Helsinki University Biomedical Dissertations No. 182

SUMO-1 conjugation in normal and stress conditions *in vivo*

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in Lecture hall 2, on May 3rd, 2013, at 12 o'clock noon.

Helsinki 2013

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ISBN 978-952-10-8671-7 (paperback)
ISBN 978-952-10-8672-4 (PDF)
ISSN 1457-8433
http://ethesis.helsinki.fi
Unigrafia Oy
Front cover: Pekka Mikkonen

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ABSTRACT

SUMOylation is an evolutionary conserved post-translational regulatory mechanism involving the covalent attachment of a Small Ubiquitin-like MOdifier (SUMO) to target proteins in reactions catalyzed by E1, E2 and E3 enzymes. The consequences of protein SUMOylation are varied; it can affect protein localization, create new interaction surfaces, exclude others, regulate transcription factor activity, and protect proteins from destruction. Loss of SUMOylation is not compatible with life, as knock-out of the only E2 enzyme (Ubc9) leads to embryonic lethality in mice. SUMOylation is possible but less specific and efficient in the absence of E3 enzymes, of which the protein inhibitors of activated STAT (PIAS) family is the most widely studied. In mammals, there are three conjugatable SUMO paralogs of which SUMO-1 differs from SUMO2/3 the most and lacks the ability to form (poly)SUMO chains. SUMO2/3 are encoded by two separate genes, but differ only by three amino acids leading to many overlapping functions.

The purpose of this thesis was to study the *in vivo* functions of SUMOylation by using knock-out animal models. PIAS2 is an E3 enzyme highly expressed in testis and involved in androgen signaling. A *Pias2* null mouse line was generated and found to be fertile and viable. Loss of *Pias2* led to a decrease in testicular weight and sperm count and increase in apoptotic cells in the seminiferous tubules. However, the sperm was qualitatively normal, potentially due to compensation by other PIAS proteins. When *Sumo1* was knocked out by targeted disruption, the mice were born in normal Mendelian ratios, and were viable and fertile. At least in the case of Ran GTPase-activating protein 1 (RanGAP1), the most heavily SUMO-1 modified protein, lack of SUMO-1 resulted in its SUMOylation by SUMO-2/3. This compensatory mechanism, however, was not reflected in the mRNA levels. In testis, SUMO-1 was localized to a nuclear structure called sex vesicle present in pachytene spermatocytes. SUMO-2/3 was also present there, and the structure was able to form in the absence of SUMO-1.

The phenotypes of the *Sumo1* null and wild-type mice did not differ under normal laboratory conditions. However, when the mice were challenged with lipopolysaccharide (LPS), a bacterial antigen, *Sumo1* null mice developed a more pronounced acute phase response in liver due to inefficient transrepressive function of

liver receptor homolog 1 (LRH-1) leading to more efficient removal of repressive factors from inflammatory gene promoters. Another nuclear receptor, liver X receptor (LXR), required SUMO-2/3 modification to function properly testifying that SUMO paralogs can partly but not in all cases compensate for each other. Another example found in this study where *Sumo1* function cannot be compensated for was adipogenesis. Adipogenesis is highly dependent on the functions of peroxisome proliferator-activated receptor (PPAR) γ. It was found that, contrary to previous reports, gene activation functions of PPARγ are compromised in the absence of SUMO-1, as shown by inefficient transactivation of a luciferase reporter in *Sumo1* null cells and incomplete response of *Sumo1* knock-out mice to rosiglitazone, a PPARγ agonist. Furthermore, embryonic fibroblasts and mesenchymal stem cells of *Sumo1* null mice and SUMO-1-depleted preadipocytes differentiated less efficiently into adipocytes, further demonstrating the importance of SUMO-1 in the activation functions of PPARγ. When *Sumo1* null mice were challenged with a high-fat diet, they gained less weight than their wild-type littermates due to a limited expansion of white adipose tissue. The results show that SUMO-1 is important for the activation functions of PPARγ either *via* modification of PPARγ or a coregulatory protein.

Taken together, the results of this thesis show that the SUMOylation pathway has many redundant functions. However, this study identified physiological phenomena where the lack of *Pias2* or *Sumo1* could not be compensated for *in vivo*.

YHTEENVETO

SUMOlaatio on evoluutiossa säilynyt translaationjälkeinen säätelymekanismi, jossa E1-, E2- ja E3-entsyymit liittävät kovalentilla sidoksella kohdeproteiineihin pienen Small Ubiquitin-like MOdifier (SUMO) -proteiinin. SUMOlaation vaikutukset ovat moninaiset, sillä se pystyy vaikuttamaan proteiinien sijaintiin, luomaan uusia kosketuspintoja tai peittämään niitä, säätelemään transkriptiotekijöiden aktiivisuutta ja suojaamaan proteiineja hajotukselta. SUMOlaatio on elämälle välttämätön, minkä osoittaa ainoan E2-entsyymin suhteen poistogeenisen hiiren alkionkehityksen aikainen letaali ilmiasu. SUMOlaatio on mahdollinen mutta epäspesifisempi ja tehottomampi ilman E3-entsyymejä, joista tunnetuimpia ovat protein inhibitors of activated STAT (PIAS) -perheen proteiinit. Nisäkkäillä on kolme SUMO-paralogia, joista SUMO-1 eroaa eniten SUMO2/3:sta, eikä pysty muodostamaan SUMO-ketjuja. SUMO2/3, joita koodittaa kaksi erillistä geeniä, eroavat toisistaan vain kolmen aminohapon osalta, mikä johtaa moniin päällekkäisiin toimintoihin.

Tämän väitöskirjatutkimuksen tavoite oli tutkia poistogeenisten eläinmallien avulla SUMOlaation *in vivo* -vaikutuksia. PIAS2 on miessukupuolihormonien signalointiin vaikuttava E3-entsyymi, joka ilmenee runsaana kiveksessä. Tutkimusta varten luotiin *Pias2*-poistogeeninen hiirimalli, joka osoittautui elin- ja lisääntymiskykyiseksi, vaikka *Pias2*-geenin puuttuminen johti pienentyneeseen kivesten painoon ja siittiöntuotantoon sekä ohjelmoidun solukuoleman lisääntymiseen siementiehyissä. Hiirten siemenneste oli kuitenkin laadullisesti normaalia johtuen todennäköisesti muiden PIAS-proteiinien kompensaatiosta. *Sumo1*-geenin kohdennettu poistaminen johti niinikään elinkelpoisiin ja lisääntymiskykyisiin hiiriin, jotka syntyivät normaaleissa mendeelisissä suhteissa. Tutkimuksissa havaittiin, että Ran GTPase-activating protein (RanGAP) 1, joka on solujen voimakkaimmin SUMO-1:llä muokattu proteiini, muokkautuu SUMO2/3:lla SUMO-1-proteiinin puuttuessa. Tämä kompensaatiomekanismi ei kuitenkaan heijastunut RNA-tasolle. Kiveksessä SUMO-1-proteiinia on erityisesti sukupuolivesikkelissä, joka on pakyteenispermatosyyteissä ilmenevä tuman rakenne. SUMO2/3 sijaitsee samassa rakenteessa, eikä SUMO-1:n puuttuminen estä sen muodostumista.

Normaaleissa laboratorio-olosuhteissa *Sumo1*-poistogeenisten hiirten ja villityypin hiirten välillä ei havaittu eroja. Sen sijaan hiirten altistaminen bakteerien pinta-antigeeni lipopolysakkaridille (LPS) johti voimakkaampaan akuutin faasin tulehdusreaktioon maksassa *Sumo1*-poistogeenisillä hiirillä johtuen Liver Receptor Homolog (LRH) 1 -tumareseptorin heikommasta transrepressiokyvystä, jolloin geenien luentaa hillitsevät tekijät poistuvat tulehdusgeenien promoottoreilta nopeammin kuin normaalisti. Toinen tumareseptori, liver X receptor (LXR), puolestaan vaati SUMO-2/3-modifikaation normaaliin toimintaansa. Nämä tulokset osoittivat, etteivät SUMO-paralogit voi kompensoida kaikkia toistensa tehtäviä. Toinen tutkimuksen paljastama esimerkki, jossa *Sumo1*:n puuttumista ei voida korvata, on rasvasolujen erilaistuminen, joka on voimakkaasti riippuvainen peroxisome proliferator-activated receptor (PPAR) γ -tumareseptorista. PPARγ:n havaittiin aktivoivan geenejä heikommin *Sumo1*-geenin puuttuessa – aiemman tiedon vastaisesti. Tämän osoittivat solukokeissa lusiferaasiraportoijageenin heikompi luenta *Sumo1*-poistogeenisissä soluissa ja *Sumo1*-poistogeenisten hiirten heikompi vaste PPARγ:n agonistille, rosiglitatsonille. Lisäksi *Sumo1*-poistogeeniset hiiren embryonaaliset fibroblastit ja mesenkymaaliset kantasolut sekä *Sumo1*-geeniä heikosti ilmentävät preadiposyytit erilaistuivat huonommin rasvasoluiksi viitaten SUMO-1:n olevan tärkeä PPARγ:n aktivaatiolle. Kun *Sumo1*-poistogeeniset hiiret laitettiin rasvaiselle ruokavaliolle, ne lihoivat vähemmän kuin villityypin hiiret johtuen niiden valkoisen rasvakudoksen heikosta laajenemisesta, mikä näkyy rasvasolujen vähäisyytenä ja pienuutena. Nämä tulokset viittavat siihen, että joko PPARγ tai sen kanssa vuorovaikuttava säätelijäproteiini tarvitsee SUMO-1-proteiinia toimiakseen normaalisti.

Tämän tutkimuksen tulokset osoittavat, että vaikka SUMOlaatioketjun proteiineilla on monia päällekkäisiä tehtäviä, tässä tutkimuksessa saatiin selville fysiologisia ilmiöitä, joissa *Pias2*- tai *Sumo1*-geenien puuttumista ei voitu kompensoida.

ORIGINAL PUBLICATIONS

I. Santti H, Mikkonen L, Anand A, Hirvonen-Santti S, Toppari J, Panhuysen M, Vauti F, Perera M, Corte G, Wurst W, Jänne OA, Palvimo JJ 2005. Disruption of the murine PIASx gene results in reduced testis weight. J Mol Endocrinol 34:645-654

II. Zhang F-P*****, Mikkonen L*****, Toppari J, Palvimo JJ, Thesleff I, Jänne OA 2008 Sumo1 function is dispensable in normal mouse development. Mol Cell Biol 28:5381-5390

III. Venteclef N, Jakobsson T, Ehrlund A, Damdimopoulos A, Mikkonen L, Ellis E, Nilsson LM, Parini P, Jänne OA, Gustafsson JÅ, Steffensen K, Treuter E 2009 GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRβ in the hepatic acute phase response. Genes Dev 24:381-395

IV. Mikkonen L, Hirvonen J, Jänne OA 2012 SUMO-1 regulates body weight and adipogenesis via PPARγ in male and female mice. Endocrinology 154:698-708

* Equal contribution

The above articles are referred to in the text as publications I–IV. In addition, some unpublished results are presented.

The manuscript of the publication I was included in the thesis by Dr. Henrikki Santti. Publication III was included in the theses of Dr. Thomas Jakobsson and Dr. Anna Ehrlund in Sweden.

ABBREVIATIONS

AF	activation function
AP	activator protein
APR	acute phase response
AR	androgen receptor
BAT	brown adipose tissue
BLM	Bloom syndrome protein
BRCA1	breast cancer 1, early onset
CBP	CREB-binding protein
C/EBP	CCAAT-enhancer-binding protein
ChIP	chromatin immunoprecipitation
CLP	cleft lip and palate
CRP	C-reactive protein
Cys	cysteine
DAX-1	dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome, gene 1
DAXX	death-domain associated protein
DBD	DNA-binding domain
DDR	DNA damage response
DeSI	deSUMOylating isopeptidase
Dex	dexamethasone
dpc	day(s) *post* conception
DSB	double strand break
ER	estrogen receptor
ESC	embryonic stem cells
FA	fatty acid
FEN	flap structure-specific endonuclease
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HD	Huntington's disease
HDAC	histone deacetylase
HIF	hypoxia-inducible factor
HIPK	homeodomain-interacting protein kinase
HR	homologous recombination
HRE	hormone response element
Htt	Huntingtin
IFN	interferon
Ik-Bα	NF-κB inhibitor alpha
IKK	inhibitor of kappa-B kinase
IL	interleukin
IRF	interferon regulatory factor
KO	knock-out
LBD	ligand binding domain
LXR	liver X receptor
LPS	lipopolysaccharide
LRH	liver receptor homolog
LXRE	LXR response elements
Lys	lysine
MEF	mouse embryonic fibroblast
MSC	mesenchymal stem cell
MYF	myogenic factor
NB	nuclear body
NCoR	nuclear receptor corepressor
NDSM	negatively charged amino acid-dependent SUMO motif
NEMO	NF-κB essential modulator
NFAT	nuclear factor of activated T-cells

NF-κB	nuclear factor κ-B
NR	nuclear receptor
NTD	amino terminal domain
P53BP	p53-binding protein
PCNA	proliferating cell nuclear antigen
PDSM	phosphorylation-dependent SUMO motif
PGC	peroxisome proliferator-activated receptor-γ coactivator
PIAS	protein inhibitors of activated STAT
PML	promyelocytic leukemia protein
Pol II	RNA Polymerase II
PPAR	peroxisome proliferator-activated receptor
PRDM	PR domain containing
PRR	post-replication repair
qRT-PCR	quantitative real-time PCR
RanBP	Ran-binding protein
RanGAP	Ran GTPase-activating protein
RCT	reverse cholesterol transport
RIP140	receptor interacting protein 140
RNF	ring finger protein
ROR	RAR-related orphan receptor
ROS	reactive oxidative species
RXR	retinoid X receptor
SAA	serum amyloid A
SEM	standard error of mean
SENP	sentrin/SUMO-specific protease
Ser	serine
SHP	small heterodimer partner
SIM	SUMO-interacting motif
SMRT	silencing mediator for retinoid and thyroid receptors
SRY	sex-determining region of the Y chromosome
STAT	signal transducers and activators of transcription
STUbL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin-like modifier
TF	transcription factor
TBP	TATA-box binding protein
TDG	thymine-DNA glycosylase
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TOPORS	topoisomerase I-binding, arginine/serine rich
TR	thyroid hormone receptor
TZD	thiazolidinedione
UBL	ubiquitin-like protein
UCP	uncoupling protein
WAT	white adipose tissue
WT	wild-type

INTRODUCTION

Hormones are molecules that are secreted into the blood stream and that act in target cells *via* specific receptors. When the hormones are lipophilic, such as steroid hormones or intermediates of fat metabolism, they diffuse through the cell membrane to reach their cognate receptors, which belong to the superfamily of nuclear receptors (Mangelsdorf et al. 1995). The hormone-occupied nuclear receptors then affect the expression of target genes by either inhibiting or activating their transcription and act in concert with coregulator complexes the assembly of which is influenced by several factors among other post-translational modifications (PTMs) (Anbalagan et al. 2012).

PTMs come in many flavors consisting of a covalent addition of a molecule, such as phosphorylation, or a small protein, which is the case in SUMOylation. SUMOylation refers to modification of substrate proteins with a Small Ubiquitin-like MOdifier, changing the conformation of the target protein allosterically or creating new interaction surfaces (Yeh 2009). Most nuclear receptors are SUMOylated leading in many instances to decreased gene activation function (Treuter and Venteclef 2011; Anbalagan et al. 2012).

Nuclear receptors (NRs) control many essential functions of the body, including development, reproduction, inflammation and metabolism. They have been targeted for the treatment of many diseases and not always without side effects (Gronemeyer et al. 2004). As every tissue has its own combination of nuclear receptors, coregulators and other modifying proteins, the development of more specific drugs requires thorough dissection of the underlying mechanisms by which the actions of these receptors are exerted. The functions of SUMOylation in different physiological contexts involving NR action form the basis of this thesis.

REVIEW OF THE LITERATURE

1. Nuclear receptors as regulators of transcription

DNA contains instructions to all proteins present in any cell of the organism. However, cells and tissues are different in the selection of genes turned on and off leading to a different mixture of proteins in each cell type varying with time and responses to intracellular and extracellular signals. An important checkpoint is the regulation of transcription, where the amount of regulatory RNA or mRNA coding for a specific protein is kept under control. mRNA stability, alternative splicing, translation, and protein activity and stability are further tightly regulated. Therefore, the dogma ”gene-mRNA-protein” and the definition of a gene have been redefined by recognizing RNA as an important end product (Djebali et al. 2012).

1.1 Transcription factors and their coregulators

Approximately three-quarters of DNA is transcribed into different kinds of RNA products in eukaryotic cells, but the transcriptome of different cell types comprises 39% of the genome on the average (Djebali et al. 2012). How some areas of the genome are transcribed while other remain silent involves regulation of chromatin accessibility and the mixture of transcription factors and their coregulators present in each cell. Minimal requirements for a protein-coding gene to be transcribed are RNA polymerase II (Pol II) on the minimal promoter along with the general transcription machinery. Previously, it was considered a hallmark of transcriptional activation that, when a gene is turned on, transcription factor (TF) IID, *via* its subunit TATA-box binding protein (TBP), binds a conserved DNA sequence composed of T and A. Recently, however, a 50-base-pair footprint was identified to define the site of transcript origination within thousands of human promoters with TBP occupancy in the center of the region but, surprisingly, lacking the classical TATA-box (Neph et al. 2012). Other general transcription factors along with Pol II assemble onto the promoter to form a transcription initiation complex. *In vivo*, where DNA is wrapped around histones to form nucleosomes, DNA needs to be unwound with the help of additional proteins, and the whole process of transcription initiation includes more than 100 proteins. The Mediator complex bridges the interaction between Pol II,

general TFs, other TFs and coactivators/corepressors together with chromatin-modifying enzymes, chromatin-remodeling complexes and histone-modifying genes (Fig. 1). The definition of a gene not only includes the transcribed DNA sequence but all the regulatory sequences often situated far away from the transcription start site (TSS). These regulatory DNAs have been mapped using the DNAse I hypersensitive sites as markers of *cis*-regulatory elements, as occupancy of TFs creates a gap in place of a canonical nucleosome that is available for enzyme cleavage (Thurman et al. 2012).

TFs are proteins that directly bind to DNA, and they usually have a specific DNA-binding motif such as helix-turn-helix, zinc finger or leucine zipper. TFs may bind to DNA alone or more often as a combination forming a delicately regulated hierarchical network (Gerstein et al. 2012). Recently, ChIP (chromatin immunoprecipitation) -on-chip and ChIP-sequencing techniques have revolutionized the TF field by allowing the identification of all DNA-binding sites ("cistromes") of a given protein or TF in a given cell or tissue type. TFs interact with coregulators, which have multiple functions. They may act as bridging factors that make the TF, the Mediator, the general transcription factors and ultimately the Pol II a stable complex, allowing the release of Pol II from the pre-initiation complex and turning on the elongation phase (Nechaev et al. 2011). In addition, TFs attract other proteins with chromatin and histone modifying properties, thus affecting the availability of DNA to yet other proteins. PTMs – discussed in detail below – are crucial in defining the combinations of different proteins and even switching a coactivator into a corepressor in a specific context.

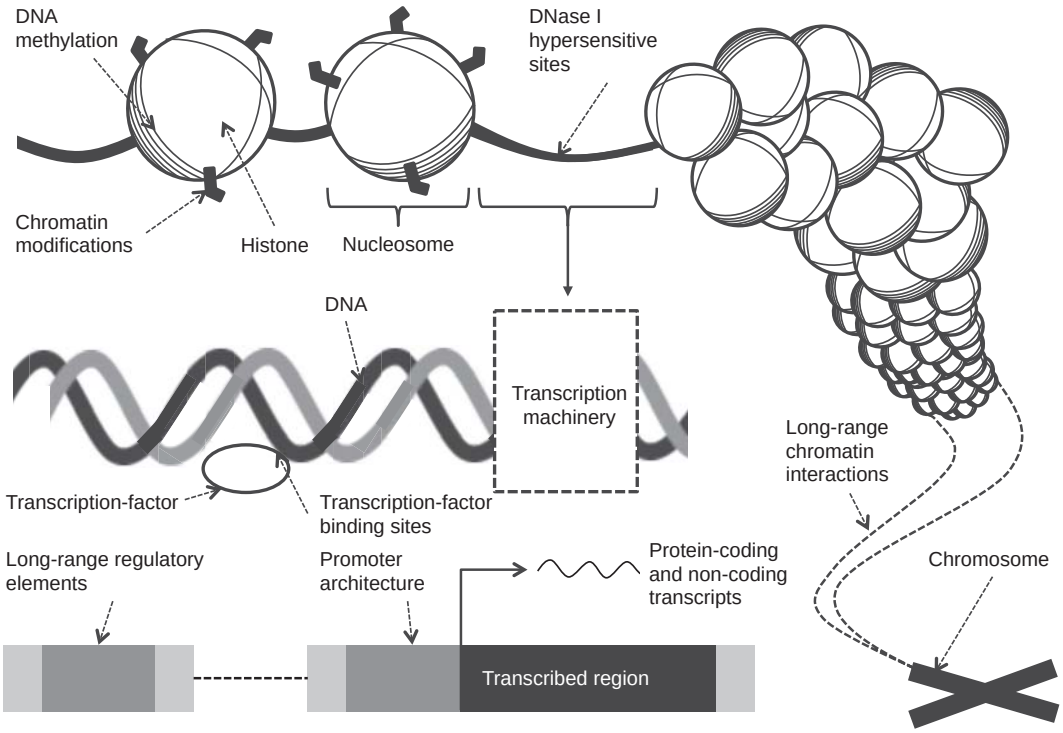

Figure 1. Overview of transcriptional regulation. Adapted from Ecker et al. 2012.

1.2 The nuclear receptor superfamily

A very special class of TFs are the nuclear receptors (NRs). Nuclear receptors are a large family of homologous proteins comprising receptors for hormones (steroids and thyroid hormones), retinoic acid and fatty acids, as well as orphan receptors for which ligands have not been identified (Gronemeyer et al. 2004; Mangelsdorf et al. 1995). There are 48 NR genes in humans encoding proteins that regulate diverse vital processes of life ranging from development and reproduction to metabolism and body homeostasis (Bertrand et al. 2004), and they have been classified based on their molecular properties (www.nursa.org). Class I NRs are receptors for steroid hormones such as androgen receptor (AR). Class II receptors have non-steroidal lipophilic ligands such as thyroid hormone receptor (TR) and peroxisome proliferator-activated receptors (PPARs). Class III receptors either do not have an identified ligand or have a closed ligand-binding pocket forming the group of orphan receptors. NRs have a

characteristic structure comprising an amino-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region and a carboxy-terminal ligand-binding domain (LBD).

The NTD (A/B domain) varies considerably between different NRs and contains a transactivation domain 1 (AF-1) interacting with coregulators (Wärnmark et al. 2003). AF-1 may work ligand-independently as a regulator of transcription or it may be affected by ligand binding of the LBD *via* intramolecular interaction, as is the case with the androgen receptor (AR) (Langley et al. 1995). The DBD recognizes the cognate hormone response elements (HREs) on DNA with its two conserved zinc finger domains each characterized by four highly conserved cysteine (Cys) residues coordinating a zinc ion (Freedman et al. 1988). The DBD contains two α-helices the first of which binds directly to the major groove of DNA, whereas the C-terminal extension of the DBD – highly variable between NRs – interacts with sequences flanking the core HRE and adding specificity to DNA binding (Melvin et al. 2004). DAX1 (dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome, gene 1; NR0B1) and SHP1 (small heterodimer partner; NR0B2) are exceptions to the general NR structure, in that they lack DBD and function through protein-protein interactions (Seol et al. 1996; Zanaria et al. 1994). The hinge region between DBD and LBD brings spatial flexibility to the receptors, participates in coregulator binding, forms part of the bipartite nuclear localization signal and is subject to PTMs (Pourcet et al. 2010). The C-terminal LBD varies to some extent among the NRs to allow ligand-specific responses and also contains the ligand-dependent activation function (AF-2) domain (Gronemeyer et al. 2004). The domain is formed by 12 helices, the helix 12 being crucial in moving over the ligand-binding pocket when a ligand is present allowing some coregulators to dispatch and others to bind. Some NRs, such as NURR1, have such a small ligand-binding pocket that they hardly can bind any ligand and may thus remain true orphans (Wang et al. 2003). In addition to binding coregulators in a ligand-dependent manner, this domain also contains part of the nuclear localization signal and is essential in NR dimerization.

The ligands of NRs are lipophilic. In the absence of ligand, NRs reside in the cytoplasm or nucleus. The ligands diffuse passively from the blood stream through the cell membrane reaching the receptor inside the cell. Alternatively, the ligand may be produced within the target cell. Some reports show that this may not be the only

mode of function for certain NRs as cell membrane bound NRs have been detected (Madak-Erdogan et al. 2008). However, it is well established that the nuclear functions related to gene regulation are the principal mode of action of this protein family.

Figure 2. Schematic structure of NRs. NRs share a common structure containing an N-terminal region (A/B), a DNA-binding domain (C), a hinge region (D), a ligand-binding domain (E) and a C-terminal domain (F).

1.3 Transcriptional activation and repression by NRs

There is a growing list of over 350 NR coregulators involved in NR-mediated gene activation and repression (O'Malley et al. 2008; Lonard et al. 2012). In the absence of an agonist, many class II NRs (e.g., PPARγ) reside in the nucleus along with corepressors, such as NCoR or SMRT, actively repressing target genes, NCoR/SMRT being part of multiprotein complexes comprising enzymes such as histone deacetylases silencing the chromatin nearby. Upon ligand binding, PPARγ heterodimerizes with the promiscuous retinoid X receptor (RXR), corepressors are changed into coactivators, and this leads to activation of target genes. Class I NRs (e.g., AR) are in the cytoplasm when not bound to a ligand. Ligand binding liberates the receptor from heat shock proteins, allowing it to homodimerize and through the nuclear localization signal move to the nucleus, where it binds to the cognate HREs along with coactivators or corepressors depending on the context, turning target genes on or off, respectively. A common mechanism for ligand-induced conformational change is the movement of helix 12 against the LBD, creating a surface for interaction with coregulators containing an LxxLL (where L is leucine and x is any amino acid) (Heery et al. 1997). For instance, the p160 family of coregulators (SRC-1–3) and RIP140 (receptor interacting protein 140), an important coregulator in the adipose

tissue, all contain an LxxLL motif. The AF-2 situated in the LBD is required for ligand-dependent activation in steroid receptors (Danielian et al. 1992).

NRs bind to DNA as monomers, homodimers (e.g., AR) or heterodimers (e.g., PPARγ/RXR). HREs for the class I receptors (steroid receptors) are mainly inverted hexanucleotide repeats with three base pair spacing (IR3) (Cotnoir-White et al. 2011). Class II receptors typically heterodimerize with RXR and recognize HREs with direct repeats with a varied spacing (DR1–5) while class III receptors mainly function as monomers. DNA has to be accessible to TFs before they can bind to their response elements. In recent years, pioneer factors making the chromatin environment accessible for NRs have been under attention. Such a pioneer factor for estrogen receptor (ER) and AR is FoxA1 (Carroll et al. 2005; Sahu et al. 2011). NR binding sites reside typically far away from transcription start sites (TSSs) upstream or downstream, in inter- or intragenic regions (Carroll et al. 2005; Sahu et al. 2011; Lefterova et al. 2008; Nielsen et al. 2008; So et al. 2007).

Mechanisms of transcriptional activation are better understood than transcriptional repression. For a long time, it was thought that binding of an agonist simply produces an interaction surface for coactivators, whereas binding of an antagonist or in the case of class II receptors, absence of ligand, involves corepressor binding leading to transcriptional repression. However, this view is a gross underestimation of the complexity of the regulatory mechanisms, since binding of an agonist may as well lead to transcriptional repression (negative regulation). ChIP-seq experiments combined with global gene expression analyses have exponentially shed light into these mechanisms. Transcriptional repression has several mechanisms. One is the still disputed existence of negative response elements where DNA changes the conformation of the ligand-bound receptor into one that cannot positively regulate the target gene (Sakai et al. 1988). It seems that the traditional coactivator/corepressor division needs to be revised, because a protein may function as either coregulator depending on the cell-type, promoter and status of its PTMs, as demonstrated by LSD1 (lysine-specific demethylase 1) acting mostly as a corepressor. However, it may also act as a coactivator on promoters where histone 3 is phosphorylated (Metzger et al. 2010). Approximately half of the differentially regulated genes are

downregulated when a NR ligand is added, but some of this regulation may be indirect (Kininis et al. 2008).

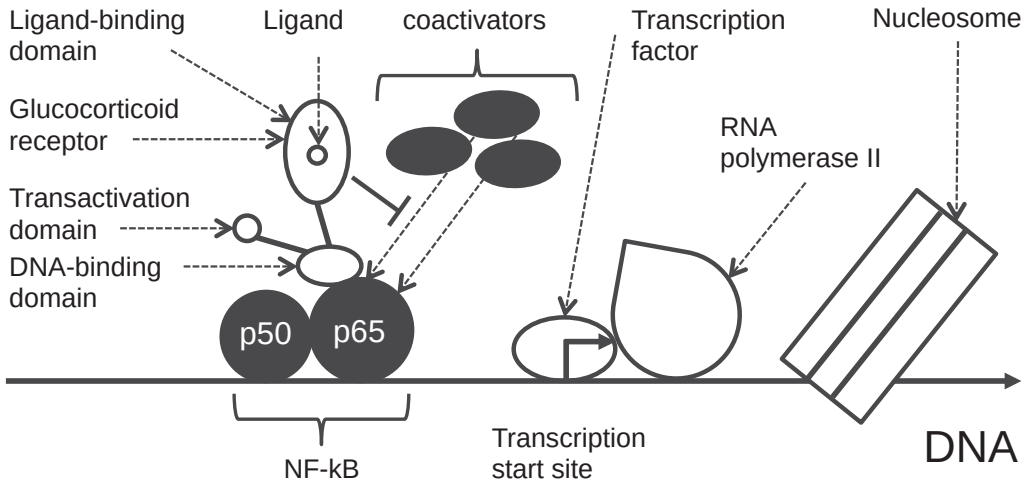

Figure 3. Simplified model of transrepression showing NF-κB on a promoter of an inflammatory gene. The presence of GR stabilizes corepressor complexes, inhibits their clearance from the promoter and interferes with the recruitment of coactivators. Adapted from Glass and Saijo 2010.

A special form of transcriptional repression is the tethering of NRs onto repressor complexes and their stabilization without their direct loading onto the DNA, a phenomenon referred to as ”transrepression” as opposed to *cis*-acting regulation (Fig. 3). Transrepression was first found for glucocorticoid receptor (GR) that can inhibit the functions of both activator protein 1 (AP1) and nuclear factor-κB (NF-κB) on a wide range of promoters. It was shown in macrophages that upon stimulation by LPS acting *via* toll-like receptor (TLR) 4, GR loading sites on DNA were devoid of glucocorticoid response elements but contained interferon regulatory factor (IRF) 3-binding motifs and that dexamethasone (Dex), a GR ligand, disrupted the interaction between p65, a subunit of NF-κB, and IRF3 or PTEFb that are needed for full activation of NF-κB target genes (Ogawa et al. 2005; Luecke et al. 2005). Later, it was found that other mechanisms exist for other NRs and they complement each other in a gene- and cell-specific manner. Peroxisome proliferator-activated receptor (PPAR) γ and liver X receptors (LXR) have been shown to repress inflammation in macrophages and liver (Pascual et al. 2005; Ghisletti et al. 2007; Blaschke et al.

2006). Transrepression has been shown to inhibit the expression of inflammatory genes governed not only by AP-1 and NF-κB but nuclear factor of activated T-cells (NFAT) and signal transducers and activators of transcription (STAT). Innate inflammation is a non-specific response against pathogens and plays a role in the low-grade inflammation aggravating several diseases including metabolic syndrome. Therefore, ligand-dependent inhibition of inflammation by NRs has opened new therapeutic opportunities.

1.4 PPARs

PPARs form a family of three members – PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) – belonging to the class II NRs and forming heterodimers with RXR. They are widely expressed and regulate lipid and carbohydrate metabolism, cell proliferation, vascular biology, tissue repair and inflammation (Wahli et al. 2012). PPARs have an isotype-specific but partially overlapping expression profiles, and they all are involved in anti-inflammatory mechanisms alleviating the deleterious effects of the low-grade inflammation present in metabolic syndrome. They use fatty acid (FA) derivatives as endogenous ligands and may therefore be considered as nutrient sensors (Forman et al. 1997).

PPARα is highly expressed in tissues with active β-oxidation (FA oxidation) and dense mitochondrial content such as brown adipose tissue (BAT), liver, kidney and heart (Escher et al. 2001). PPARα is the major regulator of the hepatic response to fasting, inducing expression of genes involved in FA catabolism and ketogenesis (Kersten et al. 1999). Fibrates are synthetic ligands of PPARα successfully used to treat hyperlipidemias.

PPARβ/δ is more ubiquitously expressed but is the predominantly expressed PPAR isoform in skeletal and cardiac muscle as well as pancreatic β-cells (Escher et al. 2001). Activation of PPARβ/δ in muscle results in increase in oxidative capacity of muscle cells and an increase in the number of type I myofibers reminiscent of physical training (Wang et al. 2004; Tanaka et al. 2003).

PPARγ is the master regulator of adipogenesis and a coordinator of whole-body insulin sensitivity (Tontonoz et al. 1994). Like other PPARs, it is considered a nutrient sensor having several FAs as ligands at a micromolar range (Forman et al. 1997; Krey et al. 1997). Synthetic PPARγ ligands – the thiazolidinediones (TZDs) – have been widely used to improve glucose tolerance in diabetics (Cariou et al. 2012). However, this class of drugs has also deleterious effects limiting their clinical use. One of these effects is a marked weight gain due to increased adiposity (Cariou et al. 2012; Nesto et al. 2004). PPARγ is transcribed as two isoforms from alternative promoters, PPARγ1 and PPARγ2, the latter of which is almost exclusively expressed in adipocytes. PPARγ2-specific knock-out mouse models have shown that PPARγ1 can compensate to some extent for the absence of PPARγ2 *in vivo*. However, mouse embryonic fibroblasts (MEFs) from these mice differentiated poorly into adipocytes under cell culture conditions (Medina-Gomez et al. 2005; Zhang et al. 2004). Besides being the most important TF in adipose tissue, PPARγ has important functions in other cell types such as macrophages, where it limits the inflammatory response and protects the body from atherosclerosis and insulin resistance. The binding sites of PPARγ on chromatin are highly cell type-specific reflecting the chromatin context and available coregulators (Lefterova et al. 2010). PPARγ is heavily influenced by PTMs, discussed in more detail in chapter 3.1.

1.5 LXR

Liver X receptors (LXR) α and β (NR1H3 and NR1H2, respectively) are adopted orphan NRs of the class II. They use oxysterols – oxidized cholesterol metabolites – as natural ligands and are encoded by two separate genes (Janowski et al. 1996). In addition to being physiological regulators of lipid and cholesterol metabolism in liver, these receptors are involved in anti-inflammation in a fashion similar to PPARs. LXRs bind to DNA through LXR response elements (LXREs) consisting of a direct repeat with a spacing of four nucleotides (DR4), but they may also regulate gene transcription through tethering onto other TFs without a direct contact to DNA. It seems that ligand binding is an important determinant of LXR function, as it increases substantially the number of LXR-binding events on chromatin of murine liver and human macrophages (Boergesen et al. 2012; Pehkonen et al. 2012). LXRβ is

ubiquitously expressed, whereas LXRα is more specifically expressed in liver, adipose tissue and intestine (www.nursa.com).

The key effects of LXR activation are related to the control of cholesterol metabolism and lipogenesis in liver (Peet et al. 1998; Repa et al. 2000; Boergesen et al. 2012). It is also important in reverse cholesterol transport (RCT) through upregulation of the cholesterol transporters ABCG1 and ABCA1 in macrophages (Naik et al. 2006). RCT is responsible for removing cholesterol away from the tissues and ultimately out of the body *via* bile, thus protecting the body from atherosclerosis. In addition to direct regulation of gene transcription programs, LXRs are also involved in transrepression of inflammatory genes. Therefore, pharmacological activation of LXR would be an attractive therapeutic target in treating metabolic diseases, but so far the side effects related to increased lipogenesis, elevation of blood triglyceride levels and neurological symptoms have prevented clinical use of LXR agonists in human patients (Bensinger et al. 2008; Katz et al. 2009).

1.6 LRH-1

Liver receptor homolog-1 (LRH-1; NR5A2) is a metabolic sensor involved in early development, bile acid synthesis, cholesterol metabolism and steroidogenesis (Fayard et al. 2004; Lazarus et al. 2012). It is mainly expressed in embryonic stem cells, liver, intestine, pancreas and ovary (www.nursa.com). Although initially identified as an orphan NR exhibiting constitutive activity and binding to DNA as a monomer, the ligands of LRH-1 now include endogenous phospholipids and synthetic compounds (Sablin et al. 2003; Ortlund et al. 2005; Lazarus et al. 2012). However, PTMs and interaction with coregulators and other NRs seem to be more important in regulating its activity. For instance, physical interaction of LRH-1 with the orphan receptor small heterodimer partner (SHP) or DAX-1 leads to a decrease in the transcriptional activation of LRH-1 (Ortlund et al. 2005; Sablin et al. 2008).

The physiological role of LRH-1 in liver is related to RCT and excretion of cholesterol metabolites into the bile (Lee et al. 2008; Chong et al. 2012). Notably, LRH-1 positively regulates CYP7A1, the key enzyme in bile acid production (Nitta et al. 1999). In the intestine, LRH-1 is involved in regulation of enterohepatic circulation

and inhibition of bile acid absorption (Chen et al. 2003). In early development, LRH-1 has a crucial role in inducing the expression of *Oct1*, one of the four genes required to maintain pluripotency and may even replace it (Gu et al. 2005; Heng et al. 2010).

2. SUMOylation pathway

PTMs include phosphorylation, acetylation, ubiquitination, to name but a few. Ubiquitin was the first protein-based modification described and soon other ubiquitin-like proteins (UBLs) were found, all conjugated to lysine (Lys) side chains of target proteins (Kerscher et al. 2006). The classical function of ubiquitin is to tag proteins for 26S proteasomal degradation via Lys48-linked polyubiquitin chains, although new functions are beginning to emerge, mainly involving monoubiquitination or different chain formation (Sadowski et al. 2012). The various ubiquitin modifications are reminiscent of a code recognized by a large machinery and leading to multiple outcomes in cells (Komander and Rape 2012). Isolated in 1975 (Goldstein et al. 1975), ubiquitin was later found to have relatives based on sequence similarity. The family of UBLs now encompasses nearly 20 proteins that all harbor a similar 3D structure, but are surprisingly diverse in function (van der Veen and Ploegh 2012). A common function for all UBLs is to alter the interaction properties of target proteins, either by promoting or inhibiting their binding to other proteins.

2.1 *Sumo1-4*

Small Ubiquitin-like MOdifier (SUMO) is an 11-kDa protein structurally very close to ubiquitin. Yeast only has one type of SUMO (Smt3), but metazoans, including mice and humans, have four SUMO paralogs each encoded by its own gene, *Sumo1-4*. SUMO-1 shares the amino acid sequence by 50% with SUMO-2 and -3, whose mature forms differ by three N-terminal amino acid residues from each other. These three amino acids reside very close to the SUMO consensus sequence involved in chain formation, and the part that is cleaved from the precursor protein is also different and slightly longer in SUMO-3 compared to SUMO-2. Therefore, the small differences are likely to contribute to dissimilar functions of the two proteins although most studies have referred to them as SUMO-2/3, and not much is known about the specific functions of these paralogs. The most obvious difference between SUMO-1

and SUMO-2/3 is that SUMO-2/3 form (poly)SUMO chains, whereas SUMO-1 is either used as a monoSUMO modification or as the last SUMO of a chain (Matic et al. 2008; Wilkinson et al. 2010). *Sumo1-3* are ubiquitously expressed in all stages of development and in all cell types (Wilkinson and Henley 2010). Most of SUMO-1 is attached to target proteins, whereas unconjugated SUMO-2/3 is suggested to serve as a reserve for stress situations (Saitoh and Hinchey 2000).

Sumo4 is expressed in spleen, lymph nodes and kidney (Guo et al. 2004). Because of a proline residue close to the diglycine motif essential for the activation step discussed below, SUMO-4 cannot be processed *in vivo* and, therefore, is not considered to be involved in PTMs (Owerbach et al. 2005). Also, lacking introns, *Sumo4* may be a pseudogene, although a polymorphism in its sequence has been associated with autoimmune diseases such as type I diabetes (Bohren et al. 2004; Guo et al. 2004).

The SUMOylation process is catalyzed by activating (E1), conjugating (E2) and ligating (E3) enzymes, analogous to the ubiquitin pathway (Fig. 4). However, where the ubiquitin system has a wide array of enzymes, the SUMOylation pathway has only one E1 enzyme (SAE1/SAE2 heterodimer in mammals, also known as AOS1/UBA2), one E2 enzyme (UBC9) and a handful of E3 enzymes. All SUMO proteins are translated as immature precursors that are activated by C-terminal cleavage revealing a Gly-Gly-motif (Gly for glycine). This step is carried out by sentrin-specific proteases (SENPs), the same enzymes that remove SUMO from target proteins, discussed below. Then the E1 enzyme (heterodimer SAE1/SAE2) adenylates SUMO and transfers it to the catalytic Cys of SAE2 forming a thioesther bond between SUMO and SAE2 (activation), followed by transfer of SUMO further to the catalytic Cys of UBC9 by a thioesther bond. UBC9 can then catalyze the conjugation of SUMO to the acceptor Lys independently of E3 enzymes (Bernier-Villamor et al. 2002). However, in most cases SUMOylation is more efficient in the presence of E3 enzymes (Tatham et al. 2003).

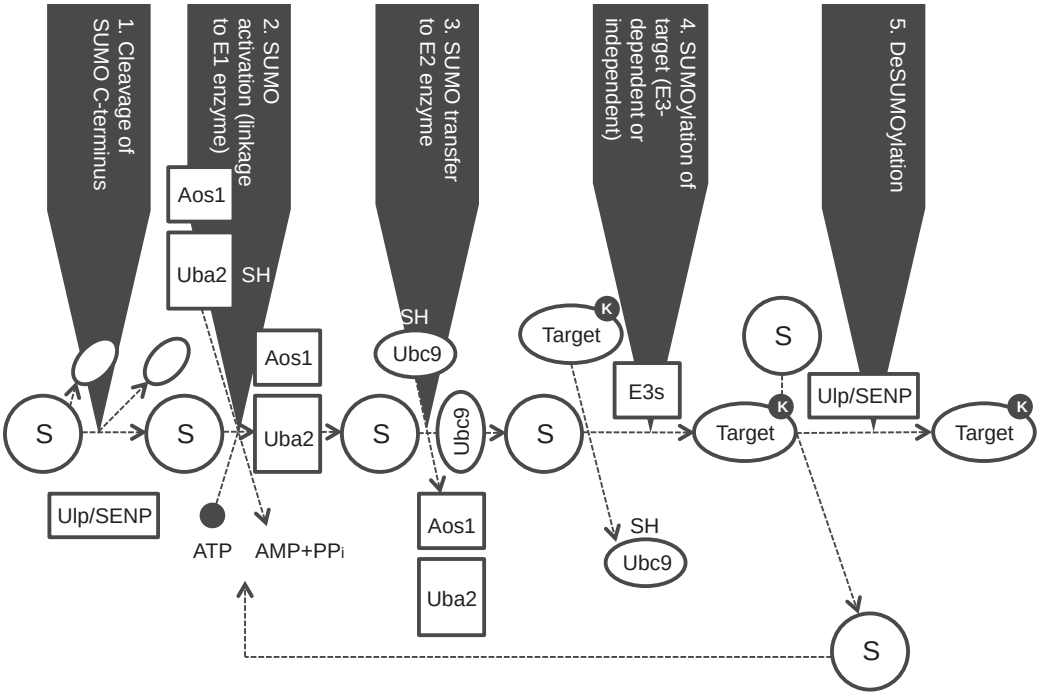

Figure 4. The SUMOylation pathway. Adapted from Wang and Dasso 2009. (S= SUMO).

2.2 E3 enzymes

Although possible with mere E1 and E2 enzymes, SUMOylation becomes clearly more efficient and more specific when catalyzed by E3 enzymes (Gareau and Lima 2010). The SUMO pathway has five classes of E3 enzymes: SIZ/PIAS family, Ran-binding protein (RanBP) 2, Polycomb 2 homologue (PC2), histone deacetylase (HDAC) 4 and TOPORS (topoisomerase I-binding, arginine/serine rich). RanBP can be found as a very stable complex throughout the cell cycle together with SUMOylated RanGAP1, the most heavily SUMOylated protein in the cell, and recent evidence suggests that it is the multiprotein complex composed of SUMOylated RanGAP1, UBC9 and RanBP2 that acts as a functional SUMO E3 ligase (Matunis et al. 1996; Werner et al. 2012). PC2 acts as an E3 ligase towards a handful of substrates (Kagey et al. 2003; Long et al. 2005; Wotton and Merrill 2007), while HDAC4 only has a few known substrates such as MEF2 (Zhao et al. 2005). The substrates of TOPORS include p53 and many chromatin-modifying proteins (Weger et al. 2005;

Pungaliya et al. 2007). Interestingly, TOPORS may also act as a ubiquitin E3 ligase, working thus at the interface of the two pathways regulated by phosphorylation (Rajendra et al. 2004; Park et al. 2008). In some instances, it is possible that the substrate functions as its own E3 ligase through intramolecular interactions (Quimby et al. 2006).

The first E3 enzymes identified were the PIAS orthologs in yeast, the Siz proteins (Johnson and Gupta 2001). Although PIAS proteins were originally identified as protein inhibitors of activated STAT, it was soon discovered that their functions were not limited to STAT signaling (Chung et al. 1997; Liu et al. 1998). They are now considered the most widely functioning group of E3 enzymes acting towards a large array of targets, and they are found ubiquitously in vertebrates. The human and murine PIAS proteins are encoded by four genes: *Pias1*, *Pias2* (*Piasx*), *Pias3*, *Pias4* (*Piasy*) of which *Pias2* and *Pias4* are highly expressed in testis. *Pias2* encodes two proteins through alternative splicing, PIASxα (ARIP3) and PIASxβ (Miz1).

All PIAS proteins have a conserved structure including an N-terminal SAP (SAF-A/B, Acinus and PIAS) domain involved in DNA and transcription factor binding (Okubo et al. 2004). The PINIT domain is involved in nuclear localization and the C-terminal S/T domain varies between the members (Duval et al. 2003). The domains linked to SUMOylation are the SP (Siz/PIAS) -RING domain and the SIM (SUMO-interacting motif). Many RING-containing proteins act as E3 enzymes of the ubiquitin pathway, and the SP-RING has been thought to have a related mode of function. The SIM domain is essential for recognition of SUMO (see below). It is interesting to note that PIAS proteins may have complex effects on TFs as demonstrated by studies on AR. SUMOylation of AR leads to transcriptional repression (Poukka et al. 2000). However, PIAS1 that mediates this modification may also act as a coactivator for AR, and this coactivator function is dependent both on AR SUMOylation sites and SP-RING domain of PIAS1 (Kotaja et al. 2000; Kotaja et al. 2002). Thus, the current view is that the PIAS proteins have different context-dependent functions some of which are related to SUMOylation. It is also interesting to note that the PIAS proteins themselves are substrates for SUMOylation and other PTMs, adding yet another layer to their regulation.

2.3 SENPs

SUMOylation is a highly reversible process. Only a small fraction of target proteins are SUMOylated at any one moment, and the cell responds to different stimuli with a rapid shift between on and off states. The first deSUMOylating enzyme identified was Ulp1 in yeast, and the mammalian deSUMOylating enzymes were identified on the basis of the sequence similarity with the yeast protein (Li and Hochstrasser 1999). The mammalian cell has six sentrin/SUMO-specific proteases (SENPs) of which SENP1 and SENP2 appear to be broader in their substrate specificity than the rest (SENP3, -5, -6 and -7). They do not discriminate between the SUMO paralogs and function as both C-terminal hydrolases (forming the mature form of SUMO) and isopeptidases (deSUMOylating target proteins). SENP3 and SENP5-7 function as isopeptidases only and prefer SUMO2/3 as substrates. Recently, a new class of deSUMOylating enzymes was described consisting of two mammalian proteins DeSI (deSUMOylating isopeptidase) 1 and DeSI-2 (Shin et al. 2012). DeSI-1 and -2 have a wide tissue distribution in mice and, unlike nuclear SENPs, are also present in the cytoplasm (Shin et al. 2012). Furthermore, Wss1, belonging to yet another class of proteases (Wss1p-like metalloproteases) was suggested to have SUMO-dependent isopeptidase activity in yeast, but direct evidence is still missing (Mullen et al. 2010).

2.4 Consensus motifs for SUMO conjugation and recognition

The carboxyl group of the C-terminal glycine in SUMO is covalently attached to the ε-amino group of a lysine residue in the target proteins at a consensus ψKXE site, where ψ is a large hydrophobic amino acid, K is lysine, X is any amino acid and E is glutamic acid (Anckar et al. 2007). SUMO-2 and -3 contain this consensus sequence through which the (poly)SUMO chains are formed. Extensions of the SUMO consensus site include phosphorylation-dependent SUMO motifs (PDSM) and negatively charged amino acid-dependent SUMO motifs (NDSMs) (Hietakangas et al. 2006; Yang et al. 2006). However, not all consensus sequences are actually SUMOylated and other SUMOylation sites not fitting the above consensus sequence have been observed (Matic et al. 2010; Pichler et al. 2005). Therefore, more studies are needed to confirm the *in silico* predicted SUMOylation sites. This is going to be a

time- and labor-consuming task, since even the *in vitro* and *in vivo* mechanisms may differ significantly (Lee et al. 2011).

Proteins recognizing SUMO contain a SIM. While SUMOylation consensus sequence is responsible for the covalent modification of the substrate protein, SIMs mediate the non-covalent interactions of SUMO and other proteins (Kerscher 2007). A SIM motif is suggested to contain a hydrophobic core sequence next to acidic residues flanked by two serine (Ser) residues, although the requirement for the Ser-residues has been disputed (Minty et al. 2000; Song et al. 2004). In many cases, a SIM is required in the SUMO substrate to mediate the SUMOylation by bringing SUMO and the substrate together before the covalent bonding. This has been shown at least with TDG (thymine-DNA glycosylase), DAXX (death-domain associated protein) and BLM (Bloom syndrome protein) (Takahashi et al. 2005; Lin et al. 2006; Zhu et al. 2008). To some other proteins, SUMOylation may take place without a SIM domain, although SIM may be important for another function. This is the case for PML (promyelocytic leukemia protein), a protein essential for the formation of a subnuclear structure, the PML nuclear body (NB). PML lacking the SIM sequence may be SUMOylated and this SUMOylation is required for the PML NB formation, but dispersion of the NB requires an intact SIM (Duprez et al. 1998; Maroui et al. 2012). The PML NBs are thought to be the SUMOylation hotspots of the cell, since most of the proteins localized to these structures are SUMOylated (Lallemand-Breitenbach et al. 2010). SIMs are also present in many proteins of the SUMOylation machinery including the E1 and many of the E3 enzymes (Minty et al. 2000; Stehmeier et al. 2009).

2.5 Crosstalk with other modifications

SUMOylation can be seen as part of a post-translational code where one modification precedes another or where SUMOylation inhibits another modification. The well-known example of the latter includes the SUMOylation of Iκ-Bα (nuclear factor -κB [NF-κB] inhibitor alpha) (Desterro et al. 1998). SUMOylation of Iκ-Bα leads to stabilization of the protein by preventing its ubiquitination and subsequent proteasomal degradation. PCNA (proliferating cell nuclear antigen) has been found to

be modified in a similar fashion by either SUMO or ubiquitin at the same Lys residue, leading to selection of different pathways in DNA replication stress depending of the modification (Hoege et al. 2002).

Functional consequences of SUMOylation include creation of new interaction surfaces or masking the existing ones. The effector proteins recognizing SUMO modification may have the ability to modify the target protein further. This is evident in the class of SUMO-targeted ubiquitin ligases (STUbLs), first identified in yeast, which changed the whole SUMO paradigm from competitor of ubiquitin into enhancer of ubiquitin chain formation and therefore promoter of proteasomal degradation in some cases. The proteins harbor ubiquitin E3 ligase activity and one or many SIMs recognizing SUMO non-covalently. The STUbLs identified so far are the mammalian RNF4 (ring finger protein 4) corresponding to Slx5/Slx8 in *Saccharomyces cerevisiae* (Sun et al. 2007; Xie et al. 2007). The functions of STUbLs include regulation of transcription factors, DNA damage and arsenic-induced degradation of the PML NB (Wang et al. 2011; Galanty et al. 2012; Tatham et al. 2008).

The aforementioned extension of the SUMO consensus sequence, the PDSM, provides an example of the interplay between phosphorylation and SUMOylation. In the sequence ψKXEXXSP, the regulatory proline-directed Ser residue (SP) is phosphorylated prior to SUMO conjugation in an E2-dependent manner (Hietakangas et al. 2006; Mohideen et al. 2009). Furthermore, when the same Ser residue is dephosphorylated in neurons, the Lys is acetylated instead of being SUMOylated, known as a SUMOylation-acetylation switch (Shalizi et al. 2006). The SUMOylation-acetylation switch has been shown in phosphorylation-independent sites as well (Stankovic-Valentin et al. 2007), and SUMO itself is sometimes modified by acetylation leading to diminished SUMO-SIM interactions (Ullman et al. 2012). An example of multiple layers of the PTM code is the human FEN1 (flap structure-specific endonuclease 1) that is post-translationally modified by phosphorylation, leading to SUMOylation by SUMO-3. Both of these modifications are needed for ubiquitylation leading to protein degradation (Guo et al. 2012). A defect in any of these modifications results in accumulation of FEN1 and a delay in the cell cycle.

3. Functions of SUMOylation

SUMOylation is essential to life, because knock-out mice lacking the only E2, *Ubc9*, are embryonically lethal at the early postimplantation stage (Nacerddine et al. 2005). Zebrafish devoid of all three SUMO paralogs is embryonically lethal, but the presence of any SUMO protein restored the phenotype (Yuan et al. 2010). Invertebrates have only one SUMO, SMT3. In yeast, mutation of *SMT3* or *UBC9* leads to arrests in cell cycle, and *C. elegans* devoid of its only *SUMO* gene results in abnormal embryogenesis (Seufert et al. 1995; Dieckhoff et al. 2004; Broday et al. 2004). On the other hand, too much of SUMO may be harmful as shown by a study in *C. elegans* (Rytinki et al. 2012). The first identified SUMO substrate was RanGAP1 involved in nuclear transport and soon a plethora of SUMO targets were identified, making SUMOylation one of the established regulators of cellular functions (Matunis et al. 1996; Mahajan et al. 1997). Animal models related to SUMOylation are summarized in Table 1.

Table 1. Animal models related to the SUMO pathway

Gene	Animal Model	Phenotype	Reference
Sumo1	KO mouse	cleft lip and palate	Alkuraya et al. 2006
	KO mouse	disturbed formation of PML bodies	Evdokimov et al. 2006
Sumo1 *Sumo2* *Sumo3*	triple knock-down zebrafish	embryonic lethality	Yuan et al. 2010
Ubc9	KO mouse	embryonic lethality at early postimplantation	Nacerddine et al. 2005
	Overexpression mouse	Normal gross anatomy, improved tolerance to brain ischemia	Lee et al. 2011
Pias1	KO mouse	partial perinatal mortality	Liu et al. 2004
Pias4	KO mouse	no apparent phenotype	Wong et al. 2004
		hypersensitivity to LPS	Tahk et al. 2007
		modulation of cytokine signaling	Roth et al. 2004
Pias1 *Pias4*	double KO mouse	embryonic lethality before dpc 11.5	Tahk et al. 2007
Ranbp2	KO mouse	-/-: embryonic lethality +/-: deficits in growth rates and glucose catabolism	Aslanukov et al. 2006
Senp1	KO mouse	embryonic lethality at midgestation due to insufficient EPO production	Cheng et al. 2007
	mouse harboring a proviral mutation	embryonic lethality, placental deficiency	Yamaguchi et al. 2005
Senp2	KO mouse	embryonic lethality, placental defects	Chiu et al. 2008
	cardiac-specific SENP2 knock-in mouse	premature death, cardiac septal defects	Kim et al. 2012
Senp6	KO mouse	embryonic lethality	European Mouse Mutant Archive, unpublished

3.1 Regulation of transcription

SUMOylation was initially associated only with inhibition of transcription (Gill 2005). However, as the number of modified TFs increased, it became clear that SUMOylation regulates transcription in a context-dependent manner with multiple mechanisms leading to positive or negative regulation of transcription.

Modification of transcription factors. A lot of TFs and their coregulators have been shown to be subject to SUMOylation, which can affect a TF's ability to bind DNA, alter the stability of a coregulator complex or attract/exclude proteins with enzymatic

activity (Lyst and Stancheva 2007). The repression by SUMO is thought to result from the recruitment of chromatin-modifying enzymes such as HDACs (Yang and Sharrocks 2004; Girdwood et al. 2003). In some cases, SUMOylation leads to sequestration of the TF to a nuclear compartment not involved with transcriptional activation (Sachdev et al. 2001). However, SUMOylation may also lead to transcriptional activation, and this has been shown at least with p53, RORα (RAR-related orphan receptor alpha) and TCF-4 (transcription factor 4) (Gostissa et al. 1999; Hwang et al. 2009; Ihara et al. 2005).

Many NRs are targets for SUMOylation (Table 2). The consequences of NR SUMOylation are varied and to make the matters more complex, PTMs often form a combination of modifications with PPARγ serving as a good example. The function of PPARγ is modulated by several PTMs, such as phosphorylation, ubiquitination and SUMOylation (van Beekum et al. 2009). Phosphorylation of Ser112 results in reduced transcriptional activity of PPARγ, which, in turn, is required for Lys107 SUMOylation leading to inhibition of PPARγ transactivation function *in vitro* (Hu et al. 1996; Ohshima et al. 2004; Yamashita et al. 2004; Floyd and Stephens 2004). Moreover, the knock-in mice with a Ser112A (A for alanine) mutation are protected from insulin resistance on high-fat diet (Rangwala et al. 2003). In transrepression, discussed in more detail below, ligand-induced SUMOylation of Lys395 stabilizes the repressor complex in macrophages (Pascual et al. 2005). The anti-diabetic actions of PPARγ are possible to achieve even ligand-independently by blocking the phosphorylation of Ser273, a modification more often observed in obese than lean individuals (Choi et al. 2011). The ligand-induced poly-ubiquitination (the exact site of which is not known) appears to be important in negative regulation of the receptor through proteasomal degradation (Hauser et al. 2000).

Modification of chromatin and histones. Chromatin is composed of DNA wrapped around histones and may be turned active or inactive by modifications involving the DNA or the histones. SUMOylation of all four major histone types was found to result in gene repression in *S. cerevisiae* (Nathan et al. 2006). In human cells, histone H4 is SUMOylated resulting in gene silencing through recruitment of HDACs (Shiio and Eisenman 2003). SUMOylation of H1 and H3 has also been observed, but

functional consequences of this modification are unknown (Matafora et al. 2009). Although SUMOylation has been associated with gene repression, a recent paper describes the occupation of SUMO-1 on HeLa cell chromatin in a genome-wide manner as being involved with active housekeeping genes and associated with active chromatin marks (Liu et al. 2012b). A similar result was already found in yeast, where ChIP experiments detected SUMO-modified proteins on actively transcribed genes and on promoters of inducible genes upon gene activation. However, silencing of *UBC9* resulted paradoxically to even higher induction of these genes, implying that multiple mechanisms exist (Rosonina et al. 2010). Thus, SUMOylation regulates chromatin in many different ways and is not a simple repressive mark as previously thought.

Table 2. Effect of SUMOylation on NR function. Adapted from Treuter and Venteclef 2011.

NR	SUMO pathway	Effect of SUMOylation	Reference
LXRα	SUMO-2/3, HDAC4	anti-inflammatory transrepression in macrophages	Ghisletti et al. 2007
		anti-inflammatory transrepression in brain astrocytes	Lee et al. 2009
LXRβ	SUMO-2/3, HDAC4	anti-inflammatory transrepression in macrophages	Ghisletti et al. 2007
	SUMO-1, PIAS	anti-inflammatory transrepression in brain astrocytes	Lee et al. 2009
LRH-1		transcriptional reprogramming of mouse somatic to stem cells	Heng et al. 2010
	SUMO-1, PIAS	sequestration of LRH-1 to PML-NBs	Chalkiadaki and Talianidis 2005; Yang et al. 2009
	SUMO-1	interaction with ARIP4	Ogawa et al. 2009
RXR	SUMO-1	repression, transrepression, cross-SUMOylation with PPAR γ in chondrosarcoma cells	Burrage et al. 2008; Choi et al. 2006
ROR	SUMO-1–2	SUMO site mutants less active	Hwang et al. 2009
PPARα	SUMO-1–3	anti-inflammatory transrepression in female liver	Leuenberger et al. 2009
	SUMO-1, PIAS	repression, recruitment of NCoR, ligand-decreased SUMOylation	Pourcet et al. 2010
PPARγ	SUMO-1, PIAS	repression	Ohshima et al. 2008
	SUMO-1, PIAS	anti-inflammatory transrepression in macrophages, inhibition of NCoR degradation	Pascual et al. 2005; Jennewein et al. 2008
	SUMO-1, PIAS	enhanced proliferation vascular smooth muscle cells, pro-atherogenic	Lim et al. 2009
AR	SUMO-1, PIAS	repression, binding site selection, SUMO site within synergy control motif	Poukka et al. 2000; Kotaja et al. 2002; Callewaert et al. 2004
		SUMOylation attenuates polyQ-mediated aggregation	Mukherjee et al. 2009
GR	SUMO-1–3	Repression, SUMO site within synergy control motif, Daxx (SIM)-binding	Tian et al. 2002; Lin et al. 2006; Holmstrom et al. 2003; Holmstrom et al. 2008
ERα	SUMO-1, PIAS	repression	Sentis et al. 2005

3.2 Regulation of inflammation

Inflammation is a complex response of the body against pathogens, damaged cells or irritants. Deregulation of inflammation leads to inflammatory diseases such as rheumatoid arthritis or atherosclerosis and therefore, inflammatory responses are tightly regulated. Inflammation can be local involving migration of monocytes into the inflamed tissue *via* increased vascular permeability mediated by local cytokines. In more severe inflammatory responses, the liver produces a variety of acute phase proteins including SAA (serum amyloid A), CRP (C-reactive protein) and haptoglobin. CRP is widely used in the clinic to assess the severity of bacterial infection or degree of inflammation.

The first hints that SUMOylation plays a part in the regulation of inflammation came from the seminal paper describing the SUMOylation of Iκ-Bα *in vitro* (Desterro et al. 1998). A key player in inflammation is NF-κB that regulates positively a set of inflammatory genes. Normally, NF-κB is kept out of the nucleus by Iκ-Bα, which, upon inflammatory signaling by e.g. TNFα (tumor necrosis factor alpha), is phosphorylated by IKK (inhibitor of kappa-B kinase) leading to its modification by ubiquitin and subsequent degradation by the proteasomal system. However, SUMOylation of Iκ-Bα protects the protein from ubiquitination at the same Lys residue stabilizing the protein and leading to retention of NF-κB in the cytoplasm. This led to a hypothesis that the main function of SUMOylation is the protection of proteins from degradation, but later studies have shown that this is rarely the case. As discussed above, modification of a substrate by SUMO may even lead to its degradation via STUbLs (Sun et al. 2007). Recently, SUMOylation was shown to negatively regulate the NF-κB in the nucleus *via* the RelA subunit as well (Liu et al. 2012a), confirming SUMO as the inhibitor of the NF-κB pathway in inflammation. It is important to note that the NF-κB may also be activated by genotoxic stress and in this setting (nucleus-to-cytoplasm signaling), SUMOylation is involved in the *activation* of the pathway *via* NEMO (NF-κB essential modulator), the IKKγ regulatory subunit of the IKK complex (Huang et al. 2003; Mabb et al. 2006). NF-κB is at the crossroads of several pathways linking apoptotic and inflammatory signals together highlighting the importance of its proper regulation.

SUMOylation has turned out to be an essential layer in the regulation of inflammation by stabilization of NRs with corepressors on the promoters of inflammatory genes (transrepression). Lipopolysaccharide (LPS) is a bacterial antigen recognized by TLR4 on the cell surface of macrophages resulting in the activation of innate immunity towards pathogens. Activation of the TLR4 results in derepression of the inflammatory genes by active clearance of the nuclear receptor corepressor complexes containing NCoR or SMRT as well as TBLR1, TBL1, HDAC3, from the promoters. Most of the results concerning SUMOylation regulating these mechanisms have been obtained from macrophages, although the same principles have been detected in microglia and astrocytes in the brain through the orphan receptor NURR1 (Pascual et al. 2005; Ghisletti et al. 2007 and 2009; Saijo et al. 2009). In addition, SUMOylation of LXRs was shown to modulate the IFN (interferon) γ-mediated inflammation in brain astrocytes with SUMO-1-specific modification of LXRβ and SUMO-2/3 modification of LXRα using the PIAS1 and HDAC4 as E3 enzymes, respectively (Lee et al. 2009). Interestingly, in macrophages, SUMOylation of both LXR paralogs is carried out by SUMO-2/3, and they block NCoR turnover by binding to a conserved SUMO2/SUMO3-interaction motif in CORO2A and preventing actin-mediated NCoR clearance (Ghisletti et al. 2007; Huang et al 2011). In macrophages, SUMO-1-modified PPARγ mediates transrepression of a subset of genes in a ligand-dependent manner (Pascual et al. 2005; Jennewein et al. 2008).

3.3 Stress and SUMO

The finding that SUMO-2/3 is largely in free form in the cell, as opposed to SUMO-1 that is usually covalently bound to substrates, was the first hint towards free SUMO-2/3 being a reserve related to stress situations and mobilized when needed (Saitoh and Hinchey 2000). The authors tested several cellular stressors on cultured monkey kidney cells and noted accumulation of high molecular weight SUMO-2/3 conjugates upon exposure to heat, oxidative stress, ethanol and osmotic stress. *In vivo*, experimental stroke models with occlusion to transient middle cerebral artery resulted in massive SUMOylation at the infarct area mostly with SUMO-2/3 (Cimarosti et al. 2008; Yang et al. 2008). Responses to hypoxia are, however, not limited to SUMO-

2/3 only, as hypoxia also increases *Sumo1* mRNA levels of *in vitro* and *in vivo* (Comerford et al. 2003; Shao et al. 2004). SUMO-1 has been shown to regulate the stability of HIF1 (hypoxia-inducible factor 1) that is a dimeric protein stabilized under hypoxic conditions and regulates directly expressions of genes involved in survival under these conditions, such as *Epo* (Wang et al. 1995a). The consequences of HIF1α SUMOylation are contradictory, with one study showing increased stability and activity of the protein upon SUMOylation while two other reports claim the contrary (Bae et al. 2004; Carbia-Nagashima et al. 2007; Berta et al. 2007). Although in general, hypoxia tends to increase the global SUMOylation, this does not necessarily apply to all proteins, as in human corneal epithelial cells it was found that hypoxia induces de-SUMOylation of CTCF despite increased universal SUMOylation (Wang et al. 2012). There is evidence from yeast that SUMOylation of multiple enzymes shifts the metabolism of cells to favor glycolysis under hypoxic conditions (Agbor et al. 2011).

Not only hypoxia but also oxidative stress has been linked to SUMOylation as H_2O_2 treatment resulted in increased SUMOylation (Saitoh and Hinchey 2000; Zhou et al. 2004; Manza et al. 2004). However, with lower concentrations of H_2O_2, global SUMOylation decreases because of inactivation of the E1 and E2 enzymes through formation of thioesther bonds by the reactive oxidative species (ROS) (Bossis et al. 2006). Again, SUMO modification of single proteins may have tremendous effects on the cell fate in oxidative stress, as demonstrated by HIPK2 (homeodomain-interacting protein kinase 2) that has redox sensor activity. With increasing levels of H_2O_2, HIPK2 is deSUMOylated, which causes dissociation of HDAC3 from the complex. The resulting hyperacetylation of HIPK2 improved cell survival and resistance to ROS (de la Vega et al. 2012). SUMOylation may thus have clinical implications in the treatment of cancer, where cancer cells have become insensitive to oxidative stress and create strategies to evade apoptosis (Hanahan and Weinberg 2011).

3.4 SUMO and chromosomal stability

Genotoxic stress such as ionizing radiation causes DNA damage that the cell tries to repair by activating one or many of the DNA damage response (DDR) pathways to

avoid passing a potentially harmful mutation forward to daughter cells and, in case this fails, the cell – if part of a multicellular organism – usually activates the programmed cell death (apoptosis) pathway. SUMOylation is involved in the regulation of this at multiple points. Intriguingly, one of the most important regulators of chromosomal stability, the tumor suppressor protein p53, is stabilized by SUMO (Gostissa et al. 1999; Rodriguez et al. 1999).

Base excision repair. Single bases in DNA may be damaged by deamination, oxidation or alkylation. These non-fitting bases are removed by glycosylases leaving behind an abasic nucleotide removed by AP (apuric or apyrimidic) endonucleases. The filling of the resulting gap is catalyzed by DNA polymerases and ligases. One of the glycosylases modified by SUMO is TDG, which removes the sugar moiety of thymine or uracil when mismatched with guanine (Takahashi et al. 2005). SUMOylation of TDG results in dissociation of TDG from the site allowing the repair pathway to proceed (Hardeland et al. 2005).

Double strand break (DSB) repair. DSBs are caused by genotoxic agents, γ-irradiation or faulty replication and are usually efficiently repaired either by non-homologous end joining or homologous recombination (HR). The cell may also create DSBs as part of normal meiosis in testes and ovaries when the chromosomes exchange genetic material. SUMOylation of several proteins accumulated in the DSB foci has been reported (Bergink and Jentsch 2009). The DSB brings about a cascade of events leading to the accumulation of repair proteins in the vicinity of damaged DNA. Phosphorylation by the ATM/ATR/DNA-PK pathway is an important event, leading to formation of phospho-H2AX, a hallmark of DNA damage, which functions as a docking site for the ubiquitin E3 ligase RNF8 (Rogakou et al. 1998; Mailand et al. 2007). The ubiquitination of several other proteins catalyzed by RNF8 allows complexes containing p53BP1 (p53-binding protein 1) and BRCA1 (breast cancer 1, early onset) to assemble. Interestingly, the presence of PIAS1 and PIAS4 is also required for p53BP and BRCA1 to load onto the damage site (Galanty et al. 2009). Furthermore, BRCA1, a ubiquitin E3 ligase itself, is modified by SUMO, and SUMO conjugates are assembled onto the damaged loci (Morris et al. 2009). p53BP1 and MDC1 (mediator of DNA-damage checkpoint 1), a scaffold protein recognizing the

phospho-H2AX, are also SUMO substrates (Vyas et al. 2012). It seems that DSB repair is a complex cascade of events requiring the correct timing of different PTMs and assembly of a large array of DNA repair proteins, a process far from being understood in full detail.

A DSB may also result during S-phase, if the replication fork collapses at an unrepaired DNA lesion. An important protein orchestrating the events at the replication fork, PCNA, has several interaction partners depending on its PTMs. The same Lys164 may be modified by mono-ubiquitination leading to error-prone post-replication repair (PRR) *via* the translesion synthesis. However, if the same Lys is poly-ubiquitinated, error-free PRR results. The same Lys may also be SUMOylated leading to inhibition of HR, which might lead to chromosomal instability at an unrepaired DSB (Hoege et al. 2002). The SUMOylated PCNA is recognized by the SIM of Srs2, the crystal structure of which has recently been resolved (Armstrong et al. 2012). The mechanisms have been studied mostly in yeast but many of the proteins involved are conserved in mammals. However, mammalian cells lack a homologue for Srs2 and, therefore, the mediator recognizing the SUMOylated PCNA remains to be identified, although the same conserved Lys164 is SUMOylated in human cells (Gali et al. 2012).

3.5 SUMO and human diseases

Cancer. As might be expected from the role of SUMOylation in DNA damage repair, genes of the SUMOylation pathway have also been implicated in human cancers (Bettermann et al. 2012). Lung adenocarcinoma, ovarian and cervical carcinoma, colon and prostate cancer have all been reported to have higher expression of *UBC9* compared to normal tissue from the same patients (McDoniels-Silvers et al. 2002; Mo et al. 2005; Moschos et al. 2010). However, both colon and prostate cancer metastatic lesions show lower levels of *UBC9* mRNA compared to normal tissue (Moschos et al. 2010). UBC9 has, therefore, been proposed to be a potential drug target. It is possible that the changes observed in SUMOylation of cancerous tissues are related to the tumor microenvironment characterized by changes in metabolism, hypoxia, inflammation and genotoxic stress, situations that are all related to SUMOylation. The relationship between SUMO and cancer is likely to be very complex, as many of the

cancers mentioned above are highly dependent on steroid hormone signaling, and therefore, SUMOylation may have a specific role in each cancer type.

Neurodegenerative diseases. Huntington's disease (HD) is a neurodegenerative disease characterized by the accumulation of a pathogenic form of huntingtin (Htt) exhibiting an expanded polyglutamine tract. Htt was shown to be SUMOylated and in the *Drosophila* model of HD, SUMOylation of Htt leads to exacerbation of the disease (Steffan et al. 2004). Other neurodegenerative diseases have been associated with SUMOylation as well. Both α-synuclein and DJ-1, proteins involved in Parkinson's disease, are known SUMO-substrates (Dorval and Fraser 2006; Shinbo et al. 2006). APP (amyloid precursor protein) and Tau, two proteins contributing to the plaques and Tau filaments accumulating in Alzheimer's disease, are both targets for SUMOylation (Li et al. 2009; Dorval and Fraser 2006). Despite several animal models and *in vitro* results, final evidence showing that SUMOylation is involved in human neurodegenerative diseases is not yet available.

Infections. Various human intracellular pathogens have evolved to modulate or benefit from the SUMOylation system. The best studied are adenoviruses and herpes viruses that have been shown to disrupt the PML-NBs, natural antiviral structures of the nucleus (Van Damme and Van Ostade 2011). DNA of Herpes simplex virus-1 moves to the nucleus upon entrance of viral particles into the host cell and localizes to the PML-NB where Daxx1, Sp100 and PML act as an antiviral defense. The virus fights this with an immediate early protein aiming at destruction of SUMOylated proteins of the PML-NB (Müller and Dejean 1999). Other viruses and even intracellular bacteria have also been shown to hijack the SUMOylation machinery (Wimmer et al. 2012).

4. Adipose tissue

PPARγ, post-translationally modified by SUMO, is the master regulator of the adipose tissue, an important endocrine organ affecting whole body energy homeostasis and insulin sensitivity by secreting adipokines. White adipose tissue

(WAT) stores energy whereas brown adipose tissue (BAT) burns it by generating heat through mitochondrial uncoupling of respiration from ATP production.

4.1 White adipose tissue

White adipose tissue comprises mainly unilocular adipocytes, fibroblasts, nerves, endothelial cells and immune cells. It is present subcutaneously and around internal organs, the latter of which is considered metabolically more important as its circulation drains directly to the liver (Smorlesi et al. 2012). All adipocytes are derived from mesenchymal stem cells (MSC) present in the stromal vascular fraction of the adipose tissue. The first transition into committed preadipocytes upon activation by messages from the extracellular matrix is not entirely clear, but is known to involve signaling *via* WNT pathway and the transforming growth factor (TGF)-β family (Cristancho and Lazar 2011). A recently found marker for committed preadipocytes is Zfp423 that sensitizes the cells to BMP (bone morphogenic protein), a member of the TGFβ family (Gupta et al. 2010). The terminal differentiation has been studied in more detail, although a majority of the results are derived from *in vitro* differentiation studies using a single cell line, the murine preadipocyte cell line 3T3-L1 (Green and Meuth 1974). A large number of TFs modulate adipogenesis, but the main players are PPARγ and the CCAAT-enhancer-binding proteins (C/EBPs) (Lefterova et al. 2008; Nielsen et al. 2008). In committed adipocytes, low level of C/EBPβ is bound on stretches of chromatin that display marks for active enhancers (hotspots) while PPARγ level is very low. Upon addition of the adipogenic cocktail – typically consisting of a glucocorticoid, cAMP agonist and insulin – these hotspots recruit binding of other adipogenic TFs such as C/EBPδ, STAT5A, GR and RXR (Siersbæk et al. 2011). The early TFs also induce the late adipogenic factors, PPARγ and C/EBPα, which together induce and maintain the expression of metabolic genes. It has been demonstrated *in vivo* and *in vitro* that adipogenesis does not occur in the absence of PPARγ, and the *Cebpa* null mice die at 8 h postnatally exhibiting adipose tissue unable to store lipids (Barak et al. 1999; Rosen et al. 1999; Wang et al. 1995). Together they also form a positive autoregulatory loop maintaining their own expression (Wu et al. 1999).

WAT stores energy as triglycerides within its unilocular fat droplets. It also communicates with the rest of the body by secreting its own hormones, adipokines, into the blood stream (Ouchi et al. 2011). The role of leptin was demonstrated by the ob/ob mouse harboring a mutation in the *leptin* gene. Leptin functions *via* the central nervous system as an anorexigenic hormone, and consequently, the mouse model exhibits hyperphagia and severe obesity, which are reversed by leptin administration (Zhang et al. 1994). Another proinflammatory adipokine is resistin, mediating insulin resistance (Steppan et al. 2001). Although secreted robustly by adipocytes in mice, resistin may not be a true adipokine in humans, where it is secreted by macrophages, although it seems to have a similar function in carbohydrate metabolism and insulin signaling as the murine counterpart (Qatanani et al. 2009; Park et al. 2011). An important anti-inflammatory adipokine is adiponectin that acts *via* adiponectin receptors in muscle and liver to boost fatty acid oxidation and inhibit gluconeogenesis, respectively (Tomas et al. 2002; Yamauchi et al. 2002).

4.2 Brown adipose tissue

There is increasing evidence that adult humans possess functional BAT, previously thought to be present only in infants and hibernating animals (Virtanen et al. 2009; van Marken Lichtenbelt et al. 2009; Cypess et al. 2009). It is located in depots in the neck and around clavicles and activated upon cold exposure (Ouellet et al. 2012). Furthermore, there is a strong inverse relationship between obesity and the amount of BAT in humans (Saito et al. 2009; van Marken Lichtenbelt et al. 2009). The ability of BAT to produce heat is dependent on uncoupling protein 1 (UCP1) which allows energy to be consumed and released as heat instead of being stored as fat. This protects the body against cold and may prevent obesity by burning calories.

Similar to white adipocytes, the brown adipocytes are derived from MSCs. However, early on in the differentiation, the two lines diverge and result in committed white preadipocytes and myogenic factor 5 (MYF5) positive myoblastic precursor cells, which have the potential to differentiate into muscle cells and brown adipocytes (Seale et al. 2008). The lineage specific TF promoting the differentiation to the direction of brown adipocytes is PRDM (PR domain containing) 16, a zinc-finger protein working in complex with C/EBPs (Seale et al. 2007). PRDM16 is such a

robust inducer of the brown adipocyte program that even fibroblasts expressing ectopically the protein assume the brown adipocyte phenotype (Kajimura et al. 2009). Other TFs required for the brown adipocyte development are again PPARγ and the C/EBPs along with a PGC1α (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), which is essential in mitochondrial biogenesis and highly expressed in BAT (Kajimura et al. 2010). Interestingly, SUMOylation of PGC1α has been found to attenuate its functions *in vitro*, possibly by enhancing its interaction with RIP140 (Rytinki and Palvimo 2009). RIP140 is a transcriptional corepressor antagonizing the effects of PGC1α and part of the white adipocyte signature (Powelka et al. 2006).

A recent breakthrough in the adipocyte field is the finding that brown-like adipocytes may appear in WAT depots (Vegiopoulos et al. 2010; Petrovic et al. 2010). Browning of the WAT has been known previously, but the novelty of the studies was the molecular signature showing that they are a distinct population of cells resembling the brown adipocytes but lacking some of their characteristics such as the expression of PRDM16. These brown-like cells are called beige or brite, and they have evoked high hopes in the battle against obesity, as they literally burn fat. A crucial question still remains whether the beige cells arise from stem cells or through transdifferentiation from white adipocytes and whether this process could be modified pharmacologically. The transdifferentiation theory was initially thought to explain the phenomenon (Barbatelli et al. 2010). However, there is recent evidence that a subpopulation of cells in the stromal vascular fraction could be beige stem cells, and the BAT depots in humans would be more beige than brown, i.e., derived from the MYF5-negative precursors (Wu et al. 2012). The white-to-beige conversion is induced by both exposure to cold and physical exercise, possibly through sympathetic activity (Barbatelli et al. 2010; Smorlesi et al. 2012). A hormone released from the muscles during physical exercise was recently shown to induce the browning of WAT. This newly identified hormone was named irisin (Boström et al. 2012).

4.3 Obesity

Consumption of excessive amounts of food combined to a sedentary lifestyle has led to a world-wide epidemic of obesity, which is associated with type 2 diabetes, fatty

liver, cardiovascular diseases and osteoarthritis. Weight gain results when calorie intake exceeds calorie consumption. WAT can expand to a certain point by hyperplasia and hypertrophy, but at some point the adipocytes become saturated with fat, leading to inflammation (Stienstra et al. 2012). The inflamed adipocytes secrete inflammatory mediators, such as TNFα and IL (interleukin)-1β, attracting macrophages and activating the ones already residing in the tissue. This is thought to contribute to the whole-body insulin resistance seen in obesity (Weisberg et al. 2003; Xu et al. 2003). Macrophages display two opposing polarization states. Lean WAT typically contains M2 polarized (alternatively activated) macrophages that secrete anti-inflammatory signals, whereas obese WAT makes macrophages to assume an M1 (classically activated) state promoting inflammation (Prieur et al. 2011; Zeyda et al. 2007). An altered inflammatory status of the WAT in obesity is also evident, in that lean but not obese mice had a specific subpopulation of regulatory T-cells in their abdominal WAT and, interestingly, PPARγ was required for them to maintain insulin sensitivity of the body (Feuerer et al. 2009; Cipolletta et al. 2012). This may have implications in the treatment of diabetes, where many of the PPARγ agonists have failed due to side-effects including weight gain (Nesto et al. 2003; Cariou et al. 2012).

Besides the pathological low-grade inflammation, obesity involves dysregulation of the adipokines. Leptin levels are elevated in obese individuals but due to leptin resistance, the hormone fails to exert its hypothalamic anorexigenic response (Friedman and Halaas 1998; Maffei et al. 1995). Accordingly, exogenously administered leptin is not a promising drug in obesity, but may turn out to be useful in treatment of diabetes (Coppari and Bjørbæk 2012). The role of resistin in the pathophysiology of obesity is somewhat unclear, but adiponectin levels show a clear decrease in obese people (Heilbronn et al. 2004; Ryo et al. 2004).

5. Testis

Testis is the site of male germ cell maturation and the main source of androgens. It has developed its own mechanisms to protect the delicate process of sperm production by tightly regulating the molecular environment by the testis-blood barrier. With a high cell division activity – approximately 100 to 200 million sperm cells are

produced daily – it is important to regulate the cell cycle and keep the chromosomes stable, functions where the SUMOylation system comes to play (Vigodner 2011). The functions of testis are ultimately orchestrated by hypothalamic hormones, the luteinizing hormone stimulating androgen production by the Leydig cells and the follicle-stimulating hormone acting on the Sertoli cells, the somatic cells that nurture the developing sperm. Knowledge about the mechanisms of spermatogenesis is needed in order to solve the mystery of declining sperm quality in the western countries (Andersson et al. 2008).

5.1 Development

Primordial germ cells migrate from the yolk sac to the posterior body wall where they induce the genital ridges early in the embryogenesis (fifth week in humans; E10.5 dpc in mice). The surrounding epithelium and the mesonephros, a temporary embryonic structure, start invading the genital ridges and form the primitive sex cords with cortical and medullary regions, similar in both sexes at this point. Testis development follows when the sex-determining region of the Y chromosome (SRY) is present and when it is absent, an ovary is formed (Sinclair et al. 1990; Gubbay et al. 1990; Vainio et al. 1999). The SRY protein makes the medulla of the sex cords to differentiate into Sertoli cells that start to produce Anti-Müllerian hormone acting in the surrounding tissues to degenerate the Müllerian ducts that in females form the oviducts. In males, the medullary sex cords (the future seminiferous tubules) connects to the mesonephric tubule that later forms the epididymis (Tilmann and Capel 1999). Simultaneously, at week 9-10 (E13.0 dpc in mice), the SRY causes mesenchymal cells of the gonadal ridge to differentiate into Leydig cells that start expressing genes needed for androgen production (Baker et al. 1999). By weeks 13-15, fetal testosterone levels have reached a high concentration, which is required for seminal vesicles, prostate and bulbourethral glands to develop and the external genitalia to assume the male phenotype. Before birth, the testes descend to the scrotum, a process guided by a ligamentous structure called gubernaculum and highly dependent on androgen action (Bay et al. 2011). Should the descent fail, the testes have to be surgically aided to their correct position to prevent testicular cancers and to preserve fertility, as spermatogenesis, starting at puberty, cannot take place in too high temperature. In mice, the germ cells undergo mitotic arrest at E13.5–E15.5, and they start dividing

again 3–4 days after birth giving rise to the first mature sperm around day 42, when the mice reach puberty (Nel-Themaat et al. 2010). Spermatogenesis in humans is also delayed until puberty, when the testes start producing large amounts of androgens in response to the pulsatile activity of the hypothalamus-pituitary-axis (Nielsen et al. 1986).

5.2 Spermatogenesis

Spermatogenesis is a complex, multistep process where cohorts of diploid cells undergo meiosis resulting in mature sperm cells. This takes place in seminiferous tubules and requires male hormones (Fig. 5A). In mice, the seminiferous tubules are histologically organized into stages I–XII, where germ cells within each layer of the seminiferous epithelium change in synchrony with the other layers over time (Fig. 5B). The spermatogonia are in the outer part of the tubule and as the differentiation proceeds, the cells move towards the center of the lumen. Spermatogenesis is divided into three phases: mitotic phase, meiotic phase and spermiogenesis (Jan et al. 2012).

Mitotic phase. The germ cells at the outer layer of the seminiferous tubules divide mitotically. These spermatogonia divide mitotically giving rise either to new stem cells or differentiating spermatogonia (Yoshida 2012). Cohorts of spermatogonia are recruited simultaneously, and they maintain connections through cellular openings. The differentiating cells divide mitotically several times further and become type B spermatogonia. When they enter the prophase of meiosis, they are called primary spermatocytes.

Meiotic phase. The pre-leptotene spermatocytes duplicate their DNA. In the following leptotene phase, chromatin condenses leading to zygotene spermatocytes, where the homologous chromosomes start pairing. The exchange of genetic material through homologous recombination takes place in the pachytene spermatocytes. The homologous autosomal chromosomes form crossovers while the sex chromosomes are protected from promiscuous recombination in the XY body (Hoyer-Fender 2003). In diplotene spermatocytes, the synaptonemal complex holding the homologous chromosomes together is decomposed, and the cells are ready for the first meiotic division (Page and Hawley 2004). Homologous recombination involves the creation

of DNA DSBs and, therefore, many of the proteins involved in DNA repair are abundant in testis. It has been shown that loss of *Rnf4* that encodes the ubiquitin ligase targeting SUMOylated proteins and essential in DSB repair not only renders mice more sensitive to ionizing radiation but also leads to defects in spermatogenesis (Vyas et al. 2012). The secondary spermatocytes resulting from the first meiotic division immediately enter the second division and become spermatids that contain a haploid genome with either the X or Y chromosome.

Spermiogenesis. During the last phase of spermatogenesis, the spermatids undergo nuclear condensation and formation of the tail and an acrosome that contains the enzymes needed to penetrate the oocyte. The chromosomes obtain a supercondensed state to protect DNA, leading to silencing of the transcription altogether. The protection of DNA at this stage involves a number of epigenetic mechanisms such as non-coding RNAs and post-translational modification of proteins in contact with DNA and direct DNA modifications (Carrell 2012; Hamatani 2012). The histones are first replaced by transition proteins, which soon are replaced by protamines (Meistrich et al. 2003; Yelick et al. 1987). The process where the haploid sperm cells are released into the lumen leaving behind part of its cytoplasm is called spermiation. Finally, the spermatozoa are moved with the help of myotubular cells towards the epididymis for maturation to gain the ability to move and fertilize an egg.

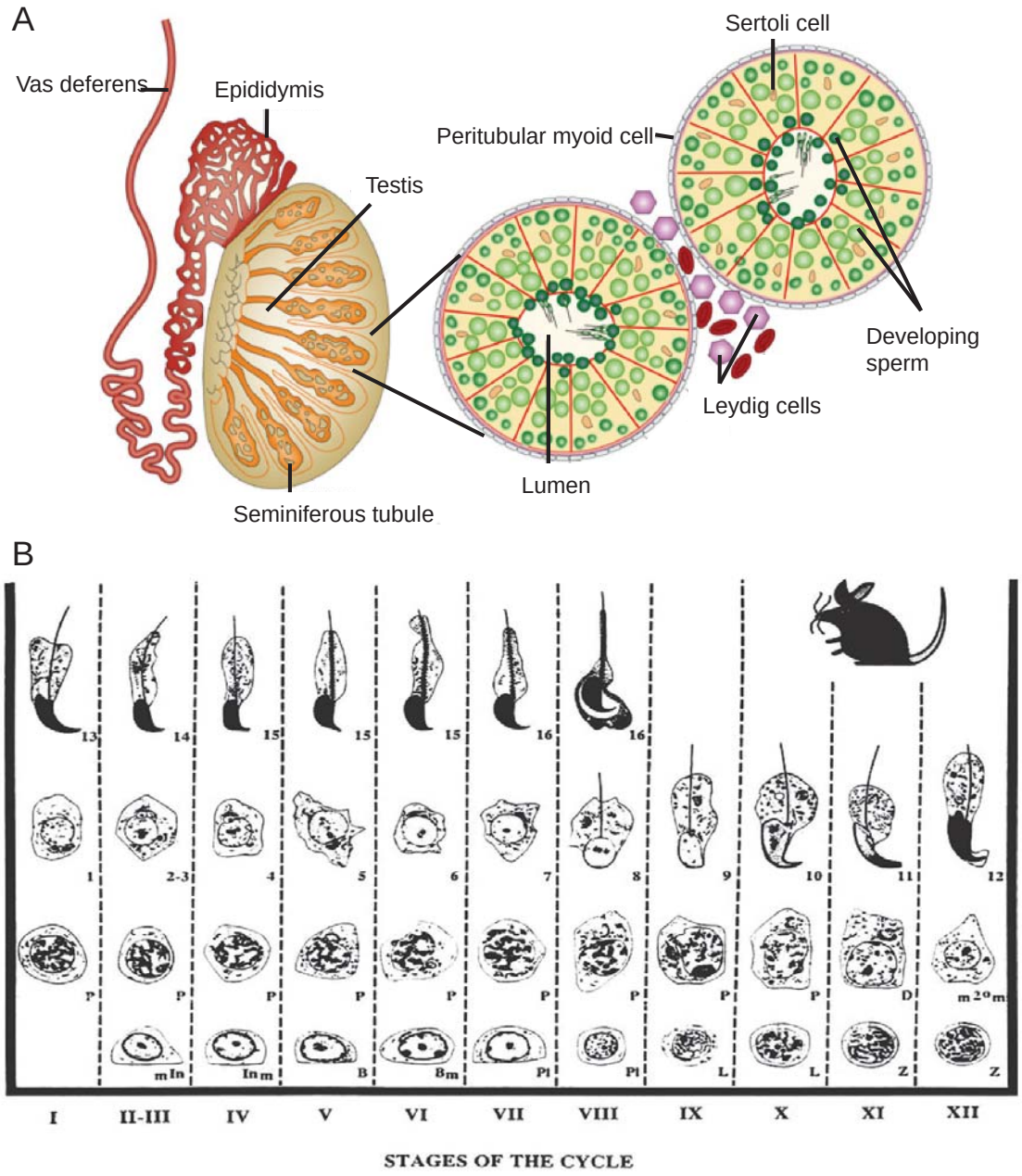

Figure 5. A. Gross anatomy of testis with a zoom into the seminiferous epithelium (Adapted from Cooke and Saunders 2002) B. Stages of the murine spermatogenic cycle as depicted in Russell et al. 1990.

AIMS OF THE STUDY

SUMOylation is an important post-translational modification affecting a plethora of cellular functions ranging from chromosomal stability to regulation of protein–protein interactions. SUMO paralogs share significant functional and structural similarities and are partially able to compensate for each other. This study aimed at understanding the *in vivo* functions of SUMOylation by creating two knock-out mouse lines and analyzing them under different conditions. The following specific aims were addressed:

1. Physiological role of the *Pias2* gene, a SUMO E3 ligase, by gene disruption in mice with emphasis on testis function
2. SUMOylation pathway in spermatogenesis in normal and *Sumo1* null mice
3. General phenotype characterization of the *Sumo1* null mice
4. SUMO-1 in inflammation *in vivo*
5. Importance of SUMO-1 in energy metabolism

MATERIALS AND METHODS

Generation of knock-out mice

Pias2 knock-out mice were generated using the gene-trap method (Wiles et al. 2000; Hansen et al. 2003). Embryonic stem cells (ESC) were electroporated with PT1βgeo gene-trap vector, which contains an En2 splice acceptor site upstream of a β-galactosidase/neomycin-resistance fusion gene (βgeo). Random gene-trap vector integration occurred in the second intron of the *Pias2* gene, as determined by sequencing of the fusion transcript. The insertion of the gene trap in the second intron results in a transcript devoid of exons 3–13 and 3–14 of PIASxα (ARIP3) and PIASxβ (Miz1), respectively (Fig. 6A). PIASxα and PIASxβ are transcripts resulting from alternative splicing at the 3' end of the *Pias2* gene. The mice were backcrossed with C57Bl/6 mice for several generations. *Sumo1* knock-out mice were created using direct gene-targeting. A targeting vector was constructed comprising sequences flanking exons 3, 4 and 5 and selection markers resulting in deletion of exons 3–5 of the *Sumo1* gene from the genome (Fig. 6B). ESCs were electroporated with the targeting vector and colonies surviving the G418 (presence of the positive selection marker Neo) and ganciclovir (absence of the *thymidine kinase* gene outside of the homologous sequence) exposure were screened by PCR and Southern blot analysis, after which the positive cells were used for morula aggregations. The *Sumo1* knock-out mice were analyzed as littermates in a mixed 129Sv/ICR background.

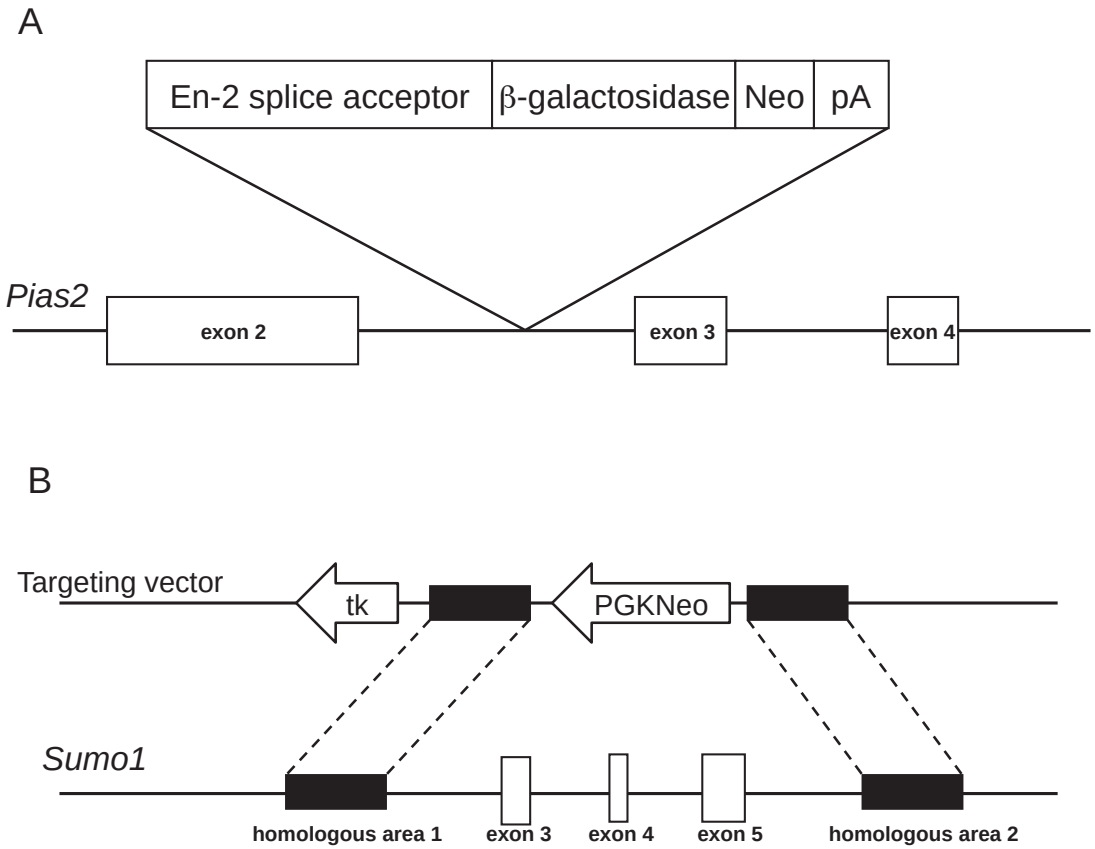

Figure 6. Constructs for generation of the mutant mice A. *Pias2* null mutant B. *Sumo1* null mutant. En-2 = *engrailed 2*, Neo = neomycin resistance gene, pA = SV40 polyadenylation signal, tk = the *thymidine kinase* gene, PGKNeo = phosphoglycerate kinase I (PGK) promoter, neomycin resistance gene (Neo)

Treatments of mice

All mice were handled in accordance with the institutional animal care policy of the University of Helsinki, and the University of Helsinki Review Board for Animal Experiments approved all animal protocols.

High-fat diet

In high-fat diet (HFD) studies, six-week-old wild-type (WT) or *Sumo1* null (KO) mice were fed chow (TD06416) or high-fat diet (TD06414) from Harlan Laboratories (Indianapolis, IN) ad libitum for 17 weeks. Food consumption was based on weekly food weight measurements for 12 weeks. Abdominal fat and lean tissue volumes were

determined by imaging the abdominal area of the mice with MRI using a 4.7 T scanner (PharmaScan, Bruker BioSpin, Germany).

Drug administration

Rosiglitazone (Cayman Chemical, Ann Arbor, MI) was given by oral gavage for 4 or 13 days at a dose of 10 mg/kg/day. LPS (10 mg/kg, Sigma-Aldrich, St. Louis, MO) was given intraperitoneally for 6 h before dissection.

Glucose and insulin tolerance tests

For the intraperitoneal glucose tolerance test, mice were fasted overnight (16 h). Each mouse was given glucose 2 g/kg i.p., and blood glucose concentration was measured from tail vein samples at 0, 15, 30, 60 and 120 min using the OneTouch UltraEasy meter (Life Scan, Milpitas, CA). For the insulin tolerance test, mice were fasted for 4 h. The mice received human insulin (Humulin Regular, Lilly, Indianapolis, IN) 1 U/kg i.p., and blood glucose concentration was determined at 0, 30, 60 and 90 min.

Other measurements

Rectal temperature was measured using the BAT-12 thermometer (Physitemp Instruments, Clifton NJ). Fecal fat was extracted from dried feces by chloroform:methanol (2:1, by vol.), and the extract evaporated to dryness and weighed. After sacrifice of the mice, their gonadal fat pads were dissected and weighed. A small sample was cut, weighed and dispersed with 500 U/ml collagenase in 2% BSA and centrifuged at 1,000 x g for 5 min. DNA content of the adipocytes was measured using the Fluorescent DNA Quantification Kit (Bio-Rad, Hercules, CA).

Cell culture

MEFs were derived from 13.5-day-old WT and *Sumo1* null embryos. After removal of the head and gastrointestinal tract, the embryos were washed with phosphate-buffered saline and minced, and the tissues were placed into a 15-ml conical tube. A total of 5 ml of trypsin solution (0.025% trypsin, 1 mM EDTA) was added to the minced tissues, and cell suspensions were incubated at 37°C for 1 h with stirring. After centrifugation (1,000 rpm, 5 min), the cell pellets were washed twice with and

resuspended in 10 ml of Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum. Single-cell suspensions were plated onto 6-cm dishes that were incubated at 37°C for 2 to 3 days until confluence. MEFs were immortalized by culturing the cells until passage 18.

Quantitative RT-PCR

Total RNA was isolated from cells or tissues using the RNEasy Mini Kit (Qiagen GmbH, Hilden, Germany) or TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. cDNA was synthesized from 1 μg of total RNA using random hexamer primers and Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed with LightCycler® 480 Real-Time PCR System (Roche, Diagnostics, Indianapolis, IN) in 20-μl reactions containing SYBR Green I Master (Roche) and 1 μM forward and reverse primers. PCR reaction included a 5-min denaturation step at 95°C followed by 40 cycles of 10-s denaturation at 95°C, 5-s annealing at 57–60°C, 20-s extension at 72°C and 5-s SYBR Green signal measurement. The results were analyzed with LightCycler analysis software (Roche) and were normalized to 18S rRNA or *Gapdh* mRNA levels.

RNA blotting

Total RNA (10 μg) collected from murine tissues were resolved on 1.2% formaldehyde agarose gels and transferred onto nylon membranes (Hybond-XL; GE Healthcare, Little Chalfont, United Kingdom). The blot was hybridized with a ^{32}P-labeled antisense RNA probe (1.7 x 10^6 cpm/ml) for 2 h at 68°C in ULTRAhyb buffer (Ambion, Austin, TX). After washes with 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) (twice for 5 min at 68°C) and with 0.1 x SSC and 0.1% SDS (twice for 15 min at 68°C), the membrane was exposed to Fuji X-ray film at -70°C for 24 to 72 h.

Histological analyses

WAT and testis samples were fixed immediately after dissection in 4%

paraformaldehyde at 4°C overnight, dehydrated and embedded in paraffin. Five-µm sections were mounted onto Superfrost Plus slides (Menzel GmbH, Braunschweig, Germany), dewaxed with xylene, rehydrated and counterstained using Mayer's hemalum solution (Merck, Darmstadt, Germany). The slides were dehydrated and mounted using Permount (Fisher Chemicals, Fair Lawn, NJ).

In situ hybridization

Sections (5 µm) of testis tissues at the different developmental stages were used for *in situ* hybridization. Sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol. Hybridization with antisense or sense probes was carried out in prehybridization solution containing 10% dextran sulfate and 0.5 x 10^5 to 1 x 10^5 cpm of cRNA probe/ml at 50°C for 4 h. After hybridization, tissue sections were treated for 30 min at 37°C with 10 µg of RNase A/ml, washed twice for 15 min at 50°C with 1 x SSC, and dehydrated in a graded series of ethanol containing 0.3 M ammonium acetate (pH 5.2). The slides were coated with Kodak NTB-3 emulsion (Eastman Kodak Company, Rochester, NY) and stored at 4°C in light-tight boxes for 1 to 3 weeks. After development, the slides were counterstained with hematoxylin.

Immunohistochemistry

Testes from 10-day-old and adult mice were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Sections (5 µm thick) were mounted onto SuperFrost Plus slides (Menzel), dewaxed, and rehydrated. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol. Slides were boiled for 15 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval, washed in Tris-buffered saline (TBS), and blocked in TBS containing 1% bovine serum albumin (Sigma-Aldrich) and 3% normal house or goat serum (Vector Laboratories, Burlingame, CA). Slides were incubated with primary antibody (anti-SUMO-1 [Santa Cruz Biotechnology, Santa Cruz, CA]; anti-SUMO2/3 [ab3742; Abcam, Cambridge, United Kingdom]) overnight at 4°C. After three washes in TBS, biotinylated anti-mouse immunoglobulin G was applied on sections and incubated for 1 h at room temperature. Visualization of the reaction was carried out by using the

Vectastain Elite ABC and peroxidase DAB substrate kits (Vector Laboratories) according to the manufacturer's instructions. Slides were dehydrated in ascending ethanol series and mounted in Permount mounting medium (Fisher Chemicals).

Protein isolation and immunoblotting

Proteins were isolated from cells or tissues using a buffer containing 50 mM Tris-HCl (pH 7.8), 300 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM N-ethylmaleimide (Sigma-Aldrich), and 1 x complete protease inhibitor set (Roche). Lysates were centrifuged, and soluble protein concentration was quantified with the BioRad protein assay. Protein samples (50 μg) were resolved by 10% SDS-PAGE and transferred onto ECL membrane (GE Healthcare). Immunoblotting was performed using rabbit polyclonal PPARγ antibody (81B8, Cell Signaling Technology, Danvers, MA), mouse monoclonal SUMO-1 antibody (SC-5308, Santa Cruz Biotechnology), β-actin antibody (sc-47778, Santa Cruz Biotechnology) or α-tubulin antibody (sc-5286, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG was used as a secondary antibody. ECL reagent (GE Healthcare) was used for visualization.

Immunoprecipitation

For immunoprecipitation experiments, MEFs and tissues were homogenized in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 1% protein inhibitor cocktail and 20 mM N-ethylmaleimide. Homogenates were clarified by centrifugation at 4°C for 10 min at 5,000 rpm and precleared by incubation with 50 μl of GammaBind Sepharose (Amersham Biosciences) for 30 min at 4°C. After centrifugation, the precleared supernatants were incubated with anti-SUMO-1 monoclonal antibody (Santa Cruz Biotechnology), or anti-SUMO-2 MAb (Abnova) overnight at 4°C. After the addition of 50 μl of GammaBind Sepharose, the samples were incubated at 4°C for 1 h. The resin was washed four times, and the pellets were resuspended in 2 x SDS sample buffer. Immunoprecipitated proteins were resolved by SDS–12% polyacrylamide gel electrophoresis (PAGE), and immunoblotting was carried out by using polyclonal anti-RanGAP1 antibody (a gift from Frauke Melchior, Max-Planck Institute for

Biochemistry, Munich, Germany).

Chromatin immunoprecipitation

3T3-L1 cells (10^6 cells) were seeded onto 10-cm dishes and differentiated into adipocytes for 7 days (see below). The cells or livers of LPS-treated WT and *Sumo1* KO mice were cross-linked in 1% formaldehyde, washed with phosphate-buffered saline, collected by scraping, pelleted by centrifugation and lysed in buffer containing 10 mM EDTA, 50 mM Tris-HCl and 1% SDS. Sonicated chromatin samples (200–500 bp in size) were precleared with normal rabbit serum and GammaBind G Sepharose (GE Healthcare) and immunoprecipitated with PPARγ (Santa Cruz Biotechnology), C/EBPα (Santa Cruz Biotechnology), C/EBPβ (Santa Cruz Biotechnology), GPS2 (Santa Cruz Biotechnology), LRH-1 (a gift from Iannis Talianidis, Biomedical Sciences Research Center Alexander Fleming, Vari, Greece) or NCoR (17-10260, Millipore, Billerica, MA) antibodies. Antibody-bound complexes were adsorbed to GammaBind G Sepharose that was sequentially washed with TSE I, TSE II, TSE III, and TE buffers (Kang et al. 2004). DNA was eluted from the matrix with 1% SDS in 0.1 M $NaHCO_3$, cross-linking was reverted at 65°C overnight, and DNA isolated using QIAquick PCR purification system (Qiagen). Input samples were treated the same way except that no immunoprecipitation was performed. qRT-PCR was performed with LightCycler 480 System in 20-μl reactions containing SYBR Green I Master and 1 μM forward and reverse primers for the *aP2* promoter. Input values were used for normalization.

Adipocyte differentiation

Primary MEFs and MSCs were differentiated into adipocytes for 12 days and 3T3-L1 cells for 7 days. Adipogenesis was induced using a mixture comprising 1 μM dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich) and 1 μM rosiglitazone (Cayman Chemical) in DMEM supplemented with 10% FBS and antibiotics. After 48 h, the medium was changed into DMEM containing 10 μg/ml insulin and 1 μM rosiglitazone; this medium was replenished every two days.

Transfections

Immortalized MEFs were transfected with FuGene (Roche) according to the manufacturer's instructions. WT and KO MEFs (250,000 cells in both cases) were seeded onto six-well dishes and transfected next day with 2.7 μg of the PPRE-Luc reporter construct together with 0.15 μg of PPARγ and 0.45 μg of β-galactosidase expression plasmids. Luciferase and β-galactosidase activities were measured from cell pellets after 48 h using the Reporter Lysis Buffer (Promega, Madison WI) and β-galactosidase assays, respectively. The measurements were conducted with a Luminoskan RT reader (Labsystems, Helsinki, Finland).

Statistical analyses

Statistical analyses were calculated with Student's *t*-test from at least three independent experiments or biological replicate samples. The weight gain was analyzed by measuring the area under curve for each individual and comparing the different groups with Student's *t*-test.

RESULTS AND DISCUSSION

1. Disruption of *Pias2* results in smaller testis weight while maintaining fertility through functional redundancy of the *Pias* genes (I)

Pias2 null mice were generated with the gene trap method as part of a large-scale gene trap library experiment (Hansen et al. 2003). Disruption of the *Pias2* gene resulted in viable and fertile mice born in normal Mendelian ratios. Their body weight did not differ from that of their littermates. However, the mice exhibited reduced testis weight by 23% and decreased sperm quantity, although the quality of the sperm was considered normal (Fig. 7A). The number of apoptotic cells, as measured by TUNEL staining, was increased by 74% at all stages of spermatogenesis. This is interesting, since *Piasxa* mRNA, a splice variant of the *Pias2* gene, is very abundantly and almost exclusively expressed in Sertoli cells and developing spermatogonia and spermatocytes (Moilanen et al. 1999; Yan et al. 2003). The mice had normal circulating levels of FSH and LH, hormones that stimulate Sertoli cells and Leydig cells, respectively, and the intratesticular testosterone concentrations were normal, not explaining the observed phenotype. In search of compensatory changes in gene expression of other members of the gene family, *Pias1*, *Pias3* and *Piasy* mRNA levels were measured by quantitative real-time PCR (qRT-PCR): *Pias1* and *Pias3* mRNA levels did not change, whereas *Piasy* mRNA level was reduced by 30% (Fig. 7B). AR was the first NR shown to be SUMOylated and SUMOylation was found to decrease its transcriptional activation (Poukka et. al 2000). Therefore, accumulation of *Pem* mRNA encoded by the Sertoli cell-specific, highly AR-dependent gene, *Pem* (Lindsey and Wilkinson 1996), was studied and found to be elevated, possibly resulting from decreased inhibition of AR function by SUMO (Fig. 7B).

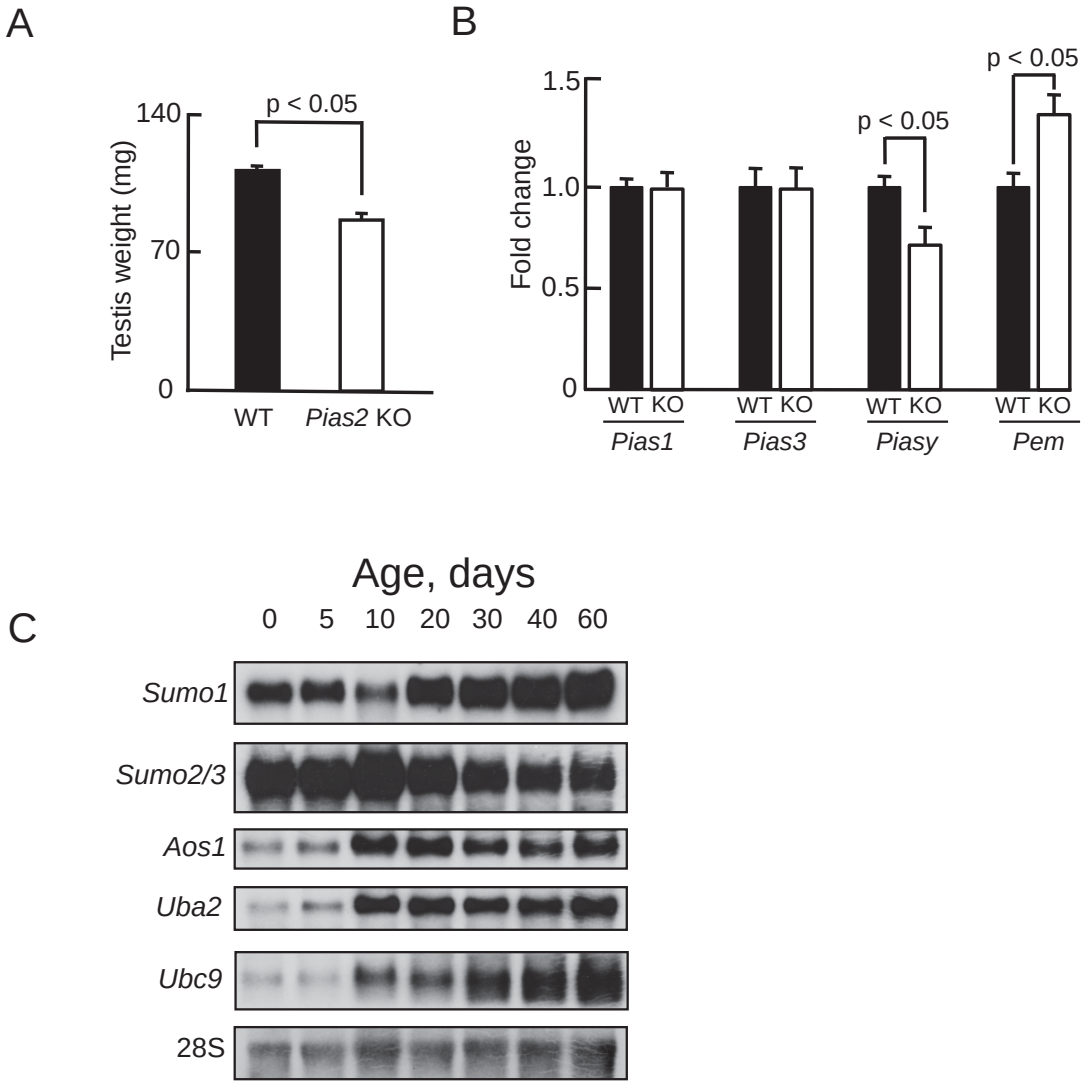

Figure 7. Testicular expression of genes related to SUMOylation. A. Testis weight of adult WT and *Pias2* KO mice B. *Pias* and *Pem* mRNA levels in WT and *Pias2* KO testis C. mRNA levels encoded by the SUMOylation pathway genes in WT postnatal testis.

2. Genes of the SUMOylation pathway are expressed in different parts of the developing and adult testis (II)

On the basis of the studies in the *Pias2* null mice, SUMOylation was suspected to have an important role in testicular function. Since SUMOylation has been shown to regulate chromosomal stability in cell division (Takahashi et al. 2006; Dawlaty et al. 2008), the expression of different genes belonging to the SUMOylation pathway in a tissue with abundant cell division activity and high demand for accuracy in

chromosomal segregation, the testis, was found interesting. To this end, the role of SUMOylation in adult and developing murine testis was studied by *in situ* hybridization, RNA blotting, qRT-PCR and immunohistochemistry. During development, expression of *Sumo1*, *Sae1* (AOS1), *Sae2* (UBA2), and *Ubc9* increased with age starting from very low in newborn testes. By contrast, *Sumo2/3* levels were high in the newborn testes and diminished with age, with the levels dropping around day 10 postnatally (Fig. 7C). This likely reflects a dilution effect, as the number of germ cells begins to increase at that time, and the first pachytene spermatocytes appear around that time. *In situ* hybridization analyses of the adult testis revealed that *Sumo1* mRNA was present in all germ cells except elongating spermatids, whereas the presence of *Sumo2/3* mRNA was restricted to pachytene spermatocytes. It is important to note that the probe used could not distinguish between *Sumo2* and *Sumo3* mRNAs. *Sae1*, *Sae2* and *Ubc9* were expressed to a moderate level in the germ cells, and all of the genes that were studied (*Sumo1-3*, *Sae1-2 and Ubc9*) were expressed in the Sertoli cells or Leydig cells at low levels. At the protein level, SUMO-1 and SUMO-2/3 antigens appeared to be concentrated in the pachytene spermatocytes in a structure called XY body (also known as the sex vesicle), a subnuclear compartment involved in sex chromosome silencing (Hoyer-Fender 2003) in both developing (day 10) and adult testis (Fig. 8A, left panel and II). The results showed that the different genes in the SUMOylation pathway have distinct expression patterns and they are involved in sperm production.

Studies on human testis by another group reported a decrease in SUMO levels in infertile men and showed the presence of SUMO-1 in the XY body in human spermatocytes (Vigodner et al. 2006). Also studies from human testicular biopsies confirmed the presence of SUMO-1–3 in the germ cells and showed a role for SUMO in the chromosome maintenance through modification of the synaptonemal complex that holds the homologous chromosomes together in meiosis I (Brown et al. 2008). The results from this study complement these previous studies by showing expression of the genes of the SUMO pathway primarily in the spermatocytes undergoing meiosis I. The testes of *Pias2* null mice with an increased number of apoptotic cells may be due to chromosomal disturbances resulting from disturbed functions of the SUMOylation machinery, possibly combined to SUMO-independent functions of PIAS2.

3. Sumo1 KO mice are viable and fertile (II)

Sumo1 null mice were generated to study the *in vivo* role of SUMOylation. The overall phenotype of the mice was apparently normal, and the mice were sent to the German Mouse Clinic, a facility to screen the phenotypes of mutant mouse lines. The gross anatomy did not reveal any differences between the phenotypes, and tests carried out at the German Mouse Clinic showed no difference in different organ systems and their functions. *Sumo1* null mice were born according to the expected Mendelian ratios, and they were viable and fertile.

There is a previous report in the literature claiming that *Sumo1* haploinsufficiency leads to cleft lip and palate (CLP) (Alkuraya et al. 2006). Therefore, the fusion of the palate was carefully studied, but no CLP in the *Sumo1* knock-out mice was detected throughout embryonic development. The secondary palate was not totally closed at E15, but there was no clear morphological difference among $Sumo1^{-/-}$, $Sumo1^{+/-}$, and $Sumo1^{+/+}$ embryos. At E18.5, the secondary palate was properly fused in 43 of 44 embryos examined; the only cleft palate was detected in a WT embryo. Serial coronal sections of homozygous and heterozygous *Sumo-1* embryos at E18.5 confirmed that the closure of the palate was complete. This discrepancy between the previous work (Alkuraya et al. 2006) and our study (article II) may be explained by the different background of the mice, but this explanation seems unlikely, since another group reported in agreement with our findings that *Sumo1* knock-out mice do not develop CLP and the mice are viable (Evdokimov et al. 2008). They even revived mice from the same gene-trap cell line used by Alkuraya et al., but failed to reproduce the results and detected normal amounts of SUMO-1 protein in the mice due to genomic rearrangements. There are reports on *Sumo1* polymorphisms or deletions causing CLP in humans (Alkuraya et al. 2006; Song et al. 2008; Shi et al. 2009), but experiments in mice have failed to show any evidence for the *Sumo1* deletion directly causing CLP. However, this does not exclude *Sumo1* from being a contributing gene in a multifactorial disease.

Since the SUMO-1 protein was shown to localize to the XY body, the testes of the *Sumo1* null mice were studied in more detail. Immunohistochemistry showed no

signal with SUMO-1 antibody in the testes, confirming the lack of SUMO-1 protein in these mice. Interestingly, also SUMO-2/3 localized to the sex vesicle both in WT and *Sumo1* null testes, indicating a compensatory mechanism for the loss of *Sumo1* by *Sumo2* and *Sumo3* (Fig. 8A). However, *Sumo2* or *Sumo3* mRNA levels, as studied by qRT-PCR from testicular mRNA, were not changed, suggesting that *Sumo2* and *Sumo3* are expressed abundantly in WT mice and no further compensatory mechanisms are needed. Immunoblotting of testicular lysates with anti-SUMO-1 antibody revealed one major SUMOylated band with a molecular mass of 90 kDa in WT but not in *Sumo1* null lysates assumed to be the SUMOylated RanGAP1 (Fig. 8B). In addition, a band with the molecular size of free SUMO-1 was visible, especially after a longer exposure time in both testis and MEF lysates of WT but not of *Sumo1* null mice.

MEFs generated from *Sumo1* null embryos proliferated to a similar degree to WT MEFs. Immunoblotting of lysates from MEFs with anti-SUMO-1 antibody showed a similar pattern with testis, having one major band at 90 kDa. Immunoblotting of MEF and testis lysates with anti-SUMO-2 antibody revealed not only the presence of a band corresponding to the molecular size of free SUMO-2 but also a more intensively stained 90-kDa band in *Sumo1*-null cells than in WT cells (Fig. 8C and II). RanGAP1 is the most abundant cellular protein modified by SUMO-1 conjugation (Müller at al. 2001; Matunis et al. 1996). To confirm that the 90-kDa band seen in testis and MEF lysate blots corresponds to the SUMOylated form of RanGAP1, anti-RanGAP1 antibody was used on the same blot and further, proteins from testis and MEFs were immunoprecipitated with SUMO-1 and SUMO-2/3 antibodies. The SUMOylated RanGAP1 band was present in WT but not in *Sumo1* null MEFs after immunoprecipitation with anti-SUMO-1 antibody. In line with the immunoblotting experiments, the samples immunoprecipitated with SUMO-2/3 antibody showed a stronger band in *Sumo1* null MEFs, suggesting that in WT cells, a only a small fraction of SUMOylation of RanGAP1 is represented by SUMO-2/3 conjugation. A similar conclusion was also reached in quantitative proteomics analyses of HeLa cells expressing His6-tagged SUMO-1 and SUMO-2 (Vertegaal et al. 2006). Later, it was suggested that RanGAP1 is equally modified by SUMO-1 and SUMO-2 but the SUMO-1:RanGAP1 would be more stably protected against deconjugation (Zhu et al. 2009). In view of this, there could be some compensation by the deconjugation

enzyme machinery to allow RanGAP1 – and possibly other proteins – to be more abundantly SUMOylated by SUMO-2 in the absence of SUMO-1.

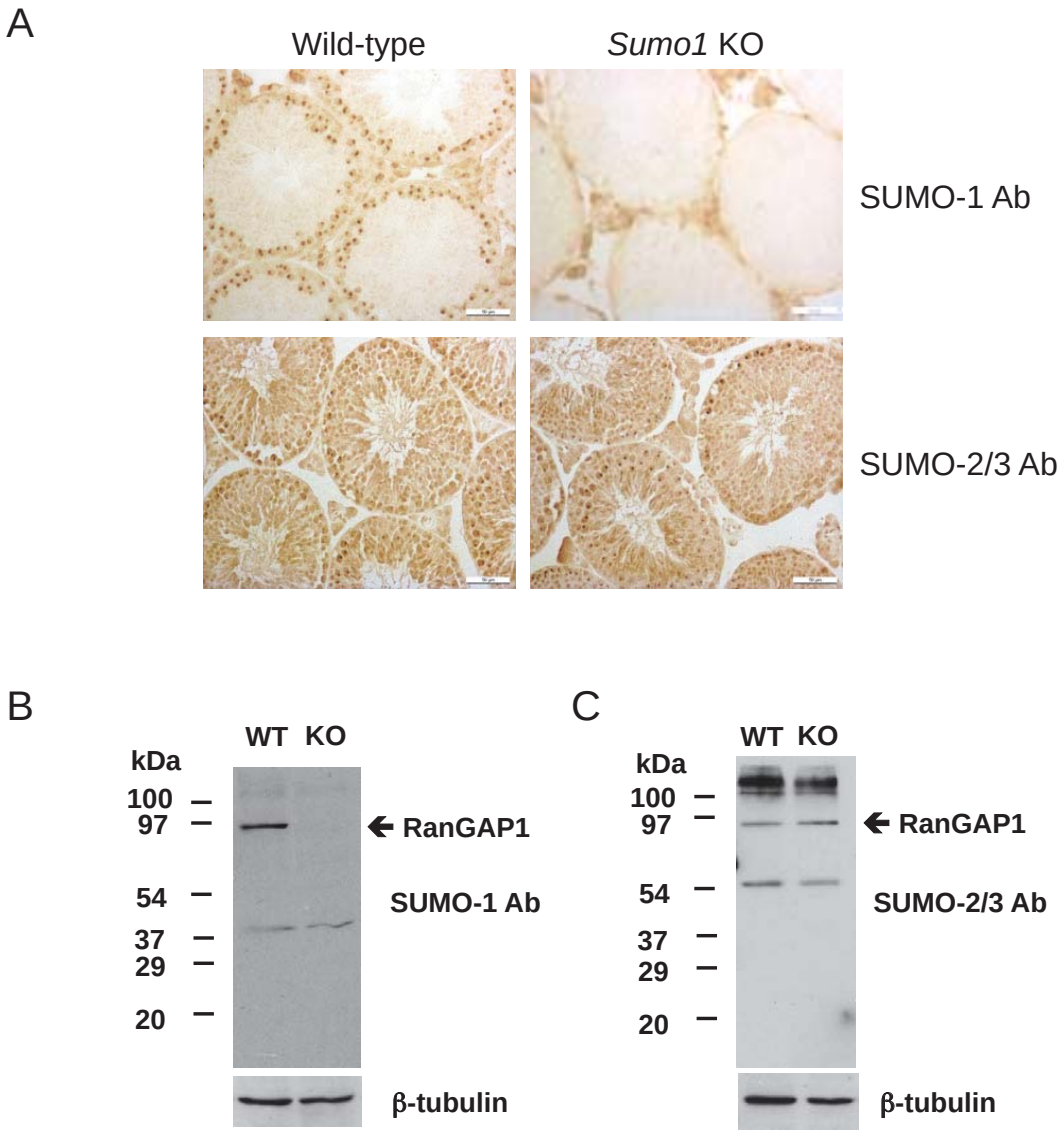

Figure 8. Compensation of SUMO-1 by SUMO-2/3. A. Testes of WT and *Sumo1* null mice were stained with SUMO-1 and SUMO-2/3 antibodies. B and C. Immunoblotting of WT and *Sumo1* KO MEF lysates with SUMO-1 (B) and SUMO-2/3 antibodies (C).

4. *Sumo1* KO mice have an exaggerated acute phase response upon LPS stimulation (unpublished results and III)

Previous experiments have indicated that SUMOylation plays a role in inflammatory responses by decreasing the expression of several inflammatory genes. Reports from *in vitro* experiments suggest that PPARγ prevents the clearance of corepressors from the promoters of inflammatory genes when SUMOylated by SUMO-1 (Pascual et al. 2005; Ghisletti et al. 2007). To test this hypothesis *in vivo*, the mice were challenged with 10 mg/kg LPS i.p. for 6 h to induce a mild general inflammatory response. The results showed that *Sumo1* null mice have elevated levels of TNFα in their serum after the LPS exposure compared to WT mice (Fig. 9A). Likewise, *Tnfα* mRNA levels measured from spleens of *Sumo1* null mice were elevated in comparison to those of WT spleens (Fig. 9B). Ghisletti et al. (2007) further showed in macrophages that a subset of genes were specific to PPARγ-SUMO-1 (including *Tnfa*), whereas some genes belonged to the LXR-SUMO-2 pathway (*Il1b*) and some genes were affected by both pathways (*Nos2*). Experiments carried out in *Sumo1* KO mice confirmed these results by showing elevated *Tnfa* mRNA levels in the spleen but normal levels of *Il1b* and *Nos2* mRNAs upon LPS challenge (Fig. 9C-D). The expression of *Nos2* showed a trend, but not statistical significance of upregulation in *Sumo1* null mice. This was the first evidence that some SUMO-1 functions cannot be compensated for by other SUMO paralogs.

LRH-1 attenuates the hepatic acute phase response (APR) together with the well-established metabolic regulator LXR (Venteclef et al. 2006). Since the transrepression capacity of LXR had been shown to involve conjugation by SUMO-2/3, and the transrepression by PPARγ was SUMO-1 specific in macrophages (Ghisletti et al. 2007), we were interested in the generality of SUMOylation in the regulation of NRs in transrepression. To investigate the roles of LXRs, LRH-1 and SUMO in hepatic APR *in vivo*, *Sumo1* null mice were treated with 10 mg/kg LPS i.p., and expression levels of hepatic acute phase genes were analyzed by qPCR. The inflammatory response was significantly more pronounced in *Sumo1* KO mice than in WT mice, as shown by increased *haptoglobin*, *SAA* and *CRP* mRNA levels in liver. However, the LPS-mediated expression of *Pai1*, which is not repressed by LRH-1 or LXR, was

indistinguishable between *Sumo1* KO and WT mice. Because the absence of SUMOylated NRs on the inflammatory gene promoters in *Sumo1* KO mice could explain the exacerbated inflammatory response, recruitment of NCoR, GPS2, SUMO-1, SUMO-2/3, LRH-1, LXRs, and PPARα onto the *haptoglobin* promoter was examined. In WT mice, LRH-1 and SUMO-1 were recruited onto the *haptoglobin* promoter five-fold and four-fold compared to IgG, respectively, suggesting that SUMO-1-conjugated LRH-1 interacts with the NCoR complex already in the absence of inflammatory stimuli. LPS treatment induced partial dissociation of NCoR and GPS2, but not of SUMO-1 and LRH-1 (Fig. 9E, left panel). In contrast, LRH-1 was not recruited onto the *haptoglobin* promoter in *Sumo1* KO mice, demonstrating the importance of SUMO-1 conjugation for the recruitment of LRH-1 *in vivo,* and LPS challenge induced a more complete clearance of the NCoR complex from the promoter in *Sumo1* KO mice in comparison with WT mice (Fig. 9F, right panel). Expression levels of endogenous LRH-1 target genes (*Shp* and *Cyp7a1* mRNAs), as well as LRH-1 recruitment to their promoters, were also increased in *Sumo1* null mice, indicating that SUMO-1 modification influences LRH-1 repression of direct target genes as well. Collectively, these data indicate that SUMOylated LRH-1 interacts with the NCoR/GPS2 complex, and prevents its dismissal from the *haptoglobin* promoter *in vivo*.

This study also determined the SUMOylation status of LXRs in suppressing the hepatic APR. Evidence from *Lxra* and *Lxrb* null mice confirmed that transrepression of the *haptoglobin* gene in liver was LXRβ-specific. Further, it was shown that LXRβ co-occupied the promoter with HDAC4 and SUMO-2/3. Accordingly, no significant differences were detected regarding the recruitment of LXRs onto the *haptoglobin* promoter in the liver of *Sumo1* null mice, providing support for the specificity of SUMO-1 and LRH-1. However, GPS2 was shown to mediate NR-, ligand- and SUMOylation-dependent interaction of both LXRβ and LRH-1 in liver. It remains to be determined whether GPS2 is the universal SUMO-NR sensor of the transrepression complex or whether each tissue has their specific mechanisms. In macrophages, GPS2 was not detected on *Nos2* or *Il1b* promoters, although LXRs were present, suggesting that other mechanisms exist (Huang et al. 2011).

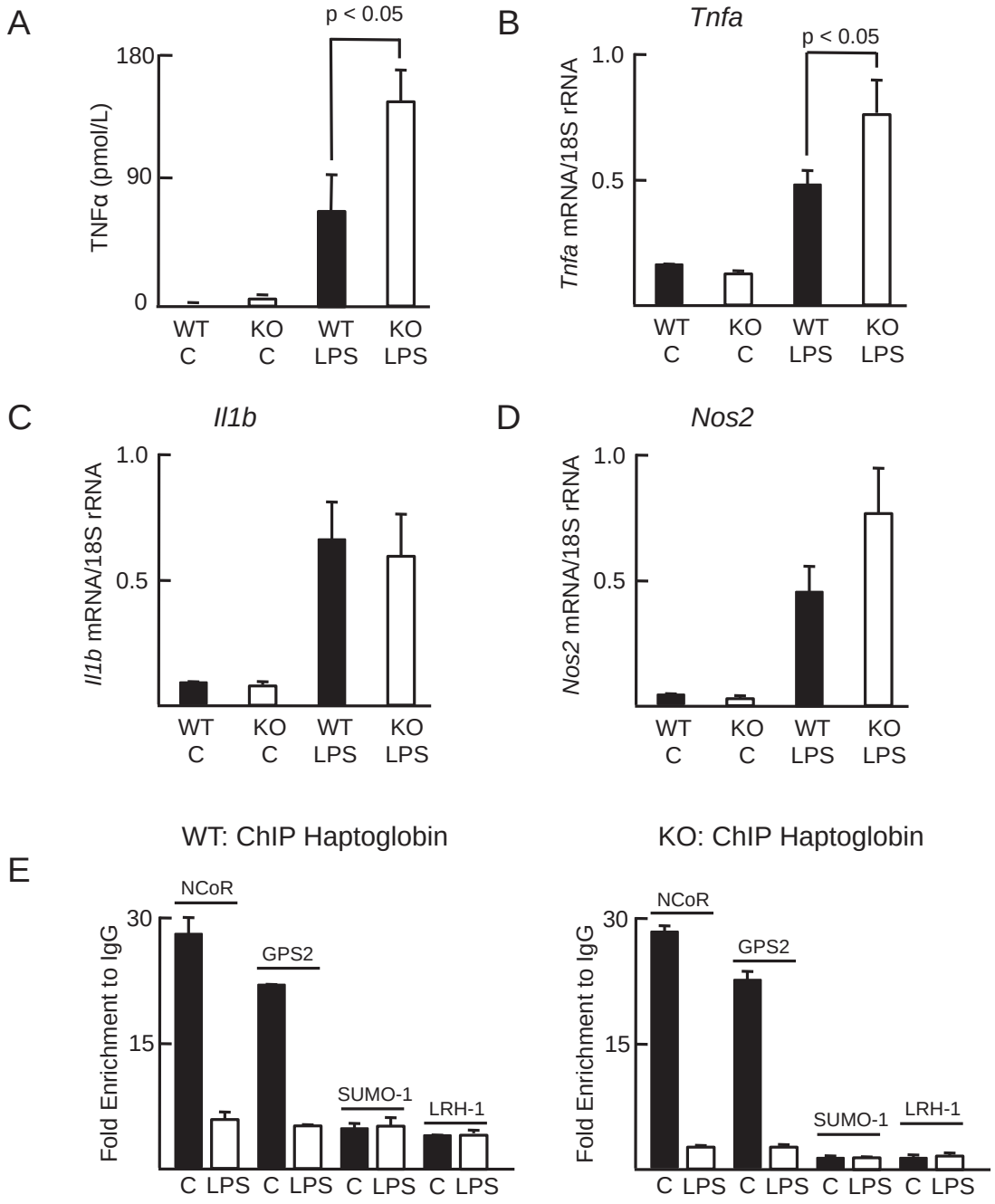

Figure 9. Inflammatory response of *Sumo1* null mice upon LPS treatment for 6 h. A. Serum levels of TNFα. B–D. mRNA levels of *Tnfa* (B), *Il1b* (C) and *Nos2* (D) in spleen. E–F. Loading of the repressor complex onto the *haptoglobin* promoter in the liver of WT (E) and *Sumo1* KO (F) mice. The results are expressed as mean + SEM. WT = wild type, KO = *Sumo1* knock-out, C = control, LPS = lipopolysaccharide, ChIP = chromatin immunoprecipitation

5. Loss of *Sumo1* impairs adipogenesis and activation functions of PPARγ (II, IV)

It has been suggested that PPARγ is specifically SUMOylated by SUMO-1 in transrepression (Ghisletti *et al*. 2007). As PPARγ is the master regulator of adipose tissue and the protein indispensable for adipogenesis directly regulating many genes involved in the adipogenic program (Rosen et al. 1999; Nielsen et al. 2008), the ability of *Sumo1* null MEFs to differentiate into adipocytes was studied. Surprisingly, no difference was observed between *Sumo1* null and WT MEFs as measured by Oil Red O staining of the adipocytes and by qPCR analysis of expression of adipocyte marker genes, *Pparg* or *aP2,* after a 8-day differentiation protocol. However, when a longer adipogenic protocol (12 days) was used, there was a clear inhibition of adipogenesis in the absence of SUMO-1, as judged by Oil Red O staining of lipids and expression of four adipocyte marker genes, *aP2*, *Pparg*, *Adipoq* and *Lpl*, suggesting that lack of SUMOylation attenuates terminal differentiation of adipocytes more than early adipogenesis (Fig. 10A-B, II, IV). As was noted previously in MEFs and testes (II), *Sumo2* or *Sumo3* mRNA levels were not up-regulated in *Sumo1* KO cells, leaving the possibility that there may be some compensation between the paralogs at the protein level. Since the efficiency of adipocyte differentiation using MEFs was quite low, the results were repeated using bone marrow MSCs of *Sumo1* null and WT mice. To study the mechanisms further in a well-established model system, a SUMO-1-depleted 3T3-L1 cell line was created by using lentivirus-mediated expression of shRNA to target *Sumo1* mRNA, and a cell line with a *Sumo1* mRNA depletion of ~80% was chosen for subsequent experiments. Expectedly, the control cells differentiated into adipocytes more efficiently than SUMO-1-depleted 3T3-L1 cells, showing a 50% reduction in *aP2* mRNA level. Collectively, these results from MEFs, MSCs and 3T3-L1 cells confirmed that SUMO-1 function is important for adipogenesis. Another group has shown that knock-down of *Ubc9* in 3T3-L1 cells also results in diminished adipogenic efficiency (Cignarelli et al. 2010). However, as UBC9 is the E2 enzyme for all SUMO paralogs, it is possible that lower levels of overall SUMOylation affect multiple cellular functions and cell viability. There is also evidence that deSUMOylation is important for adipogenesis, as SENP2

was found essential for adipogenesis and maintenance of C/EBPβ levels (Chung et al. 2010).

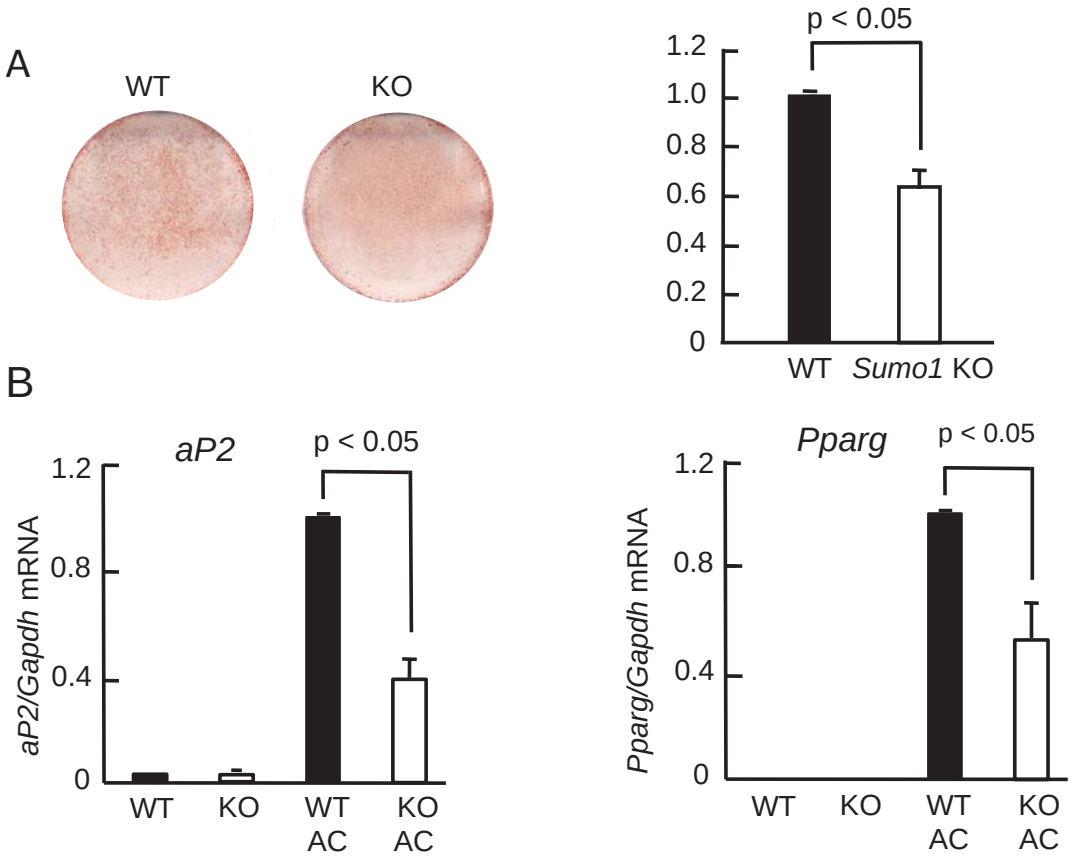

Figure 10. Adipogenesis from WT and *Sumo1* null MEFs. A. Oil Red O staining of adipocytes and their quantification. B and C. mRNA levels of two adipocyte markers, *aP2* and *Pparg*.

Reports in literature show that mutation of the SUMOylation site Lys107 in PPARγ *in vitro* leads to diminished gene activation function of the receptor (Hu et al. 1996; Ohshima et al. 2004; Yamashita et al. 2004; Floyd and Stephens 2004). Therefore, the expression of PPARγ target genes, *Plin1* and *Fsp27,* was studied and found to be lower in *Sumo1* null than WT adipocytes differentiated from MEFs, in addition to *aP2* and *Pparg* mRNA levels. It is known that PPARγ forms an autoregulatory loop

where it, together with C/EBPα, upregulates its own expression levels (Wu et al. 1999). Since PPARγ1 and PPARγ2 protein levels were also lower in mature adipocytes differentiated from *Sumo1* null than from WT MEFs, this could partly explain the attenuated expression of PPARγ target genes. To circumvent this potential caveat, PPARγ protein was transiently expressed together with a PPARγ response element (PPRE) reporter in immortalized *Sumo1* null and WT MEFs. Transactivation of the reporter gene by PPARγ was significantly attenuated in *Sumo1* null MEFs compared to WT MEFs, indicating that SUMO-1 is indeed required for optimal transactivation function of PPARγ. To assess whether this was related to PPARγ binding to chromatin, ChIP assays were used to examine loading of PPARγ onto the PPRE of the *aP2* promoter in 3T3-L1 cells differentiated into adipocytes. There was no difference in the loading of PPARγ onto the *aP2* promoter between control and SUMO-1-depleted 3T3-L1 cells, despite the fact that *aP2* mRNA levels were decreased in SUMO-1-depleted cells and the PPARγ levels did not differ.

MEFs require the presence of PPARγ agonist for efficient adipogenesis. To assess the responsiveness of the cells to agonist treatment, rosiglitazone was omitted from the MEF adipocyte differentiation medium after four days to be reintroduced after a five-day pause. In control cells, with no rosiglitazone, *aP2* mRNA levels did not differ between KO and WT MEFs. However, when rosiglitazone was reintroduced, *aP2* mRNA accumulation was significantly higher in WT than in *Sumo1* null MEFs (Fig. 11A). These results in cultured cells suggested that the function of ligand-occupied PPARγ is attenuated in the absence of SUMO-1. To test this *in vivo*, *Sumo1* null mice and their WT littermates were treated with rosiglitazone (10 mg/kg). Increased *Ucp1* mRNA accumulation was clearly observed after the 13-day rosiglitazone treatment in BAT of WT mice but not of KO mice, and a similar trend was seen in WAT (Fig. 11B). The treatment also brought about a significant increase in *Sumo1* mRNA level in WT BAT but, interestingly, not in WAT.

The finding that the transactivation function of PPARγ and responses to agonist treatment required SUMO-1 both *in vitro* and *in vivo* was not predictable, since mutation of the SUMOylation site has been reported to activate PPARγ function *in vitro* (Hu et al. 1996; Ohshima et al. 2004; Yamashita et al. 2004; Floyd and Stephens

2004). By and large, SUMOylation of NRs has been associated with repression of the NR function (Pourcet et al. 2010; Poukka et al. 2000; Sentis et al. 2005). However, in thyroid hormone receptor (TR) function, SUMO-1 is required for ligand-induced recruitment of the co-activator CREB-binding protein (CBP) and release of nuclear receptor co-repressor (NCoR) on a response element, but SUMOylation had no significant effect on TR DNA binding (Liu et al. 2012c). It is likely that SUMO-1 requirement for turning on *aP2* expression (and possibly that of other genes) is not related to the SUMOylation of PPARγ itself, but is needed for binding of a coactivator or exclusion of a corepressor. Furthermore, it is important to study each promoter in each cell type separately before drawing generalized conclusions. Genome-wide techniques will be essential in determining the binding sites of PPARγ and its coregulators under SUMO-less conditions to examine whether the mechanism determined at the *aP2* promoter apply to other sites as well.

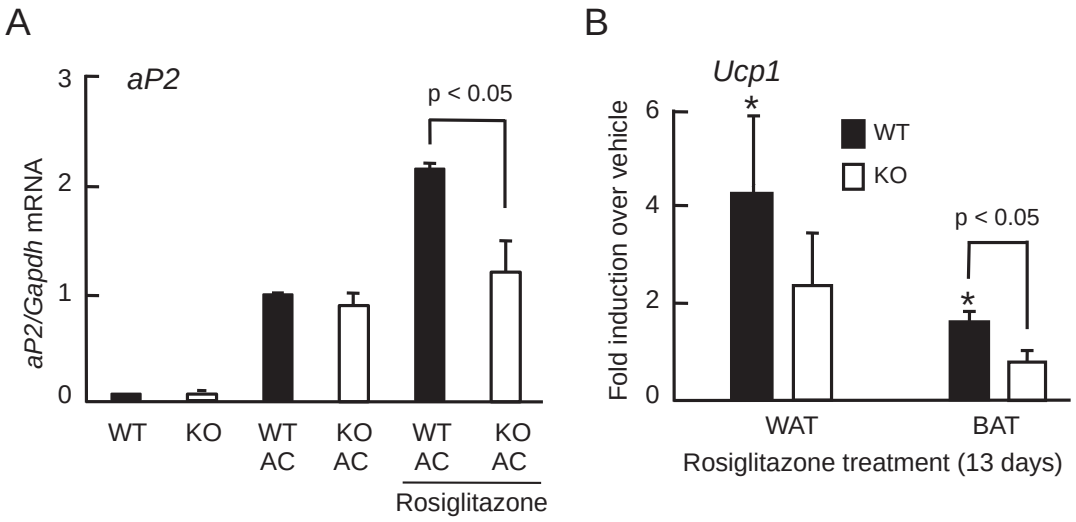

Figure 11. PPARγ ligand responsiveness in the absence of SUMO-1 A. Effects of rosiglitazone on adipocyte differentiation of MEFs. After four days of differentiation, rosiglitazone was omitted from the medium for five days and reintroduced for two days. *aP2* mRNA levels were measured with qPCR. B. WT and *Sumo1* null mice were treated with rosiglitazone. Expression of *Ucp1*, a PPARγ target gene, was measured from WAT and BAT. WT = wild type, KO = *Sumo1* knock-out, AC = adipocyte, WAT = white adipose tissue, BAT = brown adipose tissue, * = $p < 0.05$ WT rosiglitazone vs. WT vehicle.

6. *Sumo1* null mice are protected from obesity on high-fat diet (IV)

As SUMO-1 was shown to play a role in adipogenesis *in vitro* and rosiglitazone treatment of *Sumo1* null mice suggested that activation functions of PPARγ would be SUMO-1-specific *in vivo*, *Sumo1* null mice were subjected to high-fat diet (HFD). HFD normally results in weight gain and expansion of WAT, both thought to require PPARγ activity, as adipose tissue expansion and weight gain are major side-effects in patients receiving rosiglitazone treatment (Cariou et al. 2012). The HFD, containing 60% of the calories from fat, was found to result in lower weight-gain in KO than WT mice, both in males and females (Fig. 12A and IV). MRI studies indicated that this weight-gain was due to limited accumulation of WAT in the KO mice, whereas the lean mass did not differ between the phenotypes. *Sumo1* null mice consumed a smaller amount of food per mouse than WT mice. However, this cannot explain the phenotype entirely, as the food consumption normalized to body weight did not differ between KO and WT mice.

Weight gain is dependent on calorie consumption, food absorption, and burning of the calories. There was no difference in fat absorption from the gut, as judged by a similar fecal fat content in WT and *Sumo1* null mice. BAT depots were therefore examined more carefully, as BAT is a specialized tissue where the respiratory chain is partially uncoupled from ATP production by uncoupling proteins (UCPs) thus generating heat. However, BAT appeared indistinguishable between the two phenotypes histologically, the weight of the interscapular BAT depots was similar, and the expression of *Ucp1* or *Pgc1α* did not differ between the two phenotypes. *Sumo1* null and WT mice showed no difference in rectal temperature, implying that their overall BAT functions were not significantly different.

In obesity, WAT expands both by hypertrophy and hyperplasia. When the capacity of the WAT to store FA reaches its limit, the tissue starts to secrete inflammatory signals, which causes whole body insulin resistance. The gonadal fat pad was chosen for closer examination, because it represents metabolically relevant visceral fat depots. Gonadal fat consists mainly of white adipocytes and is not easily converted into beige cells, brown-like adipocytes that have been shown to emerge from inguinal white adipose tissue (Fisher et al. 2012). There was a significantly decreased amount

of DNA in fad pads of *Sumo1* null mice on HFD, reflecting a reduced adipocyte number. The amount of DNA in fat pads on control diet was similar between the two phenotypes, suggesting that the expansion of the depot was disturbed in KO mice (Fig. 12B). The adipocytes in the gonadal fat depot were also smaller in *Sumo1* KO than WT mice on HFD (Fig. 12C), implying that both hyperplasia and hypertrophy are dysregulated in the *Sumo1* null mice. Taken together, the results obtained from the HFD experiments confirm that the disturbances in adipogenesis seen in *Sumo1* depleted cells also apply to *in vivo* conditions by preventing the expansion of WAT in obesity.

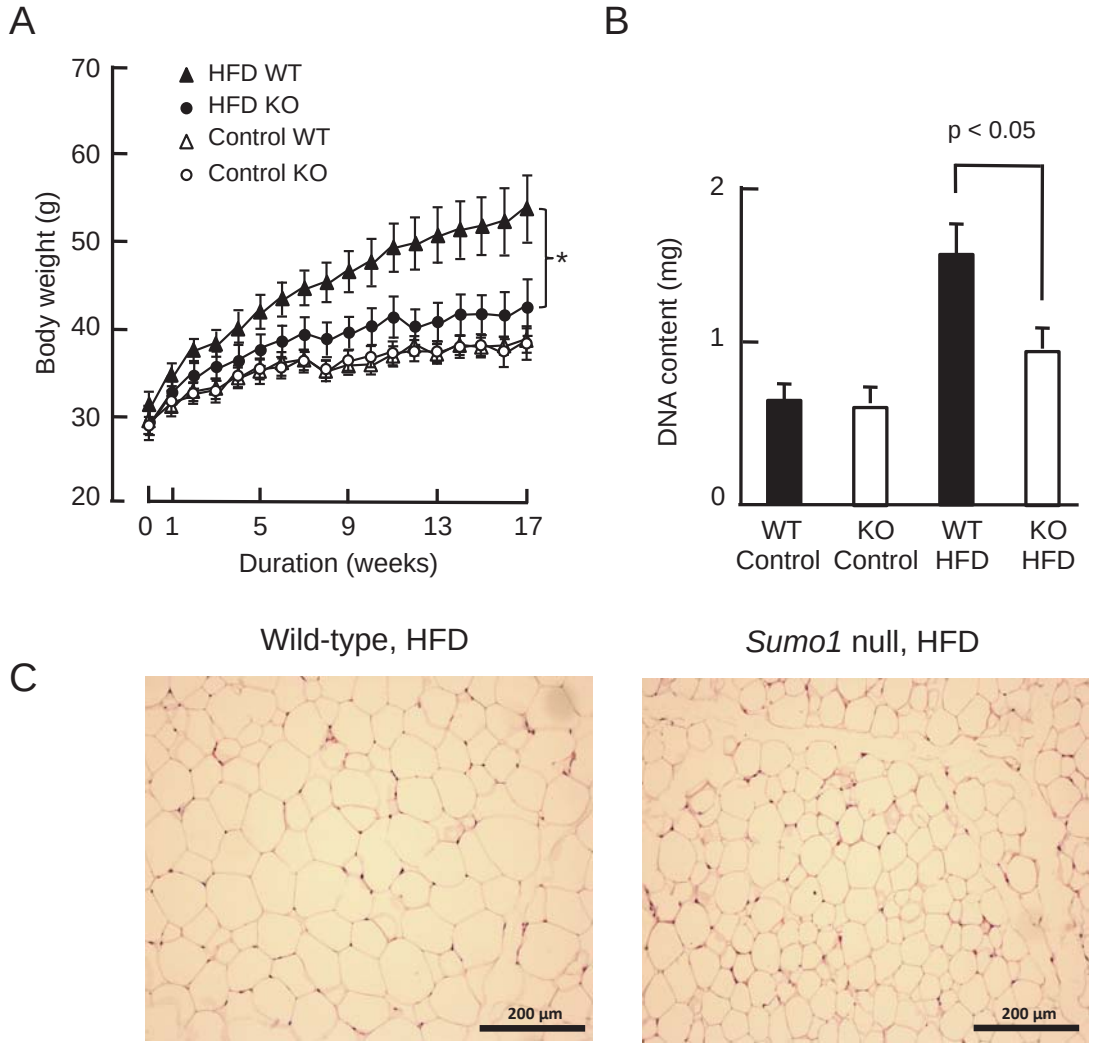

Figure 12. WT and *Sumo1* null mice on HFD. A. Weight gain. B. Quantification of DNA from gonadal WAT. C. Histology of WAT. WT = wild-type, KO = *Sumo1* null, HFD = high-fat diet.

The HFD experiments were carried out with mice lacking SUMO-1 in all tissues at all times. Therefore, it is possible that some of the consequences detected in WAT are modulated by other tissues or are due to developmental defects. It has been shown that many outcomes of TZD treatment are due to PPARγ action in brain (Lu et al. 2012), which may as well be affected by the lack of SUMO-1, but this phenomenon not explored in the present study. Furthermore, since inflammation accounts for many of the adverse effects of the metabolic syndrome and aggravates insulin resistance, the inflammatory status of WAT and other tissues is likely to add one more piece to the complex puzzle pertaining to the effects of SUMO-1 in metabolism.

FUTURE PERSPECTIVES

This study established SUMOylation as an essential modulator of the *in vivo* responses to inflammation and obesity, and maintenance of normal testicular architecture. The thesis work used two knock-out mouse lines, lacking *Sumo1* or *Pias2* from all cells of the body at all developmental stages. The gene-targeted mice were viable and fertile, although specific functions for each gene was found in this study, that is, PIAS2 is required for normal testicular volume and SUMO-1 is a regulator of LRH-1 in liver upon LPS-induced inflammation and of PPARγ in WAT on HFD and adipogenesis. As SUMOylation is indispensable for life, the different members of the SUMO pathway must exhibit significant redundancy in most functions. It is also important to note that the PIAS proteins function not only as E3 enzymes but also as regulators of STAT signaling, implying that some of the effects of knocking out PIAS proteins may not be linked to SUMOylation. Therefore, more *in vivo* studies using different knock-out combinations are needed to find out the specific functions of each gene. Also, knock-in mouse models expressing unSUMOylatable forms of different SUMO targets – such as PPARγ and LRH-1 – will be needed.

Obesity is a complex phenomenon that integrates metabolic and inflammatory responses in several tissues, including adipose tissue, liver, muscle and macrophages. It will be interesting, in the future, to specify the functions of SUMO-1 in inducible knock-out mice by turning the expression off in different tissues. *Sumo1* knock-out mice showed resistance to HFD-induced obesity, but nevertheless exhibited decreased glucose tolerance, to a level comparable to that of WT mice. Therefore, a drug that targets SUMO in all tissues is expected to have no benefit in treating the adverse metabolic effects of obesity. However, tissue-specific mechanisms may provide more possibilities in the future, as preventing the SUMOylation in adipose tissue alone could be beneficial in treating obesity without increasing the inflammation in macrophages and liver.

The *in vivo* functions of SUMO-2 and SUMO-3 are still largely unknown, although there is evidence that both are involved in stress responses (Tempé et al. 2008). Currently, the two proteins have been largely referred to as SUMO-2/3 due to the lack of paralog-specific antibodies. Animal models lacking SUMO-2 or SUMO-3 have not yet been described, but will hopefully provide many answers concerning the paralog selectivity. Our laboratory made an effort to produce a *Sumo2* knock-out mouse line using different gene trap ESCs, but all attempts resulted in a failure of the chimeric mice to transfer the gene trap to their progeny, suggesting that SUMO-2 is important for haploid sperm cells and their ability to fertilize an egg. However, ESCs have to be carefully kept in undifferentiated form before the morula aggregation, leaving the possibility of a technical problem. In addition, as the gene trap construct is randomly integrated into the ESC genome, disruption of another gene by a second integration event has to be excluded. Preliminary results from *Sumo3* knock-out mice generated by the gene trap method in our laboratory suggest a partial embryonic lethality. It seems, therefore, that evolution has had its reasons to maintain two nearly identical genes, an explanation for this is still to be discovered.

SUMMARY AND CONCLUSIONS

SUMOylation is a conserved post-translational modification system present from yeast to humans. Mammals have three functional *Sumo* genes and a selection of SUMOylation enzymes. This study aimed at determining the *in vivo* functions of SUMOylation under normal conditions, in inflammation and on high-fat diet. The main conclusions can be summarized as follows:

- Disruption of the *Pias2* gene in mice results in a reduced testis weight and epididymal sperm count. However, normal fertility is maintained without compensatory increases in the expression of the other *Pias* genes.

- *Sumo1* null mice are viable, fertile and indistinguishable from wild-type mice under normal conditions for laboratory animals. The lack of SUMO-1 can be compensated for by SUMO-2 and SUMO-3 proteins, which are abundantly, present in the unconjugated form.

- *Sumo1* null mice exhibit a pronounced acute phase response upon LPS stimulation due to inefficient loading of LRH-1 onto inflammatory gene promoters. This result expands the role of SUMOylation in transrepression and shows a SUMO-1-specific effect *in vivo*.

- Lack of *Sumo1* results in impaired terminal adipogenesis *via* diminished activation functions of PPARγ, the master regulator of adipose tissue. These PPARγ functions require SUMO-1 *in vivo* as well, as rosiglitazone fails to activate PPARγ target genes in adipose tissue of *Sumo1* null mice.

- *Sumo1* null mice are resistant to high-fat diet-induced obesity due to inefficient expandability of their white adipose tissue (WAT), highlighted by the presence of smaller and fewer adipocytes. This has no effect on glucose tolerance but reduces the inflammation of their WAT.

ACKNOWLEDGEMENTS

This work was carried out in the Androgen Receptor Laboratory in the Institute of Biomedicine, Physiology, at the University of Helsinki. I would like to express my gratitude to my supervisor professor Olli A. Jänne who guided me through the thesis work starting from a first-year medical student. Professor Jorma Palvimo is warmly thanked for all his expertise and help during the first years of this work. Docent Fu-Ping Zhang is thanked for all the help and support. Dr. Henrikki Santti, thank you for friendship and my first five years in the lab. The pre-examiners assistan professor Ville Hietakangas and docent Anna-Liisa Levonen are warmly thanked for valuable comments. Members of my thesis committee, professor Heikki Rauvala and professor Matti Poutanen are thanked for their time and support.

Helsinki Biomedical Graduate Program, Academy of Finland, Sigrid Jusélius Foundation, Biocentrum Helsinki, Helsinki University Central Hospital, CRESCENDO, Synergy, Finnish Cultural Foundation, Biomedicum Helsinki Foundation, University of Helsinki Funds, The Finnish Medical Foundation, The Chancellor of the University of Helsinki, Paulo Foundation, Emil Aaltonen Foundation, Finnish Cancer Foundations and Oskar Öflund Foundation are thanked for funding.

Professor Jorma Toppari, professor Eckardt Treuter and professor Irma Thesleff are thanked for fruitful collaboration. All the coauthors, especially Henrikki Santti, Johanna Hirvonen, Nicolas Venteclef, Knut Steffensen and Tomas Jakobsson are thanked for their work.

Mice are thanked for sacrificing their life and freedom to science.

Saijalle suurkiitos kaikesta! All the past and present members of the AR-lab are thanked for all the good times, especially Maria B, Maria W, Päivi, Sahu, Andrii, James, Johanna H, Hanna, Annariikka, Noora, Marianne, Sha, Sirpa, Ulla, Zhigang, Marika L, Marika H, Tanya, Tom, Rakibul, Ievhen… Docent Piia Aarnisalo and her group: Johanna H, Johanna V and Ann-Marie. Professor Taneli Raivio is thanked for

his support and the sex team for adopting me: thank you Johanna T, Eve, Kirsi, Johanna K and the rest. Linda not only for Swedish lessons and yoga but friendship. Anne and Johanna N for administration.

Friends who I couldn't have survived without: Maria B, Anna Sofia, Maria W, Anna H, Tuomas, Marianne, Riikka K, Anna P, Lotta, Riikka H, Heli, Sanna, Sari. Thank you all! And finally, to my family, Mom, Dad, Pekka, Sini and Maija – thank you for everything.

Helsinki, April 2013

Laura Mikkonen

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