PARP-1 közötti kapcsolatot már több szerző is valószínűsítette (Pillai és mtsai, 2005, Rajamohan és mtsai, 2009, Zhang, 2003) ezért megvizsgáltuk, hogy a PARP-1$^{-/-}$ egerekben magasabb-e a SIRT1 aktivitás a BAT-ban és a harántcsíkolt izomban. Mindkét szervben a PARP-1 deléciója csökkentette a PARP aktivitást (5A ábra), megnövelte a NAD$^+$ szintet (5B ábra) és megemelte a SIRT1 aktivitását, amit a PGC1α és a FOXO1 deacetilációja jelzett (5D és 5E ábra). Emellett a SIRT1 fehérje mennyiségének jelentős növekedését is tapasztaltuk (5D és 5E ábra). Ezekből a megfigyelésekből arra következtettünk, hogy a PARP-1 deléciója a NAD$^+$ szint megnövelésén keresztül a SIRT1 aktivitását és/vagy expresszióját indukálja.

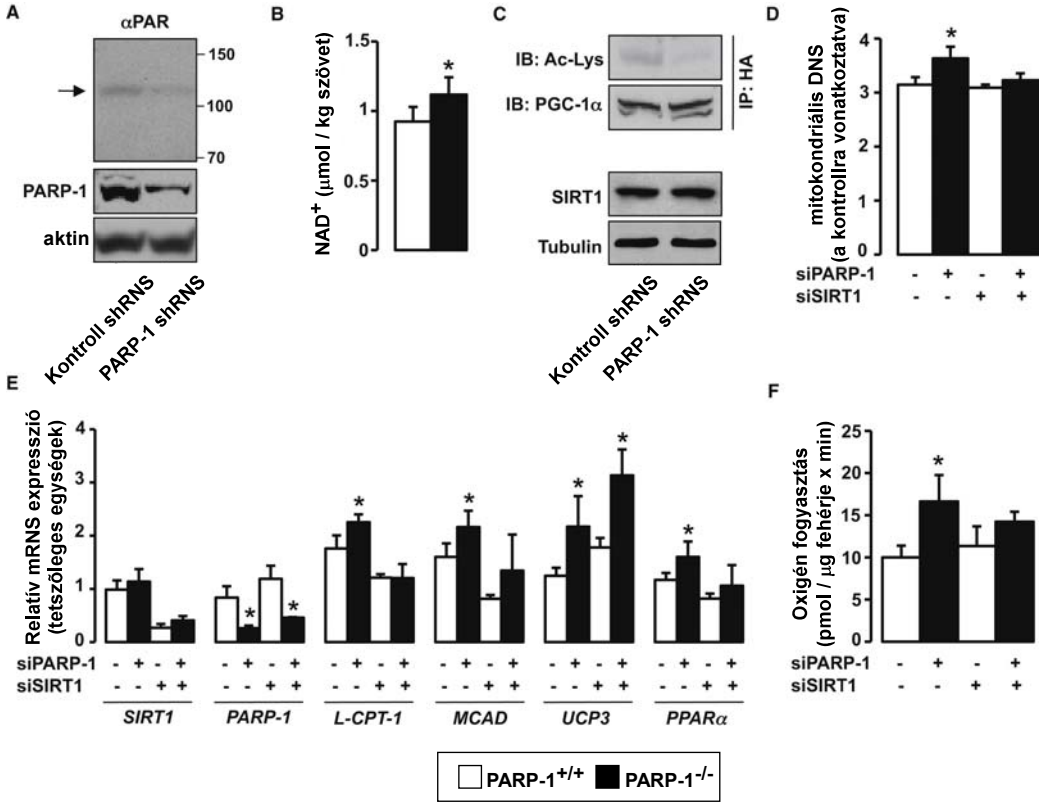


5. ábra. A PARP-1 deléciója megnöveli a NAD$^+$ szintet és a SIRT1 aktivitást
(A) A PAR mennyiségét immunhisztokémiai módszerrel mutattuk ki BAT-ban és harántcsíkolt izomban (a fehér vonal 10 μm). (B) Ugyanezekben a szövetekben meghatároztuk MS technikával a NAD$^+$ mennyiségét, majd (C-D) immunprecipitáció és Western blot segítségével jellemeztük a PARP-1 és SIRT1 fehérje mennyiségi arányait és a SIRT1 aktivitást.

4.1.2 A PARP-1 deléció hatásának vizsgálata sejtes modellekben

A jelenség vizsgálatát sejtes modelleken folytattuk. HEK293T sejtekben sh/siRNS segítségével depletáltuk a PARP-1-et (6A ábra), ami a NAD$^+$ szint (6B ábra) és a SIRT1 aktivitás (6C ábra) emelkedésével járt hasonlóan az egerekben tapasztalt változásokhoz. Érdekes módon azonban bár a SIRT1 mennyisége *in vivo* növekedett a PARP-1 depléciója kapcsán (5D és 5E ábra), a HEK293T sejtekben nem tapasztaltunk különbséget (6C ábra). Ezt a megfigyelést támasztja alá, hogy a PARP-1 depléciója nem aktiválja a SIRT1 promóterét riporter kísérletekben (ezeket az adatokat nem mutatom be). Ez arra utal, hogy valószínűleg a NAD$^+$ szint emelkedése az elsődleges oka a SIRT1 aktivitás növekedésének. Úgy tűnik, hogy ez a modell hasonlóan működik az egerekben tapasztaltakhoz, ezért megvizsgáltuk a mitokondriális

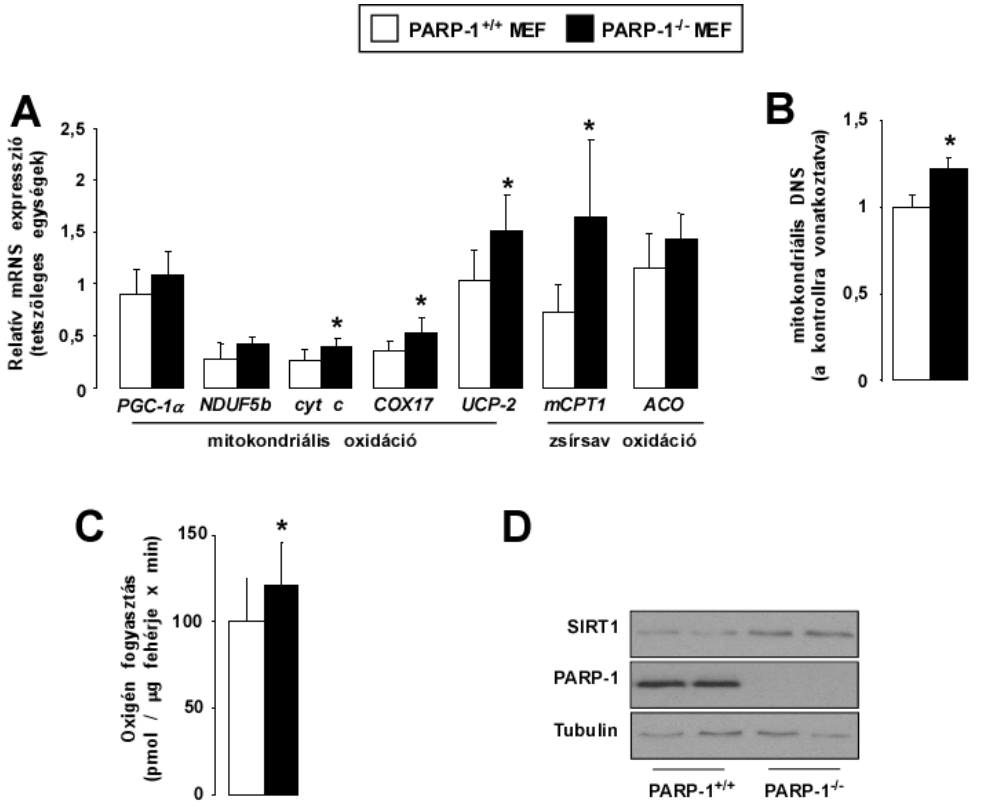
funkció markereit. Azt tapasztaltuk, hogy a PARP-1 depléció hatására a mitokondriális DNS mennyisége (6D ábra), egyes mitokondriális gének expressziója (karnitin palmitoil transzferáz-1 (LCPT-1), Medium-chain acyl-CoA dehydrogenase (MCAD), UCP3 és PPARα) (6E ábra), illetve a sejtek oxigénfogyasztása (6F ábra) megnő. Ha a SIRT1-et is depletáltuk, akkor az előbbi fiziológiás paraméterek változása elmaradt (6D - 6F ábra). Ez arra utal, hogy a PARP-1 deléciója által kiváltott mitokondriális biogenezis indukcióban központi szerepe van a SIRT1 aktivitás emelkedésének.



6. ábra. A PARP-1 csendesítése a SIRT1 aktivitás és a mitokondriális biogenezis növekedéséhez vezet
(A-C) HEK293T sejteket PARP-1 shRNS-sel transzfektáltuk, illetve az immunprecipitációs kísérletben ezen felül HA-PGC-1α expressziós kontsrukttal is, majd meghatároztuk (A) a PARP-1 és a PAR szinteket, (B) kolorimetriás módszerrel a NAD^+ szintet, illetve (C) a SIRT1 fehérje mennyiségét és a SIRT1 aktivitást. A nyíl a PARP-1 autoPARilációját mutatja. (D-F) HEK293T sejteket siPARP-1, illetve siSIRT1 siRNS-ekkel transzfektáltuk, majd meghatároztuk (D) a mitokondriális DNS mennyiségét, (E) több mitokondriális gén expresszióját, illetve (F) az oxigén fogyasztást.

Ugyanezeket a paramétereket megvizsgáltuk primer $PARP\text{-}1^{+/+}$ és $PARP\text{-}1^{-/-}$ MEF sejtekben. A $PARP\text{-}1^{-/-}$ sejtekben nőtt a mitokondriális aktivitás markergénjeinek (PGC1α, nduf5a, cyt c, COX17 UCP2, mCPT1 és acil-KoA oxidáz (ACO)) expressziója (7A ábra), ami

magasabb mitokondriális DNS mennyiséggel (7B ábra) és oxigénfogyasztással járt együtt (7C ábra). Ezek az adatok a SIRT1 aktivitás növekedésére utaltak, ami a sejtekben egybeesett a SIRT1 fehérje mennyiségének megnövekedésével (7D ábra).

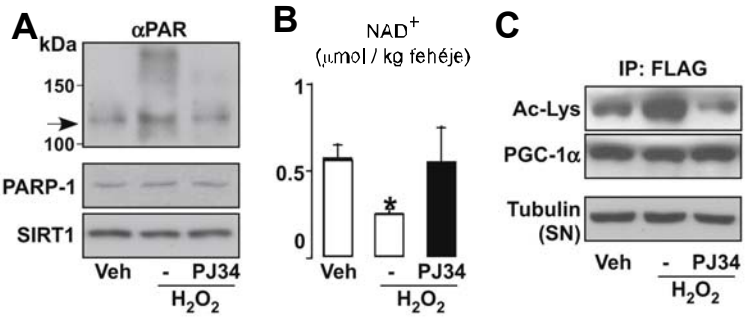


7. ábra. A SIRT1 aktiválódik a PARP-1$^{-/-}$ primer MEF-ekben
PARP-1$^{+/+}$ és PARP-1$^{-/-}$ primer MEF sejtekben (n=3/3) meghatároztuk (A) néhány mitokondriális biogenezishez szükséges gén expresszióját, (B) a mitokondriális DNS mennyiségét, (C) a sejtek oxigén fogyasztását, illetve (D) a PARP-1 és SIRT1 fehérje expresszióját.

4.1.3. A PARP gátlás metabolikus hatásai

Az eddigi eredmények arra utalnak, hogy a PARP-1 depléciója a NAD^+ szint emelkedéséhez és a SIRT1 indukciójához vezet, ami a mitokondriális biogenezist aktiválja. A PARP-1 felelős a sejtek PARP aktivitásának 85-90%-ért (Schreiber és mtsai, 2002), illetve a PARP-1 gyors és hatékony NAD^+ fogyasztó (Ame és mtsai, 1999) ezért aktivitásának gátlása megemelheti a NAD^+ szintet („NAD^+ spórolás"). Logikusan adódik a kérdés, hogy PARP inhibitor adása hasonló fenotípust alakít-e ki, mint a PARP-1 deléciója.

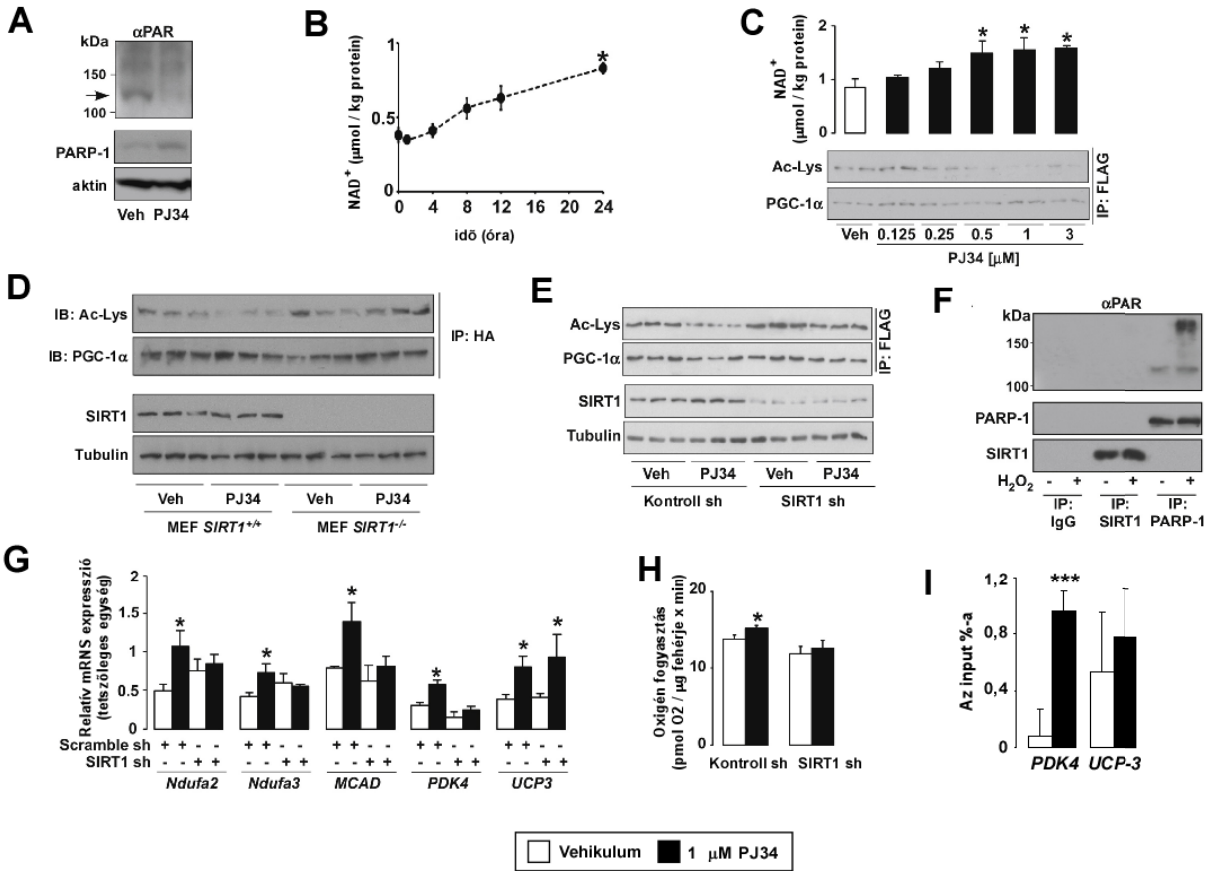
Izomrostokká differenciált C2C12 mioblasztokat kezeltünk 1 µM PJ34 PARP inhibitorral (Soriano és mtsai, 2001). A PARP-1 aktivációja H_2O_2-vel gátolja a SIRT1 aktivitást és csökkenti a NAD^+ szintet (8A-C ábra), ami ellen a PJ34 kezelés védelmet nyújt.



8. ábra. Az oxidatív stressz PARP függő módon gátolja a SIRT1 aktivációt
C2C12 sejteket H_2O_2-vel kezeltük PJ34 előkezelés mellett, vagy anélkül, majd meghatároztuk (A) a PARP-1 és PAR szinteket, (B) a NAD^+ mennyiségét, illetve (C) a SIRT1 aktivitást.

A PJ34 gátolta a PARP-1 aktivitást (9A ábra), ezzel párhuzamosan idő- (9B ábra) és dózisfüggő (9C ábra) módon a NAD^+ szint emelkedését okozta, illetve aktiválta a SIRT1 enzimet (9D ábra), azaz hasonlóan működött, mint a PARP-1 genetikai inaktiválása. A SIRT1 expresszió nem változott PJ34 kezelés hatására (9D és 9E ábra). A SIRT1 deléciója, vagy csendesítése felfüggesztette a PJ34 hatását (9D és 9E ábra). Ezek az adatok arra utalnak, hogy a PARP aktivitás gátlása is a PARP-1 deléciójához hasonlóan a NAD^+ szint emelésén keresztül a SIRT1 aktivációját okozzák. Ezzel párhuzamosan kizártuk annak a lehetőségét, hogy a SIRT1 PARilációja okozza a SIRT1 aktivitásban kimutatott változást (9F ábra). A PJ34 a SIRT1 aktiváción keresztül tehát a mitokondriális biogenezis indukciójához vezet, amit a mitokondriális gének (Ndufa2, Ndufa3, MCAD és PDK4) expressziójának és a sejtek oxigénfogyasztásának emelkedése jellemzett (9G és 9H ábra).

SIRT1 shRNS és a SIRT1 knockout MEF-ek segítségével bizonyítottuk, hogy a metabolikus változások SIRT1 függőek (9D, 9F-H ábra), bár vannak SIRT1 független hatások is, mint az UCP3 indukció (9G ábra). A SIRT1 aktiváció hatására az útvonal SIRT1-től lefelé helyeződő elemei is aktiválódnak. Például jelentősen megnő a PGC1α kötődése a SIRT1 függő promóterekhez (pl. PDK4 promótere) (9I ábra). Ezzel ellentétben a HA antitest jele (i.e. a PGC1α jelenléte) nem változott az UCP3 promóterén, amely SIRT1-független módon indukálódott (9I ábra).

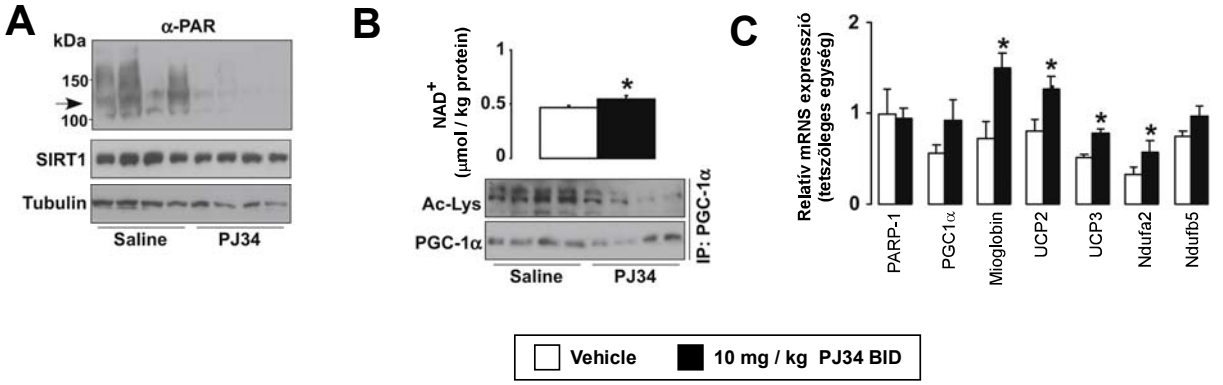


9. ábra. A PARP-1 farmakológiai gátlása a SIRT1 aktivitás emelkedéséhez vezet C2C12 sejtekben

C2C12 sejteket PJ34-gyel (1 µM) kezeltük, majd meghatároztuk (A) a PARP-1 és a PAR szintjét, (B) a NAD^+ mennyiségének időbeli változását, illetve (C) a NAD^+ mennyiségének és a SIRT1 aktivitásának változását a PJ34 koncentrációjának függvényében. (D) $SIRT1^{+/+}$ és $SIRT1^{-/-}$ MEF sejtekben, illetve (E) SIRT1 csendesített C2C12 sejtekben meghatároztuk a SIRT1 fehérje mennyiségét és a SIRT1 aktivitását. (F) FLAG-HA-PGC-1α-t overexpresszáló C2C12 sejtek lizátumából az ábrán megjelölt antitestekkel immunprecipitációt végeztünk el, majd anti-PAR antitesttel vizsgáltuk az ábrán megjelölt fehérjék PARilációját. (G-I) PJ34 kezelt C2C12 sejteket kontroll (nem specifikus), vagy SIRT1 specifikus shRNS-sel kezeltünk, majd meghatároztuk (G) több, a mitokondriális biogenezishez szükséges gén expresszióját és (H) a sejtek oxigén fogyasztását. (I) PJ34 kezelt C2C12 sejtek felhasználásával ChIP kísérletekben jellemeztük a PGC-1α jelenlétét a PDK4 (PARP-1 függő promóter) és az UCP3 (PARP-1 független promóter) promóterein.

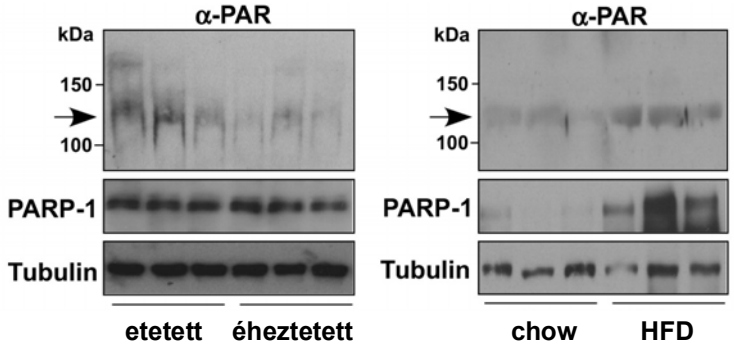
C57/Bl6J hím egereket PJ34-gyel (10 mg/kg BID 5 napig), vagy fiziológiás sóoldattal kezeltünk, majd a *m. gastrocnemiusban* megvizsgáltuk, hogy a PARP gátlás hatását. A PJ34 kezelés hatékonyan gátolta a *m. gastrocnemiusban* a PARP aktivitást (10A ábra), ami a NAD^+ szint és a SIRT1 aktivitás növekedésével járt együtt (10B ábra), ugyanúgy, mint PARP-1 deléciója, vagy a C2C12 sejtek PJ34-gyel történő kezelése esetében. A SIRT1 fehérje mennyisége nem növekedett (10A ábra). A PJ34 kezelt állatokban a szérum triglicerid (1.21 ±

0.08 mM vehikulum vs. 1.11 ± 0.04 mM PJ34; p = 0.08) és szabad zsírsav (1.59 ± 0.06 mEq/l vehikulum vs. 1.44 ± 0.03 mEq/l PJ34; p = 0.03) szintje lecsökkent. Ezek a változások együtt jártak a *m. gastrocnemius* expressziós mintázatának a változásával. A mioglobin, az UCP2, az UCP3 és az ndufa2 expressziójának szignifikáns emelkedését tapasztaltuk, míg a PGC1α és az Ndufa5 expresszió változása hasonló trendet mutatott (10C ábra), ami az izmokban a PJ34 kezelés hatására megnövekvő mitokondriális biogenezisre utal.



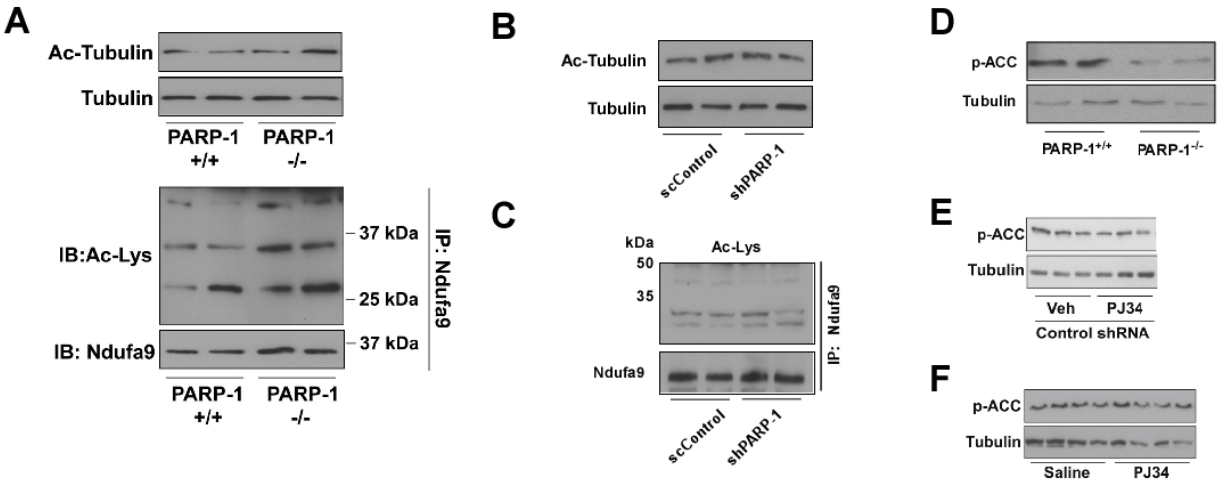
10. ábra A PJ34 kezelés a mitokondriális biogenezis megemelkedéséhez vezet C57/Bl6J egerek harántcsíkolt izmaiban.
C57/Bl6J egereket öt napon át i.p. 10 mg/kg PJ34–gyel oldottuk kétszer naponta, majd a *m. gastrocnemiusból* meghatároztuk (A) a PARP-1 autoPARilációját, a SIRT1 expresszióját, (B) az izom NAD^+ koncentrációját, a SIRT1 aktivitást, illetve (C) több, a mitokondriális aktivitáshoz szükséges gén expresszióját.

Felvetődik, hogy létezik-e olyan metabolikus hatás, ami befolyásolja a PARP aktivációt. Vizsgáltuk a PARP aktivitás változását a tápláltság függvényében. Meghatároztuk a PARP aktivitást C57/Bl6J egerek *m. gastrocnemiusában* 24 órás éhezés, illetve 12 hetes HFD diéta (hiperkalorikus, 60% zsírtartalom) után. Az éhezés hatására a PARP aktivitás lecsökkent, míg a kalorikus terhelés hatására a PARP aktivitás és a PARP-1 szintje megnő (11. ábra), vagyis úgy tűnik a PARP-1 valamilyen módon képes reagálni a tápanyag ellátottságra is.



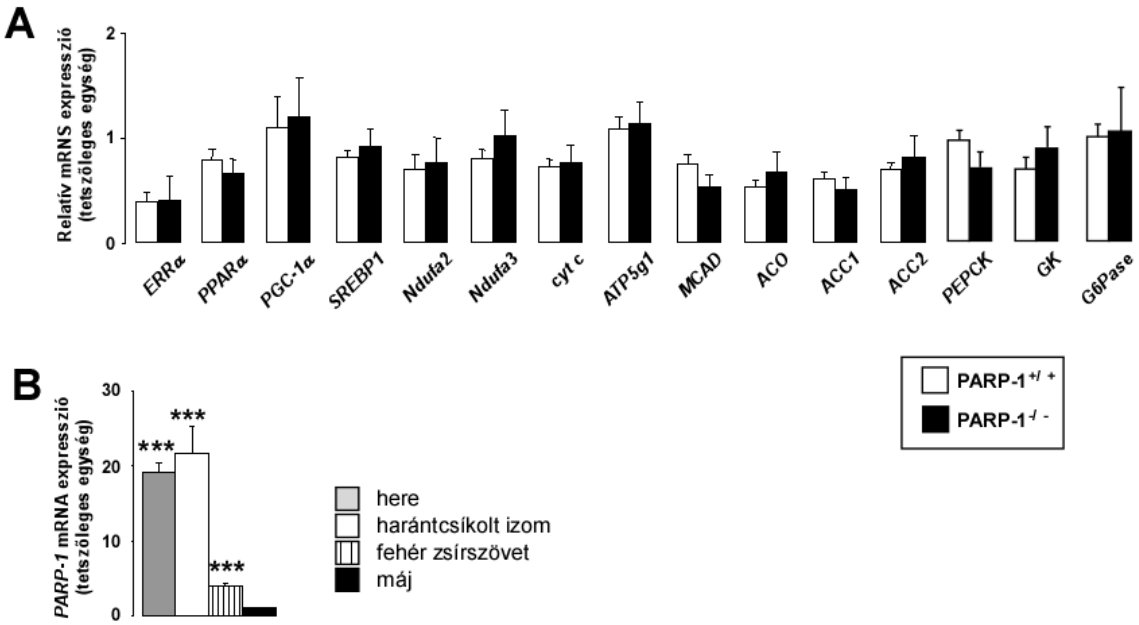
11. ábra. A PARP aktivitás és expresszió változik a tápláltság függvényében
C57/Bl6J egerek *m. gastrocnemiusában* 24 órás éhezést, vagy 3 hónap HFD-t követően Western blottal meghatároztuk a PARP-1 fehérje expressziót és a PARP aktivitást a PARP-1 autoPARilációján keresztül.

Felvetődött, hogy más sirtuinok, a citoplazmatikus SIRT2 és a mitokondriális SIRT3 aktivitása megváltozik-e a PARP-1 delációja hatására. Jellegzetes szubsztrátjaik (SIRT2 – tubulin, SIRT3 – Ndufa9) acetiláltsága azonban nem mutatott eltérést a két genotípus között egyik modell esetében sem (12A-C ábra). Ez arra utal, hogy a SIRT2 és a SIRT3 aktivitását nem befolyásolja a PARP-1 hiánya. Hasonlóképpen felvetődött, hogy a SIRT1 aktivitás megváltozása befolyásolhatja az AMPK aktivitását. Bizonyos kísérleti körülmények között (PARP-$1^{+/+}$ vs. PARP-$1^{-/-}$ MEF sejtek, MEF-ek, C2C12 sejtek PJ34 kezelése – 12D-E ábra) a pACC jel csökkent a PARP gátlás hatására, míg a Bl6 egerek PJ34 kezelése során nem volt nyilvánvaló változás a pACC szintben (12F ábra).



12. ábra A PARP-1 deléciója, vagy gátlása nem befolyásolja a SIRT2 és SIRT3, azonban gátolja az AMPK aktivitást.
(A-B) A tubulin és az Ndufa9 acetilációját Western blot technikával határoztuk meg (A) PARP-$1^{+/+}$ és PARP-$1^{-/-}$ egerek *m. gastrocnemiusában*, illetve (B-C) PARP-1 csendesített HEK293T sejtekben. (D-F) A pACC szintet Western blot technikával határoztuk meg (D) MEF sejtekben, (E) C2C12 sejtekben és C57/Bl6J egerek *m. gastrocnemiusában*.

Végül megvizsgáltuk, hogy a májban okoz-e metabolikus eltérést a PARP-1 deléciója. PARP-$1^{+/+}$ és PARP-$1^{-/-}$ egerek több metabolikus gén expresszióját hasonlítottuk össze, de nem tapasztaltunk szignifikáns különbséget a két genotípus között (13A. ábra). Úgy tűnik, hogy a PARP-1-nek elhanyagolható metabolikus hatása van a májban, ami valószínűleg arra vezethető vissza, hogy expressziója nagyon alacsony ebben a szervben (13B. ábra).

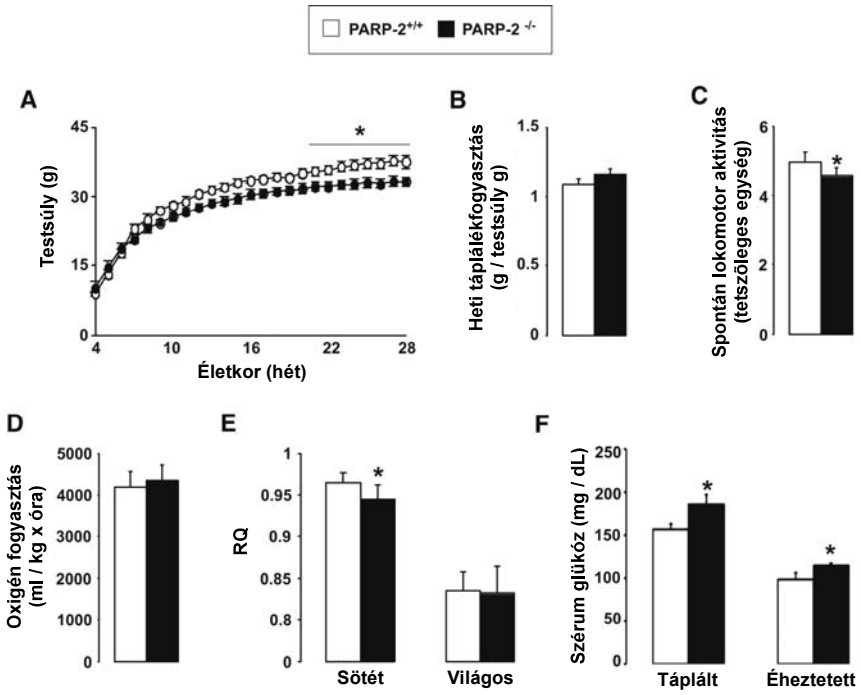


13. ábra A PARP-1 deléciója nem okoz metabolikus változást a májban.
(A) PARP-$1^{+/+}$ és PARP-$1^{-/-}$ egerek (n=8/9) májában megvizsgáltuk több metabolikus fehérjét kódoló gén expresszióját. (B) C57/Bl6J egerek (n=3) szerveiben összehasonlítottuk a PARP-1 expresszióját RT-qPCR technikával.

4.2. Hogyan befolyásolja a PARP-2 enzim a metabolikus szervek, szövetek működését?

4.2.1. A PARP-2$^{-/-}$ egerek metabolikus fenotipizálása

A PARP-2 enzim, mint a bevezetőben is említettem, szerkezetében és több funkciójában hasonlít a PARP-1-re, ezért hasonló metabolikus fenotipizálást végeztünk el a PARP-2 törzsön is. A PARP-2$^{-/-}$ egerek alacsonyabb testsúlyúak vad típusú társaiknál (14A ábra) azonos táplálékfelvétel (14B ábra) és kisebb mértékű spontán mozgás (14C ábra) ellenére. A PARP-2$^{-/-}$ egerek oxigénfogyasztása nem volt szignifikánsan magasabb, mint a PARP-2$^{+/+}$ állatoké, bár tendenciájában növekedést mutatott (14D ábra). A sötét (aktív) periódusban a PARP-2$^{-/-}$ állatok RQ értéke a vad típusú egerekhez képest csökkent, ami emelkedett zsírsav oxidációra utalt (14E ábra), azonban a várakozásunkkal ellentétben a szérum glükóz értéke mind a táplált, mind az éheztetett állapotban magasabb volt a PARP-2 knockout állatokban (14F ábra).

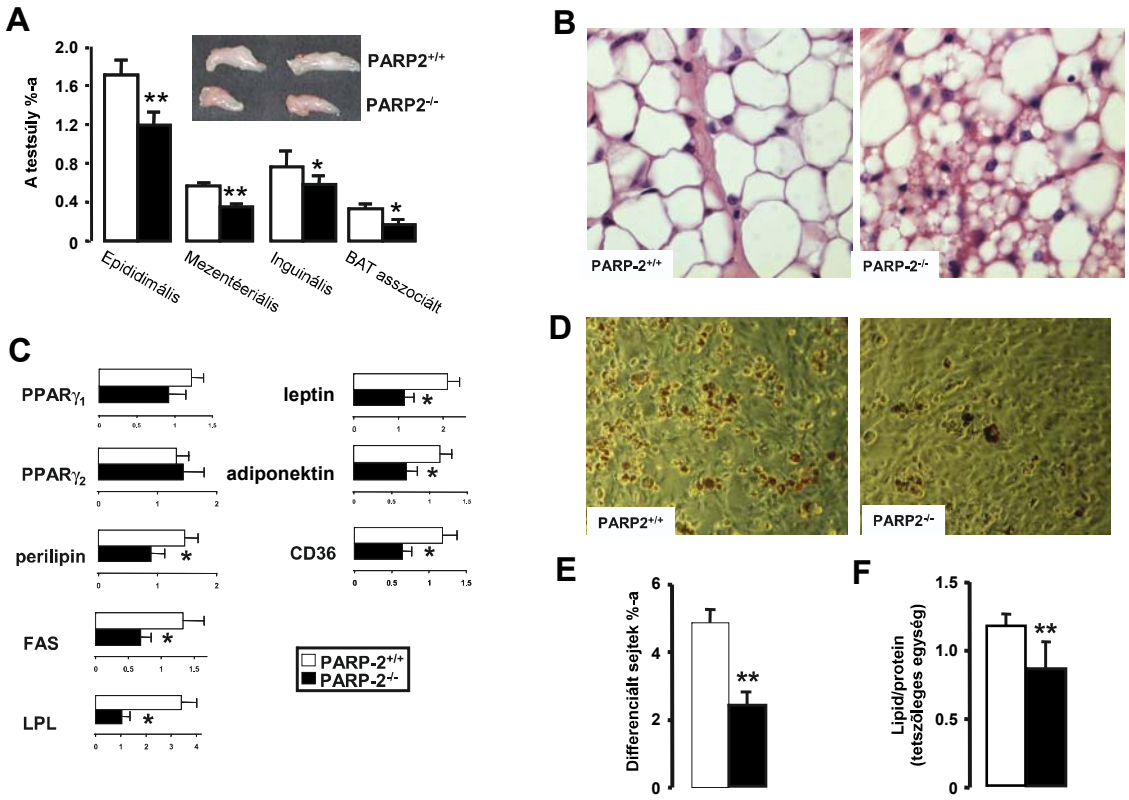


14. ábra A PARP-2 knockout egerek metabolikus fenotipizálása.
(A-B) PARP-2$^{+/+}$ és PARP-2$^{-/-}$ egerek (n=6/6) (A) testsúlyának változását, illetve (B) táplálékfogyasztását vizsgáltuk az ábrán jelölt időtartamban. (C-F) A vizsgálat végén jellemeztük (C) az egerek spontán mozgását, (D) meghatároztuk oxigén fogyasztásukat, (E) RQ értéküket, illetve (F) a szérum glükóz koncentrációt.

4.2.2 A PARP-2 deléció hatása a fehér zsírszövet funkciójára

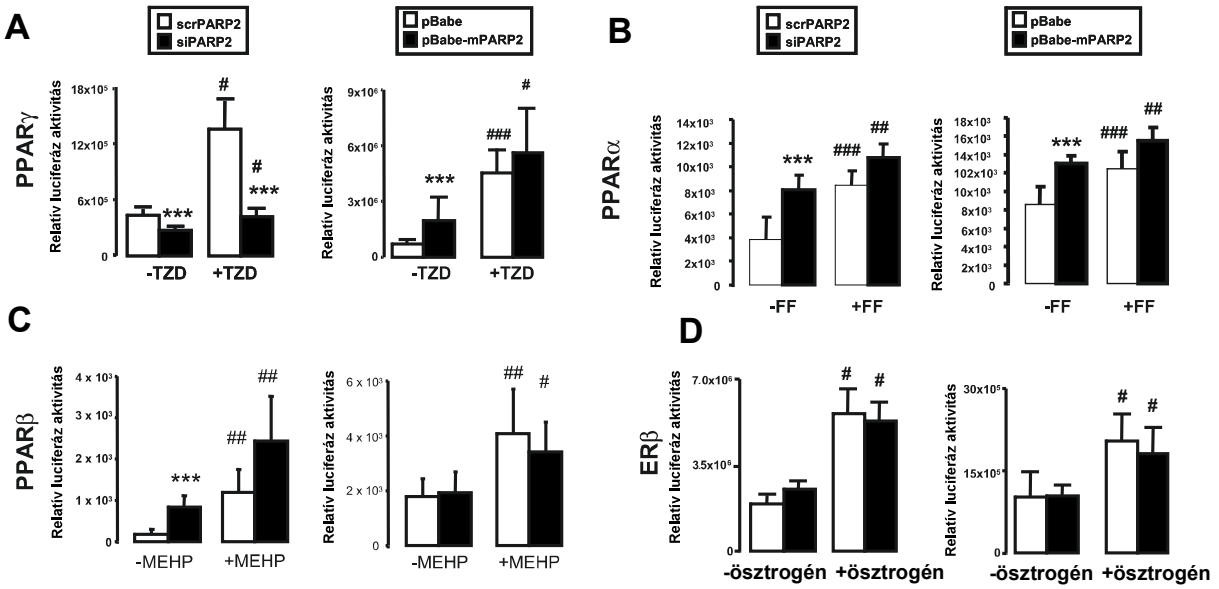
Vizsgáltuk azokat a metabolikus szerveket is, amelyek felelősek lehetnek a fenti fiziológiai eltérésekért. A kisebb testsúly egyik kézenfekvő oka lehet, hogy csökken a szervezetben a tárolt

zsír mennyisége. Ezt a feltételezést ellenőrizendő megmértük PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek zsírdepóinak a testsúlyra normalizált súlyát: a vizsgált depók kisebbek voltak a PARP-$2^{-/-}$, mint a PARP-$2^{+/+}$ egerekben (15A ábra), sőt a modellként kiválasztott epididimális zsírszövetben a zsírsejtek mérete is kisebb volt (15B ábra), ami az adipociták diszfunkciójára utal. A zsírszövet talán legfontosabb transzkripciós regulátora a PPARγ, ezért megvizsgáltuk a PPARγ izoformák és a PPARγ által vezérelt gének expresszióját. A PPARγ izoformák expressziója nem változott, azonban a PPARγ függő gének expressziója jelentősen lecsökkent (15C ábra). Sejtes modellben is vizsgáltuk a PARP-2 hiányának hatását a zsírsejt irányú differenciációra. PARP-$2^{+/+}$ és PARP-$2^{-/-}$ MEF-eket differenciáltattunk zsírsejtekké és azt tapasztaltuk, hogy a PARP-2 hiányában a differenciáció mértéke lecsökken, mint azt az Oil-Red O festés, a FACS mérés, a lipid tartalom, vagy PPARγ függő gének expressziójának meghatározása mutatja (15D-F ábra).



15. ábra. Lipodisztófia és csökkent zsírsejt irányú differenciáció a PARP-$2^{-/-}$ egerekben
(A-C) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerekben (n=6/7) (A) meghatároztuk több zsírdepó arányát a testtömeghez viszonyítva, (B) megvizsgáltuk a zsírszövet szerkezetét HE festés után, illetve (C) meghatároztuk több PPARγ függő gén expresszióját. A (C) panelen az y tengelyen a relatív expressziót tüntettük fel tetszőleges egységekben. PARP-$2^{+/+}$ és PARP-$2^{-/-}$ MEF-eket (n=6/7) differenciáltattunk zsírsejtekké. (D-E) A terminálisan differenciált zsírsejteket (E) ORO festéssel tettük láthatóvá, illetve (F) Nílusvörös festés után FACS méréssel jellemeztük. (F) Meghatároztuk ugyanezen sejtek triglicerid tartalmát is.

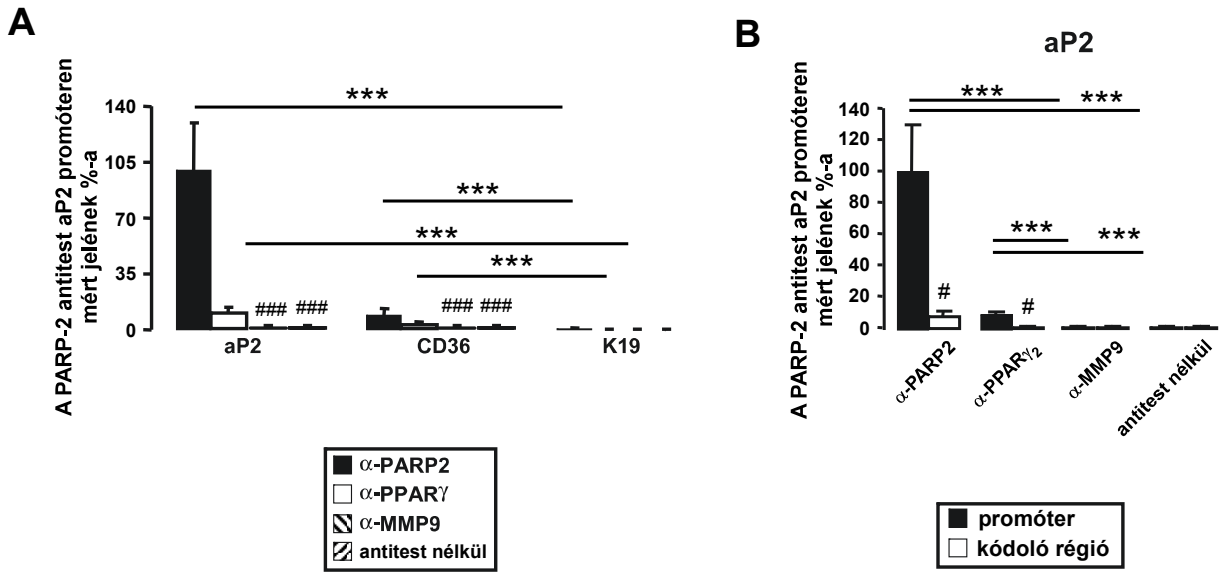
Az eddigi eredmények a PPARγ rendszer csökkent mértékű működésére utaltak, további kísérleteinkben bizonyítékot kerestünk arra, hogy valóban a PPARγ rendszer defektív működése áll a feltárt jelenség mögött. Riporter assay-ket végeztünk PPAR-specifikus riporter konstrukttal HEK293T sejtekben, amelyekben shRNS-sel depletáltuk a PARP-2-t. A PARP-2 depléció drasztikusan csökkentette a PPARγ specifikus transzaktivációt, sőt a PPARγ specifikus aktivátora a TZD sem képes a PARP-2 nélkül hatékonyan aktiválni a PPARγ-t (16A ábra). A PARP-2 overexpressziója megemeli a PPARγ alapaktivitását, azonban a maximális aktivitást nem befolyásolja (16A ábra). Megvizsgáltuk, hogy szükséges-e a PARP-2 más magreceptorok működéséhez is. Érdekes módon a PPARα és a PAPRβ/δ függő transzaktivációt a PARP-2 a PPARγ-hoz képest ellentétesen befolyásolta (16B és 16C ábra). Azonban az ösztrogén receptor-β (ERβ) esetében sem a PARP-2 depléciója, sem az overexpressziója nem befolyásolta a receptor transzaktivációt (16D ábra).



16. ábra. A PARP-2 deléció és overexpresszió hatása egyes magreceptorok aktivitására
HEK293T sejtekben a PARP-2-t shRNS segítségével csendesítettük, illetve a pBabe-PARP-2 konstrukt segítségével overexpresszáltuk. Ezekben a sejtekben luciferáz riporter kísérletekben meghatároztuk a (A) PPARγ, (B) PPARα, (C) PPARβ/δ és az (D) ERβ aktivitását.

A riporter assay-k eredményei arra utalnak, hogy a PARP-2 számos más fehérjéhez hasonlóan a PPARγ transzkripciós rendszer tagja és mivel rendelkezik DNS-kötő doménnal valószínűleg közvetlenül a DNS-hez kapcsolódik közel a PPARγ-RXR dimerhez. Ennek ellenőrzésére kromatin immunprecipitációt végeztünk el a PARP-2-re, PPARγ-ra, illetve kontrollként a nem magi mátrix metalloproteináz 9 (MMP9) specifikus antitestekkel és antitest nélkül is. Két PPARγ függő gén (CD36 és aP2), illetve egy ERβ függő gén (keratin 19 (K19))

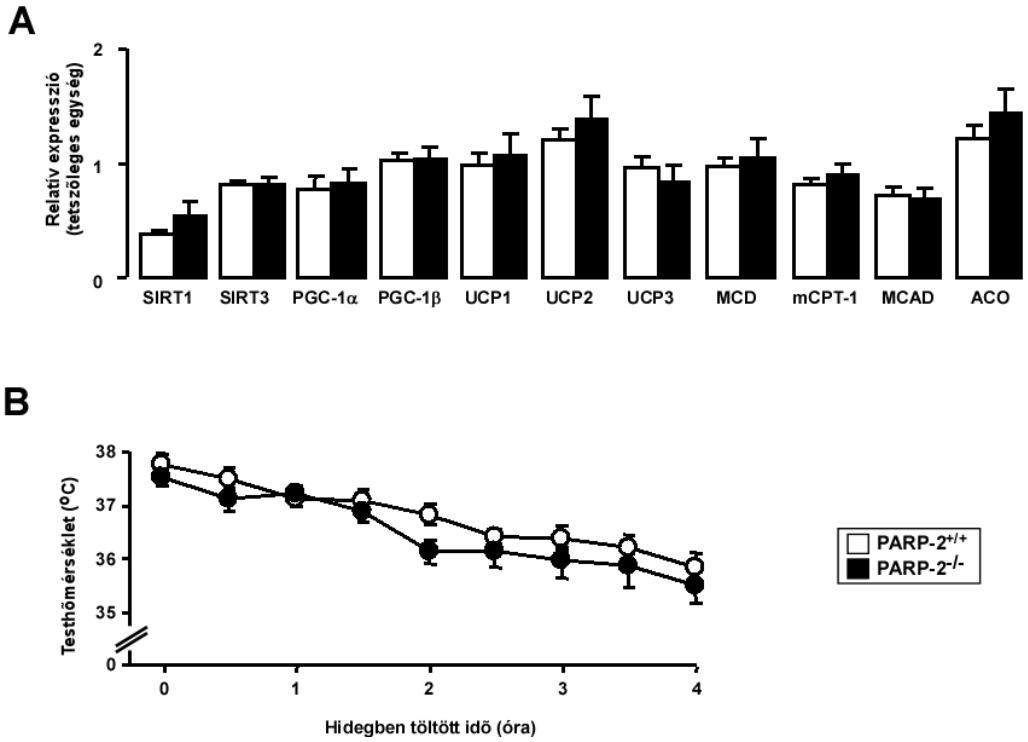
promóterét vizsgáltuk meg. Az α-PARP-2 és az α-PPARγ antitest magasabb jelet adott az aP2 és a CD36 promóterén (17A ábra). További kontrollként megvizsgáltuk a PARP-2 és a PPARγ jelenlétét az aP2 promóterén és a kódoló szekvenciájában és azt találtuk, hogy az aP2 promóteren magasabb jelet ad mindkét antitest, mint a kódoló régióban (17B ábra). Mindezek arra utalnak, hogy a PARP-2 a PPARγ-val együtt foglalja el a fenti promótereket, vagyis a PARP-2 a PPARγ kofaktora és a PARP-2 hiányában az RXR-PPARγ dimer aktivitása lecsökken, ami a zsírszövet diszfunkciójához vezet.



17. ábra. A PARP-2 a PPARγ függő promóterekhez kapcsolódik
(A-B) 3T3-L1 sejtekben ChIP kísérleteket végeztünk el az ábrán jelzett antitestekkel. A kísérletekben összegyűjtött kromatin darabokat qPCR technikával erősítettük az ábrán feltüntetett DNS szakaszokra specifikus primerek között. A kapott értékeket először a kísérlet „input" mintájára normalizáltuk, majd a kísérletben a legmagasabb jelet adó mintára.

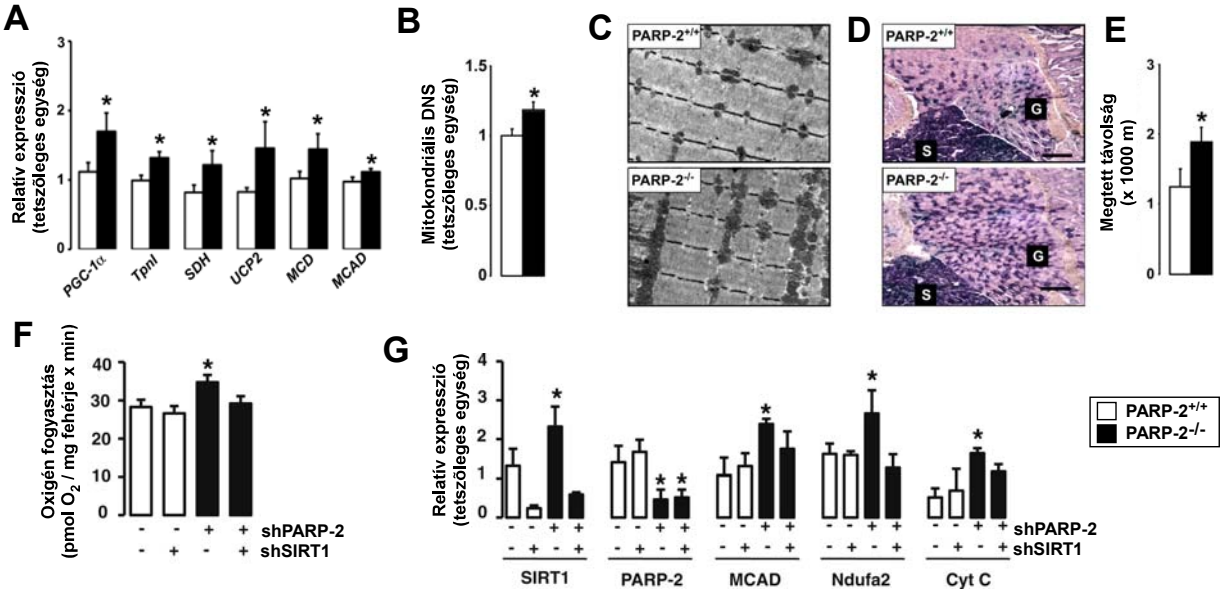
4.2.3. Az energialeadás szerveinek (barna zsírszövet, harántcsíkolt izom) vizsgálata

A kisebb testsúly adódhat a szervezet energia leadásának a megemelkedéséből is, ennek megfelelően megvizsgáltuk az energia leadásban szerepet játszó szerveket, mint a BAT, a harántcsíkolt izom és a máj. Bár a PARP-1 knockout egerekben, illetve a PARP inhibitor kezelés hatására a BAT aktivitása megemelkedik, azonban a PARP-2 – annak ellenére, hogy deléciója a SIRT1 fehérje mennyiségének növekedéséhez vezet (18A ábra) - úgy tűnik, nem befolyásolja a BAT működését (18B-C ábra).



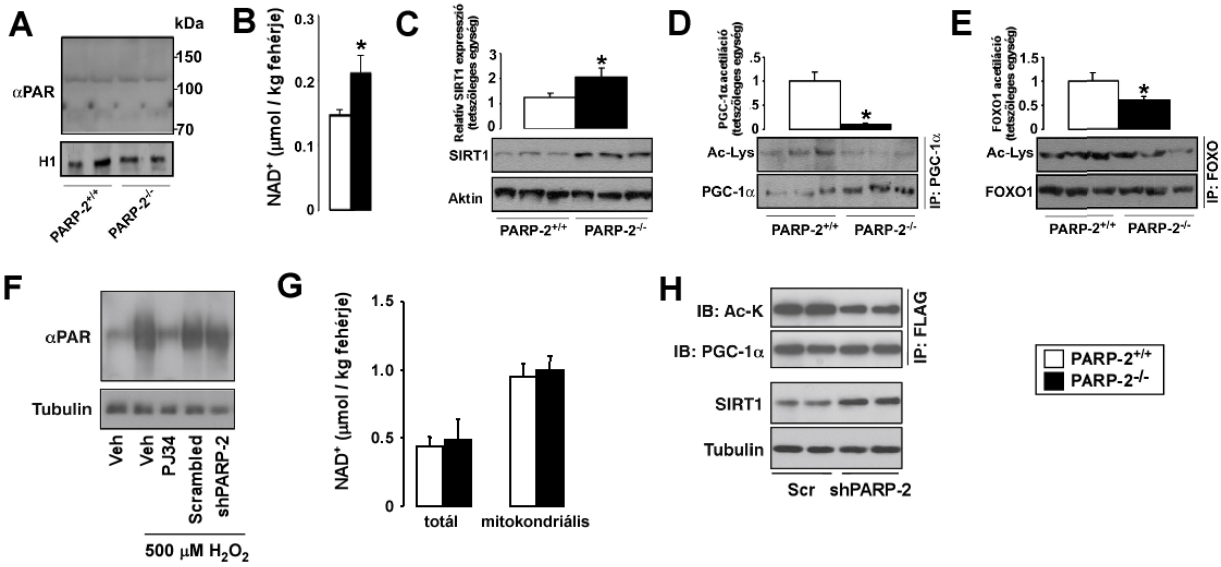
18. ábra. A PARP-2 deléció nem vezet a BAT működés növekedéséhez
PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek (n=6/6) BAT-jában meghatároztuk (A) Western blot technikával a SIRT1 fehérje mennyiségét, (B) több mitokondrium és BAT-specifikus gén expresszióját RT-qPCR technikával. (C) Az egerek BAT funkcióját hideg stressz kísérletben jellemeztük.

Jellegzetes energia leadásra alkalmas szövet a harántcsíkolt izom, amely vizsgálatára PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek *m. gastrocnemius* izmát, illetve PARP-2 csendesített (shPARP-2) és kontroll (scPARP-2) C2C12 sejteket használtunk. A PARP-2 depléciója a harántcsíkolt izmokban az izotípusváltás jeleit mutatja, megjelennek az I. típusú, mitokondriumokban gazdag, lassú rostok, amit a génexpresszió átrendeződése (19A ábra), az mtDNS mennyiségének növekedése (19B ábra), az elektronmikroszópos és az SDH hisztokémiai vizsgálatok jeleznek (19C és 19D ábra). Ezt megerősíti, hogy az egerek kifáradásig tartó futtatása (forced running) során a PARP-$2^{-/-}$ egerek hosszabb távot tesznek meg, mint vad típusú társaik (19E ábra). A sejtes modellben hasonló fenotípus változást okozott a PARP-2 csendesítése: SIRT1 függő módon megnőtt a sejtek oxigénfogyasztása (19F ábra) és több, a mitokondriális funkcióhoz köthető gén (MCAD, Ndufa2, cyt c – 19G ábra) expressziója is fokozódott.



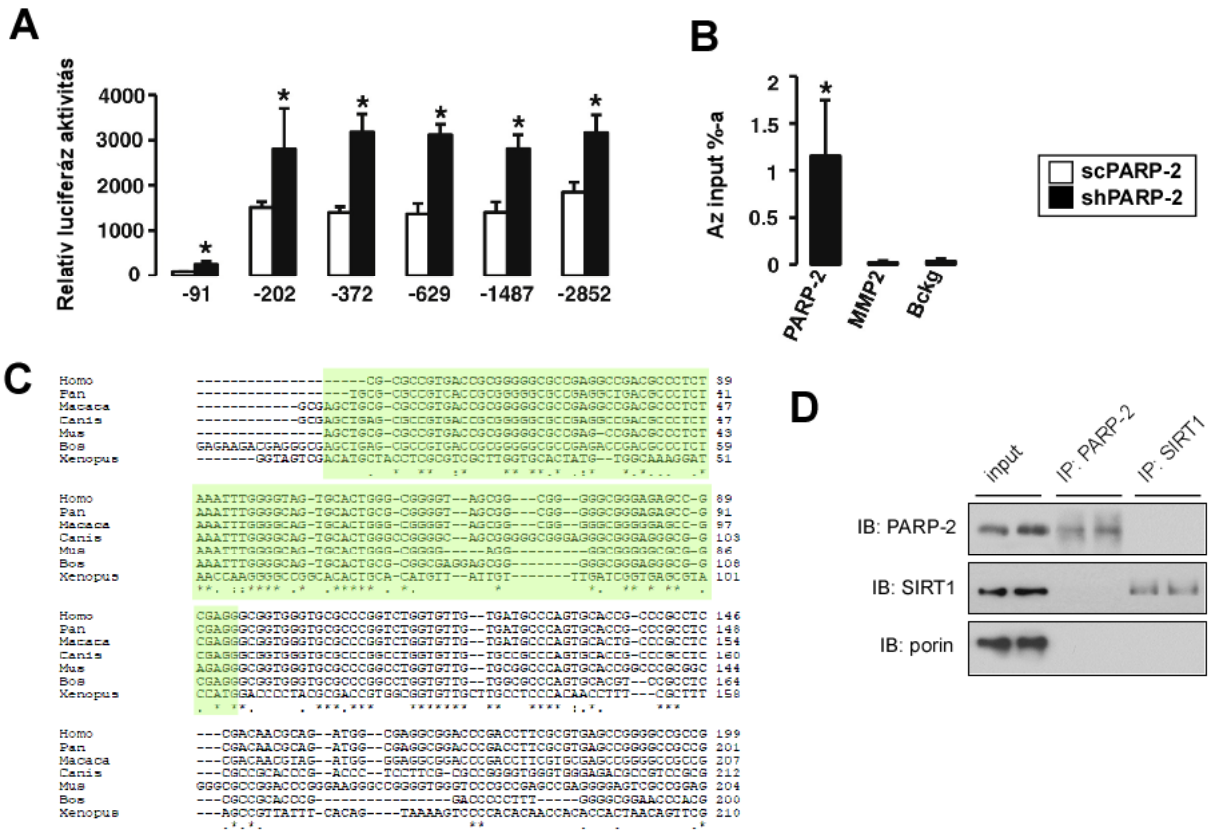
19. ábra. A PARP-2 deléciója a mitokondriális aktivitás növekedéséhez vezet harántcsíkolt izomban és C2C12 sejtekben
PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek (n=6/6) *m. gastrocnemiusában* meghatároztuk (A) a mitokondriális funkció kulcsgénjeinek expresszióját, (B) a mitokondriális DNS mennyiségét, illetve (C) elektronmikroszkópos vizsgálattal és (D) SDH hisztokémiai vizsgálattal a mitokondriumok mennyiségét jellemeztük. (E) Az egereket kifáradásig futtattuk. (F-G) C2C12 sejtekben csendesítettük a PARP-2 és a SIRT1 expressziót és meghatároztuk a sejtek (F) oxigénfogyasztását és (G) több mitokondrium-specifikus gén expresszióját.

A PARP-2 deléciója nem változtatja meg jelentősen az izomrostok össz-PARP aktivitását (20A ábra), a NAD^+ szint, a SIRT1 expresszió és a SIRT1 aktivitás fokozódik (20B-E ábra). Ez arra utal, hogy a PARP-2 deléciója, a PARP-1 deléciótól eltérő módon képes a SIRT1 aktiválására. Elvégeztük az előbbiekben részletezett méréseket PARP-2 csendesített C2C12 sejtekben is. Ebben a modellben nem vezetett a PARP-2 depléciója sem a PARP aktivitás (20F ábra), sem a NAD^+ szint növekedéséhez (sem a teljes, sem a mitokondriális poolban) (20G ábra). Az egerekben tapasztaltakhoz hasonlóan a PARP-2 depléciója a SIRT1 mRNS és fehérjeszint és a SIRT1 aktivitás növekedéséhez vezetett (20H ábra), ami arra utalt, hogy a PARP-2 valószínűleg a SIRT1 expressziót szabályozza.



20. ábra. A PARP-2 deléciója a SIRT1 expressziós és aktivitás növekedéséhez vezet
PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek (n=6/6) *m. gastrocnemiusában* meghatároztuk (A) a PARP aktivitást, (B) a NAD^+ koncentrációt, (C) a SIRT1 mRNS és fehérje szintjét, illetve (D-E) a SIRT1 aktivitást. PARP-2 csendesített C2C12 sejtekben meghatároztuk a (F) PARP aktivitást, (G) a totál és a mitokondriális NAD^+ koncentrációját és (H) a SIRT1 fehérje expresszióját és a SIRT1 aktivitást.

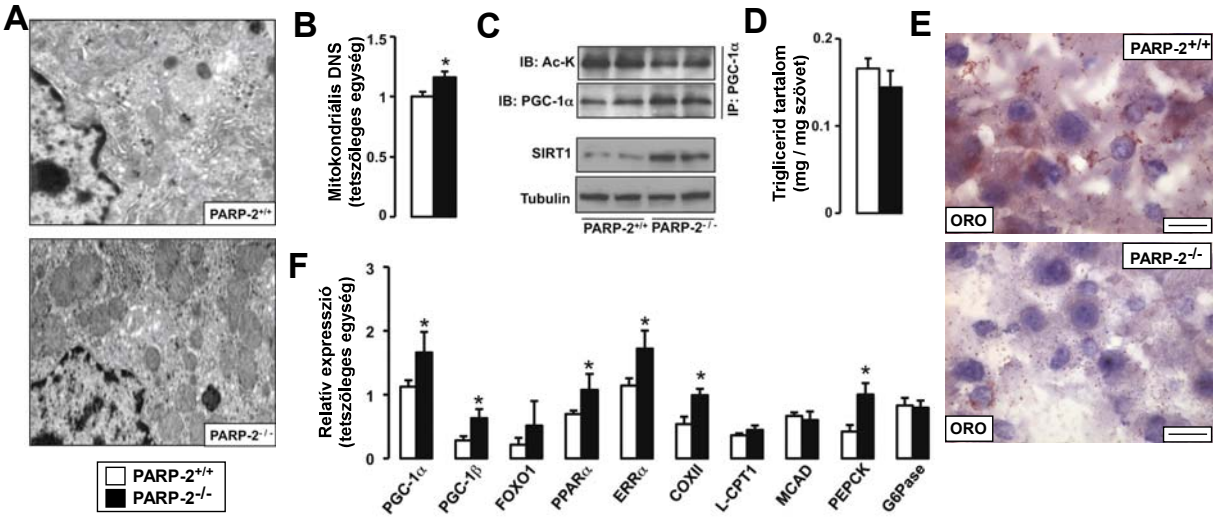
Riporter kísérletekben kimutattuk, hogy a PARP-2 depléciója indukálja a SIRT1 promóterét (21A ábra). A legrövidebb konstrukt (¯1-¯91 régiót tartalmazó) a hosszabbakkal azonos módon válaszol, ami arra utal, hogy ebbe a régióba kötődik a PARP-2. Ezzel a feltételezéssel összhangban kromatin immunprecipitációval kimutattuk, hogy a PARP-2 valóban fizikailag kötődik a humán SIRT1 ¯1-¯91 régiójába (21B ábra). Az NCBI honlapján (http://www.ncbi.nlm.nih.gov/gene) elérhető SIRT1 promóter szekvenciákat összehasonlítva azt tapasztaltuk, hogy a SIRT1 promóter transzkripciós origóhoz közel eső része nagymértékben konzervált az emlősök között, kismértékű homológiát még a *Xenopus* esetében is találtunk (21C ábra). Immunprecipitációs kísérletek nem utaltak a PARP-2 és a SIRT1 közvetlen fizikai kölcsönhatására (21D ábra).



21. ábra A PARP-2 a SIRT1 promóter represszora.
(A) HEK293T sejtekben depletáltuk a PARP-2-t, majd megvizsgáltuk a SIRT1 promóter különböző deléciós mutánsainak (a számok a promóter szakaszok hosszát jelzik ⁻1-től kezdve) aktivitását luciferáz riporter kísérletekben. (B) ChIP kísérletekben a jelzett antitestekkel kromatin darabokat gyűjtöttünk össze és a SIRT1 promóter ⁻1-⁻91 részére specifikus primerek közötti szakaszt felerősítettük qPCR reakcióban, majd a kapott értéket az input-ra normalizáltuk. (C) Az NCBI-ban fellelhető SIRT1 promóter szekvenciák első 300 bázisának szekvenciáját a ClustalW program segítségével összehasonlítottuk. (D) C2C12 sejtekből immunprecipitációt végeztünk el a megjelölt antitestekkel és az összegyűjtött fehérjéket az ábrán jelzett antitestekkel vizsgáltuk.

4.2.4. A PARP-2 deléciója után a májban végbemenő biokémiai változások

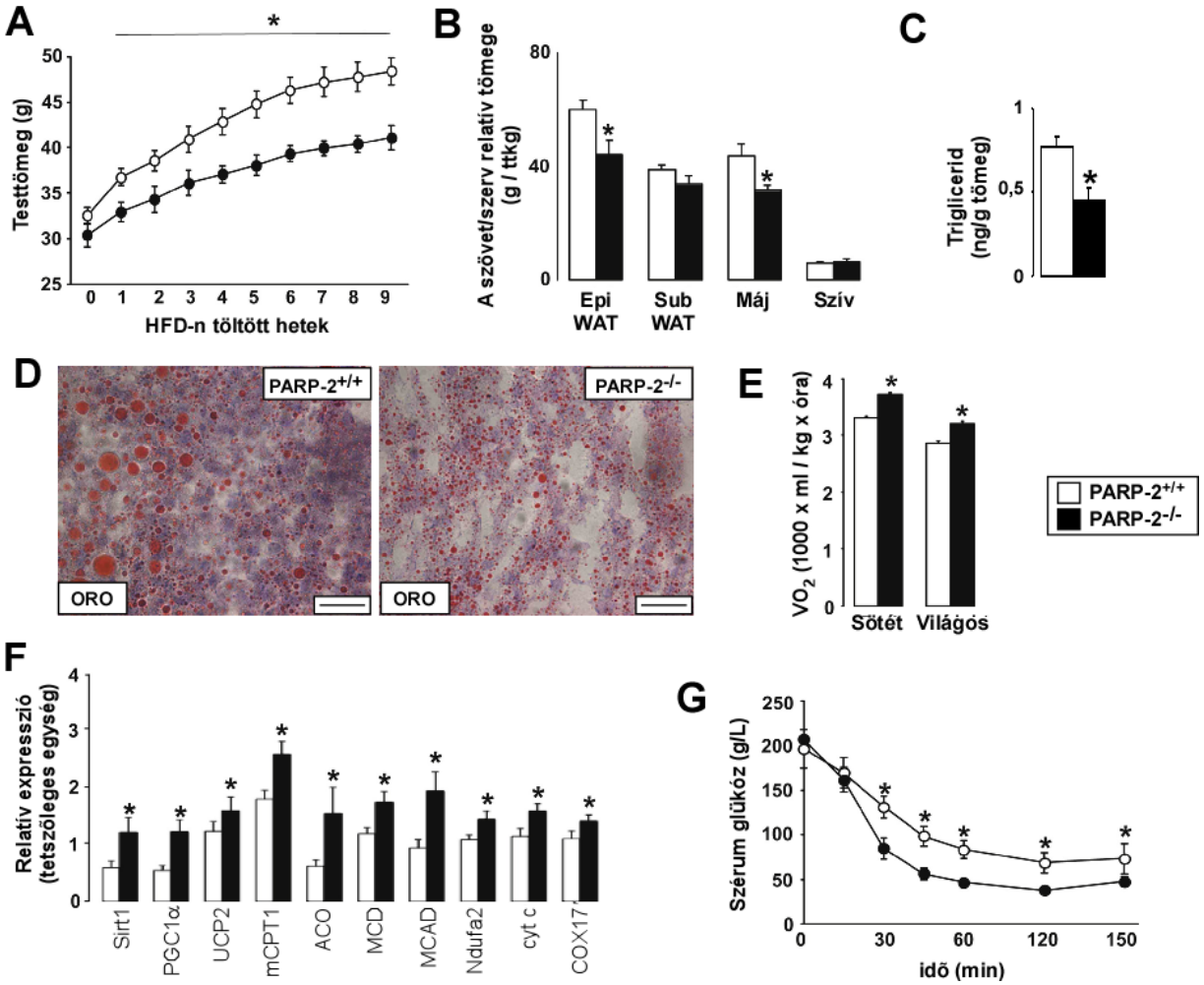
A májban is megvizsgáltuk indukálódik-e a mitokondriális biogenezis. Elektronmikroszkópos vizsgálattal és a mitokondriális DNS mennyiségének meghatározásával kimutattuk, hogy a mitokondriális altivitás megnő a PARP-2$^{-/-}$ egerekben a PARP-2$^{+/+}$ állatokhoz képest (22 A és 22B ábra). A májban azt tapasztaltuk, hogy a SIRT1 fehérjemennyisége és ennek megfelelően a SIRT1 aktivitása is megnő (22C ábra). A PARP-2$^{-/-}$ egerek májában alacsonyabb tárolt triglicerid szintet találtunk, ami megemelkedő energia leadásra utal (22D és 22E ábra). Ezt alátámasztja, hogy a májban megnőtt az oxidatív metabolizmusra jellemző gének expressziója (22F ábra). Úgy tűnik tehát, hogy a PARP-2 deléciója következtében fellépő energialeadás célszerve a harántcsíkolt izom és a máj.



22. ábra. A PARP-2$^{-/-}$ egerek májában megnő a mitokondriális aktivitás
(A-B) PARP-2$^{+/+}$ és PARP-2$^{-/-}$ egerek (n=16/13) májában a mitokondriumok számának változását (A) elektronmikroszkópos vizsgálattal és (B) a mitokondriális DNS mennyiségének a változásával jellemeztük. Ugyanezen egerek májában immunprecipitációs és Western blot kísérletekben meghatároztuk a SIRT1 fehérjemennyiségét és a SIRT1 aktivitását. (D-E) A máj triglicerid tartalmát (D) Floch-extrakciót követően kolorimetriás módszerrel, illetve ORO festéssel határoztuk meg. (F) Ugyanezen egerek májában meghatároztuk több mRNS expresszióját RT-qPCR technikával.

4.2.5. A PARP-2$^{-/-}$ egerek magas zsírtartalmú diétára adott válaszának vizsgálata

A PARP-2 deléciója a metabolikus profil javulásához vezetett, amiből adódott a kérdés, hogy a metabolikus kihívásokra, mint a magas zsírtartalmú diéta, adott választ milyen mértékben befolyásolja a PARP-2 hiánya. Az állatokat magas zsírtartalmú diétával (HFD, 60% hiperkalorikus diéta) etettük kilenc héten át, majd több metabolikus paramétert megvizsgáltunk. A PARP-2$^{-/-}$ állatok testtömege vad típusú társaikhoz képest kevésbé nőtt a kezelés során (23A ábra), ami a fehérzsír depók arányosan kisebb méretével magyarázható (23B ábra). A fehér zsír depók kisebb mérete azonban nem vezetett a májban fokozott lipid lerakódáshoz (23C és 23D ábra), ami a magasabb energia leadásra (23E ábra) vezethető vissza. Ezzel párhuzamosan a harántcsíkolt izomban több, az energia leadáshoz vezető gén expresszió magasabb volt, mint a vad típusú állatokban (23F ábra), ami azt valószínűsíti, hogy ez a szövet – nagy tömege miatt - elsődleges szerepet játszik a szervezet energia leadásban. Ezzel párhuzamosan javult az állatok inzulin szenzitivitása (23G ábra), ami szintén a metabolizmus oxidatív irányba tolódásával magyarázható.



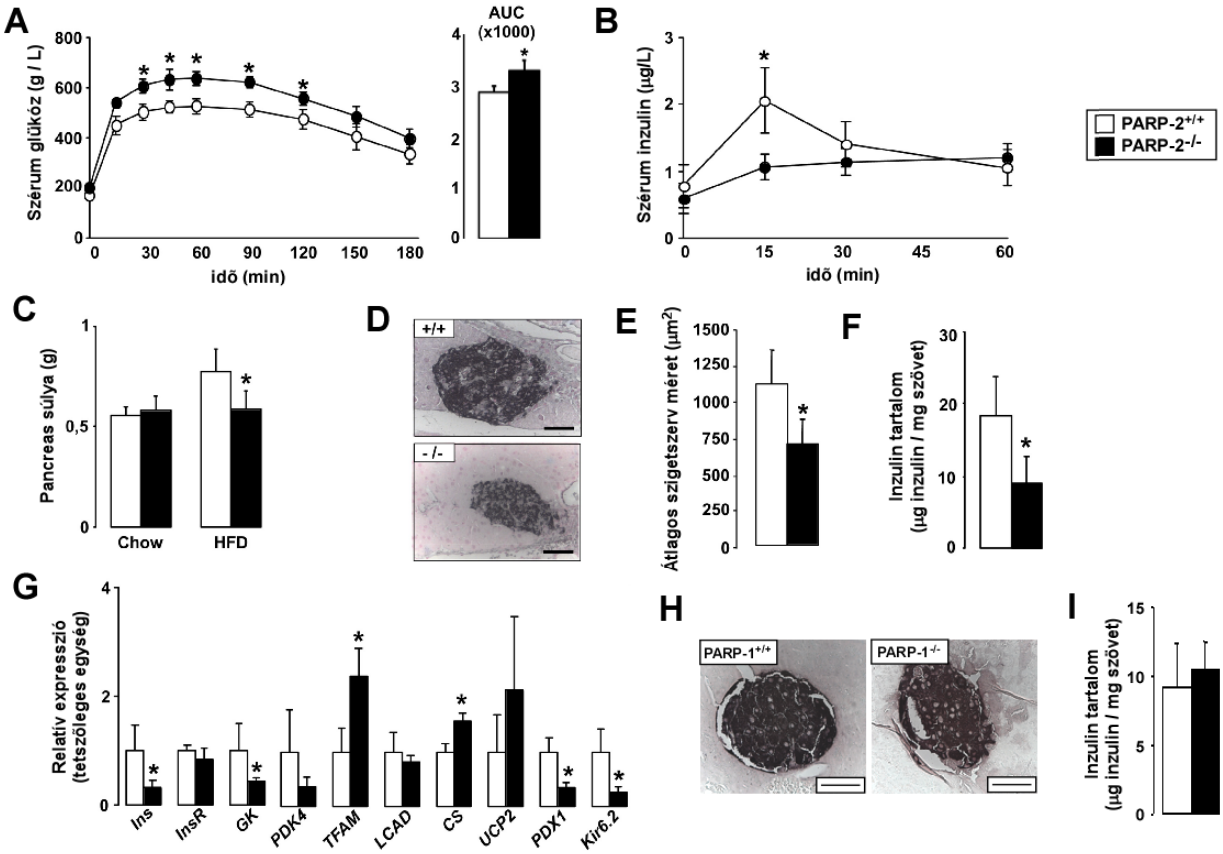
23. ábra. A PARP-2 deléciója védelmet nyújt a fokozott táplálékbevitel indukálta elhízás és inzulin rezisztencia ellen

PARP-2$^{+/+}$ és PARP-2$^{-/-}$ egereket (n=7/9) HFD-vel tápláltuk az (A) panelen jelzett időtartamban. (A) Az egerek testsúlyát minden héten megmértük, majd (B) meghatároztuk több fehérzsír zsírdepó méretét, illetve a máj triglicerid tartalmát egyrészt (C) Floch-extrakciót követően kolorimetriás módszerrel, másrészt (D) szövettani módszerrel ORO festést követően. (E) Indirekt kalorimetriával mértük az egerek oxigén fogyasztását. (F) RT-qPCR technikával megmértük több, a mitokondriális funkcióhoz szükséges gén expresszióját a *m. gastrocnemiusban*, illetve (G) az inzulin szenzitivitásra jellemző ipITT vizsgálatot végeztünk el.

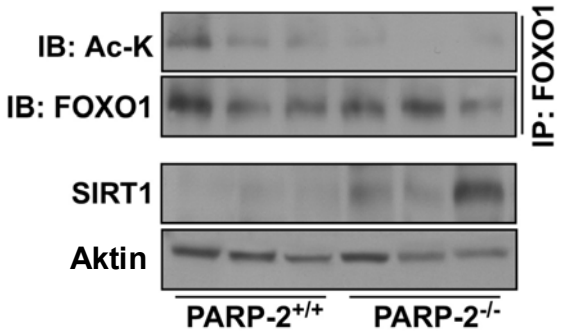
4.2.6. Az endokrin pankreász működésének vizsgálata PARP-2$^{-/-}$ egerekben

Bár a PARP-2$^{-/-}$ egerek inzulinérzékenysége javuló tendenciát mutatott, a glükóz érzékenység azonban meglepő módon romlott (24A ábra), ami a glükóz-indukált inzulin felszabadulás zavarára volt visszavezethető (24B ábra). A PARP-2$^{-/-}$ egerek pankreászában normál (chow) tápon nem tapasztaltunk változást, azonban HFD adása esetében a PARP-2$^{-/-}$ egerek béta-sejtjei nem voltak képesek hiperpláziával kompenzálni az emelkedő inzulin iránti igényt. Vagyis a PARP-2$^{-/-}$ egerek szigetszerve HFD adása esetén nem nőtt meg, amire a

pankreász súlyának változásából, szövettani és biokémiai vizsgálatokból következettünk (24C-G ábra). Fontos megjegyezni, hogy a PARP-1 deléciója nem okozott hasonló változásokat a szigetszerv működésében (24H és 24I ábra).



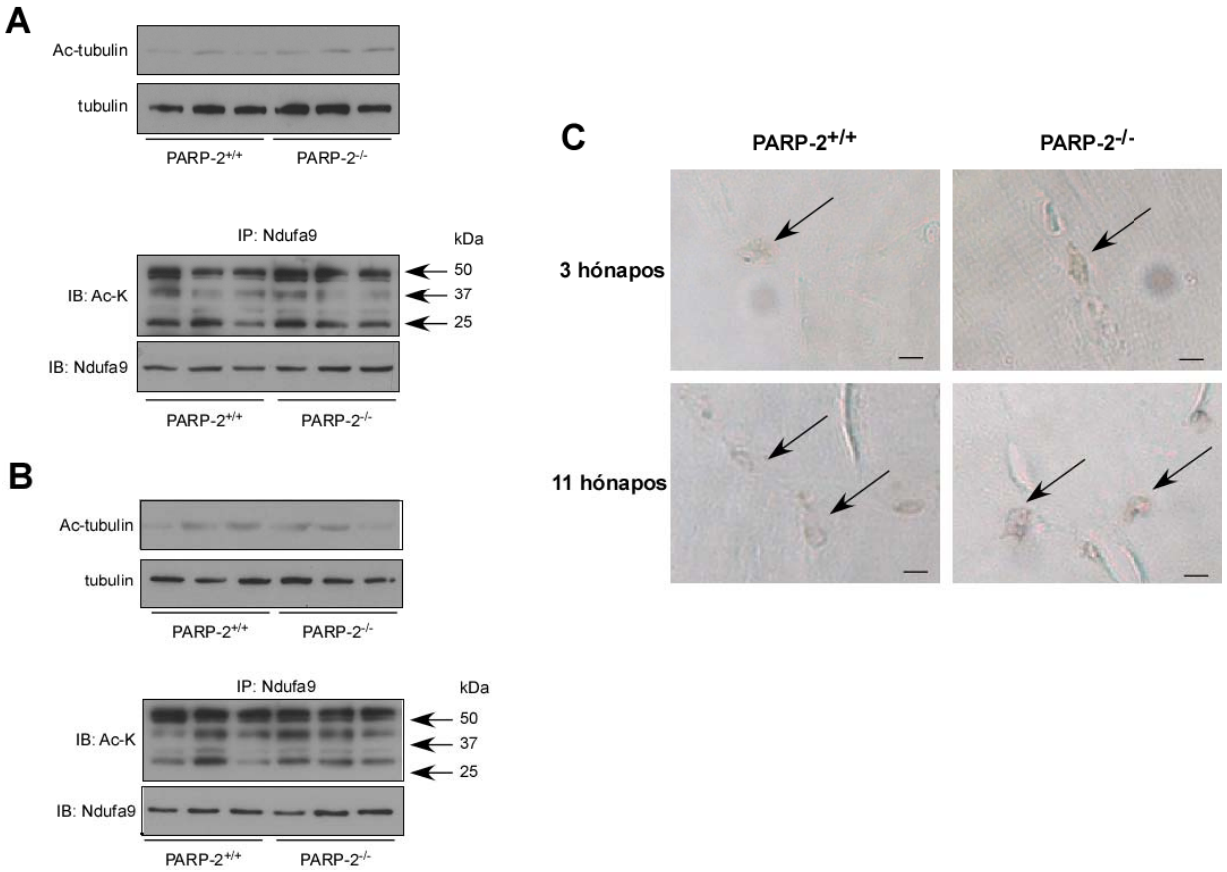
24. ábra A PARP-2 deléciója az endokrin pankreász diszfunkcióját okozza.
(A-B) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egereken (n=7/9) (A) a glükóz érzékenység jellemzésére OGTT vizsgálatot végeztünk el, eközben (B) vért gyűjtöttünk, amiből szérum inzulin szintet határoztunk meg. Meghatároztuk az egerek pankreászának (C) súlyát, (D-E) inzulin immunfestéssel a szigetszerv méretét és morfológiáját (a vonal 50 µm-t jelez), (F) savas extrakció után a pankreász inzulin tartalmát és (G) az inzulin szekréció kulcsgénjeinek az expresszióját. (H-I) PARP-$1^{+/+}$ és PARP-$1^{-/-}$ egerek (n=3/3) pankreászában (H) inzulin immunfestést végeztünk (a vonal 50 µm-t jelez), illetve (I) savas extrakció után meghatároztuk az inzulin tartalmat.



25. ábra. A PARP-2 deléciója a pankreászban a SIRT1 fehérje mennyiségének és a SIRT1 aktivitásának emelkedéséhez vezet

Mivel a feltárt fenotípus abból adódik, hogy a pankreász béta-sejtjei nem képesek a proliferációra, úgy gondoltuk, hogy egy, a béta-sejtek proliferációjához szükséges transzkripciós faktor diszfunkciója lehet a fenotípus oka. A pankreatikus-duodenális homeobox-1 (PDX1) acetilált állapotban működő fehérje, amelynek megfelelő működése elengedhetetlen a béta-sejt proliferációhoz. A PARP-$2^{-/-}$ egerek pancreasában a PDX1 expressziója alacsonyabb volt, mint vad típusú társaikban (24G ábra), míg a SIRT1 mRNS mennyisége és fehérje expressziója magasabb volt (25 ábra). A SIRT1 deacetilálja és aktiválja a FOXO1 enzimet, ami a PDX1 expresszió csökkenéséhez és a hiperplasztikus válasz elmaradásához vezet (Kitamura és mtsai, 2002), ami esetünkben is lehetséges magyarázat.

A PARP-1 knockout egereken végzett kísérletsorozathoz hasonlóan megvizsgáltuk a SIRT2 és SIRT3 aktivitásának változását a PARP-2 deléciója esetén is. Sem a harántcsíkolt izomban (26A ábra), sem a májban (26B ábra) nem változott a SIRT2 és SIRT3 célfehérjék acetiláltsága. Itt szeretném kiemelni, hogy az alkalmazott metabolikus modellekben nem tapasztaltuk a DNS törések akkumulációját a PARP-2 deléciója során (26C ábra). Ehhez hasonlóan a PARP-1 deléciója sem vezetett a DNS törések számának növekedéséhez (nem közölt eredmény).



26. ábra. A PARP-2 deléciója nem befolyásolja a SIRT2 és a SIRT3 aktivitását, illetve nem vezet a DNS hibák akkumulációjához

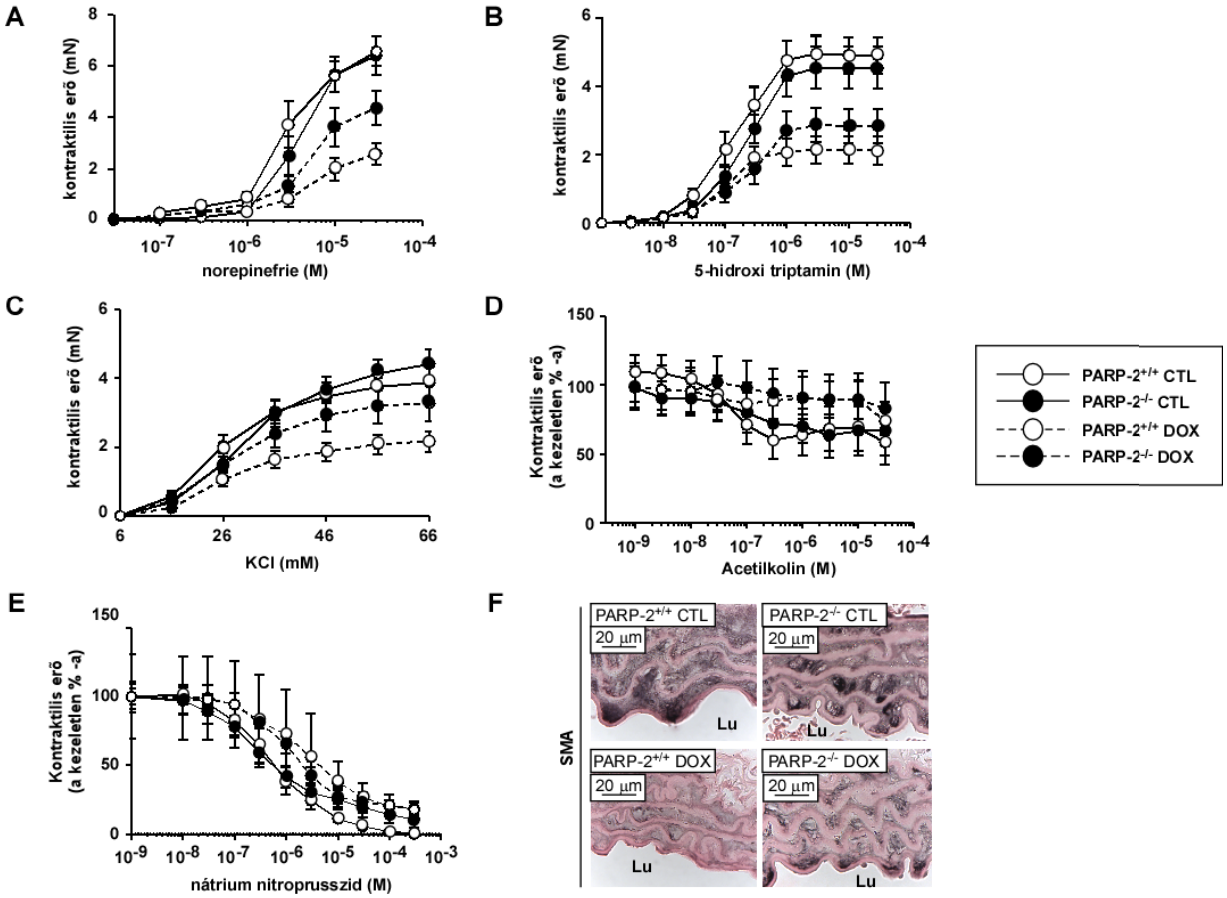
(A-B) Az (A) *m. gastrocnemiusban* és a (B) májban immunprecipitációs kísérletekben vizsgálatuk a SIRT2 szubsztrát tubulin és a SIRT3 szubsztrát Ndufa9 acetiláltságát. (C) A *m. gastrocnemiusban* TUNEL assay-vel vizsgáltuk a DNS törések jelenlétét.

4.3 Képes védelmet nyújtani a PARP-2 delécióját oxidatív károsodás ellen?

A mitokondriális funkció károsodása gyakori kísérőjelensége az oxidatív stresszel jellemezhető betegségeknek (Liaudet és mtsai, 2000, Pacher és mtsai, 2007). Több csoport is kimutatta, hogy a mitokondriális funkció javítása elősegíti a túlélést az oxidatív stresszel kapcsolt betegségekben (Danz és mtsai, 2009, Hasinoff és mtsai, 2003, Tao és mtsai, 2007, Xu és Ashraf, 2002, Yen és mtsai, 1996). Jól ismert jelenség, hogy a PARP-1 delécióját, vagy farmakológiai gátlása véd az oxidatív stresszel jellemezhető patológiás állapotok ellen (Virag és Szabo, 2002). Az előzőekben bemutatott eredményeink arra utaltak, hogy a PARP-2 delécióját, vagy deplécióját felszabadítja a SIRT1 promóterét a represszió alól és a megemelkedő SIRT1 aktivitás a mitokondriális biogenezis indukciójához vezet – vagyis a PARP-2 delécióját védelmet nyújthat az oxidatív stressz-indukált sejtelhalás ellen.

Ezt az elméletet akut doxorubicin (DOX)-indukált vaszkuláris károsodás modellben próbáltuk ki. A doxorubicin egy ciklikus kinon-szemikinon átalakuláson keresztül gyökök képződéséhez vezet, amelyek károsítják – többek között – a kardiovaszkuláris rendszert (Davies és Doroshow, 1986, Doroshow és Davies, 1986). A modellben az egerek egyszeri 25 mg/kg doxorubicin dózist kapnak, ami után pár nappal megjelennek a kardiovaszkuláris rendszer károsodását jelző markerek (Bai és mtsai, 2004, Pacher és mtsai, 2003). Ismert, hogy a doxorubicin kezelés a szívben PARP aktivációt okoz és a PARP-1 delécióját kardioprotektív hatású (Pacher és mtsai, 2002).

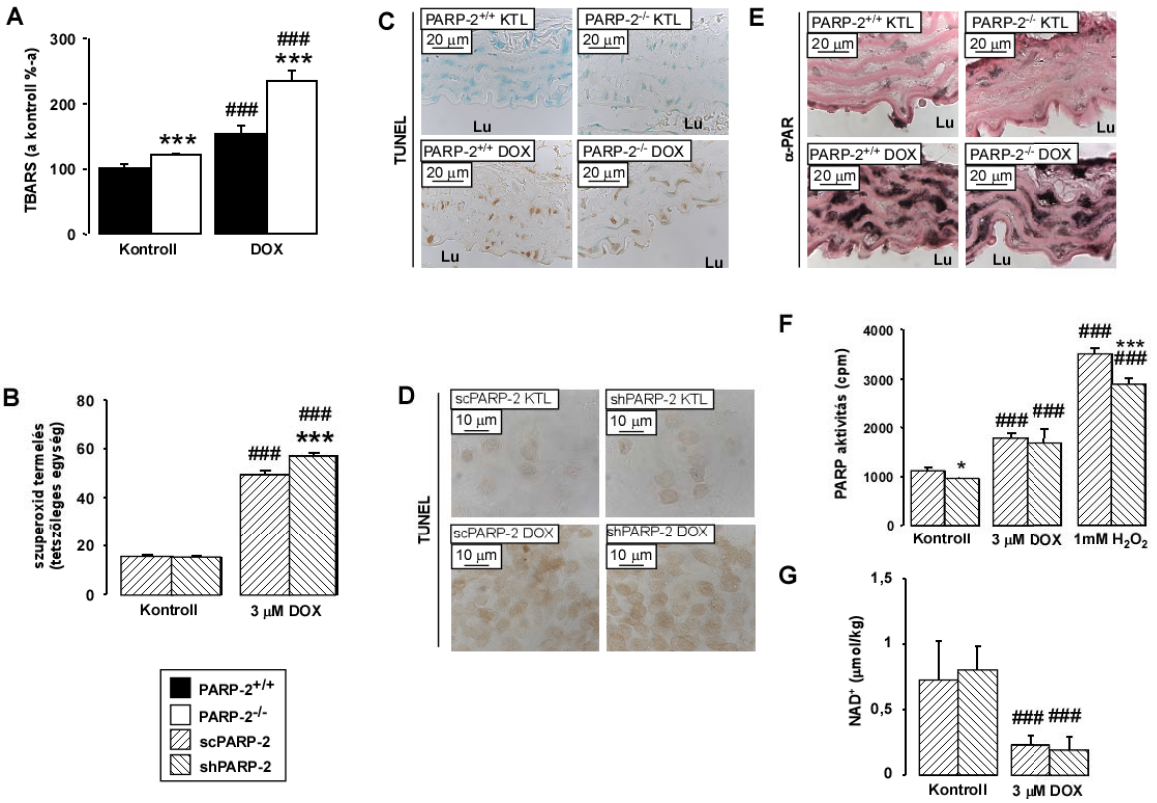
PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egereket kezeltünk 25 mg/kg DOX-szal, vagy vehikulummal (VEH, fiziológiás sóoldat) és a kezelés után két nappal megvizsgáltuk az aorták kontraktilitását norepinefrin, 5-hidroxi-triptamin (szerotonin) és KCl hatására. Bár a vehikulum kezelt PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek ereinek kontraktilitása között nem találtunk különbséget, a DOX kezelés mindhárom vazokonstriktor anyag hatását rontotta, ami ellen a PARP-$2^{-/-}$ egerek részleges védelmet élveztek (27A-C ábra). Nem befolyásolta az acetilkolin vagy külső NO donor által indukált vazorelaxációt a PARP-2 delécióját (27D-E ábra). A KCl koncentráció változtatása a simaizom sejtek depolarizálásán keresztül okoz érösszehúzódást, vagyis a PARP-$2^{-/-}$ egerek részleges védettsége (27C ábra) a simaizom sejtek érintettségére utal. Ezt ellenőrzendő simaizom aktinra (SMA) specifikus antitesttel, ami a simaizom sejtek markere, megfestettük a kísérletben részt vevő állatok aortáját (27F ábra) és azt tapasztaltuk, hogy a simaizom marker jele lecsökken a PARP-$2^{+/+}$ egerek aortájában, ami az előző eredményekkel összhangban a simaizom sejtek pusztulására utal két napos DOX kezelés után, amit a PARP-2 hiánya részlegesen kivéd.



27. ábra. A PARP-2 deléciója védelmet nyújt a DOX vaszkulatúrát károsító hatása ellen
PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egereket kezeltünk vehikulummal, vagy DOX-szal (PARP-$2^{+/+}$ CTL n=10, PARP-$2^{-/-}$ CTL n=7, PARP-$2^{+/+}$ DOX n=8, PARP-$2^{-/-}$ DOX n=8), majd az állatok aortáit vizsgáltuk két nappal a kezelés után. Az érgyűrűk kontraktilis válaszát (A) norepinefrin, (B) 5-hidroxi-triptamin és (C) KCl kumulatív adásával izometrikus kontraktilis erőmérő apparátus segítségével határoztuk meg. A dilattatív válasz vizsgálatához az érgyűrűket norepinefrinnel előfeszítettük, majd emelkedő koncentrációban (D) acetilkolinnal és (E) nátrium-nitroprusszidal kezeltük. (F) Az aortákon formalinos fixálást és paraffinba történő beágyazást követően SMA festést végeztünk. Lu –érlumen

A DOX által termelt gyökök DNS töréseket hoznak létre, ami PARP aktivációhoz vezet és a sejtek NAD^+ és ATP készletét elhasználva sejthalált indukál (Bartha és mtsai, 2011, Mizutani és mtsai, 2005). A PARP-1 deléciója vagy a PARP gátlás a NAD^+ és ATP készlet elhasználását akadályozza meg (Virag és Szabo, 2002). A PARP-2 deléció esetében is felmerül, hogy hasonló módon fejti ki védő hatását. A további kísérleteinket PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egereken, illetve MOVAS (egér aorta simaizom) sejteken végeztük el. A MOVAS sejtekben shRNS technikával stabilan csendesítettük a PARP-2-t (shPARP-2 sejtvonal) és létrehoztunk egy transzdukált, azonban normális PARP-2 szinttel rendelkező sejtvonalat is (scPARP-2). Mind az aortákban, mind a sejtekben a DOX kezelés gyöktermelést okozott (28A-B ábra), ami DNS törésekhez

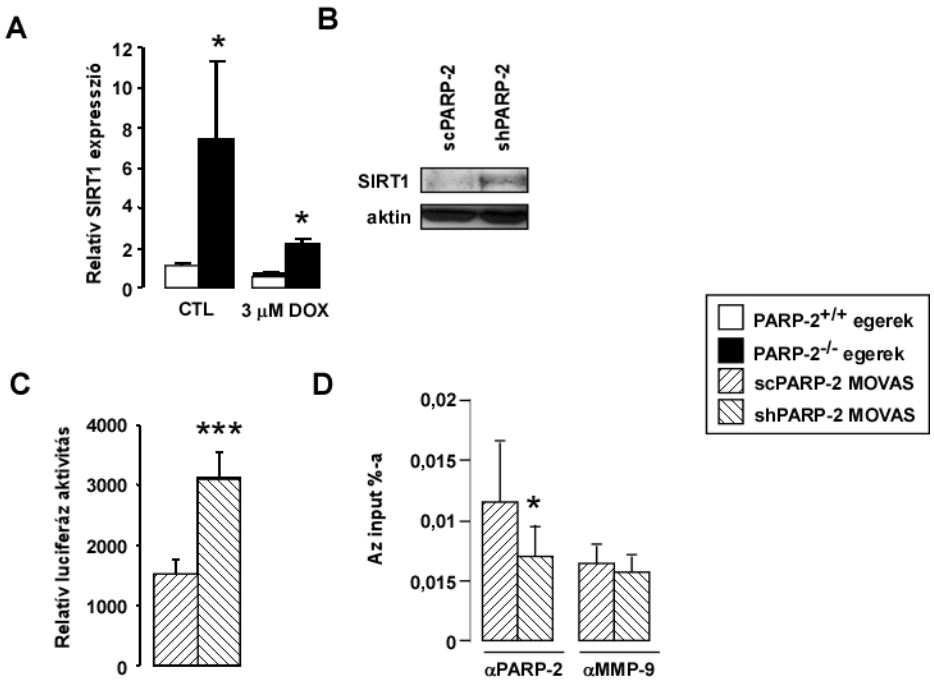
(28C-D ábra), PARP aktivációhoz (28E-F ábra) és a NAD^+ szint radikális csökkenéséhez vezetett (28G ábra). A PARP-2 hiánya nem okozott jelentős különbséget egyik vizsgált paraméterben sem: az alap vagy az indukált (DOX és H_2O_2) PARP aktivitás (28E és 2F ábra), és a NAD^+ szintek nem változtak (28G ábra), ami arra utal, hogy a védelem mechanizmusa eltér a PARP-1 deléciójával elérhető védelemtől.



28. ábra A PARP-2 deléciója nem befolyásolja érdemben a sejtek össz-PARP aktivitását és nem a NAD^+ szint csökkenésének megakadályozásán keresztül fejti ki védő hatását. (A) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek (n=10/7/8/8) aortáiban TBARS assay-vel jellemeztük a szabadgyök termelést. (B) scPARP-2 és shPARP-2 MOVAS sejtekben (n=3/3) hidroetidin festéssel határoztuk meg a DOX által indukált szuperoxid gyök termelést. (C-D) A DNS töréseket TUNEL assay-vel mutattuk ki (C) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerekben, illetve (D) scPARP-2 és shPARP-2 MOVAS sejtekben. (E) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek aortáiban a PARP aktivitást anti-PAR immunhisztokémiával mutattuk ki. (F-G) scPARP-2 és shPARP-2 MOVAS sejtekben (n=3/3) (F) ^{3}H-NAD^+ beépülésével határoztuk meg az össz-PARP aktivitást, illetve (G) kolorimetriás assay-vel a NAD^+ koncentrációt. Lu – érlumen.

Feltételeztük, hogy a PARP-2 deléciója az előzőekben leírtakhoz hasonlóan a SIRT1 indukciójához vezet és ezen keresztül a mitokondriális funkció stabilizálásához. Mind az aortában, mind a MOVAS sejtekben a PARP-2 deléciója a SIRT1 mennyiségének növekedést eredményezett (29A és 29B ábra) hasonlóan az eddig vizsgált modellekhez. A SIRT1

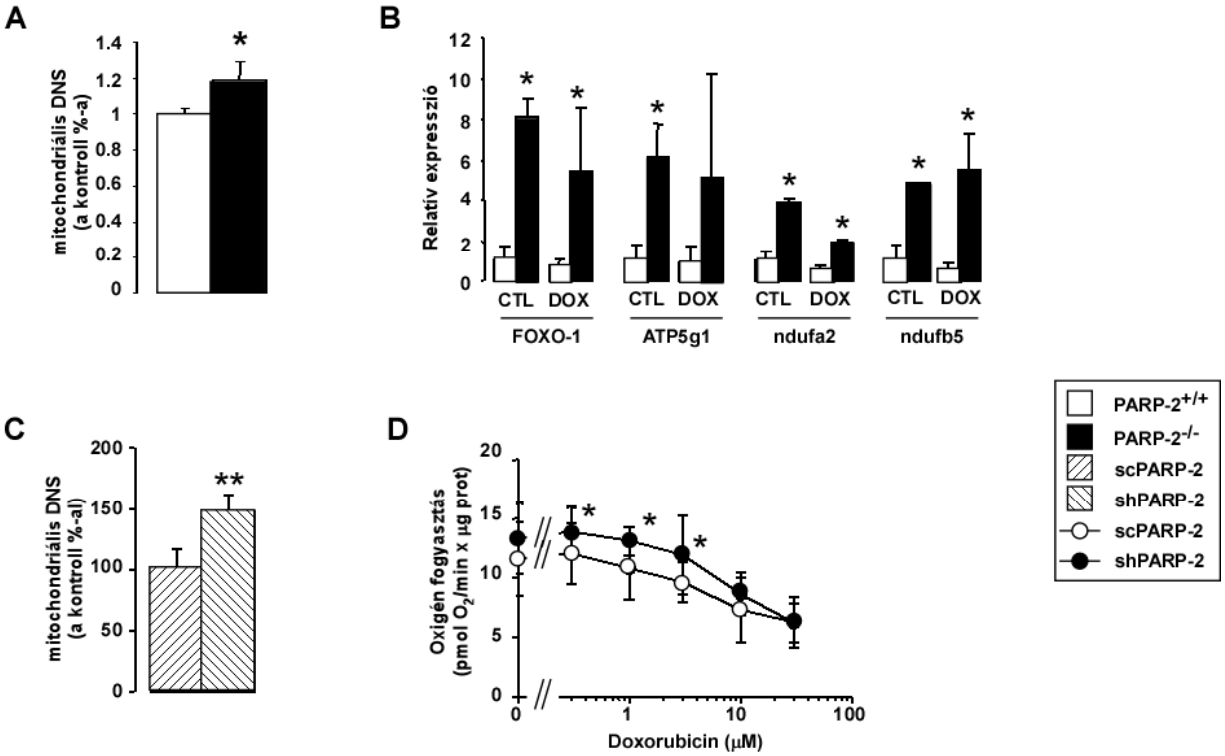
promóterének ¯1-¯91 régióját tartalmazó konstrukt az shPARP-2 MOVAS sejtekben magasabb aktivitást mutatott, mint a kontroll scPARP-2 MOVAS sejtekben (29C ábra). ChIP technika segítségével kimutattuk a PARP-2 jelenlétét a SIRT1 promóterén (29D ábra).



29. ábra A PARP-2 deléciója a simaizomban is a SIRT1 expresszió emelkedését okozza. (A) scPARP-2 és shPARP-2 MOVAS sejtekben (n=3/3) RT-qPCR technikával meghatároztuk a SIRT1 expressziót. (B) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek (n=10/7/8/8) aortájában Western blot segítségével határoztuk meg a SIRT1 fehérje mennyiségét. (C) A SIRT1 promóterének ¯1 - ¯91 darabját tartalmazó luciferáz riporter konstrukt felhasználásával megmértük scPARP-2 és shPARP-2 MOVAS sejtekben a PARP-2 deléciójának hatását a promóter aktivitására. (D) ChIP kísérletekben jellemeztük a PARP-2 jelenlétét a SIRT1 promóter ¯1-¯91 szakaszán scPARP-2 és shPARP-2 MOVAS sejtek (n=3/3) felhasználásával.

A SIRT1 expresszió fokozódása más szervekben (pl. harántcsíkolt izom, máj) a mitokondriális biogenezis fokozódásával járt, ezért megvizsgáltuk az aortában is a mitokondriális aktivitás mértékét. Az aortában fokozódott a mitokondriális DNS mennyisége (30A ábra) és több, a mitokondriális oxidációhoz szükséges gén (FOXO1, ATP5g1, ndufa2, ndufb5) expressziója is (30B ábra). Fontos megjegyezni, hogy bár DOX kezelés hatására csökken a fenti mitokondriális markerek (FOXO1, ATP5g1, ndufa2, ndufb5) expressziója, a PARP-2 deléciója esetén ezeknek a géneknek az expressziója magasabb maradt, mint a DOX kezelt kontroll állatokban mért érték. Hasonló változásokat tapasztaltunk a sejtes modellben is: a PARP-2 csendesítés hatására a mitokondriális DNS mennyisége és a sejtek oxigénfogyasztása (a mitokondriális aktivitás egyik

jellemző markere) megnőtt (30C és 30D ábra). Az állatmodellben tapasztaltakkal megegyezően a PARP-2 deléció által indukált mitokondriális aktivitás DOX kezelés mellett is magasabb volt, mint a kontroll sejtekben.



30. ábra. A PARP-2 delréciója védi a mitokondriumot a DOX kiváltotta károsodással szemben

(A-B) PARP-$2^{+/+}$ és PARP-$2^{-/-}$ egerek aortájában (n=10/7/8/8) meghatároztuk (A) a mitokondriális DNS tartalmat, illetve (B) több, a mitokondriális funkcióhoz szükséges gén expresszióját. (C-D) scPARP-2 és shPARP-2 MOVAS sejtekben (C) megmértük a mitokondriális DNS mennyiségét (n=3/3), míg (D) az XF96 készülék segítségével meghatároztuk a sejtek oxigén fogyasztását (n=46/46).

5. Megbeszélés és perspektívák

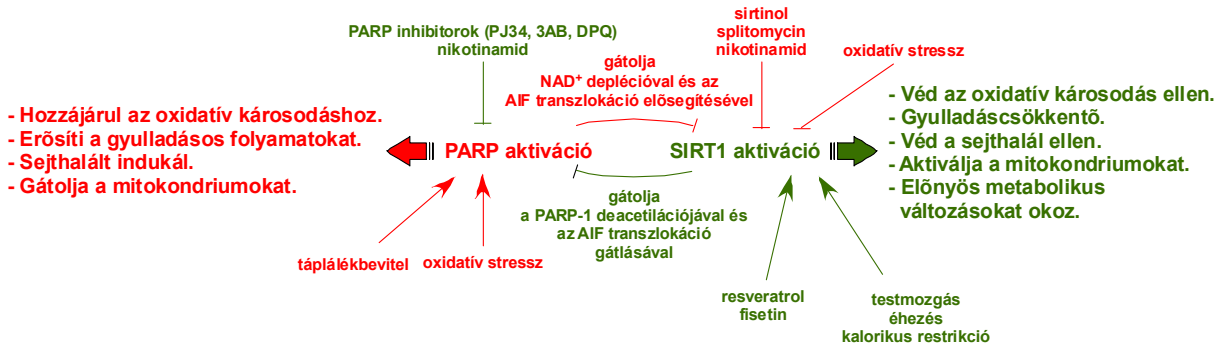
5.1. A PARP-1 és PARP-2 metabolikus szerepének molekuláris mechanizmusa

Munkám során a PARP-1 és PARP-2 enzimek metabolikus szerepét vizsgáltuk. A PARP-1 hiánya előnyös metabolikus fenotípust hoz létre, amit a harántcsíkolt izomban és a BAT-ban megnövekvő energialeadás jellemez. Eredményeink arra utalnak, hogy PARP-1 hiányában a SIRT1 aktivitása megemelkedik. A SIRT1 több fontos transzkripciós faktor deacetilálásával átrendezi a génexpressziót és több mitokondriális gén, mint a mitokondriális oxidáció vagy a zsírsav oxidáció génjei, expresszióját indukálja (Feige és mtsai, 2008, Lagouge és mtsai, 2006). Ezért a SIRT1 diszfunkciója károsítja a mitokondrium működését, amiben központi szerepe van a mitokondriális fehérjék csökkent expressziójának is. A folyamat során a mitokondriális fehérjék sejten belüli aránya lecsökken és több stressz útvonal indukálódik, többek között a mitokondriumból kiinduló, fel nem tekeredett, vagy rosszul feltekeredett fehérjékre adott stresszválasz (mitochondrial unfolded protein response) (Houtkooper és mtsai, 2013, Mouchiroud és mtsai, 2013).

Értekezésemben bemutatott egér és sejtes modellekben a PARP-1 delécióját, vagy PARP gátlószer adása esetében a NAD^+ szint emelkedését tapasztaltuk. Erre a legelfogadhatóbb magyarázatnak az tűnik, hogy a PARP-1 aktivitás (még stimulálatlan állapotban is!) jelentős mértékben használja a sejtek NAD^+ készletét ezért a PARP-1 gátlása jelentős NAD^+ megtakarítással jár. A PARP-1 affinitása a NAD^+ iránt magas (Km = 20-60 μM (Ame és mtsai, 1999)), ami majd egy nagyságrenddel alacsonyabb a sejtek nyugalmi NAD^+ szintjénél (200-500 μM) (Houtkooper és mtsai, 2010). Ez arra utal, hogy a NAD^+ szint fiziológiás fluktuációja, sőt a PARP-1 aktiváció által előidézett NAD^+ szint csökkenés sem képes jelentős mértékben csökkenteni a PARP-1 aktivitását. Könnyen elképzelhető tehát, hogy a PARP-1 aktivitás kiesése jelentős mértékben csökkenti a NAD^+ lebontását, ami a NAD^+ szint emelkedéséhez vezet. A PARP-1 felelős a sejtek, szövetek össz-PARP aktivitásának 85-90%-ért (Schreiber és mtsai, 2002), amit MOVAS és C2C12 sejtekben végzett kísérleteink igazoltak is, így érthető, hogy a PARP gátlószerek alkalmazása a PARP-1 delécióra jellemző fenotípust hoz létre.

A megemelkedő NAD^+ szint aktiválja a SIRT1-et, mivel a SIRT1 érzékeny a NAD^+ szint változásaira, Km értéke (150-200 μM) közel áll a nyugalmi NAD^+ szinthez, azaz a NAD^+ szint fluktuációja befolyásolhatja a SIRT1 aktivitását is. Ez arra is utal, hogy a PARP-1 és a SIRT1 között a molekuláris kapocs a NAD^+ szint változása. A nagyobb affinitású és hatékonyabb PARP-1-gyel versenyzik a kisebb affinitású SIRT1 a közös szubsztrátért, amit alátámaszt az is, hogy minden alkalmazott modellben a NAD^+ szint emelkedését tapasztaltuk függetlenül attól, hogy hogyan gátoltuk a PARP-1-et (molekuláris biológiai, vagy farmakológiai módszerekkel).

Ha megvizsgáljuk, hogy a SIRT1 reciprok módon képes-e befolyásolni a PARP-1 működését, azt tapasztaljuk, hogy a SIRT1 - az előbbiekben említett kinetikai jellemzői miatt - nem képes hatékonyan gátolni a PARP-1 aktivitását a NAD^+ szintek befolyásolásával. Mahesh Gupta csoportja azonban kimutatta, hogy a SIRT1 aktiváció valóban gátolja a PARP-1 aktivitást (és ezen keresztül védelmet nyújt a DOX kardiotoxicitás ellen), de nem a NAD^+ szubsztrát limitálásával, hanem a PARP-1 deacetilálásával éri el gátló hatását (Pillai és mtsai, 2005, Rajamohan és mtsai, 2009). Belátható, hogy a SIRT1 és a PARP-1 kölcsönösen szabályozza egymás működését (31. ábra). Fontos kiemelni, hogy a fiziológiás/patofiziológiás hatások, amelyeket a PARP-1 aktivációhoz köthetőek javarészt átfednek a SIRT1 gátlásához, vagy hiányához köthető fiziológiás/patofiziológiás folyamatokkal, ami arra utal, hogy a két enzim aktivitása közti „egyensúly" megbomlása több betegség kialakulásában is szerepet játszhat.

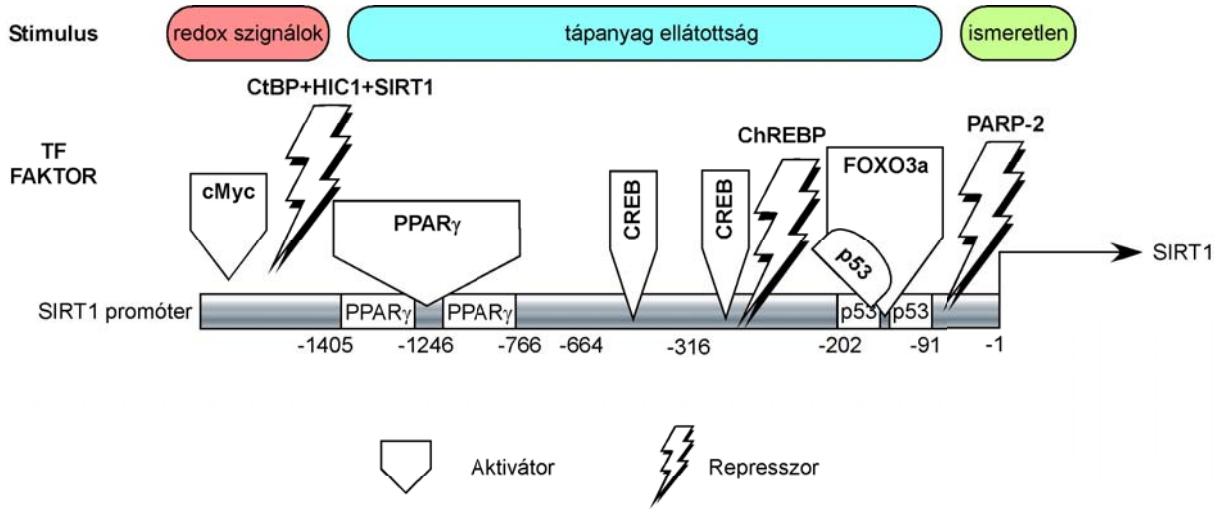


31. ábra. A PARP-1 és a SIRT1 közötti kapcsolat

A PARP-2 a PARP-1-hez hasonlóan NAD^+-függő enzim, így joggal vetődött fel, hogy azonos mechanizmus szerint a PARP-2 deléciója a NAD^+ szinteket képes növelni és ezen keresztül aktiválja a SIRT1-et. A PARP-$2^{-/-}$ egerek szöveteiben és sejtes modellekben a NAD^+ szintet meghatározva azonban diverz képet kaptunk. Ellentétben a PARP-1 aktivitás modulálásával nem minden modellben emelkedett a NAD^+ szint a PARP-2 delécója esetében, ami a NAD^+ szint változásától eltérő mechanizmusra utalt. A PARP-2 kinetikai jellemzői hasonlítanak a SIRT1-re: NAD^+-ra vonatkoztatott $Km_{PARP-1} > Km_{PARP-2} \sim Km_{SIRT1}$ (Ame és mtsai, 1999, Houtkooper és mtsai, 2010), ezért valószínűtlen, hogy PARP-2 aktiváció megfelelő mértékben csökkenti a NAD^+ szubsztrátot a SIRT1 elől, ezért feltehető, hogy a PARP-1 deléció hatásától eltérő molekuláris mechanizmussal állunk szemben.

Minden alkalmazott modellben a PARP-2 delécója esetén a SIRT1 expresszió növekedését tapasztaltuk és kimutattuk, hogy a PARP-2 a SIRT1 promóter represszora. Hiányában a

promóter aktiválódik és a SIRT1 fehérje mennyiségének növekedése vezet a SIRT1 aktivitás indukciójához. A SIRT1 promóterét szabályzó transzkripciós faktorok közül többet ismerünk, mint azt a Bevezetőben tárgyaltuk. A tápanyag ellátottságot integrálják a PPAR-ok (Han és mtsai, 2010), a CREB, a ChREBP (Noriega és mtsai, 2011), a p53 és a FOXO-k (Nemoto és mtsai, 2004), míg a redox környezet jeleit a cMyc (Yuan és mtsai, 2009) és a HIC1 (hypermethylated in cancer-1 (Chen és mtsai, 2005, Zhang és mtsai, 2007)) (32. ábra). A PARP-2 jelenlétét és transzkripciós aktivitását moduláló hatások egyelőre nem ismertek. Feltételezhető, hogy hasonlóan a Myc-hez és a HIC1-hez, a környezet redox állapotát közvetíti. A SIRT1 expressziója, illetve a egyes SNP-k a SIRT1 génben összefüggést mutatnak az energialeadás, az inzulin szenzitivitás (Rutanen és mtsai, 2010), az inzulin szekréció (Dong és mtsai, 2011) és az elhízásra való hajlam mértékével emberben (Clark és mtsai, 2012, Zillikens és mtsai, 2009, Zillikens és mtsai, 2009), vagyis a SIRT1 promóterének aktivitása úgy tűnik fontos faktora a metabolikus adaptációnak, amiben a promótert szabályzó transzkripciós faktoroknak központi szerepe lehet.



32. ábra. A SIRT1 promóter szerkezete a transzkripciós faktorok kötődési helyének megjelenítésével
A számok a humán SIRT1 promóter nukleotid sorrendjének megfelelő helyeket jelzik. Azokat a transzkripciós faktorokat, amelyek kapcsolódási helye nem ismert úgy ábrázoltam, hogy ne kapcsolódjon a DNS szálhoz. Az ismert működő konszenzus szekvenciákat világos téglalapok jelölik a DNS szálban.

A PARP-2 más metabolikus transzkripciós faktorok működésébe is beavatkozik, a fehér zsírszövetben a PARP-2 hiányában az RXR-PPARγ dimer diszfunkcióját tapasztaltuk, ami lipodisztrófiához vezetett. A PPARγ az RXR receptorral heterodimerként kapcsolódik a DNS-hez és így fejti ki a hatását (Fajas és mtsai, 1997). Eredményeink alapján nem lehet egyértelműen

megítélni, hogy a PARP-2 csak a PPARγ-hoz, csak az RXR-hez, vagy a dimer mindkét tagjához kacsolódva fejti-e ki a hatását. Úgy tűnik, hogy a PARP-2 az RXR-PPARγ pozitív kofaktora, a PARP-2 jelenléte szükséges mind a PPARγ alapaktivitásához és ligandfüggő aktivációjához. A rendelkezése álló eredmények azt valószínűsítik, hogy a PARP-2 állandó kofaktora a RXR-PPARγ dimer környezetében kialakuló fehérjekomplexnek. Bár a PARP-2 nem minden magreceptorral képes kölcsönhatásba lépni (pl. az ERβ receptor aktivitását nem befolyásolja a PARP-2 expresszió változása, míg a PPAR-ok, vagy az ERα aktivitását igen), várhatóan más magreceptorok működésébe is beleszólhat a PARP-2, ennek megismerése, azonban további vizsgálatokat igényel.

A PARP-1 több magreceptorral is kölcsönhat, amit a Bevezetés 1. táblázatában összesítettünk. A PARP-1-en belül több magreceptorokkal kölcsönható régiót is leírtak, mint a második cink ujj motívumot (Miyamoto és mtsai, 1999, Pavri és mtsai, 2005) és a BRCT domént (Pavri és mtsai, 2005). A PARP-2 fehérjében található egy nagymértékben konzervált magreceptor aktivációs motívum (LIQLL szekvencia az E doménben). Feltételezhető, hogy a PARP-2 – magreceptor kölcsönhatás során ennek a motívumnak a jelenléte elsődleges fontosságú.

Milyen molekuláris mechanizmuson keresztül aktiválja az RXR-PPARγ dimert a PARP-2? A kromatin immunprecipitációs kísérletek arra utalnak, hogy a PARP-2 transzkripciós események során a DNS-hez kötődik. A PARP-2 több abnormális DNS szerkezethez is kötődhet (Kutuzov és mtsai, 2013), amelyek közül több megjelenik a transzkripció során, mint a DNS kétszálú törése (Ju és mtsai, 2006). Az ER aktivációja során a topoizomeráz II működése kettős szálú DNS töréseket hoz létre a topológiai stressz feloldása során (Ju és mtsai, 2006), amelyek javítása szükséges a hatékony transzkripcióhoz. A kettős törések javításának megindításában központi szerepe van a PARP-1 által katalizált PARilációnak (Ju és mtsai, 2006, Le May és mtsai, 2012). Mivel a PARP-1 és a PARP-2 is szerepet játszik a kettőszálú DNS törések javításában (Huber és mtsai, 2004, Langelier és mtsai, 2012, Schreiber és mtsai, 2002, Yelamos és mtsai, 2008) feltételezhető, hogy a PARP-1-hez hasonló mechanizmussal vesz részt a PARP-2 magreceptorok működésének regulációjában. Ezen felül a PARP-2 a transzkripciós események beindításához valószínűleg az epigenetikai faktorok megváltoztatásán keresztül is hozzájárul. Erre utal, hogy a hiszton PARiláció megváltozik a magreceptor aktiváció során, ami befolyásolja a környezetének hiszton metilációs mintázatát is (Fujiki és mtsai, 2013, Quenet és mtsai, 2009).

A PARP-2 által közvetített represszió molekuláris mechanizmusa valamivel jobban tisztázott, mint az aktiváció mechanizmusa. Quenet és munkatársai (Quenet és mtsai, 2008)

kimutatták, hogy a PARP-2 a kormatin szerkezet zártabbá válását, a heterokromatin megjelenését segíti elő. Liang és munkatársai (Liang és mtsai, 2013) kimutatták, hogy a PARP-2 az általa represszált promótereken az HDAC5 és HDAC7 hiszton-deacetilázok, illetve a G9a hiszton metiltranszferáz akkumulációjához vezet és a represszált kromatinra jellemző acetilációs és metilációs mintázatot hoz létre. Ugyanez a munkacsoport (Liang és mtsai, 2013) kimutatta továbbá, hogy ezen hiszton módosító enzimek akkumulációja független a PARP-2 enzimatikus aktivitásától. Hozzá kell tennem, hogy a PARP-2 hiányában nagyfokú génexpressziós átrendeződést írtak le Farres és munkatársai (Farres és mtsai, 2013). Saját, közlés alatt lévő, microarray kísérleteinkben a PARP-2 depléciója után több, mint 600 gén expressziójának változását tapasztaltuk. Közülük ~450 gén expressziója csökkent, míg ~150 gén expressziója nőtt. Ismert, hogy a PARP-1 nagyon sokrétűen avatkozik be a transzkripciós folyamatokba (Kim és mtsai, 2004, Kraus, 2008, Kraus és Hottiger, 2013, Krishnakumar és mtsai, 2008) és a PARP-2-vel fennálló szerkezeti és funkcionális analógia, illetve az, hogy a PARP-2 delécióját széleskörű génexpressziós változásokat okoz arra utal, hogy a PARP-2-ről a PARP-1-hez hasonló, szerteágazó transzkripciós tulajdonságokat fognak leírni a jövőben.

5.2. PARP-1 és a PARP-2 enzimek szerepe metabolikus szövetekben

A PARP-1, vagy a PARP-2 depléciója a harántcsíkolt izomban izomrost izotípus váltást okozott, az I. típus (lassú), oxidatív rostok aránya megnőtt a SIRT1 aktiváció következtében. Ezek a változások hasonlóak a SIRT1 farmakológiai aktiválásához (Feige és mtsai, 2008, Lagouge és mtsai, 2006). Itt kell, hogy rámutassak, egyedül a harántcsíkolt izomban azonos a két enzim deléciója által kialakított fenotípus.

A BAT-ban a PARP-1 deléciója – a SIRT1 farmakológiai szerekkel történő aktivációjához hasonlóan – megemelte a mitokondriális biogenezist. A BAT, a harántcsíkolt izomhoz hasonlóan, az energialeadás igen fontos szerve (Seale és mtsai, 2009). A BAT-ra jellemző magas mitokondriális aktivitás jelentős szabadgyök képződéssel jár (Barja de, 1992), ami magas PARP aktivitást feltételez ebben a szövetben. Így nem meglepő, hogy a PARP-1 gátlása jelentős NAD^+ szint emelkedéssel jár, ami a SIRT1 aktivációjához vezet. Érdekes módon a PARP-2 deléciója nem fokozza a mitokondriális biogenezist a BAT-ban, ami arra utal, hogy további szövetspecifikus transzkripciós faktorok szabályozzák a SIRT1 expresszióját.

A májban a PARP-2 deléciója a mitokondriális biogenezis indukciójához vezetett és védett a lipid lerakódás ellen mind normál, mind HFD diétán. A PARP-1 deléciója ezzel szemben nem okoz SIRT1 aktivációt a májban, sőt PARP-1 knockout egerekben hepatosteatosis lép fel kalorikus terhelés esetén (Erener és mtsai, 2012) valószínűleg azért, mert a nem indukálódik a

PARP-2 deléciójához hasonló mitokondriális védekezési mechanizmus. Ennek az oka valószínűleg az, hogy a PARP-1 expresszió igen alacsony a májban (~5%-a a harántcsíkolt izomnak, vagy a herének), ami arra utal, hogy a májban a PARP-1 aktivitása kisebb mértékben befolyásolja a NAD^+ szintet, mint az említett szervekben. Vagyis a PARP-1 deléció által okozott NAD^+ szint változás túl kicsi ahhoz, hogy jelentősebben növelje a SIRT1 aktivitását. Más jellegű, hosszú távú hepatotoxikus hatások ellen (mint a tiroid státusz (Cesarone és mtsai, 1994, Cesarone és mtsai, 2000, Merlo és mtsai, 1998, Scarabelli és mtsai, 1998), az alkoholfogyasztás (Nomura és mtsai, 2001, Yang és mtsai, 2004) vagy a diabétesz (Pang és mtsai, 2011)) a PARP-1 hiánya véd, aminek feltételezhetően nem metabolikus háttere van, hanem a sejtpusztulás vagy a gyulladás mértéknek a csökkentésén keresztül éri el a PARP-1 deléciója a védő hatását.

A PARP-2 deléciója a fehér zsírszövet csökkent működéséhez vezet az RXR-PPARγ dimer funkciójának a redukciója miatt. Ugyanakkor ez nem vezet a szérum TG és FFA szint emelkedéséhez valószínűleg a harántcsíkolt izomban történő lipid oxidáció mértékének növekedése miatt.

A PARP-1 fehér zsírszövetben betöltött szerepe több kérdőjelet vet fel. Több szerző is a zsírsejtek differenciációjának a csökkenését tapasztalta (Erener és mtsai, 2012, Janssen és Hilz, 1989, Smulson és mtsai, 1995), saját eredményeink arra utalnak, hogy kalorikus terhelés esetén a PARP-$1^{-/-}$ egerekben kevesebb zsír halmozódik fel. Az általunk alkalmazott knockout egérmodelltől eltérő módon létrehozott és SV129 háttéren tartott PARP-1 knockout egértörzset magas zsírtartalmú diétán tartva azok elhíztak (Devalaraja-Narashimha és Padanilam, 2010, Wang és mtsai, 1995). A különbség oka valószínűleg a genetikai háttér (Champy és mtsai, 2008), ami arra figyelmeztet, hogy a PARP enzimek metabolikus szerepét egyelőre feltáratlan genetikai faktorok is befolyásolják.

A pankreász béta-sejtjei esetében a PARP-1 és a PARP-2 antagonisztikus hatásúak. A PARP-1 hiánya véd az oxidatív stresszel jellemezhető állapotokban bekövetkező béta-sejt pusztulás ellen, mint a streptozotocin indukált diabétesz (Burkart és mtsai, 1999) vagy a részleges pankreász eltávolítás (Yonemura és mtsai, 1984, Yonemura és mtsai, 1988). Sőt a PARP-1 gátlása javítja az inzulin promóter aktivitását is (Ye és mtsai, 2006). Saját adataink arra utalnak, hogy a kalorikus stressz nem befolyásolja a PARP-$1^{-/-}$ egerek béta-sejtjeinek működését, vagyis a PARP-1 elsősorban oxidatív stressz ellen véd.

A PARP-2 deléciója csökkentette a béta-sejtek proliferatív kapacitását, ezért elmaradt a HFD által indukált kompenzatórikus béta-sejt hiperplázia, ami a pankreász csökkent endokrin funkciójához vezetett. A tapasztalt hatásokat a SIRT1 expresszió emelkedését követő FOXO1

aktiváció és a következményes PDX1 expresszió csökkenéshez kötöttük (Kitamura és mtsai, 2002). Mindenképpen váratlan a pankreász hipofunkció a PARP-$2^{-/-}$ egerekben, mivel a SIRT1 overexpresszió a mitokondriális funkció javulásán keresztül a pankreászban az inzulin szekréciót fokozza (Bordone és mtsai, 2006, Moynihan és mtsai, 2005). Magyarázat lehet az ellentmondásra, hogy a két hatás eredője (PDX1 gátlás és a mitokondriális biogenezis indukciója) szabja meg a béta-sejtek viselkedését, amelyek eltérő arányban vannak jelen a két modellben. A PARP-2 delécióját követő kismértékű SIRT1 overexpresszió következtében fellépő FOXO1 deacetiláció (és következményes PDX1 gátlás) túlsúlya miatt a proliferáció gátlás válik dominánssá. Ezzel szemben nagymértékű SIRT1 overexpresszió esetén (a két megjelölt tanulmányban az expresszió ~30-szoros növekedését tapasztalták) kifejezettebbé válik a mitokondriális funkció javulása. Természetesen nem zárható ki, hogy ismeretlen, PARP-2 specifikus útvonalak játszanak szerepet a két egymástó eltérő hatás kialakításában.

5.3. A farmakológiai PARP gátlás következményei

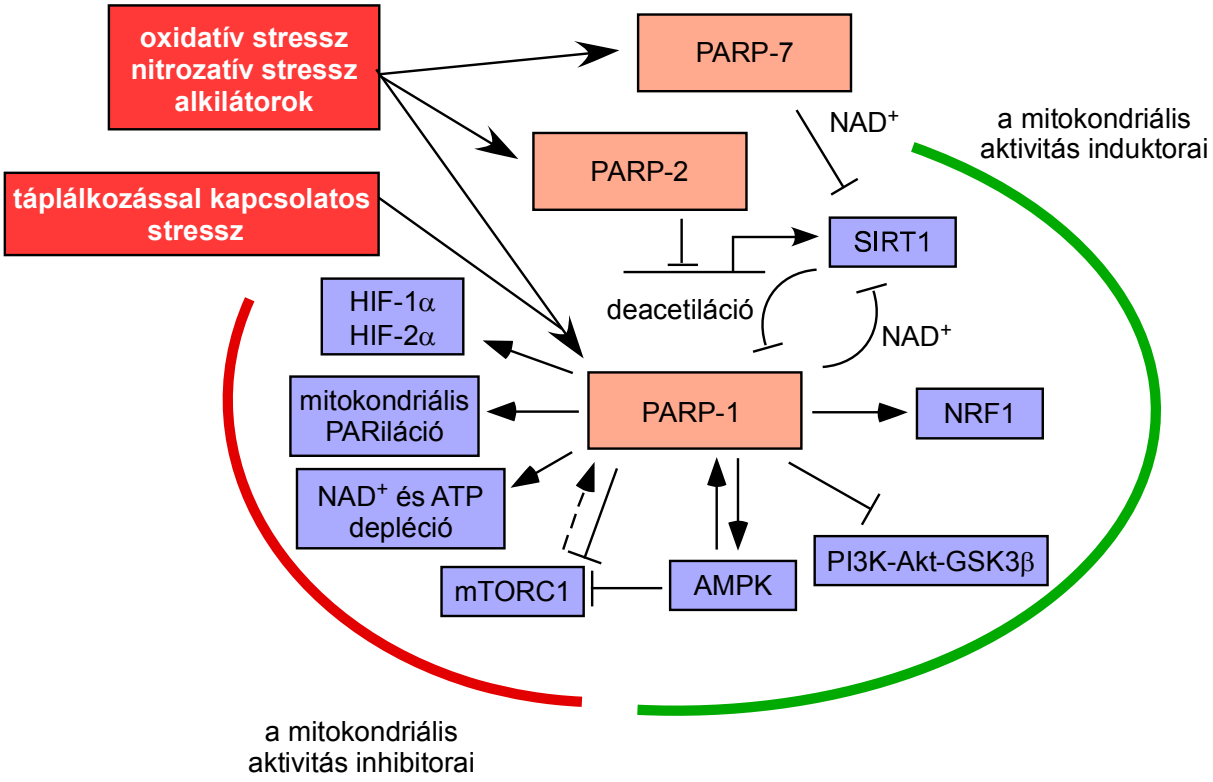
A rövid távú PARP inhibitor kezelés előnyös metabolikus változásokat okozott, ami felveti a PARP inhibitorok ilyen irányú alkalmazhatóságát, azonban kérdésként vetődik fel, hogy milyen mértékben okozunk ezzel genomi instabilitást. Széles körben elfogadott tény, hogy a PARP-1 és a PARP-2 aktivitása szükséges a hatékony DNS hibajavításhoz stressz esetén (Huber és mtsai, 2004, Schreiber és mtsai, 2006), azonban úgy tűnik, hogy a sejtek normál állapotában (külső stresszhatás nélkül) a hatékony DNS hibajavításhoz nem szükséges a két enzim működése (Allinson és mtsai, 2003). Az általunk alkalmazott modellekben nem tapasztaltunk DNS hiba akkumulációt. A metabolikus betegségek azonban megemelkedett oxidatív stresszel jellemezhetőek. Ezért a metabolikus betegségekben a hosszú távú PARP inhibitor kezelés biztonságosságát külön vizsgálni kell.

A jelenleg ismert, illetve kereskedelmi forgalomban lévő PARP inhibitorok nem szelektívek az egyes PARP izoformákra (Wahlberg és mtsai, 2012). Emiatt a PARP inhibitorok hosszú távú alkalmazása során számíthatunk a szövetspecifikus hatások kevert megjelenésére annak ellenére, hogy az általunk elvégzett rövid távú PARP inhibitor kezelés során ezeket nem tapasztaltuk (pl. nem károsodott az endokrin pankreász).

5.4. A PARP enzimek és energiaszenzor útvonalak kölcsönhatásai

PARP-1 az irodalmi adatok alapján olyan jelátviteli útvonalakkal alakít ki kölcsönhatást (33. ábra), amelyek részt vesznek a mitokondriális biogenezis szabályzásában. Jelen munkában a PARP-1 és az AMPK közötti kapcsolatot vizsgáltuk. Mint a Bevezetőben tárgyaltuk, a PARP-1

és az AMPK egymással fizikailag kölcsönhat és képesek egymást kölcsönösen aktiválni (Walker és mtsai, 2006, Zhou és mtsai, 2012). Ennek megfelelően a PARP-1 deléció, illetve a PARP enzimek farmakológiai gátlása esetén több esetben az AMPK aktivitás enyhe csökkenését tapasztaltuk. A továbbiakban vizsgálni kell, hogy milyen körülmények között következik be az AMPK aktivitás csökkenése, mivel egyrészt nem tapasztaltuk minden esetben, másrészt Gongol és munkatársai (Gongol és mtsai, 2013) kimutattak fordított arányosságot is az AMPK és a PARP-1 aktivitás között. A PARP-1 gátlás által indukált AMPK aktivitás változások metabolikus következményei ismeretlenek.



33. ábra. A PARP enzimek és az energiaszenzor útvonalak kölcsönhatásai
A nyilak pozitív, a tompa végek gátló hatást jeleznek. A szaggatott vonal feltételezett hatást jelez.

5.5 A PARP enzimek és a metabolikus betegségek közti összefüggések

Az általunk leírt biokémiai változások több fiziológiai folyamatba vagy betegségek patomechanizmusába is beavatkoznak. Ha az egész egyed szintjén vizsgáljuk a PARP enzimek energiaforgalomra gyakorolt hatását, azt figyelhetjük meg, hogy a PARP-1 vagy a PARP-2 eltávolítása eltolja az energiaegyensúlyt a leadás irányába, ami a mitokondriális biogenezis indukciójához köthető. Másrészt a PARP-1 deléciója megnövelte a táplálékfelvételt, ami a

hipotalamusz érintettségére utal. Ismert, hogy a PARP-1 deléciója esetén szétkapcsol a szervek saját cirkadián ritmusa az állat környezetének cirkadián ritmusától (Asher és mtsai, 2010). A cirkadián ritmus szabályzásában a NAD^+ szintek és ezen keresztül a SIRT1 aktivitás modulálásával történik a metabolikus jelek integrálása (Nakahata és mtsai, 2008, Nakahata és mtsai, 2009). A NAD^+ szintek befolyásolásán keresztül a PARP-1-nek jelentős szerepe lehet a hipotalamusz metabolikus integrátor funkciójának szabályzásában, illetve más perifériális szövet belső cirkadián ritmus a finomhangolásában. A PARP-2 deléció esetében nem tapasztaltunk változásokat a cirkadián ritmus szabályzásában, illetve a táplálékfelvételben.

A PARP-1 és a PARP-2 hiánya csökkenti a fehér zsírszövetben, illetve a PARP-2 hiánya csökkenti a májban történő lipid raktározást. Ennek megfelelően magas zsírtartalmú diétán ezen szövetekben kisebb mértékben növekszik a lipidek mennyisége a knockout egértörzsekben a vad típushoz képest. A nem raktározott lipidek azonban nem dúsulnak fel a szérumban. A szérum triglicerid és szabad zsírsav tartalma a PARP-$1^{-/-}$ és a PARP inhibitor kezelt egerekben alacsonyabb, mint vad típusú, illetve vehikulum kezelt társaikban. A PARP-2 deléciója esetén nem találunk emelkedést a szérum triglicerid és szabad zsírsav tartalmában. A triglicerid és a zsírsav szint emelkedésének elmaradása valószínűleg annak tulajdonítható, hogy a mitokondriális biogenezis indukciója miatt a zsírsavak oxidációja emelkedik. Ezt támasztja alá, hogy a PARP-2 deléciója az RQ érték csökkenéséhez vezet.

A lipid háztartás egy másik ágát, a koleszterin homeosztázist nem vizsgáltuk. Hamid Boulares kutatócsoportja kimutatta, hogy a PARP-1 deléciója, illetve a hosszú távú PARP gátlószer kezelés csökkenti a szérum koleszterin, illetve az LDL szintjét és emeli a HDL szintet atherosclerosis modellekben (Hans és mtsai, 2008, Hans és mtsai, 2009, Hans és mtsai, 2009). A jelenség pontos mechanizmusa nem ismert, azonban eredményeink valószínűsítenek egy útvonalat. A SIRT1 – ami a PARP-1 deléciója, vagy gátlása esetén aktiválódik – csökkenti a koleszterol szintézisét (Defour és mtsai, 2012, Ponugoti és mtsai, 2010, Walker és mtsai, 2010). A SIRT1 deacetilálja és ezzel inaktiválja a koleszterin *de novo* bioszintézisét és transzportját reguláló SREBP fehérjéket csökkentve a koleszterin szintézisét.

A glükóz metabolizmusra kifejtett hatásban jelentős eltérés van a PARP-1 és a PARP-2 között. Az inzulin szenzitivitás javul a PARP-1 vagy a PARP-2 deléciója esetében, ami szintén a harántcsíkolt izomban bekövetkező mitokondriális biogenezis indukcióhoz köthető. A PARP-1 knockout egerek szérum glükózszintje alacsonyabb és az RQ értéke magasabb, mint a vad típusú állatokban, ami fokozott glükóz oxidációra utal. A glükóz eltávolításáért elsősorban a harántcsíkolt izmok felelősek. Ezzel szemben a PARP-$2^{-/-}$ egerekben a szérum glükózszint emelkedik. A PARP-2 deléciója eredményeink szerint csökkenti a pankreász béta-sejtjeinek a

magas zsírtartalmú diétára adott hiperplasztikus válaszát, ami a glükóz-indukált inzulin felszabadulás csökkenéséhez vezet. Emiatt a PARP-2$^{-/-}$ egerek magasabb szérum glükóz koncentrációval jellemezhetőek.

A PARP-1 delációja és a rövid távú PARP inhibitor kezelés javuló metabolikus profilt eredményez ami védelmet nyújt a hiperkalorikus táplálékbevitel által indukált elhízás és az ennek a talaján kialakuló II. típusú diabétesz ellen. Fontos kiemelni, hogy a PARP-1 deléció, vagy a PARP gátlás a diabétesz vaszkuláris diszfunkcióhoz köthető komplikációi (endoteliális diszfunkció, neuropátia, nefropátia és retinopátia) ellen is védelmet nyújt (Minchenko és mtsai, 2003, Obrosova és mtsai, 2004, Soriano és mtsai, 2001, Virag és Szabo, 2002). A PARP-1 deléció vagy a PARP gátlószerek alkalmazása anti-aterogén hatású, ami a PARP-1 gátlás anti-inflammatorikus hatásán kívül metabolikus változásokhoz is köthető (Xu és mtsai, 2013). ApoE$^{-/-}$ környezetben aterogén diéta mellett PARP-1 gátlás hatására csökken az LDL és nő a HDL szint (Hans és mtsai, 2008, Hans és mtsai, 2009, Hans és mtsai, 2009).

A mitokondriális biogenezis csökkenése jellemzi az öregedést (Braidy és mtsai, 2011, Massudi és mtsai, 2012). Az átlagoshoz képest magasabb PARP-1 aktivitás jellemzi az idős embereket (Burkle és mtsai, 1994, Muiras és mtsai, 1998), ami az életkorral megemelkedő mértékű DNS károsodáshoz köthető (Braidy és mtsai, 2011, Massudi és mtsai, 2012). A magasabb PARP-1 aktivitás csökkenti szövetek a NAD^+ szintjét, ami a SIRT1 aktivitás csökkenéséhez vezet (Braidy és mtsai, 2011, Massudi és mtsai, 2012). Felvetődik, hogy a PARP-1 – SIRT1 – NAD^+ tengely egyensúlyának az eltolódása szerepet játszhat mitokondriális aktivitás időskori csökkenésében és ezen keresztül hozzájárulhat több, az időskorra jellemző betegség kialakulásához (pl. diabétesz). Ezt a feltételezést megerősíti a PARP-1 knock-in egértörzs vizsgálata (Mangerich és mtsai, 2010). A PARP-1 extra kópiája bár javította a DNS javítás hatékonyságát, azonban emelte a mitokondriális diszfunkcióval jellemezhető betegségek (pl. diabétesz) incidenciáját (Mangerich és mtsai, 2010). Ezek az eredmények arra utalnak, hogy a PARP-1 – SIRT1 – NAD^+ tengely egyensúlyának visszaállítása alkalmas lehet az egészségben eltöltött idő növelésére, illetve az életminőség javítására (Canto és Auwerx, 2011). A PARP-1 – SIRT1 arány befolyásolása talán a legperspektivikusabb az időskori mitokondriális diszfunkció javítására, mivel idős korban a DNS javítás deficienciával összefüggő malignitások megjelenése és proliferációja jóval lassúbb, mint fiatalkorban, vagyis a PARP inhibitoroknak várhatóan kevesebb mellékhatása lehet.

A PARP-1 fontos proinflammatórikus fehérje. Elsőként a PARP-1 és az NFκB közti kölcsönhatást írták le (Oliver és mtsai, 1999). A közleményben leírt folyamat azt sugallta, hogy a PARP-1 PARilálja az NFκB-t, ami feltétele a gyulladásos citokinek NFκB-n keresztül történő

átírásnak, azonban ezt cáfolták (Hassa és Hottiger, 1999). Hosszú időn át volt a terület megoldatlan ellentmondása, hogy bár a PARP-1 *in vitro* kísérletekben nem módosítja az NFκB-t, a PARP inhibitor kezelés azonban gyulladáscsökkentő hatású. Kauppinen és munkatársai (Kauppinen és mtsai, 2013) kimutatták, hogy a PARP-1 gátlás a NAD^+ szint emelésével a SIRT1 aktivitás emelkedéséhez vezet, a SIRT1 pedig az NFκB p65 alegységének deacetilálásával inaktiválja az NFκB dimert. Ez az egyetlen ismert példája annak, amikor a SIRT1 és PARP-1 kölcsönhatása közvetlenül szabályoz gyulladásos folyamatot, várhatóan más, hasonló útvonalakat is felismerhetnek a közeljövőben – erre utal, hogy a SIRT1 (anti-inflammatórikus hatású) és a PARP-1 (proinflammatórikus hatású) antagonisztikus a gyulladás szabályzásában (Bai és Virag, 2012, Winnik és mtsai, 2012, Xie és mtsai, 2013).

5.6. A PARP enzimek és a sirtuin enzimek kölcsönhatása oxidatív stresszre adott válasz során

Az oxidatív stresszre adott válaszban szintén megfigyelhető a PARP-1 és a SIRT1 közti kölcsönhatás. A PARP-1-et aktiválja az oxidatív stressz, sőt a PARP-1 aktiváció – több útvonalon keresztül – az oxidatív stressz fokozódásához is vezet (Virag és Szabo, 2002). Ezen felül a PARP-1 aktiváció – mint azt a Bevetésben áttekintettük – sejthalál folyamatokat indít be. A SIRT1 aktiváció antagonisztikus hatású a PARP-1 aktivációhoz képest, mert (1) a sejtciklus megállása esetén apoptózist indukál (Brunet és mtsai, 2004, Han és mtsai, 2008, Luo és mtsai, 2001); (2) fokozza több antioxidáns enzimek expresszióját mint a mangán szuperoxid diszmutáz (Danz és mtsai, 2009) vagy a kataláz (Hasegawa és mtsai, 2008); (3) visszaállítja az oxidatív stressz által károsított mitokondriális aktivitást (Danz és mtsai, 2009); (4) illetve autofágiát indukál (Alcendor és mtsai, 2007). Ezen felül a SIRT1 redox szenzitív enzim.

A SIRT1 karbonilációja, illetve a tiol-csoportok redox állapotának a megváltozása gátolja a SIRT1 enzimet (Caito és mtsai, 2010, Caito és mtsai, 2010). Számos esetben tapasztalható a SIRT1 és a PARP-1 kölcsönhatása magas oxidatív stresszel jellemezhető patológiás állapotokban, amelyet az 3. táblázatban foglaltam össze. Ki szeretném emelni, hogy a SIRT1 aktivitás emelése, vagy a PARP aktivitás gátlása több – egyébként nehezen befolyásolható – neurológiai kórképben (pl. Huntington, Alzheimer vagy Parkinson betegség) javította a kísérleti állatok állapotát (Abbott és mtsai, 2003, Chong és mtsai, 2012, Cui és mtsai, 2006, Donmez és Outeiro, 2013, Karuppagounder és mtsai, 2009, Kim és mtsai, 2007, Liu és mtsai, 2009, Virag és Szabo, 2002, Weydt és mtsai, 2006, Zhang és mtsai, 2012), ami ezekben az idősödő társadalmakat egyre jobban érintő betegségcsoportban jó kezelési alternatívát kínál.

3. táblázat. A PARP-1 és a SIRT1 egyensúlyának megbomlásával jellemezhető betegségek

Szervrendszer	Betegség	Kölcsönhatás a SIRT1 és a PARP-1 között	Ref.
Kardiovaszkuláris rendszer	Angiotenzin II-indukált kardiális hipertrófia	A PARP-1 aktiváció gátolja a SIRT1-et a NAD^+ depléciójával, és ezen keresztül hozzájárul a kardiomiociták angiotenzin-II indukált elhalásához.	(Pillai és mtsai, 2006)
	Szívelégtelenség (aorta lekötési modell)	A PARP-1 a NAD^+ szint csökkenésén keresztül gátolja a SIRT1-et és hozzájárul a kardiomiociták elhalásához.	(Pillai és mtsai, 2005)
	Endotél sejteken fellépő nyírási stressz	Az endotél sejteken ható nyíróerők hatására csökken a NAD+ szint és a SIRT1 aktivitás, amit a PARP-1 csendesítése vagy gátlása kivéd.	(Qin és mtsai, 2012)
Központi idegrendszer	Szérum megvonás	A PARP-1 aktiváció gátolja a SIRT1-et a NAD^+ mennyiségének csökkentésével és így hozzájárul a kialakuló neurotoxicitáshoz.	(Sheline és mtsai, 2010)
	Szerzett epilepszia modell, hippokampális primer neuron sejtkultúra	Mg^{2+} megvonás PARP-1 aktivációt okoz, ami csökkenti a NAD^+ koncentrációt és gátolja a SIRT1 aktivitást.	(Wang és mtsai, 2013)
	NMDA neurotoxicitás	Az NMDA receptor aktiváció gátolja a SIRT1 aktivitást, valószínűleg a PARP aktiváción és a NAD+ koncentráció csökkenésen keresztül.	(Liu és mtsai, 2008) (Liu és mtsai, 2009)
Emésztő szervrendszer	Glükóz toxicitás hepatocitákon	A PARP-1 aktiváció gátolja a SIRT1 aktivitását valószínűleg a NAD^+ elhasználásával.	(Pang és mtsai, 2011, Pang és mtsai, 2013)

A PARP-2 az oxidatív stresszre adott válaszban a PARP-1-hez hasonlóan viselkedik, deléciója azonban csak parciális védelmet nyújt oxidatív stresszel jellemezhető betegségekben, mint a cerebrális iszkémia (Kofler és mtsai, 2006, Li és mtsai, 2010) vagy a kolítisz (Popoff és mtsai, 2002). Valószínűtlen, hogy a PARP-1 delécióhoz (Pacher és mtsai, 2002) hasonló lenne a PARP-2 delécióhoz köthető védelem, mivel az irodalmi adatokkal összhangban azt tapasztaltuk, hogy szinte nagyságrendi különbség van a PARP-1 és a PARP-2 aktivitása között akár stimulált, akár stimulálatlan körülmények között (Schreiber és mtsai, 2002, Shieh és mtsai, 1998), illetve doxorubicin kezelés után nem találtunk különbséget a kontroll és PARP-2 csendesített MOVAS sejtek NAD^+ szintjében. A legvalószínűbb magyarázat, hogy a SIRT1 expresszió növekedése a PARP-2 delécióját követően javítja a DOX által indukált mitokondriális diszfunkciót. Ez a felismerés megmagyarázhatja miért nyújt a PARP-2 deléció csak parciális védelmet amint azt más modellekben is megfigyelték (Kofler és mtsai, 2006, Li és mtsai, 2010, Popoff és mtsai, 2002). A SIRT1 promóterében található egyes SNP-k összefüggést mutatnak az oxidatív stresszel jellemezhető Parkinson kór megjelenésének esélyével emberben (Zhang és mtsai, 2012), ami arra utal, hogy az oxidatív stresszel jellemezhető kórképek kialakulásában szerepet játszhat a SIRT1 promóter diszfunkciója, illetve a SIRT1 expresszió modulálása.

Jelenleg több PARP inhibitor van a klinikai kipróbálás különböző fázisában. Különböző tumorok kemo- és radioterápiájában kerülnek ezek a szerek bevezetésre, mint kemo- és radioszenzitizáló szerek (Curtin és Szabo, 2013). Nem célom itt a klinikai vizsgálatok eredményeinek és az egyes tumorfajtáknak az áttekintése, azonban szeretnék egy érdekes koncepciót bemutatni az inhibitorok kemoszenzitizáló tulajdonságával kapcsolatban. A tumorsejteket jellegzetes metabolikus átrendeződések jellemzik, amelyet Warburg hatásként ismerünk (Warburg és mtsai, 1927). A tumorsejtek energiatermelésükben a glikolízisre támaszkodnak, míg a mitokondriális oxidáció limitált, a mitokondriumok több, a tumorsejtek osztódásához elengedhetetlen intermedier szintézisét végzik (Bayley és Devilee, 2012). A mitokondriális oxidáció induktorai (pl. AMPK) csökkentik a mitokondriumokban zajló metabolitok szintézisét ezáltal leállítják a sejtciklust és csökkentik a mitotikus potenciált (Colombo és mtsai, 2011, Faubert és mtsai, 2013, Icard és Lincet, 2012). Eredményeink arra utalnak, hogy a PARP inhibitoroknak is lehet anti-Warburg hatása, mivel indukálják a mitokondriális biogenezist. A PARP inhibitorok anti-Warburg hatása új támadáspontot (a metabolizmus befolyásolása) jelenthet, ami hozzáadódhat a kemo- és radioptencírozó hatásokhoz.

5.7 További kölcsönhatások sirtuin és PARP enzimek között

Feltehetjük azt a kérdést, hogy a SIRT1 enzimen kívül más sirtuin enzimek is lépesek-e kölcsönhatásba lépni a PARP-1 vagy a PARP-2 enzimmel. Kimutattuk, hogy a PARP-1 deléció, vagy a PARP inhibitor adása nem változtatta meg a citoplazmatikus SIRT2 és a mitokondriális SIRT3 aktivitását. Valószínűleg azért nem tapasztaltunk változást, mert a PARP-1 aktivitás gátlása által okozott NAD^+ szint emelkedés a magi NAD^+ kompartmentre korlátozódott és nem befolyásolta az izoláltnak tekintett citoplazmatikus vagy a mitokondriális kompartmentek NAD^+ koncentrációját. A PARP-2 hatása szintén a SIRT1-re korlátozódott, ami a transzkripciós változások specificitására vezethetőek vissza.

A PARP család más enzimeinek is szerepe lehet a metabolikus szabályzásban. Az ismert metabolikus szereppel bíró PARP enzimeket az 4. táblázatban foglaltam össze.

4. táblázat. A metabolikus, vagy feltételezhető szereppel bíró PARP enzimek

PARP enzim	Modell	Metabolizmusra gyakorolt hatás	Ref.
PARP5a (TNK1)	3T3-L1 sejtek Knockout egerek	- A TNK1 kölcsönhat a Glut4 tartalmú vezikulumokkal az IRAP fehérjén keresztül. - A TNK1 delécióját megemeli a harántcsíkolt izmokban az energialeadást, a májban a ketogenezist, a zsírsav oxidációt és a pankreász hiperfunkcióját okozza a béta-sejtek proliferációjának emelésével.	(Chi és Lodish, 2000, Sbodio és mtsai, 2002, Yeh és mtsai, 2007, Yeh és mtsai, 2009)
PARP5b (TNK2)	3T3-L1 sejtek	A TNK2 befolyásolja a Glut4 pozitív vezikulumok transzlokációját.	(Chi és Lodish, 2000, Sbodio és mtsai, 2002)
PARP-7 (TiPARP)	Csirke embrióból származó hepatociták	A PARP-7 aktivációja csökkenti a NAD^+ koncentrációt, ami valószínűleg a SIRT1 aktivációján keresztül a PGC1α hiperacetilációjához vezet.	(Diani-Moore és mtsai, 2010)
PARP-10	Fehérje microarray vizsgálatok	A PARP10 mono-ADP-ribozilálja a GSK3β enzimet.	(Feijs és mtsai, 2013)
PARP-14	HT-1080 humán fibroszarkóma sejtek, Hs68 humán bőr fibroblasztok	Megakadályozza a foszfoglükóz izomeráz ubiquitinilációját és ezzel lebontását.	(Yanagawa és mtsai, 2007)

Bár a PARP enzimek és a metabolikus szabályzó elemek kölcsönhatásának vizsgálata még csak a kezdeti lépéseket jelentik, látható, hogy több, nagy populációt érintő betegség (metabolikus, neurodegeneratív és tumoros megbetegedések) esetében központi szerepük lehet. A betegségek széles köre és az érintett betegek óriási száma, illetve a rövidesen megjelenő, és emberben alkalmazható PARP inhibitorok abba az irányba mutatnak, hogy az általunk bemutatott terület potenciálisan kiaknázható a terápiában.

7. Az értekezésben ismertetett új tudományos eredmények összefoglalása

1. A PARP-1 deléciója vagy gátlása a NAD^+ koncentráció emelésével aktiválja a SIRT1 enzimet, ami a mitokondriális biogenezis emelkedéséhez vezet a barna zsírszövetben és a harántcsíkolt izomban.

2. A PARP-1 deléciója nem befolyásolja a SIRT2 és a SIRT3 enzimek aktivitását valószínűleg azért, mert a PARP-1 deléció csak a magi NAD^+ kompartmentet érinti.

3. A PARP-1 deléciót követő mitokondriális biogenezis emelkedés védelmet nyújt több metabolikus betegséggel szemben, mint az elhízás, vagy a II. típusú diabétesz.

4. A rövid távú PARP gátlás a PARP-1 delécióhoz hasonló előnyös metabolikus változásokat hoz létre a harántcsíkolt izomban.

5. A PARP-2 a SIRT1 promóterének represszora. A PARP-2 deléciója a SIRT1 expresszió és ezen keresztül a SIRT1 aktivitás növekedéséhez vezet.

6. A PARP-2 deléciója és az ennek következtében megnő a SIRT1 aktivitás és mitokondriális biogenezis a harántcsíkolt izomban és a májban, ami az energialeadás irányába tolja el a szervezet energiaháztartását.

7. A PARP-2 deléciója az RXR-PPARγ dimer aktivitásának a gátlásán keresztül csökkenti a fehér zsírszövetben a lipid raktározást egerekben. A PARP-2 deléciója védelmet nyújt az obezitással szemben egér modellben.

8. A PARP-2 deléciója a PDX1 aktivitásának gátlásán keresztül akadályozza a pankreász béta-sejtjeinek a magas zsírtartalmú diétára adott hiperplasztikus válaszát és így glükóz intoleranciához vezet.

9. A PARP-2 deléciója részleges védelmet nyújt az doxorubicin kezelés ellen a SIRT1 indukció és a mitokondriális biogenezis következményes stabilizálódása miatt.

8. Kapcsolódó közlemények

A PhD fokozat megszerzése óta megjelent 22 közlemény (a közlő folyóiratok impakt faktora 139,5)

Ebből az értekezést megalapozó 7 közlemény (a közlő folyóiratok impakt faktora 69,59)

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A disszertációban tárgyalt közlemények

PARP-2 Regulates SIRT1 Expression and Whole-Body Energy Expenditure

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SUMMARY

SIRT1 is a NAD^+-dependent enzyme that affects metabolism by deacetylating key transcriptional regulators of energy expenditure. Here, we tested whether deletion of *PARP-2*, an alternative NAD^+-consuming enzyme, impacts on NAD^+ bioavailability and SIRT1 activity. Our results indicate that PARP-2 deficiency increases SIRT1 activity in cultured myotubes. However, this increase was not due to changes in NAD^+ levels, but to an increase in SIRT1 expression, as PARP-2 acts as a direct negative regulator of the SIRT1 promoter. *PARP-2* deletion in mice increases SIRT1 levels, promotes energy expenditure, and increases mitochondrial content. Furthermore, *PARP-2*$^{-/-}$ mice were protected against diet-induced obesity. Despite being insulin sensitized, *PARP-2*$^{-/-}$ mice were glucose intolerant due to a defective pancreatic function. Hence, while inhibition of PARP activity promotes oxidative metabolism through SIRT1 activation, the use of PARP inhibitors for metabolic purposes will require further understanding of the specific functions of different PARP family members.

INTRODUCTION

SIRT1 has recently emerged as a promising target in the battle against a number of metabolic disorders, including obesity and type 2 diabetes (Banks et al., 2008; Pfluger et al., 2008; Yu and Auwerx, 2010). The ability of NAD^+ levels to control SIRT1 activity gave rise to the hypothesis that artificial modulation of NAD^+ levels could be used to regulate SIRT1 (Houtkooper et al., 2010; Sauve, 2009). Indeed, most physiological situations where NAD^+ is increased correlate with higher SIRT1 activity (Cantó et al., 2009; Houtkooper et al., 2010; Sauve, 2009). One interesting possibility to artificially modulate NAD^+ levels relies on the inhibition of alternative NAD^+ consumers, such as the poly(ADP-ribose) polymerases (PARPs) (Houtkooper et al., 2010). Confirming this hypothesis, a number of different labs have shown that the activity of the PARP-1 enzyme, a major cellular NAD^+ consumer, and SIRT1 are interrelated due to the competition for the same limiting intracellular NAD^+ pool (Kolthur-Seetharam et al., 2006; Pillai et al., 2005; Rajamohan et al., 2009). It is, however, not known whether other PARP family members have similar effects on SIRT1 activity and global metabolism.

The poly(ADP-ribose) polymerase-2 (PARP-2) is a 66.2 kDa nuclear protein with PARP activity capable of binding to aberrant DNA forms (Amé et al., 1999). DNA binding of PARP-2 results in its activation, and PARP-2 subsequently catalyzes the formation of poly(ADP-ribose) polymers (PAR) onto itself and to different acceptor proteins (Amé et al., 1999; Haenni et al., 2008; Yélamos et al., 2008). PARP-2 has a catalytic domain (amino acids 202–593) structurally similar to PARP-1 (Oliver et al., 2004), the founding member of the PARP family. However, while it is known that PARP-1 activity critically influences NAD^+ bioavailability (Sims et al., 1981), the possible effects of a secondary PARP activity, like PARP-2, on intracellular NAD^+ levels and global metabolism in cells or organs has not yet been fully determined.

Previous studies already provide evidence that PARP-2 is involved in metabolic homeostasis, as it regulates the peroxisome proliferator-activated receptor γ (PPARγ), therefore influencing adipocyte differentiation (Bai et al., 2007). The potential relevance of PARP-2 for NAD^+ homeostasis, which would impact on SIRT1 activity and global metabolism, prompted us hence to fully examine the metabolic phenotype of germline *PARP-2*$^{-/-}$ mice. We show here how the absence of PARP-2 activates SIRT1 and promotes mitochondrial biogenesis in muscle. However, our data also reveal that the absence of PARP-2 leads to pancreatic failure upon high-fat feeding, underscoring the possibility of developing drugs that selectively inhibit specific PARP proteins for metabolic indications.

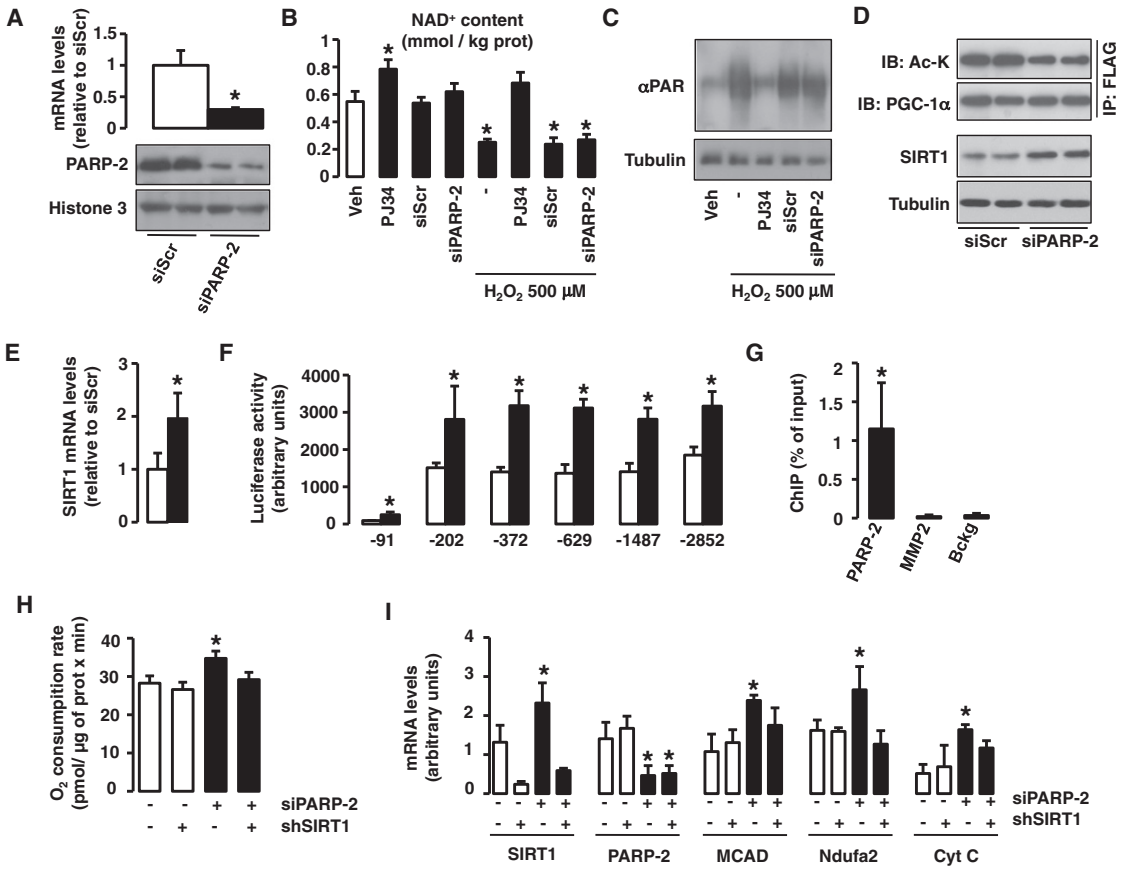

Figure 1. PARP-2 Regulates Oxidative Metabolism by Acting as a Transcriptional Repressor of SIRT1

(A) PARP-2 protein and mRNA levels were analyzed in C2C12 myotubes carrying a stably transfected scramble or PARP-2 shRNA.

(B) NAD^+ content was evaluated in C2C12 myotubes treated with PJ34 (24 hr, 1 mM) or carrying a stable transfection of a scramble or a PARP-2 shRNA. H_2O_2 treatment was performed for 1 hr.

(C) Total protein extracts from C2C12 myotubes treated as in (B) were used to test total PARylation.

(D) Scramble or PARP-2 shRNA were stably transfected in C2C12 myotubes that were infected with FLAG-PGC-1α. After 48 hr, total protein extracts were obtained and used for FLAG immunoprecipitation and to test the markers indicated.

(E) SIRT1 mRNA levels were analyzed in C2C12 myotubes carrying a stable transfection with either scramble or a PARP-2 siRNA.

(F) The activity of nested deletions of the SIRT1 promoter was measured after PARP-2 depletion in C2C12 cells.

(G) The presence of PARP-2 on the SIRT1 (−1 through −91) promoter was assessed in C2C12 cells by ChIP assays, using MMP2 as negative control.

(H and I) O_2 consumption (H) and mRNA levels of the markers indicated (I) were measured in C2C12 myotubes carrying a stable transfection with either a scramble (−) or a PARP-2 (+) shRNA and infected with adenovirus encoding for either a scramble (−) or a SIRT1 (+) shRNA. Unless otherwise indicated, white bars represent scramble shRNA-transfected myotubes and black bars represent PARP-2 shRNA-transfected myotubes. All results are expressed as mean ± SD. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

RESULTS

PARP-2 Regulates Oxidative Metabolism by Repressing SIRT1 Transcription

In order to examine the potential role of PARP-2 as a regulator of SIRT1 activity, we generated C2C12 myocytes stably transfected with either a scrambled or a PARP-2 shRNA. PARP-2 mRNA and protein content is reduced by 80% in myotubes from cells carrying the PARP-2 shRNA (Figure 1A). We next evaluated whether this deficiency in PARP-2 activity affects NAD^+ homeostasis. While inhibition of total PARP activity with the inhibitor PJ34 leads to increased intracellular NAD^+ content, a reduction in PARP-2 by itself did not affect total (Figure 1B) or mitochondrial (Figure S1A) NAD^+ levels. Similarly, knocking down PARP-2 did not prevent H_2O_2-induced NAD^+ depletion, while global inhibition of PARP activity with PJ34 did (Figure 1B). To further sustain our observations, we analyzed the impact of the PARP-2 knockdown on global PARP activity by checking H_2O_2-induced protein PARylation. While PJ34 completely reversed H_2O_2-induced PARylation, the knockdown of PARP-2 could not prevent protein hyperPARylation (Figure 1C). These results confirm that PARP-2 is a secondary PARP activity in the cell, as previously demonstrated (Amé et al., 1999; Shieh et al., 1998). Furthermore, it also suggests that PARP-2 depletion has little impact on NAD^+ homeostasis.

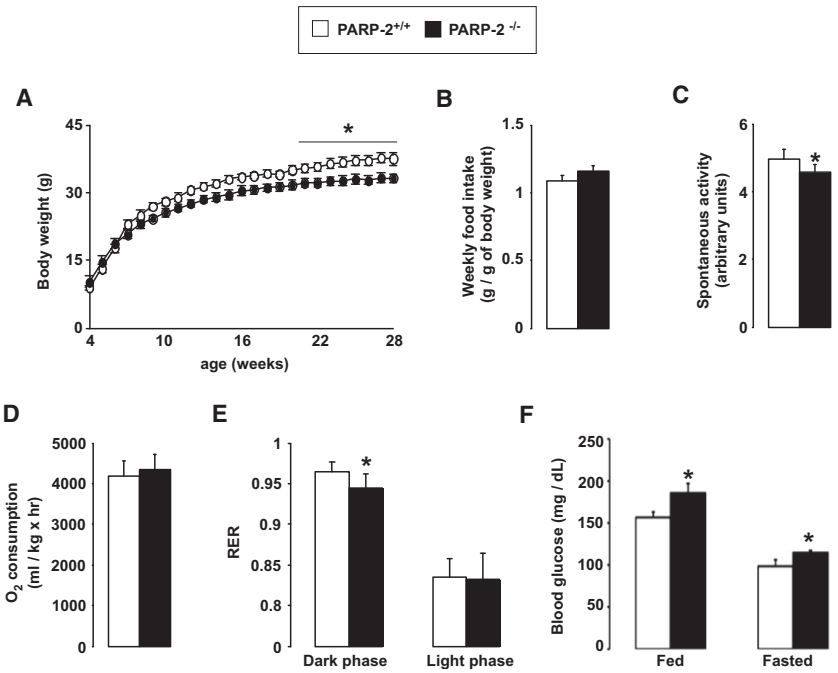

Figure 2. General Physiologic Characteristics of *PARP-2*$^{-/-}$ Mice

(A and B) *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n = 15/13) were weighed weekly (A), and food consumption was measured (B).

(C–E) *PARP-2*$^{+/+}$ and $^{-/-}$ male mice on a chow diet (n = 6/6, age of 3 months) were subjected to indirect calorimetry, where locomotor activity (C), O_2 consumption (D), and RER (E) were determined.

(F) Fed and fasted blood glucose levels. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

Given the absence of an impact on NAD^+ homeostasis, it was surprising to observe that myotubes in which PARP-2 had been knocked down displayed higher SIRT1 activity, as demonstrated by reduced PGC-1α acetylation (Figure 1D, top panels). We could not find any direct interaction between PARP-2 and SIRT1 (Figure S1B), indicating that changes in SIRT1 activity are not likely to happen through direct posttranslational modification by PARP-2. Rather, the increase in SIRT1 activity was linked to increased SIRT1 content (Figure 1D, bottom panels). The increase in SIRT1 protein was concomitant to an increase in SIRT1 mRNA levels (Figure 1E). To explore why SIRT1 mRNA levels were increased by transcriptional induction, we used a reporter construct in which serial deletions of the mouse SIRT1 promoter region controlled luciferase expression (Figure 1F). These studies demonstrated that knocking down PARP-2 promoted a ~2-fold increase in SIRT1 transcription through the very proximal promoter region (−91 bp), an effect that was maintained for the whole upstream regulatory region that was analyzed (Figure 1F). In chromatin immunoprecipitation (ChIP) assays, PARP-2 was shown to bind directly to the proximal SIRT1 promoter (region between the transcription start site and −91 bp) in C2C12 myotubes (Figure 1G). The direct binding of PARP-2 on the SIRT1 promoter was also observed in a nonmurine cell line, like 293HEK cells (Figure S1C), as this proximal −91 bp region is extremely conserved along evolution (Figure S1D). All these results suggest that PARP-2 acts as a direct negative regulator of the SIRT1 promoter. Consequently, a reduction of PARP-2 levels induces SIRT1 transcription, leading to higher SIRT1 protein levels and activity. An expected consequence of this increase in SIRT1 activity is that a reduction in PARP-2 content should lead to higher mitochondrial gene expression, by the activation of PGC-1α through deacetylation, and to increased O_2 consumption. This hypothesis turned out to be correct, as cellular O_2 consumption was increased in PARP-2 knockdown cells (Figure 1H), concomitant to the increase in expression of genes related to lipid and mitochondrial metabolism, such as *medium chain acyl coenzyme A dehydrogenase* (*MCAD*), *NADH dehydrogenase* (*Ubiquinone*) *1 alpha subcomplex subunit 2* (*Ndufa2*), and *cytochrome C* (*Cyt*) (Figure 1I). Furthermore, using adenoviruses encoding for a shRNA for SIRT1, we demonstrated that the increase in SIRT1 activity contributed in a major fashion to the oxidative phenotype of PARP-2-deficient myotubes (Figures 1H and 1I).

General Physiological Characterization of the *PARP-2*$^{-/-}$ Mice

All the experiments above illustrate that reducing PARP-2 activity might be useful to increase SIRT1 activity and, consequently, potentiate oxidative metabolism. In order to gain further insight into this mechanism, we next examined the metabolic profile of the *PARP-2*$^{-/-}$ mice. *PARP-2*$^{-/-}$ mice were smaller and leaner then their *PARP-2*$^{+/+}$ littermates (Figure 2A). The fact that there was no difference in food intake between the *PARP-2*$^{-/-}$ and *PARP-2*$^{+/+}$ mice (Figure 2B) and that spontaneous locomotor activity was lower in the *PARP-2*$^{-/-}$ mice (Figure 2C) suggested that the difference in weight gain was due to altered energy expenditure (EE). Indirect calorimetry, however, indicated only a slight tendency toward a higher O_2 consumption in chow-fed *PARP-2*$^{-/-}$ mice compared to wild-type littermates under basal conditions (Figure 2D). Interestingly, RQ values indicate that during the dark phase, *PARP-2*$^{-/-}$ mice use lipid substrates as energy source at proportionally higher rates than the *PARP-2*$^{+/+}$ littermates (Figure 2E). Strikingly, *PARP-2*$^{-/-}$ mice were mildly hyperglycemic in both fed and fasted states (Figure 2F), linked to a tendency toward lower blood insulin levels in both fed (2.52 ± 0.24 μg/l for *PARP-2*$^{+/+}$ versus 1.77 ± 0.13 μg/l for *PARP-2*$^{-/-}$ mice) and fasted (0.77 ± 0.07 μg/l for *PARP-2*$^{+/+}$ versus 0.71 ± 0.11 μg/l for *PARP-2*$^{-/-}$ mice) states. Overall, these results illustrate that *PARP-2* deletion promotes an increase in the use of fat as main energy source, associated with a leaner phenotype.

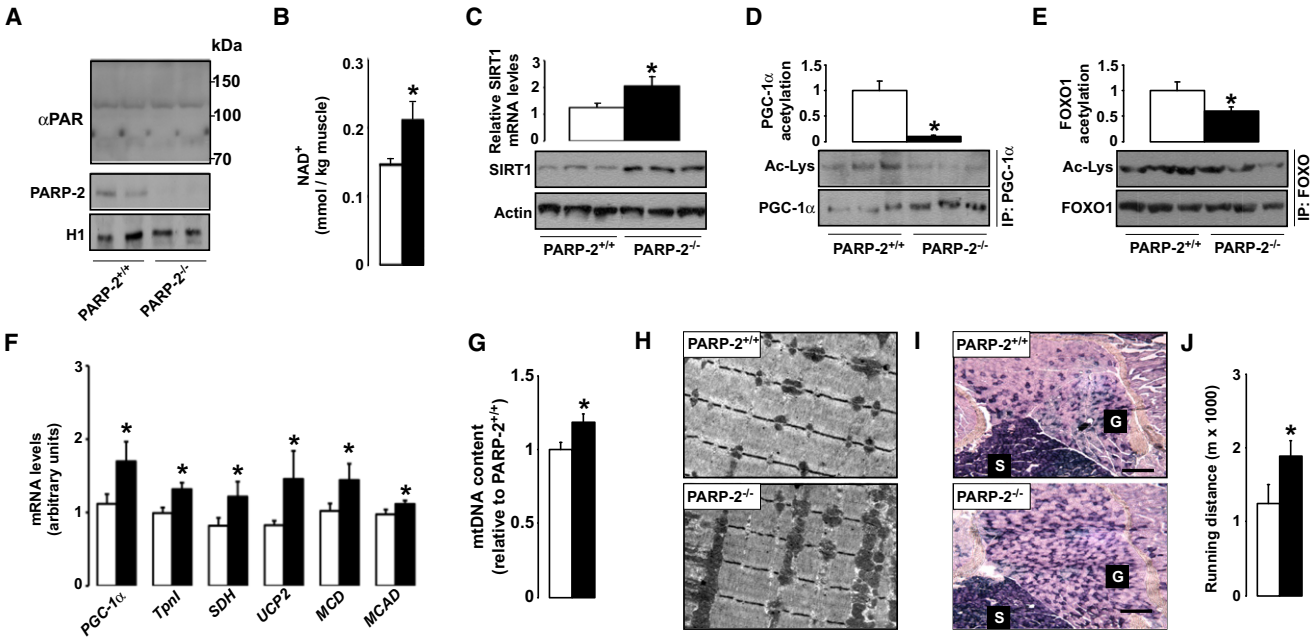

Figure 3. *PARP-2*$^{-/-}$ Muscles Have Higher SIRT1 Activity, Mitochondrial Content, and Oxidative Profile

(A) PARylation and PARP-2 levels in gastrocnemius muscle were determined by western blot. PARP-2 levels were determined in nuclear extracts, and histone 1 (H1) was used as loading control.

(B) NAD^+ levels in gastrocnemius muscle of 4-month-old *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n = 4 and 8, respectively) were determined by HPLC/MS.

(C) SIRT1 mRNA and protein levels were determined in total muscle mRNA or protein extracts.

(D and E) PGC-1α (D) and FOXO1 (E) acetylation lysine levels were examined after immunoprecipitation. Quantifications are shown on top of the respective images.

(F) Gene expression of the indicated genes in the gastrocnemius muscle of *PARP-2*$^{+/+}$ and $^{-/-}$ mice was evaluated by RT-qPCR.

(G) Quantification of mitochondrial DNA by qPCR.

(H and I) Transmission electron micrographs (H) and SDH staining (I) of representative gastrocnemius muscle sections show increased mitochondrial content (*PARP-2*$^{+/+}$ and $^{-/-}$ male mice n = 15 and 13, respectively; age of 7 months). Scale bar in (I) = 100 μm.

(J) Endurance treadmill test was performed as described. White bars represent *PARP-2*$^{+/+}$ mice, while black bars represent *PARP-2*$^{-/-}$ mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

Higher SIRT1 Activity, Mitochondrial Content, and Oxidative Profile in *PARP-2*$^{-/-}$ Tissues

At the molecular level, *PARP-2* deletion was not linked to higher DNA damage in either young or old mice (Figure S2A). In line with these in vitro data, we could not detect a significant change in protein PARylation in *PARP-2*$^{-/-}$ mice, as determined by western blotting (Figure 3A). In contrast to the data from C2C12 myotubes, *PARP-2*$^{-/-}$ muscles contained more NAD^+ (Figure 3B). The data from cultured myotubes suggest that the increase in NAD^+ levels observed in muscle tissue might be secondary to the leaner phenotype rather than a direct consequence of the reduction in PARP-2 function per se. In line with the role of PARP-2 as a negative regulator of the *SIRT1* promoter, *SIRT1* mRNA and protein levels were increased in muscles from *PARP-2*$^{-/-}$ mice (Figure 3C). The combination of higher NAD^+ and higher SIRT1 protein provides an excellent scenario to increase SIRT1 activity. Confirming this hypothesis, the acetylation levels of two different SIRT1 substrates, the PPARγ coactivator-1α (PGC-1α) (Figure 3D) and the forkhead box O1 (FOXO1) transcription factor (Figure 3E), were markedly decreased in muscles from *PARP-2*$^{-/-}$ mice. Importantly, the acetylation status of SIRT2 and SIRT3 targets, such as tubulin and Ndufa9, respectively, was not affected by *PARP-2* deletion, indicating that *PARP-2*$^{-/-}$ deletion is not affecting the activity of the closest SIRT1 homologs (Figure S2B). PGC-1α and FOXO1 are transcriptional activators strongly linked to the regulation of mitochondrial biogenesis and oxidative metabolism. Consequent to their activation through deacetylation, the expression of transcriptional regulators of oxidative metabolism (*PGC-1α*), of biomarkers of oxidative muscle fibers (*troponin I* [*tpnI*]), and of mitochondrial proteins (*succinate dehydrogenase* [SDH], *uncoupling protein 2* [*UCP2*]) and lipid oxidation enzymes (*malonyl-CoA decarboxylase* [*MCD*], *MCAD*) was increased in gastrocnemius muscle of the *PARP-2*$^{-/-}$ mice (Figure 3F). The increase in mitochondrial content was further evidenced by the higher mitochondrial DNA content (Figure 3G) and by the more prominent mitochondria observed upon transmission electron microscopy (TEM) analysis of the gastrocnemius muscle (Figure 3H). The increased mitochondrial biogenesis clearly promoted a more oxidative phenotype of the *PARP-2*$^{-/-}$ muscles, as reflected by the prominent increase in SDH-positive oxidative muscle fibers (Figure 3I). As a physiological consequence of this increased oxidative muscle profile, *PARP-2*$^{-/-}$ mice performed much better than their *PARP-2*$^{+/+}$ littermates

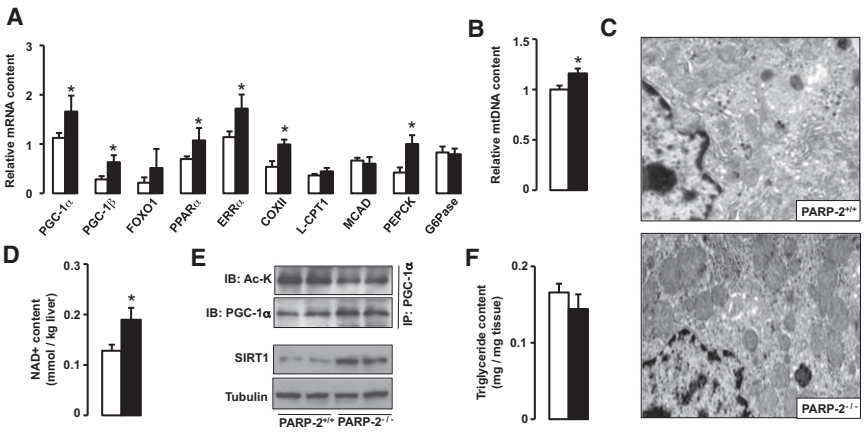

Figure 4. PARP-2$^{-/-}$ Mice Display Higher Mitochondrial Content in Liver

(A) mRNA expression analysis in livers from *PARP-2*$^{+/+}$ and $^{-/-}$ male (n = 16/13, respectively; 6 months of age) mice fed a chow diet.

(B) Relative liver mitochondrial DNA (mtDNA) content was estimated by RT-qPCR.

(C) Transmission electron microscopic images of liver sections demonstrate higher mitochondrial number in *PARP-2*$^{-/-}$ mice.

(D) Total intrahepatic NAD^+ content was measured by HPLC/MS.

(E) Total liver protein extracts were used to evaluate SIRT1 protein levels and immunoprecipitate PGC-1α to examine PGC-1α acetylation levels.

(F) Liver triglyceride content was estimated after methanol/chloroform lipid extraction as described.

White bars represent *PARP-2*$^{+/+}$ mice, while black bars represent *PARP-2*$^{-/-}$ mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

on a treadmill endurance test (Figure 3J). As a whole, these results indicate that *PARP-2* deletion promotes mitochondrial biogenesis in muscle, increasing the oxidative and endurance profile of the fibers.

We also explored whether *PARP-2* deletion could also influence mitochondrial biogenesis in other highly metabolic tissues, such as brown adipose tissue (BAT) and liver. In BAT, despite higher SIRT1 content (Figure S3A), we were unable to detect changes in the expression of the main metabolic genes (Figure S3B). Supporting the minor impact of *PARP-2* deletion on BAT function, body temperature dropped similarly in *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice upon cold exposure (Figure S3C). This suggested that BAT is unlikely to contribute significantly to the differences in EE observed in the *PARP-2*$^{-/-}$ mice. In contrast, *PARP-2* deletion had strong effects on the expression of diverse regulators of mitochondrial metabolism in the liver, including *PGC-1α*, *PGC-1β*, *FOXO1*, *PPARα*, *estrogen-related receptor α* (*ERRα*), and *Cytochrome C oxidase subunit II* (*COXII*) (Figure 4A). Consistently, *PARP-2*$^{-/-}$ livers displayed a higher mitochondrial content, as evidenced by the increase in mitochondrial DNA levels (Figure 4B) and by the appearance of more mitochondria upon electron microscopy (Figure 4C). As in muscle, liver NAD^+ content was higher in *PARP-2*$^{-/-}$ mice (Figure 4D), which, together with the higher amounts of SIRT1 protein, translated into increased SIRT1 activation (Figure 4E). In line with what was observed in muscle, no changes in the activity of SIRT2 and SIRT3, the closest SIRT1 homologs, were detected (Figure S4A). The observation that *PARP-2*$^{-/-}$ livers had a tendency toward a reduced triglyceride content both upon oil red O staining (Figure S4B) and direct biochemical measurement (Figure 4F) is consistent with the induction of oxidative metabolism. Despite the increase in *phosphoenolpyruvate carboxykinase* (*PEPCK*) expression in *PARP-2*$^{-/-}$ mice and the increased capacity of liver for oxidative metabolism, *PARP-2*$^{-/-}$ mice responded similarly to *PARP-2*$^{+/+}$ littermates upon a pyruvate tolerance test (Figure S4C), probably due to the similar expression of another rate-limiting enzyme, the glucose-6-phosphatase (G6Pase) (Figure 4A).

PARP-2$^{-/-}$ Mice Are Protected against Diet-Induced Obesity and Insulin Resistance

The increased mitochondrial biogenesis and oxidative phenotype observed in the skeletal muscle and liver of *PARP-2*$^{-/-}$ mice incited us to test how these mice would respond to high-fat diet (HFD) feeding. *PARP-2*$^{-/-}$ mice were protected against weight gain when fed a HFD (Figure 5A), despite a similar food intake (Figure 5B). This leaner phenotype was associated with a reduced body fat mass, as evidenced by EchoMRI analysis (Figure 5C). This reduction in fat content was clearly more pronounced (~20% decrease) in the epididymal fat depots, which are equivalent to visceral fat in man, than in the subcutaneous fat pads (Figure 5D). The weight of the *PARP-2*$^{-/-}$ livers was also markedly reduced (Figure 5D), consequent to a lower triglyceride accumulation (Figures S5A and S5B). Accentuating what was observed in chow-fed mice, *PARP-2*$^{-/-}$ mice on HFD displayed now significantly higher O_2 consumption rates (Figure 5E). The increase in VO_2 was not due to increased activity (Figure 5F), indicating that high-fat-fed *PARP-2*$^{-/-}$ mice have higher basal EE. As expected, the expression of the transcriptional regulators governing EE (*SIRT1*, *PGC-1α*) was increased in gastrocnemius from *PARP-2*$^{-/-}$ mice when compared to their *PARP-2*$^{+/+}$ littermates after the HFD (Figure 5G). The expression of several genes involved in fatty acid uptake and oxidation (*muscle carnitine palmitoyltransferase 1b* [*mCPT1b*], *peroxisomal acyl-coenzyme A oxidase 1* [*ACOX1*], *MCD*, *MCAD*), mitochondrial electron transport, and oxidative phosphorylation (*Ndufa2*, *Cyt C*, *COXIV*) followed a pattern similar to these transcriptional regulators and were maintained at a higher level in the *PARP-2*$^{-/-}$ muscle (Figure 5G). Consequent to the much leaner and oxidative phenotype, *PARP-2*$^{-/-}$ mice remained more insulin sensitive than their wild-type littermates after high-fat feeding (Figure 5H), and their endurance performance was markedly better (data not shown). These results clearly indicate that *PARP-2*$^{-/-}$ mice are protected from body weight gain and insulin resistance upon high-fat feeding, linked to a better muscle oxidative phenotype.

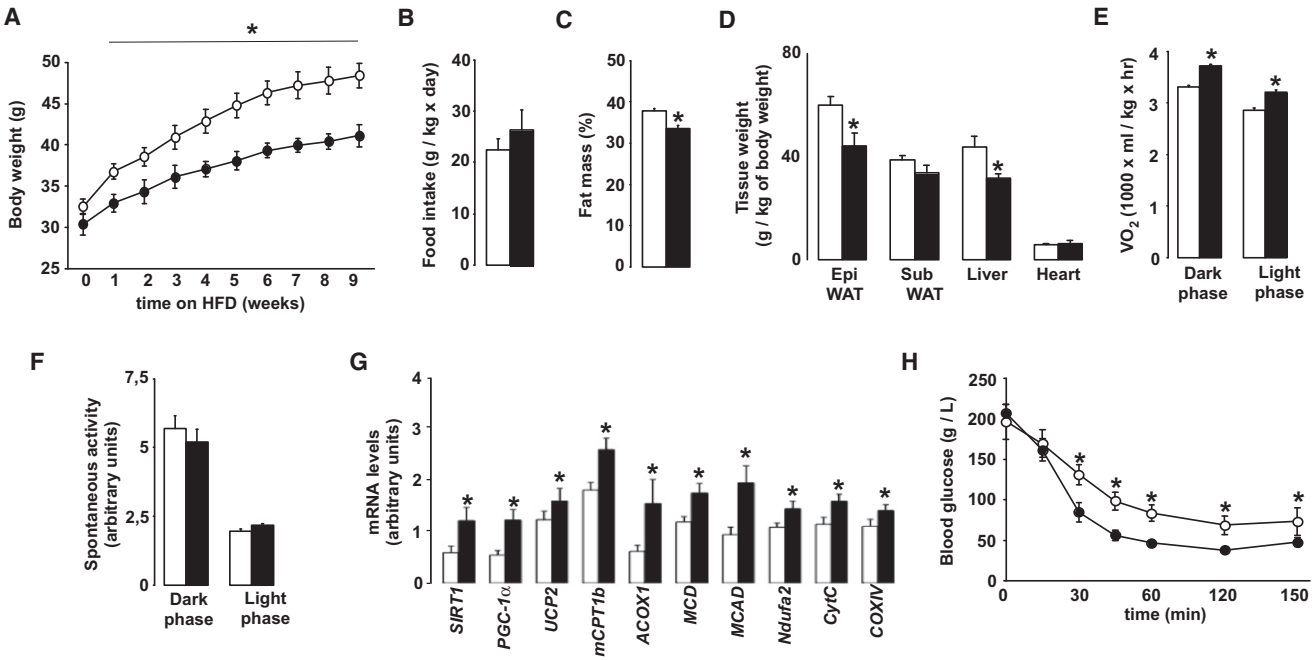

Figure 5. PARP-2$^{-/-}$ Mice Are Protected against Diet-Induced Body Weight Gain and Insulin Resistance

(A) Six-month-old *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n = 7 and 9, respectively) fed on high-fat diet were weighed weekly.

(B) Food intake was monitored during high-fat feeding.

(C) Body fat mass composition was evaluated through EchoMRI.

(D) The weight of the tissues indicated was determined upon autopsy at the end of the high-fat-feeding period.

(E and F) VO_2 (E) and spontaneous activity (F) were determined by indirect calorimetry. Quantification of the mean values during light and dark phases is shown.

(G) mRNA expression levels in gastrocnemius muscles from *PARP-2*$^{+/+}$ and $^{-/-}$ mice after 12 weeks of high-fat diet was determined by RT-qPCR.

(H) Glucose excursion after an intraperitoneal insulin tolerance test. White bars and circles represent *PARP-2*$^{+/+}$ mice, while black bars and circles represent *PARP-2*$^{-/-}$ mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

Increased SIRT1/FOXO1 Activity Renders *PARP-2*$^{-/-}$ Mice Glucose Intolerant after High-Fat Feeding due to Pancreatic Dysfunction

To our surprise, despite their lower body weight and higher insulin sensitivity, *PARP-2*$^{-/-}$ mice were more glucose intolerant compared to their *PARP-2*$^{+/+}$ littermates after high-fat feeding (Figure 6A) and still displayed fasting hyperglycemia (172.44 ± 20.11 mg/dl for *PARP-2*$^{+/+}$ versus 203.34 ± 10.26 mg/dl for *PARP-2*$^{-/-}$). The fact that *PARP-2*$^{-/-}$ mice are also more insulin sensitive (Figure 5H) suggested that this glucose intolerance could be related to defects in the insulin release upon a glucose load. Confirming this hypothesis, the insulin peak after an intraperitoneal glucose injection in *PARP-2*$^{+/+}$ mice was blunted in *PARP-2*$^{-/-}$ mice (Figure 6B). Furthermore, fasting blood insulin levels were lower in *PARP-2*$^{-/-}$ mice (0.87 ± 0.24 μg/l for *PARP-2*$^{+/+}$ versus 0.58 ± 0.16 μg/l for *PARP-2*$^{-/-}$ mice). These observations led us to examine the pancreas from *PARP-2*$^{-/-}$ mice. HFD increased the pancreatic mass in wild-type mice, but not in *PARP-2*-deficient mice (Figure 6C). Histological analysis of the pancreas of *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice revealed that islet size was smaller in *PARP-2*$^{-/-}$ mice after high-fat feeding (Figures 6D and 6E). This reduction in islet size translated into a robust reduction in pancreatic insulin content (Figure 6F). When pancreatic gene expression was analyzed in pancreas from *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice, it became evident that, in addition to an increase in some mitochondrial-related genes (*mitochondrial transcription factor A* [*TFAm*], *citrate synthase* [*CS*]), the pancreas of *PARP-2*$^{-/-}$ mice had severe reductions in the expression of a number of key genes for pancreatic function (such as *glucokinase* [*GK*] and *Kir6.2*) and proper β cell growth (*pancreatic and duodenal homeobox 1* [*pdx1*]) (Figure 6G). Given the reduced insulin content and *pdx1* expression, it was also not surprising that expression of the insulin gene (*Ins*) was decreased in the *PARP-2*$^{-/-}$ pancreas (Figure 6G). As in other tissues, *PARP-2* deletion led to higher SIRT1 protein levels in pancreas (Figure 6H), which translated not only into higher mitochondrial protein content, as manifested by complex I (39 kDa subunit) and complex III (47 kDa subunit) levels (Figure 6H), but also in the constitutive deacetylation of FOXO1 (Figure 6H). NAD^+ levels were similar in pancreas from *PARP-2*$^{+/+}$ and $^{-/-}$ mice (Figure S6A). The deacetylation and activation of FOXO1 could underpin the pancreatic phenotype of the *PARP-2*$^{-/-}$ mice, as FOXO1 activity compromises insulin content and pancreatic growth by acting as a negative regulator of *pdx1* (Kitamura et al., 2002). To further consolidate the hypothesis that higher SIRT1 and/or FOXO1 function is detrimental for pancreatic function by the downregulation of *pdx1*, we tested whether activation of FOXO1 or SIRT1 could decrease *pdx1* expression in the MIN6 mouse pancreatic β cell line. Overexpression of either FOXO1 or SIRT1 was enough to decrease *pdx1* mRNA and protein levels (Figure 7A). Similarly, resveratrol treatment, which indirectly activates endogenous SIRT1, leading

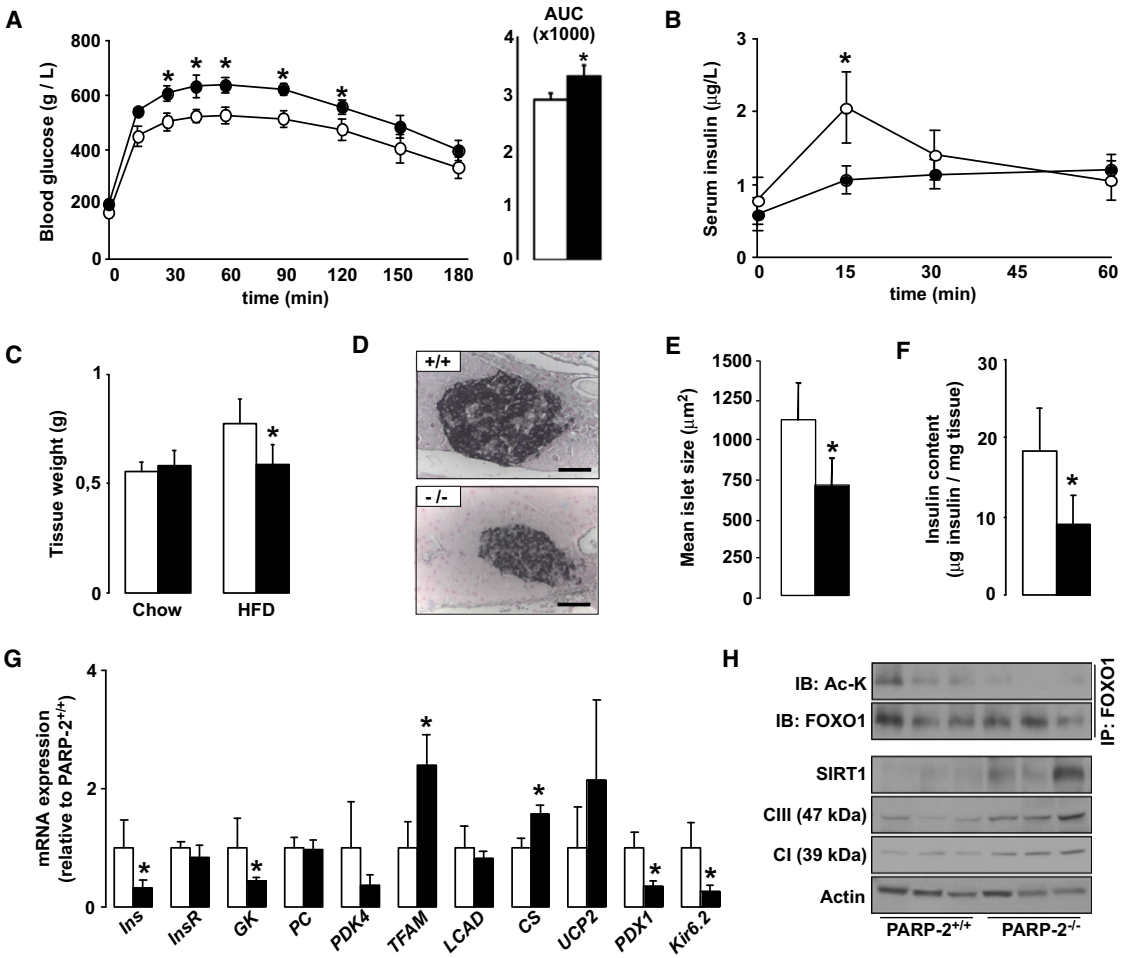

Figure 6. Pancreatic Abnormalities Render *PARP-2*$^{-/-}$ Mice Glucose Intolerant after High-Fat Feeding

(A) Plasma glucose levels during an intraperitoneal glucose tolerance test (IPGTT) in 9-month-old *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n = 7 and 9, respectively) fed a high-fat diet for 12 weeks. The area under the curve of the glucose curves is shown at the right.

(B) Insulin levels during the first hour of the IPGTT in (A).

(C) Comparison of total pancreas weight between *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice on chow and high-fat diet.

(D) Pancreas from *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice after high-fat diet were stained for insulin (scale bar = 50 µm).

(E) Mean islet size was quantified.

(F) Total insulin content in pancreas was measured as described.

(G) Gene expression in the pancreas of *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice was measured by RT-qPCR.

(H) Pancreatic total protein extracts were used to test the abundance of SIRT1, and subunits from the respiratory complexes I and III. FOXO1 was also immunoprecipitated to determine relative FOXO1 acetylation levels. White bars and circles represent *PARP-2*$^{+/+}$ mice, while black bars and circles represent *PARP-2*$^{-/-}$ mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-2*$^{+/+}$ mice at $p < 0.05$.

to FOXO1 deacetylation (Figure 7B), also decreased *pdx1* content (Figure 7A). Altogether, these results illustrate that *PARP-2*$^{-/-}$ mice have impaired pancreatic hyperplasia upon HFD, due to the lower expression of key genes involved in pancreatic β cell proliferation and function, such as *pdx1*, as a consequence of higher SIRT1 and/or FOXO1 activity.

DISCUSSION

In our present study, we have shown that PARP-2 is a negative regulator of the SIRT1 promoter and that the reduction or ablation of PARP-2 expression induces SIRT1 expression, protein content, and activity. As expected from the activation of SIRT1 (Cantó et al., 2009; Lagouge et al., 2006), *PARP-2*$^{-/-}$ mice displayed higher whole-body EE and were protected against HFD-induced obesity. Previous studies on PARP-1, the predominant PARP activity in most tissues, have demonstrated that the modulation of intracellular NAD^+ levels by PARP-1 critically influences SIRT1 activity (Kolthur-Seetharam et al., 2006; Pillai et al., 2005; Rajamohan et al., 2009; Bai et al., 2011). Our data demonstrate that deletion of an alternative PARP activity, *PARP-2*, also impacts on SIRT1 activity. Unexpectedly, our results clearly indicate that, unlike what happens with PARP-1 (Bai et al., 2011), the impact of PARP-2 on SIRT1 activity is not necessarily based on changes in NAD^+ content. This is in line with previous evidence indicating that PARP-2 is not a major

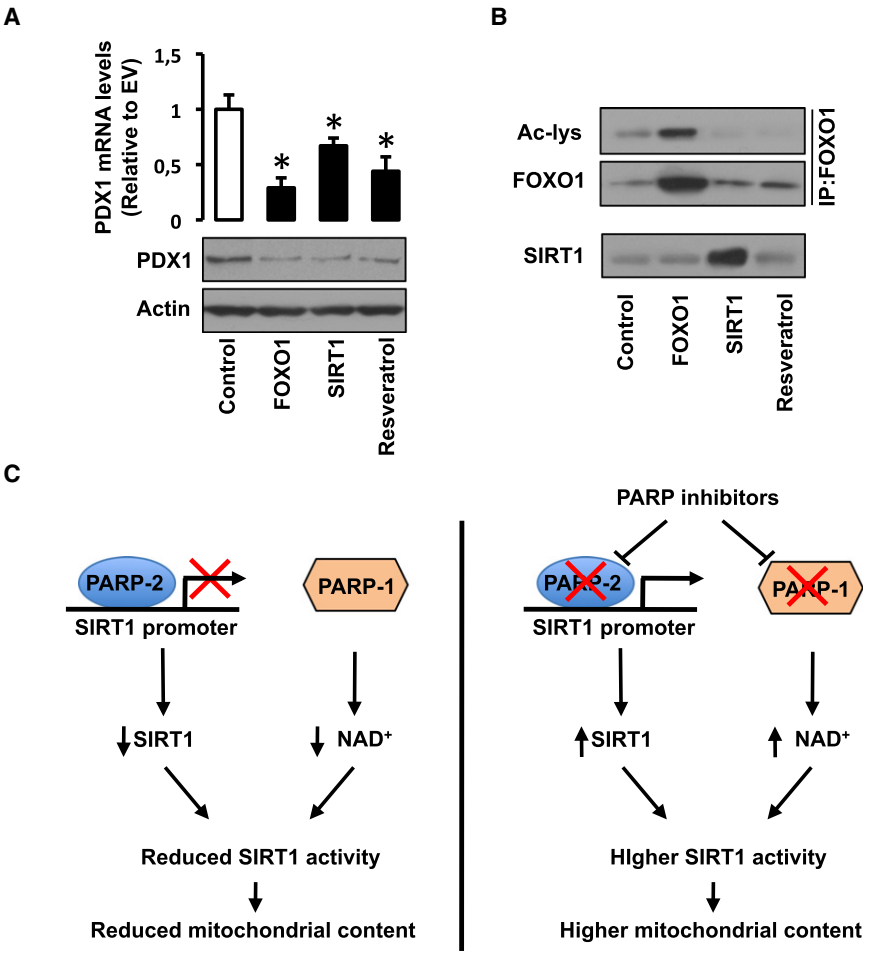

Figure 7. Increased SIRT1/FOXO1 Function Reduces *pdx1* Expression

(A and B) MIN6 cells were transfected with either empty vector (Control), human FLAG-FOXO1, or human FLAG-SIRT1. Additionally, one empty vector group was treated with resveratrol (50 mM, 5 hr/day for 2 days). Then, total protein and RNA extracts were obtained to test *pdx1* mRNA and protein levels (A) and total FOXO1 and acetylated FOXO1 levels (B), as well as SIRT1 levels.

(C) Scheme illustrating how the activation of PARP enzymes can downregulate SIRT1 function through different means. On one hand, PARP-1 activation limits SIRT1 activity by decreasing NAD^+ bioavailability, and on the other, we now report how PARP-2 acts as a negative regulator of the SIRT1 promoter. The PARP-1 and PARP-2 knockout mouse models indicate that PARP inhibition can be useful to promote SIRT1 activity and enhance mitochondrial content. This strategy might be exploited therapeutically in the context of metabolic disease, commonly linked to impaired mitochondrial content or function. Values are expressed as mean ± SEM unless otherwise stated.

PARP enzyme in the cell and therefore is not likely to significantly influence NAD^+ homeostasis (Amé et al., 1999; Shieh et al., 1998). Rather, PARP-2 impacts on SIRT1 by directly interacting with the proximal SIRT1 promoter, where it acts as a potent transcriptional repressor. Accordingly, in all models and tissues tested, PARP-2 deletion or depletion induces SIRT1 mRNA expression, protein content, and activity.

PARP-2 can act as a transcriptional regulator (Yélamos et al., 2008). We have previously shown that PARP-2 acts as a positive regulator of PPARγ, leading to triglyceride accumulation (Bai et al., 2007). In line with this and supported by the present data, *PARP-2* deficiency led to decreased lipid accumulation. Our current work furthermore expands the potential mechanisms by which PARP-2 activity might impact on lipid accumulation. First, the activation of SIRT1 in *PARP-2*$^{-/-}$ mice would provide a second way to reduce PPARγ activity, as SIRT1 docks the transcriptional corepressors NCoR and SMRT to PPARγ-regulated promoters (Picard et al., 2004). Second, and most importantly, the increase in EE and fat burning observed in *PARP-2*$^{-/-}$ mice would also prevent fat accumulation. Reinforcing this second hypothesis, the reduced size of adipose depots is not associated with lipid accumulation in other tissues, such as liver (Figures S5A and S5B), indicating that enhanced fat burning rather than fat redistribution accounts for the decreased fat mass.

Our results illustrate how pharmacological approaches to promote oxidative metabolism by inhibiting PARP activity could benefit from targeting specific PARP enzyme forms. Certainly, *PARP-2* deletion recapitulates all the previous beneficial effects observed with different strategies aimed to promote SIRT1 activation, i.e., enhanced oxidative metabolism, endurance phenotype, and protection against body weight (Feige et al., 2008; Lagouge et al., 2006). These positive outcomes seen in the absence of *PARP-2* are, however, overshadowed by the deleterious effects that the absence of *PARP-2* has on pancreatic function. Our results show that, upon high-fat feeding, pancreatic proliferation and insulin production are blunted in *PARP-2*$^{-/-}$ mice. This could be a consequence of the impaired expression of *pdx1*, a crucial regulator of pancreatic growth and insulin expression (Kaneto et al., 2008), as well as of other genes that fine-tune pancreatic function, such as *Kir6.2* and *glucokinase* (MacDonald et al., 2005). While many reasons could account for such defect, a likely possibility is that the increased SIRT1 activity in *PARP-2*$^{-/-}$ pancreas activates FOXO1 (Figure 6H) (Brunet et al., 2004; Frescas et al., 2005), which is known to downregulate *pdx1* expression (Kawamori et al., 2006). Interestingly, decreased FOXO1 activity, through its nuclear exclusion, is necessary for the compensatory pancreatic proliferation observed in situations of insulin resistance, as induced by HFD (Buteau and Accili, 2007). FOXO1 activation also is detrimental for the pancreatic proliferative effects of hormones such as glucagon-like peptide 1 (GLP-1) (Buteau and Accili, 2007). Hence, the constitutive activation of FOXO1 provides a plausible mechanism to explain the pancreatic phenotype of the *PARP-2*$^{-/-}$ mice upon HFD. Given that overexpression or

activation of SIRT1 or overexpression of FOXO1 in the MIN6 β cell line recapitulates the downregulation of *pdx1* in the *PARP-2*$^{-/-}$ mice, it is surprising that this phenotype was not observed in other models of SIRT1 activation in the pancreas (Banks et al., 2008; Moynihan et al., 2005). Unfortunately, the fact that FOXO1 deacetylation, as a read-out of effective pancreatic SIRT1 activation, was not tested in those studies (Banks et al., 2008; Moynihan et al., 2005) makes it difficult to compare it with our work. Another possibility would be that, besides SIRT1-mediated FOXO deacetylation, other PARP-2-specific functions might contribute to the pancreatic alterations in the *PARP-2*$^{-/-}$ mice. For example, PARPs can modulate the enzymatic activities of a number of proteins through direct interaction or by poly(ADP-ribosyl)ation (Yélamos et al., 2008). In this sense, PARP-1 is known to directly interact with, poly(ADP-ribosyl)ate, and inhibit FOXO (Sakamaki et al., 2009), but we could not demonstrate an interaction between PARP-2 and FOXO in the pancreas (Figure S6B). Notably, we also could not find evidence for a direct interaction between PARP-2 and SIRT1 (Figure S1B) or poly(ADP-ribosyl)ation of SIRT1 (Bai et al., 2011). Furthermore, PARP-2 deletion affects blood adipokine levels (Bai et al., 2007), which might also impact pancreatic function. Unraveling the additional mechanisms by which PARP-2 influences pancreatic function warrants future investigation. Importantly, it must be noted that this pancreatic phenotype is specific for the *PARP-2*$^{-/-}$ mice, as *PARP-1*$^{-/-}$ mice, while sharing many common characteristics with the *PARP-2* mutants, do not develop glucose intolerance after high-fat feeding (Bai et al., 2011), and islet size and insulin content are similar between wild-type and *PARP-1*$^{-/-}$ littermates upon high-fat feeding (Figures S6C and S6D), indicating that the pancreatic phenotype is specific to *PARP-2* deletion.

In conclusion, our results complement the picture by which PARP activation is detrimental for mitochondrial function by decreasing SIRT1 activity (Figure 7C). On the one hand, we recently reported that the activation of PARP-1 depletes intracellular NAD^+, which limits SIRT1 activity (Bai et al., 2011). On the other hand, we describe here how PARP-2 acts as a negative regulator of SIRT1 expression. In combination, these results prompt the speculation that PARP inhibitors could be used not only for cancer, but also in the context of metabolic disease to enhance mitochondrial content.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were from Sigma-Aldrich (St. Louis) unless stated otherwise.

Animal Studies

All animal experiments were carried out according to local, national, and EU ethical guidelines. Homozygous *PARP-2*$^{-/-}$ and littermate *PARP-2*$^{+/+}$ mice (Ménissier de Murcia et al., 2003) and *PARP-1*$^{-/-}$ and *PARP-1*$^{+/+}$ mice (de Murcia et al., 1997) on a mixed C57BL/6J / SV129 background (87.5%/12.5%) background from heterozygous crossings were used. Mice were housed separately, had ad libitum access to water and food, and were kept under a 12/12 hr dark/light cycle. Mice were selected for the study at the age of 4 weeks and were kept on chow diet (10 kcal% of fat, Safe, Augy, France). For a part of the animals, food was changed by a HFD (60 kcal% of fat, Research Diets, New Brunswick, NJ) at the age of 16 weeks. Each week on the same day, mice were weighed and food consumption was measured. O_2 consumption, CO_2 production, and actimetry were measured by CLAMS of Columbus Instruments; total body fat was measured by EchoMRI as described (Lagouge et al., 2006; Watanabe et al., 2006). Endurance test was performed as described in Cantó et al. (2009). Intraperitoneal glucose and insulin tolerance tests (IPGTT and IPITT, respectively) were performed as previously described (Feige et al., 2008). The animals were killed by CO_2 asphyxiation after 6 hr of fasting, and tissues were collected. Total body fat content was then examined at autopsy by weighing the subcutaneous, gonadal, mesenterial, retroperitoneal, and BAT-associated fat depots. Liver triglyceride was determined after Folch extraction using a commercial triglyceride kit (Roche). Pancreas and plasma insulin content was determined from acidic extracts using a commercial ELISA kit (Mercodia) (Champy et al., 2004).

Histology and Microscopy

Hematoxylin-eosin (HE), oil red O, and SDH stainings were performed on 7 μm tissue sections as described (Lagouge et al., 2006; Picard et al., 2002). Immunohistochemistry was performed on 7 μm frozen tissue sections using anti-insulin antibody (Dako). Aspecific binding of the secondary antibody was controlled on sections where the primary antibody was omitted. Islet size was determined on insulin-stained sections. TUNEL assay was performed using a commercial kit (Millipore). TEM was performed on glutaraldehyde-fixed, ismium tetroxide-stained ultrafine sections (Watanabe et al., 2006).

Cell Culture

HEK293T cells were cultured as described in Bai et al. (2007). The MIN6 mouse pancreatic β cell line was grown in DMEM 4.5 g/l glucose with 15% inactivated FBS, 1% penicillin/streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol. C2C12 cells were cultured and differentiated as described in Cantó et al. (2009). Lentiviral short hairpin (shRNA) and control scramble constructs against PARP-2 were from Sigma-Aldrich (MISSION Custom Vector) incorporating the interfering sequence described in Bai et al. (2007). C2C12 cells were transduced with 20 moi of virus, and transduced clones were selected with 2.5 μg/ml puromycin. Puromycin selection was maintained during subsequent routine cell culture.

Constructs, Reporter Assays, and Chromatin Immunoprecipitation

SIRT1 promoter constructs were described in Nemoto et al. (2004). pSuper-siPARP2, pSuper-scrPARP2, and pBabe-PARP2 were described in Bai et al. (2007). Adenovirus for SIRT1 knockdown is reported in Rodgers et al. (2005). Reporter assays and ChIPs were performed as in Bai et al. (2007). Primers for ChIP are summarized in Table S3.

mRNA Preparation, Reverse Transcription, and qPCR

Messenger RNA preparation, cDNA synthesis, and RT-qPCR were performed as described in Bai et al. (2007). Expression was normalized to the expression of cyclophilin. Mitochondrial DNA was determined in qPCR reactions as described in Lagouge et al. (2006). Primers are summarized in Tables S1 and S2.

NAD^+ Determination

NAD^+ was determined by either a general alcohol-dehydrogenase-coupled colorimetric reaction (Cantó et al., 2009) or, where indicated, by mass spectrometry, as described (Sauve et al., 2005).

Protein Extraction and Western Blotting

Protein extracts were prepared as described in Cantó et al. (2009); nuclear extraction was prepared as described (Edelman et al., 1965). Extracts were subjected to western blotting as in Bai et al. (2007) and Cantó et al. (2009). Blots were probed with the following primary antibodies: SIRT1 (Millipore), actin (Sigma), PARP-2 (rabbit polyclonal antibody raised against full-length mouse PARP-2), and H1 (kindly provided by S. Muller, IBMC, Strasbourg).

Determination of PGC-1α Acetylation Status

PGC-1α and FOXO1 acetylation was analyzed by immunoprecipitation as described in Cantó et al. (2009).

Statistical Analysis

For the statistical analysis of the animal studies (body weight gain, food consumption), the data were checked for normal distribution. To determine

significance, Student's t test was applied, and $p < 0.05$ was considered significant. Error bars represent SEM unless stated otherwise.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, six figures, and two tables and can be found with this article online at doi:10.1016/j.cmet.2011.03.013.

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Supplemental Information
Cell Metabolism, *Volume 13*

PARP-2 Regulates SIRT1 Expression and Whole-Body Energy Expenditure

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Supplemental Experimental Procedures

DNA Constructs

The SIRT1 promoter reporter constructs were described in (Nemoto et al., 2004). PARP-2 was overexpressed (by pBabe-PARP-2 using pBabe as control), or depleted (using pSuper-shPARP-2 and pSuper-scrPARP-2 as control) as described in (Bai et al., 2007). The pCMV-βGal construct was used to control transfection efficiency. The pCMX-ERβ construct was described in (Tremblay et al., 1997). Adenoviral construct for SIRT1 silencing was described in (Rodgers et al., 2005).

Cell Culture

HEK293T cells were maintained in 4.5 g/l glucose DMEM, 10% FCS. For the transfection of HEK293T cells JetPei was used (*Polyplus transfections*, Illkirch, France) according to the description of the company. PARP-2 depletion and overexpression took place as described in (Bai et al. 2007).

C2C12 cells were maintained in 4.5 g/l glucose DMEM, 10% FCS and were differentiated in 4.5 g/l glucose DMEM, 2% horse serum for 2 days, when cells were considered myofibers. PARP-2 was depleted from C2C12 cells using lentiviral shRNA system (MISSION Lentiviral Vectors, *Sigma-Aldrich*). The vectors contained the interfering and control sequences described in (Bai et al. 2007). C2C12 cells were transduced with 20 MOI virus and were selected with 2.5 μg/ml puromycin. Cells withstanding puromycin selection were subcultured. In all subsequent cell culture steps puromycin selection was constantly maintained. The efficiency of knock-down at the mRNA level was assessed by RT-qPCR.

Cell Fractionation for NAD^+ Determination

Cells from 2x10 cm dishes were scrapped in ice-cold PBS, pooled together and transfered and each sample was subdivided equally in 2 independent tubes and cells were pelleted. The pellet from one of the tubes was resuspended in acidic buffer and used for total NAD^+ stimation. The other was resuspended in 1 ml of an ice-cold buffer containing sucrose 0.25M, EGTA 2mM, HEPES 20 mM and protease inhibitors. Then they were transfered into a Teflon-glass homogenized and homogenized with a rotating mechanic piston. Two microliters from the homogenate were used to estimate total protein using the Bradford reagent. The remaining homogenate was then centrifuged at 600 rpm at 4ºC to pellet nuclei and cell debris. Equal volumes of the resulting supernatant was transfered into two independent tubes and both were centrifuged at 7000 rpm. The resulting pellets were considered the mitochondrial fraction. One of the tubes was resuspended in acidic lysis buffer to measure NAD^+, while the other was resuspended in the buffer described above in order to measure protein.

SIRT1 Promoter Reporter Assay

HEK293T cells seeded in 6 well plates, after the depletion or overexpression of PARP-2, were transfected with 1.6 μg SIRT1 promoter reporter construct, 1 μg of pBabe/pBabe-PARP-2/pSuper-shPARP-2/pSuper-scrPARP-2 and 0.4 μg pCMV-βGal. Ten hours after transfection cells were scraped then luciferase and β-galactosidase activity were determined. Luciferase activity was expressed as luciferase activity/β-galactosidase activity.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in (Bai et al., 2007; Balint et al., 2005). HEK293T, or C2C12 cells were fixed, lysed and subjected to ChIP using anti-PARP-2 and anti-MMP9 (*Santa Cruz*) antibodies (both of rabbit origin; no antibody

control was equally introduced). The DNA pool obtained was assayed in qPCR using primers specific for the -1 - -91 portion of the SIRT1 promoter and primers specific for the K19 promoter (that is independent of PARP-2 (Bai et al., 2007)). The values obtained from the qPCR run were normalized for the value of the corresponding input (SIRT1/K19) and expressed as % of input. The human K19 primers were validated on HEK cells transfected with pCMX-ERβ (data not shown); the murine primers were already validated (Bai et al., 2007). Primers are summarized in Supplemental Table 3.

Intraperitoneal Pyruvate Tolerance Test (ipPTT)

Intraperitoneal pyruvate tolerance test was performed as described in (Yao et al., 2006). Increase in blood glucose was expressed as percentage of baseline glucose level.

Sequence Alignment

Sequences of SIRT1 promoter was acquired for the indicated vertebrate species from Pubmed. The initial 300 bp segment (-1 to -300) was aligned using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the sequences homologous to the murine -1 - -204 (corresponding to the two shortest SIRT1 promoter construct) were displayed.

Cold Exposure

Cold exposure was performed as described in (Lagouge et al., 2006).

Endurance Test

Chow fed PARP-2 mice were subjected to a resistance running test, using a variable speed belt treadmill enclosed in a plexi glass chamber with a stimulus device consisting of a shock grid attached to the rear of the belt (*Columbus*). Animals were acclimatized to the chamber for 5 days preceding the running test. For the habituation, mice run at 12 m/min for 10 minutes with a 5º incline. For the actual test, we used a protocol at 5º incline where, beginning at 10 m/min, speed increased gradually by 1 m/min every 5 minutes. The distance

run and the number of shocks were monitored during the test, and exhaustion was assumed when mice received more than 50 shocks in a 2.5 minutes interval. Mice were removed from the treadmill upon exhaustion. After high-fat diet, a few modifications were introduced: habituation was performed for 5 days preceding the test running at 10 m/min for 10 minutes with no inclination. For the actual test, we used a protocol with no incline where, beginning at 8 m/min, speed increased gradually by 1 m/min every 5 minutes.

Islet Size Determination

Islet size was determined on insulin-stained slides. From each pancreas several consecutive sections (3-11) were made and all were stained for insulin. All islets on each section were photographed on a Zeiss Axioscope microscope and a Zeiss Axiocam camera with special care to avoid duplicate photographing the same islet multiple times. The area of the islets was measured using the Image J freeware that was converted into μm^2 by determining the original size of a large islet.

mRNA Preparation, Reverse Transcription and qPCR

Total RNA was prepared using TRIzol (*Invitrogen*) according to the manufacturer's instructions. RNA was treated with DNase, and 2 μg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (*Qiagen*, Valencia, CA). 50X diluted cDNA was used for quantitative PCR (qPCR) reactions (Bai et al., 2007). The qPCR reactions were preformed using the Light-Cycler system (*Roche Applied Science*) and a qPCR Supermix (*Qiagen*) with the primers summarized in Table S1.

Mitochondrial DNA Measurement

Assay was performed as described in (Lagouge et al., 2006). DNA was extracted using the standard proteinase K digestion following phenol-chloroform extraction. Mitochondrial and genomic DNA was determined using specific primers in qPCR reactions (primers

summarized in Table S2.) and was expressed as percentage of WT.

Muscle Nuclear Protein Isolation

Protocol was slightly modified as described in (Edelman et al., 1965). Gastrocnemius muscle was removed from animals after 6 hours of fasting and was frozen in liquid N_2 immediately. All manipulations were carried out on ice. Tissues were homogenized in 2 ml homogenization buffer H1 (0.32 M sucrose; 1 mM $MgCl_2$; 0.2 mM K_2HPO_4; 0.6 mM KH_2PO_4; pH 6.8) by Ultra TURAX (~1000 rpm). The homogenate was pottered in a Potter-Elvhejem tissue grinder. Next the suspension was filtered through a 70 μm then a 40 μm nylon net (*BD Falcon*). After each filtration homogenate volume was adjusted to 2 ml with H1 buffer. After filtration the homogenate was centrifuged at 800 g for 5 minutes and then the pellet was washed with additional 1 ml H1. The pellet was exsiccated at room temperature for 5 minutes and resuspended in N2 buffer (2.15 M sucrose; 1 mM $MgCl_2$; 3.5 mM K_2HPO_4; pH 6.8). Suspension was centrifuged at 16000g for 2 hours and the pellet was suspended in lysis buffer (62.5 mM TRIS pH 6.8; 2 % SDS; 10 % glycerol; 1 mM PMSF; 50 mM DTT; 1x protease inhibitor cocktail). The pellet was resuspended in 200 μl lysis buffer. The lysate was passed through a 22G needle (20 X) then centrifuged at 10000 rpm for 10 min. The supernatant was used in further experiments as nuclear extract.

Determination of Protein Acetylation Status

PGC-1α, FOXO1 and Ndufa9 acetylation was analyzed by immunoprecipitation of PGC-1α, FOXO1 and Nduf9a from nuclear lysates of tissues with anti-PGC-1α (Millipore), anti-FOXO1 (1 mg, *Cell Signalling*, Danvers, MA, USA), or Ndufa9 (*Abcam*) antibody followed by Western blot using an acetyl-lysine antibody (*Cell Signalling*) that was normalized to total PGC-1α/FOXO1/Ndufa9 (Canto et al., 2009). Tubulin acetylation was assessed by Western blotting of total protein samples by acetylated tubulin-specific antibody (*Sigma*) that was then normalized for total tubulin (*Santa Cruz*).

Supplementary Figure 1.

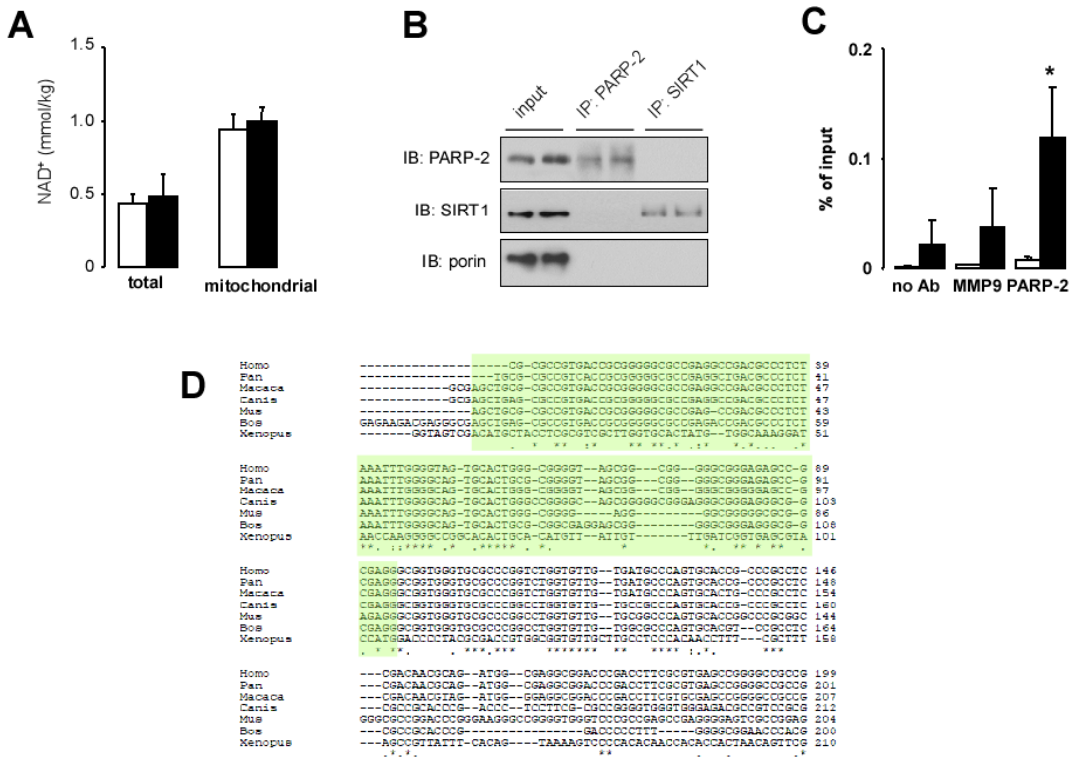

Figure S1. *PARP-2* Influences SIRT1 Activity by Directly Regulating the SIRT1 Promoter

(A) Total and mitochondrial NAD^+ was determined as described in Experimental procedures in C2C12 cells transduced with either scramble (white bars) or a PARP-2 (black bars) shRNA.

(B) PARP-2 and SIRT1 were immunoprecipitated from C2C12 cells and blotted for the markers indicated.

(C) ChIP assay was performed in HEK293T cells and tested for SIRT1 the SIRT1 -91 bp promoter region (black bars) or the K19 promoter (white bars).

(D) Alignment of the SIRT1 promoter of different vertebrate species was performed using the ClustalW software. The green field indicates the murine -1 - -91 region, where PARP-2 binds. * indicates statistical difference between the PARP-2 IP and the unspecific antibody binding at $P < 0.05$.

Supplementary Figure 2.

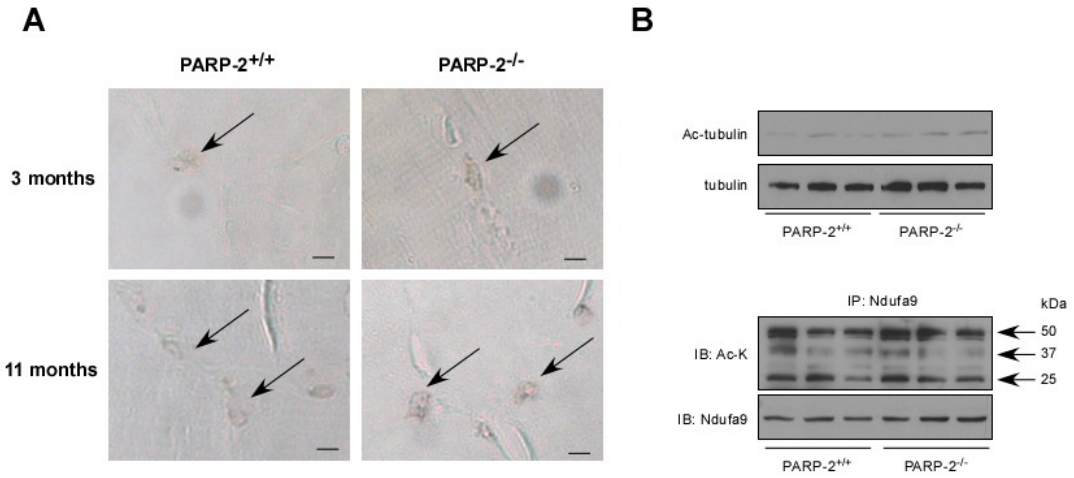

Figure 2. *PARP-2* Deletion Does Not Lead to the Accumulation of DNA Damage and Does Not Influence SIRT2 and SIRT3 Activity in Muscle

(A) TUNEL reaction was performed in the gastrocnemius muscle of young (3 months of age) and old (11 months of age) *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n=3/3/4/3; young *PARP-2*$^{+/+}$/young *PARP-2*$^{-/-}$/old *PARP-2*$^{+/+}$/old *PARP-2*$^{-/-}$) to determine the amount of DNA strand breaks. The bar represents 1μm. Arrows represent TUNEL-positive nuclei indicative of DNA damage.

(B) SIRT2 and SIRT3 activity was assessed by detecting the acetylation status of tubulin, using specific antibodies, and Ndufa9, by immunoprecipitation.

Supplementary figure 3.

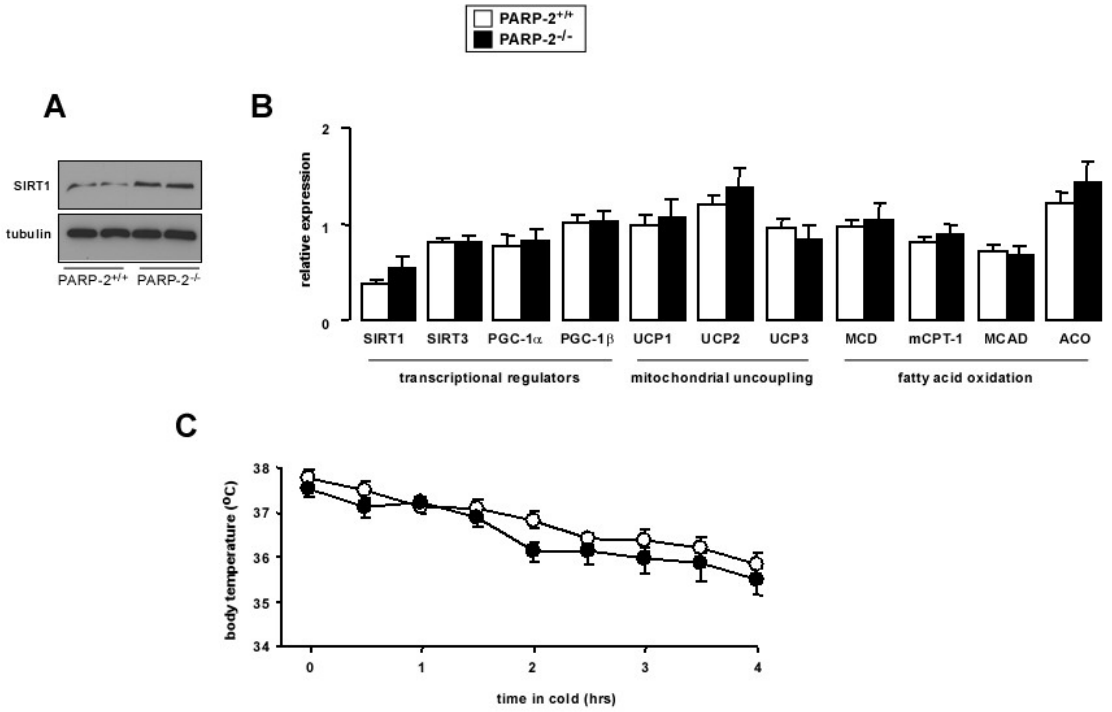

Figure S3. *PARP-2* Deletion Does Not Have a Major Impact on BAT Gene Expression and Function

(A) SIRT1 protein levels were detected in the BAT of *PARP-2*$^{+/+}$ and $^{-/-}$ male mice by Western boltting.

(B) BAT mRNA expression pattern was determined in *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n=16/13) by RT-qPCR.

(C) *PARP-2*$^{+/+}$ and $^{-/-}$ mice (n=6/6) were subjected to cold shock as described in Experimental procedures. Abbreviations are in the text. White bars and circles represent *PARP-2*$^{+/+}$ mice and black bars or circles represent *PARP-2*$^{-/-}$ mice. All results are expressed as mean ± SD.

Supplementary figure 4.

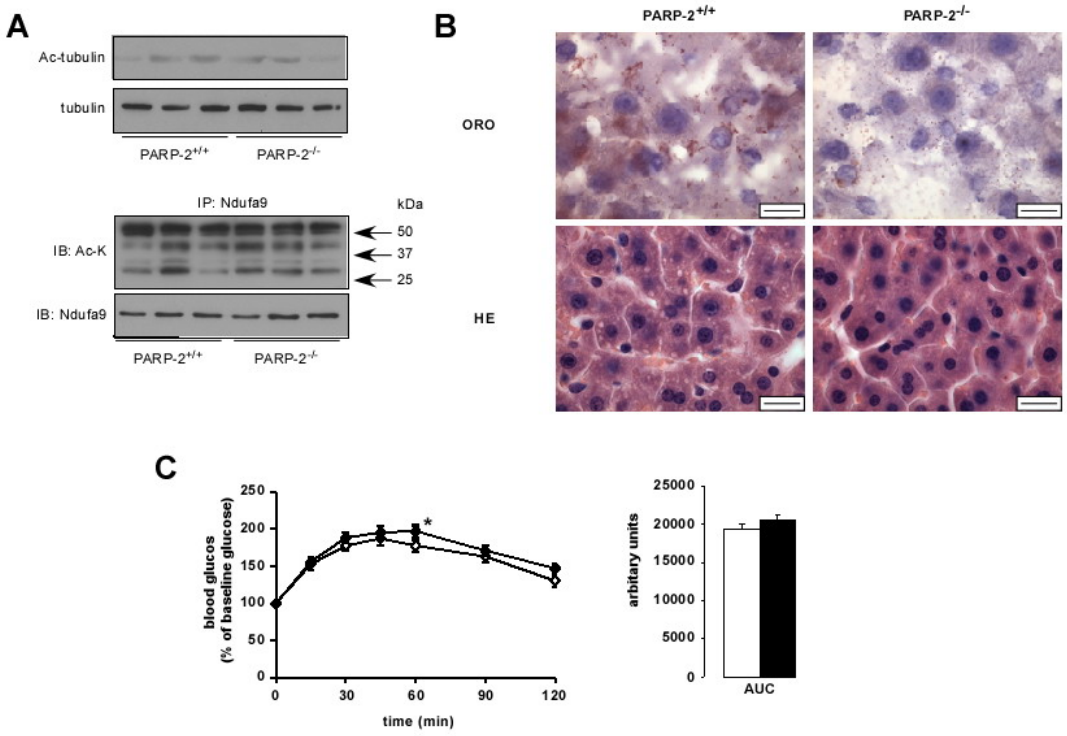

Figure S4. The Liver of *PARP-2*$^{-/-}$ Mice Display Reduced Lipid Accumulation, in the Absence of Changes in SIRT2 and SIRT3 Activity or Gluconeogenic Potential

(A) The acetylation of SIRT2 and SIRT3 targets (tubulin and Ndufa9, respectively) were determined by the use of specific antibodies (tubulin) or by immunoprecipitation (Ndufa9).

(B) Liver morphology and lipid content was assessed by hematoxilin-eosin (HE) and Oil-Red O (ORO) stainings. The bar represents 10 μm.

(C) Liver gluconeogenesis was assessed by intraperitoneal pyruvate tolerance test in *PARP-2*$^{+/+}$ (white bar and circles) and $^{-/-}$ (black bar and circles) male mice (n=10/9) as described in Materials and methods. * indicates statistical difference *PARP-2*$^{+/+}$ vs. PARP-2$^{-/-}$ mice at $p<0.05$

Supplementary figure 5.

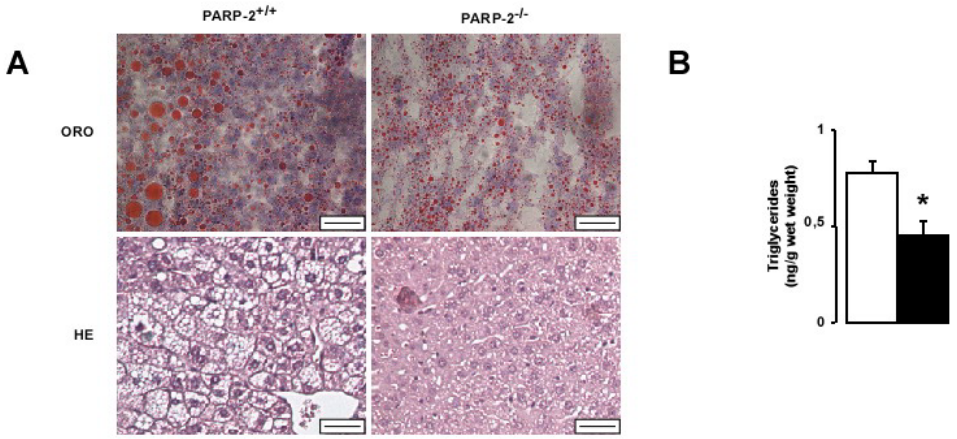

Figure S5. Livers from *PARP-2*$^{-/-}$ Mice Are Protected from High-Fat-Diet-Induced Lipid Accumulation

(A) Morphology and lipid accumulation in the liver of *PARP-2*$^{+/+}$ and $^{-/-}$ male mice (n=16/13) after 12 weeks of high-fat diet was visualized with hematoxilin-eosin (HE) and Oil Red-O colorations. The bar represents 20 μm.

(B) Triglyceride quantity was determined after lipid extraction from *PARP-2*$^{+/+}$ (white bar) or *PARP-2*$^{-/-}$ (black bar) as described in methods.* indicates statistical difference *PARP-2*$^{+/+}$ HFD vs. PARP-2$^{-/-}$ HFD mice at $p<0.05$

Supplementary figure 6.

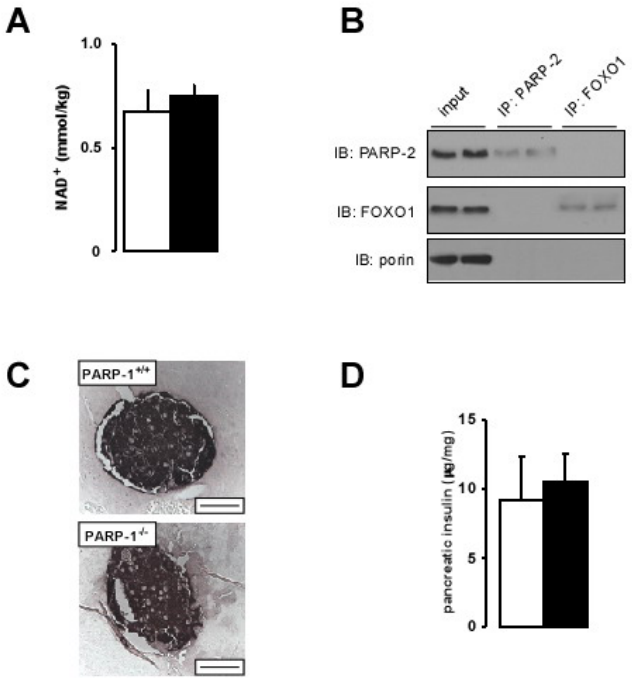

Figure S6. The Pancreas of *PARP-2*$^{-/-}$, but Not *PARP-1*$^{-/-}$ Mice, Is Hypofunctional upon High-Fat Feeding

(A) Total NAD^+ was determined from the pancreas of *PARP-2*$^{+/+}$ (white bar) and $^{-/-}$ (black bar) male mice (n=7/5).

(B) The interaction of pancreatic PARP-2 and FOXO-1 has been investigated in immunprecipitation experiments.

(C) The pancreas of *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (n=3/3) were stained for insulin (bar = 50 µm).

(D) Insulin content in pancreas from *PARP-1*$^{+/+}$ (white bar) or $^{-/-}$ (black bar) mice was determined by ELISA.

Table S1. RT-qPCR Primers for Quantification of Gene Expression

Gene	Primers
ACO	CCC AAC TGT GAC TTC CAT T GGC ATG TAA CCC GTA GCA CT
ATP5g1	GCT GCT TGA GAG ATG GGT TC AGT TGG TGT GGC TGG ATC A
COX17	CGT GAT GCG TGC ATC ATT GA CAT TCA CAA AGT AGG CCA CC
Citrate synthase	GGA GCC AAG AAC TCA TCC TG TCT GGC CTG CTC CTT AGG TA
Cyclophyllin B	TGG AGA GCA CCA AGA CAG ACA TGC CGG AGT CGA CAA TGA T
Cytochrome C	TCC ATC AGG GTA TCC TCT CC GGA GGC AAG CAT AAG ACT GG
Dio2	GCA CGT CTC CAA TCC TGA AT TGA ACC AAA GTT GAC CAC CA
ERRα	ACT GCC ACT GCA GGA TGA G CAC AGC CTC AGC ATC TTC AA
FOXO1	AAG GAT AAG GGC GAC AGC AA TCC ACC AAG AAC TCT TTC CA
G6Pase	CCG GAT CTA CCT TGC TGC TCA CTT T TAG CAG GTA GAA TCC AAG CGC GAA AC
GK	ACA TTG TGC GCC GTG CCT GTG AA AGC CTG CGC ACA CTG GCG TGA AA
Kir6.2	CTG TCC CGA AAG GGC ATT AT CGT TGC AGT TGC CTT TCT TG
Insulin	GTG GGG AGC GTG GCT TCT TCT A ACT GAT CCA CAA TGC CAC GCT TCT
Insulin receptor	CGA GTG CCC GTC TGG CTA TA GGC AGG GTC CCA GAC ATG
LCAD	GTA GCT TAT GAA TGT GTG CAA CTC GTC TTG CGA TCA GCT CTT TCA TTA
L-CPT1	GCA CTG CAG CTC GCA CAT TAC AA CTC AGA CAG TAC CTC CTT CAG GAA A
MCD	TGG ATG GCT GAC AGC AGC CTC AA CTG AGG ATC TGC TCG GAA GCT TTG
MCAD	GAT CGC AAT GGG TGC TTT TGA TAG AA AGC TGA TTG GCA ATG TCT CCA GCA AA
mCPT1	TTG CCC TAC AGC TGG CTC ATT TCC GCA CCC AGA TGA TTG GGA TAC TGT
Ndufa2	GCA CAC ATT TCC CCA CAC TG CCC AAC CTG CCC ATT CTG AT
PEPCK	CCA CAG CTG CTG CAG AAC A GAA GGG TCG CAT GGC AAA
PPARα	AGG AAG CCG TTC TGT GAC AT TTG AAG GAG CTT TGG GAA GA
PC	AGG GGC TGC TGT TGA TGG AC CAG GGG CAC TCG TAC AGG AAG C

PDK4	AAA GGA CAG GAT GGA AGG AAT CA ATT AAC TGG CAG AGT GGC AGG TAA
SDH	GAA CTG CAC ACA GAC CTG C GAC TGG GTT AAG CCA ATG CTC
SIRT1	TGT GAA GTT ACT GCA GGA GTG TAA A GCA TAG ATA CCG TCT CTT GAT CTG AA
SIRT3	AGG TGG AGG AAG CAG TGA GA GCT TGG GGT TGT GAA AGA AA
PDX1	AAT CCA CCA AAG CTC ACG CGT GGA A TGA TGT GTC TCT CGG TCA AGT TCA A
PGC-1α	AAG TGT GGA ACT CTC TGG AAC TG GGG TTA TCT TGG TTG GCT TTA TG
PGC-1β	TGG AGA CTG CTC TGG AAG GT TGC TGC TGT CCT CAA ATA CG
TFAm	CCA AAA AGA CCT CGT TCA GC ATG TCT CCG GAT CGT TTC AC
Troponin I	CCA GCA CCT TCA GCT TCA GGT CCT TGA T TGC CGG AAG TTG AGA GGA AAT CCA AGA T
UCP1	GGC CCT TGT AAA CAA CAA AAT AC GGC AAC AAG AGC TGA CAG TAA AT
UCP2	ACC AAG GGC TCA GAG CAT GCA TGG CTT TCA GGA GAG TAT CTT TG
UCP3	ACT CCA GCG TCG CCA TCA GGA TTC T TAA ACA GGT GAG ACT CCA GCA ACT T

Abbreviations are in the text.

Table S2. Primers for mtDNA Determination

mtDNA specific (murine)	5’-CCG CAA GGG AAA GAT GAA AGA C-3’ 5’-TCG TTT GGT TTC GGG GTT TC-3’
nuclear specific (murine)	5’-GCC AGC CTC TCC TGA TTT TAG TGT-3’ 5’-GGG AAC ACA AAA GAC CTC TTC TGG-3’

Table S3. Primers for ChIP

SIRT1 -91	5’-TCC CGC AGC CGA GCC GCG GGG-3’ 5’-TCT TCC AAC TGC CTC TCT GGC CCT CCG-3’
human K19	5’-CAT TTC TCC ACC TCA CTG AAA CTG-3’ 5’-AAT GTG TTA GTG CAT GCA CAC AC-3’
murine K19	5’-AAG GGT GGA GGT GTC TTG GT-3’ 5’-GCT TCT TTA CAC TCC TGC T AAA-3’

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Cell Metabolism
Short Article dc_792_13

PARP-1 Inhibition Increases Mitochondrial Metabolism through SIRT1 Activation

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SUMMARY

SIRT1 regulates energy homeostasis by controlling the acetylation status and activity of a number of enzymes and transcriptional regulators. The fact that NAD^+ levels control SIRT1 activity confers a hypothetical basis for the design of new strategies to activate SIRT1 by increasing NAD^+ availability. Here we show that the deletion of the poly(ADP-ribose) polymerase-1 (*PARP-1*) gene, encoding a major NAD^+-consuming enzyme, increases NAD^+ content and SIRT1 activity in brown adipose tissue and muscle. *PARP-1*$^{-/-}$ mice phenocopied many aspects of SIRT1 activation, such as a higher mitochondrial content, increased energy expenditure, and protection against metabolic disease. Also, the pharmacologic inhibition of PARP in vitro and in vivo increased NAD^+ content and SIRT1 activity and enhanced oxidative metabolism. These data show how PARP-1 inhibition has strong metabolic implications through the modulation of SIRT1 activity, a property that could be useful in the management not only of metabolic diseases, but also of cancer.

INTRODUCTION

Intracellular NAD^+ levels control the activity of the type III deacetylase SIRT1, allowing it to act both as a metabolic sensor and effector (Yu and Auwerx, 2009). Overexpression studies indicated how activation of SIRT1 or of its orthologs extends life span in lower eukaryotes and protects against high-fat-diet (HFD)-induced metabolic disease in mice (Yu and Auwerx, 2009). These attractive properties spurred a quest to identify small-molecule SIRT1 agonists that could be used in situations of metabolic stress and damage. This strategy identified compounds like resveratrol or SRT1720 (Howitz et al., 2003; Milne et al., 2007), whose ability to directly interact with and activate SIRT1 is, however, debated (Pacholec et al., 2010). Therefore, a strong interest exists to develop alternative strategies to activate SIRT1. Given the NAD^+ dependency of SIRT1, another potential way to activate it is by increasing NAD^+ availability. This could be achieved by specifically inhibiting other NAD^+-consuming activities.

Poly(ADP-ribose) polymerase (PARP)-1 is a major cellular NAD^+ consumer (Sims et al., 1981). PARP-1 is activated upon binding to damaged or abnormal DNA (Durkacz et al., 1980) and catalyzes the formation of poly(ADP-ribose) polymers (PAR) onto different acceptor proteins, including PARP-1 itself (autoPARylation), using NAD^+ as substrate (Adamietz, 1987). PARP-1 activation depletes cellular NAD^+ levels, using it to form PAR (Sims et al., 1981). This led us to test the influence of PARP-1 on SIRT1 activity and metabolic homeostasis. Our results show how a reduction/ablation of PARP-1 activity boosts NAD^+ levels and SIRT1 activity, which in turn enhances mitochondrial content and function, culminating in a solid protection against metabolic disease.

RESULTS

PARP-1$^{-/-}$ Mice Are Leaner and Have Increased Energy Expenditure

Chow-fed *PARP-1*$^{-/-}$ mice (de Murcia et al., 1997) weighed less (Figure 1A) and accumulated less fat than *PARP-1*$^{+/+}$ littermates upon aging (Figure 1B), despite eating significantly more (Figure 1C). During indirect calorimetry, *PARP-1*$^{-/-}$ mice consumed more O_2 (Figure 1D), suggestive of higher energy expenditure (EE). Resting EE was not different (Figure S1A), indicating that the increase was due to changes at night, when mice are active. Accordingly, *PARP-1*$^{-/-}$ mice were more active at night (Figure S1B). In addition, the respiratory quotient was also higher in *PARP-1*$^{-/-}$ mice during the dark phase, indicating enhanced glucose oxidation during the feeding period (Figure 1E). *PARP-1*$^{-/-}$ mice also maintained a higher body temperature upon cold exposure (Figure 1F), were more glucose tolerant (Figure 1G), and had a trend toward lower fasting blood glucose levels (4.30 ± 0.17 mM in *PARP-1*$^{+/+}$ mice versus 3.98 ± 0.18 mM in *PARP-1*$^{-/-}$ mice; $p = 0.058$), despite similar insulin levels (data not shown). During euglycemic-hyperinsulinemic clamp, glucose infusion rates or hepatic glucose production were similar to *PARP-1*$^{+/+}$ mice,

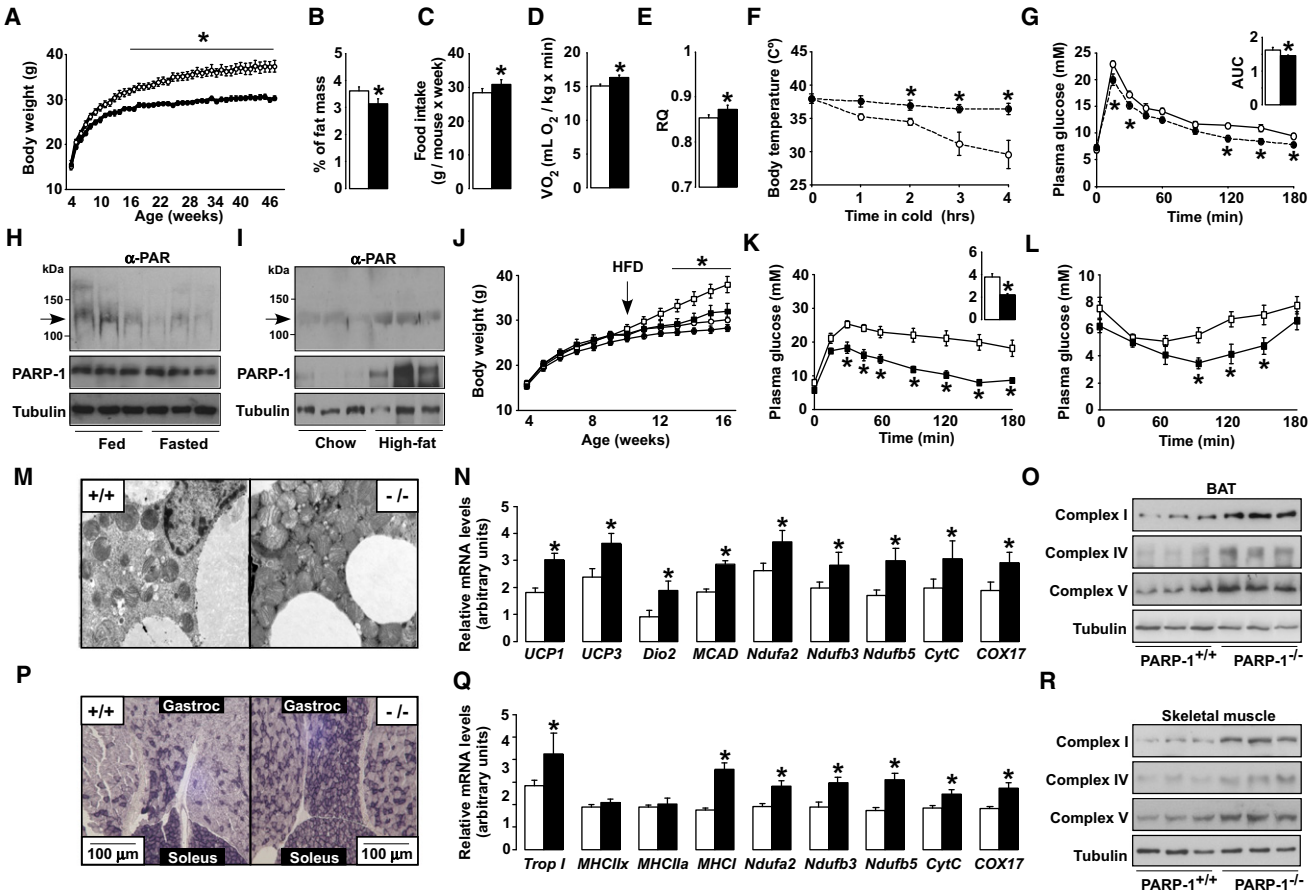

Figure 1. Phenotyping the PARP-1$^{-/-}$ Mice

(A) Body weight (BW) evolution in *PARP-1*$^{+/+}$ and $^{-/-}$ mice (n = 8/9).

(B) Epididymal white adipose tissue mass.

(C) Average food consumption.

(D and E) O_2 consumption (D) and respiratory quotient (RQ) (E) of *PARP-1*$^{+/+}$ and $^{-/-}$ mice (n = 9/9) determined by indirect calorimetry.

(F) Body temperature after exposure to 4°C (n = 6/5).

(G) Oral glucose tolerance test (OGTT) (n = 5/5) and the area under curve (AUC).

(H) PARP-1 autoPARylation (arrow), analyzed in 100 µg of protein extract from gastrocnemius muscles of 16-week-old mice fed ad libitum or fasted (24 hr). PARP-1 and tubulin levels were checked using 50 µg of protein extract.

(I) Gastrocnemius from mice on chow diet (CD) or HFD (12 weeks) were analyzed as in (H).

(J) BW evolution in *PARP-1*$^{+/+}$ and $^{-/-}$ mice (n = 10/10) fed a CD (circles) or HFD (squares) from 8 weeks of age.

(K and L) OGTT (K) and an insulin tolerance test (L) in HFD-fed *PARP-1*$^{+/+}$ and $^{-/-}$ mice at 16 weeks of age (n = 10/10). The AUC of the OGTT is shown on the top right.

(M–O) BAT from *PARP-1*$^{+/+}$ and $^{-/-}$ mice on CD was extracted and mitochondrial biogenesis was analyzed by transmission electron microscopy (M), mRNA expression of the genes indicated (N), and the abundance of mitochondrial complexes (O) in 25 µg of total protein extracts.

(P) SDH staining of sections from gastrocnemius and soleus of *PARP-1*$^{+/+}$ and $^{-/-}$ mice on CD.

(Q and R) Gastrocnemius was also used to analyze mRNA levels of the indicated genes (Q) and the abundance of mitochondrial complexes in 25 µg of total protein extracts (R). White bars represent *PARP-1*$^{+/+}$ mice; black bars represent *PARP1*$^{-/-}$ mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-1*$^{+/+}$ mice at $p < 0.05$. For abbreviations, see Table S1.

but supporting the idea of their better glucose tolerance, glucose uptake in *PARP-1*$^{-/-}$ muscle trended up (Figures S1C–S1E). In line with the lower fat mass and improved glucose tolerance, serum triglycerides (1.04 ± 0.07 mM in *PARP-1*$^{+/+}$ versus 0.84 ± 0.05 mM in *PARP-1*$^{-/-}$ mice; p = 0.048) and free fatty acids (FFA) (0.93 ± 0.09 mEq/l in *PARP-1*$^{+/+}$ versus 0.72 ± 0.03 mEq/l in *PARP-1*$^{-/-}$ mice; p = 0.040) were reduced in *PARP-1*$^{-/-}$ mice.

PARP-1 Is Induced by Nutrient Availability and Contributes to HFD-Induced Diabesity

The metabolic impact of *PARP-1* deletion made us evaluate whether nutrient scarcity (fasting) or overload (HFD) affects PARP-1 activity. Despite similar PARP-1 protein levels, a 24 hr fast sharply reduced PARP-1 autoPARylation levels, which reflect global PARP activity (Adamietz, 1987), suggesting a lower enzymatic activity (Figure 1H). In contrast, HFD robustly

increased PARP-1 protein levels and activity (Figure 1I), indicating a positive correlation between PARP-1 activity and nutrient availability.

As nutrient availability induces PARP-1 activity and *PARP-1* deletion prompts a leaner phenotype, we next explored how *PARP-1*$^{-/-}$ mice responded to HFD-induced metabolic disease. *PARP-1*$^{-/-}$ mice gained less weight after 2 months of HFD (Figure 1J), due to a lower fat accumulation (Figure S1F). Moreover, *PARP-1*$^{-/-}$ mice on HFD were more glucose tolerant (Figure 1K) insulin sensitized (Figure 1L) and had lower serum FFAs (0.66 ± 0.05 mEq/l versus 0.53 ± 0.03 mEq/l; p = 0.026).

Mitochondrial Activation in Brown Adipose Tissue and Muscle from PARP-1$^{-/-}$ Mice

The above results suggested improved mitochondrial activity in key metabolic tissues of *PARP-1*$^{-/-}$ mice, such as skeletal muscle and brown adipose tissue (BAT). *PARP-1*$^{-/-}$ mice had a relatively higher amount of BAT, with a more intense red appearance (Figure S1G). Transmission electron microscopy revealed higher mitochondrial content in *PARP-1*$^{-/-}$ BAT (Figure 1M), which was further corroborated by the increased mitochondrial DNA content (Figure S1H) and mRNA expression of genes involved in mitochondrial respiration (*Ndufa2*, *Ndufb2*, *Ndufb5*, *Cyt C*, *COX17*), uncoupling (*UCP1*, *UCP3*), fatty acid oxidation (*MCAD*), and thyroid hormone activation (*Dio2*). Mitochondrial biogenesis was also evidenced by the higher protein content of subunits from different respiratory complexes in the BAT from *PARP-1*$^{-/-}$ mice (Figure 1O).

Also, *PARP-1*$^{-/-}$ skeletal muscle had a marked oxidative profile. Succinate dehydrogenase (SDH) staining (Figure 1P) and the expression of muscle fiber isotype genes (*Trop I*, *MHCI*) (Figure 1Q) exposed an increase in oxidative fibers with a high mitochondrial content. As in BAT, the increased mitochondrial content was linked to an induction of the mRNA (Figure 1Q) and protein levels (Figure 1R) of mitochondrial components. In contrast to BAT and muscle, the expression of key metabolic genes was not altered in *PARP-1*$^{-/-}$ livers (Figure S1I), reflecting a minor role of PARP-1 in the liver, probably due to its very low expression (Figure S1J).

Higher NAD$^+$ Content and SIRT1 Activity in BAT and Muscle from PARP-1$^{-/-}$ Mice

The *PARP-1*$^{-/-}$ mice phenocopy many features seen after SIRT1 activation (Yu and Auwerx, 2009). As PARP-1 is a major NAD$^+$ consumer (Sims et al., 1981), we speculated that the lack of PARP-1 activity might increase NAD$^+$ content, in turn activating SIRT1. Illustrating how PARP-1 drives most PARP activity, the ablation of *PARP-1* reduced PARylation in both BAT and muscle (Figure 2A). The expression of the other PARP enzymes was not increased in *PARP-1*$^{-/-}$ BAT and muscle (Figures S2A and S2B), explaining the lack of compensation on PARylation. Confirming previous studies (Allinson et al., 2003; Fong et al., 2009), terminal dUTP nick-end labeling indicated that DNA damage was not increased in *PARP-1*$^{-/-}$ tissues (data not shown). In line with the attenuated NAD$^+$-consuming PARP activity, NAD$^+$ content was robustly increased in *PARP-1*$^{-/-}$ BAT and muscle (Figure 2B), while the levels of nicotinamide (NAM), a NAD$^+$-derived metabolite that inhibits sirtuin activity (Bitterman et al., 2002), remained unaffected (Figure 2C).

We next tested if the increase in NAD$^+$ correlated with SIRT1 activation. Indicative of SIRT1 activation, and supporting the higher mitochondrial content, PGC-1α acetylation levels in BAT and muscle of *PARP-1*$^{-/-}$ mice were reduced by ~40% and ~90%, respectively (Figures 2D and 2E). The acetylation of another SIRT1 target, forkhead box O1 (FOXO1), was also reduced by ~60% in BAT and ~40% in muscle (Figures 2D and 2E), supporting the idea that *PARP-1* deficiency leads to SIRT1 activation. Remarkably, SIRT1 protein was also robustly induced in *PARP-1*$^{-/-}$ BAT and muscle (Figures 2D and 2E), further amplifying SIRT1 activity.

As altered NAD$^+$ levels could also potentially impact other sirtuins, we also tested the activity of SIRT2 and SIRT3, which act as cytoplasmic and mitochondrial sirtuins, respectively. The acetylation level of tubulin, a SIRT2 target (North et al., 2003), was not altered in muscle from *PARP-1*$^{-/-}$ mice (Figure 2F). Likewise, the acetylation of complex I, a target for SIRT3 (Ahn et al., 2008), even showed a slight tendency to increase in *PARP-1*$^{-/-}$ muscles (Figure 2G). These results indicate that not all sirtuins are activated in *PARP-1*$^{-/-}$ tissues.

Reduced PARP-1 Activity in Cellular Models Enhances Oxidative Metabolism

Next, we knocked down *PARP-1* in HEK293T cells to evaluate whether an acute reduction in PARP-1 activity enhances oxidative metabolism. In these conditions, PARP-1 protein levels were reduced by ~80%, and the low PARP-1 autoPARylation demonstrated that PARP activity was largely blunted (Figure 3A). The reduced PARP activity was associated with enhanced NAD$^+$ content and SIRT1 function, as illustrated by decreased PGC-1α acetylation (Figures 3B and 3C). Importantly, this happened despite unchanged SIRT1 protein levels (Figure 3C) or changes in the activity of SIRT2 or SIRT3, as manifested in tubulin, Ndufa9, or total mitochondrial acetylation levels (Figures S3A–S3C). The induction of SIRT1 and PGC-1α activity culminated in a robust increase in mitochondrial DNA content (Figure 3D), mitochondrial-related gene expression (Figure 3E), and O_2 consumption (Figure 3F). Importantly, most of the metabolic effects elicited by PARP-1 depletion were lost when SIRT1 was simultaneously knocked down (Figures 3D–3F).

In line with the results in HEK293T cells, the expression of genes involved in mitochondrial function, mitochondrial DNA content, and O_2 consumption were also induced in *PARP-1*$^{-/-}$ compared to *PARP-1*$^{+/+}$ MEFs (Figures S3D–S3F). Consistent with our observations in tissues from *PARP-1*$^{-/-}$ mice, SIRT1 protein was also induced in *PARP-1*$^{-/-}$ MEFs (Figure S3G).

Pharmacological PARP Inhibition Enhances Oxidative Metabolism via SIRT1

To test the relation between SIRT1 and PARP-1 activities, we exposed C2C12 myotubes to H_2O_2 (500 μM, 6 hr), a well-known inducer of PARP-1 activity (Schraufstatter et al., 1986). H_2O_2 treatment vigorously increased PARP-1 (Figure 4A) and global protein PARylation levels (Figure 4B), as manifested by the slow migrating bands, in the absence of changes in PARP-1 levels (Figures 4A and 4B). Importantly, SIRT1 was not PARylated in response to H_2O_2 (Figure 4A), indicating that it is not a PARylation substrate. As reported (Schraufstatter et al., 1986), the H_2O_2-induced increase in PARP-1 activity sharply

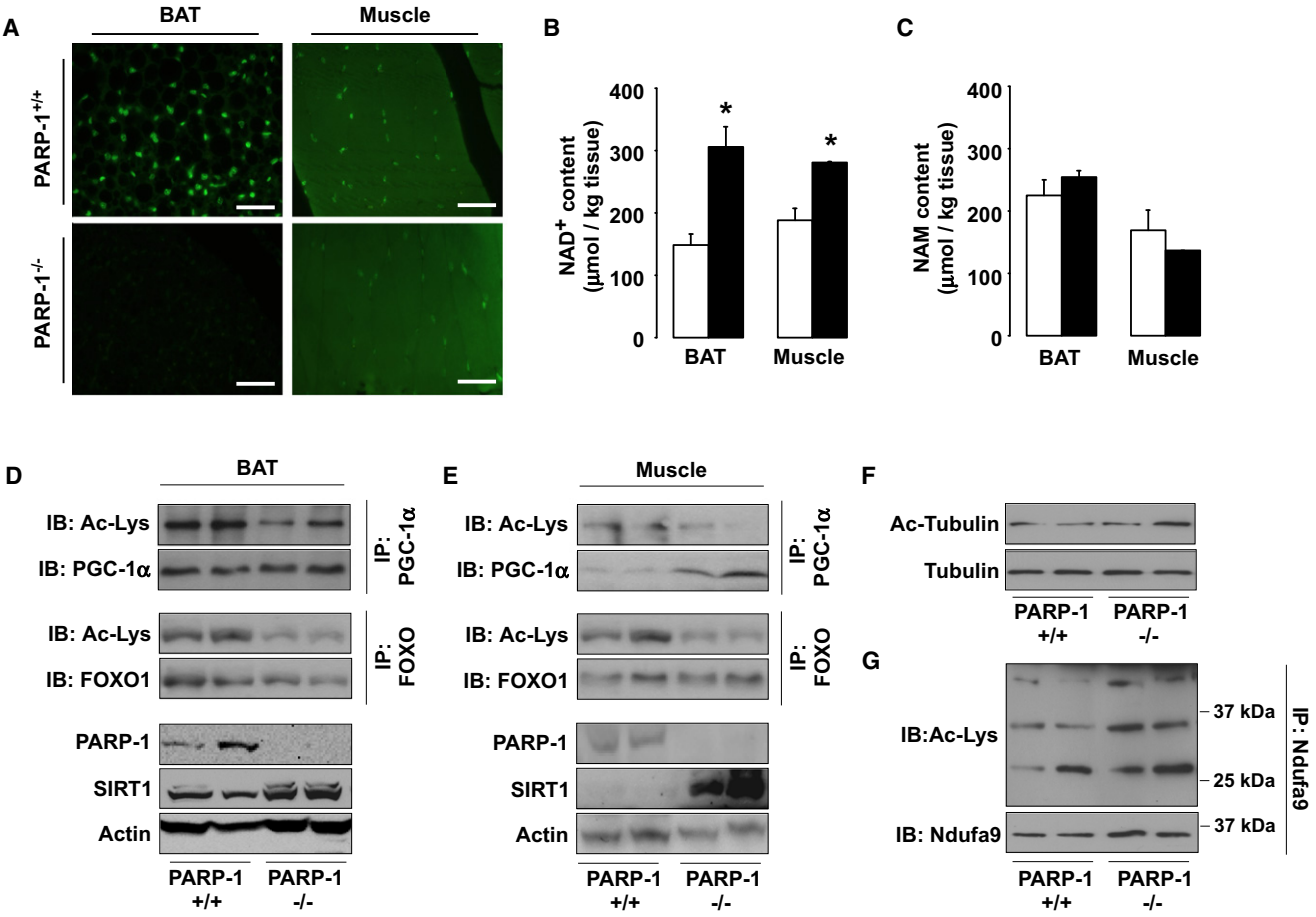

Figure 2. PARP-1 Deletion Raises NAD^+ Levels and Activates SIRT1

(A) Protein PARylation determined by α-PAR staining on formalin-fixed 7 μm BAT and muscle tissue sections of *PARP-1*$^{+/+}$ and $^{-/-}$ mice. White bar = 10 μm.

(B and C) NAD^+ (B) and NAM (C) levels in BAT and muscle from *PARP-1*$^{+/+}$ (white bars) and *PARP-1*$^{-/-}$ (black bars) mice determined by mass spectrometry.

(D and E) PARP-1, SIRT1, and actin protein content in BAT (D) and muscle (E) were determined by western blot, using 100 μg of protein lysate. PGC-1α and FOXO1 acetylation were examined by immunoprecipitation.

(F) Tubulin and acetylated-tubulin levels were tested in *PARP-1*$^{+/+}$ and $^{-/-}$ gastrocnemius.

(G) The Ndufa9 subunit of mitochondrial complex I was immunoprecipitated from 400 μg of total protein from gastrocnemius, and acetylation levels were analyzed by western blot. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus *PARP-1*$^{+/+}$ mice at $p < 0.05$.

depleted NAD^+ content (Figure 4C), but did not affect SIRT1 protein levels (Figures 4A and 4B). This lower NAD^+ availability limited SIRT1 activity, as reflected in PGC-1α hyperacetylation (Figure 4D). Interestingly, the inhibition of PARP activity with PJ34 (Garcia Soriano et al., 2001) rescued the drop in NAD^+ and recovered SIRT1 function during H_2O_2 exposure (Figures 4B–4D). These results indicate that PARP-1 activation restrains SIRT1 activity and that PARP inhibitors relieve this limitation.

PARP-1 activity is not necessarily linked to DNA damage, and it has been shown to fluctuate in a circadian fashion (Asher et al., 2010). Therefore, we wondered whether prolonged PARP inhibition, even in the absence of DNA damage, would favor NAD^+ accumulation and, potentially, SIRT1 activity. Supporting this premise, PARP inhibition by PJ34 (Figure 4E) or a structurally unrelated compound, TIQ-A (data not shown), gradually raised NAD^+ levels, becoming significant after 24 hr. At that time, PARP activity, but not PARP-1 protein levels, was robustly decreased (Figure 4F). PJ34 increased NAD^+ levels dose dependently, in correlation with SIRT1 activity, as illustrated by PGC-1α deacetylation (Figure 4G). Similar effects also happened in vivo, as treatment of mice with PJ34 (10 mg/kg, b.i.d. for 5 days) blunted basal PARP activity in muscle (Figure 4H), while increasing NAD^+ and SIRT1 activity (Figure 4I). Despite the short duration of the treatment, serum triglyceride (1.21 ± 0.08 mM vehicle versus 1.11 ± 0.04 mM PJ34; $p = 0.08$) and FFA levels (1.59 ± 0.06 mEq/l vehicle versus 1.44 ± 0.03 mEq/l PJ34; $p = 0.03$) were reduced in PJ34-treated mice. Of note, while compounds like resveratrol impact SIRT1 through AMP-activated protein kinase (AMPK) (Cantó et al., 2010), PJ34 did not alter AMPK activity, as reflected by the unchanged acetyl-CoA carboxylase (ACC) phosphorylation in either C2C12 myotubes (Figure 4J) or gastrocnemius muscle (Figure 4H). PJ34 did not affect SIRT1 protein levels either (Figures 4H and 4J), but robustly induced the expression of mitochondrial and lipid oxidation genes, both in C2C12 myotubes and muscle (Figures 4K and 4L). Consistent with PGC-1α deacetylation and activation,

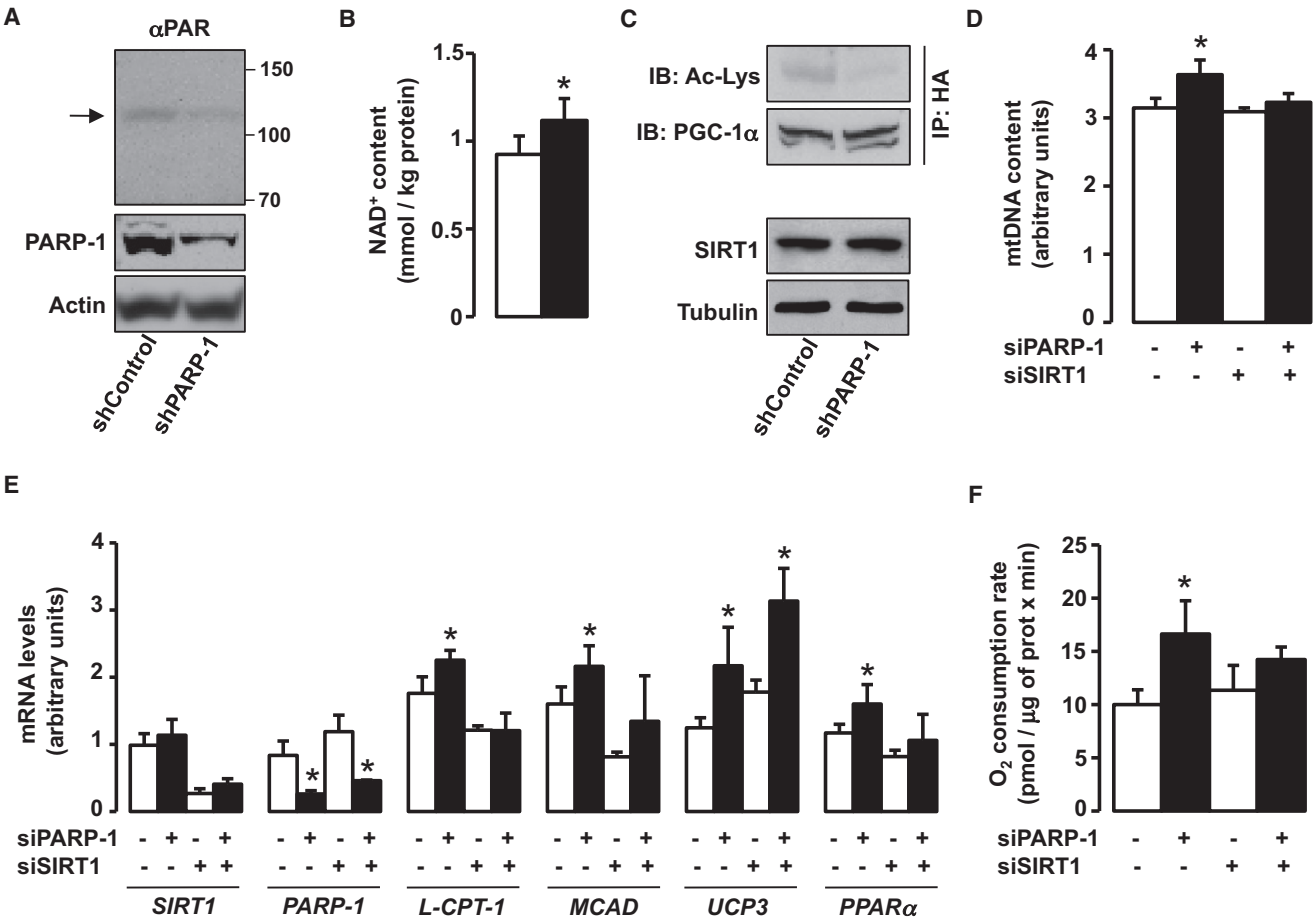

Figure 3. PARP-1 Knockdown Promotes SIRT1 Activity and Oxidative Metabolism

(A–C) HEK293T cells were transfected with either scramble (control) or *PARP-1* shRNA and HA-PGC-1α expression vector for 48 hr. Then, PARP-1 protein levels and autoPARylation (arrowhead) were analyzed in total protein lysates (A). Intracellular NAD^+ was measured on total acid extracts (B). PGC-1α acetylation was analyzed in HA immunoprecipitates (C).

(D–F) HEK293T cells were transfected with either a pool of *PARP-1* siRNAs, a pool of *SIRT1* siRNAs, or different combinations of both using the corresponding scramble siRNAs as control (−). The cells were simultaneously transfected with HA-PGC-1α for 48 hr. Then, relative mitochondrial DNA content (D), mRNA levels of the genes indicated (E), and total O_2 consumption (F) were analyzed. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus respective control sh/siRNA-transfected cells at $p < 0.05$.

PJ34 promoted the recruitment of PGC-1α to target gene promoters (e.g., PDK4) (Figure S4A). Finally, the activation of SIRT1/PGC-1α by PJ34 culminated in higher O_2 consumption rates (Figure 4M), testifying for enhanced oxidative metabolism.

The effect of PJ34 on PGC-1α acetylation in C2C12 myotubes was blunted upon *SIRT1* knockdown (Figure 4J). The role of SIRT1 in mediating PJ34-induced PGC-1α deacetylation was further confirmed in *SIRT1*$^{-/-}$ MEFs, where PJ34 was unable to decrease PGC-1α acetylation (Figure S4B). In line with impaired PGC-1α activation, mitochondrial gene expression and O_2 consumption were largely unresponsive to PJ34 upon SIRT1 depletion in C2C12 cells (Figures 4K and 4M) and in *SIRT1*$^{-/-}$ MEFs (Figures S4C and S4D), indicating that SIRT1 is a key mediator of PJ34 action. However, PJ34 also had SIRT1-independent effects, as reflected by the persistent increase in *UCP3* mRNA even after the *SIRT1* knockdown (Figure 4K). This could be explained by the fact that PJ34 does not regulate *UCP3* expression by recruitment of PGC-1α to its promoter (Figure S4A). The pharmacological inhibition of PARP recapitulates the phenotypic characteristics of the *PARP-1*$^{-/-}$ mice and reveals that these effects are largely mediated by SIRT1.

DISCUSSION

The difficulty of identifying compounds that specifically and directly bind and activate SIRT1 led us to test whether the modulation of NAD^+ availability could be an alternative path to activation of SIRT1. Our present work supports this concept by showing how the attenuation of PARP-1, another NAD^+-consuming enzyme, increases intracellular NAD^+ levels and enhances SIRT1 activity. This prompts the deacetylation and activation of key metabolic transcriptional regulators, such as PGC-1α and FOXO1, leading to increased mitochondrial content and metabolism.

Our data suggest that PARP-1 limits NAD^+ availability for SIRT1 activation. This concept originates from the differences

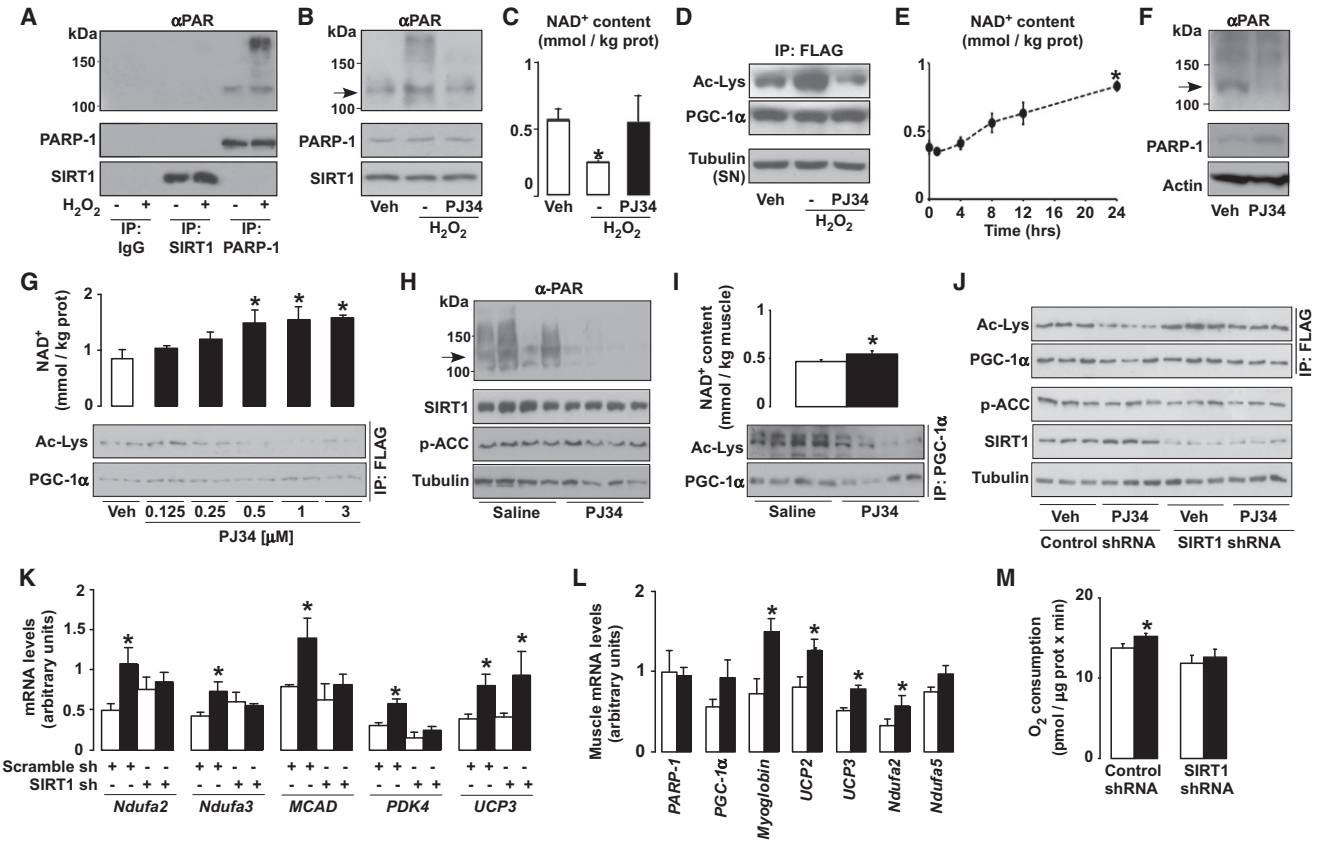

Figure 4. PARP-1 Inhibition Enhances Mitochondrial Function through SIRT1

(A–D) C2C12 myotubes expressing FLAG-HA-PGC-1α were treated for 6 hr with either PBS (vehicle [Veh]), H_2O_2 (500 μM), or H_2O_2 and PJ34 (1 μM). Then, SIRT1, PARP-1, and unspecific IgG immunoprecipitates from 500 μg of protein extracts were used to test PARylation and the proteins indicated (A). Proteins were analyzed in total cell extracts, and the arrow indicates PARP-1 autoPARylation (B). NAD^+ content was measured (C), and PGC-1α acetylation was tested in FLAG immunoprecipitates (D). Tubulin was checked on the supernatants as input.

(E) C2C12 myotubes were treated with PJ34 (1 μM) for the times indicated, and NAD^+ levels were evaluated in acidic extracts.

(F and G) C2C12 myotubes expressing FLAG-HA-PGC-1α were treated for 24 hr with PBS (Veh) or with PJ34 (1 μM, unless stated otherwise). PARP-1 protein and autoPARylation (arrow) were determined by western blot (F), and NAD^+ content and PGC-1α acetylation were measured (G).

(H and I) Ten-week-old mice received PJ34 (10 mg/kg b.i.d, i.p.) or saline (Veh) for 5 days before sacrifice (n = 10/10); then PARP-1 autoPARylation (arrow), p-ACC, and SIRT1 levels were determined in 100 μg of total protein extracts from gastrocnemius (H), and NAD^+ and PGC-1α acetylation were determined (I). PGC-1α was immunoprecipitated using 2 mg of protein from gastrocnemius muscle and 5 μg of antibody.

(J and K) C2C12 myotubes expressing FLAG-HA-PGC-1α and either a control or a SIRT1 shRNA were treated with PJ34 for 48 hr. Then, PGC-1α acetylation levels were quantified in FLAG immunoprecipitates (J), and 50 μg of total protein extracts was used to measure the other markers indicated; mRNA levels of selected genes were quantified (K).

(L) Mice were treated as in (H), and mRNA of selected genes was determined in gastrocnemius.

(M) C2C12 myotubes were treated as in (J), and cellular O_2 consumption was measured. White bars represent Veh; black bars represent PJ34 treatment. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus Veh group at $p < 0.05$. For abbreviations, see Table S1.

in the K_M and k_{cat}/K_M of both enzymes for NAD^+, which indicate that PARP-1 is a faster and more efficient NAD^+ consumer than SIRT1 (Knight and Chambers, 2001; Smith et al., 2009). Therefore, it is likely that PARP-1 activity maintains NAD^+ at limiting levels for SIRT1 function. The prediction that *PARP-1* deletion would increase NAD^+ levels and activate SIRT1 is perfectly matched by our data. While previous work already speculated on a link between PARP-1 and sirtuin activities (Kolthur-Seetharam et al., 2006; Pillai et al., 2005), our study expands the consequences of this link to energy homeostasis. In apparent discrepancy, one report (Devalaraja-Narashimha and Padanilam, 2010) suggested that *PARP-1*$^{-/-}$ mice could be more susceptible to HFD-induced obesity. However, that study used mice on an SV129 background, which are less suited for metabolic studies than C57BL/6J mice. The convergent results of our genetic, physiological, pharmacological, and in vitro studies clearly support our conclusions.

Results from our lab indicate that the activation of SIRT1 after a bout of exercise or cold exposure is not linked to decreased PARP-1 activity (data not shown). Rather, only robust and/or protracted changes, such as pharmacological (PJ34) or genetic (knockdown or deletion) PARP-1 inhibition, influence SIRT1 activity. While PARP-1 might not always participate in the physiological modulation of SIRT1 activity, our data suggest that the

interplay between both proteins could be exploited pharmacologically.

Our work illustrates the way in which SIRT1 is a key mediator of the PARP-1-deficient phenotype, but the link between PARP-1 and SIRT1 activities is still unclear. Several models used in this work show that reducing PARP-1 activity controls SIRT1 function, independent of changes in SIRT1 protein levels. In all these cases, the levels of NAD^+, the rate-limiting coenzyme for SIRT1, correlated with SIRT1 activity, suggesting that NAD^+ availability might influence SIRT1 activity. If this were true, boosting NAD^+ content through alternative strategies should elicit similar metabolic phenotypes to those of the *PARP-1*$^{-/-}$ mice. Supporting this notion, deletion of another NAD^+ consumer, CD38, also activates SIRT1 (Aksoy et al., 2006), resulting in protection against HFD-induced obesity (Barbosa et al., 2007). Nutrient scarcity and AMPK activation also lead to increased NAD^+ levels and SIRT1 activation coupled to the induction of oxidative metabolism (Cantó et al., 2009, 2010). This correlative evidence indicates that the increased NAD^+ availability might be a key mechanism by which PARP deficiency activates SIRT1. However, we cannot exclude the possibility that PARP inhibition also impacts SIRT1 via other means, even though our results rule out direct PARylation of SIRT1 as the mechanism (Figure 4A). In addition, SIRT1 content was increased in *PARP-1*$^{-/-}$ tissues and MEFs, further amplifying SIRT1 activity. The reason for this increase in SIRT1 levels remains elusive, but is independent of changes in SIRT1 mRNA (P.B., C.C., and J.A., unpublished data).

Of note, *PARP-1* depletion affects the activity of SIRT1, but not that of SIRT2 and SIRT3, which occupy nonnuclear compartments. If increased SIRT1 activity was mainly driven by changes in NAD^+, the unchanged SIRT2 and SIRT3 activities in *PARP-1*$^{-/-}$ tissues suggest that the increase in NAD^+ is either not enough to enhance SIRT2 and SIRT3 activities or that it only happens in specific cellular compartments, supporting an independent regulation of different subcellular NAD^+ pools (Yang et al., 2007). Alternatively, PARP-1 and SIRT1 activities might not be linked by changes in NAD^+, and some yet unfound mechanism drives the specificity toward this sirtuin.

Some results indicate that the effects of PARP deficiency cannot be completely explained by SIRT1 (Figure 4K). Future research will have to clarify the nature of these SIRT1-independent effects of PARP inhibition on metabolism. It will be interesting to explore whether PARylation can directly modulate the activity of key metabolic transcriptional regulators, as PARP-1 may contribute to nuclear processes other than DNA repair (Krishnakumar and Kraus, 2010).

PARP inhibitors are currently in clinical development as antitumoral drugs (Fong et al., 2009). While our data encourages a possible utilization of PARP inhibitors as therapeutic agents to activate SIRT1 and promote oxidative metabolism, this should be taken cautiously. PARP-1 has key roles in genomic maintenance, and while neither this nor previous studies (Allinson et al., 2003) detected enhanced DNA damage in *PARP-1*$^{-/-}$ mice under basal conditions, it cannot be ignored that *PARP-1*$^{-/-}$ mice are sensitive to ionizing radiation (de Murcia et al.,1997). Hence, it will be important to analyze the impact of aging and metabolic disease on DNA damage to establish the therapeutic potential and limitations of PARP inhibition.

EXPERIMENTAL PROCEDURES

Detailed materials and procedures can be found in the Supplemental Information.

Animal Experiments

Pure C57BL/6J male mice were used for the study. *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ were described (de Murcia et al., 1997). Mice were housed separately, had ad libitum access to water and chow (10 kcal% of fat) (SAFE, Augy, France) or HFD (60 kcal% of fat) (Research Diets, Inc., New Brunswick, NJ), and were kept in a 12 hr dark/light cycle. Animal experiments were carried out according to local, national, and EU ethical guidelines. Animals were sacrificed after 6 hr of fast, and tissues were collected and processed as specified.

NAD^+ and NAM Determination

NAD^+ levels in cultured cells were determined using an enzymatic method (EnzyChrom, BioAssays Systems, Hayward, CA), whereas for tissues (Figure 4), NAD^+ and NAM levels were determined as described (Sauve et al., 2005).

Statistics

All data were verified for normal distribution. Statistical significance was assessed by Student's t test for independent samples. Values are expressed as mean ± SEM unless otherwise stated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, four figures, and three tables and can be found with this article online at doi:10.1016/j.cmet.2011.03.004.

ACKNOWLEDGMENTS

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Supplemental Information

Cell Metabolism, *Volume 13*

PARP-1 Inhibition Increases Mitochondrial Metabolism through SIRT1 Activation

Péter Bai, Carles Cantó, Hugues Oudart, Attila Brunyánszki, Yana Cen, Charles Thomas, Hiroyasu Yamamoto, Aline Huber, Borbála Kiss, Riekelt H. Houtkooper, Kristina Schoonjans, Valérie Schreiber, Anthony A. Sauve, Josiane Menissier-de Murcia, and Johan Auwerx

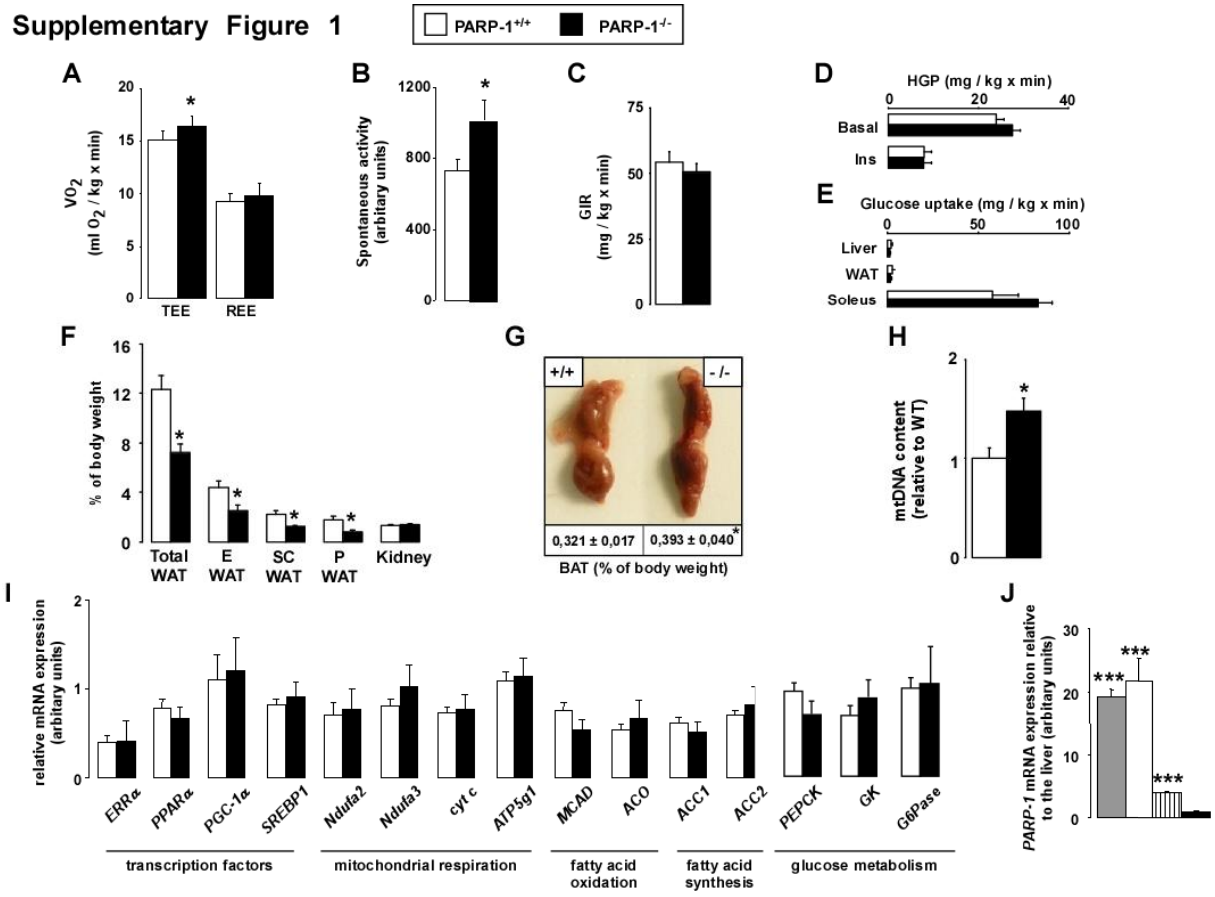

Figure S1, Related to Figure 1. Increased Spontaneous Locomotor Activity and Energy Expenditure in the *PARP-1*$^{-/-}$ Mice during Night

(A) Oxygen consumption was determined in *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (n=6/6) as described in the Materials and Methods. TEE – total energy expenditure, REE – resting energy expenditure.

(B) Spontaneous activity was determined during indirect calorimetry in CLAMS using *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (n=9/9).

(C-E) Peripheral and hepatic insulin responsiveness of *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ male mice was assed by euglycemic-hyperinsulinemic clamp. (C) Glucose infusion rates (GIR), (D) hepatic glucose production (HGP) and (E) glucose uptake in different tissues are all shown as mean +/- SEM.

(F) The total WAT mass, individual WAT depots, and organ weights were determined upon autopsy (E – epididymal, SC – subcutaneous, P – perirenal).

(G) BAT was photographed and weighed after autopsy of *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (11,5 months of age, n=8/9 males). BAT content (relative to total body weight), is shown at the bottom of the image.

(H) BAT mitochondrial DNA (mtDNA) was quantified by qPCR in the BAT.

(I) mRNA expression levels of selected genes were quantified by RT-qPCR reactions in the liver of *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (n=9/9). Abbreviations are listed in the text and in Table S1.

(J) mRNA expression of *PARP-1* were quantified in different metabolic tissues of C57Bl/6J male mice (n=5) (black bar – liver; dashed bar – BAT; white bar – skeletal muscle; grey bar – testis). Asterisks indicate significant difference at * $p<0,05$; *** $p<0.001$.

Supplementary Figure 2

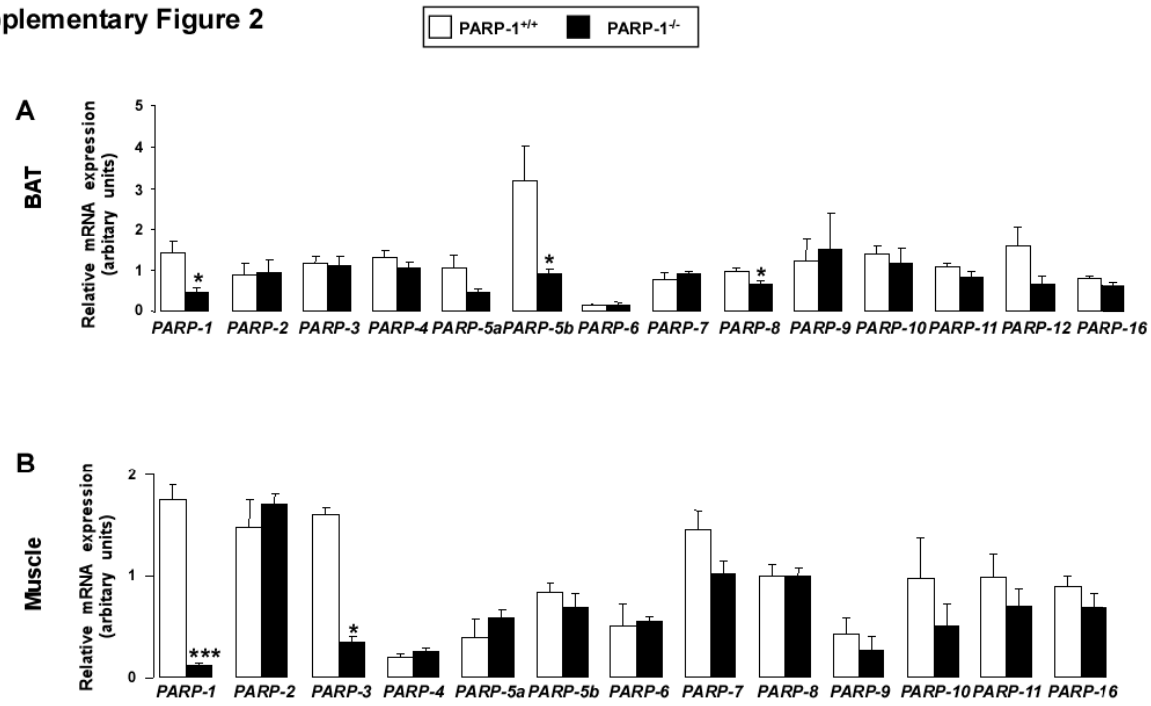

Figure S2, Related to Figure 2. Gene Expression Pattern of the Different Members of the PARP Family in the BAT and Gastrocnemius Muscle

(A-B) RT-qPCR reactions were performed on cDNA populations from the BAT (A) and the gastrocnemius muscle (B) of *PARP-1*$^{+/+}$ and $^{-/-}$ male mice (n=7/5). Asterisks indicate significant difference between cohorts, where * p<0,05; *** p<0.001.

Supplementary Figure 3

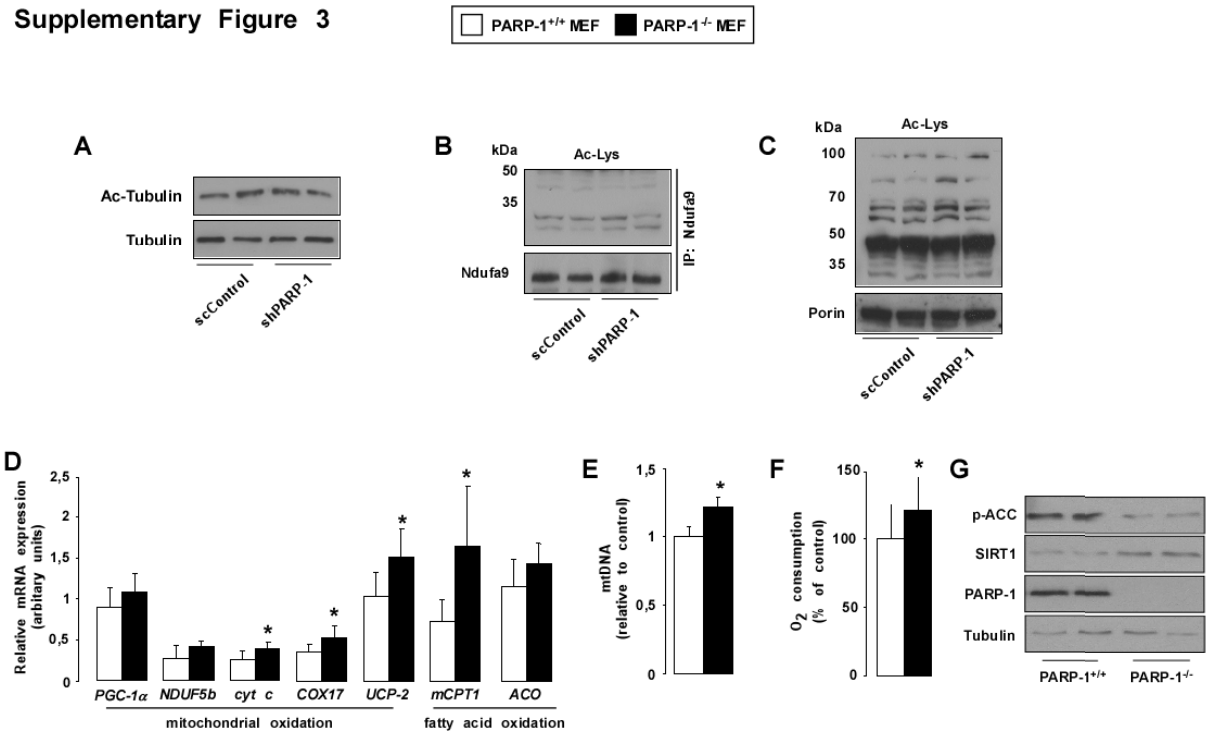

Figure S3, Related to Figure 3. Assessment of Protein Acetylation in HEK293T Cells and Measurement of Mitochondrial Function and Protein Levels in *PARP-1*$^{+/+}$ *and* $^{-/-}$ MEFs

(A-C). HEK293T were transfected with either control or PARP-1 shRNAs. 48 hrs later, total and mitochondrial extracts were obtained.

(A) 50 mg of total extracts were used to test tubulin acetylation levels.

(B) 400 mg of total extracts were used to immunoprecipitate Ndufa9, using 3 mg of antibody, and evaluate lysisne actylation on the immunoprecipitates.

(C) 50 ug of mitochondrial extracts were used to test global lysine acetylation and porin levels (as input).

(D-F) In *PARP-1*$^{+/+}$ and $^{-/-}$ primary MEFs (n=3/3) mRNA expression (D), mitochondrial DNA content (E), oxygen consumption (F) pACC, SIRT1 and PARP-1 protein levels (G) was determined. Abbreviations are listed in the text. Asterisks indicate significant difference between cohorts, where * $p<0,05$, ** $p<0,01$.

Supplementary Figure 4

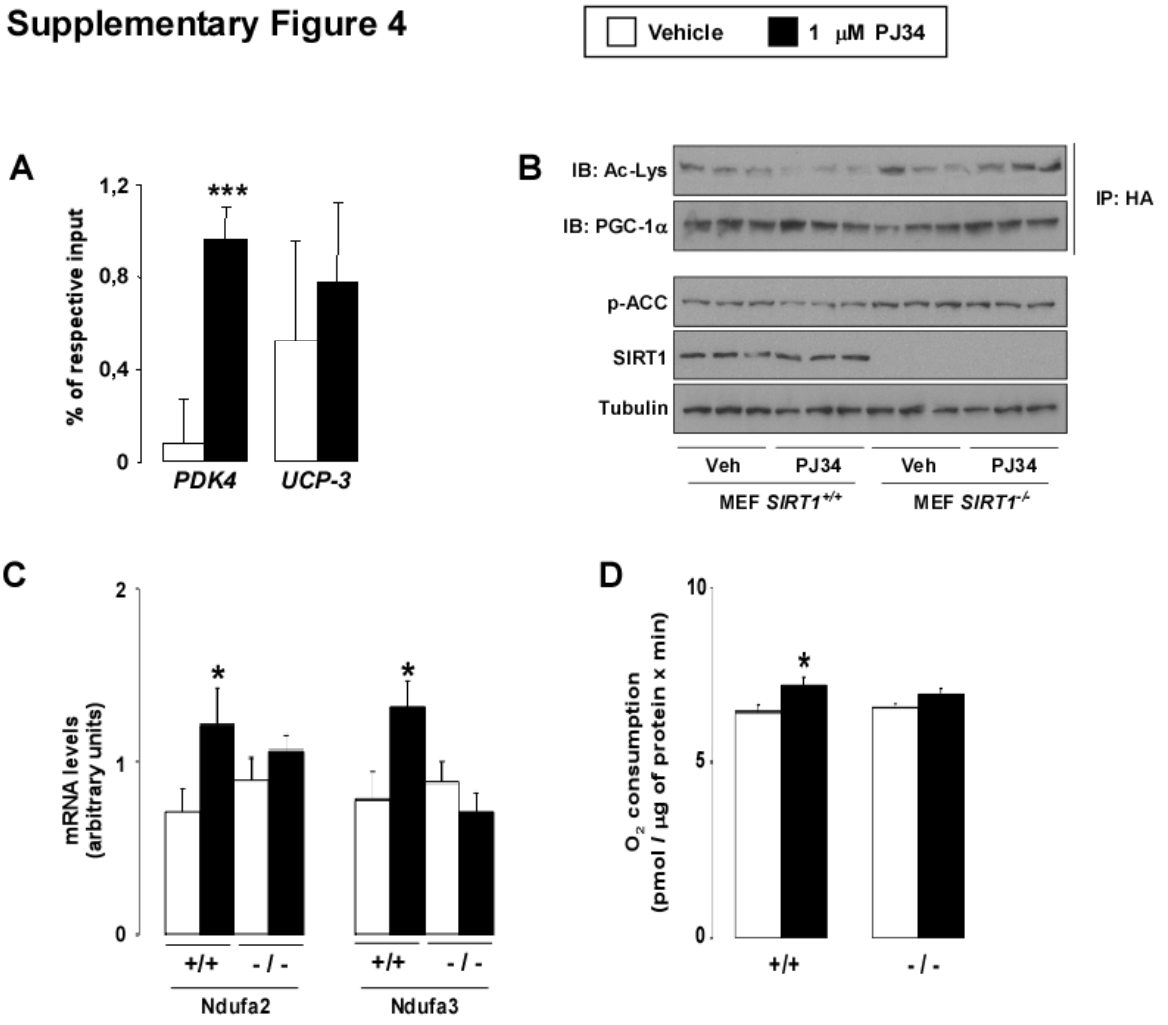

Figure S4, Related to Figure 4. Assessment of Mitochondrial Function upon Pharmacological PARP Inhibition in C2C12 Cells and SIRT1$^{-/-}$ MEFs

(A) Promoter occupancy of PGC-1α was quantified after PJ34 treatment on the *PDK4* and *UCP-3* promoters (1 μM, 48h) (n=3/3).

(B-D) PGC-1α acetylation, ACC phosphorylation, SIRT1 protein levels (B), expression of mitochondrial protein mRNAs (C) and O_2 consumption (D) were determined in *SIRT1*$^{+/+}$ and $^{-/-}$ MEF cells , treated with PBS (Veh) or PJ34 for 48 hrs.

Table S1. qRT-PCR Primers for Quantification of Gene Expression

All primers are designed for murine sequences unless otherwise specified.

Gene	**Primers**
18S (human)	5'-CGG CTA CCA CAT CCA AGG AA-3' 5'-CCT GTA TTG TTA TTT TTC GTC ACT ACC T-3'
ACC1 ***acetyl-CoA carboxylase -1***	5'-GAC AGA CTG ATC GCA GAG AAA G-3' 5'-TGG AGA GCC CCA CAC ACA-3'
ACC2 ***acetyl-CoA carboxylase-2***	5'-CCC AGC CGA GTT TGT CAC T-3' 5'-GGC GAT GAG CAC CTT CTC TA-3'
ACO ***Acyl-CoA oxidase***	5'-CCC AAC TGT GAC TTC CAT T-3' 5'-GGC ATG TAA CCC GTA GCA CT-3'
ATP5g1	5'-GCT GCT TGA GAG ATG GGT TC-3' 5'-AGT TGG TGT GGC TGG ATC A-3'
COX17	5'-CGT GAT GCG TGC ATC ATT GA-3' 5'-CAT TCA CAA AGT AGG CCA CC-3'
Cyclophyllin B	5'-TGG AGA GCA CCA AGA CAG ACA -3' 5'-TGC CGG AGT CGA CAA TGA T-3'
cyt c ***Cytochrome C***	5'-TCC ATC AGG GTA TCC TCT CC-3' 5'-GGA GGC AAG CAT AAG ACT GG-3'
Dio2 ***Deiodinase-2***	5'-GCA CGT CTC CAA TCC TGA AT-3' 5'-TGA ACC AAA GTT GAC CAC CA -3'
ERRα ***estrogen receptor related receptor α***	5'-ACTGCCACTGCAGGATGAG-3' 5'-CACAGCCTCAGCATCTTCAA-3'
GK ***Glucokinase***	5'-ACA TTG TGC GCC GTG CCT GTG AA-3' 5'-AGC CTG CGC ACA CTG GCG TGA AA-3'
G6Pase ***- glucose-6 phosphatase***	5'-CCG GAT CTA CCT TGC TGC TCA CTT T-3' 5'-TAG CAG GTA GAA TCC AAG CGC GAA AC-3'
L-CPT-1 (human) ***Liver Carnitine Palmitoyl Transferase***	5'-CAG GCG AGA ACA CGA TCT TC-3' 5'-GCG GAT GTG GTT TCC AAA G-3'
MCD ***malonyl-CoA decarboxylase***	5'-TGG ATG GCT GAC AGC AGC CTC AA-3' 5'-CTG AGG ATC TGC TCG GAA GCT TTG-3'
MCAD (human) ***Medium chain acyl-coenzyme A dehydrogenase***	5'-AGA ATT GGC TTA TGG ATG TAC AGG-3' 5'-TTT GTT GAT CAT TTC CAG CAA TAA T-3'
MCAD ***Medium chain acyl-coenzyme A dehydrogenase***	5'-GAT CGC AAT GGG TGC TTT TGA TAG AA-3' 5'-AGC TGA TTG GCA ATG TCT CCA GCA AA-3'
mCPT1 ***Carnitine Palmitoyl Transferase-1***	5'-TTG CCC TAC AGC TGG CTC ATT TCC -3' 5'-GCA CCC AGA TGA TTG GGA TAC TGT-3'
MHCI ***Myosin heavy chain 1***	5'-GAG TAG CTC TTG TGC TAC CCA GC -3' 5'-AAT TGC TTT ATT CTG CTT CCA CC -3'
MHCIIA ***Myosin heavy chain 2A***	5'-GCA AGA AGC AGA TCC AGA AAC-3' 5'-GGT CTT CTT CTG TCT GGT AAG TAA GC-3'
MHCIIX ***Myosin heavy chain 2X***	5'-GCA ACA GGA GAT TTC TGA CCT CAC-3' 5'-CCA GAG ATG CCT CTG CTT C-3'
Ndufa2	5'-GCA CAC ATT TCC CCA CAC TG-3' 5'-CCC AAC CTG CCC ATT CTG AT-3'
Ndufb3	5'-TAC CAC AAA CGC AGC AAA CC-3'

	5’-AAG GGA CGC CAT TAG AAA CG-3’
Ndufb5	5’-CTT CGA ACT TCC TGC TCC TT-3’ 5’-GGC CCT GAA AAG AAC TAC G-3’
PARP-1 (human)	5’-GCT CCT GAA CAA TGC AGA CA-3’ 5’-CAT TGT GTG TGG TTG CAT GA-3’
PARP-1	5’-GGA GCT GCT CAT CTT CAA CC-3’ 5’-GCA GTG ACA TCC CCA GTA CA-3’
PARP-2	5’-GGA AGG CGA GTG CTA AAT GAA-3’ 5’-AAG GTC TTC ACA GAG TCT CGA TTG-3’
PARP-3	5’-CCT GCT GAT AAT CGG GTC AT-3’ 5’-TTG TTG TTG TTG CCG ATG TT-3’
PARP-4	5’-GTT AAA TTT TGC ACT CCT GGA G-3’ 5’-AAT GTG AAC ACT GTC AAG AGG AAC A-3’
PARP-5a	5’-TAG AGG CAT CGA AAG CTG GT-3’ 5’-CAG GCA TTG TGA AGG GG-3’
PARP-5b	5’-GGC CCT GCT TAC ACC ATT G-3’ 5’-CGT GCT TGA CCA GAA GTT CA-3’
PARP-6	5’-TTT CCA GCC ATC GAA TAA GG-3’ 5’-ACC ACT TGC CTT GAA CCA AC-3’
PARP-7	5’-AAA ACC CCT GGA AAT CAA CC-3’ 5’-AGA AGG ATG CGC TTC TGG TA-3’
PARP-8	5’-TCC ACC ATT AAA TCG CAC AA-3’ 5’-GCT CCA TTT TCG ATG TCT TG-3’
PARP-9	5’-ACC TGA AGA ATG GCC TAT TAC ATG G-3’ 5’-ACA GCT CAG GGT AGA GAT GC-3’
PARP-10	5’-CAA GAT CCT GCA GAT GCA AA-3’ 5’-TTG GAG AAG CAC ACG TTC TG-3’
PARP-11	5’-CAA TGA GCA GAT GCT ATT TCA TG-3’ 5’-CAC CAA TTA GCA CTC GAG CA-3’
PARP-12	5’-CGG ATC CAG AAC ATG GGC-3’ 5’-GGC ATC TCT CGC AAA GTA GC-3’
PARP-14	5’-GGC AAA CGC AAT GGA ACT AT-3’ 5’-AGC ACG TTC CTA AGC CTT GA-3’
PARP-16	5’-CCG TGT GCC TTA TGG AAA CT-3’ 5’-TGG ATT GTG TCT GGG CAC-3’
PDK4 ***pyruvate dehydrogenase kinase, isoenzyme 4***	5’-AAA GGA CAG GAT GGA AGG AAT CA-3’ 5’-ATT AAC TGG CAG AGT GGC AGG TAA-3’
PEPCK ***Phosphoenolpyruvate carboxykinase***	5’-CCA CAG CTG CTG CAG AAC A-3’ 5’-GAA GGG TCG CAT GGC AAA-3’
PGC-1α ***peroxisome proliferator-activated receptor gamma, coactivator 1α***	5’-AAG TGT GGA ACT CTC TGG AAC TG-3’ 5’-GGG TTA TCT TGG TTG GCT TTA TG-3’
PPARα (human) ***peroxisome proliferator-activated receptor α***	5’-TCA TCA AGA AGA CGA GTC G-3’ 5’-CGG TTA CCT ACA GCT CAG AC-3’
PPARα ***peroxisome proliferator-activated receptor α***	5’-CCT GAA CAT CGA GTG TCG AAT AT-3’ 5’-GGT TCT TCT TCT GAA TCT TGC AGC T-3’
SIRT1 (human)	5’-TAG GCG GCT TGA TGG TAA TC-3’

	5’-TCT GGC ATG TCC CAC TAT CA-3’
SREBP1 ***sterol regulatory element binding transcription factor 1***	5’-GGC CGA GAT GTG CGA ACT-3’ 5’-TTG TTG ATG AGC TGG AGC ATG T-3’
Trop I ***Troponin I***	5’-CCA GCA CCT TCA GCT TCA GGT CCT TGA T-3’ 5’-TGC CGG AAG TTG AGA GGA AAT CCA AGA T-3’
UCP1 ***Uncoupling protein-1***	5’-GGC CCT TGT AAA CAA CAA AAT AC-3’ 5’-GGC AAC AAG AGC TGA CAG TAA AT-3’
UCP2 ***Uncoupling protein-2***	5’-TGG CAG GTA GCA CCA CAG G-3’ 5’-CAT CTG GTC TTG CAG CAA CTC T-3’
UCP3 (human) ***Uncoupling protein-3***	5’-GTG ACC TAC GAC ATC CTC AAG G-3’ 5’-GCT CCA AAG GCA GAG ACA AAG-3’
UCP3 ***Uncoupling protein-3***	5’-ACT CCA GCG TCG CCA TCA GGA TTC T-3’ 5’-TAA ACA GGT GAG ACT CCA GCA ACT T-3’

Table S2. Primers for mtDNA Determination

mtDNA specific (murine)	5’-CCG CAA GGG AAA GAT GAA AGA C-3’ 5’-TCG TTT GGT TTC GGG GTT TC-3’
nuclear specific (murine)	5’-GCC AGC CTC TCC TGA TTT TAG TGT-3’ 5’-GGG AAC ACA AAA GAC CTC TTC TGG-3’
mtDNA specific (human)	5’-CTA TGT CGC AGT ATC TGT CTT TG-3’ 5’-GTT ATG ATG TCT GTG TGG AAA G-3’
nuclear specific (human)	5’-GTT TGT GTG CTA TAG ATG ATA TTT TAA ATT G-3’ 5’-CAT TAA ACA GTC TAC AAA ACA TAT-3’

Table S3. ChIP Primers

PDK4	5’-AAC CCT CCT CCC TCT CAC CCT-3’ 5’-ACA CCA ATC AGC TCA GAG AA-3’
UCP-3	5’-GAA TGT CAG GCC TCT AAG AA-3’ 5’-CAG GAG GTG TGT GAC AGC AT-3’

Supplemental Experimental Procedures

Animal Experiments

To monitor body weight, mice were weighed and the food consumption was measured each week on the same day. In case of PJ34 treatment, mice received each 12h (at 7:00 and 19:00) 10 mg/kg PJ34 by intraperitoneal injection for 5 continuous days. Oral glucose tolerance test, intraperitoneal insulin tolerance test, free fatty acid (FFA) and triglycerides were determined as described (Lagouge et al., 2006). Plasma insulin and was determined in heparinized plasma samples using specific ELISA kits (*Mercodia*). Thermoadaptation was performed as described (Lagouge et al., 2006). We measured O_2 consumption, CO_2 production, and spontaneous locomotor activity in an open-circuit indirect calorimetry system (*Sabre systems*, Las Vegas, NV, USA) over 24-48h as described (Dali-Youcef et al., 2007; Lagouge et al., 2006; Watanabe et al., 2006). Energy expenditure was obtained by using an energy equivalent of 20.1 J / ml O_2. The respiratory quotient was the ratio of CO_2 production over O_2 consumption. During actimetry, beamline crossings were summarized each 15 minutes. The sum of beamline crosses for each 15 min period were plotted against time and AUC was calculated for each mouse that was averaged in each experimental cohort. Euglycemic-hyperinsulinemic clamps were performed in *PARP-1*$^{-/-}$ and $^{+/+}$ male mice (n = 4 mice per genotype; age = 4 months) exactly as previously described (Feige et al., 2008). In all studies animals were killed (at 14:00) either after CO_2 inhalation or cervical dislocation after 6h of fasting (starting at 8:00), and tissues were collected and their weight was expressed relative to the body weight.

Histology and Microscopy

Haematoxylin-eosine (HE) and succinate dehydrogenase (SDH) staining was performed on frozen-section- and paraffin-fixed 7 μm sections, as described (Lagouge et al., 2006). Transmission electron microscopy (TEM) investigation was performed on glutaraldehyde-

fixed, osmium tetroxyde stained ultrafine sections (Watanabe et al., 2006). Aspecific binding of the secondary antibody was controlled on sections where the primary antibody was omitted.

Cell Culture, Transfection, Adenoviral Infection and Mitochondrial Characterization

HEK293T, MEF and C2C12 cells were cultured in DMEM (4,5 g/l glucose, 10% FCS). *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ MEFs were prepared as described in (Menissier-de Murcia et al., 1997). *SIRT1*$^{-/-}$ and *SIRT1*$^{+/+}$ MEFs were kindly provided by Fred Alt (Chua et al., 2005). HEK293T cells were transfected using JetPei reagent (*Polyplus Transfections*, Illkirch, France) according to the manufacturer's instructions. C2C12 cells were differentiated in DMEM (4,5 g/L glucose, 2% horse serum) after reaching confluency for 2 days, followed by 2 days of PJ34 treatment (10 μM). PARP-1 shRNA constructs were described in (Shah et al., 2005). Human PARP-1 and SIRT1 siRNAs were obtained from Dharmacon (Thermo Scientific). The adenovirus encoding for FLAG-HA-PGC-1α, control and SIRT1 shRNAs were a kind gift from Pere Puigserver and were used (MOI = 100) in C2C12 myotubes as described (Canto et al., 2009). DNA strand breaks were quantified by TUNEL assays according to the manufacturer's instructions (*Millipore*).

mRNA and mtDNA Analysis

Total RNA was prepared using TRIzol (*Invitrogen*) according to the manufacturer's instructions. RNA was treated with DNase, and 2 μg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (*Qiagen*, Valencia, CA, USA). 50X diluted cDNA was used for RT-quantitative PCR (RT-qPCR) reactions (Bai et al., 2007). The RT-qPCR reactions were preformed using the Light-Cycler system (*Roche Applied Science*) and a qPCR Supermix (*Qiagen*) with the primers summarized in Table S1. mtDNA quantification was performed as described (Lagouge et al., 2006) with the primers indicated in Table S2.

Immunoprecipitation, SDS-PAGE, Western Blotting

Cells were lysed in lysis buffer (50 mM Tris, 100 mM KCl, EDTA 1 mM, NP40 1%, nicotinamide 5 mM, Na-butyrate 1 mM, protease inhibitors pH 7,4). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The origin of the primary and secondary antibodies used can be found bellow. Reactions were developed by enhanced chemiluminescence (*Amersham*, Little Chalfont, UK). PGC-1α, FOXO1 and Ndufa9 acetylation levels were analyzed by immunoprecipitation from cellular or nuclear lysates of tissues with anti-PGC-1α (Millipore), anti-FOXO1 (*Cell Signalling*, Danvers, MA, USA) and anti-Ndufa9 (*Abcam*) antibody followed by Western blot using an acetyl-lysine antibody (*Cell Signalling*) that was normalized to total PGC-1α/FOXO1/Ndufa9 levels. In HEK293T cells HA-tagged PGC-1α was overexpressed and was immunoprecipitated using an anti-HA. In C2C12 myotubes, FLAG-HA tagged PGC-1α was introduced through adenoviral delivery 2 days before treatments, then IP was performed using anti-FLAG antibody and samples were processed as described. All blots were quantified by densitometry using ImageJ software.

Antibodies Used for Western Blot Applications

PARP-1 (Erdelyi et al., 2009), PAR (*Alexis*, Lausanne, Switzerland), SIRT1 (*Millipore*), FOXO1 (*Cell Signalling*), haemagglutinin (*Sigma*), p-ACC (*Upstate*), Complex I (Ndufa9) (*Abcam*), Complex IV (COXI) and V (α subunit) (*Molecular probes*), FLAG (*Sigma*), and actin (*Sigma*) were detected using a polyclonal rabbit antibodies. The secondary antibody was IgG-peroxidase conjugate (*Sigma*, 1:10000).

Poly(ADP-Ribose) Detection

PAR was detected either by using a monoclonal anti-PAR antibody (Alexis) and Mouse-on-mouse kit (Vector Laboratories) on 7 μm formalin-fixed tissues using as described in (Garcia et al., 2001) or by Western blotting of total protein lysates (Erdelyi et al., 2009).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed according to (Bai et al., 2007). FLAG-HA-PGC-1α (Rodgers et al., 2005) was introduced by adenoviral transfer into C2C12 myotubes after 48h of differentiation and cells were cultured for an additional 2 days. Cell were then exposed to 1 μM PJ34 in saline for 24h. Thereafter ChIP was performed using anti-FLAG (*Sigma*) and anti-TNF-R1 (*Santa Cruz*) as described (Bai et al., 2007). Pelleted DNA was quantified by qPCR using the primers against PDK4 and UCP-3 promoters flanking the nuclear receptor site (Table S3). The results were normalized for the signal of the respective inputs (vehicle/PJ34–treated) and were expressed as a percentage. The signal of anti-TNF-R1 (non-specific antibody) was subtracted from the anti-FLAG signal (specific) and the specific signal was plotted.

NAD^+ and NAM Determination

NAD^+ levels in cultured cells were determined using a commercial kit (*Enzychrom*, BioAssays Systems, CA). For tissue samples NAD^+ and NAM levels were determined as described in (Sauve et al., 2005). In brief, to a weighed aliquot of frozen pulverized tissue we added as standards, O^{18}-NAD^+ (typically 2,00 nmol) and O^{18}-NAM (typically 2,00 nmol). 70 μL of ice-cold 7% perchloric acid was then added and the sample was vortexed and sonicated three times, then centrifuged. Clear supernatant was removed and neutralized by additions of 3 M NaOH and 1 M phosphate buffer (pH=9), then centrifuged. Clear supernatant was injected onto HPLC C-18 column with 20 mM ammonium acetate eluent to separate NAD^+ and NAM from other cellular components, NAD^+ and NAM peaks (260 nm absorbance) were collected. Collections were lyophilized to dryness and subjected to MALDI-TOF analysis. For NAD^+ measurement, ratio of intensities for m/z = 664 and 666 peaks, corresponding to ^{16}O- and ^{18}O-NAD^+ isotopomers, was multiplied by 2,00 nmol and then divided by tissue weight to determine NAD^+ concentration in the sample. For NAM the ratio of intensities for m/z = 123 and 125 peaks, corresponding to ^{16}O- and ^{18}O-NAM isotopomers, was multiplied by 2,00

nmol and then divided by tissue weight to determine NAM concentration in the sample. Corrections were applied for isotopic abundance.

Oxygen Consumption in Cultured Cells

Cellular O_2 consumption was measured using a Seahorse bioscience XF24 analyzer with thirty biological replicates per condition, in 24 well plates at 37°C, exactly as described (Canto et al., 2009). C2C12 were infected with an adenovirus encoding for FLAG-HA-PGC-1α and either scramble or SIRT1 shRNAs 48h previous to O_2 consumption measurements. Then myotubes were treated with 1 μM PJ34 for 48h.

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Poly(ADP-ribose) polymerase-2 depletion reduces doxorubicin-induced damage through SIRT1 induction

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Aims Doxorubicin (DOX) is widely used in cytostatic treatments, although it may cause cardiovascular dysfunction as a side effect. DOX treatment leads to enhanced free radical production that in turn causes DNA strand breakage culminating in poly(ADP-ribose) polymerase (PARP) activation and mitochondrial and cellular dysfunction. DNA nicks can activate numerous enzymes, such as PARP-2. Depletion of PARP-2 has been shown to result in a protective phenotype against free radical-mediated diseases, suggesting similar properties in the case of DOX-induced vascular damage.

Methods and results *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice and aortic smooth muscle (MOVAS) cells were treated with DOX (25 mg/kg or 3 μM, respectively). Aortas were harvested 2-day post-treatment while MOVAS cells were treated with DOX for 7 hours. Aortas from *PARP-2*$^{-/-}$ mice displayed partial protection against DOX toxicity, and the protection depended on the conservation of smooth muscle but not on the conservation of endothelial function. DOX treatment evoked free radical production, DNA breakage and PARP activation. Importantly, depletion of PARP-2 did not quench any of these phenomena, suggesting an alternative mechanism. Depletion of PARP-2 prevented DOX-induced mitochondrial dysfunction through SIRT1 activation. Genetic deletion of PARP-2 resulted in the induction of the SIRT1 promoter and consequently increased SIRT1 expression both in aortas and in MOVAS cells. SIRT1 activation enhanced mitochondrial biogenesis, which provided protection against DOX-induced mitochondrial damage.

Conclusion Our data identify PARP-2 as a mediator of DOX toxicity by regulating vascular SIRT1 activity and mitochondrial biogenesis. Moreover, to the best of our knowledge, this is the first report of SIRT1 as a protective factor in the vasculature upon oxidative stress.

Keywords PARP-2 • Doxorubicin • SIRT1 • Mitochondria • Vascular smooth muscle

1. Introduction

Doxorubicin (adriamycin, DOX) is a widely used antitumor drug, exerting cardiovascular side effects that may culminate in irreversible degenerative cardiomyopathy and heart failure.[1] DOX-induced cardiovascular injury is linked to increased oxidative stress[2] as DOX can export electrons from the mitochondria via redox cycling, creating radical species.[3] Although cardiac effects of DOX toxicity are well characterized, vascular damage is scarcely mapped and the molecular background is largely unknown. Vascular damage upon DOX treatment affects the aorta[4,5] and smaller arteries in the liver[6] and kidney.[7,8] Furthermore, DOX impairs endothelial function[9] and promotes thrombosis.[10]

Free radical-evoked DNA damage activates poly(ADP-ribose) polymerase (PARP)-1, which catabolizes NAD^+ in order to create poly(ADP-ribose) (PAR) polymers. PARP-1 activation leads to

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NAD^+ and consequently ATP depletion, provoking cell dysfunction and cell death.[11] This PARP-1-mediated cell death pathway is active in DOX-induced toxicity and it is a major contributor to cardiac and vascular dysfunction.[12,13] Therefore, pharmacological inhibition of PARP-1 proved to be advantageous under such circumstances.[12,13] Upon DOX damage, PARP inhibition partially restored vascular contractility.[13]

PARP-2 is a nuclear enzyme that can bind to DNA nicks and thereby becomes active.[14] Upon activation, it is capable of synthesizing PAR at the expense of NAD^+.[14] Moreover, *PARP-2*$^{-/-}$ mice are resistant to numerous oxidative stress-related pathologies in the brain and intestines.[15–17] These properties of PARP-2 prompted us to examine whether PARP-2 inactivation may also provide protection against DOX-induced vascular damage.

2. Methods

All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

2.1 Animal experiments

All animal experiments were carried out according to the national and EU ethical guidelines and were authorized by the Institutional Ethics Committee (7/2010 DE MÁB). Homozygous female *PARP-2*$^{-/-}$ and littermate *PARP-2*$^{+/+}$ mice[18] derived from heterozygous crossings were kept in a 12/12 h dark–light cycle with *ad libitum* access to water and food. Mice were randomly assigned to four groups: *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ control (CTL), and *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ DOX-treated. DOX treatment was performed by the injection of 25 mg/kg DOX or saline ip as described.[12] Aortas were harvested 2-day post-injection for further assessment.

2.2 Aorta ring studies

For aorta ring studies, mice were anaesthetized by thiopental (50 mg/kg, iv). Mice were dissected after they did not respond to pain. Thoracic aortas were cut into 4 mm rings in an organ chamber and were fixed on an isometric contractile force measurement system (DMT 510A, Aarhus, Denmark) by metal wires. Aortic rings were stretched according to the manufacturer's instructions. Fixed, stretched aortic rings were treated with the indicated agents for the indicated times and contractile force was recorded.

2.3 Histology and microscopy

Staining was performed on 7 μm tissue sections or cells using antibodies against PAR, PARP-2 (both from Alexis, Lausen, Switzerland), and smooth muscle actin (SMA; Novocastra, Newcastle upon Tyne, UK) as in Bai *et al.*[19]

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was performed following the instructions of the manufacturer (Millipore).

2.4 Cell culture

MOVAS murine aortic smooth muscle cells were obtained from ATCC and were cultured in DMEM (4.5 g/L glucose, 10% FCS, and 0.2 mg/mL G418). PARP-2 silencing was performed as in Bai *et al.*[19] employing shPARP-2 (small hairpin) and scPARP-2 (scrambled) shRNA by lentiviral delivery. Cellular measurements took place 7 h after addition of DOX.

2.5 Measurement of thiobarbituric acid reactive species

The extent of oxidative stress was assessed by determining lipid peroxidation. Aortas were homogenized in 1.15% KCl. Homogenates were cleared and supernatants were used. Supernatants were incubated at 90°C for 45 min with thiobarbituric acid. After cooling to room temperature, absorbance at 532 nm was measured and normalized to protein content. The level of lipid peroxides was expressed as a percentage of control.

2.6 PARP activity measurement

PARP activity in MOVAS cells was measured by the incorporation of ^{3}H-NAD^+. Cells stimulated with DOX (3 μM, 7 h) or H_2O_2 (1 mM, 10 min) and were incubated with ^{3}H-NAD^+ (10 min, 37°C). After incubation, cellular proteins were precipitated and subsequently washed with ice-cold TCA (50 and 5% w/v, respectively). Precipitates were solubilized in 250 μL 2% w/v SDS/0.1 N NaOH at 37°C overnight. Tritium incorporation was measured by liquid scintillation counting.

2.7 Measurement of superoxide production

Superoxide was measured using hydroethidine (HE) staining as described in Bai *et al.*[20] Cells were induced by DOX (7 h, 3 μM) and were stained by 2 μM HE for 30 min. Fluorescence was analysed by flow cytometry (FACSCalibur, BD Biosciences). Superoxide production was indicated as a mean of HE fluorescence in each sample.

2.8 Oxygen consumption

Oxygen consumption was measured using an XF96 oximeter (Seahorse Biosciences, North Billerica, MA, USA). scPARP-2 and shPARP-2 MOVAS cells were seeded in 96-well assay plates. After recording the baseline oxygen consumption, cells received a single bolus dose of DOX (0.3–30 μM final concentration). Then, oxygen consumption was recorded every 30 min to follow DOX toxicity. Final reading took place at 7 h post-treatment. The oxygen consumption rate was normalized to protein content and normalized readings were displayed.

2.9 NAD^+ measurement

Cells were seeded in six-well plates and treated with 3 μM DOX for 7 h. Control and DOX-treated cells were homogenized in 500 μL 0.5 N $HClO_4$ solution and the homogenates were then neutralized with 150 μL 3 M KOH. The concentration of NAD^+ was measured photometrically at 560 nm after enzymatic reaction, which is based on an alcohol dehydrogenase cycling reaction in which a tetrazolium dye is reduced by NADH in the presence of phenazine methosulfate. Samples were normalized to protein content and NAD^+ concentration was determined using an NAD^+ standard curve.

2.10 Luciferase activity measurement

Luciferase activity measurement took place as described in Bai *et al.*[19] with modifications as follows. MOVAS cells carrying a stable transfection of a scrambled or specific PARP-2 shRNA were seeded in six-well plates. Transfection took place (8 μg SIRT1 −91 promoter construct[21] and 4 μg β-galactosidase expression plasmid per 9 μL JetPEI transfection reagent (Polyplus Transfection, Illkirch, France) after cells reached ~60% confluence. Cells were scraped 48 h post-transfection and luciferase activity was determined by standard procedures. The activity was expressed as luciferase activity/β-galactosidase activity.

2.11 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined by tetramethylrhodamine ethyl ester (TMRE) staining.[22] MOVAS cells carrying a stable transfection of a scrambled or specific PARP-2 shRNA were seeded in 96-well plate (25 000 cells/well) and induced by DOX (0.3–30 μM, 7 h). After DOX treatment, cells were stained with 25 nM TMRE for 30 min and then were washed with Hanks' balanced salt solution (0.138 M NaCl, 5.33 mM KCl, 0.338 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, and

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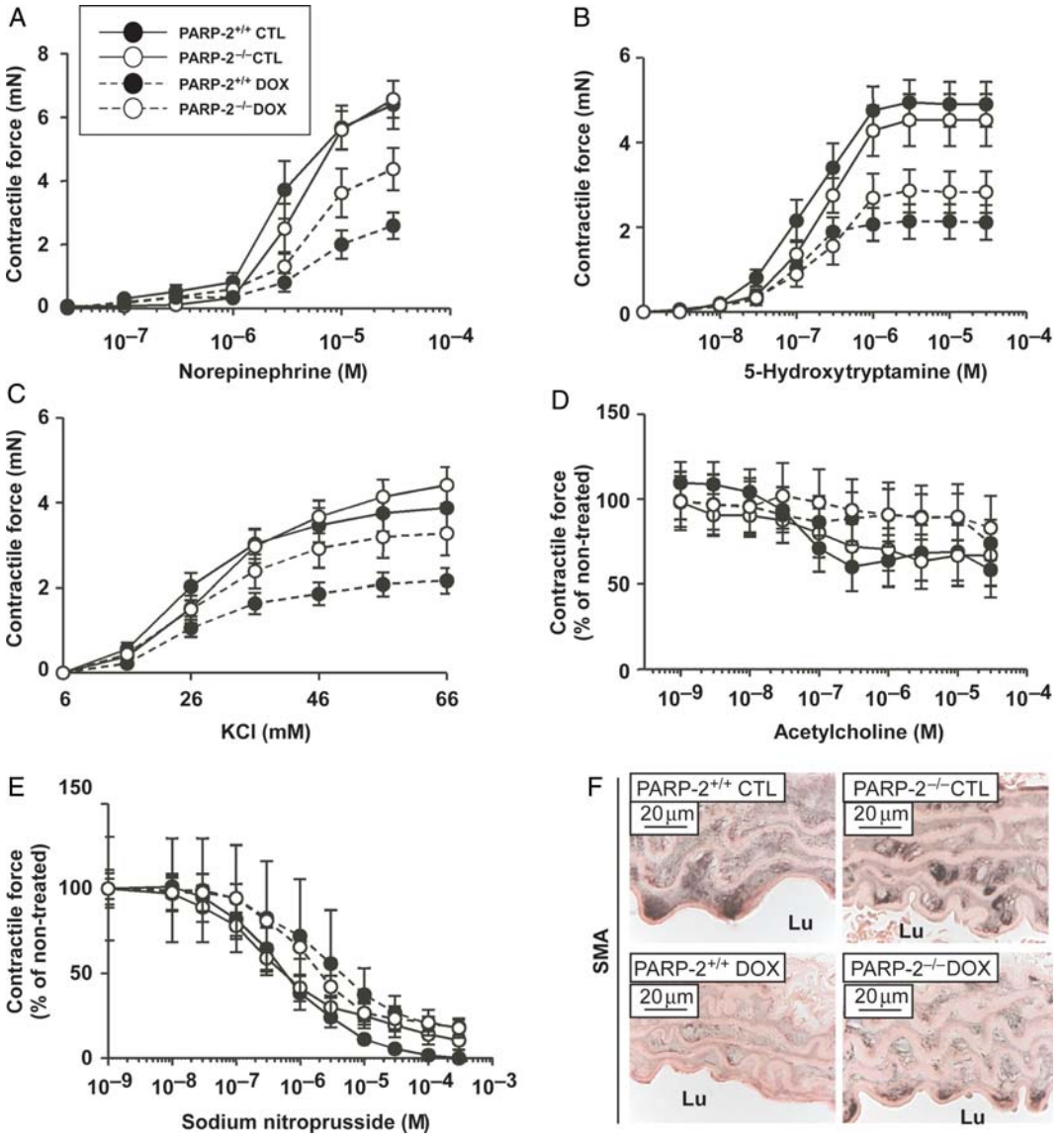

Figure 1 Genetic deletion of PARP-2 protects against DOX-induced aortic dysfunction. *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice (3 months of age) were injected with saline (CTL) or with 25 mg/kg DOX ($n = 10$ for *PARP-2*$^{+/+}$ CTL, $n = 7$ for *PARP-2*$^{-/-}$ CTL, $n = 8$ for *PARP-2*$^{+/+}$ DOX, and $n = 8$ for *PARP-2*$^{-/-}$ DOX). Aortas were harvested 2-day post-injection. Aortic rings were mounted on an isometric contractile force measurement system (*A*–*E*) or embedded in paraffin (*F*). Aortic contractile responses (smooth muscle function) were tested by the cumulative application of norepinephrine (*A*), 5-hydroxytryptamine (*B*), and potassium chloride (*C*) and endothelial function was tested by acetylcholine (*D*) and smooth muscle NO sensitivity by sodium nitroprusside (*E*). SMA was visualized by immunohistochemistry (*F*). Luminal side is indicated (Lu), scale bar equals 20 μm.

4.17 mM $NaHCO_3$). Fluorescence was measured on 530 nm as excitation and 590 nm as emission wavelength. TMRE fluorescence was normalized to protein content.

2.12 Protein extraction and western blotting

Cells were lysed with RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, and freshly added protease inhibitor cocktail (1:100, Sigma, St Louis, USA). Cells were left on ice for several minutes in the buffer and then were homogenized with a 22 G needle. Lysates were cleared by centrifugation. Extracts were separated by SDS–PAGE and were blotted as in Erdelyi *et al.*[23] Blots were probed with the following antibodies: SIRT1 (1:1000, Millipore-Upstate, Billerica, MA, USA), actin (1:1000, Sigma), and PARP-2 (1:1000, PARP-2, Alexis).

2.13 Total RNA preparation, reverse transcription, and RT-qPCR

Total RNA preparation, reverse transcription, and RT-qPCR were performed similarly as in Brunyanszki *et al.*[24] Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of RNA were used for reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Diluted cDNA was used for RT-qPCR. Expression was normalized to the geometric mean of murine 36B4, 18S, and cyclophylin.

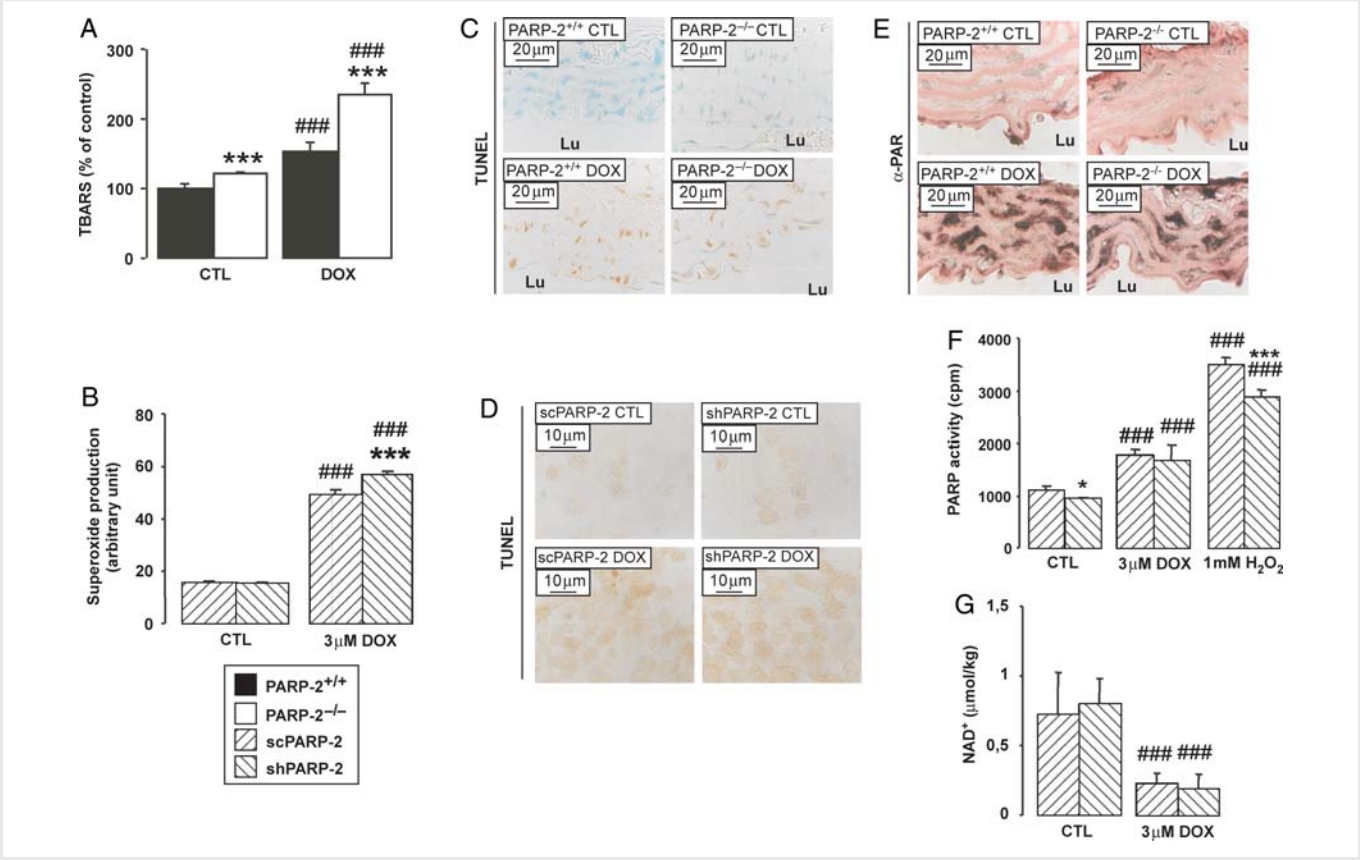

Figure 2 Deletion of PARP-2 does not affect free radical-induced PARP activation or NAD^+ depletion. *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice were injected with saline (CTL) or with 25 mg/kg DOX at 3 months of age ($n = 5$ for *PARP-2*$^{+/+}$ CTL, $n = 5$ for *PARP-2*$^{-/-}$ CTL, $n = 5$ for *PARP-2*$^{+/+}$ DOX, and $n = 4$ for *PARP-2*$^{-/-}$ DOX; *A*, *C*, and *E*). An aortic smooth muscle cell line (MOVAS) was also tested (*B*, *D*, *F*, and *G*; $n = 3$ parallel measurements). MOVAS cells were transduced with a PARP-2-silencing (shPARP-2) or -scrambled (scPARP-2) shRNA and treated with solvent (CTL), 3 μM DOX or with 1 mM H_2O_2. Measurements were performed 2-day post-DOX injection (aortas) or 7 h (MOVAS) after DOX treatment. Free radical formation was measured by determining thiobarbituric acid reactive species (TBARS) (*A*) and HE fluorescence (*B*). DNA breaks were detected using TUNEL assay; scale bar represents 20 or 10 μm, respectively (*C* and *D*). PAR formation in paraffin-embedded aortas was assessed with an anti-PAR antibody; scale bar represents 20 μm (*E*), or in MOVAS cells by the PARP enzyme activity assay (*F*). NAD^+ concentrations were determined in MOVAS cells using an alcohol dehydrogenase-coupled colorimetric assay (*G*). Lu, lumen; ### indicate statistically significant difference between CTL and DOX/H_2O_2-treated samples, at $P < 0.001$, * or *** indicate statistically significant difference between *PARP-2*$^{+/+}$ mice/scPARP-2 cells and *PARP-2*$^{-/-}$ mice/shPARP-2 cells at $P < 0.05$ or <0.001, respectively. On (*B*), (*F*), and (*G*), error is represented as SD.

Mitochondrial DNA content was determined in qPCRs using total DNA extract of cells or tissues as described in Bai *et al.*[19] Primers are summarized in Supplementary material online, *Tables S1* and *S2*.

2.14 Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed similarly as in Bai *et al.*[25] In MOVAS cells, chromatin-bound proteins were fixed on DNA by formaldehyde. Nuclei were prepared and chromatin was sonicated in order to split DNA into shorter, ~500 bp fragments. PARP-2-bound chromatin fragments were collected by immunoprecipitation using α-PARP-2 (Alexis) and α-matrix-metalloproteinase-9 (Santa Cruz, Santa Cruz, CA, USA) antibodies. Formaldehyde crosslinking was reversed by heating and then DNA fragments were purified and amplified using SIRT1 promoter-specific primers by qPCR (primers are indicated in the respective section of Supplementary material online). The results were expressed as a percentage of input.

2.15 Statistical analysis

Statistical significance was determined using Student's *t*-test. Error bars represent SEM unless stated otherwise.

3. Results

3.1 PARP-2 depletion counteracts vascular dysfunction

We investigated vascular functions after DOX treatment in *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice first. We did not detect major differences in aortic reactivity between untreated (CTL) *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice. In contrast, DOX treatment significantly decreased norepinephrine and 5-hydroxytryptamine (serotonin)-induced contractility of the vessels in *PARP-2*$^{+/+}$ mice, while *PARP-2*$^{-/-}$ mice were partially protected (*Figure 1A* and *B*). KCl-induced contraction of aortas from *PARP-2*$^{+/+}$ mice was reduced upon DOX treatment,

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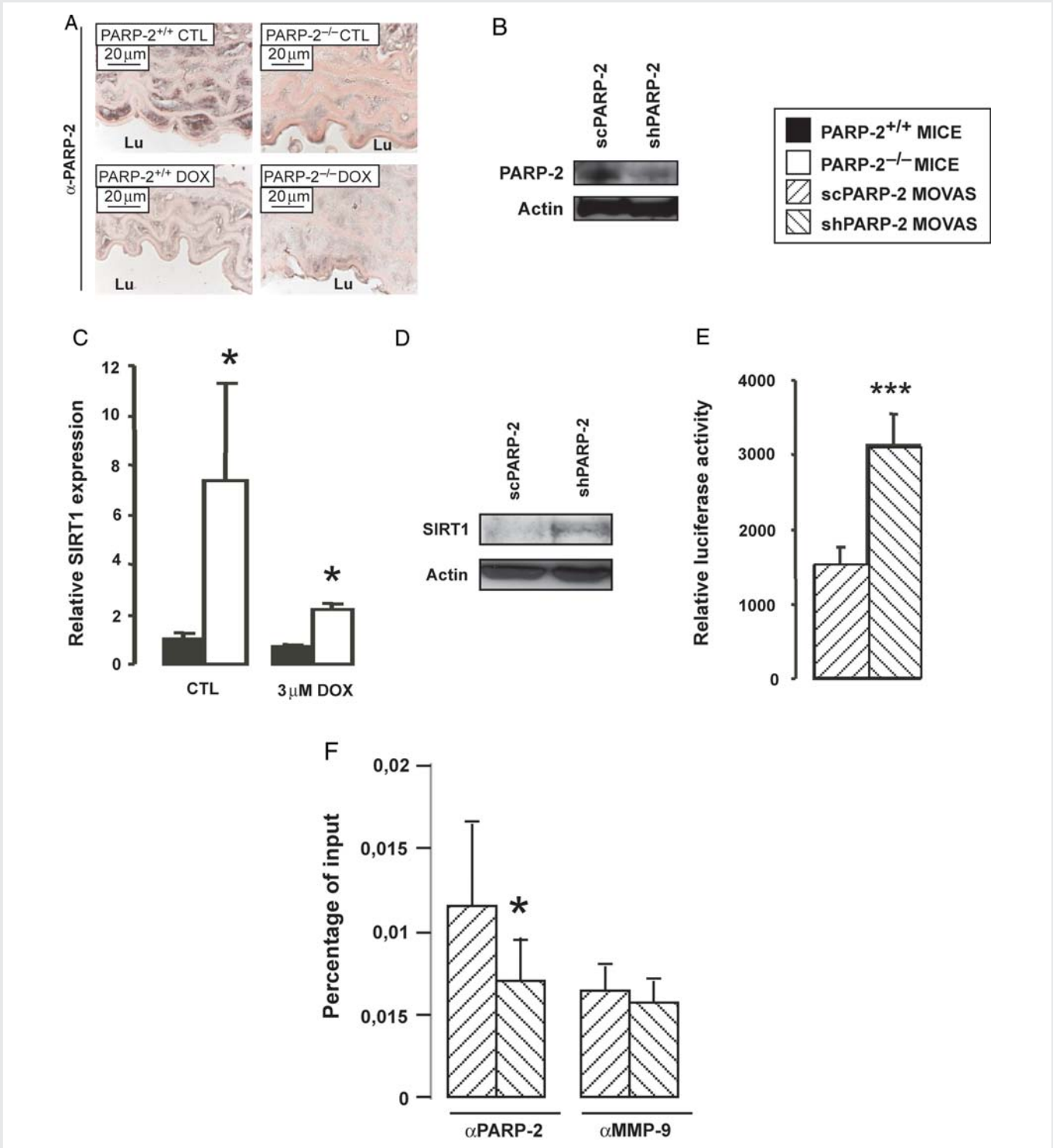

Figure 3 Depletion of PARP-2 induces SIRT1 levels in aortic smooth muscle cells. *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice were injected with saline (CTL) or with DOX at 3 months of age ($n = 7$, 5, 5, and 5, respectively), and then aortic samples were collected on day 2 (*A* and *C*). An aortic smooth muscle cell line (MOVAS) was also tested (*B*, *D*, *E*, and *F*). MOVAS cells were transduced with a PARP-2-silencing (shPARP-2) or -scrambled (scPARP-2) shRNA and treated with solvent (CTL) or DOX. PARP-2 expression was determined in aortas by immunohistochemistry, scale bar represents 20 μm (*A*), and in MOVAS cells by western blotting (*B*). SIRT1 expression was determined in aortas by RT-qPCR (*C*), while in MOVAS cells by western blotting (*D*). The activity of the −1 to −91 portion of the SIRT1 promoter was determined in luciferase assays (*E*, $n = 6$). PARP-2 binding to the SIRT1 promoter was determined using ChIP assays (*F*, $n = 3$). Lu, lumen, * and *** indicate statistically significant difference between scPARP-2 cells/*PARP-2*$^{+/+}$ mice and shPARP-2 cells/*PARP-2*$^{-/-}$ mice at $P < 0.05$ or <0.001, respectively. On (*E*) and (*F*), error is represented as SD.

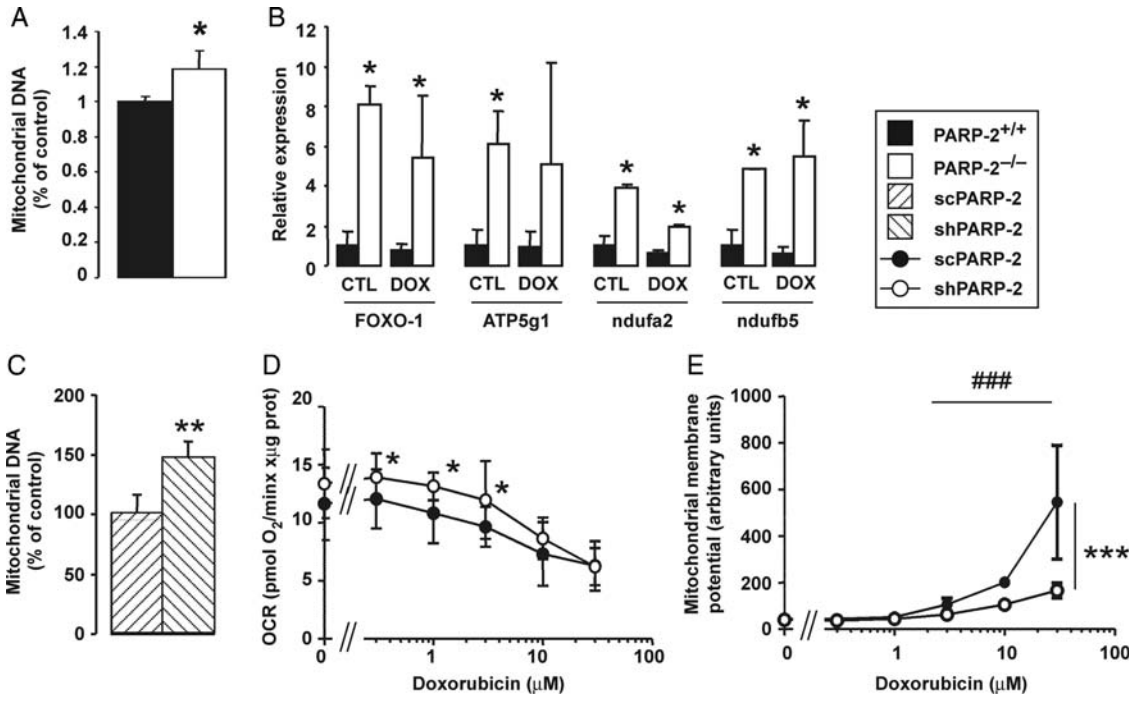

Figure 4 PARP-2 regulates mitochondrial function: possible involvement of SIRT1. *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice (3 months of age) were injected with saline (CTL) or with DOX ($n = 7$ for *PARP-2*$^{+/+}$ CTL, $n = 5$ for *PARP-2*$^{-/-}$ CTL, $n = 5$ for *PARP-2*$^{+/+}$ DOX, and $n = 7$ for *PARP-2*$^{-/-}$ DOX), and then aortic samples were collected on day 2 (*A* and *B*). An aortic smooth muscle cell line (MOVAS) was also tested (*C*, *D*, and *E*). MOVAS cells ($n = 3$ parallel measurements) transduced with a PARP-2-silencing (shPARP-2) or -scrambled (scPARP-2) shRNA were treated with solvent (CTL) or DOX. (*A* and *C*) Mitochondrial DNA content was determined by qPCR. (*B*) Expression of a set of mitochondrial genes was determined by RT-qPCR. (*D*) Oxygen consumption rate was measured as described in Section 2 [open symbols represent CTL ($n = 8$); filled symbols represent DOX ($n = 8$)]. (*E*) Membrane potential was determined using TMRE dye. ### indicate statistically significant difference between DOX-treated samples and their respective controls, at $P < 0.001$; *, ** and *** indicate statistically significant difference between *PARP-2*$^{+/+}$ mice/scPARP-2 cells and *PARP-2*$^{-/-}$ mice/shPARP-2 cells at $P < 0.05$, < 0.01 and < 0.001, respectively. On (*C*) and (*D*), error is presented as SD.

whereas the contractile responses of aortas from *PARP-2*$^{-/-}$ mice were unaffected (*Figure 1C*). These findings suggested a PARP-2-dependent deterioration of vascular smooth muscle function after DOX treatment in mice.

Endothelial function was assessed by the application of acetylcholine. Impaired reactivity of aortas to acetylcholine after DOX treatment in both *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice indicated that the DOX-induced deterioration of endothelial function was independent of PARP-2 (*Figure 1D*). Moreover, sodium nitroprusside-induced vasorelaxation was not affected by either DOX treatment or by the deletion of PARP-2 (*Figure 1E*), suggesting that blunted acetylcholine responses were not related to impaired NO reactivity of smooth muscle cells.

Taken together, our physiological data suggested a role for PARP-2 in DOX-evoked vascular dysfunction and implicated smooth muscle as a potential target. This notion was further supported by decreased smooth muscle actin (SMA) immunoreactivity in *PARP-2*$^{+/+}$ mice after DOX treatment, suggesting a loss of smooth muscle cells, which was not the case in *PARP-2*$^{-/-}$ mice (*Figure 1F*). Importantly, these data identify smooth muscle dysfunction as a novel mechanism of DOX-induced vascular damage. In our following experiments, we employed an aortic smooth muscle cell line (MOVAS) to reveal the potential mechanisms for PARP-2 involvement in DOX-evoked functional deterioration.

3.2 PARP activity is not altered upon PARP-2 depletion

Reactive oxygen species produced by mitochondrial redox cycling of DOX has previously been shown to trigger PARP activation and PAR synthesis. Moreover, ablation of PARP-1 resulted in suppressed PAR synthesis and protection against DOX-evoked cardiovascular damage.[12,13] Since PARP-2 also possesses PARP activity,[14] we set out to investigate the role of PARP-2 in DOX-evoked PAR synthesis and vascular smooth muscle dysfunction.

We detected free radical production in both aortas and MOVAS cells upon DOX treatment (*Figure 2A* and *B*). Moreover, free radical production was increased upon PARP-2 ablation. Enhanced free radical production resulted in DNA-strand breakage in both types of samples as shown by increased TUNEL staining (*Figure 2C* and *D*). However, there was no apparent change in TUNEL positivity upon PARP-2 ablation or depletion neither in mice nor in MOVAS cells (*Figure 2C* and *D*). The level of DOX-induced PARP activity was not different between *PARP-2*$^{+/+}$ and *PARP-2*$^{-/-}$ mice (*Figure 2E*). Similarly, there was no marked difference in PARP activation between MOVAS cells in which PARP-2 was silenced by specific shRNA (shPARP-2) or its unspecific scrambled version (scPARP-2) (*Figure 2F*). These results suggested a dominant role for PARP-1 in DOX-evoked PAR formation. PAR was detected only in smooth muscle cells, suggesting that

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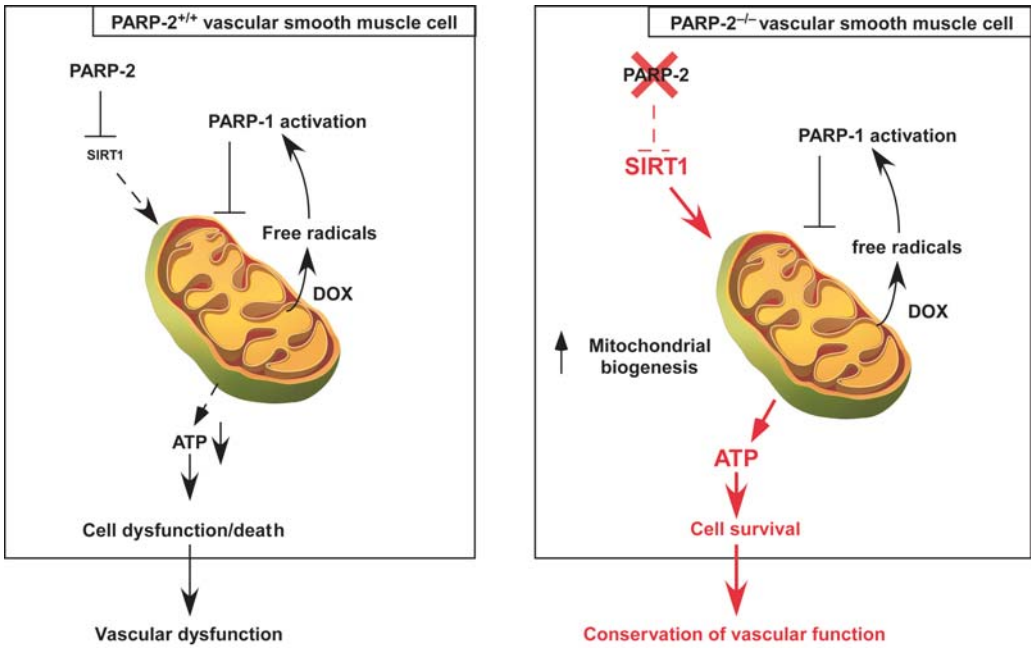

Figure 5 Proposed mechanism for PARP-2-dependent protection against DOX-evoked vascular dysfunction. DOX treatment evokes vascular dysfunction, which is mediated by an increase in free radical formation, resulting in DNA breaks, PARP-1 activation, and cellular energetic catastrophe. However, DOX-induced toxicity is partially antagonized by PARP-2 depletion. PARP-2 down-regulation evokes an increase in SIRT1 expression, which results in the activation of mitochondrial biogenesis. Elevated mitochondrial function protects aortic smooth muscle from DOX-induced toxicity without affecting PARP-1-mediated functions.

DOX-evoked endothelial dysfunction is both PARP-1- and PARP-2-independent. Cellular NAD^+ depletion was also similar in control and PARP-2-depleted cells (*Figure 2G*), suggesting again a minor, if any, role for PARP-2 in this process. Taken together, PARP-2 depletion or deletion results in vascular protection without affecting DOX-induced overall PARP activity.

3.3 SIRT1 overexpression counteracts DOX toxicity

Apparently, the protection provided by the depletion of PARP-2 has a different mechanism than the one responsible for the protective effect of PARP-1 inhibition. Mitochondrial function and structure is deteriorated upon DOX treatment.[26] Moreover, preservation of mitochondrial function is associated with protection against DOX toxicity.[27–29] These observations prompted us to investigate pathways that modulate mitochondrial function. SIRT1 is an NAD^+-dependent class III deacetylase that promotes mitochondrial biogenesis,[30–32] while PARP-2 has been identified as a repressor of SIRT1 expression.[19] Danz *et al.* have shown that pharmacological activation of SIRT1 by resveratrol enhances mitochondrial biogenesis in cardiomyocytes. Thus, induction of SIRT1 by PARP-2 depletion might be capable of counteracting the free radical-evoked mitochondrial dysfunction that occurs upon DOX treatment.[33] Accordingly, we tested whether increased SIRT1 expression might be the key protective mechanism against DOX treatment in *PARP-2*$^{-/-}$ mice.

Disruption or depletion of PARP-2 in aortas or in MOVAS cells (*Figure 3A* and *B*) resulted in an increase in *SIRT1* mRNA and SIRT1 protein levels (*Figure 3C* and *D*). This increase in SIRT1 expression was associated with the induction of the SIRT1 promoter (*Figure 3E*), which was mediated by a decrease in the occupancy of the SIRT1 promoter by PARP-2 (*Figure 3F*).

SIRT1 activation enhances mitochondrial oxidative capacity of multiple metabolic tissues.[31] We detected increased mitochondrial DNA content both in the aorta and in MOVAS cells upon the deletion or depletion of PARP-2 (*Figure 4A* and *C*). Increased mitochondrial biogenesis was further supported by increased expression of genes involved in biological oxidation (*Foxo1*, *ATP5g1*, *ndufa2*, and *ndufb5*) in aortas (*Figure 4B*). Higher expression of oxidative genes was maintained in *PARP-2*$^{-/-}$ mice even after DOX treatment compared with $^{+/+}$ mice (*Figure 4B*). To provide further evidence for the involvement of increased mitochondrial activity, mitochondrial membrane potential and oxygen consumption were determined in MOVAS cells upon DOX treatment (*Figure 4D* and *E*). Without DOX treatment, scPARP-2 and shPARP-2 MOVAS cells displayed similar mitochondrial membrane potential, but PARP-2 silencing resulted in a slightly higher oxygen consumption rate. Oxygen consumption and free radical production increased at 7 h after DOX treatment (*Figures 2B* and *4D*), suggesting mitochondrial uncoupling and mitochondrial dysfunction. Mitochondrial membrane potential in scPARP-2 MOVAS cells increased in line with the increment of DOX concentration, suggesting mitochondrial hyperpolarization (*Figure 4E*). Hyperpolarization of mitochondrial membrane has been described as an early event in apoptosis supporting impaired mitochondrial biogenesis.[34] Overall oxygen consumption was higher in shPARP-2 MOVAS cells than in scPARP-2 cells. The difference in oxygen consumption increased upon DOX treatment similarly, while mitochondrial hyperpolarization was much lower suggesting milder mitochondrial damage (*Figure 4D* and *E*).

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4. Discussion

DOX toxicity affects both cardiac and vascular functions.[1] In the vasculature, DOX treatment has been shown to affect endothelial function[9] but no definitive data are available for its effect on the vascular smooth muscle and the extracellular matrix.[4,5,10] We have affirmed the deterioration of endothelial function in DOX-treated animals but it seems to be independent of both PARP-2 and PARP-1, at least 2 days post-treatment. Importantly, however, early upon DOX administration, vascular smooth muscle is also damaged. We have shown that the deletion of PARP-2 provided protection against the vascular failure induced by DOX. Moreover, this protective phenotype was linked to the preservation of vascular smooth muscle. PARP-2 depletion did not modulate the DNA breakage–PARP-1 activation–cell death pathway. Therefore, it is likely that PARP-2 does not affect PARP-1 activation that may exert its deleterious effects.

As a possible alternative protective mechanism, we have investigated the preservation of mitochondrial functions, as it has been shown to protect against DOX toxicity.[26–29,33] PARP-2 depletion has been recently associated with the induction of mitochondrial oxidation through the induction of SIRT1 expression.[19] The link between PARP-2 depletion–SIRT1 activation–enhancement of mitochondrial biogenesis was further supported by the fact that SIRT1 activation has been shown to induce mitochondrial biogenesis in various tissues.[30–32] Indeed, we have detected increased SIRT1 content after PARP-2 ablation both *in vivo* and in cultured aortic smooth muscle cells which was the consequence of the induction of the SIRT1 promoter.

The augmented expression and activity of SIRT1 caused enhanced mitochondrial biogenesis, which may have contributed to the resistance of *PARP-2*$^{-/-}$ animals against vascular DOX toxicity. It appears that higher level of mitochondrial activity provides an advantage for PARP-2-depleted cells over control cells upon oxidative stress-induced mitochondrial dysfunction (e.g. DOX treatment). Mitochondrial biogenesis, induced by PARP-2 depletion, may counterbalance the DOX-induced loss of mitochondrial activity (*Figure 5*).

To the best of our knowledge, this is the first study reporting the protective properties of SIRT1-induced mitochondrial biogenesis in blood vessels. Indeed, SIRT1 has been shown to act as a cardioprotective factor.[33,35–38] Pharmacological activation of SIRT1 by resveratrol has been demonstrated to induce mitochondrial activity in cardiomyocytes.[33] However, it must be noted that results obtained with resveratrol cannot simply be attributed to SIRT1 activation, as the specificity of resveratrol towards SIRT1 is debated.[39] Moreover, it may act as a free radical scavenger;[40] hence, it may eliminate reactive species released by DOX. Therefore, resveratrol may interfere with PARP-1 activation by quenching free radicals and hence protect mitochondria, or may act on unknown pharmacological targets to confer protection. As Danz *et al.*[33] have detected a complete loss of free radical production upon resveratrol treatment, it cannot be ruled out that resveratrol treatment acted upstream of mitochondrial biogenesis induction interfering with PARP-1 activation and provided protection through blunting PARP-1-mediated cell death.

In the heart, SIRT1 can activate other protective mechanisms. SIRT1 may deacetylate p53 and induce the expression of Bax family proteins.[41] SIRT1 may also impair PARP-1 activation and the consequent cellular dysfunction through deacetylating PARP-1[38] or by consuming NAD^+.[37] However, we did not observe differences in PARP activity or NAD^+ consumption in PARP-2-depleted smooth muscle cells or in the *PARP-2*$^{-/-}$ aorta, but observed an up-regulation of SIRT1 and increased mitochondrial protein expression and function. These apparent differences suggest that the mechanism of vascular protection may consist of different pathways than the ones observed in the heart. Importantly, however, preservation of mitochondrial function seems to be a key issue in the protection against DOX toxicity.

It is tempting to speculate that SIRT1-mediated induction of mitochondrial biogenesis may also have contributed to the protective phenotype of PARP-2 ablation in oxidative stress-mediated diseases such as colitis[16] or cerebral ischaemia[15,17] and could be exploited in other oxidative stress-related diseases. This hypothesis calls for the continuation of the development of PARP-2-specific inhibitors that may be used in such settings.[17]

In summary, here, we provide evidence for the protective role of SIRT1 in the vasculature and implicate PARP-2 as a new target to limit DOX-induced vascular damage. Furthermore, we propose that modulation of the PARP-2–SIRT1 axis to enhance mitochondrial activity may be a new therapeutic pathway to revert mitochondrial hypofunction in the cardiovascular system or in other organs.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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Supplementary Data

Poly(ADP-ribose) polymerase-2 depletion reduces doxorubicin-induced damage through SIRT1 induction

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Supplementary Experimental Procedures

Aorta ring studies

Thoracic aorta was excised after over-anaesthesia (50mg/kg thiopental i.v.) then placed into an organ chamber containing a cold (4^{o}C) Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS) composed of: 110 mM NaCl, 5.0 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM KH_2PO_4, 5.0 mM glucose and 24.0 mM $NaHCO_3$ equilibrated with a gas mixture of 5% CO_2, 10% O_2, 85% N_2 at pH 7.4. Aorta was cut into approximately 4-5 mm rings, then two metal wires (diameter 40 μm) were inserted into the lumen of the arteries and fixed on an adjustable clamp and a force transducer (Helpern-Mulvany arteriograph, DMT510A system, *Danish Myotechnology*, Aarhus, Denmark). The buffer was changed to PSS after fixing the vessels on the system. After the PSS reached 37 ^{o}C, each ring was systematically stretched by increasing the force by 1.5 mN in every half minute until the pressure reached 13.3 kPa (normalization protocol). Then rings were kept in the oxygenated organ bath for 60 min. The tension was recorded in mN at each 0.5 s. Normalization was followed by testing the viability of the arteries with KCl. Effects of serotonin and norepinephrine were tested in a cumulative manner. Rings were precontracted with norepinephrine for relaxation experiments. Endothelium dependent relaxation was induced by acetylcholine and the endothelium independent relaxation was induced by sodium nitroprusside.

TUNEL assay

Dewaxed and rehydrated sections were treated with 20 g per ml proteinase K (in 10 mM Tris/HCl pH 7.8) for 30 min at 37°C. DNA breaks were labeled with terminal deoxyribonucleotidyl transferase (TdT) and a deoxyribonucleotide mix containing digoxigenin labeled dUTP for 60 min at 37°C. After washing, sections were incubated with anti- digoxigenin peroxidase conjugated antibody (30 min at room temperature) and peroxidase was detected by diamino-benzamide reaction. Sections were counterstained with methyl green.

Measurement of thiobarbituric acid reactive species (TBARS)

Malondialdehyde (MDA) formation was utilized to quantify the lipid peroxidation and oxidative stress in tissues and was measured as thiobarbituric acid-reactive components. Tissues were homogenized in 1.15% KCl buffer. Homogenates (200 μl) were then added to a reaction mixture consisting of 1.5 ml of 0.8% thiobarbituric acid, 200 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5) and 600 μl of distilled H_2O that was incubated at 90°C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10000 × g, 10 min), and absorbance at 532 nm was measured and normalized to protein content. The level of lipid peroxides was expressed as percentage of control.

PARP activity measurement

PARP activity was measured in MOVAS cells using the assay based on the incorporation of isotope from ^{3}H-NAD into trichloroacetic acid (TCA) precipitable proteins as in[1]. Cells were induced with 3 μM DOX for 7 hours. H_2O_2 induction was used (10 minutes, 1 mM) as positive control. Then control and doxorubicin/H_2O_2-treated cells were scraped in 1 ml assay

buffer (56 mM HEPES pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% w/v digitonin, 0.125 μM NAD^+ and 0.5 μCi/ml ^3H-NAD^+). Following incubation (10 min at 37°C) 400 μl ice-cold 50% w/v TCA was added and samples were incubated for 4 h at 4°C. Samples were then spun (10000 × *g*, 10 min) and pellets washed twice with ice-cold 5% w/v TCA and solubilized overnight in 250 μl 2% w/v SDS/0.1 N NaOH at 37°C. Contents of the tubes were added to 5.0 ml ScintiSafe Plus scintillation liquid (*Fisher Scientific*, Pittsburgh, PA, USA) and radioactivity was determined using a liquid scintillation counter (TriCarb2800TR, Perkin Elmer, Waltham, MA, USA).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described in[3]. Control and doxorubicin-treated MOVAS cells were fixed with 1% formaldehyde for 10 min at room temperature. Cells were washed with phosphate-buffered saline (PBS) then scraped and collected by centrifugation in Eppendorf tubes. Cells were resuspended in a lysis buffer containing 5 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), pH 8, 85 mM KCl, 0.5% NP-40, and protease inhibitor cocktail to prepare chromatin. After centrifugation at 3000 x *g* for 10 min at 4°C, nuclei were resuspended in sonication buffer (1% sodium dodecyl sulfate, 0.1 M $NaHCO_3$, and protease inhibitor cocktail), incubated on ice for 10 min and sonicated. Cell debris was pelleted by centrifugation at 10000 x *g* for 30 min at 4°C. Equal amounts of the soluble chromatin was diluted in immunoprecipitation (IP) buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 16.7 mM NaCl, and protease inhibitor cocktail) for immunoprecipitation. Immunoprecipitation was carried out with antibodies against PARP-2 and matrix metalloproteinase-9 (as non-specific control). Complexes were collected with blocked protein A+G sepharose beads (*GE Healthcare*) by overnight incubation at 4 °C. Beads were pelleted and washed twice with each of the following buffers: buffer A (low salt)

(0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl, protease inhibitor cocktail), buffer B (high salt) (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl, protease inhibitor cocktail), buffer C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1, protease inhibitor cocktail), and TE buffer (10 mM Tris, 10 mM EDTA, pH 8). Cross-links were reversed by incubation overnight at 65 °C meanwhile treatment with RNaseA was applied (1 μg). Then samples were treated with 0,5% SDS and 2 μg proteinase K and incubated for 2 hours at 45 °C. Purification of the samples was carried out by ion exchange purification (*Qiagen*). Purified DNA was analyzed by qPCR using the following primers:

SIRT1 -91 FWD: 5'-TCC CGC AGC CGA GCC GCG GGG-3'

SIRT1 -91 REV: 5'-TCT TCC AAC TGC CTC TCT GGC CCT CCG-3'

Supplementary Tables

Supplementary Table S1. RT-qPCR Primers for Quantification of Gene Expression

ATP5g1 (murine)	5'-GCT GCT TGA GAG ATG GGT TC-3' 5'-AGT TGG TGT GGC TGG ATC A-3'
FOXO1 (murine)	5'- AAG GAT AAG GGC GAC AGC AA-3' 5'- TCC ACC AAG AAC TCT TTC CA-3'
Ndufa2 (murine)	5'-GCA CAC ATT TCC CCA CAC TG-3' 5'-CCC AAC CTG CCC ATT CTG AT-3'
Ndufb5 (murine)	5'-CTT CGA ACT TCC TGC TCC TT-3' 5'-GGC CCT GAA AAG AAC TAC G-3'
PARP-2 (murine)	5'-GGA AGG CGA GTG CTA AAT GAA-3' 5'-AAG GTC TTC ACA GAG TCT CGA TTG-3'
SIRT1 (murine)	5'- TGT GAA GTT ACT GCA GGA GTG TAA A-3' 5'-GCA TAG ATA CCG TCT CTT GAT CTG AA-3'

Supplementary Table S2. Primers for mtDNA Determination

mtDNA specific (murine)	5'-CCG CAA GGG AAA GAT GAA AGA C-3' 5'-TCG TTT GGT TTC GGG GTT TC-3'
nuclear specific (murine)	5'-GCC AGC CTC TCC TGA TTT TAG TGT-3' 5'-GGG AAC ACA AAA GAC CTC TTC TGG-3'

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Poly(ADP-ribose) polymerase-2 controls adipocyte differentiation and adipose tissue function through the regulation of the activity of the retinoid X receptor/peroxisome proliferator-activated receptor-γ heterodimer.

Péter Bai, Sander M. Houten, Aline Huber, Valérie Schreiber, Mitsuhiro Watanabe, Borbála Kiss, Gilbert de Murcia, Johan Auwerx, and Josiane Ménissier-de Murcia

There was an error in the title of the article. The correct title is shown above.

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Novel binding site for Src Homology 2-containing protein-tyrosine phosphatase-1 in CD22 activated by B lymphocyte stimulation with antigen.

Chenghua Zhu, Motohiko Sato, Teruhiko Yanagisawa, Manabu Fujimoto, Takahiro Adachi, and Takeshi Tsubata

Dr. Adachi was inadvertently omitted as an author of this article. The correct authors are listed above. Dr. Adachi's affiliation is the Laboratory of Immunology, School of Biomedical Science, and the Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 113-8510 Tokyo, Japan.

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The localization and activity of sphingosine kinase 1 are coordinately regulated with actin cytoskeletal dynamics in macrophages.

David J. Kusner, Christopher R. Thompson, Natalie A. Melrose, Stuart M. Pitson, Lina M. Obeid, and Shankar S. Iyer

On Page 23157, the final sentence of the legend to Fig. 8 should read as follows: Data represent the mean ± S.D. of duplicate determinations from a single representative experiment of a total of four identical experiments. On Page 23158, there is an error in the data in Fig. 9 (*A–C*), and these three panels should be retracted.

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Peroxisome Proliferator-activated Receptor (PPAR)-2 Controls Adipocyte Differentiation and Adipose Tissue Function through the Regulation of the Activity of the Retinoid X Receptor/PPARγ Heterodimer*

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The peroxisome proliferator-activated receptor-γ (PPARγ, NR1C3) in complex with the retinoid X receptor (RXR) plays a central role in white adipose tissue (WAT) differentiation and function, regulating the expression of key WAT proteins. In this report we show that poly(ADP-ribose) polymerase-2 (PARP-2), also known as an enzyme participating in the surveillance of the genome integrity, is a member of the PPARγ/RXR transcription machinery. PARP-2$^{-/-}$ mice accumulate less WAT, characterized by smaller adipocytes. In the WAT of PARP-2$^{-/-}$ mice the expression of a number of PPARγ target genes is reduced despite the fact that PPARγ1 and -γ2 are expressed at normal levels. Consistent with this, PARP-2$^{-/-}$ mouse embryonic fibroblasts fail to differentiate to adipocytes. In transient transfection assays, PARP-2 small interference RNA decreases basal activity and ligand-dependent activation of PPARγ, whereas PARP-2 overexpression enhances the basal activity of PPARγ, although it does not change the maximal ligand-dependent activation. In addition, we show a DNA-dependent interaction of PARP-2 and PPARγ/RXR heterodimer by chromatin immunoprecipitation. In combination, our results suggest that PARP-2 is a novel cofactor of PPARγ activity.

Adipose tissue is composed of adipocytes that store energy in the form of triglycerides. Excessive accumulation of white adipose tissue (WAT)[2] leads to obesity, whereas its absence leads to lipodystrophic syndromes. The peroxisome proliferator-activated receptor-γ (PPARγ, NR1C3) is the main protein orchestrating the differentiation and function of WAT, as evidenced by the combination of *in vitro* studies, the analysis of mouse models, and the characterization of patients with mutations in the human PPARγ gene (1, 2). PPARγ acts as heterodimer with the retinoid X receptor (RXR) (3). The PPARγ/RXR receptor dimer is involved in the transcriptional control of energy, lipid, and glucose homeostasis (4, 5). The actions of PPARγ are mediated by two protein isoforms, the widely expressed PPARγ$_1$ and adipose tissue-restricted PPARγ$_2$, both produced from a single gene by alternative splicing and differing only by an additional 28 amino acids in the N terminus of PPARγ$_2$ (3, 6).

PPARγ is activated by binding of small lipophilic ligands, mainly fatty acids, derived from nutrition or metabolic pathways, or synthetic agonists, like the anti-diabetic thiazolidenediones (2, 7, 8). Docking of these ligands in the ligand binding pocket alters the conformation of PPARγ, resulting in transcriptional activation subsequent to the release of corepressors and the recruitment of coactivators. Many corepressors and coactivators have been described such as the nuclear receptor corepressor and the steroid receptor coactivators, also known as p160 proteins (9–11). These corepressors and coactivators determine transcriptional activity by altering chromatin structure via enzyme such as histone deacetylases and histone acetyltransferases (CREB-binding protein/p300). Other mechanisms include DNA methylation, ATP-dependent remodeling, protein phosphorylation, sumoylation, ubiquitinylation, and poly(ADP-ribosyl)ation.

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This work is dedicated to the memory of Josiane Ménissier-de Murcia, who passed away (July 15, 2007).

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[2] The abbreviations used are: WAT, white adipose tissue; PPAR, peroxisome proliferator-activated receptor; PARP-1 and -2, poly(ADP-ribose) polymerase-1 and -2; TTF1, thyroid transcription factor-1; WT, wild type; RT-qPCR, reverse transcription-coupled quantitative PCR; aP2, adipocyte fatty acid-binding protein 2; ERβ, estrogen receptor β; K19, keratin-19; ChIP, chromatin immunoprecipitation; RXR, retinoid X receptor; HEK 293, human embryonic kidney 293; TNFα, tumor necrosis factor α; CREB, cAMP-response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; siRNA, small interference RNA; BES, 2[*bis*(2-hydroxyethyl)amino]ethanesulfonic acid.

TABLE 1

Oligonucleotides used to generate pSuper-siPARP-2 and pSuper-scrPARP-2

The interfering sequences are in bold.

Name	Sequence (5′-3′)	Structure
siPARP-2 sense	GATCT**AAGATGATGCCCAGAGGAACT**TTCAAGAGA**AGTTCCTCTGGGCATCATCTT**TTTTTA	BglII/**sense**/loop/**antisense**/T(5)/HindIII
siPARP-2 antisense	AGCTTAAAAA**AAGATGATGCCCAGAGGAACT**TCTCTTGAA**AGTTCCTCTGGGCATCATCTT**A	HindIII/T(5)/**antisense**/loop/**sense**/BglII
scrPARP-2 sense	GATCT**TTCGGGGAACAAACGTGCAAC**TTCAAGAGA**GTTGCACGTTTGTTCCCCGAA**TTTTTA	BglII/**sense**/loop/**antisense**/T(5)/HindIII
scrPARP-2 antisense	AGCTTAAAAA**TTCGGGGAACAAACGTGCAAC**TCTCTTGAA**GTTGCACGTTTGTTCCCCGAA**A	HindIII/T(5)/**antisense**/loop/**sense**/BglII

Poly(ADP-ribose) polymerase-2 (PARP-2) was described by Ame *et al.* (12) in 1999 as a 66.2-kDa nuclear protein with poly-(ADP-ribosyl)ating activity. Through its DNA-binding domain in the N terminus (amino acids 1–62), PARP-2 can bind to DNase I-treated DNA and to aberrant DNA forms, and its subsequent activation results in poly(ADP-ribose) polymer formation (12). According to the general scheme of PARP activation, the active enzyme catalyzes the polymerization of poly(ADP-ribose) polymer onto different acceptor proteins and itself using NAD^+ as a substrate (13). PARP-2 shares a similar catalytic domain (amino acid 202–593) as poly(ADP-ribose) polymerase-1 (PARP-1) (14), the founding member of the PARP family, though PARP-2 has a smaller reaction velocity compared with PARP-1 (12).

PARP-2 has multiple *in vivo* functions comprising DNA surveillance and DNA repair processes (reviewed in Ref. 15), spermatogenesis (16, 17), inflammation, and oxidative injury (18–20). Most of these functions are accomplished through protein-protein interactions. In PARP-2, the interaction platforms can be mapped to the DNA-binding domain and to the domain E (amino acids 63–202) (21–25). A role for PARP-2 in the regulation of transcription has already been described. In lung epithelial cells PARP-2 interacts with thyroid transcription factor-1 (TTF1). TTF1 is a homeodomain-containing transcription factor of the Nkx-2 family. In these cells, PARP-2 regulates the expression of the surfactant protein-B by affecting TTF1 activity (25). In this study we show that PARP-2 affects the transcriptional activity of PPARγ both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were from Sigma-Aldrich unless stated otherwise.

Animals—PARP-$2^{-/-}$ mice and their wild-type (WT) littermates (26) coming from heterozygous crossings were used. Mice were housed separately, had *ad libitum* access to water and chow, and were kept under a 12-h dark-light cycle. The animals were killed at the age of 7 months by cervical dislocation after 4 h of fasting, and tissues were collected.

Cell Culture—3T3-L1 cells were maintained in DMEM (Invitrogen), 10% newborn calf serum (Invitrogen), Gentamicin (Invitrogen), and HEK, and mouse embryonic fibroblasts (MEFs) were maintained in DMEM, 10% fetal calf serum (Adgenix, Voisins le Bretonneux, France), and Gentamicin (Invitrogen). The 3T3-L1 cells were maintained subconfluent.

MEF Preparation and Differentiation—MEFs were prepared from embryos as described elsewhere (26). For the differentiation studies 4×10^5 MEFs were seeded in 12-well plates and maintained in DMEM, 10% fetal calf serum. The medium was changed every 2 days until confluence. The cells were maintained at confluency for 2 days. Cells were then differentiated in DMEM, 10% newborn calf serum, 5 μM troglitazone, 5 μM dexamethasone, 500 μM isobutylmethylxanthine, and 10 μg/ml insulin (later defined as differentiation mix), while the control cells received DMEM, 10% fetal calf serum, and Me_2SO as vehicle. The medium with the differentiation mix was replaced every 2 days, and the cells were differentiated for 8 days. Control cells after confluency were cultured in DMEM plus 10% fetal calf serum containing only vehicle (Me_2SO, 0.21%).

DNA Constructs—To create an siRNA-expressing construct, double stranded oligonucleotides were cloned into the pSuper vector (for sequences see Table 1) (27). The oligonucleotides siPARP-2sense and siPARP-2antisense (containing the siRNA sequence), as well as the control scrPARP-2sense and scrPARP-2antisense (scrambled version of the siRNA sequence), respectively, were annealed in annealing buffer (150 mM NaCl, 1 mM EDTA, 50 mM Hepes, pH. 8.0). The resulting duplexes carried BglII and HindIII sites and were cloned into pSuper using these sites resulting in pSuper-siPARP-2 (oligonucleotides siPARP-2sense plus siPARP-2antisense) and pSuper-scrPARP-2 (oligonucleotides scrPARP-2sense plus scrPARP-2antisense). An EcoRV/SmaI fragment encoding mouse PARP-2 was isolated from pBC-mPARP-2 (23) and inserted into the SnaBI site of pBABEpuro (Addgene, Cambridge, MA), giving the pBABE-mPARP-2 vector. All other constructs pGL3-$(J_{wt})_3$TKluc reporter construct (28), pSG-PPARγ_2 (3), pSG5-PPARα (29), pSG5-PPARβ (30), pCMX-ERβ, and vitellogeninA2-ERE-TKLuc (ER-luc) (31) were described before. The pCMV-βGal construct was used to control the transfection efficiency.

Transfections—Transfections were preformed either by the BES-buffered saline method (26) or by JetPei (Polyplus Transfections, Illkirch, France).

Luciferase Activity Measurement—3×10^5 HEK cells were seeded in 6-well plates and were transfected with pSuper-siPARP-2, pSuper-scrPARP-2, pBabe, or pBabe-PARP-2 using the BES-buffered saline method. Two days later the cells were once more transfected with the constructs mentioned above. Cells were transfected 24 h later with 0.6 μg of pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2, 0.4 μg of β-galactosidase expression plasmid, 1 μg of pSG-PPARα/pSG-PPARβ/pSG-PPARγ_2/pCMX-ERβ expression vector, and 1 μg of PPAR-/ER-responsive construct. Six hours after transfection, cells were scraped, and luciferase activity was determined. For the determination of PPAR activity, just before transfection, cells were washed in serum-free DMEM medium, and the transfection was carried out in DMEM plus 10% fat-free serum. As ligand we used, fenofibrate (50 μM), monoethylhexyl phthalate (100 μM), troglitazone (5 μM), and β-estradiol (10 μM). After 6 h of transfection, cells were washed with phosphate-buffered saline, scraped, and stored at −80 °C. Luciferase assay was car-

TABLE 2
qPCR primers
FAS, fatty acid synthase; LPL, liproprotein lipase; HSL, hormone-sensitive lipase.

Name	Sequence (5′-3′)	Accession number
Adiponectin	F 5′-AAG AAG GAC AAG GCC GTT CTC TT-3′ (652–674) R 5′-GCT ATG GGT AGT TGC AGT CAG TT-3′ (875–853)	NM_009605.4
aP2	F 5′-TGC CAC AAG GAA AGT GGC AG-3′ (132–151) R 5′-CTT CAC CTT CCT GTC GTC TG-3′ (294–275)	BC054426
CD36	F 5′-GAT GTG GAA CCC ATA ACT GGA TTC AC-3′ (1378–1403) R 5′-GGT CCC AGT CTC AAT TAG CCA CAG TA-3′ (1527–1502)	NM_007643
Cyclophylin B	F 5′-TGG AGA GCA CCA AGA CAG ACA-3′ (561–581) R 5′-TGC CGG AGT CGA CAA TGA T-3′ (626–608)	M60456
FAS	F 5′-GCT GCG GAA ACT TCA GGA AAT-3′ (6612–6632) R 5′-AGA GAC GTG TCA CTC CTG GAC TT-3′ (6695–6673)	BC046513
LPL	F 5′-AGG ACC CCT GAA GAC AC-3′ (317–333) R 5′-GGC ACC CAA CTC TCA TA-3′ (465–449)	BC003305
Leptin	F 5′-GAC ACC AAA ACC CTC AT-3′ (147–163) R 5′-CAG AGT CTG GTC CAT CT-3′ (296–280)	NM_008493
Perilipin	F 5′-GCT TCT TCC GGC CCA GC-3′ (1511–1527) R 5′-CTC TTC TTG CGC AGC TGG CT-3′ (1580–1561)	NM_175640
PPARγ$_1$	F 5′-CCA CCA ACT TCG GAA TCA GCT-3′ (158–178) R 3′-TTT GTG GAT CCG GCA GTT AAG A-3′ (591–570)	NM_011146
PPARγ$_2$	F 5′-ATG GGTG AAA CTC TGG GAG ATT CT-3′ (46–69) R 5′-CTT GGA GCT TCA GGT CAT ATT TGT A-3′ (346–322)	AY243585
HSL	F 5′-CCT CAT GGC TCA ACT CC-3′ (1633/2075–1649/2091) R 5′-GGT TCT TGA CTA TGG GTG A-3′ (2067/2509–2049/2491)	NM_001039507.1/NM_010719.5
TNFα	F 5′-GCC ACC ACG CTC TTC TG-3′ (286–302) R 3′-GGT GTG GGT GAG GAG CA-3′ (627–611)	NM_013693.2

ried out by standard procedures. Luciferase activity was expressed as luciferase activity/β-galactosidase activity.

Nile Red Flow Cytometry—To assess the extent of MEF differentiation, cytosolic triglyceride content was assessed by determining Nile red uptake (modified from Ref. 32) followed by flow cytometry using a FACSCalibur machine (BD Biosciences). Cells were harvested by adding trypsin/EDTA, and the detached cells were stained with Nile red (20 μg/ml, 5 min). Cells were subjected to flow cytometric analysis with 10,000 events collected for each sample; each measurement point was repeated in 4 parallel replicates. Samples for each cell line were normalized against the non-differentiated cells of the same line. The rate of differentiation was expressed as the percentage of the differentiated cells *versus* total number of cells.

SDS-PAGE and Western Blotting—Cells were lysed in lysis buffer (50 mM Tris, 500 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 8.0). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. For the detection of PARP-2, a polyclonal rabbit antibody was used 1:2,000, Alexis, Lausen, Switzerland), and actin was as detected using a rabbit polyclonal antibody (Sigma, 1:200). The secondary antibody was IgG-peroxidase conjugate (Sigma, 1:10,000). Reactions were developed by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

Total RNA Preparation, Reverse Transcription, and qPCR—Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase, and 2 μg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (Qiagen, Valencia, CA). 50× diluted cDNA was used for quantitative PCR (qPCR) reactions. The qPCR reactions were preformed using the LightCycler system (Roche Applied Science) and a qPCR Supermix (Qiagen) with the primers summarized in Table 2.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed according to a previous study (33) on 3T3-L1 cells using α-PARP-2, α-PPARγ$_2$ (Alexis), and α-matrix metalloproteinase-9 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. We used also a no antibody control. The chromatin fragments collected upon precipitation with the above antibodies were amplified using promoter-specific primers by qPCR. For the analysis of the coding sequence the same qPCR primer set was used as the one for the quantitation of the given gene. The respective primers are listed in Tables 2 and 3. The results were normalized for the signal of the input and were expressed as a percentage of the aP2 signal with the PARP-2 antibody.

TABLE 3
Chip primers

Name	Sequence	Reference
aP2	F 5′-CCC AGC AGG AAT CAG GTA GC-3′ R 5′-AGA GGG CGG AGC AGT TCA TC-3′	52
CD36	F 5′-TTT GCT GGG ACA GAC CAA TC-3′ R 5′-GCC ATG TTC CCA TCC AAG TA-3′	39
K19	F 5′-AAG GGT GGA GGT GTC TTG GT-3′ R 5′-GCT TCT TTA CAC TCC TGC T AAA-3′	AF237661

For the testing of the K19 primer set we used non-confluent 3T3-L1 cells transfected with pCMX-ERβ. Chromatin immunoprecipitation was performed using the α-ERβ (Santa Cruz Biotechnology), and as controls we used an α-MRE11 (Santa Cruz Biotechnology) and a no antibody control. The chromatin fragments collected upon precipitation with the above antibodies were amplified using K19 promoter-specific primers by qPCR.

Microscopy—Formaldehyde-fixed, paraffin-embedded sections (7 μm) were made from WAT samples and were stained with hematoxylin and eosine. The same sections were stained with a biotin-conjugated F4/80 antibody (Serotec, Raleigh, NC, 1:100 dilution), and the bound primary antibodies were detected using streptavidin-peroxidase (Vector ABC kit) and diaminobenzidine as chromogenic substrate. Terminally differentiated MEFs were stained by Oil red O as described elsewhere.

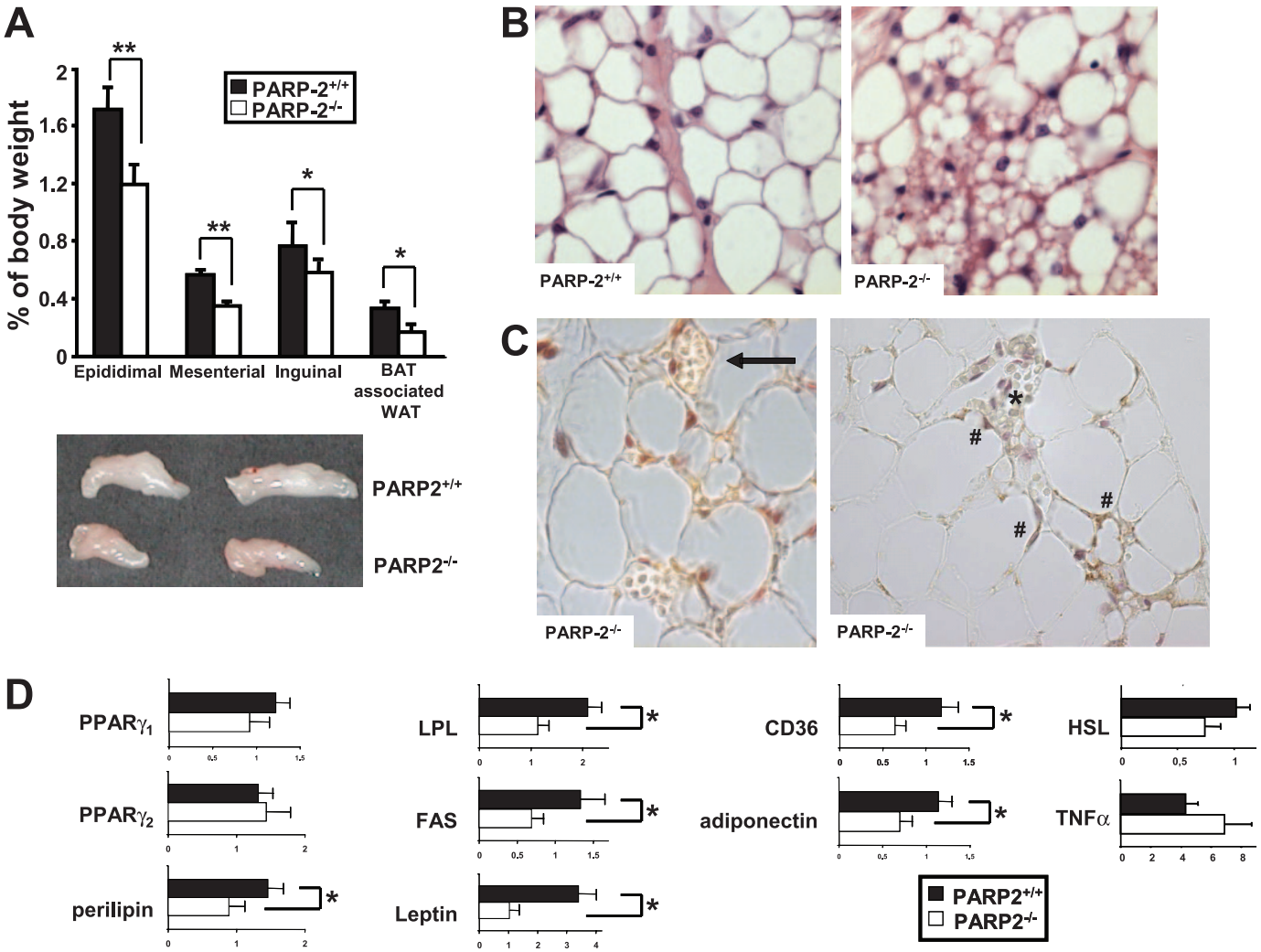

FIGURE 1. **Abnormal WAT function in PARP-2$^{-/-}$ mice.** *A*, weight and macroscopic view of different adipose tissue depots in PARP-2$^{+/+}$ and PARP-2$^{-/-}$ mice (age of 7 months). In the PARP-2$^{-/-}$ mice there is a significant reduction of the different fat depots. *Error bars* represent ± S.E. *, $p < 0.05$; **, $p < 0.01$. *B*, the epididymal WAT stained with H&E (100× magnification). *C*, the *arrow* points toward a dilated capillary in the PARP-2$^{-/-}$ epididymal WAT (100× magnification, H&E). Staining with the F4/80 antibody detects macrophages (marked by #) in the vicinity of the dilated capillaries (*). *D*, gene expression in epididymal WAT. *, $p < 0.05$.

Triglyceride Measurement—The triglyceride content of the MEFs was determined using a commercially available Sigma kit according to the manufacturer's instructions.

Statistical Analysis—Significance was analyzed by Student's *t* test. *Error bars* represent ± S.E., unless noted otherwise.

RESULTS

In Vivo Dysfunction of the PPARγ/RXR Heterodimer in the WAT of PARP-2$^{-/-}$ Mice—The different fat depots (epididymal, mesenteric, and inguinal) and the interscapular brown adipose tissue-associated WAT were measured in 7-month-old PARP-2$^{-/-}$ mice and their wild-type littermates. A proportional loss of the weight of all adipose tissue depots was observed in the PARP-2$^{-/-}$ mice (Fig. 1*A*).

Histological examination of the PARP-2$^{-/-}$ epididymal WAT showed adipocytes with reduced and irregular size. This tissue contained diluted capillaries, indicative of inflammation, which was confirmed by a faint staining with the macrophage-specific F4/80 antibody in the PARP-2$^{-/-}$ (Fig. 1, *B* and *C*) and the macroscopic appearance of the WAT (Fig. 1*A*). The F4/80-positive cells were present in the vicinity of the blood vessels.

To identify the molecular changes that contribute to the decreased fat accumulation and abnormal adipocyte morphology, we determined the expression of the PPARγ target genes, TNFα, and hormone-sensitive lipase by RT-qPCR in the epididymal WAT.

TNFα expression was undetectable in 8 of the 22 mice used for this study (4 out of 14 PARP-2$^{+/+}$ and 4 out of 8 PARP-2$^{-/-}$). In the TNFα-positive mice, expression levels were not different, ruling out a major role for inflammation in the adipose tissue dysfunction in PARP-2$^{-/-}$ mice. The expression level of hormone-sensitive lipase, which is responsible for lipolysis, was also not different between the two genotypes. The expression of several PPARγ target genes, however, was markedly decreased. These include genes involved in chylomicron and very low density lipoprotein triglyceride hydrolysis (lipoprotein lipase), free fatty acid uptake (CD36), *de novo* fatty acid synthesis, and endocrine signaling (leptin and adiponectin) (Fig. 1*D*). Interestingly,

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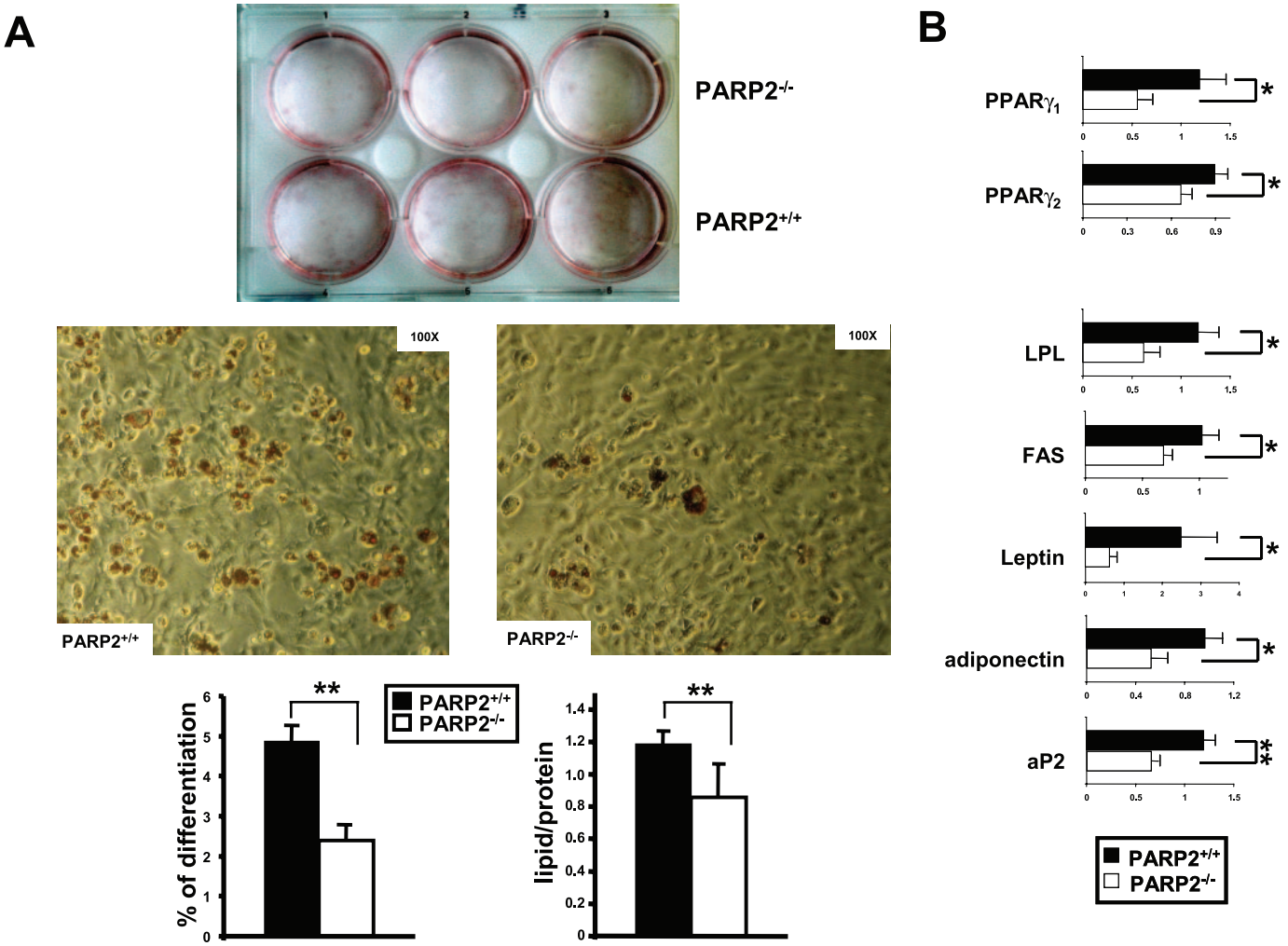

FIGURE 2. **Effect of PARP-2 on MEF differentiation into adipocytes.** *A*, MEFs were differentiated into adipocytes and stained with Oil red O. On the terminally differentiated MEFs, Nile red fluorescence-activated cell sorting analysis and lipid measurements were performed. The *left histogram* shows the percentage of differentiation as measured with Nile red, and the *right histogram* shows the accumulation of lipids in the culture. *, $p < 0.05$; **, $p < 0.01$. *B*, expression of selected marker genes of adipocyte differentiation as measured by RT-qPCR on MEF cDNA samples (*, $p < 0.05$; **, $p < 0.01$).

no difference was detected in PPARγ$_1$ and PPARγ$_2$ mRNA levels between the different genotypes.

MEF Differentiation Is Affected by PARP-2 Ablation—We next aimed to determine whether MEFs differentiation toward adipocytes was affected by the PARP-2 deletion. Differentiation of PAPR-2$^{-/-}$ MEFs into adipocytes was decreased as judged by Oil red O staining, determination of lipid content, and Nile red staining followed by fluorescence-activated cell sorting analysis (Fig. 2*A*).

The expression of genes involved in adipocyte differentiation and function such as PPARγ$_1$ and PPARγ$_2$ were decreased in the PARP-2$^{-/-}$ MEFs (34). Because the PPARγ transcripts are primarily present in the differentiated cells, these data confirm that PARP-2$^{-/-}$ cells differentiate less into adipocytes. The expression of PPARγ target genes, such as lipoprotein lipase, fatty acid synthase, leptin, adiponectin, and adipocyte fatty acid-binding protein 2 (aP2), were decreased in parallel (Fig. 2*B*).

PARP-2 Expression Modulates Transactivation of PPARs—To measure whether changes in PARP-2 expression affect PPAR transactivation, we used HEK 293 cells transfected with a PPARγ$_2$ expression vector and a PPARγ-responsive luciferase construct. In these experiments we modulated the expression of PARP-2 expression by overexpression and siRNA depletion. For the siRNA depletion of PARP-2 we used the pSuper-siPARP-2 construct, whereas for PARP-2 overexpression we used the pBabe-PARP-2. The pSuper-scrPARP-2 and the empty pBabe vector served as the respective controls. PARP-2 levels were assessed by Western blotting using a PARP-2-specific antibody. For both constructs, the cells were transfected twice, on day 0 and on day 2. On day 3, the specific siRNA decreased PARP-2 protein levels significantly, whereas the scrambled PARP-2 siRNA did not alter the PARP-2 levels. A strong increase in PARP-2 protein was observed on day 3 of the overexpression experiment (Fig. 3).

PARP-2 depletion diminished the basal PPARγ activity and abrogated receptor activation by its synthetic ligand, troglitazone. Conversely, PARP-2 overexpression induced by 3-fold the basal PPARγ activity, although it does not significantly change the ligand-dependent activation by troglitazone (Fig. 4*A*). To verify whether this effect of PARP-2 was specific for PPARγ, we performed similar experiments for the related nuclear receptors PPARα (NR1C1) and PPARβ (NR1C2), and

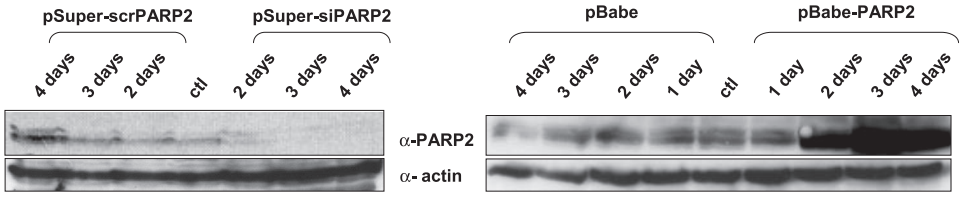

FIGURE 3. **Characterization of the pSuper-scrPARP-2 and the pSuper-siPARP-2 constructs.** 3×10^7 HEK cells were plated in Petri dishes and were BES-buffered saline transfected on day 0 and on day 2. Cells were scraped from day 2 daily. These samples were analyzed by Western blotting. PARP-2 was depleted by the pSuper-siPARP-2 construct, but was unmodified by the pSuper-scrPARP-2 construct. Whereas the transfection with pBabe-PARP-2 resulted on day 3 and day 4 in a robust induction of PARP-2 expression, the transfection with pBabe alone did not modify PARP-2 expression.

the unrelated estrogen receptor β (ERβ, NR3A2). Interestingly, siRNA depletion of PARP-2 increased the basal activity of both PPARα and -β (Fig. 4, *B* and *C*). PARP-2 overexpression did not affect PPARβ but increased PPARα activity. The activation of PPARα and -β with fenofibrate and monoethylhexyl phthalate, respectively, was not modified by the modulation of PARP-2 expression. In addition, neither PARP-2 depletion, nor PARP-2 overexpression had an effect on the basal or ligand-induced activity of ERβ (Fig. 4*D*). Combined these results indicate specificity of the PARP-2-dependent effect on PPARγ.

PARP-2 Is the Member of the RXR/PPARγ Transcription Complex—To demonstrate an interaction between PPARγ and PARP-2 we used ChIP assays. To precipitate chromatin from undifferentiated 3T3-L1 cells we used antibodies against PARP-2 and PPARγ$_2$. An anti-matrix metalloproteinase-9 antibody and a sample without antibody served as negative controls. We used qPCR to amplify the promoters of the aP2 (6) and CD36 (35) as promoters driven by PPARγ, and keratin-19 (K19), as a non-related, ERβ-regulated promoter (36). PARP-2 and PPARγ gave a strong signal on PPARγ-regulated promoters. These signals were significantly higher compared with the signal from the K19 promoter (Fig. 5*A*). We also performed qPCR reactions to cover the coding sequences of aP2 using the chromatin fragments obtained in the ChIP experiments. The signal of PARP-2 and PPARγ coding sequences in the immunoprecipitates was strongly decreased compared with the signal of the corresponding promoter. Apparently, both PARP-2 and PPARγ are present on the PPARγ-driven promoters but not in the coding sequence (Fig. 5*B*). In addition, our results suggest that PARP-2 possesses specificity toward the PPARγ-driven promoters, because the signal from ERβ-driven K19 promoter was significantly lower than that from PPARγ-driven promoters.

Despite the huge difference in the signal of the specific promoters and the nonspecific regions (K19 promoter, coding sequence) we observed some background signal from the nonspecific region. It is likely that this represents the real presence of PARP-2 in these regions, which is probably linked to the formaldehyde-induced DNA damage.

To provide proof that the interaction of ERβ with the K19 promoter is basically detectable we complemented 3T3-L1 cells with ERβ, and we performed ChIP probing with the K19 primer set. To precipitate chromatin from ERβ-complemented 3T3-L1 cells we used an antibody against ERβ, an anti-MRE11 antibody and a sample with no antibody served as negative controls. The precipitate of the ERβ-specific antibody gave significantly higher signal then the non-specific MRE11 (2.7-fold increase) as well as with the non-antibody control (6.1-fold increase) proving that the K19 primer pair is capable of detecting the K19 promoter if present in the precipitate (Fig. 5*C*).

DISCUSSION

PPARγ plays an important role in adipose tissue differentiation and function. In PARP-2 knock-out mice we have identified a defect of adipose tissue function and a decrease of adipocyte differentiation. *In vivo*, the adipose tissue depots had smaller weight and histologically showed an adipodegenerative phenotype.

We have detected a mild inflammation in the WAT of the PARP-2$^{-/-}$ mice. The capillaries were dilated, and we have detected F4/80-positive cells in the vicinity of the capillaries suggesting the presence of macrophages. The areas more distant from the capillaries are devoid of staining. Similar coloration was not observed in the WAT of the wild-type mice. Activated macrophages and adipocytes may secrete pro-inflammatory cytokines, such as TNFα that may induce adipocyte cell death (37). Because TNFα expression was not detectable in many mice and, if it was detected, its expression was not significantly increased by the absence of PARP-2, it is less likely that inflammation is a leading cause of the adipodegeneration in the PARP-2$^{-/-}$ mice. It is also unlikely that increased lipolysis may contribute to the phenotype in the PARP-2$^{-/-}$ mice, because there was no difference in the expression of hormone-sensitive lipase between the wild-type and PARP-2$^{-/-}$ mice.

We did observe decreased expression of multiple PPARγ target genes involved in adipocyte function. Expression of both PPARγ isoforms was normal, suggesting effects on PPARγ/RXR transactivation. *In vitro*, the differentiation of the PARP-2$^{-/-}$ MEFs into adipocytes was delayed when compared with the differentiation of wild-type MEFs. At the end of the differentiation the expression of both PPARγ$_1$ and PPARγ$_2$ was decreased in the PARP-2$^{-/-}$ cells indicating the lack of differentiation. Similarly, the expression of the PPARγ target genes was decreased.

In transfection assays, the ablation of PARP-2 results in the diminution, whereas PARP-2 overexpression raises transactivation by PPARγ. The effect of PARP-2 seems specific for PPARγ, because opposite or no effects were observed for the related PPARα and PPARβ, and the non-related ERβ.

PARP-2 achieves these activities, because it is part of the PPARγ/RXR transcription complex as shown by ChIP assays, suggesting that PARP-2 could act as a PPARγ/RXR receptor cofactor.

Both members of the PPARγ/RXR nuclear receptor dimer might be the effector behind the phenotype of the PARP-2$^{-/-}$ mice. If PARP-2 would directly influence RXR, all PPAR isoforms should respond the same way to the modulation of PARP-2 expression. PPARγ was differentially regulated when compared with PPARα and -β, suggesting that PARP-2 acts on

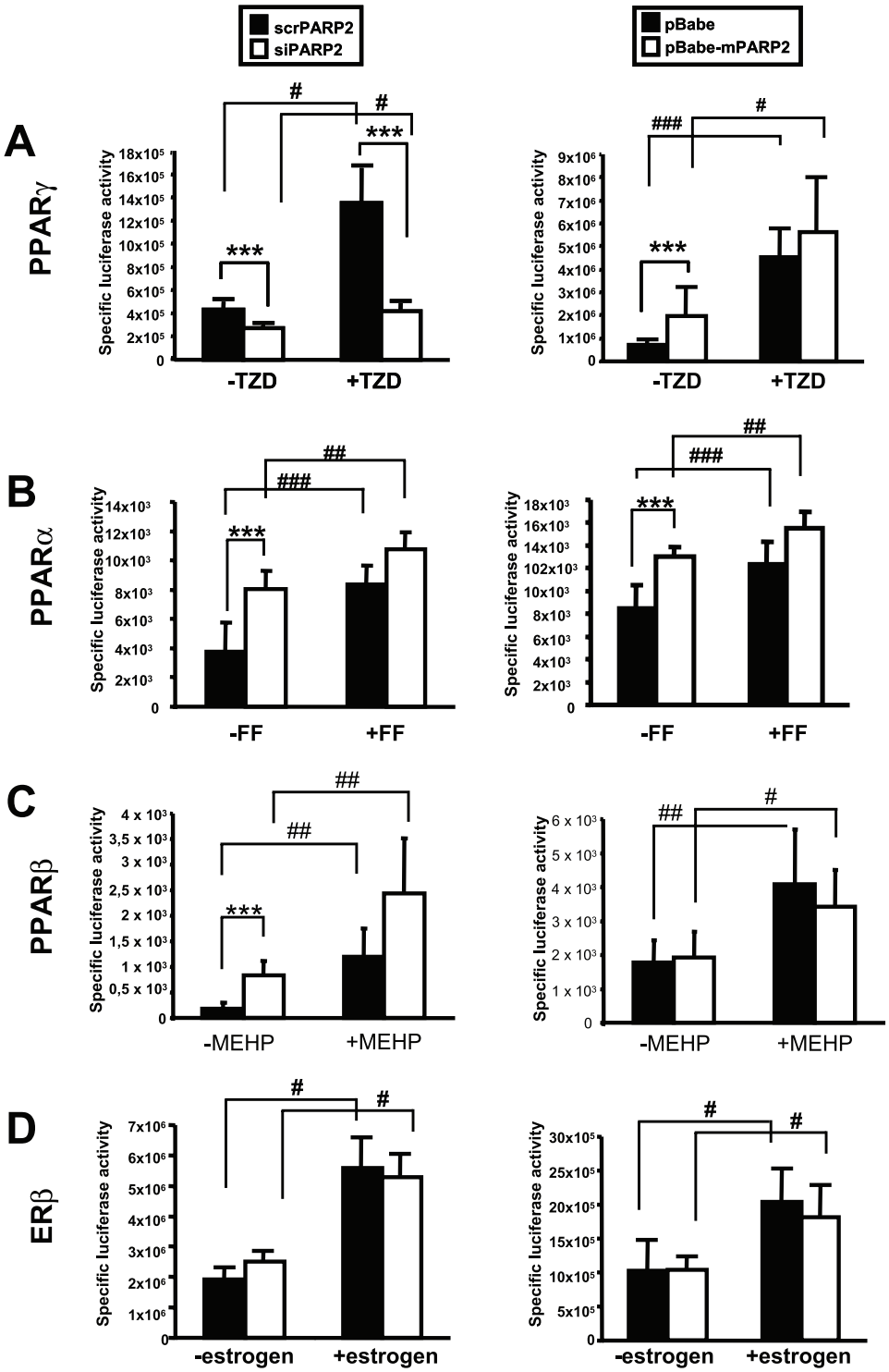

FIGURE 4. **Effect of PARP-2 expression levels on PPARγ$_2$ transactivation.** Effect of PARP-2 depletion and overexpression on the basal activity and receptor activation of PPARγ (*A*), PPARα (*B*), PPARβ (*C*), and ERβ (*D*) receptors. *Error bars* represent ±S.D. ### and ***, $p < 0.001$; **, $p < 0.01$; #, $p < 0.05$.

PPARγ. Our report comprises *in vitro* data and gives first time *in vivo* evidence that PARP-2 may be considered a cofactor of nuclear receptor transcription.

PARP-2 is a multidomain protein with multiple functions. These functions comprise DNA repair (reviewed in Ref. 15), spermatogenesis (16, 17), T-cell development (38), inflammation, and oxidative injury (18–20). Most of these functions are accomplished through protein-protein interactions. The N terminus, with the following domain E are apparently important protein-protein interaction domains, serving as an interaction platform for TRF-2 (21), B23 (22), PARP-1, XRCC1, and DNA polymerase β (23), and TTF1 (25). PARP-2 also homodimerizes with itself through its domain E (23).

PARP-1 has been described as a cofactor for numerous transcription factors (reviewed in 40 and 25), including for some members of the nuclear receptor family, such as the progesterone receptor (41), RXR (42, 43), androgen receptor (44), and the thyroid receptor (42). A recent study, based on *in vitro* results, suggested that PARP-2 acts as a cofactor of a homeodomain-containing transcription factor, TTF1, which belongs to the Nkx-2 family. Binding of PARP-2 through its E domain to the C terminus of TTF1 regulates the expression of the surfactant protein-B in lung epithelial cells. TTF1-mediated transcription encompasses similar mechanisms, including chromatin modification, and involves some of the same cofactors such as the steroid receptor coactivators as described for PPARγ-coupled transcription. This suggests that similar molecular mechanisms exist both in the case of PPARγ- and TTF1-mediated transcription (25). Our results hence confirm the observation of Maeda and colleagues (25), that PARP-2 is a cofactor of some transcription factors, and extend these conclusions by showing that PARP-2 is involved in nuclear receptor-mediated transcriptional control *in vivo*. Recent evidence has suggested that the interaction between PARP-1 and the promoter of target gene could be mediated via double strand breaks, which are produced by activation of a nuclear receptor followed by the unwinding of DNA by topoisomerase II (45). Our results do provide evidence that interaction with DNA is important for the interaction between PPARγ and PARP-2. ChIP assays that depend on DNA binding show strong interaction. In contrast, immunoprecipitation experiments performed on cell

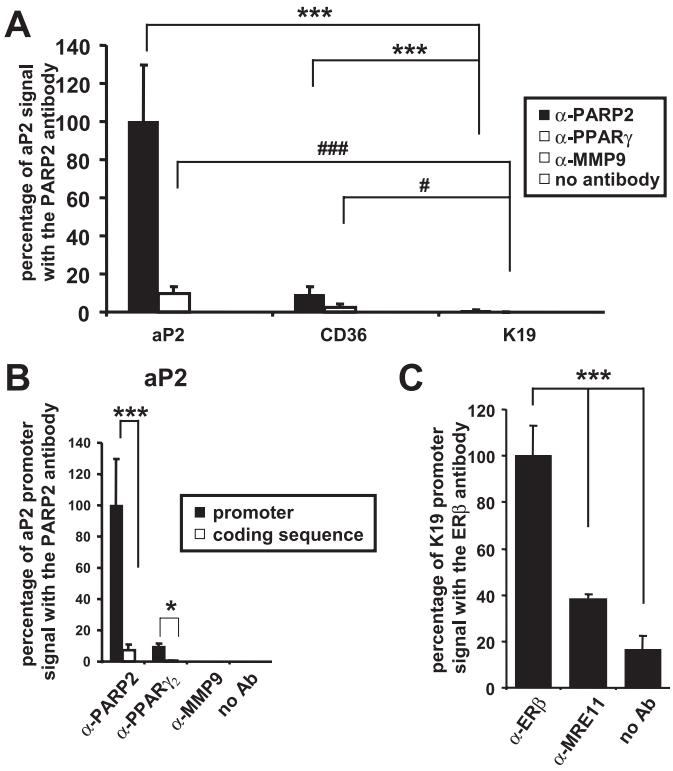

FIGURE 5. **PPARγ$_2$ and PARP-2 occupy together the PPARγ-dependent promoters.** *A*, PARP-2 is present on PPARγ-driven promoters (aP2 and CD36) as demonstrated in ChIP assays. The PARP-2 signal is increased in the PPARγ-driven promoters compared with the non-related, ERβ-driven K19 promoter. *Error bars* represent ±S.D. (### and ***, $p < 0.001$; # and *, $p < 0.05$). *B*, a similar ChIP assay was performed to compare the presence of PARP-2 on the promoters *versus* the coding sequence of the same gene. PARP-2 is present preferentially on the promoter rather then the coding sequence of the aP2 gene. *Error bars* represent ±S.D. (***, $p < 0.001$; *, $p < 0.05$). *C*, as a positive control, the K19 primer set was tested for the ability to detect the presence of ERβ in ERβ-transfected 3T3-L1 cells. The α-ERβ signal was significantly higher then the signal of the negative control α-MRE11 and the control without antibody. These data confirm that the K19 primer set is capable of detecting the presence of the ERβ receptor on the K19 promoter. *Error bars* represent S.D. (### and ***, $p < 0.001$; # and *, $p < 0.05$).

extracts showed only a weak interaction between PARP-2 and PPARγ (data not shown), which was abrogated by low concentrations of Nonidet P-40 (>0.1%). Furthermore, like in the case for PARP-1, the N terminus of PARP-2, comprising its DNA-binding domain, seems also to play the most important role in the interaction with PPARγ. Consistent with this observation a nuclear receptor-binding consensus sequence (^{113}LIQLL^{117}) was present in the E domain of PARP-2.

Concerning the mode of action of PARP-2, it is possible that not only the physical presence but also the activity of PARP-2 is necessary for the nuclear receptor function. Poly-(ADP-ribosyl)ation is reported to increase throughout the differentiation process of 3T3-L1 cells (46). Interestingly, this poly(ADP-ribosyl)ation activity is not completely inhibited by PARP-1 depletion (47), suggesting the involvement of other member(s) of the PARP family, such as PARP-2. Indeed both PARP-1 and PARP-2 are reported to poly(ADP-ribosyl)ate histones (13). Similarly to histone acetylation, poly(ADP-ribosyl)ation of the high mobility group of proteins and histones loosens chromatin structure enabling transcription initiation (48). Consistent with this line of thinking, there is molecular and *in vivo* evidence that the enzymatic activity of PARP-1 is necessary for efficient gene transcription, and inhibition of PARP activity impairs the transcription of a number of different genes, including different chemokines and inflammation-related genes (*e.g.* iNOS, TNFα, ICAM-1, IL-8, MIP-1α, and IL-12) (40, 49, 50).

PARP-2 specifically occupies the promoter of PPARγ target genes, because it bound efficiently to the regulatory sequence, whereas binding to the corresponding coding sequences was strongly decreased. Despite this rather specific binding, we observed a background signal rising most likely from non-coding regions or from non-PPARγ-dependent promoters, such as that of the K19 gene, which is under the control of ERβ. When comparing the specific to the above mentioned nonspecific signal, it is at least 10- to 100-fold increased, which can be considered as a significant difference. It is likely that the nonspecific presence of PARP-2 on the K19 promoter and in the non-coding regions is explained by the fact that PARP-2 binds to the DNA-damage sites created by the formaldehyde treatment during the cross-linking of the cells. The cross-linking-related DNA damage is present throughout the entire genome, equally affecting coding regions and promoters, thus theoretically providing a background signal throughout the genome.

The present study indicates that PARP-2 modulates the activity of PPARγ/RXR nuclear receptor complex, a key transcription factor involved in the pathogenesis of several important diseases such as obesity, insulin resistance, type II diabetes atherosclerosis, and lipodystrophy. Because many of these diseases affect a large part of the population and have high costs to society, our data, linking the activation of PPARγ and PARP-2, show it is possible to modulate PPARγ activity via PARP-2. It is therefore tempting to speculate that the various PARP inhibitors that are currently being developed and tested in clinical trials (51) could also be useful in the metabolic disease arena.

Acknowledgments—We thank Dr. Maria Malanga and Dr. Felix Althaus (Institute of Pharmacology and Toxicology, University of Zurich-Tierspital) for the siRNA sequence against PARP-2. We also acknowledge the help of Dr. Máté Demény, Dr. László Tora, and Dr. Jean-Christophe Ame in the assays performed.

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Review

Crosstalk between poly(ADP-ribose) polymerase and sirtuin enzymes

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ABSTRACT

Poly(ADP-ribose) polymerases (PARPs) are NAD^+ dependent enzymes that were identified as DNA repair proteins, however, today it seems clear that PARPs are responsible for a plethora of biological functions. Sirtuins (SIRTs) are NAD^+-dependent deacetylase enzymes involved in the same biological processes as PARPs raising the question whether PARP and SIRT enzymes may interact with each other in physiological and pathophysiological conditions. Hereby we review the current understanding of the SIRT–PARP interplay in regard to the biochemical nature of the interaction (competition for the common NAD^+ substrate, mutual posttranslational modifications and direct transcriptional effects) and the physiological or pathophysiological consequences of the interactions (metabolic events, oxidative stress response, genomic stability and aging). Finally, we give an overview of the possibilities of pharmacological intervention to modulate PARP and SIRT enzymes either directly, or through modulating NAD^+ homeostasis.

Contents

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1. Introduction

Adaptative responses are the product of critical balances integrating a myriad of molecular changes. These molecular changes include covalent modifications of diverse protein and changes in enzymatic substrate bioavailability, amongst others.

NAD^+ (Fig. 1) and its redox counterpart, NADH, are key metabolites influencing a large constellation of metabolic reactions. The most largely studied poly(ADP-ribose) polymerase (PARP) family members, PARP-1 and PARP-2, use NAD^+ as a co-substrate in their catalytic activity. It has been observed that persistent PARP activation can deplete total intracellular levels by 80% and elevates nicotinamide (NAM), its reaction product. This depletion can have major metabolic impacts, due to the large spectrum of metabolic activities depending on NAD^+ bioavailability (Bai and Canto, 2012).

Sirtuins are a family of NAD^+-dependent protein deacetylases with critical metabolic roles (Houtkooper et al., 2012). Early observations indicated that the decline of NAD^+ and the rise of NAM promoted by enhanced PARP activity correlates with a downregulation of sirtuin activity (Bai et al., 2011b; Pillai et al., 2005). Similarly, the activation of the most well-known mammalian sirtuin, SIRT1, led to reduced PARP activity (Kolthur-Seetharam et al., 2006). These observations supported a hypothesis raised a decade ago, where it was postulated that the activity of sirtuins and PARPs might compete for the availability of a common NAD^+ pool (Zhang, 2003). In this review we will dissect the possible linkage of these two ancient pathways, PARPs and sirtuins, their possible competition for NAD^+, and the physiological, or pathophysiological impact of these interactions. Furthermore, we will discuss how the vertexes of these interactions could be approached pharmacologically.

1.1. NAD^+ metabolism

The metabolism of the pyridine dinucleotides is a long studied one with reports dating to the early parts of the 20th century (Harden and Young, 1906) wherein Harden coined the term "co-zymase" to indicate NAD^+. The redox activity of the dinucleotide compounds (NAD^+ and $NADP^+$) were first described to be a consequence of the pyridine moiety by Warburg in 1936 (Warburg and Christian, 1936). For most of the last century, the chemistry that converted the dinucleotides to their reduced counterparts (NADH and NADPH) constituted nearly the entire focus of interest on these important players in metabolism. In fact, cell metabolism has a plentitude of redox transformations that interconvert NAD^+ and NADH (or $NADP^+$ and NADPH), ranging from catabolism to biosynthesis (Pfleiderer, 1970).

In the latter part of the 20th century, the non-redox reactivity of NAD^+ was recognized as a second major function of NAD^+, wherein ADP-ribose (ADPR) is transferred to cellular nucleophiles, such as proteins, in chemistry called ADP-ribosyl transfer (Honjo et al., 1968; Nishizuka et al., 1968). This "newer" chemistry of NAD^+ is diversified and has been expanded in mammalian organisms, where seven sirtuins (Sauve et al., 2006) and 17 PARP enzymes (Ame et al., 2004) harness this chemistry for signaling and cell adaptation.

The central role of NAD^+ in metabolic transformations, as well as its incorporation into signaling pathways has made the study of NAD^+ and how it is made in cells a rejuvenated topic of interest (Houtkooper et al., 2010; Koch-Nolte et al., 2009). The manner in which NAD^+ is made and utilized constitutes "NAD^+ metabolism" and is a modern subject, with open ended questions on how it is biosynthesized, maintained in cells, incorporated into signaling, etc. This introduction surveys these topics in brief, but also provides an opportunity to highlight the variety of ways in which the study of NAD^+ has blossomed over the years.

1.1.1. Redox properties of NAD^+

The role of NAD^+ as a direct player in catabolic metabolism is well known. NAD^+ participates as a co-substrate in several steps of glycolysis, lactate pyruvate interconversion, pyruvate oxidation to acetyl-CoA catalyzed by pyruvate dehydrogenase

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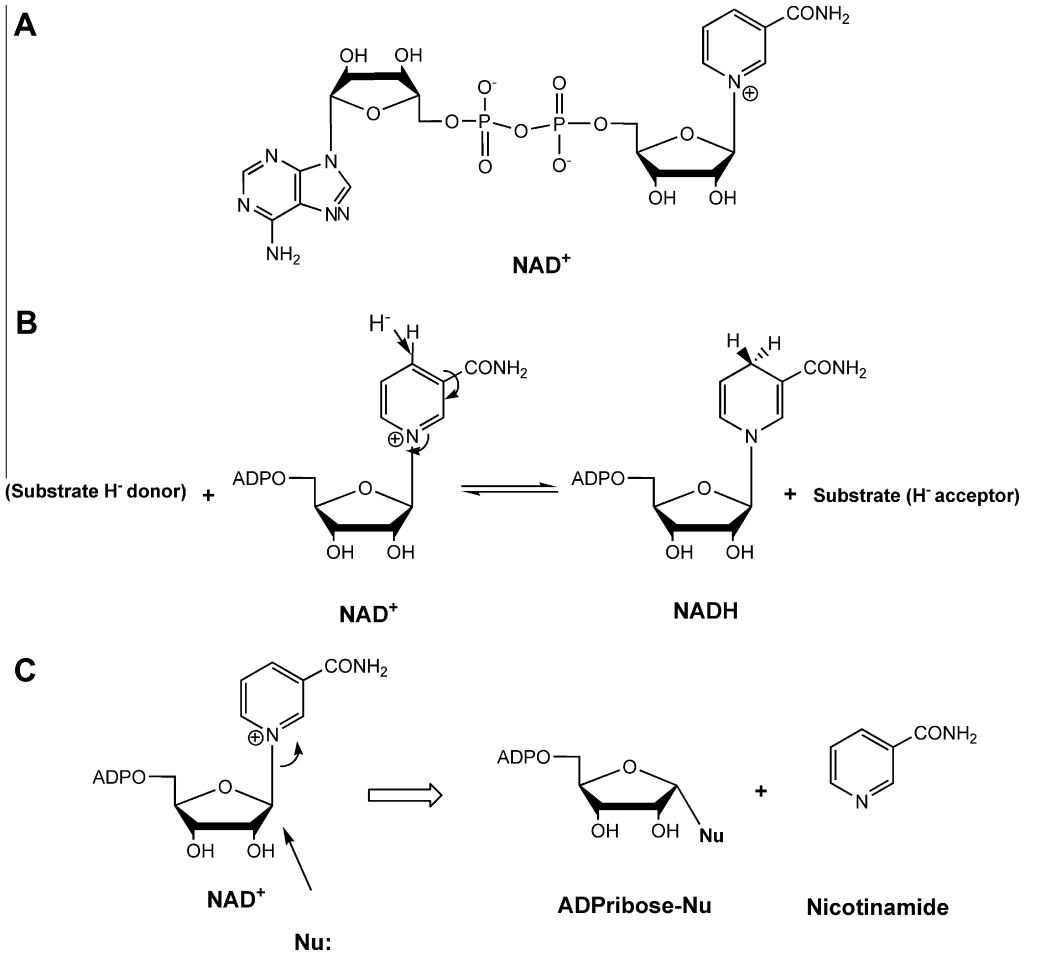

Fig. 1. NAD and reactions of NAD^+. (A) Depiction of the chemical structure of NAD^+. (B) Transfer of hydride ion to nicotinamide of NAD^+ to form NADH. (C) ADP-ribosyltransfer reaction of NAD^+ to a cellular nucleophile (acetyllysine, aspartate, glutamate, protein, etc.).

complex, TCA cycle and is the donor of electron equivalents to Complex I in the electron transport chain (Ramakrishna et al., 2001). NAD^+ is integrated centrally into energy metabolism. Consequently, NAD^+ level is crucial for the proper maintenance of metabolic functions in cells. NAD^+ level is normally maintained at a relative abundance to NADH level in cells (Williamson et al., 1967), and the NAD^+/NADH ratio regulates numerous metabolic pathways, including glycolysis (Sun et al., 2012). NADH accumulation generally leads to feedback inhibition of metabolic processes upstream of the electron transport chain, except lactate production. Lactate production provides means to mitigate unbalanced NAD^+/NADH ratio (Sun et al., 2012) and basis for the Cori cycle, in which lactate is released into the bloodstream and delivered to the liver for gluconeogenesis (Katz and Tayek, 1998). Not surprisingly, when excess NADH accumulates, lactate also accumulates and typically, high lactate is associated with hypoxia (Rimachi et al., 2012) or other mitochondrial deficiencies (Yamada et al., 2012). Lactate pyruvate ratio is a clinical surrogate for NAD^+/NADH ratio in physiology.

The redox properties of NAD^+ originate from the deficiency in electron density in the NAM ring. When conjugated to ribose, this electron deficiency becomes further accentuated by the quaternization of the pyridine nitrogen in the heterocycle (Fig. 1B). This quaternary pyridine group is made even more electron deficient by the carboxamide, which is a good electron withdrawing group. The positive charge on the nicotinamide group in NAD^+ has been calculated to be 0.541 charge units (Cen and Sauve, 2010). The electron deficiency of the pyridine ring provides a driving force for acceptance of hydride ion at C4 (Fig. 1B). Hydride ion acceptance breaks the aromaticity in the pyridine ring, but the energy expense is compensated by increased negative charge into the ring. The removal of the hydride ion in the reverse direction is driven by restoration of aromaticity. This redox chemistry reflects fine balancing of acceptance and removal of hydride ion, and evolution has centrally incorporated NAD^+ into many metabolic processes requiring hydride ion transfer.

1.1.2. Non-redox properties of NAD^+

The ribose ring is conjugated to nicotinamide in NAD^+ via the anomeric carbon. The construction of this bond can occur via several pathways, as discussed in the next section; however, the decomposition of this bond is crucial to the action of

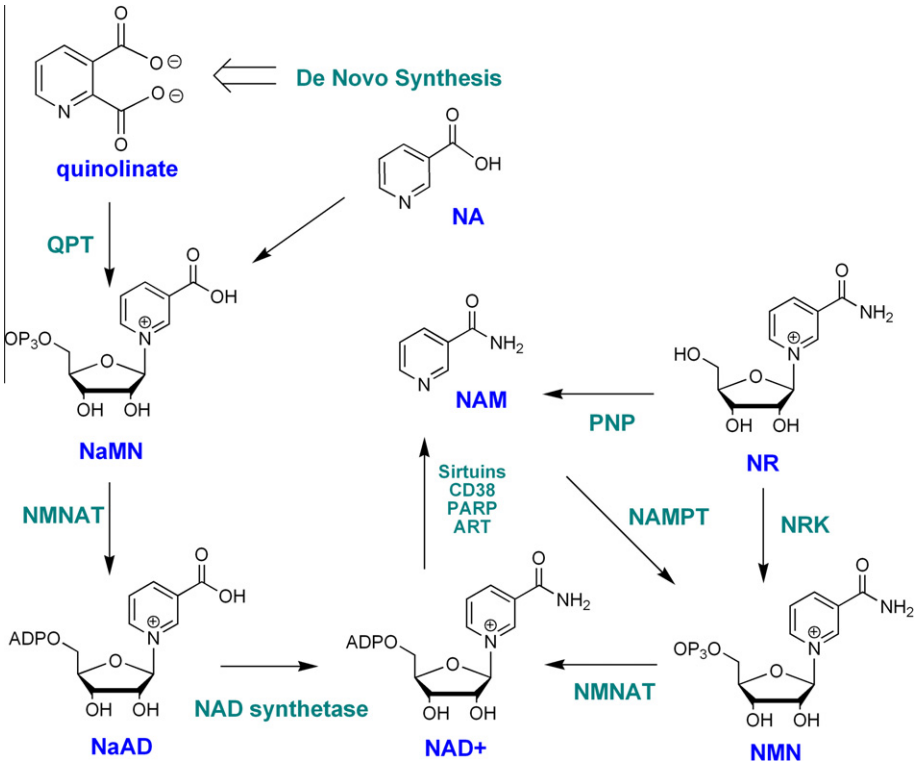

Fig. 2. NAD^+ biosynthetic pathways in mammals. Naming derives from mammalian abbreviations. NA: nicotinic acid; NR: NAM riboside; NAM: nicotinamide; NAM NMN: NAM mononucleotide; NaMN: nicotinic acid mononucleotide; NaAD: nicotinic acid adenine dinucleotide; QPT: nicotinic acid phosphoribosyl-transferase; NRK: NAM riboside kinase; ART: ADP-ribosyl transferase; PARP: poly-ADP-polymerase; Nampt: NAM phosphoribosyltransferase; PNP: purine nucleoside phosphorylase; NMNAT: NMN/NaMN adenylyltransferase.

ADPR transfer (ART) enzymes (Fig. 1C). NAD^+ is the electrophile, and the ADPR moiety is transferred to a variety of cellular nucleophiles, including proteins (Fig. 1C). This ADPR transfer chemistry is facilitated by the property of NAM as a good leaving group, with a pK_a value of near 3.5 (Jackson et al., 2003). PARPs and sirtuins harness this general reactivity in addition to the NAD^+ glycohydrolase/ADP-ribosyl cyclases CD38 (Sauve and Schramm, 2004) and CD157 (Ortolan et al., 2002). In addition there are a number of other putative ART enzymes encoded by the human genome with possible effects in modulating protein activities by ADP-ribosylation (Hottiger et al., 2010). The diverse ADP-ribosylation enzymes encoded by the mammalian genome suggest that ADP-ribosyl modifications are of fundamental importance for shaping mammalian physiology.

1.1.2.1. NAD^+ biosynthesis. NAD^+ is biosynthesized by a number of different pathways in humans (Bogan and Brenner, 2008; Sauve, 2008; Xu and Sauve, 2010). In broader terms, these can be broken down into *de novo* and salvage pathways. The *de novo* pathway in humans derives from the essential amino acid tryptophan, which is catabolized through the kynurenic pathway to quinolinic acid (Fig. 2) (Satyanarayana and Rao, 1980). Quinolinic acid is the universal metabolite in biology that generates the aromatic pyridine ring of NAD^+ (Colabroy and Begley, 2005; Kurnasov et al., 2003). This metabolite is coupled to the activated sugar metabolite 5-phospho-ribosyl-1-pyrophosphate (PRPP) to produce nicotinic acid mononucleotide (NaMN) with decarboxylation (Gholson et al., 1964). NaMN intersects the salvage pathway of nicotinic acid (niacin, NA), which was first characterized by Preiss and Handler in human erythrocytes (Preiss and Handler, 1958a,b). NA is coupled to PRPP via a separate enzyme nicotinic acid phosphoribosyltransferase which has interesting biochemical properties, in that it appears to couple NA and PRPP in a coupled reaction with ATP hydrolysis (Galassi et al., 2012; Vinitsky and Grubmeyer, 1993). This ATPase activity assists forward progress of the reaction via energy coupling (Vinitsky and Grubmeyer, 1993). NaMN is subsequently adenylated to nicotinic acid adenine dinucleotide by one of three mammalian adenylyltransferases (Lau et al., 2009; Schweiger et al., 2001) (NMNAT1, NMNAT2 or NMNAT3) and then the acid is converted to an amide via a glutamine dependent NAD^+ synthetase (Bembenek et al., 2005; Bieganowski and Brenner, 2003). These reactions complete the biosynthetic process that culminates in NAD^+ synthesis from *de novo* NA synthesis and NA salvage.

A separate salvage pathway is known, although only recently characterized, wherein NAM is coupled to PRPP to form NMN, via an enzyme called nicotinamide phosphoribosyltransferase (Revollo et al., 2004; Rongvaux et al., 2002) (Nampt). This latter enzyme has a weakly coupled ATPase activity, thereby having some similarity to the corresponding nicotinate coupling enzyme (Burgos and Schramm, 2008). This enzyme has a very low K_m for NAM, ranging from 1 μM to 5 nM (Burgos

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and Schramm, 2008) and is key to setting NAD^+ levels in cells (Revollo et al., 2004; Yang et al., 2007a). NAM recycling is of singular importance, because of the abundance of ART activities in cells, which generate continuous flux of NAM, which sustain *in vivo* tissue concentrations of NAM well above 20 μM (Qin et al., 2006). The importance of NAM recycling activity in regulating NAD^+ biosynthesis is discussed in the following section. Importantly, the Nampt activity is not found in lower metazoans, suggesting that this enzyme is a mammalian adaptation (although the last common ancestor is unidentified) (Yang et al., 2007a).

In addition there has been an identification of two kinases encoded in mammalian cells called nicotinamide riboside kinase 1 and 2 (Nrk1 and 2) (Bieganowski and Brenner, 2004; Tempel et al., 2007). These enzymes catalyze the efficient phosphorylation of nicotinamide riboside (NR) and nicotinic acid riboside (NaR) *in vitro* (Tempel et al., 2007). The K_m and k_{cat} parameters are as follows: human Nrk1 NR: $k_{cat} = 0.6\ s^{-1}$, $K_m = 88$ μM, NaR: $k_{cat} = 0.21\ s^{-1}$, $K_m = 51$ μM; human Nrk2 NR $k_{cat} = 0.34\ s^{-1}$ $K_m = 190$ μM; NaR: $k_{cat} = 0.34\ s^{-1}$, $K_m = 63$ μM (Tempel et al., 2007). A structural study of the human Nrk1 enzyme complexed with NR and a non-hydrolyzable ATP analog has confirmed that the enzyme accommodates NR into a geometry that places the 5-OH of the ribose into close proximity to the terminal phosphate position of the ATP for efficient phosphorylation (Tempel et al., 2007). Studies of the Nrk1 and Nrk2 roles in mammalian NAD^+ biosynthesis are very limited, although studies of the human enzymes in yeast establish that they can complement loss of the corresponding Nrk1 in yeast (Bieganowski and Brenner, 2004). Moreover, yeast can grow on NR if NAD^+ synthetase is deleted (Δ*qns1*), indicating that NR is metabolized differently from nicotinamide or nicotinic acid (Bieganowski and Brenner, 2004). The putative role of NR as a mammalian metabolite is supported by detection of NR in milk (Bieganowski and Brenner, 2004), although quantitative information on its abundance in milk is currently unavailable. Detection of NR in liver tissues has been reported by the Imai laboratory although quantitation was not provided (Yoshino et al., 2011).

The relative contributions of the different pathways of NAD^+ synthesis in mammals is only generally understood, and is subject to many factors including diet (Rodgers and Puigserver, 2007; Yang et al., 2007a). Humans do not encode efficient pathways for nicotinic acid synthesis, suggesting that nicotinic acid is not an abundant cellular metabolite. On the other hand, plant and fermented food sources are likely fortified with NA, since plants, yeast and bacteria encode nicotinamidases (French et al., 2010). Meats are enriched in NAM and have less NA.

1.1.2.2. Regulation of NAD^+ biosynthesis. The ability of cells to regulate NAD^+ synthesis was only recently appreciated. Consistent with the centrality of NAM recycling as the ultimate regulator of NAD^+ levels in cells and tissues, it has been determined that the enzyme Nampt is subject to dynamic regulation (Fulco et al., 2008; Yang et al., 2007a), and that it is also subject to circadian influences (Nakahata et al., 2009; Ramsey et al., 2009). The NAM salvage pathway is thought to be central to mammalian NAD^+ homeostasis, since NAD^+ has a limited lifetime in tissues. For example NAD^+ has a reported half-life of 5–10 h in liver (Ijichi et al., 1966). Sauve laboratory experiments in cell culture measure NAD^+ half-lives in the timeframe of 3–5 h in unstressed cells. Several laboratories have examined the responsiveness of NAD^+ metabolism to level of Nampt expression, and have determined that Nampt level determines cellular NAD^+ level (Yang et al., 2007a). Nampt appears to be induced by different stresses, such as reduced nutrient availability and exercise (Costford et al., 2010). Fulco et al. established a link with Nampt transcription linked to AMP-activated protein kinase (AMPK) activation (Fulco et al., 2008). Recent work by the Chang laboratory has also established that cAMP production can activate NAD^+ biosynthesis, presumably also through AMPK activation (Park et al., 2012). Some other enzymes that may be dynamically regulated include NMNAT-2. This adenylyltransferase is limiting in injured axons, and its targeted degradation may lead to rapid NAD^+ depletion and may stimulate axon degeneration (Gilley and Coleman, 2010).

Key questions of interest include why NAD^+ metabolism should be regulated in the first place? One possible explanation is that NAD^+ levels are important for optimizing metabolic performance during different nutritional situations; in light of the key involvement of NAD^+ in key metabolic pathways (glycolysis, fermentation, pyruvate dehydrogenase, TCA cycle and oxidative phosphorylation). In fact, dynamic regulation of NAD^+ metabolism by nutritional stress, while not preserved in specific details, is phylogenetically conserved from yeast to humans. The downstream coupling of powerful signaling enzymes called sirtuins, which are sensitive to NAD^+ concentrations, establishes a second set of effectors that are cued by these NAD^+ biosynthetic changes.

1.2. Sirtuins as NAD^+ consuming enzymes

Sirtuins have emerged in the last decade as an essential family of enzymes in the regulation of eukaryotic metabolism. In mammalians, sirtuins control whole body metabolic homeostasis and are postulated as promising targets for multiple pathophysiological states, including insulin resistance, cardiovascular disease, neurodegeneration and cancer (Houtkooper et al., 2012; Nakagawa and Guarente, 2011).

The first sirtuin, Sir2 (silent information regulator 2) was identified almost three decades ago as a protein contributing to gene silencing (Ivy et al., 1986; Shore et al., 1984). However, Sir2 remained as a largely overseen protein until Kaeberlein and collaborators demonstrated in 1999 how Sir2 could influence yeast replicative lifespan (Kaeberlein et al., 1999). Additional copies of Sir2 increased yeast replicative lifespan by 30%, while ablation of the Sir2 gene had the opposite effects, reducing life span by 50% (Kaeberlein et al., 1999). A critical breakthrough in the sirtuin world came immediately after, when Sir2 was demonstrated to be an NAD^+-dependent deacetylase enzyme (Imai et al., 2000). Unlike all previously described deacetylases (HDACs Type I and II), Sir2 coupled the removal of acetyl modifications on lysine residues to the consumption of NAD^+,

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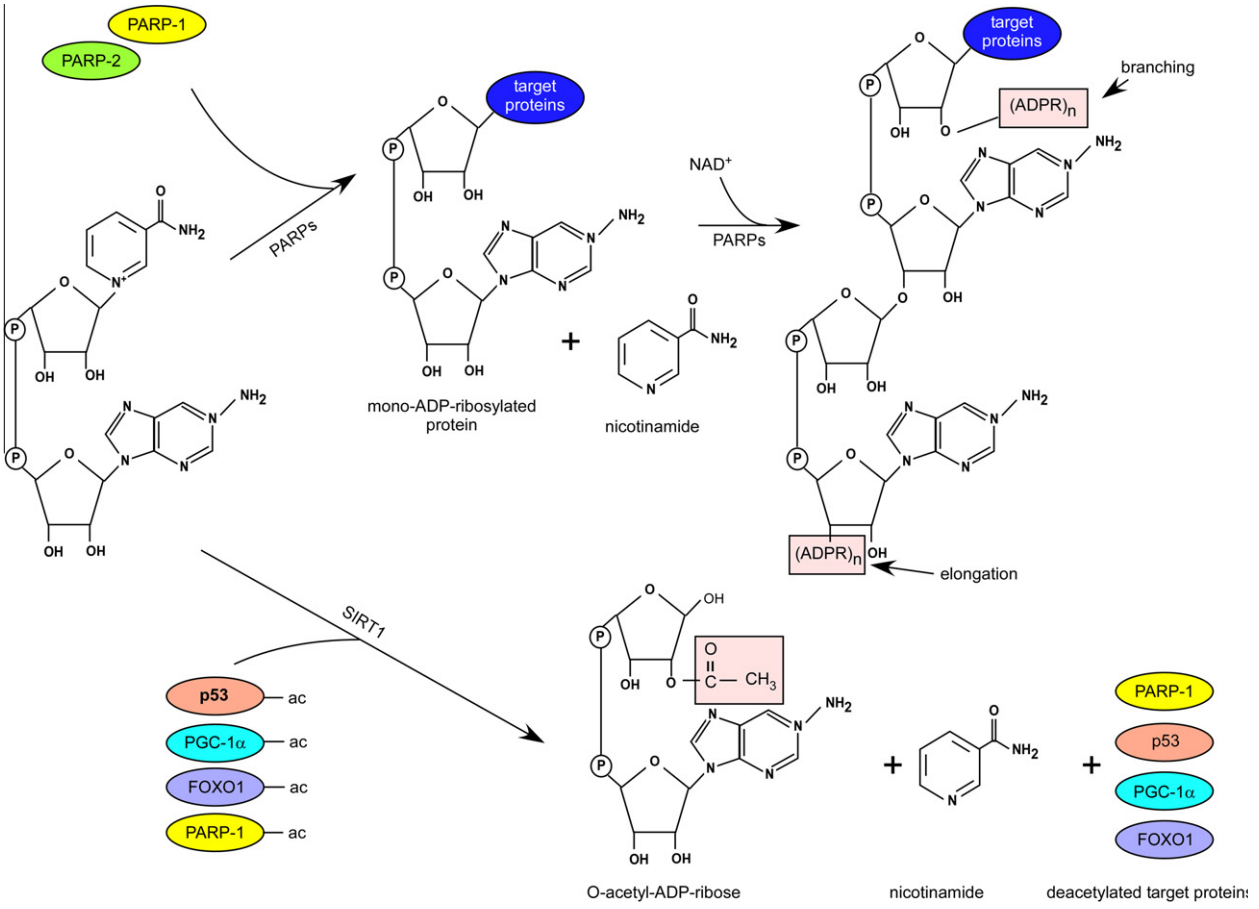

Fig. 3. An overview of the deacetylation reaction catalyzed by SIRT1 and mono/poly(ADP-ribosyl)ation reactions catalyzed by PARPs.

providing NAM and O-acetyl-ADP ribose as side products (Fig. 3). The coupling of the deacetylase reaction to NAD^+ at a K_m around the intracellular concentration of NAD^+ immediately suggested a potential link between Sir2 activity and the metabolic/redox status of the cell (Guarente, 2000; Imai et al., 2000). This notion was further supported by a possible implication of Sir2 and its invertebrate orthologs as effectors of the metabolic adaptations triggered by caloric restriction (see Canto and Auwerx, 2009, for review). However, the consistency and amplitude of the effects of Sir2 orthologs in organismal lifespan and their role as key mediators by which calorie restriction enhances lifespan in lower eukaryotes are still a matter of debate (Burnett et al., 2011; Kaeberlein and Powers, 2007; Lombard et al., 2011; Viswanathan and Guarente, 2011). While there are also some caveats on the mammalian translation of the link between Sir2 and lifespan, it is nevertheless true that the mammalian sirtuins have key role in metabolic regulation, as will be discussed below.

Sir2 unfolded into seven mammalian homolog family members (SIRT1–7). The seven mammalian sirtuins share a conserved catalytic domain of 275 aminoacids and their expression is quite ubiquitous (Michan and Sinclair, 2007). The different members of the sirtuin family, however, show distinct features that might endow them with specific functions. An initial difference can be found in their subcellular localization: SIRT1 can shuttle between the nucleus and the cytosol, and its predominant localization varies depending on the cell type and environmental cues (Michishita et al., 2005; Tanno et al., 2007). SIRT2 is predominantly cytosolic (Michishita et al., 2005). In contrast, SIRT3, SIRT4 and SIRT5 are considered mitochondrial proteins (Hallows et al., 2008; Michishita et al., 2005), whereas SIRT6 and SIRT7 are nuclear. However, while SIRT6 is located in the heterochromatin, SIRT7 is mostly found in the nucleolus (Michishita et al., 2005).

A second key difference between sirtuins can be found at the level of their catalytic activity. Originally, Sir2 was characterized as a deacetylase enzyme (Imai et al., 2000). However, its spectrum of functions has largely expanded in mammals. SIRT1, SIRT2 and SIRT3 maintain a strong (North et al., 2003; Schwer et al., 2002; Vaziri et al., 2001), while, SIRT4–6 display weak deacetylase activity. Instead, SIRT4 and SIRT6 might rather act as NAD^+-dependent mono-ADP-ribosyltransferases (Haigis et al., 2006; Liszt et al., 2005). SIRT5 has recently been reported to amplify the spectrum or sirtuin functions, being able to act as a demalonylase and desuccinylase enzyme (Du et al., 2011). In this sense, it would not be surprising if new deacylation activites are identified within the sirtuin family in the near future. SIRT7 seems to predominantly act as a deacetylase, but only a few substrates have been identified, such as p53 (Vakhrusheva et al., 2008b) and H3K18 (Barber et al., 2012).

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The activity of sirtuins is characterized by its NAD^+ dependence. Kinetic studies have determined that the K_m of most sirtuins for NAD^+ are in the range of 100–300 μM (see Houtkooper et al., 2010, for review). Intracellular concentrations of bioavailable NAD^+ are still to this date not easy to determine. While most papers report fluctuations of NAD^+ concentrations between 200 and 500 μM (Houtkooper et al., 2010), these estimations do not take into account cellular compartmentalization or whether the measured NAD^+ is freely available or protein-bound. Considering that freely available NAD^+ is only a fraction of the total NAD^+ content of the cell, it is likely that the activity of sirtuins could truly be rate-limited by NAD^+ in certain scenarios.

A number of interventions aimed to increase NAD^+ bioavailability have been shown to impact on sirtuin activity. For example, dietary supplementation with NAD^+ precursors, such as NMN or NR enhances sirtuin, at least SIRT1 and SIRT3, activation in rodent tissues (Canto et al., 2012; Yoshino et al., 2011). Physiologically, NAD^+ levels generally fluctuate within a twofold range (Chen et al., 2008; Houtkooper et al., 2010; Rodgers et al., 2005), which is a fine range to affect sirtuin activity. In general, it has been observed that sirtuins are activated in situations of energy stress, including exercise (Canto et al., 2009, 2010), and nutrient deprivation (fasting or caloric restriction) (Canto et al., 2010; Rodgers et al., 2005). All these situations are also characterized by increases in NAD^+ levels (Canto et al., 2009, 2010; Chen et al., 2008; Costford et al., 2010; Rodgers et al., 2005). In addition, NAD^+ fluctuates in a circadian fashion according to feeding/fasting cycles (Nakahata et al., 2009; Ramsey et al., 2009). While a causal link has not been demonstrated to date, the fact that SIRT1 activity also changes in a circadian fashion (Asher et al., 2008; Nakahata et al., 2008) strongly suggest that NAD^+ levels could act as a determinant for these shifts.

It is important to note that sirtuins are also tightly regulated by NAM, a product of their catalytic activity. Actually, NAM is also a reaction product of other NAD^+ consuming enzymes, such as PARPs or cADP-ribose synthases (CD38 and CD157) (Houtkooper et al., 2010). This way, enhanced activity of non-sirtuin NAD^+ consuming enzymes might not only influence sirtuin activity by reducing the availability for NAD^+, but also by increasing NAM levels. In this sense, it is important to note that NAD^+ can be generated from NAM via salvage pathways, initiated and rate-limited in mammals by Nampt (Revollo et al., 2004). The overexpression of Nampt favors NAD^+ synthesis while lowering NAM levels in virtually any mammalian cell tested (Fulco et al., 2008; Pittelli et al., 2010; Rongvaux et al., 2008; van der Veer et al., 2005). Consequently, overexpression, or knock-down of Nampt were associated with increases or reductions, respectively, of, at least, SIRT1 activity (Fulco et al., 2008; Revollo et al., 2007; van der Veer et al., 2005, 2007).

Amongst the whole family of mammalian sirtuins, SIRT1 is the one more deeply studied. SIRT1 might play a crucial role in metabolic homeostasis by regulating the activity of a number of transcriptional regulators (Canto and Auwerx, 2012). The deacetylation by SIRT1 can lead to direct activation or inhibition of the target transcriptional regulator, as well as the modification of their interaction profiles. The spectrum of transcriptional targets for SIRT1 includes key controllers of mitochondrial biogenesis (peroxisome proliferator activated receptor γ coactivator (PGC)-1α), lipid and carbohydrate metabolism (peroxisome proliferator activated receptors (PPARs), sterol regulatory element binding protein (SREBP)-1, liver X receptor (LRX), FOXOs, cAMP response element binding protein (CREB), CREB regulated transcription coactivator 2 (CRTC2), etc.) and cellular proliferation (p53). Given the dual localization of SIRT1 in both the cytoplasmatic and nuclear compartment, it is not surprising that SIRT1 also deacetylates a constellation of cytosolic proteins, including acetyl-coA synthase 1, endothelial nitrogen monoxide synthase (eNOS) and components of the authophagy machinery, including the Atg family of proteins. For an extensive overview on SIRT1 targets, we refer the reader to other recent reviews (Canto and Auwerx, 2012). Broadly, the activation of SIRT1 leads to changes in the acetylation status of these targets, which co-ordinately orchestrate cellular and whole-body metabolism to extract energy from non-carbohydrate sources and using respiration based-routes. This perfectly matches the fact that SIRT1 is activated in situations of nutrient scarcity. Further pinpointing the interaction between SIRT1 and the metabolic status, SIRT1 expression is triggered by nutrient scarcity and other energy stresses, while blocked by nutrient abundance. A number of transcription factors can regulate the expression of SIRT1 under fasting conditions, such as CREB, PPARs, FOXOs or p53 (see Canto and Auwerx, 2012). Conversely, transcription factors activated by high glucose availability, such as ChREBP, downregulate SIRT1 levels (Noriega et al., 2011).

SIRT2 is the only sirtuin residing primarily in the cytoplasm (Michishita et al., 2005). An initial functional clue was provided by the finding that SIRT2 acts as a tubulin deacetylase (North et al., 2003). At the same time, SIRT2 was demonstrated to be downregulated in human gliomas (Hiratsuka et al., 2003), the most frequent malignant brain tumors, which suggested a tumor suppression role. The interest on SIRT2 has re-emerged recently as the identification of SIRT2 targets unfolds. SIRT2 has been shown to target also key metabolic regulators, such as FOXOs (Jing et al., 2007), the p65 subunit of NF-kB (Rothgiesser et al., 2010) and phosphoenolpyruvate carboxykinase (PEPCK) (Jiang et al., 2011), suggesting a role in the regulation of inflammation, gluconeogenesis and the responses to caloric restriction. In addition, SIRT2 has been linked to Hungtinton disease (HD), by acting as a key regulator of sterol biosynthesis (Luthi-Carter et al., 2010). Surprisingly, experiments in SIRT2 knock-out mice do not support a major role of SIRT2 in tubulin acetylation, cholesterol biosynthesis or the progression of HD (Bobrowska et al., 2012), indicating that either it is dispensable or that compensatory activities might exist. In all, the role of SIRT2 in mammalian biology is still far from established. Transgenic models currently arising will help uncovering the roles of SIRT2.

Probably SIRT3 is the sirtuin that has attracted most attention in the last few years. SIRT3, together with SIRT4 and SIRT5, was identified as a mitochondrial sirtuin. Interestingly, only the deletion of SIRT3, but not other mitochondrial sirtuins, led to mitochondrial protein hyperacetylation (Lombard et al., 2007). The target proteins of SIRT3 include mitochondrial respiratory complexes, TCA cycle proteins and enzymes related to lipid metabolism and reactive oxygen intermediates (ROI)

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detoxification (for review, see Giralt and Villarroya, 2012). While no robust phenotype is found on SIRT3$^{-/-}$ mice in normal conditions, they show many layers of defects when nutritionally challenged. For example, fasted SIRT3$^{-/-}$ mice show defects in fatty acid oxidation (Hirschey et al., 2010) and ketogenesis (Shimazu et al., 2010). Upon caloric restriction, SIRT3 also determines isocitrate dehydrogenase (IDH)2 (Someya et al., 2010) and superoxide dysmutase (SOD)2 (Qiu et al., 2010) acetylation, which act as key controllers of ROI levels. The impact of SIRT3 in the function of these proteins also provides a possible explanation on why SIRT3 seems protective against cancer development (Bell et al., 2011; Kim et al., 2010a). In general, the activation of SIRT3 procures optimal mitochondrial function and energy synthesis. In agreement with this notion, SIRT3 is positively regulated at the transcriptional level by PGC-1α, a master orchestrator of mitochondrial biogenesis, and in response to fasting and other energy stresses (Hirschey et al., 2010; Kong et al., 2010; Palacios et al., 2009). Fully confirming the critical role of SIRT3 in energy homeostasis, SIRT3$^{-/-}$ mice were more prone to obesity and metabolic disease upon a fat regime (Hirschey et al., 2011). Of note, the defects of the SIRT3 null mice do not seem to be explained by a single tissue deficiency (i.e. liver-specific or muscle-specific defects) (Fernandez-Marcos et al., 2012), suggesting that the coordinated defect of SIRT3 in multiple tissues might be required to prompt these metabolic phenotypes.

The role of another mitochondrial sirtuin, SIRT4, is far less known. Initial studies identified SIRT4 as a mono-ADP-ribosylase for the glutamate dehydrogenase (GDH) enzyme. Mono-ADP ribosylation by SIRT4 impaired GDH activity, compromising amino-acid induced insulin secretion (Haigis et al., 2006). SIRT4 deficient mice display no gross phenotyping abnormalities, but have increased plasma insulin levels in fed, fasted and aminoacid-stimulated situations (Haigis et al., 2006). Recently, SIRT4 has also been shown to act as a modulator of fat metabolism in hepatocytes and myocytes. In an opposed fashion to SIRT3, the downregulation of SIRT4 potentiates fatty acid oxidation (Nasrin et al., 2010). Given that SIRT4 promotes opposite effects to those of SIRT1 on insulin secretion (Bordone et al., 2006; Moynihan et al., 2005), or SIRT3 on fat oxidation (Hirschey et al., 2010), it will be crucial to understand how the activation of these enzymes is regulated and physiologically integrated. In addition, it suggests that mitochondrial sirtuin activation might not just depend on NAD^+ availability, and that multiple other regulatory layers might exist.

As SIRT4, SIRT5 is a mitochondrial sirtuin with weak deacetylase activity (Du et al., 2011). Still, SIRT5 has been shown to regulate the activity of the carbamoyl phosphate synthase 1 (CPS-1) enzyme through direct deacetylation (Nakagawa et al., 2009). CPS-1 plays a crucial role in ammonia detoxification, as it is a critical step in the urea cycle. The deacetylation of CPS-1 by SIRT5 enhances CPS-1 catalytic activity in situations of fasting, allowing to handle ammonia detoxification during this higher amino acid catabolism state (Nakagawa et al., 2009). A major breakthrough in the sirtuin field came recently with the finding that the primary function of SIRT5 might not be to act as a deacetylase, but rather as a demalonylase and desuccinylase (Du et al., 2011). The relevance of malonylation and succinylation events in the mitochondria will be fertile ground for research in the upcoming years.

SIRT6 is another sirtuin that is gaining a lot of attention recently, due to its crucial roles in genomic DNA stability, metabolism and aging. Initially, SIRT6 was described as a mono-ADP-ribosylation enzyme (Liszt et al., 2005). Later studies, however, indicated that SIRT6 had also critical actions as a histone deacetylase (Michishita et al., 2008). SIRT6 null mice die prematurely, displaying severe defects, such as lymphopenia, loss of subcutaneous fat, decreased bone mineral density, hypoglycemia and reduced levels of insulin-like growth factor (IGF)-1 (Mostoslavsky et al., 2006). At least some of these effects might be explained by the overactivation of the hypoxia-inducible factor 1α (HIF-1α), which leads to abnormally high glycolytic rates (Zhong et al., 2010). In this scenario, SIRT6 was found to act as a co-repressor of HIF-1α function (Zhong et al., 2010). In line with the above results, liver-specific deletion of SIRT6 led to increased glycolysis, triglyceride synthesis, reduced beta oxidation, and fatty liver formation (Kim et al., 2010b). Strikingly, mice with a neuron-specific defect of SIRT6 are also smaller at birth, but recover normal body weight later and even develop obesity in late life stages (Schwer et al., 2010). The mechanisms regulating these phenotypes are not clear yet. Additional knowledge on SIRT6 has been provided by gain-of-function strategies. Overexpression of SIRT6 renders protection against high-fat diet obesity (Kanfi et al., 2010) and has been recently shown to increase lifespan in mice (Kanfi et al., 2012). SIRT6, therefore, becomes the first sirtuin with genetic evidence for a direct effect on mammalian lifespan.

Finally, SIRT7 might still be the less known sirtuin. SIRT7 is localized in the nucleolus and was described as a component o the RNA polymerase I (Pol I) transcriptional machinery (Ford et al., 2006). However, the specific enzymatic activity of SIRT7 and its targets in these complexes remain unclear. Initial hints of a likely deacetylase activity of SIRT7 were confirmed when SIRT7 was reported to be a p53 deacetylase in cardiomyocytes (Vakhrusheva et al., 2008b). This way, mice lacking SIRT7 display cardiac hypertrophy, linked to p53 hyperacetylaion. The defects in cardiac morphology dampen the mean and maximum lifespan of SIRT7 null mice (Vakhrusheva et al., 2008b). In addition, a role for SIRT7 in cancer, while hypothesized a few years ago (Vakhrusheva et al., 2008a), has been recently confirmed by elegant studies showing how the deacetylation of H3K18Ac by SIRT7 is necessary for maintaining essential features of human cancer cells (Barber et al., 2012). The possible roles of SIRT7 in chromatin regulation, cellular transformation programs and tumour formation *in vivo* warrants future research and might also unveil further links between metabolic sensing and tumor development.

When viewed as a whole, it is clear that sirtuins play a key role in metabolic adaptation and in all the processes in the cell that are governed or require changes in energy substrate utilization: from caloric restriction to cell growth and proliferation control. Still, the many ways by which sirtuins might be regulated are still unclear. Their catalytic reaction is NAD^+-dependent, but to this date it is still difficult to unequivocally demonstrate that sirtuin activity is determined by physiological fluctuations in NAD^+. This does not rule out, however, than in extreme toxicity situations, where NAD^+ levels sharply drop by 50–70% (Goodwin et al., 1978; Pillai et al., 2005; Skidmore et al., 1979), NAD^+ might truly become rate-limiting

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for the sirtuin reaction. The K_m of most sirtuins for NAD^+ is still not well-determined (Houtkooper et al., 2010), however seems different for the members of the sirtuin family suggesting that all sirtuins are not activated at the same time that seems logical given the often opposing biological effect of these proteins. Compartmentalization of NAD^+ bioavailability may also refine sirtuin activation, as it might allow sirtuin activation in a compartment specific fashion. Additionally, there are a few examples of proteins whose deacetylation *in vivo* is primed or impeded by other post-translational marks (for examples, see Canto et al., 2009; Murray-Zmijewski et al., 2008) which could help refining subsets of targets to be deacetylated. A canonical example of how sirtuin activity specification must be required is the one constituted by SIRT3 and SIRT4, both of which share cellular compartment and NAD^+-dependence, but drive apparently opposite metabolic adaptations. Altogether, logic dictates that sirtuins activity might be influenced by NAD^+, but that many additional regulatory layers must exist in order to achieve specific substrate deacetylation and fine-tune their activity to the cellular metabolic needs.

1.3. Enzymology, function and biological significance of poly(ADP-ribose) polymerases

Poly(ADP-ribosyl)ation (PARylation) was identified by Pierre Chambon and colleagues (Chambon et al., 1963) initiating a half century long quest of understanding PARP enzyme action. PARP-1, the main enzyme responsible for that biochemical activity, was recognized in 1967 (Shimizu et al., 1967). Recently several other PARP enzymes, possessing a catalytic domain similar to that of PARP-1, were identified (PARP-1 to -17 in humans, PARP-1 to -16 in mice) (Ame et al., 2004). Besides the PARP domain, responsible for catalytic activity, PARPs are equipped with numerous other domains enabling the execution of a plethora of molecular functions (reviewed in Ame et al., 2004; Hottiger et al., 2010). Among others, there are domains for DNA binding (e.g. the zinc fingers in PARP-1 (Langelier et al., 2008; Mazen et al., 1989; Menissier-de Murcia et al., 1989), or SAP domain in PARP-2 (Huber et al., 2004)), protein–protein interaction (e.g. BRCT domain in PARP-1 (de Murcia et al., 1994), or ankyrin repeats in tankyrases (Smith et al., 1998)), or the macro domain in the macro-PARPs for PAR binding (Karras et al., 2005). In certain PARP enzymes nuclear, or nucleolar localization signals guide protein transport between organelles (Meder et al., 2005; Schreiber et al., 1992).

Poly(ADP-ribosyl)ation (PARylation) is considered to be an ancient and evolutionarily conserved biochemical reaction. In line with that PARP catalytic domain is highly conserved throughout evolution as shown in sequence analysis studies (Otto et al., 2005) and by the discovery of PARP enzymes in plants (Doucet-Chabeaud et al., 2001; Lepiniec et al., 1995), in lower animals (Tewari et al., 1995), or certain eubacteria, arhaebacteria and double-stranded DNA viruses (Hassa et al., 2006; Otto et al., 2005). The catalytic domain of the chicken PARP-1 enzyme had been crystallized first (Ruf et al., 1996) giving insight into PARP action. The structure of the known catalytic domains of other members of the PARP superfamily displayed high sequence and structural homology with each other (Hottiger et al., 2010). Moreover, despite the poor sequence homology, considerable structural homology was observed with the catalytic domain of bacterial ARTs (Hottiger et al., 2010; Ruf et al., 1996).

PARP-1, considered as the prototypical PARP enzyme, cleaves NAD^+ and forms large, negatively charged poly(ADP-ribose) (PAR) polymers on a large set of target proteins. The poly(ADP-ribosyl)ation reaction (PARylation) can be divided into three steps: initiation, elongation and branching (Fig. 3) (Alvarez-Gonzalez and Mendoza-Alvarez, 1995). In the initiation phase, reaction the glycosidic bond between nicotinamide and ribose is cleaved due to the nucleophylic attack of glutamate, aspartate residues, or the carboxy terminal of acceptor proteins (Bellocchi et al., 2006) (positively charged lysine residues were also shown to be PAR acceptors (Altmeyer et al., 2009)). Then the mono-ADPR units are bonded via an ester bond (Altmeyer et al., 2009; Burzio et al., 1979; Ogata et al., 1980). The ADPR moiety remains bound to the acceptor protein, while NAM is released in the reaction. Subsequently, the enzymes catalyze elongation and branching reactions using additional ADPR units from NAD^+ leading to formation of branched polymers up to 200 ADPR units (Hayashi et al., 1983). The half-life of the polymer is estimated to be less than 1 min, it is rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase (Kawaichi et al., 1983; Ueda et al., 1972).

How are PARPs activated? The first known activator of PARP-1 was DNA strand breaks (Benjamin and Gill, 1980) and irregular DNA structures (Kun et al., 2002). PARP-1 binds to these structures through its zinc fingers that subsequently leads to its activation. To date PARP-1, -2 and -3 had been shown to be induced by DNA damage (Ame et al., 1999; Boehler et al., 2011; Menissier-de Murcia et al., 1989; Rulten et al., 2011). The majority of DNA-induced PARP activity is covered by PARP-1 (85–90%), while PARP-2 is considered to be responsible for the rest (Schreiber et al., 2002; Szanto et al., 2011). It seems that not all PARPs are active, or build polymers: PARP-13 is inactive, PARP-7, PARP-10 and PARP-16 perform only mono-ADP-ribosylation (Di Paola et al., 2012; Kleine et al., 2008; Leung et al., 2012; Ma et al., 2001), while PARP activity of PARP-9 and PARP-13 is under debate.

PAR molecules may be introduced onto PARP-1 itself (autoPARylation), or onto other proteins (transPARylation). PARP-1 autoPARylation efficiently inhibits PARP-1 activity (Kawaichi et al., 1981; Zahradka and Ebisuzaki, 1982) due to strong electrostatic repulsion between DNA and PAR. PARP-2 has also been reported to perform autoPARylation (Ame et al., 1999) suggesting the existence of a similar autoPARylation cycle as PARP-1. Inhibition of PARP-1 by autoPARylation seems an exquisite mechanism to avoid uncontrolled and excessive PARP-1 activity. The inhibitory effect of autoPARylation can be reverted by PARG that removes PAR polymers creating a reversible PARylation cycle for PARP-1 (Erdelyi et al., 2009; Ying and Swanson, 2000). Indeed, inhibition, or deletion of PARG blocks PARP-1 in a PARylated state and therefore protect against PARP-1 mediated NAD^+ and ATP depletion and the consequent cell death (Bakondi et al., 2004; Erdelyi et al., 2009; Ying and Swanson, 2000).

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There are numerous posttranslational pathways through which the activity of PARP enzymes can be regulated. Reversible phosphorylation regulates PARP-1 (for a proteomic approach see Gagne et al., 2009, for review, see Virág and Bürkle, in this series) and tankyrases (Ha et al., 2012; Li et al., 2012; Yeh et al., 2006). PARP-1 is acetylated and activated by p300/CBP-association factor (PCAF) and p300 (Hassa et al., 2005; Rajamohan et al., 2009), while deacetylation by SIRT1 leads to radical decrease in PARP activity (Rajamohan et al., 2009). PARP-2 is acetylated by PCAF and GCN5L (Haenni et al., 2008). PIASy, a SUMO ligase physically interact and modify PARP-1 upon heat shock (Martin et al., 2009). PARP-1 can be mono-ADP-ribosylated and activated by PARP-3, or SIRT6 (Loseva et al., 2010; Mao et al., 2011). PARP-1 activity seems to be linked to cellular calcium homeostasis (Bakondi et al., 2003; Wyrsch et al., 2012).

PARP enzymes were related to numerous biological processes. The first function to be discovered for PARP-1 (and later PARP-2 and -3) was its involvement in DNA repair (Durkacz et al., 1980; Purnell and Whish, 1980). Later, the involvement of these PARP enzymes and tankyrases in the maintenance of genomic integrity was evidenced (reviewed in this series by Valérie Schreiber and Francoise Dantzer). In our current understanding, under non-stress conditions the action of PARP-1 and -2 are not essential for efficient DNA repair (Allinson et al., 2003; Bai et al., 2011a,b; De Vos et al., 2012). However, deletion of PARP-1, PARP-2, or the application of PARP inhibitors leads to sensitization against DNA damaging agents (MNNG, ionizing radiation, etc.) (Menissier-de Murcia et al., 1997, 2003; Wang et al., 1995). It seems that PAR polymers act as a scaffold matrix around DNA damage sites that other DNA repair enzymes bind to (Karras et al., 2005; Mortusewicz et al., 2007; Tartier et al., 2003). PARP-1 and -2 participate in the resolution of single strand breaks, base excision repair (Dantzer et al., 2000; Schreiber et al., 2002) and double strand break repair (Langelier et al., 2012; Szanto et al., 2012). PARP-1 has antirecombinogenic activity (Morrison et al., 1997) that consequently protects against retroviral infections (Ha et al., 2001).

Insufficient DNA repair on a longer timeline leads to either cell death, or to the accumulation of mutations, genomic instability that ultimately induce tumorigenic transformation. Indeed, the lack of PARP-1 enhanced the number of sister chromatid exchange events when challenged by DNA damaging agents (Menissier-de Murcia et al., 1997; Schreiber et al., 1995; Wang et al., 1995), however, to date, it seems that the lack of PARP-1, or -2 alone, under non-stress conditions, does not lead to tumorigenic transformation (Menissier-de Murcia et al., 2003; Wang et al., 1995). It suggests that other parallel DNA repair pathways cope with DNA damage in the absence of PARPs. However, the simultaneous removal of PARP-1 and -2, or other DNA repair enzymes, such as ataxia-telangiectasia mutated (ATM) leads to embryonic lethality (Huber et al., 2004; Menissier-de Murcia et al., 2003), or tumorigenic transformation as in the case of $p53^{-/-}$ PARP-$1^{-/-}$, or $p53^{-/-}$ PARP-$2^{-/-}$ mice (Nicolas et al., 2010; Tong et al., 2001).

The extent of PARP activation has major influence on the fate of the cell (reviewed in this series by László Virág, Agnieska Robaszkievicz, Jose Vargas and Javier Oliver). Obviously, if DNA damage is repairable, PARP activation contributes to the survival of the cell as discussed above. Unrepairable DNA damage induces apoptosis that is an energy intensive process that disposes of cells in a safe manner. Extensive PARP activation – as suggested by Berger and colleagues (Berger, 1985) – may markedly reduce cellular NAD^+ content. NAD^+ resynthesis through NMNAT and phosphoribosyl pyrophosphate synthetase (PPS) is energy consuming therefore reducing cellular ATP content. Further metabolic rearrangements encumber the replenishment of ATP: slowdown of glycolytic flow due to NAD^+ loss (Ying et al., 2002), the reversal of the activity of F1/F0 ATPase (synthase activity shifts to ATPase activity) (Ha and Snyder, 1999) and the opening of mitochondrial transition pores (Virag et al., 1998). The lack of energy prevents the progression of the apoptotic program and turns cell death into necrosis (Leist et al., 1997, 1999). The fact that PARP activation affect cell survival, moreover switches apoptosis into necrosis suggested that the application of PARP inhibitors may have beneficial effects in pathological states associated with oxidative stress (e.g. reperfusion injuries, or inflammatory pathologies) (Virag and Szabo, 2002).

PARP-1 is involved in transcriptional regulation at numerous levels (reviewed in this series by Lee Kraus and Michael Hottiger): it may modulate chromatin structure (de Murcia et al., 1986; Quenet et al., 2009), bind to enhancer sequences, or promoters (Krishnakumar et al., 2008), act as a transcriptional cofactor (Oliver et al., 1999), or may promote chromatin insulation (Yu et al., 2004) leading to complex gene expression rearrangements (Frizzell et al., 2009; Simbulan-Rosenthal et al., 2000). It is under debate whether the catalytic activation of PARP-1 is necessary in every transcriptional event (e.g. in the case of NFkB activation (Hassa et al., 2001)), however it seems that global and local NAD^+ levels affect PARP action at transcription foci (Kraus, 2008; Zhang et al., 2012). Besides, PARP-1, other PARP enzymes influence transcription (e.g. PARP-2 (Szanto et al., 2012), or PARP-14 (Mehrotra et al., 2011)).

The above detailed biological functions of PARPs act jointly in complex physiological, or pathophysiological scenarios. PARP enzymes have major impact on inflammatory diseases (Bai and Virag, 2012; Levaot et al., 2011; Mehrotra et al., 2012; Yelamos et al., 2006). PARPs influence the maturation and function of immune cells (Bai and Virag, 2012). PARP-1 is necessary for the appropriate activation of numerous proinflammatory transcription factors (e.g. NFAT, NFκB, AP-1, YY1, or sp1) that have key role in producing chemokines (Bai and Virag, 2012; Oliver et al., 1999), cytokines, adhesion factors and other inflammatory mediators (matrix metalloproteinases, cyclooxygenase-2, or inducible NO synthase) (Virag and Szabo, 2002). Under inflammatory conditions oxidative stress is largely enhanced that leads to cell death that is diverged towards necrosis by PARP activation that further enhance the inflammatory response (Virag and Szabo, 2002). Vast amount of data had been assembled suggesting that inflammatory processes can be quenched by the application of PARP inhibitors (reviewed in Bai and Virag, 2012).

Recent data suggests the involvement of PARPs in metabolic regulation (Bai and Canto, 2012) that – similarly to the inflammatory role of PARPs – stem from multiple roots. As discussed above, prolonged PARP activation through depleting cellular NAD^+ pools hamper cellular energy metabolism: glycolytic slowdown (Ying et al., 2002) and a rapid shutdown of

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mitochondrial function (Bai et al., 2001, 2007a; Cipriani et al., 2005; Virag et al., 1998). Inversely, upon the lack, or inhibition of PARP-1, or -2 mitochondrial activity is not only preserved, but are further enhanced due to the activation SIRT1 (Bai et al., 2011a,b). It seems therefore that the level and activity of PARP-1, or -2 activity is in strong correlation with mitochondrial activity (Bai and Canto, 2012). PARPs are related to other metabolic processes by interacting with several metabotropic receptors (Bai and Canto, 2012) and by influencing energy intake (Asher et al., 2010; Bai et al., 2011b). These metabolic changes together influence insulin and glucose sensitivity, adipogenesis and body weight (Bai et al., 2007b, 2011a,b; Erener et al., 2012a,b; Mangerich et al., 2010). PARP-5a, -5b, -7 and -14 also seems to influence metabolism, however their action is yet blurry (Bai and Canto, 2012).

2. Levels of SIRT–PARP interaction

2.1. Interaction of PARPs and SIRTs through the common NAD^+ substrate

As discussed in Sections 1.2 and 1.3 both PARPs and SIRTs are NAD^+ dependent enzymes that makes it likely that they may compete for the limiting NAD^+ substrate. Most studies report 200–500 μM intracellular NAD^+ concentrations, however the NAD^+ levels in different compartments (mitochondria, nucleus, or cytosol) are still debated (Houtkooper et al., 2010). As discussed in detail in Section 1.2, SIRT1 activity (and probably the activity of further members of the sirtuin family) is linked to fluctuations in NAD^+ levels (Asher et al., 2008; Canto et al., 2012; Imai et al., 2000; Nakahata et al., 2008) as the K_m of SIRT1 falls in the range of physiological cellular NAD^+ changes (Houtkooper et al., 2010).

The K_m of PARP-1 towards NAD^+ falls in the low micromolar range (20–60 μM) (Ame et al., 1999; Mendoza-Alvarez and Alvarez-Gonzalez, 1993) suggesting that physiological fluctuations in NAD^+ levels are unlikely to affect PARP-1 activity. In contrast, the K_m of PARP-2 towards NAD^+ is higher (around 130 μM) (Ame et al., 1999) that is comparable to the one of SIRT1 (Houtkooper et al., 2010). Also, PARP-1 had been described as an effective enzyme in NAD^+ degradation (Ame et al., 1999) displaying high catalytic turnover when compared to SIRT1 (Bai and Canto, 2012; Bai et al., 2011b). This is further highlighted by the fact that the maintenance of local NAD^+ levels seems important upon PARP-1 activation. NMNAT-1 has been shown to recruit to sites of PARP-1 activation upon oxidative stress (Berger et al., 2007), or in transcriptional events (Zhang et al., 2012). It seems that NMNAT-1 recruitment does not only enhance local NAD^+ availability, but activate PARP-1 in an NAD^+-independent manner (Zhang et al., 2012). PARP-1 is responsible for the majority of PARP activity (Section 1.3, Schreiber et al., 2002; Szanto et al., 2011), while the rest is mostly covered by PARP-2.

The drop in NAD^+ levels upon excessive DNA damage due to PARP activation is a long-known fact (Berger, 1985). Under such conditions NAD^+ levels may drop to 20–30% of the original that is likely to rate limit sirtuin enzymes (Houtkooper et al., 2010). SIRT1 activity is largely reduced under these conditions (Pillai et al., 2006; Qin et al., 2012; Rajamohan et al., 2009) that might be followed by decreased SIRT1 expression (Qin et al., 2012). It is logical to assume that the activity of other nuclear sirtuins will drop under these conditions, however it is not known whether extranuclear sirtuins would respond to these insults, or would remain intact.

When the biochemical changes upon deletion, or inhibition of PARP-1 were analyzed we found that NAD^+ levels were induced (20–100% as a function of cell model, or tissue) in animal and cellular models (Bai et al., 2011b). It is likely that PARP-1 activity is a major activity in NAD^+ degradation and consequently in NAD^+ turnover (Houtkooper et al., 2010), therefore the lack of PARP-1 activity elevates NAD^+ levels. That induction is translated into higher SIRT1 activity and better metabolic performance (Bai et al., 2011b).

As previously mentioned, the affinity of PARP-2 to NAD^+ and the rate of NAD^+ degradation is similar to SIRT1, therefore it is unlikely that these enzymes could limit NAD^+ for one another. In line that we were unable to detect differences NAD^+ levels of PARP-$2^{+/+}$ and $^{-/-}$ cells and tissues under non-stress and oxidative stress conditions (Bai et al., 2011a; Szanto et al., 2011).

A particularity on the interaction between PARP-1 and PARP-2 with sirtuins is that these two models seem to specifically target SIRT1: neither cytoplasmic SIRT2, nor mitochondrial SIRT3 activities were increased by the absence of PARP-1 or PARP-2 (Bai et al., 2011a,b). In the case of PARP-2, the nature of this selectivity is clearer, as it roots on the direct regulation of the SIRT1 promoter (discussed in the following section). The case of PARP-1 is a bit more complicated, as the modulation of NAD^+ levels could potentially impact on all sirtuins. The reasons for the specificity might be that the changes in NAD^+ levels promoted the reduction of PARP-1 activity could be restricted to the nucleus (Bai et al., 2011b). This is logical, as PARP-1 is predominantly a nuclear protein. Another possible explanation is that different sirtuins might have different windows of sensitivity for NAD^+. Confirming this, recent efforts from the Denu lab have demonstrated that SIRT6 binds to NAD^+, even in the absence of acetylated substrate, at a K_d around 27 μM, which is a concentration far lower than that of intracellular NAD^+ content (Pan et al., 2011). This means that NAD^+ might rarely be rate-limiting. Hence, SIRT6 activity might not act as an NAD^+ sensor and, rather, other regulatory mechanisms, such as specific protein binding or post-translational modifications, determine SIRT6 activity. In fact, it is conceivable that NAD^+ could just be permissive for certain sirtuins, and that the true switch for their activity is found in changes in their protein interactions or post-translational modifications. This might explain why only a subset of sirtuins (such as SIRT1 in the PARP-1 KO mice) is responsive to fluctuations in NAD^+. Of note, also recent studies have highlighted how post-translational modifications might change the affinity of sirtuins for NAD^+,

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therefore enhancing or blocking their sensing capabilities. This is the case actually for SIRT1, as phosphorylation by PKA during fasting enhances the sensitivity of SIRT1 for NAD^+ (Gerhart-Hines et al., 2011).

2.2. Post-translational modifications

While SIRT1 and PARP activities might influence each other through the competition for a limited NAD^+ pool, other events, such as their interaction with different proteins and the impact of diverse post-translational modifications, act also as key determinants.

A first crucial possibility would be the cross-action of both activities, i.e. that PARPs could PARylate SIRT1 and, conversely that SIRT1 could deacetylate PARPs. Very little information exists on whether SIRT1 could be a substrate for PARylation. However, SIRT1 is not PARylated in C2C12 myotubes when PARP activity is triggered by exposure to genotoxic hydrogen peroxide concentrations (Bai et al., 2011b). This suggests that endogenous SIRT1 might not be a direct PARylation target, even though additional scenarios of enhanced PARP activity will have to be tested in order to solidify this conclusion.

Conversely, it could be hypothesized that PARP-1 might be targeted by SIRT1 deacetylase activity. In line with this hypothesis, reduction of PARP activity is observed upon SIRT1 activation (Kolthur-Seetharam et al., 2006). Given the relatively high K_m and low V_{max} of sirtuins, it is unlikely that sirtuin activity ever rate-limits NAD^+ availability for PARP-1, characterized by a fivefold lower K_m and much stronger V_{max} of PARP-1 then the one of SIRT1 (Houtkooper et al., 2010) and Section 2.1. Clues to our understanding on how SIRT1 might impact on PARP activity were given when PARP-1 was identified to be an acetylated protein (Hassa et al., 2005; Rajamohan et al., 2009) and Section 1.3. In cardiomyocytes, PARP-1 acetylation was increased by mechanical stress, phenylephrine or angiotensin-II (Rajamohan et al., 2009). This increase in PARP-1 acetylation was coupled to enhanced catalytic activity and was enough to trigger PARP-1 activation in the absence of DNA damage (Rajamohan et al., 2009).

Following the discovery that PARP-1 activity is influenced by its acetylation status, Rajamohan and colleagues demonstrated that SIRT1 could directly deacetylate PARP-1. Overexpression of SIRT1 or treatment with resveratrol, as a SIRT1 agonist, both led to the deacetylation of PARP-1 in cell cultured models (Rajamohan et al., 2009). Finally, the authors also demonstrated that SIRT1-mediated deacetylation blocks PARP-1 catalytic activity (Rajamohan et al., 2009). Altogether, these observations set a scenario in which enhanced SIRT1 activity would reduce PARP-1 activity via direct deacetylation. However, if PARP-1 activity is prompted through DNA damage, this will reduce NAD^+ availability, hence blocking the ability of SIRT1 to retain PARP-1 in a deacetylated (low activity) state.

The direct influence of sirtuins on PARP activity was further reinforced when trying to elucidate why SIRT6 deficient mice display genomic instability. Remarkably, it was found that SIRT6, but not other nuclear sirtuins, is directly recruited to the sites of DNA double-strand breaks and enhances the efficiency of non-homologous end joining and homologous recombination after paraquat treatment (Mao et al., 2011). In these experiments, PARP-1 was found to be a mono-ADP-ribosylation substrate for SIRT6 (Mao et al., 2011). Both proteins bind to each other, and the binding is somehow potentiated by DNA damage. SIRT6 overexpression did not stimulate DNA repair in PARP-1 knock-out cells, indicating that PARP-1 is required to mediate the effects of SIRT6 (Mao et al., 2011). Key experiments demonstrated that while PARP-1 can be mono-ADP-ribosylated in at least six sites, only K521 is the only one affected by SIRT6. Of note, SIRT6 did not seem to affect the acetylation status of PARP-1 (Mao et al., 2011).

As mentioned in previous chapters, it will be of crucial interest to understand in which scenarios sirtuins might be selectively activated and how this is molecularly channeled. Illustrating this point, the cases above show how SIRT1 and SIRT6 exert theoretically opposite effects on PARP-1 activity (inhibition and activation, respectively). Hence, it should be expected that the docking of specific sirtuins to DNA locations or differential protein interaction might crucially determine sirtuin activity. An example of the latter case can be found in Deleted in Breast Cancer-1 (DBC-1), a protein that can selectively bind the catalytic domain of SIRT1, negatively regulating its activity (Kim et al., 2008; Zhao et al., 2008). Upon genotoxic stress, a condition that triggers PARP-1 activation, DBC-1 is phosphorylated by ATM at Thr454, creating a second binding site for SIRT1 (Yuan et al., 2012; Zannini et al., 2012). This leads to enhanced binding between SIRT1 and DBC-1, hence abolishing SIRT1 activity (Yuan et al., 2012; Zannini et al., 2012). This would provide a very elegant mechanism for shutting down SIRT1 and relieve the inhibition of PARP-1 exerted via deacetylation, while promoting simultaneously PARP-1 activation via SIRT6 mediated mono-ADP-ribosylation.

Acetylated residues have also been identified in other PARP enzymes, such as PARP-2 (Haenni et al., 2008). However, whether the acetylation status of these residues is modulated by sirtuins is not currently clear. Similarly, the identification of mitochondrial PARP activity (Du et al., 2003; Lai et al., 2008; Pankotai et al., 2009), opens a whole new world for possible direct cross-regulation through posttranslational modifications between PARP enzymes with mitochondrial sirtuins (SIRT3–5).

2.3. SIRT–PARP interaction through the regulation of gene expression

Marked changes in SIRT1 expression is capable of influencing metabolic and energetic balance. In humans, SIRT1 mRNA levels and certain SNPs in the SIRT1 gene correlated well with enhanced energy expenditure, insulin sensitivity (Rutanen et al., 2010), insulin secretion (Dong et al., 2011), or predisposition to obesity (Clark et al., 2012; Zillikens et al., 2009a).

The activity of the SIRT1 promoter had been shown to be controlled by several transcription factors, such as CREB (cAMP response element-binding protein), ChREBP (carbohydrate response element binding protein) (Noriega et al., 2011), FOXOs

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(forkhead box transcription factor O), p53 (Nemoto et al., 2004), HIC1 (hypermethylated in cancer 1) (Chen et al., 2005; Zhang et al., 2007), PPARs (peroxisome proliferator-activated receptors) (Han et al., 2010) and c-Myc (Yuan et al., 2009). Most of these transcription factors integrate nutritional signal (Nemoto et al., 2004; Noriega et al., 2011). We have described the presence of PARP-2 on the SIRT1 promoter and provided evidence that PARP-2 acts as a suppressor of SIRT1 transcription (Bai et al., 2011a; Szanto et al., 2011).

PARP-2 binds to DNA in the proximal region of the SIRT1 promoter ($^{-}$1 to $^{-}$91 region of the mouse SIRT1 promoter) (Bai et al., 2011a). This region is on one hand directly adjacent to the region where FOXOs bind ($^{-}$91 to $^{-}$202 region of the mouse SIRT1 promoter) (Nemoto et al., 2004), while on the other it's sequence is highly conserved among mammals and shows conservation when compared to the distantly related sequence of the promoter of SIRT1 in Xenopus (Bai et al., 2011a). Depletion of PARP-2 enhanced the activity of the SIRT1 promoter that translated into higher SIRT1 mRNA and protein levels in skeletal and smooth muscle, liver, brown adipose tissue and pancreas as shown in murine and cellular models (Bai et al., 2011a; Szanto et al., 2011). Interestingly, although in brown adipose tissue SIRT1 protein levels are enhanced the induction of mitochondrial activity was not detected (Bai et al., 2011a) suggesting yet unknown tissue-specific mechanisms that limit the phenotypical manifestation of PARP-2, or SIRT1 action. Likewise, tissue specific gene expression changes alter the effects of SIRT1 induction in PARP-2$^{-/-}$ mice, wherein in contrast to pancreatic SIRT1 overexpression that ameliorates β cell function (Moynihan et al., 2005) PARP-2 deficiency hampers β cell expansion leading to pancreatic dysfunction (Bai et al., 2011a) (discussed in detail in Section 3.1.4).

Alterations in NAD^+ levels upon the depletion of PARP-2 were minor or negligible in cellular models and inconsistent in *in vivo* experiments (Bai et al., 2011a; Schreiber et al., 2002; Szanto et al., 2011). That suggest that activation of SIRT1 upon PARP-2 depletion seems to rely primarily on transcriptional effects and unlikely on activation through enhanced NAD^+ availability. PARP-2 seems specific for the SIRT1 promoter, as the depletion of PARP-1 did not alter promoter activity (Bai et al., 2011a). To date, no further direct regulation of other sirtuin genes by PARPs has been clearly evaluated.

3. Physiological processes influenced by SIRT–PARP interaction

3.1. Metabolism

Experiments in cell lines and animal models have shown that sirtuins act as key regulators of oxidative metabolism and global metabolic homeostasis. The multiple levels of interaction between PARP enzymes and sirtuins (see Section 2), predict, therefore, that the modulation of PARP activity could also have a strong impact on energy metabolism.

While many of the original studies showed a negative correlation of PARP activity and sirtuin activity in situations of supraphysiological oxidative stress or DNA damage, it is worth mentioning that this relation has recently been found also in physiological scenarios. For example, PARP activity is largely increased upon high-fat feeding, when SIRT1 activity is lower (Bai et al., 2011b). Oppositely, PARP activity is lower in muscle after an overnight fast, where enhanced SIRT1 activity is observed (Bai et al., 2011b). A recent report has also highlighted how higher PARP activity is observed in aged rodent tissues, leading to decreased NAD^+ content and limiting SIRT1 activity, even though SIRT1 protein content is higher (Braidy et al., 2011). All these observations indicate how genetical and physiological variations in PARP activity might have a large impact on sirtuin activity, and, consequently, on global metabolism.

3.1.1. PARP–SIRT1 interactions in food intake behavior

PARP-1 null C57Bl/6 mice display a clear metabolic phenotype, characterized by lower body weight gain upon aging and high-fat feeding. Strikingly, this happens despite the increased food intake observed in PARP-1 knockout mice (Bai et al., 2011b; Devalaraja-Narashimha and Padanilam, 2010). Moreover, recent data indicates that PARP-1 plays role in the regulation of the circadian entrainment of feeding behavior and body temperature cycles (Asher et al., 2010). Interestingly, also SIRT1 is a key regulator of the core circadian clock molecular machinery (Asher et al., 2008; Nakahata et al., 2008). The regulation of NAD^+ bioavailability might constitute an attractive mechanism tying the circadian fluctuations of PARP-1 and SIRT1 activities. Essentially, the expression levels of Nampt, the critical rate limiting enzyme in the mammalian NAD^+ salvaging pathway, display a robust diurnal oscillation, with a peak around the beginning of the dark period in mice, in line with the maximal peak for the circadian fluctuation of SIRT1 activity (Nakahata et al., 2009; Ramsey et al., 2009). SIRT1 negatively regulates CLOCK:BMAL-1 transcriptional activity, which is a key positive controller of Nampt expression (Nakahata et al., 2009; Ramsey et al., 2009). Hence, the activation of SIRT1 shuts down Nampt expression. This will likely promote a decrease in NAD^+ levels low enough to limit SIRT1. It is likely that PARP-1 activity could also rise simultaneously, as the decrease in SIRT1 activity should lead to increased PARP-1 acetylation and activity. This would further limit NAD^+ availability for SIRT1, completely shutting down its activity. Once SIRT1 activity is low enough, CLOCK:BMAL-1 activity will be increased, and Nampt expression will be slowly recovered, reaching full circle.

3.1.2. PARP–SIRT1 interaction in the regulation of energy expenditure

A key element driving the metabolic phenotype of the PARP-1 knock-out mice is their enhanced energy expenditure (Bai et al., 2011b). This effect likely derives, at least in part, from a potentiation in SIRT1 activity and the activation of key transcriptional metabolic regulators, such as the transcriptional coactivator PGC-1α (Rodgers et al., 2005). It has been shown that

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PGC-1α activation is linked to enhanced mitochondrial biogenesis and a more oxidative profile of skeletal muscle fibers (Lin et al., 2002). Another key downstream effector of SIRT1 contributing to the regulation of oxidative metabolism is the FOXO family of transcription factors. FOXOs are deacetylated by SIRT1 (Brunet et al., 2004), prompting their activation and the transcriptional activation of genes linked to lipid oxidation and stress resistance (Banks et al., 2011; Gross et al., 2008). It was therefore reassuring to see that, consistent with SIRT1 activation, mice where PARP activity is impaired, either by genetic or pharmacological means, show a marked deacetylation of PGC-1α and FOXO1 in a key metabolic tissue such as skeletal muscle (Bai et al., 2011b). Consistent with the activation of gene programs related to mitochondrial biogenesis, the muscles from PARP-1 deficient mice displayed a large increase in mitochondrial content and an enhanced oxidative profile of their muscle fibers (Bai et al., 2011b).

Another key tissue influencing whole body energy expenditure is the brown adipose tissue (BAT), which has a key role in thermogenesis. As seen in muscle, the BAT from PARP-1 deficient mice is characterized by increased NAD^+ content and SIRT1 activity, as manifested in the deacetylation and activation of PGC-1α (Bai et al., 2011b). This leads to a marked increase in mitochondrial content in the BAT of PARP-1 deficient mice (Bai et al., 2011b). Physiologically, this renders the PARP-$1^{-/-}$ mice with a stronger ability to maintain body temperature when exposed to cold compared to their wild-type littermates.

In agreement with the observations in mice, the knock-down of PARP-1 in cultured HEK293 or inhibition of PARP activity, using PJ34 (a pan-PARP inhibitor), in C2C12 myotubes is enough to drive an increase in mitochondrial gene expression and O_2 consumption (Bai et al., 2011b). Noteworthy, the simultaneous knock-down of SIRT1 largely prevented the increase in cellular respiration triggered by the reduction of PARP activity (Bai et al., 2011b). Importantly, when analyzing the expression of a panel of genes related to oxidative metabolism in response to PARP inhibition, it was clear that SIRT1 only participated in the regulation of certain subsets, but not all (Bai et al., 2011b). This indicates that reductions in PARP activity leads to a plethora of effects, and that SIRT1 solely controls a few contributing to enhanced mitochondrial respiration and energy expenditure.

The evaluation of PARP-2 deficient mice further consolidated the link between sirtuins and PARP enzymes on energy expenditure. PARP-2 mice also display resistance against high-fat diet-induced obesity, linked to increased energy expenditure and an enhanced oxidative profile of skeletal muscle (Bai et al., 2011a). As mentioned in Section 2.3, defects in PARP-2 expression also enhance SIRT1 activity through enhancing SIRT1 expression. As PARP-2 is a repressor of the SIRT1 promoter, PARP-2 deletion relieves the repression on the SIRT1 promoter and enhances SIRT1 mRNA and protein levels (Bai et al., 2011a; Szanto et al., 2011). Experiments in C2C12 myotubes demonstrated that the knock-down of PARP-2 triggered mitochondrial gene expression in a SIRT1 dependent fashion.

3.1.3. PARP–SIRT1 interaction in the regulation of fat deposition

This leaner phenotype of PARP-$1^{-/-}$ and PARP-$2^{-/-}$ mice can be explained, at least in part, due to their enhanced energy expenditure when compared to wild-type littermates. However, another attractive mechanism by which PARP deficiency might impact on body weight relies on the direct regulation of fat deposition in white adipose tissues (WAT). Indeed, PARP-1 and -2 deficient mice present a largely reduced size of their WAT depots (Bai et al., 2007b, 2011a,b). PPARγ is a nuclear receptor that is mainly expressed in white adipose tissue and plays key roles in adipocyte differentiation, lipid synthesis and storage (Heikkinen et al., 2007). The lower fat deposition in the PARP-1 and PARP-2 knock-out mice, therefore, might be explained by affecting the activity of PPARγ. PARP-1 and -2 had been shown to physically interact with PPARγ (Bai et al., 2007b; Miyamoto et al., 1999) and were already correlated with WAT tissue mass (Bai et al., 2007b; Erener et al., 2012a). PARylation can be observed in differentiating 3T3-L1 preadipocyte cells and in subcutaneous adipose tissue (Gehl et al., 2012; Janssen and Hilz, 1989), likely consequent to PARP-1 activation (Erener et al., 2012a; Janssen and Hilz, 1989; Simbulan-Rosenthal et al., 1996; Smulson et al., 1995). Indeed, PARP-1 is recruited to PPARγ target genes in a PAR-dependent manner, allowing a sustained expression of PPARγ and its target genes (Erener et al., 2012a). Also PARP-2 can contribute to the adipogenic program, as the lack of PARP-2 hampers the adipocytic differentiation of embryonic fibroblasts and 3T3-L1 cells (Bai et al., 2007b). PARP-2 binds to the same sites on promoters as PPARγ and apparently acts as positive cofactor (Bai et al., 2007b). Interestingly, the expression of some adipokines, such as leptin or adiponectin, is regulated by PARP-1 and -2 (Bai et al., 2007b; Erener et al., 2012a),

Besides regulation through direct physical interaction the higher SIRT1 activity in PARP-1 and PARP-2 deficient models might have a key role. SIRT1 is known to decrease PPARγ transcriptional activity through direct interaction and docking of transcriptional co-repressors, such as NCoR and SMART (Picard et al., 2004) and more recently, PPARγ has been identified as a deacetylation target for SIRT1 (Qiang et al., 2012). Activation of PPARγ through TZD decreased PPARγ acetylation levels on K268 and K293 by prompting the binding of SIRT1 (Qiang et al., 2012). The deacetylation of PPARγ at these two residues allows the recruitment of the transcriptional coactivator PRDM16 and promotes adipokine production and an upregulation of BAT-like gene expression (Qiang et al., 2012). Physiologically, the deacetylation of PPARγ is also triggered by cold exposure and blunted when mice are fed a high-fat diet (Qiang et al., 2012). This way, SIRT1-induced deacetylation of PPARγ will promote a brown-like phenotype of the WAT, enhancing energy expenditure, lowering fat deposition and favoring insulin sensitivity. Of note, PARP activity is enhanced upon high-fat feeding, which could limit SIRT1 activity and compromise PPARγ deacetylation that is in line with the enhanced insulin sensitivity and lower fat storage of PARP-1 and PARP-2 null mice, even if browning effects have never been closely examined. Of note, the regulation of SIRT1 alone might not be enough to directly impact on PPARγ activity, as the binding of both proteins seems to be ligand-dependent. This is in line with previous observations suggesting that SIRT1 *in vivo* does not deacetylate its substrates in an undiscriminated manner. Rather, substrates

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might be primed for deacetylation via different means, such as conformational changes upon ligand binding or through the modulation of other post-translational modifications. Altogether, it seems clear that both SIRT1-dependent and -independent mechanisms might contribute to the lower PPARγ activity and the blunted fat deposition in PARP-1 and PARP-2 knock-out mice (Bai et al., 2011a,b).

An interesting question is whether reduced WAT depots could potentially lead to ectopic lipid deposition. A recent report identified increased fat deposition in the livers of PARP-$1^{-/-}$ mice when fed a HFD (Erener et al., 2012b). PARP-1 is poorly expressed in the liver and global deletion of PARP-1 does not seem to have a major influence on hepatic expression of mitochondrial and lipid oxidation genes (Bai et al., 2011b), which might create a permissive scenario for lipid deposition. However, it is difficult to match this observation with the notion that PARP-1 deficiency dampens PPARγ activity and with the lower body weight of PARP-$1^{-/-}$ mice. Similarly, the possible activation of sirtuins would be theoretically at odds with a predisposition for lipid accumulation. Further evaluation of these models or the generation of tissue-specific deletions will be required to clarify this apparent discrepancy.

Another apparent discrepancy lies in the fact that PARP-1 deletion on an SV129 background renders the mice susceptible to obesity (Devalaraja-Narashimha and Padanilam, 2010). It is to be noted that the SV129 background is less suited for metabolic studies than C57Bl/6J mice (Champy et al., 2008) that may provide a plausible explanation for the misalignment of observations. Illustrating this latter point, the pharmacological inhibition of PARP activity in diverse human and murine cell types prompts an increase in oxygen consumption and mitochondrial biogenesis, very much in line with the results obtained in C57Bl/6J mice (Bai et al., 2011b; Modis et al., 2012). Furthermore, the expression of an additional copy of PARP-1 in mice leads to enhanced adiposity, perfectly mirroring once more the data obtained in the C57Bl6/J mice (Mangerich et al., 2010). The convergent results of these genetic, physiological, pharmacological and *in vitro* studies clearly support that a reduction in PARP activity would result in the enhancement of energy expenditure and prevention against HFD-induced body weight gain. The particular reasons by which the deletion of PARP-1 in the SV129 rendered an opposite phenotype are still elusive. Analyses on disturbances on NAD^+ and sirtuin activity might bring some light into this question and will warrant further investigation.

3.1.4. PARP–sirtuin interaction in whole body glucose metabolism

The large influence of PARP enzymes on highly metabolic tissues, such as muscle and brown adipose tissue, predicts that PARP enzymes should have a major impact on whole body glucose homeostasis. PARP-1 and PARP-2 deficient mice displayed increased glucose clearance in response to an insulin tolerance test compared to their wild-type littermates (Bai et al., 2011a,b). This is likely to be consequent to increased insulin-stimulated muscle glucose uptake, as skeletal muscle accounts for ~80% of the whole body glucose disposal in insulin-stimulated conditions (DeFronzo et al., 1985) involving many different factors. For example, oxidative muscle fibers are generally more insulin sensitive than glycolytic fibers (Hom and Goodner, 1984), and, as described above, PARP-1, or -2 deficiency is linked to a higher oxidative profile of muscle fibers. A second key factor is that PARP-1 and -2 deficient mice are leaner due to enhanced energy expenditure and have an impaired ability to accumulate fat (Bai et al., 2011a,b). This grants the muscle protection against the chronic deposition of lipid species that could be detrimental for an efficient insulin signaling (Petersen and Shulman, 2006). Finally, the higher mitochondrial content in oxidative fibers gives the organism a greater potential to obtain energy from fatty acids, also contributing to the prevention of fat deposition. While no causality links can yet be established between these observations and sirtuin activation, there are a number of interesting correlations. Notably, most transgenic and pharmacological approaches aimed to increase SIRT1 activity also lead to enhanced insulin sensitivity (Banks et al., 2008; Canto et al., 2012; Feige et al., 2008; Lagouge et al., 2006; Pfluger et al., 2008). The link between other sirtuins and insulin sensitivity is not so well established. While SIRT3 might be protective against insulin resistance and obesity upon high-fat feeding (Hirschey et al., 2011), most evidences do not support the activation of SIRT3 or any other non-nuclear sirtuin upon PARP inhibition (Bai et al., 2011a,b). However, we cannot rule out that other nuclear sirtuins could contribute to the insulin-sensitizing effect of reducing PARP activity. In this sense, it should be pointed out that mice overexpressing SIRT6 are not more insulin sensitive (Kanfi et al., 2010). These observations points towards SIRT1 as a very likely mediator of the insulin sensitizing effects of PARP deficiency. It will be important in the future to evaluate whether PARP inhibition can render insulin-sensitization in SIRT1-deficient tissues.

The enhanced insulin sensitivity of PARP-1 and PARP-2 deficient mice should theoretically be aligned with a better glucose tolerance. While this is certainly the case in PARP-$1^{-/-}$ mice (Bai et al., 2011b), it was surprising to observe that PARP-$2^{-/-}$ mice became markedly glucose intolerant upon high fat feeding (Bai et al., 2011a). This glucose intolerance was rooted in pancreatic β-cell dysfunction. Upon high-fat feeding, the pancreatic β-cell mass increases in order to compensate for peripheral insulin resistance (Buteau and Accili, 2007). In the PARP-$2^{-/-}$ mice, however, this hyperplasic response is largely impaired, resulting in a blunted ability to release insulin upon a glucose load. This way, PARP-$2^{-/-}$ mice have a lower average β-cell islet size and pancreatic insulin content (Bai et al., 2011a). The molecular mechanism by which PARP-2 deletion impairs β-cell proliferation might be consequent to a constitutive SIRT1 activation, which leads to FOXO1 deacetylation and activation. FOXO1 is a well-known repressor of *Pdx-1*, a key regulator for β-cell proliferation and development (Bai et al., 2011a; Buteau and Accili, 2007). An interesting question, yet unresolved, is why this is not observed in the PARP-$1^{-/-}$ mice. PARP-1 is also expressed in the β-cell and might have also have a critical role on its functionality. A role for PARP-1 in β-cells was first unraveled by studies showing how PARP inhibitors improved diabetes mellitus in partially depancreatized rats (Yonemura et al., 1984, 1988). These studies revealed that PARP inhibitors allowed more efficient β-cell regeneration after

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pancreatectomy and a faster normalization of blood glucose. Posterior studies reinforced such concept by demonstrating that mice lacking PARP-1 are completely resistant to the development of diabetes upon streptozocin (Burkart et al., 1999b). When compared to their wild-type littermates, PARP-1$^{-/-}$ mice remained normoglycemic and maintained normal pancreatic insulin content and islet morphology (Bai et al., 2011a; Burkart et al., 1999b). Parallel research demonstrated that PARP-1 is actually a master controller of β-cell death upon exposure to nitric oxide or oxygen radical generating compounds and that the inhibition of PARP-1 allows to retain β-cell survival in such circumstances (Burkart et al., 1999a). Also important for pancreatic β-cell function, PARP inhibition prevents the detrimental effects of glucotoxicity on insulin promoter activity and biosynthesis (Ye et al., 2006). Despite the major effects of PARP-1 in situations of toxicity for β-cells, PARP-1 ablation does not seem to have a major impact on the endocrine pancreas β-cell function in the basal state (Bai et al., 2011a) suggesting that the protective phenotype of PARP-1 inhibition is only effective under β cell stress. Given the above observations on the PARP deficient mice models, would the inhibition of PARP activity be beneficial or detrimental for β-cell function? A priori one would argue that in the absence of β-cell toxicity PARP activity would be detrimental, as supported by the marked β-cell dysfunction in PARP-2$^{-/-}$ mice and the lack of phenotype in the PARP-1$^{-/-}$ mice. However, in situations of β-cell toxicity, PARP inhibitors might prevent β-cell death and allow a better regeneration of the tissue.

Overall, the ability of PARP enzymes to modulate SIRT1 activity and the fact that human studies have provided evidence on the connection between SIRT1 gene expression and insulin sensitivity (Rutanen et al., 2010; Zillikens et al., 2009b), further strengthens the promising possibility of modulating PARP activity in the management of metabolic disease. In this sense, preliminary evidence indicates that short term pharmacological inhibition of PARP activity is able to decrease plasma glucose, triglyceride and free fatty acid levels (Bai et al., 2011b). However, more protracted treatments will be required to evaluate the feasibility of engaging long-term inhibition of PARP activity and whether this might have a negative impact on DNA damage and chromosome maintenance. Furthermore, while the regulation of SIRT1 activity might help granting insulin sensitivity in the PARP deficient models, this does not exclude that other mechanism yet to be found might be equally important in explaining this phenotype. Amongst them, it will be important to evaluate possible direct PARylation targets that could influence glucose homeostasis.

3.2. Interplay between PARPs and SIRTs in oxidative stress response

Reactive species are partially reduced, highly reactive molecules (radicals and non-radicals). Most reactive species can be classified as reactive oxygen intermediates (ROI), or reactive nitrogen intermediates (RNI) depending on the central atom. The major source of ROIs in unstimulated cells is the leakage of mitochondrial electron transport chain that creates superoxide anion that serves as a parent molecule of downstream ROIs, such as hydrogen peroxide, or hydroxyl radical. Besides the mitochondrial origin, ROIs can stem from the oxidation of certain xenobiotics, activated neutrophils and macrophages, or from xanthine oxidase (Ray et al., 2012; Virag and Szabo, 2002).

The parent molecule of RNIs is nitrogen monoxide (NO) that is formed by nitrogen monoxide synthase enzymes termed neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (Forstermann and Sessa, 2012; Nathan, 1992). While NO is an important signaling molecule and considered cytoprotective (Pacher et al., 2007), its downstream derivatives such as peroxynitrite (formed in reaction with superoxide (Pacher et al., 2007)), or nitroxyl radical (formed by NO reduction (Bai et al., 2001)) are cytotoxic. In oxidative stress-related diseases the balance between free radical species and antioxidant defence systems is hampered. Free radicals, in abundance, damage lipids, DNA and proteins, or may react with protein-bound metals affecting all vital components of cells and tissues (Pacher et al., 2007). Various enzyme systems are on duty to cope with free radical-induced damage and hence to protect against free radical-provoked diseases, among them PARPs and SIRTs play prominent roles.

As reactive species cause DNA damage and consequently induce PARP activation in the effort to restore DNA integrity (Virag and Szabo, 2002). As discussed previously (Section 1.3), PARP activation has pleiotropic effects (induction of necrosis, mitochondrial damage, proinflammatory actions, reprogramming of gene expression) that worsen free radical-mediated pathologies (Modis et al., 2012; Szanto et al., 2012; Virag and Szabo, 2002). The idea that PARP inactivation may provide protection against free radical production-mediated PARP activation and the consequent cell, or tissue stress is almost 15 years old (Szabo et al., 1998) and it had been successfully tested in a plethora of animal and cellular models (reviewed in Virag and Szabo, 2002).

In contrast to PARP activation the induction of SIRT1 protects against oxidative stress. The protective effects of SIRT induction involve numerous mechanisms.

(1) SIRT1 modify numerous components of the cell cycle coordination machinery (e.g. p53 and FOXO) upon oxidative injury that leads to cell cycle arrest and suppression of apoptosis (Brunet et al., 2004; Han et al., 2008; Luo et al., 2001).
(2) SIRT1 activation induces antioxidant defence systems, such as manganese superoxide dismutase (MnSOD) (Danz et al., 2009), or catalase (Hasegawa et al., 2008).
(3) Mitochondrial biogenesis is hampered upon oxidative stress that is restored upon SIRT1 induction (Danz et al., 2009; Szanto et al., 2011).
(4) In oxidative stress SIRT1 induction induces autophagy (Alcendor et al., 2007).

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Table 1
Occasions of SIRT1–PARP interaction in oxidative stress-related pathologies.

Organ system	Disease	Partners	Models	Mechanism	Effect	Ref.
Cardiovascular system	Angiotensin-induced cardiac hypertrophy	PARP-1 SIRT1	PARP-1$^{-/-}$ mice, primary cardiomyocytes treated with resveratrol, SIRT1 siRNA, or by SIRT1 overexpression	PARP-1 activation inhibits SIRT1 through NAD$^+$ depletion	Both SIRT1 activation and PARP inhibition protect against angiotensin II-mediated and oxidant-mediated cardiomyocyte cell death	Pillai et al. (2006)
	Heart failure (aortic banding model)	PARP-1 SIRT1	Cardiomyocytes overexpressing PARP-1, SIRT1, or treated with resveratrol, sirtinol, SIRT1 siRNA	PARP-1 limits NAD$^+$ levels and hence SIRT1 activity through limiting NAD$^+$ for SIRT1 action and probably by repressing SIRT1 expression	SIRT1 induction protect against oxidant-induced cardiomyocyte cell death	Pillai et al. (2005)
	Shear stress on endothelial cells	PARP-1 SIRT1	HUVEC cells undergoing shear stress treated with ABT888 and PARP-1 siRNA	Shear stress decrease NAD$^+$ levels, SIRT1 activity and expression that is reverted by PARP-1 depletion, or inhibition	Proinflammatory conditions and cell death provoked by shear stress is reduced	Qin et al. (2012)
	DOX-induced vascular damage	PARP-2 SIRT1	PARP-2 knockout mice and PARP-2 knockdown MOVAS cells	SIRT1 promoter is released from suppression upon PARP-2 depletion that induces mitochondrial biogenesis	Vascular protection against DOX damage upon SIRT1 induction after PARP-2 depletion	Szanto et al. (2011)
Central nervous system	Trophic deprivation and oxidant mediated neuronal cell death	PARP-1 SIRT1	Near-pure cortical neuronal cell cultures from PARP-1$^{-/-}$ mice. Same neurons treated with fisetin, resveratrol and sirtinol	PARP-1 activation inhibits SIRT1 through NAD$^+$ depletion. Protective effect of SIRT1 activation is not explained	Both SIRT1 activation and PARP inhibition protect against neurotoxicity	Sheline et al. (2010)
	Glutamate/ NMDA neurotoxicity	PARP-1 SIRT1	Dissociated cerebral cortical cell cultures from embryonic rat treated with sirtinol and resveratrol	NMDA treatment reduces SIRT1 activation probably due to PARP activation and NAD$^+$ depletion. Protective effect of SIRT1 activation is not explained	Reduction of cell death upon SIRT1 activation and PARP inhibition	Liu et al. (2008, 2009)
Gastrointestinal tract	Glucose toxicity on hepatocytes	PARP-1 SIRT1	HepG2 cells treated with PJ34 and PARP-1 siRNA	PARP-1 activation decreases SIRT1 activity that is restored by PARP inhibition probably through conservation of NAD$^+$ levels	Glucose toxicity is reverted by PARP inhibition	Pang et al. (2011)

Abbreviations in text. Fistein and resveratrol are SIRT1 activators, sirtinol is a SIRT1 inhibitor, 3-amino-benzamide (3AB) and PJ34 are PARP inhibitors.

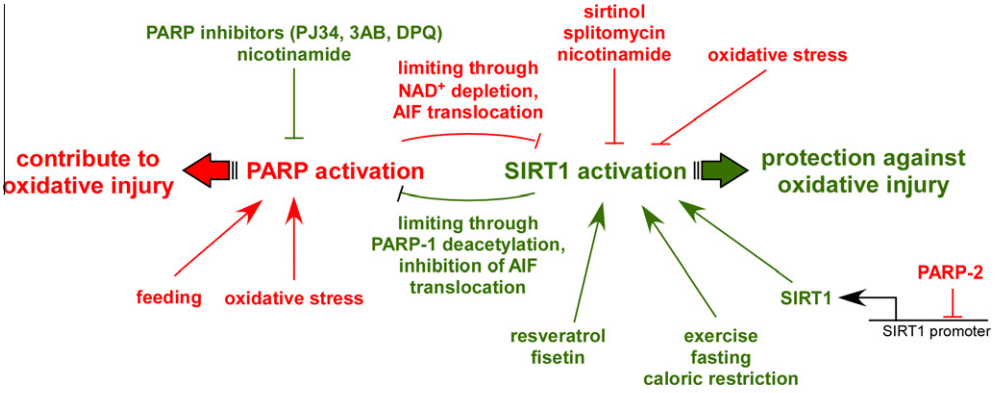

Fig. 4. The molecular level interactions between SIRT1 PARP-1 and -2 under oxidative stress conditions. The pathways enhancing oxidative stress-mediated tissue damage are in red, in turn, protective pathways are in green. Oxidative stress induces PARP-1 that through the depletion of NAD$^+$ pools inhibits SIRT1. That pathway seems to participate in the tissue damage inflicted by PARP activation. On the contrary, SIRT1 activation by pharmacological agents (e.g. resveratrol, fistein) or by induction of its expression (e.g. PARP-2 ablation) leads to SIRT1-mediated deacetylation and inactivation of PARP-1 that seems a crucial pathway in the cytoprotective action of SIRT1 activation.

SIRT1 itself is redox sensitive, its protective actions can be hampered under oxidative stress conditions by carbonylation of SIRT1 (Caito et al., 2010a) and the disregulation of thiol redox balance (Caito et al., 2010b) both impairing SIRT1 activity. Recent data by Caito and colleagues (Caito et al., 2010b) have shown the redox-dependent phosphorylation of SIRT1 that may have a role in the oxidant-mediated reduction of SIRT1 activity.

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Apparently SIRT1 and PARP activation have opposing characteristics under oxidative stress conditions. As PARP activation seems to be a key factor of cellular and tissue damage under oxidative stress (Pacher and Szabo, 2008) these observations prompted research to understand PARP–SIRT interaction under pathological scenarios. The interaction of SIRT1 and PARPs had been associated with pathologies of the cardiovascular system, the central nervous system and the liver in the gastrointestinal tract (summarized in Table 1).

The pathological states listed are characterized by increased production of reactive species either from an internal source like angiotensin II (AngII), N-methyl-D-aspartic acid (NMDA) receptor activation, high glucose levels, pressure overload in the heart, shear stress on endothelial cells and from an external noxa such as oxidants, or doxorubicin treatment (Moncada and Bolanos, 2006; Pacher et al., 2002b, 2003; Qin et al., 2012; Soriano et al., 2001; Wilson, 1990) that lead to PARP activation contributing to cellular and organ dysfunction (Moncada and Bolanos, 2006; Pacher et al., 2002a; Pillai et al., 2006; Soriano et al., 2001; Szabo et al., 2004). In the case of trophic deprivation, heart failure, AngII, NMDA and glucose toxicity extensive PARP activation decreased SIRT1 activity (Liu et al., 2009; Pang et al., 2011; Pillai et al., 2005, 2006; Qin et al., 2012; Sheline et al., 2010) through reducing NAD^+ levels that compromised SIRT1 activation and hence the protective effects of SIRT1 activation (Fig. 4). This hypothesis is further underlined by the fact that preventing NAD^+ depletion was protective in most of these pathologies (Houtkooper et al., 2010; Liu et al., 2008). As discussed in Sections 1.3 and 2.1, enhanced PARP activation depletes cellular NAD^+ (Berger, 1985) that limits substrate availability for SIRT1 (Kolthur-Seetharam et al., 2006). On the other hand SIRT1 induction limits PARP-1 through deacetylating and hence inactivating it (Rajamohan et al., 2009) (Fig. 4).

Alternative, non-NAD^+ dependent pathways may also exist between SIRT1 and PARP-1. It would be easy to speculate that PARP-1 could inhibit SIRT1 activity through direct PARylation of SIRT1, although we were unable to show SIRT1 PARylation under oxidative stress (Bai et al., 2011b and Section 2.2). It is also plausible that other transcription factors are PARylated (e.g. c-fos/c-jun PARylation upon angiotensin II treatment (Huang et al., 2009)) that modify oxidative stress sensitivity of cells, however that field is largely unexplored.

Kolthur-Seetharam and colleagues (Kolthur-Seetharam et al., 2006) have identified a possible alternative connection between SIRT1 and PARP-1 that may influence oxidative stress-induced cell death. PARylation has been shown to induce the nuclear translocation of apoptosis inducing factor (AIF) in certain cell lines contributing to cell death (Yu et al., 2002). In the absence of SIRT1 enhanced PARylation was observed that is further accentuated upon oxidative damage (Kolthur-Seetharam et al., 2006) (Fig. 4). In line with that observation in $SIRT1^{-/-}$ cells nuclear translocation of AIF is induced that may contribute to cell death.

The depletion of PARP-2 protected partially the vasculature against doxorubicin toxicity (Szanto et al., 2011). The protective enhancement of SIRT1 activity upon PARP-2 depletion relies on the induction of the SIRT1 promoter (Szanto et al., 2011) (Fig. 4). Importantly, PARP-2 depletion and the consequent enhancement of SIRT1 activity did not reduce PARP-1 activation under oxidative stress conditions (Szanto et al., 2011).

Apparently PARP-1 and SIRT1 under oxidative conditions regulate the activity of each other through various mechanisms. SIRT1 induction leads to protection against oxidative damage, while PARP-1 activation is a detrimental consequence of oxidative stress. There is a large overlap between the oxidative stress-mediated pathologies that are corrected by SIRT1 induction (Chong et al., 2012; Chung et al., 2010), or PARP inhibition (Virag and Szabo, 2002) due to joint regulation of key enzymes involved in these pathologies (e.g. matrix metalloproteinase activation in cardiovascular and dermatological diseases (Bai et al., 2004, 2009; Brunyanszki et al., 2010; Choi et al., 2005; Lee et al., 2010; Nakamaru et al., 2009; Ohguchi et al., 2010; Pacher et al., 2002a) suggesting that there are way more pathologies (e.g. diabetic complications, stroke, multiple sclerosis, inflammation) where PARPs and SIRTs collaborate on the outcome.

3.3. PARP–SIRT interaction in the maintenance of genomic stability

PARP-1, -2, -3 and tankyrases are involved in DNA repair and the maintenance of genomic integrity, as discussed in Section 1.3, SIRT1 and SIRT6 had been shown to be involved in DNA repair (Mostoslavsky et al., 2006; Oberdoerffer et al., 2008). The interaction between PARylation and SIRT1 in DNA repair events were first suggested by Zhang in 2003 (Zhang, 2003). First experimental evidence for that interaction in chromatin remodeling was presented by Tulin and co-workers (Tulin et al., 2006) showing that PARG and SIR2 co-localized in cell nuclei of Drosophila larvae and localization of SIR2 was dependent on PARG expression suggestive of the involvement of PAR levels (Tulin et al., 2006).

The apparent functional convergence of SIRT1 and PARP activation in DNA repair and genomic maintenance was assessed in detail by El-Ramy and colleagues (El Ramy et al., 2009) by studying the double deletion of PARP-1 and SIRT1 in mice. The deletion of SIRT1 induced early postnatal lethality, chromosomal and DNA repair defects as expected from previous studies (Cheng et al., 2003; McBurney et al., 2003; Oberdoerffer et al., 2008). Although, the concurrent deletion of PARP-1 did not prevent postnatal lethality, it did influence DNA repair defects (El Ramy et al., 2009).

The absence of SIRT1 have led to telomere dysfunction, the spreading of heterochromatic regions, rearranged nucleolar architecture by increasing the number of nucleoli and hampered mitotic cell division by inducing the number of mitotic divisions, however also enhanced the number of aberrant divisions, the incidence of unequal distribution of chromosomes between daughter cells and the extent of DNA damage associated with mitosis (occurrence of micronuclei) (El Ramy et al., 2009). PARP-1 had been shown to act as an actor in maintaining genome stability under genotoxic stress and to protect against the above features (De Vos et al., 2012), however unexpectedly it was the deletion of PARP-1 that was protective

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against the SIRT1-induced genome instability except for maintenance of telomere integrity (El Ramy et al., 2009). Yet the exact molecular mechanism of the above detailed phenomenon is unknown.

Among sirtuins, besides SIRT1, SIRT6 is involved in the regulation of DNA repair events (Mostoslavsky et al., 2006). The absence of SIRT6 enhances the sensitivity of fibroblasts to ionizing radiation and brings about genomic instability (increased number of chromosome fragmentation, centromere default, abnormal metaphase, chromosome translocations) (Mostoslavsky et al., 2006). SIRT6 translocate to the damage sites where it promotes DNA repair (Mao et al., 2011; Mostoslavsky et al., 2006). SIRT6 has pivotal role in the appropriate function of base excision and double strand break repair (Mao et al., 2011; Mostoslavsky et al., 2006). SIRT6 mono-ADP-ribosylate PARP-1 on K521, whereby the activity of PARP-1 is induced that contribute to successful resolution of double strand breaks, however the deacetylase activity of SIRT6 has equal contribution to DNA repair as its mono-ADP-ribosyl transferase activity (Mao et al., 2011) suggesting unknown parallel DNA repair pathways influenced by SIRT6.

It remains a question whether other PARPs involved in genome maintenance (PARP-2, -3, or tankyrases) would similarly interact with SIRT1 in DNA repair events, or whether the interaction of SIRT1 and PARP-1 would take place also in the resolution of double and single strand breaks. It is plausible that the interaction of SIRTs and PARPs in the maintenance of genomic integrity has prominent role in senescence, apoptosis, cell cycle regulation and tumorigenesis.

3.4. PARP–sirtuin interaction in aging

Higher PARP activation capacity had been associated with successful aging in mammals (Burkle et al., 1994) and in humans (Muiras et al., 1998). It was postulated that the benefit of the higher PARylation capacity is more reliable DNA repair that prevents the occurrence of DNA damage-associated diseases (e.g. neoplasms). Although, as of yet the lack of PARP-1 has not been associated with spontaneous tumorigenesis without tumorigenic challenge *in vivo* unless other DNA repair enzymes (e.g. p53) were removed too (Nicolas et al., 2010; Tong et al., 2001). Recent data, however validate that concept: the hPARP-1 mouse strain, that overexpresses an extra copy of PARP-1, are protected against neoplastic diseases (Mangerich et al., 2010). However the incidence of other age-related pathologies increase upon PARP-1 overexpression: obesity, glucose intolerance and certain inflammatory pathologies (Mangerich et al., 2010) suggesting that the previous model might need to be refined.

Aging is associated with the dysregulation of the oxidative balance enhancing oxidative stress. Upon aging PARylation capacity increases (Braidy et al., 2011; Massudi et al., 2012) that upon stress puts heavy burden on NAD^+ homeostasis. Indeed, lower NAD^+ levels were detected in aged animals and humans that coincided with lower SIRT1 activity despite the induction of SIRT1 expression (Braidy et al., 2011; Massudi et al., 2012). As decrease in SIRT1 activity leads to decrease in mitochondrial biogenesis it is tempting to speculate that the crosstalk between PARP-1 and SIRT1 might be a cause of the age-associated loss of mitochondrial function and vice versa, influencing that angle could be exploited to combat age-associated loss of mitochondrial function. The dysregulation of the PARP-1–NAD^+–SIRT1 balance may stay behind some of the pathologies observed in the hPARP-1 mice (Mangerich et al., 2010). As hampered mitochondrial biogenesis is a hallmark of aging and it is probably a major cause of several age-associated metabolic and central nerve system diseases (Lopez-Lluch et al., 2008) fine tuning of the PARP-1–SIRT1 interaction may prove to be a successful strategy to counteract these diseases and provide longer healthspan (Canto and Auwerx, 2011b).

4. Pharmacology of NAD^+, SIRT1 and PARPs

4.1. NAD^+ modulating agents

The idea that PARPs and sirtuins may be affected by NAD^+ metabolism, and that in turn, activities of these enzymes modulate NAD^+ levels is cells has indicated that NAD^+ metabolism itself is an interesting target for pharmacological modulation. It is important to bear in mind that NAD^+ itself and associated metabolites (Fig. 5) are active forms of Vitamin B3, whose levels are sensitive to pharmacologic interventions featuring different forms of Vitamin B3. Importantly, as was discussed in Section 1.1, different vitamin forms are metabolized through distinct pathways, indicating that each has unique pharmacologic and metabolic properties. This has been noted particularly for nicotinic acid, which has remarkable anti-lipogenic effects, as well as the ability to lower LDL cholesterol and raise HDL cholesterol. These effects are not found for pharmacologic use of NAM. Thus, different precursors to NAD^+ have distinct therapeutic as well as nutritional significance.

4.1.1. Niacin

The distinctive effects of NA (niacin) supplementation are thought to include systemic NAD^+ increase, as revealed by supplementation studies of NAM or NA in rats (Jackson et al., 1995). These authors supplemented each of the two Vitamin B3 compounds in 30, 100, 500, and 1000 mg/kg for three weeks and then tissue NAD^+ was analyzed by HPLC. Animals fed at the highest dose NA experienced 1.44 (packed RBC), 1.54 (liver), 1.62 (heart), 1.12 (lung), and 1.88 (kidney) increased NAD^+ contents (measured as fold over control), whereas NAM at the highest dose yielded 1.44 (packed RBC), 1.47 (liver), 1.20 (heart), 1.18 (lung), and 1.03 (kidney) increases in NAD^+ contents (measured as fold over control). It is apparent from these data that NA generally provides greater NAD^+ enhancements than NAM in mammalian tissues. This observation has been rationalized

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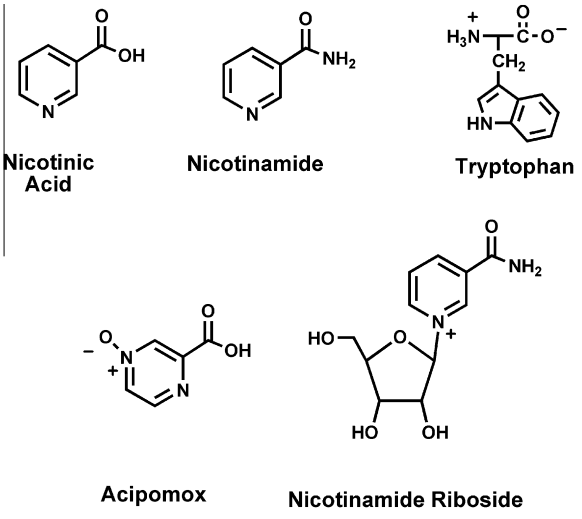

Fig. 5. Compounds that modulate NAD^+ concentrations or modulate GPR109A.

by observations that NAM and NA are metabolized to NAD^+ differently, and that NA production is typically low in mammalian tissues, enabling enhanced biosynthesis of NAD^+ when NA is provided via a pharmacologic route. NA administration enhances NAD^+ availability, therefore is protective in PARP-mediated pathologies (Benavente et al., 2009; Hageman and Stierum, 2001; Weidele et al., 2010).

NA is distinct versus NAM in its ability to bind a G-protein coupled receptor (GPR109A, or HM74A) (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003), which increases vasodilation and leads to uncomfortable flushing (Benyo et al., 2005). This effect appears to be mediated by release of prostaglandins D2 and E2 from epidermal Langerhans cells (Benyo et al., 2006). In fact, three distinct receptors for NA have been identified in mammalian cells. The high affinity receptor has a measured affinity to NA of 63–250 nM (Soga et al., 2003; Wise et al., 2003). The knockout of the murine homologue PUMA-G appears to ablate many of the effects of NA in lipid lowering (Tunaru et al., 2003). Some authors have argued that GPR109A activation is responsible for the entirety of the anti-lipidemic effects of NA (Wanders and Judd, 2011). This view is in part supported by the effects of a NA mimic acipomox, which has been used extensively in Europe for treatment of hyperlipidemias. Acipomox does not have an apparent pathway for stimulation of systemic NAD^+ increase, suggesting it parces the non-NAD^+ component in NAs effects. However, acipomox has been reported to exhibit smaller effects than NA in lipid modulation (Seed et al., 1993), and acipomox did not cause statistically significant increase of HDL in one study (Seed et al., 1993).

The latter result suggests that some of NA effects could act through NAD^+ metabolism, and through NAD^+ modulation of signal transducers (such as sirtuins and PARPs) that can impinge on lipid metabolism. Even those who are strong advocates of GPR109A as responsible for mediating NA's effects acknowledge that some of NA effects are unclear, such as how exactly NA causes the magnitudes of lipid modulations as well as how NA causes increases in HDL levels (Wanders and Judd, 2011). It is important to bear in mind that NAD^+ metabolism could play key, but yet to be determined roles, in these effects.

Niacin's lipid altering effects, were first reported in 1955 (Altschul et al., 1955) and niacin has been widely used clinically since that time for modulating serum lipids. As such, niacin supplementation therapy is the oldest known therapy for modulating cholesterol and lipids, and predates the development of the statin drugs. High dose niacin is still widely prescribed as a treatment for lipidemia, in the form of Niaspan (Abbott Laboratories), for treating elevated cholesterol and for raising HDL levels. Niacin increases HDL cholesterol, better than any other known pharmacologic agent, and decreases LDL and VLDL cholesterol (Capuzzi et al., 2000). It also can decrease serum fatty acids. Data on its effectiveness indicate that niacin reduces long-term cardiac disease mortality compared with untreated patients (Berge and Canner, 1991). Because, niacin can profoundly increase HDL cholesterol, high dose niacin was evaluated as a supplement to statin therapy (simvistatin) for possible benefit in a large 3414 patient study called AIM-HIGH (www.aimhigh.com). This study was halted in 2011 after no benefit was found in the Niaspan treated arm of the study as measured by rates of cardiovascular events. There was a slightly increased risk of stroke in the Niaspan arm (28 events versus 12 events) which prompted the study to be terminated.

4.1.2. Nicotinamide

Nicotinamide does not behave like NA as a serum cholesterol modulation agent (Altschul et al., 1955). It also cannot activate the GPR109A receptor (Wise et al., 2003). Not surprisingly, NAM does not activate flushing in people. However, as already discussed, data suggests that NAM is not particularly effective for increasing NAD^+ levels in tissues, suggesting it has limited potential as an NAD^+ enhancement agent. Nevertheless, NAM can increase liver NAD^+ contents substantially (Jackson et al., 1995). NAM is a known inhibitor of several ADP-ribosyl transferase enzymes, including sirtuins (Jackson et al., 2003;

Sauve and Schramm, 2003) and PARPs (Preiss et al., 1971; Virag and Szabo, 2002), causing its effects on NAD^+ metabolism to be in part due to decreasing rates of NAD^+ turnover.

The ability of NAM to attenuate rate of depletion of NAD^+ by inhibition of PARP probably accounts for some of the protective effects identified for NAM in models of disease (Virag and Szabo, 2002). NAM has been shown to provide protection in stroke and hypoxic neural injury (Feng et al., 2006; Liu et al., 2009), fetal alcohol syndrome (Ieraci and Herrera, 2006) and in acute neurotrauma (Hoane et al., 2003, 2006a,b,c). NAM also provides protection against streptozotocin induced β cell loss in the pancreas (Lazarus and Shapiro, 1973). A large clinical study using NAM to treat prevent Type I diabetes in Europe failed to show a clinical benefit (Gale et al., 2004), but reinforced the safety of longterm high dose NAM administration to healthy patients.

4.1.3. Tryptophan

Tryptophan is a precursor of NAD^+ via degradation through the oxidative kynurenine pathway. The value of tryptophan as an NAD^+ precursor is limited. It has been estimated that only 1 of 67 mg of tryptophan is shunted into NAD^+ synthesis in human females. This indicates that 1 mg of niacin is equivalent to 67 mg of tryptophan for NAD^+ biosynthesis (Fukuwatari et al., 2004). Nevertheless defects in tryptophan metabolism to NAD^+ have been suggested in some diseases, such as neurodegenerative disorders, through accumulation of quinolinic acid (Schwarcz et al., 1983). Inhibition of a key enzyme in this pathway, kynurenine 3-monooxygenase provides relief in a transgenic model of Alzheimer's disease (Zwilling et al., 2011).

4.1.4. Nicotinamide riboside

Limited data exists for the use of NR as an NAD^+ precursor in human or mammalian cell types. Sauve and co-workers were first to establish that NR can be broadly useful for increasing NAD^+ levels in a variety of mammalian cell types (Yang et al., 2007b). NR was found to be protective in a model of optic neuritis by direct injection into the eye (Shindler et al., 2007). More recently, NR was shown to increase NAD^+ levels in muscle and other tissues when administered to mice in food (Canto et al., 2012). NR was able to stimulate mitochondrial biogenesis, increase insulin sensitivity, lower cholesterol, and reduce weight gain in mice fed a high fat diet (Canto et al., 2012). NR supplementation was able to provide *in vivo* activation of sirtuins SIRT1 and SIRT3 (Canto et al., 2012). The beneficial effects observed *in vivo*, in combination with high levels of NAD^+ enhancements on cells (Yang et al., 2007b) suggests this compound could be of interest for nutritional supplements and for possible therapeutic formulations.

4.2. Sirtuin modulating agents

The quest for sirtuin activators (Fig. 6) began short after the finding that Sir2 could modulate replicative lifespan in yeast. These efforts culminated when, in 2003, Howitz and collaborators identified resveratrol and a few other polyphenols, including quercetin and piceatannol, as natural compounds that could directly bind and enhance SIRT1 activity (Howitz et al., 2003). A number of studies have subsequently shown that resveratrol treatment leads to enhanced SIRT1 activity in diverse cells, tissues and organisms (Baur, 2010). In line with this, resveratrol has been shown to promote calorie-restriction like health benefits in numerous organisms. Notably, resveratrol largely prevented the onset of diet-induced obesity and metabolic disease upon high-feeding, ultimately protecting against the lifespan curbing associated with high caloric intake (Baur et al., 2006; Lagouge et al., 2006). The same study demonstrated that resveratrol also improved mitochondrial function and fatty acid oxidation (Lagouge et al., 2006). In line with the possible inhibitory role of SIRT1 on PARP activity, it has been reported that resveratrol-treatment can lead to reductions in PARP activity (Kolthur-Seetharam et al., 2006).

A major caveat in the identification of resveratrol as a direct SIRT1 activator relies in the use of a fluorescently labeled substrate in the original screening. These results were questioned by convincing evidence demonstrating that the nonphysiological fluorescent "Fluor de Lys" substrate can lead to artifactual results (Borra et al., 2005; Kaeberlein et al., 2005). This suggested that the actions of resveratrol on SIRT1 might be indirect, igniting a new quest for the possible early drivers of resveratrol action. A likely candidate to initiate the metabolic actions of resveratrol is the AMPK. Many works have reported how resveratrol treatment leads to AMPK activation (see Canto and Auwerx, 2011a, for review). Elegant studies by the Hardie lab demonstrated that AMP-insensitive forms of AMPK are resistant to activation by resveratrol, clearly indicating that AMPK activation in response to resveratrol relies on an AMP/ATP imbalance. In line with this hypothesis, resveratrol has been shown to interfere with the mitochondrial respiratory chain (Zini et al., 1999), providing a likely mechanism by which resveratrol might affect AMP/ATP balances and activate AMPK. The link between AMPK and SIRT1 activation is provided at least by a couple of different mechanisms. First, AMPK activation is followed by an increase in NAD^+ levels. On an initial phase this increase is powered by increased fatty acid oxidation flux (Canto et al., 2009), which is later sustained by enhanced Nampt expression (Canto et al., 2009; Fulco et al., 2008). A second mechanism recently proposed relies on the direct phosphorylation of SIRT1 by AMPK, which would disrupt the ability of DBC-1 to interact and inhibit SIRT1 (Nin et al., 2012). Another layer of complexity is provided by the possible impact of SIRT1 in AMPK activity. Indeed, some labs have shown that SIRT1 might also influence AMPK activity (Hou et al., 2008; Lan et al., 2008; Price et al., 2012; Suchankova et al., 2009). This would create a positive feedback loop between both signaling events to amplify the signal., The fact that AMPK is activated by resveratrol in SIRT1 defective cells and that defective AMPK activity compromises resveratrol-induced SIRT1 activation (Canto et al., 2010; Dasgupta and Milbrandt, 2007; Um et al., 2010), indicates that AMPK might be the initial trigger of this signal loop.

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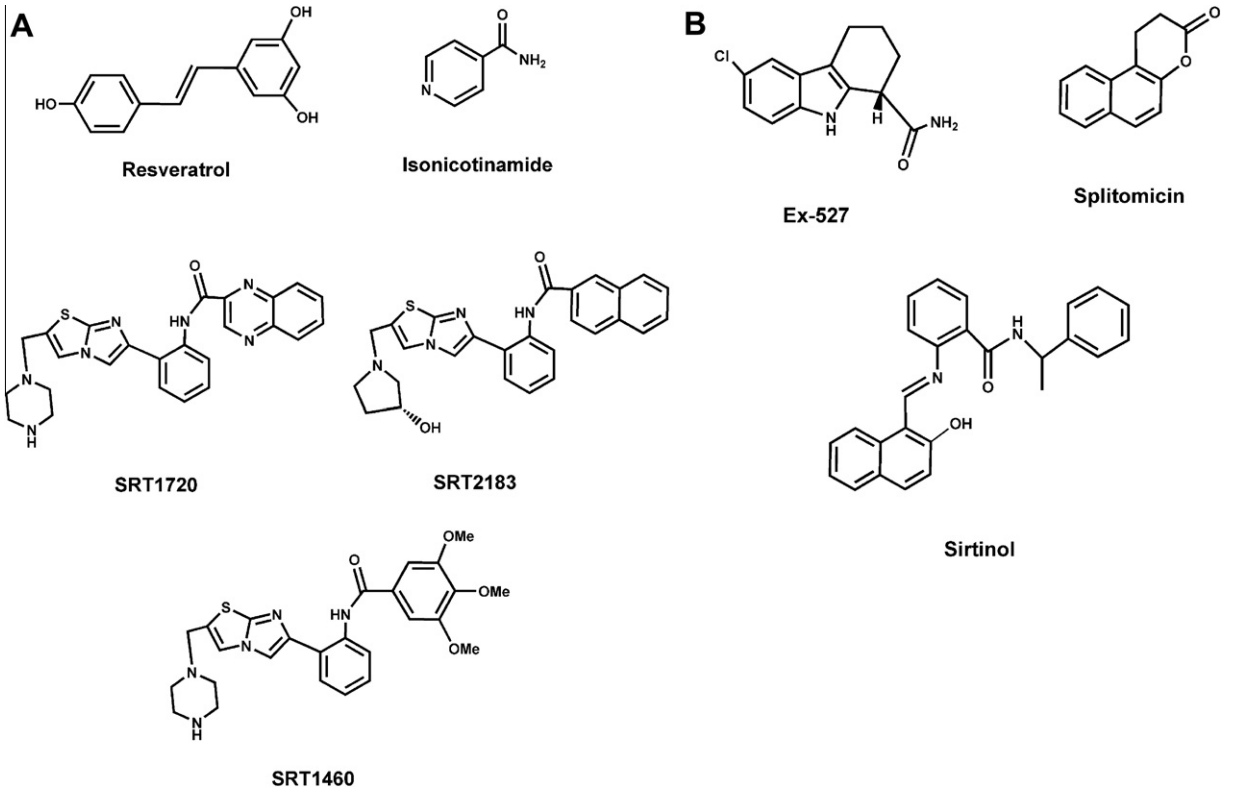

Fig. 6. Examples of compounds that (A) activate and (B) inhibit SIRT1 or other sirtuins.

This, however, might largely depend on the dose of resveratrol used (Price et al., 2012). In any case, a clear implication of this relationship is that AMPK activation should have similar silencing effects on PARP activity as SIRT1 activation. Surprisingly, however, the only study directly studying a possible relation between AMPK and PARPs described how AMPK could phosphorylate PARP-1 *in vitro* and enhance its activity in certain scenarios (Walker et al., 2006). These observations are in apparent contradiction with the metabolic observations in animal models, which indicate that AMPK rather mimics the transcriptional adaptations promoted by decreased PARP activity (Bai et al., 2011a,b; Canto and Auwerx, 2010). These discrepancies might be explained by the very different contexts and degrees of PARP and AMPK activation reached in the diverse experimental settings. In this sense, it is common that the conditions used in cell culture-based experiments activate these enzymes to supra-physiological levels. Therefore, the possible existence of interaction and phosphorylation events between AMPK and PARP-1 requires further confirmation and evaluation to validate its physiological relevance.

Following the footsteps of resveratrol, a more recent and ambitious screening for SIRT1 activators provided a new collection of compounds, amongst which N-[2-[3-(piperazin-1-ylmethyl)imidazo[2,1-*b*][1,2]thiazol-6-yl]phenyl]quinoxaline-2-carboxamide (SRT1720, Fig. 6) has been the one receiving most attention. SRT1720 is structurally different from resveratrol and acts as a more potent and efficient SIRT1 activator (Milne et al., 2007). As expected from a SIRT1 activator, the treatment of mice with SRT1720 enhanced oxidative metabolism and mitochondrial biogenesis, leading to protection against obesity and benefits on glucose homeostasis (Feige et al., 2008; Milne et al., 2007). However, it was recently pointed out that the screen that identified SRT1720 as a direct SIRT1 agonist had similar flaws as the one that identified resveratrol, namely: confusing results derived from the use of the "Fluor de Lys" fluorescent moiety (Huber et al., 2010; Pacholec et al., 2010). Therefore, a shadow of doubt stands on whether SRT1720 can be truly considered a direct SIRT1 agonist. Of note, the much higher potency of SRT1720 as a SIRT1 activator found in the *in vitro* screen was not translated *in vivo* (Feige et al., 2008), suggesting a rather indirect activation of SIRT1 or poor bioavailability. Whatever the case, no study to this data has clearly evaluated any possible impact of SRT1720 on PARP activity.

4.3. Agents modulating poly(ADP-ribosyl)ation

Most PARP activity in cells is driven by PARP-1, while the rest is mostly covered by PARP-2 both under non-stress conditions and upon oxidative injury (Bai et al., 2011a; Schreiber et al., 2002; Szanto et al., 2011). PARP activation – as discussed in Section 1.3 – can be triggered by DNA damage. PARP activation is induced voluntarily mostly under experimental conditions, by oxidants (e.g. hydrogen peroxide, peroxynitrite, radical donor compounds, certain cytostatic drugs), or by DNA

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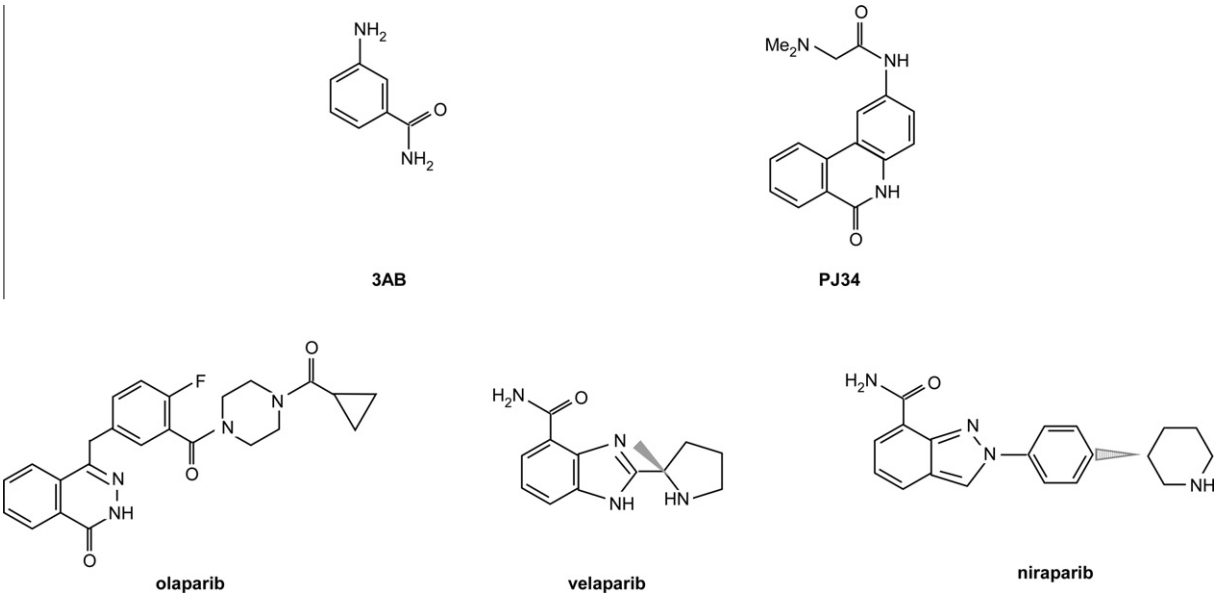

Fig. 7. Compounds that inhibit PARPs as discussed in the text.

alkylating agents (e.g. MNNG, or streptozotocin), or ionizing radiation (Schreiber et al., 2006). Besides DNA damage, PARP-1 can be activated by posttranslational modifications (see Section 1.3 and Gibson and Kraus, 2012), however it remains a question to be answered whether pharmacological modulation of these signaling pathways can efficiently modulate cellular PARP activity.

The first compound identified as PARP inhibitor was benzamide (IC_{50} = 22 μM) (Shall, 1975) and its 3-substituted versions (3-amino-benzamide is used the most frequently (IC_{50} = 33 μM)) that are simple analogs of NAM (IC_{50} = 210 μM) that is a by-product of the PARylation reaction. Benzamide and its analogs are applied in cellular assays in the millimolar range that may lead to aspecific interactions (Milam and Cleaver, 1984; Milam et al., 1986). Interestingly, certain dietary compounds (theophylline, caffeine and certain dietary flavonoids) that possess pleiotropic biological effects were also shown to inhibit PARP activity in cellular models in the submillimolar range (>100 μM) (Geraets et al., 2006, 2007; Moonen et al., 2005).

The careful modification of the backbone provided by nicotinamide and benzamide gave rise to current cutting edge PARP inhibitors (elegantly reviewed in Ferraris (2010) and (Curtin (2006)) (Fig. 7). These inhibitors bind to the NAM-binding pocket of PARP-1, hence act as competitive inhibitors of NAD^+-binding (Curtin, 2006; Ferraris, 2010; Jagtap and Szabo, 2005) with IC_{50} values in the low nanomolar range (1–5 nM) (Javle and Curtin, 2012). Targeting NAD^+ binding, to date, seems the only successful way to design specific PARP inhibitors, as an alternative approach to inhibit PARP-1 via impeding DNA binding through impairing the zinc fingers was not a viable strategy (Liu et al., 2012). PARG inhibitors would provide another alternative indirect approach for PARP inhibition (Erdelyi et al., 2009; Ying and Swanson, 2000), however the specificity of tannin derivatives used as PARG inhibitors had been questioned (Erdelyi et al., 2005).

Current PARP inhibitors are considered pan-PARP inhibitors, as they inhibit both PARP-1 and PARP-2 (Wahlberg et al., 2012). There is a current quest for the design of PARP-1/-2 specific inhibitors, however to date the best achieved specificity is 60-fold higher affinity towards PARP-2 than PARP-1 (Moroni et al., 2009), or ~10-fold preference towards PARP-1 than PARP-2 (Ferraris, 2010) in *in vitro* assays. These inhibitors are unlikely to act as highly selective agents in an *in vivo* setting. It seems that due to the large structural similarity between the PARP-1 and -2 catalytic domains (Oliver et al., 2004), the design of a highly specific inhibitor is a difficult task to accomplish (reviewed in Szanto et al., 2012).

The application of PARP inhibitors seems a promising strategy in a large number of physiological and pathophysiological states. Experimental evidence suggest the applicability of these compounds provide means to combat oxidative stress related diseases such as reperfusion injuries (gut, eye, kidney, myocardium), stroke, neurotrauma, inflammatory pathologies, shock, or diabetes and its consequent complications (Bai and Canto, 2012; Virag and Szabo, 2002, and in the present series a review by Nicola Curtin and Csaba Szabó). Several PARP inhibitors are currently making their ways through the different phases of clinical trials for the treatment of different solid and lymphoblastoid neoplasias as single agents or in combination (reviewed in Javle and Curtin, 2011, and in the present series a review by Nicola Curtin and Csaba Szabó). Importantly, in clinical studies PARP inhibitors were reported to have good tolerability that further ensure the applicability of these agents (Fong et al., 2009).

PARP inhibitors were protective as shown in multiple models of oxidative stress and aging, through guarding cellular NAD^+ levels by preventing excessive PARP activation and therefore inducing SIRT1 activity (Braidy et al., 2011; Liu et al., 2009; Massudi et al., 2012; Pang et al., 2011; Pillai et al., 2005, 2006; Qin et al., 2012; Sheline et al., 2010). PARP inhibitor

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treatment of C2C12 myotubes, or C57/Bl6J mice induced NAD^+ levels and SIRT1 activity (Bai et al., 2011b) that further consolidates the importance of the NAD^+ link between PARP-1 and SIRT1. PARP inhibitor treatment did not alter NAD^+ levels and sirtuin activity in other compartments such as cytosol, or mitochondria evidenced by the lack of SIRT2 and SIRT3 activation (Bai et al., 2011b).

Yet the effects of longer PARP inhibitor treatment on NAD^+ homeostasis and SIRT1 activity has not been studied, moreover the chronic applicability of PARP inhibitors raise concerns, due to reduction of DNA repair capacity and consequently to enhanced genomic instability that requires further studies.

5. Concluding remarks, perspectives

NAD^+ is a cofactor, or substrate of numerous enzymes that suggest widespread influence for NAD^+ over a plethora of cellular functions (Houtkooper and Auwerx, 2012; Houtkooper et al., 2010). Out of these enzymes we focused the subject of our review on two major NAD^+-dependent enzyme families: PARPs and sirtuins.

The interaction of SIRT and PARP enzymes has multiple layers. Both enzyme families consist of multiple members that show different affinities towards NAD^+. Obviously, as both enzymes are NAD^+-dependent in some occasions they may limit NAD^+ availability for one another. Furthermore, sirtuin and PARP enzymes interact through modifying acetylation levels, or in some cases these enzymes may interact through mutual regulation of gene expression. These interactions were described to modulate a series of biological processes ranging from metabolism, oxidative stress-mediated diseases through DNA repair to aging. Apparently, the appropriate balance between sirtuin and PARP activity is crucial to adequately regulate these processes. In some instances these balances are disturbed (e.g. in aging (Braidy et al., 2011; Massudi et al., 2012)) that presumably contributes to these pathologies.

Both SIRT1 and PARPs can be modulated pharmacologically (Feige et al., 2008; Fong et al., 2009; Jagtap and Szabo, 2005; Lagouge et al., 2006) enabling the modulation of both ends of this molecular seesaw. Importantly, there is ample data that not only the genetical, but pharmacological modulation of PARP, or SIRT1 activity affects the other partner (Bai et al., 2011b; Liu et al., 2008, 2009; Pang et al., 2011; Pillai et al., 2006; Rajamohan et al., 2009) suggesting that it is possible to appropriately arrange the SIRT–PARP balance by applying pharmacological agents.

It should be noted that the activity of SIRTs and PARPs can be modulated through cellular NAD^+ levels (Chambon et al., 1963; Imai et al., 2000) suggesting the applicability of NAD^+ precursors, such as NR, NA, or NAM. These agents impact profoundly on pathologies involving SIRTs or PARPs (Canto et al., 2012; Jackson et al., 1995; Schwarcz et al., 1983; Virag and Szabo, 2002) making them likely tools to fine tune PARP and SIRT enzymes, however their exact mode of action on both enzyme families had not been sufficiently mapped. The physiological and pathophysiological processes that govern NAD^+ levels are summarized in Fig. 8.

It is very likely that the occasions of sirtuin–PARP interaction is way more widespread than demonstrated experimentally – as discussed in Section 3.2 – suggesting that the circle of pathological states, where PARP–SIRT disbalance takes place can be enlarged and better defined. In fact, this notion raises the question whether other sirtuins, or PARPs may interact. It will be also important to distinguish between the gene-specific effects and the side effects of the available pharmacological agents (Antolin et al., 2012; Nicolescu et al., 2009; Pacholec et al., 2010) and to clearly define effects that are PARP, or SIRT

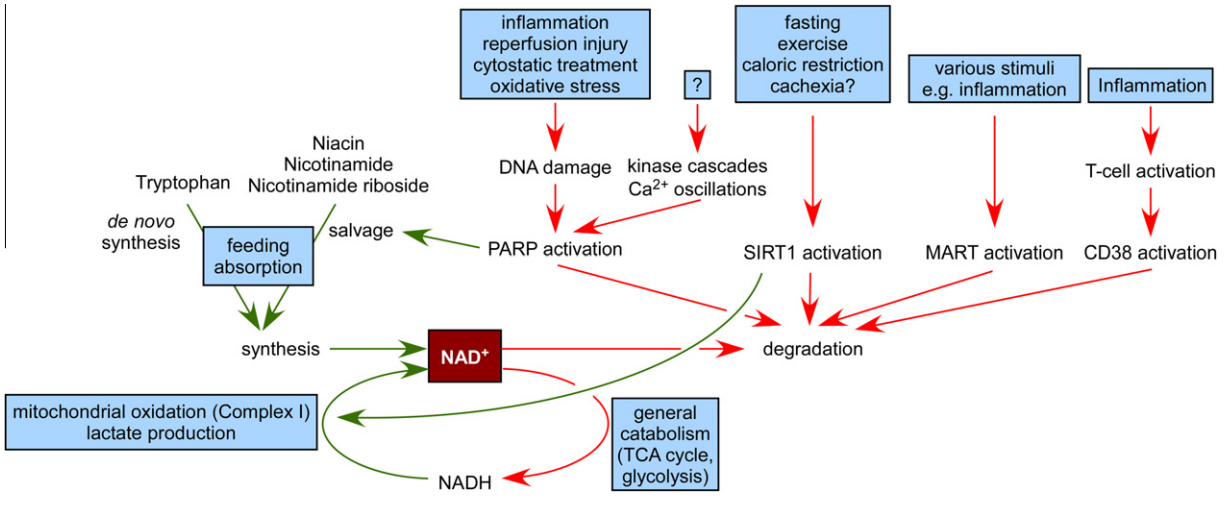

Fig. 8. Physiological and pathophysiological processes modulating NAD^+ levels. Physiological and pathological processes (in blue boxes) that enhance NAD^+ content, or availability are marked by green arrow, while those ones that lead to NAD^+ degradation are in red.

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independent in these pathologies (Bai et al., 2011b). Our current knowledge on the SIRT–PARP interaction represent only the tip of the iceberg and the field is expanding that will warrant further research in that field.

Disclosure

P.B. and A.A.S. declare no conflict of interest. C.C. is an employee of the Nestlé Institute of Health Sciences S.A. and declares no financial interests related to the work discussed in this review.

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Cell Metabolism
Minireview

The Role of PARP-1 and PARP-2 Enzymes in Metabolic Regulation and Disease

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While originally described as DNA damage repair agents, recent data suggest a role for poly(ADP-ribose) polymerase (PARP) enzymes in metabolic regulation by influencing mitochondrial function and oxidative metabolism. Here we review how PARP activity has a major metabolic impact and the role of PARP-1 and PARP-2 in diverse metabolic complications.

Introduction

Poly(ADP-ribose) polymerases (PARPs) catalyze a reaction in which the ADP-ribose moiety of NAD^+ is transferred to a receptor amino acid, building poly(ADP-ribose) (PAR) polymers. PAR polymers are an evolutionarily conserved posttranslational modification affecting a large array of proteins. However, the roles of PAR polymers remain uncertain. Recent research suggests they may constitute a protein-binding matrix, and several PAR-binding motifs have been proposed (Krishnakumar and Kraus, 2010).

Human PARPs comprise a family of 17 enzymes sharing a conserved catalytic domain (Krishnakumar and Kraus, 2010). The first PARP enzyme described, PARP-1, is responsible for the majority (85%–90%) of PARP activity in the cell, while the remainder is predominantly PARP-2. Other PARP family members bear mutations in the catalytic triad of amino acids that are crucial for polymer elongation, and these mutations critically impair their PARylating activity (Krishnakumar and Kraus, 2010). The function and localization of many PARP family members is still largely unknown.

PARP-1 and PARP-2 are highly conserved proteins, ubiquitously expressed in mammalian tissues and with predominant nuclear localization. While low in the basal state, the activity of PARP-1 and PARP-2 is enhanced allosterically through binding to several nuclear proteins and to a wide range of DNA or chromatin lesions. PARP-1 activity can also be modified through phosphorylation, acetylation, sumoylation, and ubiquitination. Importantly, PARP-1 can auto-PARylate itself, which inhibits its activity, creating an autoregulatory negative feedback (Krishnakumar and Kraus, 2010).

PARP-1 and PARP-2 have historically been described as key DNA damage repair enzymes (Krishnakumar and Kraus, 2010). However, under normal conditions, *PARP-1*$^{-/-}$ and *PARP-2*$^{-/-}$ mice show similar rates of spontaneous DNA damage to wild-type littermates, suggesting that the activity of either PARP-1 or PARP-2 is not essential for viability or DNA maintenance in the absence of genotoxic stress (Bai et al., 2011a, 2011b). Rather, these transgenic models have illustrated that PARP-1 and PARP-2 regulate a plethora of other processes, including tumorigenesis, inflammation, and cell differentiation (Krishnakumar and Kraus, 2010). Additionally, the *PARP-1*$^{-/-}$ and *PARP-2*$^{-/-}$ mouse models also uncovered a major metabolic role for PARP enzymes.

Acute Metabolic Consequences of PARP Activation

NAD^+ Metabolism and ATP Crisis

PARP-1 activity constitutes a major NAD^+ catabolic activity in the cell, depleting NAD^+ to 10%–20% of its normal levels within minutes upon DNA damage (Houtkooper et al., 2010). This unsustainable NAD^+ consumption rate forces the cell to synthesize NAD^+ using salvage pathways in order to maintain cellular viability. However, NAD^+ salvage is an ATP-consuming process (Houtkooper et al., 2010). Hence, persistent PARP activation also results in ATP depletion, creating a feedback loop that can compromise cell survival.

The reduction of NAD^+ levels promoted by PARP overactivation is sufficient to impair glycolysis, where NAD^+ is a key cofactor (Houtkooper et al., 2010). Reduction of the glycolytic rate shuts down a major source of rapid ATP formation that could be used for NAD^+ resynthesis. In addition, it reduces the flow of glucose-derived metabolites into the TCA cycle for oxidation, further compromising energy balance. This decrease in mitochondrial ATP production has a critical role in mediating the apoptotic effects of chronic PARP activation, as illustrated by the fact that addition of TCA substrates can rescue PARP overactivation-induced cell death (Ying et al., 2002).

Direct PARylation Effects on Mitochondrial Energy Production

PARP-1 activation rapidly impairs mitochondrial function (Cañuelo et al., 2012), though it is difficult to dissect whether this impairment is related to the decreased availability of NAD^+, reduced glycolysis, or to a direct effect of PARP-1 on mitochondrial respiration. Recently, elegant experiments showed that heterologous expression of a truncated, but active version of PARP-1 containing a mitochondrial localization signal, led to mitochondrial PAR accumulation and respiratory impairment, despite elevated glycolytic flux (Niere et al., 2008). This indicates that increased mitochondrial PARP activity alone is sufficient to directly impair mitochondrial function, irrespective of glycolytic rates. However, the significance of mitochondrial PARP activity in physiological scenarios is far from understood. While classically regarded as a nuclear protein, both PARP-1 and PARylation

events have been detected in mitochondria (Lai et al., 2008). Therefore, the PARylation of mitochondrial proteins could potentially affect their enzymatic activity. Supporting this hypothesis, the overexpression of mitochondrial PAR degrading enzymes, such as PAR glycohydrolase (PARG) or ADP-ribosylhydrolase 3 (ARH3), rescues mitochondrial PARylation and dysfunction upon PARP-1 activation (Lai et al., 2008; Niere et al., 2008). These findings would suggest the controversial hypothesis that direct PARylation events in the mitochondria contribute to the reduction of respiration rates upon PARP-1 activation. Understanding the physiological regulation of mitochondrial PARP activity and its impact on mitochondrial function will be an interesting field for future investigation.

PARP-1 and PARP-2 as Metabolic Transcriptional Regulators

PARP-1 localizes to the promoters of actively transcribed genes, though it only regulates a subset of them, exerting both positive and negative effects on transcription (Krishnakumar and Kraus, 2010). PARP-1 and PARP-2 act as transcriptional coregulators by influencing the recruitment of histone acetyltransferases and corepressors into different transcriptional complexes (Krishnakumar and Kraus, 2010). From a metabolic perspective, PARP-1 and PARP-2 interact with a large number of nuclear receptors and transcription factors regulating mitochondrial and lipid oxidation genes, such as PPARγ, FOXO1, and ER, among others (Bai et al., 2007; Krishnakumar and Kraus, 2010; Sakamaki et al., 2009). In some cases, PARP activation represses their transcriptional activity, for example by impeding correct DNA binding through direct PARylation of the transcription factors or their cofactors (Krishnakumar and Kraus, 2010). The PARylation of transcriptional metabolic regulators remains, however, a vastly unexplored field. PARP activity can also enhance transcription via the indirect modification of inhibitory histone marks or the facilitation of DNA cleavage and transcriptional activation (for review, see Krishnakumar and Kraus, 2010). PARP-1 may also exert noncatalytic effects on transcriptional regulation (Sakamaki et al., 2009), potentially through binding interactions in transcriptional complexes. The molecular mechanisms by which PARP-1 can display both coactivating and corepressing functions are still elusive.

The Interrelationship of PARP-1, PARP-2, and SIRT1

SIRT1 is an NAD^+-dependent type III deacetylase that regulates oxidative metabolism and global metabolic homeostasis. Pharmacologically or genetically induced increases in SIRT1 activity protect against high-fat diet (HFD)-induced metabolic damage in mice. To do so, SIRT1 deacetylates and regulates the activity of a number of crucial enzymes and transcriptional regulators (Houtkooper et al., 2010). A priori, SIRT1 has a K_M for NAD^+ that lies within the range of the most commonly reported total intracellular NAD^+ concentrations (Houtkooper et al., 2010). This could explain why most strategies designed to increase intracellular NAD^+ generally resulted in higher SIRT1 activity (Houtkooper et al., 2010). These observations suggest that NAD^+ bioavailability may control SIRT1 activity, leading to the hypothesis that SIRT1 might act as a metabolic sensor that fine tunes transcriptional programs to the use of different energetic substrates. However, the interpretation of NAD^+-related data should be taken cautiously, as most techniques used to date fail to provide complete information on subcellular NAD^+ compartmentalization or to differentiate between free and protein-bound NAD^+.

The NAD^+ dependence of SIRT1 and PARP-1 activities prompted the hypothesis that these enzymes could compete for a limited NAD^+ pool. Indeed, SIRT1 and PARP-1 activities can influence each other in different cellular and in vivo models. The mechanisms for this reciprocal regulation, however, may be different. The K_M of PARP-1 for NAD^+ is five to ten times lower than intracellular NAD^+ levels (Houtkooper et al., 2010). Therefore, it is unlikely that SIRT1 activity decreases NAD^+ concentrations to levels that are limiting for PARP-1 activity. Instead, SIRT1 has been shown to reduce PARP activity in certain contexts via direct binding and deacetylation of PARP-1 (Rajamohan et al., 2009).

PARP-1, in contrast, is an avid NAD^+ consumer (Houtkooper et al., 2010). Therefore PARP-1 may compromise SIRT1 activity by reducing NAD^+ bioavailability. This idea is further supported by recent observations indicating that pharmacological or genetic reductions of PARP activity increase intracellular NAD^+ levels and enhance SIRT1 activity (Bai et al., 2011b). Potentially, PARP-1 could also influence SIRT1 activity through direct PARylation. However, SIRT1 is not PARylated in myotubes when PARP-1 is activated in response to oxidative stress (Bai et al., 2011b). Therefore, PARP-1 most likely influences SIRT1 activity indirectly through the modulation of NAD^+ levels. Interestingly, in contrast to SIRT1, neither cytoplasmic SIRT2, nor mitochondrial SIRT3 activities were increased by *PARP-1* deletion (Bai et al., 2011b). This suggests that the increase in NAD^+ promoted by *PARP-1* deletion might be restricted to the nucleus, where PARP-1 predominantly resides. Accordingly, it will be important in future studies to monitor the activity of the other nuclear sirtuins, SIRT6 and SIRT7, upon altered PARP-1 activity.

PARP-2 deletion also enhances cellular SIRT1 activity, apparently without direct impact on NAD^+ levels (Bai et al., 2011a). Instead, *PARP-2* deletion resulted in a 2- to 3-fold increase in SIRT1 levels, both in cultured cells and mouse tissues (Bai et al., 2011a). PARP-2 binds to the *SIRT1* promoter in the basal state and represses its transcriptional activity (Bai et al., 2011a). Hence, *PARP-2* deletion relieves the repression on the *SIRT1* promoter, increasing basal *SIRT1* mRNA and protein levels (Bai et al., 2011a). Therefore, the higher SIRT1 activity observed in *PARP-2*$^{-/-}$ mice is not necessarily related to increased NAD^+ levels, but to increased *SIRT1* expression.

The negative correlation of PARP and SIRT1 activities is also found in physiological scenarios. PARP activity increases upon HFD, while SIRT1 activity is lower (Bai et al., 2011b). In contrast, PARP activity is lower in muscle after an overnight fast, when SIRT1 activity is high (Bai et al., 2011b). Interestingly, higher PARP activity is observed in aged rodent tissues, leading to decreased NAD^+ content and limited SIRT1 activity (Braidy et al., 2011). These observations suggest that physiological variations in PARP activity may have a significant impact on SIRT1.

Metabolic Actions of PARP-1 and PARP-2 in Health and Disease

Regulation of Energy Intake

Several observations suggest that PARP-1 may influence feeding behavior. *PARP-1* deletion in two different mouse strains led to increased food intake (Bai et al., 2011b; Devalaraja-Narashimha

and Padanilam, 2010). Also, PARP-1 seems crucial for the circadian entrainment of feeding behavior (Asher et al., 2010). In contrast, the deletion of *PARP-2* does not lead to significant changes in food intake or daily behavior (Bai et al., 2011a). This indicates that PARP-1 might influence food intake in two different ways: either through PARylation of substrates that PARP-2 does not target or through mass disturbance of NAD^+ metabolism, which determines circadian behavior (Nakahata et al., 2009).

Regulation of Mitochondrial Biogenesis and Energy Expenditure

Both *PARP-1*$^{-/-}$ and *PARP-2*$^{-/-}$ mice display enhanced energy expenditure (EE) (Bai et al., 2011a, 2011b). This effect could derive, at least in part, from an increase in SIRT1 activity, as described above. SIRT1 can regulate EE by deacetylating and activating master transcriptional regulators of oxidative metabolism, such as PGC-1α and the FOXO family of transcription factors (Houtkooper et al., 2010). Consistently, *PARP-1*$^{-/-}$ and *PARP-2*$^{-/-}$ muscles showed a marked deacetylation of PGC-1α and FOXO1, which was linked to enhanced mitochondrial biogenesis and a more oxidative profile of muscle fibers (Bai et al., 2011a, 2011b).

The brown adipose tissue (BAT) of *PARP-1*$^{-/-}$ mice is also characterized by increased mitochondrial content (Bai et al., 2011b). Physiologically, this renders *PARP-1*$^{-/-}$ mice more able to maintain body temperature when exposed to cold. Interestingly, the deletion of *PARP-2* does not influence mitochondrial biogenesis in BAT, despite increasing SIRT1 content (Bai et al., 2011a). It is possible that there are either limiting NAD^+ levels in the BAT of *PARP-2*$^{-/-}$ versus *PARP-1*$^{-/-}$ mice or that there are additional mediators of the *PARP-1*$^{-/-}$ phenotype other than the SIRT1/PGC-1α axis.

Regulation of Adipogenesis, Fat Deposition and Body Weight

The deletion of either *PARP-1* or *PARP-2* in C57Bl/6 mice protects against age- and HFD-induced body weight (BW) gain (Bai et al., 2011a, 2011b; Erener et al., 2012b). This phenotype is explained, at least in part, by their enhanced EE. However, a direct regulation of fat deposition by PARP enzymes may also contribute.

PARP-1 activity is necessary for white adipocyte differentiation, and increased PARylation can be observed in differentiating 3T3-L1 adipocytes (Erener et al., 2012a). PARP-1 is recruited to PPARγ target genes in a PAR-dependent manner, allowing sustained expression of PPARγ and its target genes (Erener et al., 2012a). Accordingly, *PARP-1*$^{-/-}$ mice display reduced fat mass deposition (Bai et al., 2011b; Erener et al., 2012b). The histological analysis of white adipose tissue (WAT) from *PARP-1*$^{-/-}$ mice revealed a dramatic decrease in adipocyte size (Erener et al., 2012b). In addition, adipose stem cells from *PARP-1*$^{-/-}$ mice displayed lower expression of PPARγ target genes upon differentiation, as well as a reduced ability to accumulate triglycerides (Erener et al., 2012b). Therefore, PARP-1 acts as a positive regulator of adipogenesis and adipocyte function. Fully supporting this, transgenic mice harboring an ectopic integration of human *PARP-1* (*hPARP-1* mice) display enhanced adiposity (Mangerich et al., 2010). Interestingly, the lack of *PARP-2* also hampers the adipocytic differentiation of embryonic fibroblasts and 3T3-L1 cells (Bai et al., 2007). Similarly to PARP-1, PARP-2 binds and positively regulates PPARγ-driven promoters (Bai et al., 2007), enhancing adipogenic differentiation and fat deposition.

The activation of SIRT1 may also contribute to downregulation of PPARγ activity and reduced fat storage in PARP-1- and PARP-2-deficient models. SIRT1 decreases adiposity through various mechanisms, including the reduction of PPARγ transcriptional activity by promoting the direct docking of transcriptional corepressors (reviewed in Houtkooper et al., 2010). An interesting question is whether reduced WAT depots could potentially lead to lipid redistribution. Indeed, a recent report identified increased fat deposition in the livers of PARP-1$^{-/-}$ mice when fed a HFD (Erener et al., 2012b). Interestingly, *PARP-1* is poorly expressed in the liver, and its deletion does not seem to have a major influence on hepatic expression of mitochondrial and lipid oxidation genes (Bai et al., 2011b), potentially creating a permissive scenario for lipid deposition. These observations, however, are at odds with the notion that *PARP-1* deficiency dampens PPARγ activity and with the lower BW of *PARP-1*$^{-/-}$ mice. Further research will be required to clarify this apparent discrepancy.

Strikingly, *PARP-1* deletion on an SV129 background renders mice susceptible to obesity (Devalaraja-Narashimha and Padanilam, 2010). As discussed recently (Bai et al., 2011b), however, a wealth of pharmacological, physiological and genetic data supports the observations in the C57Bl/6 strain. The particular reasons why *PARP-1* deletion in a SV129 background rendered an opposite phenotype are unknown.

PARP-1 and PARP-2 in Glucose Metabolism and Insulin Sensitivity

PARP-1$^{-/-}$ and *PARP-2*$^{-/-}$ mice display increased glucose clearance in response to an insulin tolerance test (Bai et al., 2011a, 2011b). The ability of *PARP-1* and *PARP-2* deletion to enhance the muscle oxidative profile might contribute to this phenotype, as oxidative muscle fibers are more insulin sensitive than glycolytic fibers. In addition, the greater potential to oxidize fat might prevent the chronic deposition of lipid species detrimental for insulin action.

While enhanced insulin sensitivity is generally correlated with better glucose tolerance, *PARP-2*$^{-/-}$ mice were markedly glucose intolerant when fed a HFD (Bai et al., 2011a). This glucose intolerant phenotype stems from pancreatic β cell dysfunction. Upon HFD, the pancreatic β cell mass increases in order to compensate for peripheral insulin resistance. In *PARP-2*$^{-/-}$ mice, however, this hyperplastic response is impaired. *PARP-2*$^{-/-}$ mice display smaller β cell islets and reduced insulin content, resulting in a blunted ability to release insulin after a glucose load (Bai et al., 2011a). The mechanism by which *PARP-2* deletion impairs β cell proliferation may involve constitutive SIRT1 activation, which leads to FOXO1 deacetylation and activation (Bai et al., 2011a). FOXO1, in turn, represses β cell proliferation and development. Yet why is pancreatic dysfunction not observed in *PARP-1*$^{-/-}$ mice? From one side, the actions of PARP-2 might be noncatalytic. Another likely possibility is that PARP-1 differentially affects NAD^+ homeostasis and SIRT1 activity in different tissues, depending on its basal activity. Alternatively, other actions specifically triggered by *PARP-1* deficiency might prevent β cell dysfunction.

In fact, a major role for PARP-1 in β cell physiology was revealed by experiments determining how PARP inhibitors improve

Table 1. Key Observations on How PARP-1 and PARP-2 Intertwine with Metabolic Homeostasis

Model	Phenotype	Reference
PARP-1		
Knockout in C57Bl/6 mice	Altered circadian food entrainment behavior	(Asher et al., 2010)
Knockout in C57Bl/6 mice	Increased energy expenditure and mitochondrial biogenesis	(Bai et al., 2011b)
Knockout in C57Bl/6 mice	Lower body weight gain when fed high-fat diets	(Bai et al., 2011b; Erener et al., 2012b)
Knockout in C57Bl/6 mice	Both higher and lower glucose tolerance have been reported	(Bai et al., 2011b; Erener et al., 2012b)
Knockout in C57Bl/6 mice	Higher hepatic lipid accumulation	(Erener et al., 2012b)
Knockout in C57Bl/6 mice	Increased NAD^+ content and SIRT1 activity	(Bai et al., 2011b)
Knockout in 129/SvImJ mice	Enhanced susceptibility to high-fat diet-induced obesity	(Devalaraja-Narashimha and Padanilam, 2010)
Knockout in 129SVxC57Bl/6 mice	Protection against streptozotocin-induced diabetes	(Burkart et al., 1999)
Mice harboring an ectopic integration of human PARP-1	Premature development of age-associated pathologies	(Mangerich et al., 2010)
Mice harboring an ectopic integration of human PARP-1	Enhanced adiposity	
Mice harboring an ectopic integration of human PARP-1	Glucose intolerance	
Knockdown in HEK293T cells	Enhanced mitochondrial gene expression and respiration	(Bai et al., 2011b)
Knockdown in HEK293T cells	Higher NAD^+ content and SIRT1 activity	
Knockdown in 3T3-L1 adipocytes	Reduced adipocyte differentiation and PPARγ-dependent gene expression	(Erener et al., 2012a)
PARP-2		
Knockout in C57Bl/6 mice	Increased energy expenditure and mitochondrial biogenesis	(Bai et al., 2011a)
Knockout in C57Bl/6 mice	Lower body weight when fed high-fat diets	
Knockout in C57Bl/6 mice	Glucose intolerance despite high insulin sensitivity	
Knockout in C57Bl/6 mice	Decreased pancreatic β cell mass	
Knockout in C57Bl/6 mice	Lower WAT mass on chow diet	(Bai et al., 2007)
Knockdown in C2C12 myotubes	Increased SIRT1 levels and mitochondrial gene expression	(Bai et al., 2011a)
Knockout murine embryonic fibroblasts	Reduced adipocyte differentiation and PPARγ-dependent gene expression	(Bai et al., 2007)
PARP Inhibition		
C2C12 myotubes (PJ34; 1 μM)	Enhanced mitochondrial gene expression and respiration	(Bai et al., 2011b)
Partially depancreatized rats (0.5 g/kg NAM and 0.05 g/kg 3-ABA per day)	Better β cell regeneration and prevention of diabetes mellitus	(Yonemura et al., 1984)
Mice (PJ34; 10 mg/kg/day)	Better lipid metabolic profile after a 5 day treatment	(Bai et al., 2011b)
Physiology		
High-fat diet	Enhanced PARP activity and PARP-1 protein levels	(Bai et al., 2011b)
Aging	Enhanced PARP activity	(Braidy et al., 2011)
Fasting	Lower PARP activity	(Bai et al., 2011b)

The table summarizes how changes in PARP-1 or PARP-2 activity influence metabolism and vice versa.

diabetes mellitus in partially depancreatized rats, namely by promoting faster β cell regeneration and normalization of blood glucose (Yonemura et al., 1984). Subsequent studies demonstrated that *PARP-1*$^{-/-}$ mice are resistant to the development of streptozotocin-induced diabetes and maintain normal pancreatic insulin content and islet morphology (Burkart et al., 1999). The normal pancreatic β cell function of *PARP-1*$^{-/-}$ mice in the basal state, however, suggests the detrimental effect of PARP-1 activity in the pancreas is only apparent in situations of oxidative stress.

Future Perspectives

Most classical work characterized PARP-1 and PARP-2 as genome integrity maintenance enzymes. Several findings, however, highlight novel roles for PARP-1 and PARP-2 in metabolic regulation (summarized in Table1). PARP activation impacts on cellular metabolism through diverse mechanisms, including alterations in NAD^+ metabolism, direct PARylation events and transcriptional reprogramming of the cell. In general, PARP inhibition enhances oxidative metabolism and mitochondrial content. This suggests that reducing PARP activity might prevent

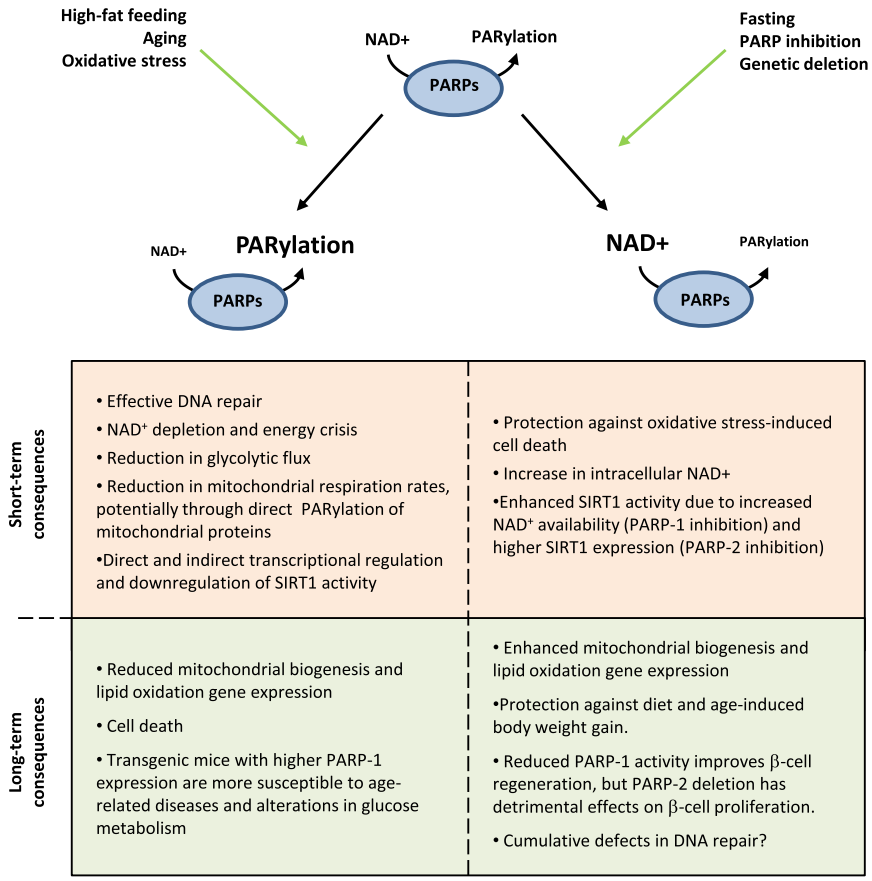

Figure 1. Metabolic Consequences of PARP Activation or Inhibition

Diverse physiological situations—such as high-fat feeding, oxidative stress, spontaneous DNA damage, and aging—increase PARP activity. In the short term, PARP activity is required for efficient DNA repair, but can also lead to NAD^+ depletion, consequently slowing down ATP production. PARP-1 and PARP-2 might also directly affect ATP production through direct PARylation of enzymes, as has been proposed for mitochondrial proteins. When prolonged, the effects of PARP activity on transcriptional regulators can compromise the maintenance of mitochondrial function and, as evidenced by transgenic models, might enhance susceptibility to age-related diseases and alterations in glucose metabolism. In contrast, nutrient deprivation decreases PARP activity. The evidence from pharmacological or genetic reductions in PARP activity indicates that lower PARP activity increases NAD^+ bioavailability and enhances mitochondrial biogenesis. These changes confer protection against age and diet-induced body-weight gain. These potential benefits of PARP inhibition need to be balanced with the detrimental effects on pancreatic β cell function caused by *PARP-2* deletion, as well as with the eventual possibility for detrimental effects on chromosome stability.

age- and metabolic-related diseases, often characterized by impaired mitochondrial function (Figure 1). In line with this, a gain of function model for PARP-1, the *hPARP-1* mice, displayed premature development of age-associated pathologies, enhanced adiposity, and glucose intolerance (Mangerich et al., 2010). However, we will need to evaluate the feasibility of long-term inhibition of PARP activity without a negative impact on genomic stability. Possible strategies to limit potential side effects on DNA damage or β cell dysfunction could rely on determining the minimum level of PARP inhibition required to reach therapeutic metabolic effects and on the design of specific inhibitors for PARP-1 and PARP-2. Importantly, while we have focused on PARP-1 and PARP-2, we need to emphasize that other PARP family members can also have important metabolic functions (Table S1 available online), further emphasizing the need for specific PARP inhibitors to elicit selective metabolic responses. Finally, most of the in vivo data gathered to date comes from germline *PARP-1* or *PARP-2* deletion. The generation of tissue-specific PARP deficient models will allow us to dissect the key tissues that should be pharmacologically targeted to achieve optimal metabolic outcomes.

Another challenge will be elucidation of the key effectors mediating the long-term benefits of PARP inhibition. While SIRT1 activation is a major candidate, PARP inhibition triggers both acute and transcriptional effects that are SIRT1-independent (Bai et al., 2011a, 2011b). Our knowledge of direct PARylation events on metabolic regulators is still weak. Of note, it will be interesting to examine which metabolic effects of PARP inhibition are mimicked by the activation of PAR degrading enzymes. In conclusion, PARP inhibition holds promising possibilities for the treatment of metabolic damage, but achieving healthy aging through PARP-based therapies will require further understanding of PAR biology and fine-tuning the dynamics and specificity of PARP inhibitors.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.06.016.

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Supplemental Information

The Role of PARP-1 and PARP-2 Enzymes

in Metabolic Regulation and Disease

Péter Bai and Carles Cantó

Table S1. Metabolic Roles Described for Other PARP Family Members, Related to the Main Text

Enzyme	Model	Metabolic effect	Ref.
PARP5a (TNK1)	3T3-L1 cells Knockout mice	- TNK1 associates with GLUT4 vesicles through the insulin –responsive aminopeptidase (IRAP). Whether TNK1 activity has the ability to influence insulin-stimulated glucose uptake is not clear. - TNK1 deletion enhances energy expenditure through the skeletal muscle, ketogenesis and fatty acid oxidation in the liver, pancreatic hyperfunction and β cell hyperproliferation.	**(Chi and Lodish, 2000; Sbodio et al., 2002; Yeh et al., 2009; Yeh et al., 2007)**
PARP5b (TNK2)	3T3-L1 cells	Influence Glut4 translocation in adipocytes through IRAP.	**(Chi and Lodish, 2000; Sbodio et al., 2002)**
PARP-7 (TiPARP)	Chicken embryos hepatocytes	PARP-7 activity decrease NAD^+ levels, leads to hyperacetylation of PGC-1α and suppress hepatic glucose production probably through influencing SIRT1.	**(Diani-Moore et al., 2010)**
PARP-14	HT-1080 human fibrosarcoma cells, Hs68 human skin fibroblast cells	Protects phosphoglucose isomerase from ubiquitination and eventual breakdown.	**(Yanagawa et al., 2007)**

The table summarizes reported metabolic roles for PARP enzymes other than PARP-1 or PARP-2.

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Cellular and Molecular Life Sciences

REVIEW

Poly(ADP-ribose) polymerase-2: emerging transcriptional roles of a DNA-repair protein

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Abstract Poly(ADP-ribose) polymerase (PARP)-2 is a nuclear enzyme that belongs to the PARP family and PARP-2 is responsible for 5–15 % of total cellular PARP activity. PARP-2 was originally described in connection to DNA repair and in physiological and pathophysiological processes associated with genome maintenance (e.g., centromere and telomere protection, spermiogenesis, thymopoiesis, azoospermia, and tumorigenesis). Recent reports have identified important rearrangements in gene expression upon the knockout of PARP-2. Such rearrangements heavily impact inflammation and metabolism. Metabolic effects are mediated through modifying PPARγ and SIRT1 function. Altered gene expression gives rise to a complex phenotype characterized primarily by enhanced mitochondrial activity that results both in beneficial (loss of fat, enhanced insulin sensitivity) and in disadvantageous (pancreatic beta cell hypofunction upon high fat feeding) consequences. Enhanced mitochondrial biogenesis provides protection in oxidative stress-related diseases. Hereby, we review the recent developments in PARP-2 research with special attention to the involvement of PARP-2 in transcriptional and metabolic regulation.

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The PARP superfamily

Poly(ADP-ribosyl)ation is a transient post-translational modification of proteins mediated by poly(ADP-ribose) polymerase (PARP) enzymes. This is a dynamic process during which the enzymes catalyze the formation of ADP-ribose polymers onto different acceptor proteins using NAD^+ as a substrate. The half-life of the polymer is very short since it is quickly degraded by poly(ADP-ribose) glycohydrolase (PARG). PARPs constitute a family of 17 members, encoded by 17 different genes sharing a conserved sequence coding for the catalytic domain that contains the PARP signature motif, a highly conserved sequence that forms the active site [1]. Based on sequence and structural homologies and the similarity of the reactions catalyzed, Hottiger and colleagues recently proposed to unite all ADP-ribose transferases (PARPs and mono-ADP-ribosyl transferases) in one protein family [2]. The same study proposed a new nomenclature for these enzymes, where PARP-2 was renamed ARTD2 (ADP-ribosyltransferase diphtheria toxin-like 2).

The prototypical enzyme of the PARP family is PARP-1 (ARTD1). PARP-1 cleaves NAD^+ to ADP-ribose and nicotinamide followed by the attachment of the first ADP-ribose moiety to a glutamate or aspartate residue of target proteins. During the elongation of the polymer further ADP-ribose moieties are attached to these protein-bound monomers. In the absence of DNA damage the constitutive polymer levels are usually very low and appear as mono- or oligo(ADP-ribose). However, in response to DNA strand breaks, the levels of poly(ADP-ribose) (PAR) polymers

increase 10–500 fold and large [3, 4] branched PAR polymers occur on different acceptor proteins and PARP-1 itself (auto-PARylation) (Fig. 1). Upon extensive PARP-1 activation, cellular NAD^+ levels are markedly reduced [5]. Poly(ADP-ribosyl)ation, at any level, is likely to have important effects on the acceptor's properties, hence PARylation and PARPs are involved in the regulation of various cellular processes [6]. In cells, polymers can be detected as early as 2 to 3 min after the one-off induction of DNA damage, and then PAR polymers are quickly degraded by PARG. In tissues, PAR levels can be detected on a longer timeline as reflecting a steady state between synthesis and degradation. Some PARP enzymes carry mutations in the catalytic domain and hence are either inactive or perform only mono-, or oligo(ADP-ribosyl)ation [1, 2, 7].

The structure of the *PARP-2* gene and PARP-2 protein

PARP-2 was discovered when residual DNA-dependent PARP activity was detected in *PARP-1*$^{-/-}$ murine embryonic fibroblasts (MEFs) [8]. So far PARP-1, PARP-2, and PARP-3 are the only PARP enzymes whose catalytic activity is stimulated by DNA strand breaks suggesting that they function as crucial members in the cellular pathways responding to DNA damage [8–11].

The *PARP-2* gene is located on chromosome 14 in humans. The gene is driven by a bidirectional promoter that *PARP-2* shares with *RNase P* [12]. Such a combination

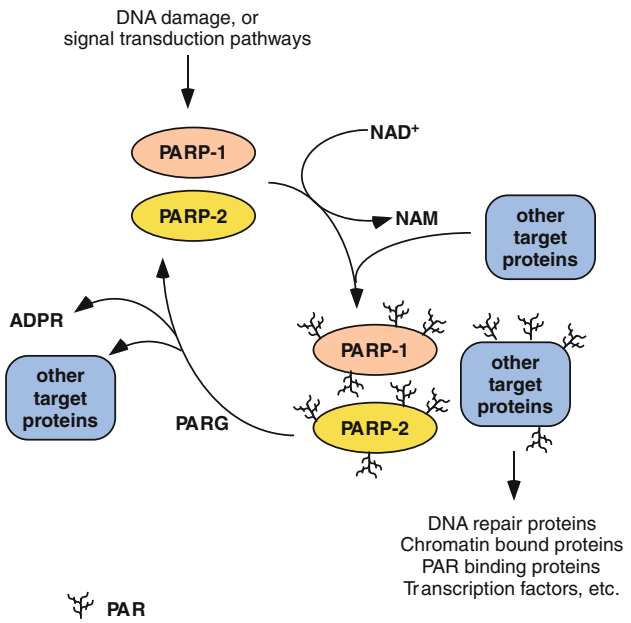

Fig. 1 The poly(ADP-ribosyl)ation cycle. *NAM* nicotinamide, *ADPR* ADP-ribose, *PAR* poly(ADP-ribose), all other abbreviations are in the text

of RNA polymerase II and RNA polymerase III genes is relatively rare. A functional TATA box and DSE/Oct-1 expression control elements were identified in the promoter regulating *PARP-2* expression [12]. Due to alternative splicing, two isoforms of *PARP-2* exist with the longer isoform containing an extra set of 13 amino acids on the border between the DNA binding domain and domain E. The longer isoform has been identified, or predicted in humans [13], common chimpanzee (*Pan troglodytes*) [14], northern white-cheeked gibbon (*Nomascus leucogenys*) [15] and sumatran orangutan (*Pongo abelii*) [16] according to the NCBI database. The sequences of different mammalian *PARP-2* genes are highly homologous (Fig. 2). Although *PARP-2* is absent in birds, sequences similar to PARP-2 can be found in lower vertebrates (*Danio rerio*, *Xenopus*), lower animals (e.g., sponges) and in *Arabidopsis thaliana* [17].

The tissue-specific expression of *PARP-2* was primarily characterized by in situ hybridization. Liver expression of *PARP-2* was high at fetal age 12.5 days, decreased at 18.5 days fetal age, and was even lower in newborn mice [8, 18]. In adult mice, the expression of PARP-2 is low in the liver (the lowest among the metabolic tissues; Bai P, unpublished data). It is tempting to speculate that the gradual decrease in *PARP-2* expression by age during fetal and postnatal development points toward the possible involvement of PARP-2 in early stage hemopoiesis that takes place in the liver.

In the central nervous system, PARP-2 content was high in the spinal ganglia and in certain parts of the brain. In the neocortical areas, *PARP-2* expression is elevated as compared to lower brain regions. High *PARP-2* expression was detected in stratum granulosum of the dentate gyrus and the stratum pyramidale of the hippocampus and was even higher in the cortex and the olfactory bulb [18]. Apart from the previously mentioned tissues, *PARP-2* is highly expressed in the cortical region of the kidneys, the spleen, adrenal glands, stomach, thymus, and intestinal epithelium [18]. The testis was also positive for *PARP-2* expression.

In humans, a slightly different expression pattern was detected. *PARP-2* was very abundant in the skeletal muscle, brain, heart, testis, it was high in pancreas, kidney, placenta, ovary, spleen, and low *PARP-2* expression was detected in the lungs, leukocytes, gastrointestinal tract (both colon and small intestine), thymus, and liver [19].

Translation of the *PARP-2* mRNA yields a protein product of 62-kDa apparent molecular weight. The PARP-2 protein can be divided into similar functional regions as PARP-1: the N-terminus of mouse PARP-2 contains the DNA binding domain (DBD), followed by domain E and the catalytic domain (domain F) [8]. The DBD is formed by a SAP domain that is responsible for DNA binding [20], and contains a functional nuclear localization signal (NLS)

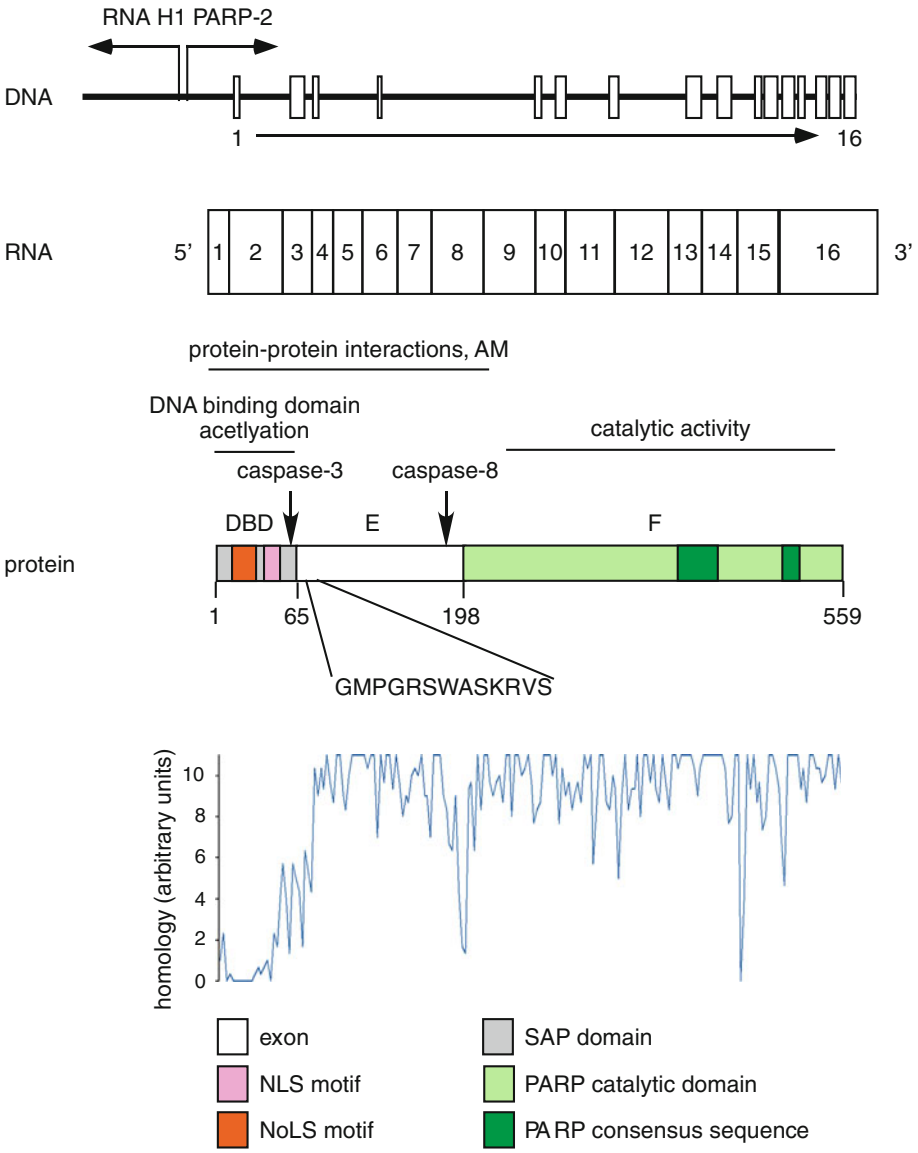

Fig. 2 The structure of the *PARP-2* gene and PARP-2 protein. The *PARP-2* gene is driven by a bidirectional promoter and consists of 16 exons. The protein product of the gene can be divided into three domains: DBD, domain E, domain F. *Numbers* below the protein product indicate amino acids on the border between domains. The *arrows* point to caspases-3 and caspases-8 cleavage sites. The highlighted amino acid sequence is the conserved 13 amino acid sequence of the longer PARP-2 isoform. Eighteen mammalian *PARP-2* sequences of the shorter isoform (*Ailuropoda melanoleuca* [118], *Bos taurus* [119], *Callithrix jacchus* [120], *Canis lupus familiaris* [121], *Cavia porcellus* [122], *Cricetulus griseus* [123], *Equus caballus* [124], *Homo sapiens* [13], *Loxodonta africana* [125], *Macaca mulatta* [126], *Monodelphis domestica* [127], *Mus musculus* [128], *Nomascus leucogenys* [15], *Oryctolagus cuniculus* [129], *Pan troglodytes* [14], *Pongo abelii* [16], *Rattus norvegicus* [130], and *Sus scrofa* [131]) were compared with Clustal W2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and relative conservation of the amino acids were plotted. Higher values indicate higher levels of conservation. *DBD* DNA binding domain, *AM* automodification, *NLS* nuclear localization signal, *NoLS* nucleolar localization signal, *SAP* SAP domain

[21] and a nucleolar localization signal (NoLS) [22]. A caspase-3 cleavage site defines the border between the DBD and domain E, which is homologous to the caspase-3 site in the E domain of PARP-1 [23]. Domain E serves as a homodimerization interface, an automodification domain and a protein–protein interaction domain as well [24]. Auto-poly(ADP-ribosyl)ation of PARP-2 takes place on domain E [25] and on lysine 36 and 37 that are targets of simultaneous acetylation [26, 27]. The PARP-2 interactome was mapped by Isabelle and coworkers [28], who identified a large number of proteins. These proteins covered a wide array of functions such as cell cycle, cell death, DNA repair, DNA replication, transcription, metabolism, energy homeostasis, and RNA metabolism.

Domain F on the C-terminus of PARP-2 harbors the PARP signature motif carrying the essential amino acid residues for catalysis [8]. Domain F is separated from domain E by a caspase-8 cleavage site [29]. PARP-2 and PARP-1 share a catalytic domain of 69 % similarity, with the exception that PARP-2 contains an additional three-amino-acid insertion in the loop connecting the β-strands *k* and *l* in PARP-1 [8, 30, 31] (Fig. 3). The three-dimensional structure of the catalytic domain also shows high similarity; however, the catalytic domain of PARP-2 has a narrower catalytic cleft that likely explains the lower substrate affinity and turnover rate of PARP-2 as compared to PARP-1 (K_M for NAD^+ 50/130 μM; k_{cat}/K_M 6,000 s^{-1} M^{-1}/323 s^{-1} M^{-1} for PARP-1/-2, respectively) [8, 30]. PARP-2 accounts for 5–15 % of total PARP activity in cells depending on the model used [8, 32, 33]. PARP-2 performs auto [18] and hetero-PARylation of proteins. Troiani and coworkers have identified possible targets of PARP-2 activity that covered proteins involved in transcription, translation and mitochondrial organization [34].

PARP-2 in DNA repair and genomic integrity

PARP-1 is a well-established DNA-repair protein [6], therefore the functional similarity with PARP-2 suggested a role for PARP-2 in the maintenance of DNA integrity. Upon the induction of DNA damage (ionizing irradiation or laser irradiation), PARP-2 accumulates at the damage foci [35] with a slower kinetics than PARP-1, and PARP-2 persisted longer at DNA damage sites [36]. In murine embryonic fibroblasts (MEFs), the loss of PARP-2 leads to hypersensitivity to ionizing irradiation and cell-cycle arrest in G1 [23], although PARP-$2^{-/-}$ cells are less sensitive to ionizing radiation than PARP-$1^{-/-}$ cells [23, 24]. In line with these observations, female lethality due to X chromosome instability was observed in *PARP-*$1^{+/-}$ *PARP-*$2^{-/-}$ mice [23].

PARP-2 preferentially binds to one-nucleotide gaps [25] and it is involved in single-strand repair processes. As shown in murine models, upon the loss of PARP-2, base excision repair (BER) slows down [18]. Moreover, PARP-2 interacts with numerous members of the BER machinery such as XRCC1, PARP-1, DNA polβ, and DNA ligase III [18] that further signifies its importance in BER. It is tempting to hypothesize that the early embryonic lethality of the *PARP-1/PARP-2* double knockout mice [23] might be due to the strong impairment of DNA repair processes.

PARP-1 has been described to participate in double-strand break repair [37]. Nicolás and coworkers [38] have identified the accumulation of double-strand breaks in *PARP-*$2^{-/-}$ murine thymocytes. This observation is in line with a previous report by Yelamos and colleagues [24] who suggested that PARP-2 interacts with the Ku proteins, mediators of double-strand break repair. Moreover, Robert and colleagues [39] have identified PARP-2 as a suppressor of recombination during immunoglobulin class switch events in murine and human B cells, while Bryant et al. have suggested that both PARP-1 and -2 are essential in resolving blocked replication forks by homologous recombination in CHO and murine embryonic fibroblasts (MEFs) [37, 40]. The fact that the ATM/PARP-2 double-knockout genotype is embryonic lethal [20] further supports the involvement of PARP-2 in double-strand break repair during replication. Moreover, the fact that mitomycin C treatment that leads to DNA double-strand breakage provoked the induction of PARP-2 expression and other double-strand break repair proteins

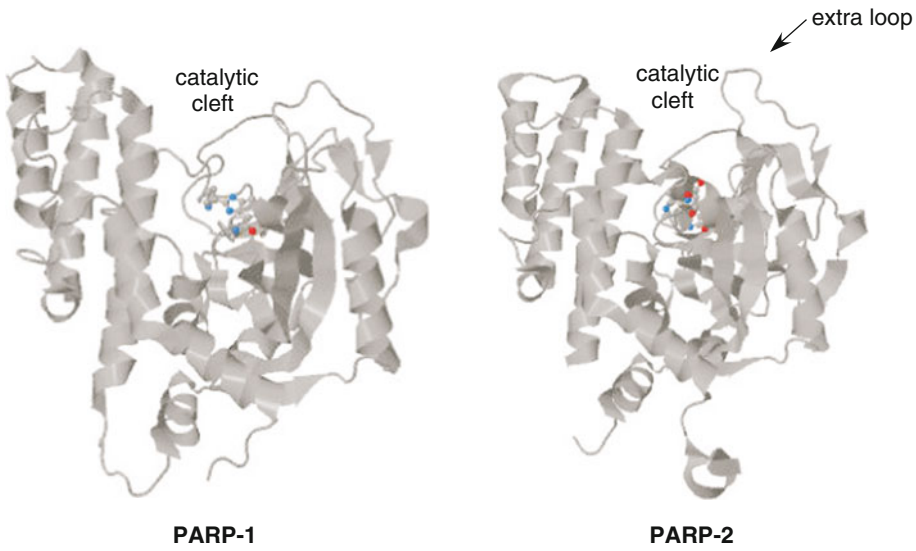

Fig. 3 Three-dimensional structure of the catalytic domain of PARP-1 and PARP-2. Crystal structure of PARP-1 (3GN7) and PARP-2 (3KJD) were retrieved from the protein data bank (PDB, http://www.rcsb.org). Both structures contain an inhibitor (*in color*), the PARP-1 catalytic domain is in complex with A861696, while the PARP-2 catalytic domain is in complex with ABT-888 [31]. The catalytic cleft and the PARP-2-specific loop is indicated

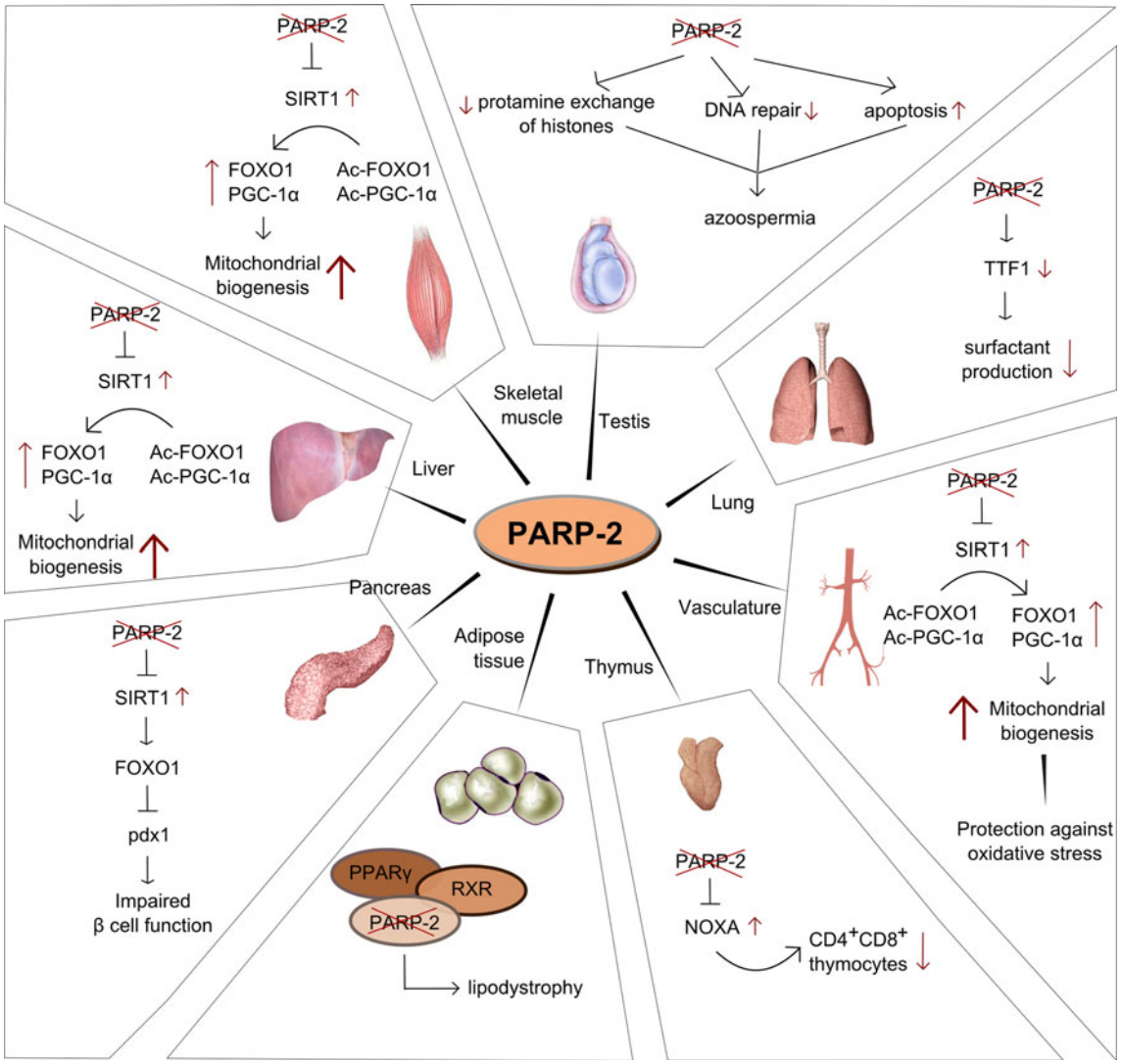

Fig. 4 Tissue-specific functions of PARP-2

in human cervical carcinoma cells also underlines the involvement of PARP-2 in double-strand break repair [41].

Appropriate telomere and centromere maintenance requires PARP-2. PARP-2 binds to and negatively regulates the DNA binding of telomere-binding protein, TRF-2 in different rodent and human cell models. The loss of PARP-2 expression increased the frequency of spontaneous chromosome and chromatid breaks and the number of DNA ends lacking detectable telomere repeats [42].

PARP-2 localizes to centromeres in human and murine cells in a cell-cycle-dependent manner and interacts with the kinetochore proteins centromere protein A (CENPA), centromere protein B (CENPB), and mitotic spindle checkpoint protein BUB3 in prometaphase and metaphase [43]. Interestingly, this centromeric accumulation of PARP-2 is increased when microtubule dynamics are disrupted, suggesting a dominant role of PARP-2 in accurate chromosome segregation [44].

Incomplete or insufficient DNA repair may ultimately lead to either cell death or cellular transformation and tumorigenesis. PARP-1 has been associated with both cell death [45] and tumorigenesis [46]. PARP-2 seems to be involved in cell death regulation similarly to PARP-1 [44, 47]. However, Cohausz and colleagues have found differences in the expression of cell death genes upon *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) treatment in PARP-1 and -2 knockdown cells. Furthermore, PARP-2 is also engaged in tumorigenesis. In mice, the double deletion of PARP-2 and p53-induced spontaneous lymphomas and certain sarcomas [38] and decreased expression of PARP-2 correlated with increased susceptibility to alkylator-induced acute myeloid leukemia (AML) [48]. Results from these experimental models suggest that PARP-2 has a dominant role in suppressing leukemias [49].

Role of PARP-2 in chromatin remodeling and genome maintenance during spermiogenesis

PARP-2 is expressed in the testis of mice [18] and rats [50], and is highly expressed in human testis [19]. Moreover, PARP-2 is found in the ejaculated spermatozoa in both mice and in humans [44, 51]. However, PARP-2 seems to be responsible for a smaller portion of PARP activity than PARP-1 in rat testis [52]. These observations prompted the study of the possible testicular functions of PARP-2 in mice. Dantzer and coworkers have revealed that upon crossing of PARP-$2^{-/-}$ males and females litter size was lower than in colonies bred by crossing wild-type mice. A smaller testis size and high number of abnormal spermatids in the distal epidymis have also been reported [44].

Decreased spermatogenesis is likely to have multiple roots that all trace back to insufficient maintenance of genomic integrity during spermatocyte differentiation (Fig. 4). The differentiation of spermatozoa was found to be hampered and large numbers of apoptotic cells were detected in murine testis [44]. Cell death is probably linked to hampered meiotic sex chromosome inactivation, and the block of cell division in meiosis I, whereby chromosome missegregation was detected [44]. Jha and colleagues [53], based on studies on 18 healthy and 12 infertile humans, also proposed a role for PARP-2 in the preservation of genomic integrity by protecting DNA against oxidative stress. Spermiogenesis involves the compaction of DNA and the exchange of histones to different protamines [54]. In this process PARP-2 (and PARP-1) regulate the activity of topoisomerase IIβ that is essential for appropriate DNA organization (e.g., removal of histone 1) [55], transition protein 2 (TP2) and the transition chaperone HSPA2 [56] as shown in mice.

Different SNPs impacting PARP-2 functionality may also hamper human spermiogenesis. Sakugawa and colleagues [51] have shown on a cohort of 18 Japanese men that such *PARP-2* SNPs coincide with azoospermia in humans. Sakugawa and colleagues identified five SNPs. Three of them fall into the coding region, while two into the 3′ UTR. The SNPs in the coding region are all in the catalytic domain. One is synonymous (1159C/T), while two others (1359G/A and 1469A/C) lead to an amino acid change: Arg/Gln and Asn/His, respectively. It is tempting to speculate that changes in the catalytic domain may affect the catalytic activity of PARP-2. SNPs in the 3′ UTR (1789A/C and 1790T/C) may interfere with mRNA stability leading to the reduction of PARP-2 mRNA copy number and consequently decreasing PARP-2 protein levels. Lower PARP-2 levels, or lower PARP-2 activity may interfere with spermiogenesis as described above.

The role of PARP-2 in thymopoiesis and inflammatory regulation

The earliest reports on PARP-2 described high *PARP-2* expression in the subcapsular zone of the thymus where lymphocyte proliferation is the most intense. *PARP-2* expression gradually decreases towards the center of the thymus as lymphocytes differentiate and mature [18, 47]. *PARP-2* transcripts were detected in the white pulp of the spleen and Peyer patches in mice, which also points toward the involvement of PARP-2 in the proliferation of lymphocytes [18].

In line with these observations, the deletion of *PARP-2* in mice led to decreases in the weight of thymus and in the total cell numbers and number of $CD4^+$, $CD8^+$ thymocytes in thymus [47] (Fig. 4). The loss of the double-positive thymocytes was due to enhanced p53-mediated apoptosis [47]. Increased expression of a pro-apoptotic, bcl-2 homolog NOXA showed correlation with the enhanced apoptosis [47]. Apoptosis can be reversed by the removal of p53 [38], suggesting that cell death is induced by unresolved DNA damage. In line with this observation, when *PARP-*$2^{-/-}$ mice were bred on a *p53*$^{-/-}$ background, spontaneous lymphomas and to a smaller extent other sarcomas developed in the double-knockout mice [38], indicating a functional interplay between these two proteins in protecting genome integrity.

It remains to be seen whether atrophy of thymus and the higher rate of thymocyte apoptosis in *PARP-*$2^{-/-}$ mice results in a restricted T cell repertoire and altered T cell responses. In fact, PARP inhibition or *PARP-1* depletion has provided marked protection in most animal models of inflammation with many of them being dependent on T cell functions [45, 57]. The defect of PARP-2 seems to be associated with a narrower spectrum of diseases in murine models. The lack of PARP-2 impairs astrocyte activation [58] and provides protection against colitis [59], while it has no effect in models of contact hypersensitivity [60], irritative dermatitis [60], or pancreatitis [61]. Interestingly, a common set of genes (iNOS, Il-1β, TNFα) has been shown to be regulated by both PARP-1 and PARP-2, suggesting similar or overlapping mechanisms in inflammatory regulation by the two PARP isoforms. However, the exact mechanism of protection by genetic PARP-2 deletion is unknown [58, 59].

PARP-2 in the regulation of gene expression

Recent reports revealed that the depletion of PARP-2 modifies the activity of multiple transcription factors [62–64]. In HepG2 cells depleted of PARP-2 by shRNA, we have found the dysregulation of more than 600 genes in

microarray experiments (Szántó and Bai, unpublished data), indicating an important role for PARP-2 in the regulation of gene expression.

PARP-2 acts at multiple levels on gene transcription. PARP-2 might be capable of modifying chromatin through regulating transcriptional intermediary factor (TIF)-1β and heterochromatin protein (HP)-1α [65]: depletion of PARP-2 modified the expression of two genes (*Mest* and *HNF4*) that are dependent on the TIF1β-HP1α complex [65]. Poly(ADP-ribosyl)ation and PARP-1 have eminent roles in epigenetic control [66–69]. Based on the similarities of PARP-1 and PARP-2-catalyzed reactions, and partially overlapping interactome and acceptor protein profile, it is tempting to assume that similar epigenetic roles may also be assigned to PARP-2.

PARP-2 can influence gene expression through more direct interactions. PARP-2 interacts with topoisomerase I and topoisomerase IIβ [40, 55] and may thus regulate the rearrangement of DNA structure in conjunction with RNA transcription. Moreover, PARP-2 has been shown to interact with nucleophosmin/B23 [22] that is involved in rRNA transcription [70]. RNA polymerase I inhibition removes PARP-2 from the nucleolus; however, the deletion of PARP-2 does not change rRNA expression. Thus, the exact mechanism whereby PARP-2 regulates rRNA expression requires further investigation. On the course of mRNA expression, also known as RNA polymerase II-mediated transcription, PARP-2 can act as either a positive co-factor, or a repressor of gene expression. Transcription factors regulated by PARP-2 are summarized in Table 1.

Nuclear receptor signaling

PARP-2 has been shown to interact with several members of the nuclear receptor superfamily such as the peroxisome proliferator-activated receptors (PPARs) and estrogen receptor (ER)α.

The group of PPARs has three members, PPARα, PPARδ, and PPARγ [71] that heterodimerize with the retinoid X receptor (RXR) and thus binds to DNA [72, 73]. PPARs bind different lipophilic ligands [74] that regulate their transcriptional activity. PPARs control the expression of a large set of genes involved in the regulation of energy, lipid, and glucose homeostasis [75]. The binding of ligands to the receptors leads to receptor activation and the release of corepressor proteins and the subsequent binding of activators [76]. PARP-1 has been suggested to be involved in nuclear receptor function. Ju and colleagues [77] have shown that upon estrogen receptor activation, topoisomerase IIβ creates DNA strand breaks that are resolved through the action of PARP-1. Moreover, inhibition of topoisomerase IIβ or PARP-1 hampered efficient gene expression [77].

PARP-2 serves as a cofactor for the members of the PPAR transcription factor family. The absence of PARP-2 impairs PPARγ activation but enhances PPARα and PPARδ activation [63]. PARP-2 binds to PPARγ-driven promoters and its absence decreases the expression of genes such as *adipocytes protein 2* (*aP2*), *CD36*, *lipoprotein lipase* (*LPL*), and *fatty acid synthase* (*FAS*) [63]. Since PARP-2 is a DNA repair protein and can interact with topoisomerase IIβ [55], it is possible that PARP-2 may also play a role in resealing transcription-related DNA breaks. The effects of PARP-2 depletion on PPARα and PPARδ activation were demonstrated only in reporter assays [63], therefore further molecular and in vivo verification of these interactions is necessary.

Estrogen receptor (ER)α activation is repressed by the depletion of PARP-2 in luciferase reporter assays (P. Bai, unpublished data). The effect of PARP-2 on PPARγ and ERα may share similar molecular characteristics. Further investigation is required to reveal possible physiological consequences of the reduced ERα activity upon PARP-2 ablation. It is important to note that PARP-2 does not interfere with the activation of ERβ, therefore ERβ and its target genes (e.g., *keratin 19*) are ideal negative controls in studies addressing the role of PARP-2 in nuclear receptor-mediated gene expression [63].

Interaction with SIRT1

SIRT1 belongs to the family of sirtuins. Sirtuins have seven homologs in humans and mice (SIRT1-7) [78, 79]. SIRT1 is considered to be a nuclear enzyme [80], although it may also appear in the cytosol [81]. SIRT1 is an NAD^+-dependent protein deacetylase [82] that enables SIRT1 to sense the energetic status of cells (e.g., changes in NAD^+/NADH ratio) [83]. SIRT1 is activated by increases in NAD^+ levels, or indirectly by different small molecule activators such as resveratrol [84], SIRT1720 [85], AMPK activators [86], or PARP inhibitors [87]. SIRT1 activation leads to the deacetylation and activation of numerous metabolic transcription factors such as PPAR gamma coactivator (PGC)-1α [88], FOXOs [89], and p53 [90]. Their activation leads to increased mitochondrial biogenesis and oxidative metabolism through enhancing the expression of key mitochondrial enzymes involved in terminal oxidation, fatty acid degradation, and mitochondrial uncoupling in several target tissues [88, 91].

It has been shown that PARP-2 can directly regulate the expression of SIRT1 [62]. PARP-2 serves as a negative regulator of SIRT1 expression, as the absence of PARP-2 induces SIRT1 expression and results in higher SIRT1 activity [33, 62]. PARP-2 binds to the murine SIRT1 promoter in a region between -1 and -91, which is a highly conserved region among mammals, showing

Table 1 Transcription factors directly regulated by PARP-2

Name	Mode of action	Effects	Model system	Known tissue specificity	References
ERα	Unknown	Depletion of PARP-2 suppress ERα activation	Luciferase reporter system in PARP-2-specific shRNA-treated HEK293T cells	Unknown	–
RXR/PPARα	Unknown	Depletion of PARP-2 enhance PPARα activation	Luciferase reporter system in PARP-2-specific shRNA-treated HEK293T cells	Unknown	[59]
RXR/PPARδ	Unknown	Depletion of PARP-2 enhance PPARδ activation	Luciferase reporter HEK293T in PARP-2-specific shRNA-treated HEK293T cells	Unknown	[59]
RXR/PPARγ	Cofactor of receptor	Modulates transcription of PPARγ target genes, Depletion of PARP-2 leads to WAT hypofunction	Luciferase reporter system in PARP-2-specific shRNA-treated HEK293T cells; PARP-2 knockout mice; embryonic fibroblasts from PARP-2 knockout mice	White adipose tissue	[59]
SIRT1	Transcriptional repressor of the SIRT1 promoter.	PARP-2 depletion induces SIRT1 and consequently enhance mitochondrial biogenesis in skeletal muscle and liver	PARP-2 knockout mice; Luciferase reporter system in PARP-2-specific shRNA-treated HEK293T cells; PARP-2 knockdown C2C12 cells	Skeletal muscle, liver	[58, 83]
TTF1	Transcriptional cofactor	Regulates the expression of surfactant protein B	Luciferase reporter system in PARP-2-specific shRNA-treated HeLa/MLE15 cells; interaction mapping in mice and in cells	Lungs	[60]

homology even in *Xenopus* [62]. It must be noted that the ablation, or pharmacological inhibition of PARP-1 also induces SIRT1 activity. However, SIRT1 activation in the absence of PARP-1 depends on enhanced NAD^+ availability and the ablation of PARP-1 does not alter the activity of the SIRT1 promoter [87].

SIRT1 induction upon PARP-2 ablation causes the deacetylation of PGC-1α and FOXO1, which in turn boost mitochondrial biogenesis by enhancing the expression of *PGC-1α*, *uncoupling protein* (*UCP*)*-2*, muscle isoform of *carnitine O-palmitoyltransferase 1* (*mCPT1*), *acyl coenzyme A oxidase I* (*ACOX1*), *medium-chain specific acyl-CoA dehydrogenase* (*MCAD*), *malonyl-CoA decarboxylase* (*MCD*), *Ndufa2*, *cytochrome c* (*cyt c*) and *COX IV* [62]. The action of PARP-2 has been shown in multiple organs and tissues such as skeletal muscle, liver, smooth muscle [33, 62], and an unexpected disadvantageous effect has been shown in the pancreas [62]. The depletion of PARP-2 was found not to interfere with SIRT2 or SIRT3 activation [62].

Similar to PARP-2 gene inactivation, SIRT1 activation has been shown to inhibit the production of inflammatory mediators and suppress certain forms of inflammation [92–94]. It is therefore plausible that the induction of SIRT1 may be responsible for the antiinflammatory effect of PARP-2 depletion in colitis [59] and in astrocyte activation [58].

Thyroid transcription factor-1

Nkx-2 transcription factors constitute a family of homeodomain-containing transcription factors. Thyroid transcription factor (TTF)-1 belongs to the Nkx-2 family and TTF-1 plays a dominant role in lung morphogenesis, respiratory epithelial cell morphogenesis, and differentiation [95, 96]. In cultured lung epithelial cells, PARP-2 interacts with TTF1 [64]. By affecting TTF1 activity PARP-2 may regulate the expression of *surfactant protein-B* (Fig. 4).

The role of PARP-2 in metabolic regulation

Alterations in gene expression accompany various biological phenomena ranging from inflammatory responses (NOXA, TNFα, IL-1β, etc.) to metabolic regulation. The transcriptional regulatory role of PARP-2 has been linked to cellular metabolism. *PARP-2*$^{-/-}$ mice are smaller and leaner as they have less body fat than their wild-type littermates [62, 63]. At the same time, *PARP-2*$^{-/-}$ mice showed higher oxygen consumption rates and lower respiratory quotients during the active (dark) phase, which points toward higher fatty acid oxidation [62].

When examining the skeletal muscle of *PARP-2*$^{-/-}$ mice, increased mitochondrial content was observed in line with higher expression of genes related to oxidative metabolism and fatty acid oxidation, which is in line with the above-described phenotype [62]. The increase in oxidative metabolism can be explained by higher SIRT1 expression due the loss of the transcriptional repressor activity of PARP-2. It is the increase in SIRT1 expression that induces mitochondrial biogenesis through PGC-1α and FOXO1 deacetylation [62] (Fig. 4).

The liver of the *PARP-2*$^{-/-}$ mice displayed characteristics similar to the ones in skeletal muscle: SIRT1 induction and consequently enhanced mitochondrial biogenesis and oxidative metabolism [62] (Fig. 4). Interestingly, the brown adipose tissue was not involved in the development of the energy expenditure phenotype in contrast to *PARP-1*$^{-/-}$ mice [62, 87].

The increased energy expenditure fueled by enhanced mitochondrial biogenesis in skeletal muscle and liver had beneficial effects on the metabolism of *PARP-2*$^{-/-}$ mice. *PARP-2*$^{-/-}$ mice are protected against diet-induced obesity, and insulin resistance of the animals was retained even after high-fat feeding [62]. Interestingly, *PARP-2*$^{-/-}$ mice proved to be glucose intolerant after high-fat feeding [62]. The pancreas in *PARP-2*$^{-/-}$ mice failed to appropriately respond to diet-induced insulin resistance as it showed no signs of hyperproliferation or reduction in pancreas weight, islet size, and pancreatic insulin content [62]. Reduced expression of pancreatic and duodenal homeobox 1 (pdx-1) is likely to be responsible for the pancreatic hypofunction in *PARP-2*$^{-/-}$ mice [62] (Fig. 4).

Functions of the white adipose tissue (WAT) are orchestrated by the RXR/PPARγ receptor [72]. We have shown that PARP-2 acts a cofactor of the RXR/PPARγ dimer [63, 97]. The loss of PARP-2 hampers RXR/PPARγ receptor activation and decreases the expression of certain PPARγ-driven genes (e.g., *LPL*, *CD36*, etc.). Due to that alterations in gene expression, the WAT of *PARP-2*$^{-/-}$ mice turned hypomorphic and hypofunctional [63] (Fig. 4). Moreover, in cellular models of adipocyte differentiation, the lack of PARP-2 resulted in decreased adipocytic differentiation [63]. It is of note that SIRT1 induction may inhibit PPARγ [98] that may provide an auxiliary mechanism underlying WAT hypofunction in the absence of PARP-2.

PARP-2 in oxidative stress-related diseases

PARP-1 depletion, or pharmacological PARP inhibition is protective against numerous oxidative stress-related diseases [45]. Depletion of PARP-2 also resulted in a protective phenotype against diseases associated with increased oxidative stress. Genetic deletion or silencing of PARP-2 has provided protection in models of focal and global cerebral ischemia [99, 100], colitis [59], and doxorubicin-induced vascular smooth muscle damage [33]. Since PARP-2 accounts for a small fraction of total cellular PARP activity [8, 32, 33], it is unlikely that the ablation of PARP-2 could protect against the loss of cellular NAD^+ and ATP, suggesting different mechanisms of cell death as compared to the case of PARP-1 ablation.

Cerebral ischemia and doxorubicin-induced vascular impairment involve mitochondrial damage [101, 102] and preventing mitochondrial damage proved to be a successful novel treatment in these pathologies [103–108]. Since SIRT1 has been demonstrated to enhance or restore mitochondrial activity in various tissues [62, 87, 91, 109–111], it is logical to assume that the interference between PARP-2 and SIRT1 expression [62] could be key for the protective phenotype. Indeed, in the case of doxorubicin-induced vascular damage, enhanced SIRT1 expression and consequent stabilization of the mitochondrial membrane potential was proposed to be responsible for the protection provided by the *PARP-2*$^{-/-}$ phenotype [33] which might be a prototypical mechanism by which PARP-2 mediates oxidative stress-related pathologies (Fig. 4). Moreover, ablation of PARP-2 led to the mitochondrial retention of apoptosis inducing factor (AIF) [100]. AIF is a mitochondrial protein that shuttles to the nucleus upon oxidative stress-evoked cell death in a PARP-1-dependent manner [112]. In a model of focal cerebral ischemia, ablation of PARP-2 only slightly reduced PAR formation but markedly inhibited the nuclear translocation of AIF [100]. This interesting finding may also be linked to the stabilization of mitochondrial membrane upon SIRT1 induction.

The involvement of PARP-2 in oxidative stress-related pathologies points towards the applicability and hence the development of PARP-2-specific inhibitors. However, all known PARP inhibitors are capable of inhibiting both PARP-1 and -2, which is not surprising since the catalytic domain of the enzymes are very similar and most PARP inhibitors bind there [30, 31]. In the quest for synthesizing PARP-2-specific inhibitors, the laboratory of Gilbert de Murcia suggested the targeting of a loop that is unique in

PARP-2 [25, 30]. Efforts to develop highly PARP-2-selective compounds have given rise to inhibitors that have 10–60 fold higher affinity for PARP-2 as compared to PARP-1 [113–117]. One of these inhibitors, UPF-1069, which has 60-fold higher affinity towards PARP-2 than PARP-1, was shown to provide protection against cerebral ischemia [117]. Although at the moment such selectivity is the highest achievable, it is possible that in cellular models or in in vivo settings these inhibitors may partially inhibit PARP-1, too. Nevertheless, the development of highly PARP-2-specific inhibitors is of current interest. Since PARP-2 is a minor PARP isoform, its inhibition is an attractive way to counteract certain drawbacks of pan-PARP inhibition or PARP-1-specific inhibitors. Since PARP-2 accounts for only 5–15 % of PARP activity [8, 18, 32, 33], it is therefore tempting to speculate that its loss probably would not drastically hamper PARylation-dependent DNA repair. Thus, highly PARP-2-specific inhibitors may provide a preferable alternative for the treatment of metabolic diseases, whereas pan-PARP inhibitors may be superior in severe oxidative injury. However, DNA damage assessment in such cases is an absolute necessity.

Conclusions and perspectives

PARP-2 has been shown to participate in multiple cellular processes such as DNA repair, maintenance of genomic integrity, spermiogenesis, and thymopoiesis. On the other hand, PARP-2 is involved in transcriptional regulation of metabolism and oxidative stress response. In fact, this plethora of functions partly overlaps with the functions of PARP-1 [24]. A better understanding of the similarities and differences between the actions of PARP-1 and -2 is of outmost importance. On the one hand, PARP-1 and -2 can act synergistically, while on the other hand isoform-specific functions of the PARP enzymes also exist. Specific targeting of PARP-2 may help overcome unwanted side-effects of pan-PARP inhibition.

Understanding the role of PARP-2 in DNA repair may hold importance in tumor biology. The better understanding of the role of PARP-2 in DNA repair may provide new knowledge on tumorigenesis, and can be capitalized in inducing synthetic lethality by joint inhibition of parallel DNA repair pathways [49].

The metabolic effects of PARP-2 can be exploited in multiple manners. Obviously, the depletion of PARP-2 can be utilized in combating metabolic diseases and mitochondrial stabilization may overcome oxidative stress-evoked damage. Better understanding the properties of PARP-2 may in turn facilitate the development of PARP-2-specific inhibitors that may have advantages over pan-PARP inhibitors.

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Conflict of interest The authors declare no conflicts of interest.

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