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SUMOs in the Regulation of Transcriptional Co-regulators RIP140 and PGC-1α and in the Development of C. elegans

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ABSTRACT:

Small ubiquitin-like modifier (SUMO) proteins are small ~100 amino acids long proteins that are covalently attached to specific lysine residues on substrate proteins. Humans express four different SUMO proteins, SUMO-1, -2, -3, and -4. The SUMO conjugation (SUMOylation) to proteins is similar to ubiquitylation pathway, but it is catalyzed by distinct enzymes. The normal SUMOylation is important at the individual protein level to maintain protein-protein interactions, subcellular and subnuclear localization, and control stability and activity. In the big picture, SUMOylation plays a role e.g. in chromatin organization, DNA repair, and cellular stress responses. The majority of studies of SUMOylation have been done in cultured cells. In the first section of this study the SUMOylation is evaluated in a whole multicellular organism, by constructing transgenic nematode *Caenorhabditis elegans* (*C. elegans*) strains expressing SUMO-1 or SUMO-2 in muscle cells. The disrupted balance of SUMO proteins by increased expression in muscle cells disturbed the normal development of the nematode muscles. The perturbation led to severe disabilities in reproduction, movement and survival. In addition, the expression of SUMO and ubiquitin-proteasome pathway genes was altered in the SUMO-expressing nematodes. The second section of this study considers the function of SUMOs in the regulation of two nuclear receptor co-regulators in cultured mammalian cells. The receptor interacting protein 140 (RIP140, NRIP1) and peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α, PPARGC1α) are co-regulators for many gene expression pathways in metabolism and energy homeostasis. It was found that the co-repressor RIP140 is SUMOylated at two distinct residues in separate C-terminal repression domains 3 and 4. SUMOylation increased the repressive potential and modulated the localization of the co-regulator. The co-activator PGC-1α was SUMO-modified at the N-terminal activation domain. The activation function of PGC-1α was depressed by SUMO modification due to the interaction of co-repressor RIP140. In conclusion, the dissertation describes the importance of balanced SUMOylation in normal development and new mechanisms of regulation of two important transcriptional co-regulators.

TIIVISTELMÄ:

Pieni ubikitiinin kaltainen proteiini (Small ubiquitin-like modifier, SUMO) on kovalentisti tiettyihin lysiinitähteisiin konjugoituva proteiini. Ihmisillä on neljä SUMO proteiinia, SUMO-1, -2, -3 ja -4. SUMO-konjugaatio säätelee proteiinien vuorovaikutuksia niiden solun- ja tumansisäisessä liikehdinnässä, sekä proteiinien stabiilisuutta ja aktiivisuutta. SUMO-konjugaatio vaikuttaa esim. kromatiinin järjestäytymiseen, DNA:n korjaukseen ja solujen stressivasteeseen. SUMO-konjugaatio muistuttaa ubikitiinin konjugaatioprosessia, mutta sitä katalysoivat entsyymit ovat erilaiset. Suurin osa SUMO-konjugaatioon liittyvistä tutkimuksista on tehty soluviljelmissä. Tämän työn ensimmäisessä osiossa tutkittiin SUMO-konjugaation vaikutuksia monisoluisessa organismissa tekemällä siirtogeenisiä sukkulamatolinjoja (*Caenorhabditis elegans*; *C. elegans*), jotka ilmentävät SUMO-1:stä tai SUMO-2:sta lihassoluissa. SUMO:n lisääntynyt ilmentyminen madon lihassoluissa johti lihasten normaalin kehittymisen häiriintymiseen, mikä näkyi heikentyneenä lisääntymis-, liikkumis- ja elinkykynä. Geenien ilmentymistasolla havaittiin muutoksia mm. SUMO-konjugaatio ja ubikitiini-proteasomisysteemi tekijöitä koodaavissa geeneissä. Työn toisessa osiossa käsiteltiin SUMO:n vaikutuksia kahden tumareseptorikoregulaattorien toimintaan nisäkässoluissa. Reseptorien kanssa interaktoiva proteiini 140 (RIP140, NRIP1) ja peroxisomin lisääntymisestä aktivoituva reseptori γ koaktivaattori 1α (PGC-1α, PPARGC1α) säätelevät monia metaboliaan ja energiatasapainoon liittyviä signaalireittejä. Korepressori RIP140:stä löytyi kaksi SUMO:lla muokkautuvaa lysiinitähdettä, jotka sijaitsevat repressioalueiden 3 ja 4 kohdalla proteiinin C-terminaalisessa päässä. SUMO-muokkauksen johdosta RIP140 repressiivinen aktiivisuus lisääntyi ja modifikaatio vaikutti samalla myös proteiinin tumansisäiseen sijaintiin. Koaktivaattori PGC-1α:n SUMO-konjugaatio kohdistui N-terminaalisessa päässä olevan aktivaatioalueen sisältämään lysiinitähteeseen. PGC-1α:n aktivaatiokyky heikkeni SUMO-konjugaation johdosta, koska se lisäsi vuorovaikutusta korepressori RIP140 kanssa. Yhteenvetona voidaan todeta tämän työn tulosten havainnollistavan SUMO-konjugaatiotasapainon tärkeyttä normaalissa monisoluisen organismin kehityksessä. Lisäksi työ kuvaa uuden tavan säädellä kahta tärkeää transkription säätelyyn osallistuvaa tekijää.

Luokitus: QU 475, QX 203

Yleinen Suomalainen asiasanasto (YSA): proteiinit; sukkulamadot; transkriptio – säätely

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Kuopio, June 2011

Miia Rytinki

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List of Original Publications

This dissertation is based on the following original publications referred to in the text by their corresponding Roman numerals (I-III). In addition, some unpublished results will be presented.

I. **Rytinki MM, Lakso M, Pehkonen P, Aarnio V, Reisner K, Peräkylä M, Wong G, Palvimo JJ.** Overexpression of SUMO perturbs the growth and development of *Caenorhabditis elegans*. Cell Mol Life Sci. 2011 Jan 21 [Epub ahead of print].

II. **Rytinki MM and Palvimo JJ.** SUMOylation modulates the transcription repressor function of RIP140. J Biol Chem. 2008 283(17):11586-95.

III. **Rytinki MM and Palvimo JJ.** SUMOylation attenuates the function of PGC-1alpha. J Biol Chem. 2009 284(38):26184-93.

The publishers of the original publications have kindly granted permission to reprint the articles in this dissertation.

Contents

APPENDIX: ORIGINAL PUBLICATIONS (I-III)

Abbreviations

C. elegans	*Caenorhabditis elegans*
DUB	Deubiquitylating enzyme
E1	Ubiquitin/SUMO activating enzyme
E2	Ubiquitin/SUMO conjugating enzyme
E3	Ubiquitin/SUMO ligase
EA	Glutamine to alanine mutation
ERα	Estrogen receptor α
ESCRT	Endosomal sorting complex required for transport
H2Bub1	Monoubiquitylation of histone H2B
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HECT	Homologous to the E6-AP carboxyl terminus
HMT	Histone methyltransferase
KR	Lysine to arginine mutation
PGC-1α	PPARγ co-activator 1α
PIAS	Protein inhibitor of activated STAT
PINIT	Amino acid sequence in PIAS involved in localization
PML	Promyelocytic leukemia protein
PPARγ	Peroxisome proliferator-activated receptor γ
PTM	Post-translational modification
RD	Repression domain
RING	Really interesting new gene
RIP140	Receptor interacting protein 140
RNF4	RING finger protein 4
SAP	Scaffold attachment factor-A/B, acinus and PIAS
SENP	SUMO-specific protease
SIM	SUMO-interacting motif
SIRT	Sirtuin
SMO-1	*C. elegans* SUMO protein
SP-RING	Siz/PIAS-RING
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
SUMOylation	Conjugation of SUMO
Ub	Ubiquitin
UBD	Ubiquitin-binding domain

UBL   Ubiquitin-like protein
UPS   Ubiquitin-proteasome system

1 Introduction

An important milestone was accomplished in research when the human genome was sequenced a decade ago. The genome is constant throughout different cell types in organisms. In strict contrast the proteome is highly variable. The proteins are the functioning elements in living cells and ultimately the most important factors. The human genome consists of ~21 000 protein-coding genes (Clamp *et al.* 2007). The majority of the genes can be alternatively spliced, resulting in a proteome several folds larger than the genome. Certainly, genes are not all expressed in the same cell at the same time; nevertheless, the amount of various proteins is huge. To add complexity, the proteins can be post-translationally modified in various ways, such as by proteolytical cleavage or by addition of chemical groups or proteins to substrates. Understandably, the systematic identification of the whole proteome is far more challenging than sequencing of the whole genome.

The post-translational modifications (PTMs) are a key factor in the regulation of signaling in cells. PTMs control every function of proteins, including activity, localization, and stability. The modifications are usually reversibly conjugated to amino acid side chains. Moreover, the PTMs covalently added to proteins can be special chemical groups (e.g. phosphate, acetyl), lipids (e.g. prenyl), carbohydrates (e.g. *O*-linked *N*-acetylglucosamine), or proteins (e.g. ubiquitin, ubiquitin-like proteins). Undoubtedly, all modifications play a role in the cells. However, the main focus in this thesis will be on the covalent protein modification by small ubiquitin-like modifier (SUMO) proteins, small ~100-amino acids long proteins, though examples of other modifications such as phosphorylation, acetylation and methylation are also discussed.

The modification by SUMOs (SUMOylation) is also linked to many pathological states in humans. A number of the enzymes involved in the SUMOylation cascade are implicated in cancers with aberrant expression levels. In addition, several tumor suppressor proteins are targets of SUMOylation. Furthermore, key proteins in many neurodegenerative diseases, huntingtin, ataxin-1, tau, α-synuclein, and Parkinson's disease 7, are also SUMOylated (Online Mendelian Inheritance in Man: OMIM). Lately, structural proteins, keratin and actin, were reported as targets of SUMOylation. The structural proteins in addition to all transcription factors and other important regulators that are SUMOylated suggest that even more disease states will be linked to the SUMOylation in the future.

In this thesis, the correctly balanced SUMOylation is identified as a key point in the normal development of the muscles in the nematode *Caenorhabditis elegans* (*C. elegans*). Furthermore, SUMOylation in two important transcriptional co-regulators, the co-repressor receptor interacting protein 140 (RIP140) and the co-activator peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), is demonstrated for the first time to enhance the repressive function and reduce the activation function, respectively.

2 Review of Literature

2.1 POST-TRANSLATIONAL MODIFICATIONS

The post-translational modifications enable a level of regulation that can rapidly be altered in response to changes in the cellular signals. The modifications are roughly divided into small and large "tags" on target proteins. The small chemical groups include e.g. acetyl, methyl, and phosphate groups. The large bulky modifications include ubiquitin and ubiquitin-like proteins (UBLs). Often the modifications are studied in isolation. However, the combinatorial effects are increasingly noticed. Obviously, different modifications targeted at a same residue in a protein can affect one another by taking the place from the other modification. In many cases, modification of a particular residue affects another site in its proximity. The best known targets of combined modifications are the histones, which maintain the packaging of the DNA in chromosomes. It is clear that many proteins are targets of several different modifications, but it is often unclear whether they reside on the same molecule at the same time or if there are pools of proteins with different modifications.

2.1.1 Small molecular modifications

2.1.1.1 Phosphorylation

Phosphorylation is the classical post-translational modification. The protein kinase activity was initially described in the 1950s. Today, the estimation is that at least 30% of all proteins in eukaryotic cells are phosphorylated (Boekhorst *et al.* 2008). Phosphorylation is a very fast way to proceed signal cascades e.g. from cell surface to nucleus (Papin *et al.* 2005).

Protein kinases catalyze the phosphorylation that transfers γ-phosphate from ATP to specific amino acid residues; mainly serine (Ser), threonine (Thr), and tyrosine (Tyr) (Figure 1). Enabling a fast turnover of the PTM, specific protein phosphatases remove the phosphate group from modified proteins. The classical protein kinases and corresponding phosphatases can be divided into two groups by specificity; the Ser/Thr-specific and Tyr-specific kinases and phosphatases. Most kinases have specific consensus sequences of approximately four amino acids that determine the specificity for their actions (Ubersax and Ferrell, 2007). A recent study of the phosphoproteome in cervical cancer (HeLa) cells shows that majority of the phosphorylated sites are at serine residues (86%). Less abundant are the modifications at threonine (12%) and tyrosine (2%) residues (Olsen *et al.* 2006). These differences may arise from the fact that phosphorylation of Tyr tends to be more unstable than of Ser/Thr. Moreover, majority of Ser/Thr kinases preferentially modify Ser residues, whereas most Ser/Thr phosphatases dephosphorylate Thr residues (Ubersax and Ferrell, 2007).

Protein phosphorylation can have activating or inhibiting effect on the substrates. For instance, phosphorylation at two sites activates the mitogen-activated protein kinase p38. The phosphorylation leads to conformational changes on p38 that makes the

substrate association possible. In its activated conformation the p38 can continue the signal cascade by phosphorylating various substrates, resulting in activation of protein phosphatase 2, and inhibition of glycogen synthase kinase 3β (Cuadrado and Nebreda, 2010).
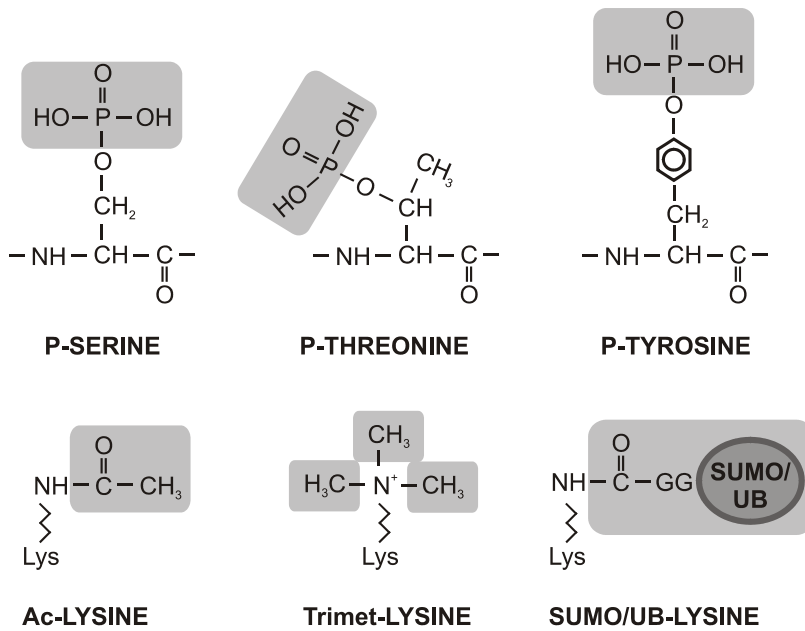
Figure 1. Illustration of selected post-translational modifications. The upper part depicts phosphorylation (P) of serine, threonine and tyrosine. The lower part shows examples of small lysine modifications by acetyl and methyl groups, and the bulky SUMO or ubiquitin (UB) modification.

2.1.1.2 Small lysine modifications

Earliest reports of histone acetylation and methylation are from the 1960s. As a matter of fact, the mechanisms of acetylation and methylation are mostly studied in the context of histone tail modifications, although they modify other proteins as well. Like many other PTMs, the acetylation and methylation are conjugated to lysine residues (Figure 1); albeit, the methylation can occur also on arginine, histidine, and proline residues. For historical reasons, the enzymes involved in these modifications are named histone acetyl-transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) even though many targets are non-histone proteins. Recently, the enzymes have been renamed to more accurate names KATs, KDACs, KMTs, and KDMs, where K indicates lysine (Allis *et al.* 2007).

Acetyltransferases that transfer acetyl groups from acetyl Coenzyme A catalyze the dynamic acetylation of target lysines. Deacetylases are responsible for the removal of the acetyl groups. It is not yet clear how acetylation is targeted to substrates, but acetyltransferases and deacetylases reside in multi-protein complexes, where associated factors may facilitate the targeting. The acetyltransferases can be divided into three main classes by their catalytic domains: Gcn5-related N-acetyltransferases (GNATs), MYST proteins, and E1A-associated protein of 300 kDa (p300)/CREB-binding protein (CBP) (Lee

and Workman, 2007). Moreover, there are four major groups of deacetylases: class I (HDAC1, -2, -3, and -8), class II (HDAC4, -5, -6, -7, -9, and -10), class III (the sirtuin family, SIRT1-7), and class IV (HDAC11) (Ekwall, 2005; Yang and Seto, 2008). The acetylation changes the charge of the lysine residues on the target proteins and therefore can have activating or inhibiting effect on the substrate. In addition, the acetylation can prevent other PTMs to the same lysine residues (Glozak *et al.* 2005). Bromodomain containing proteins recognize the acetylated lysines. The bromodomains are found in histone-modifying enzymes such as KATs, and also in many transcription factors and co-regulators (Dhalluin *et al.* 1999; Mujtaba *et al.* 2007).

For decades, the lysine methylation was considered to be a stable irreversible modification. Only recently, the discovery of the first histone demethylase LSD1 changed the methylation to a reversible modification (Shi *et al.* 2004). The lysine residue can contain up to three methyl groups in the same side chain; indicated as mono-, di-, or tri-methylation (Figure 1). All methyltransferases have a SET (Su(var), Enhancer of zeste, trithorax) domain, except Dot1L, that binds to and catalyses the transfer of methyl from the S-adenosylmethionine (SAM or AdoMet) (Varier and Timmers, 2011). Despite the short history, over 30 demethylases have been described and they can be separated into two groups: the KDM1 family with FAD-dependent amine oxidase activity, and the Jumonji C (JmjC) domain-containing enzymes dependent on Fe^{2+} and 2-oxoglutarate (Pedersen and Helin, 2010). The methylation of target lysines can have activating or inhibiting effect depending on the interacting partners that the particular modification recruits. It can also prevent other PTMs on the lysine residue or induce PTMs on other residues. Chromodomains (chromatin organization modifier) in histone modifying enzymes, transcription factors and co-regulators recognize the methylated lysines. Also, other domains that recognize methylated lysines e.g. Tudor, WD40, and plant homeodomain have been described (Bannister *et al.* 2001; Daniel *et al.* 2005).

2.1.2 Ubiquitin and ubiquitin-like modifiers

The ubiquitin was first discovered in 1975 (Schlesinger *et al.* 1975). Over a decade later, in 1987 an interferon-stimulated gene product of 15 kDa (ISG15) was identified as the first ubiquitin-like protein (Haas *et al.* 1987). Further on, several other ubiquitin-like proteins were identified. In 1996 small ubiquitin-like modifier was discovered (Boddy *et al.* 1996, Shen *et al.* 1996). Today, the list of UBLs includes SUMO, neuronal-precursor-cell-expressed developmentally downregulated protein-8 (NEDD8), ISG15, human leukocyte antigen F associated (FAT10), autophagy-8 (ATG8), autophagy-12 (ATG12), ubiquitin-related modifier-1 (URM1), and ubiquitin fold-modifier-1 (UFM1). For a long time, the highly conserved ubiquitin was considered to be a eukaryote specific protein. However, recently a prokaryotic ubiquitin-like protein (Pup) was identified in *Mycobacterium tuberculosis* (Pearce *et al.* 2008). The UBLs differ in their amino acid sequences, but the similarities arise from the three dimensional structures and analogous three-step conjugation cascades, though conjugation steps are catalyzed by separate enzymes. Ubiquitin and SUMOs have hundreds to thousands of known target proteins, but other UBLs have only few identified targets so far.

2.1.3 Ubiquitin pathway

The ubiquitin is a small 76-amino acids long protein that forms a globular three dimensional structure (Figure 5) (Huang *et al.* 2011). It is an essential and highly conserved protein in eukaryotes. Ubiquitin is expressed as three different precursors in cells. The first precursor, a concatemer of ubiquitin repeats fused end to end (polyubiquitin); the number of repeats is variable between species. The other precursors the Ub_{L40} and Ub_{S27} are two N-terminal ubiquitins fused to ribosomal polypeptides L40 and S27, respectively. These otherwise identical ubiquitins are released by specific endopeptidases (Catic and Ploegh, 2005).

The ubiquitylation describes the covalent isopeptide bond formation between substrate Lys residue and ubiquitin C-terminal diglycine motif. The reversible three step conjugation cascade requires specific enzymes (Figure 2) (Hershko *et al.* 1983). The C-terminally processed ubiquitin that has the diglycine residues revealed and ready for conjugation is bound by a thioester bond to E1 activating enzyme Uba1 (or Uba6) in an ATP-dependent manner (Disteche *et al.* 1992; Jin *et al.* 2007). This is followed by the transfer of ubiquitin through transthiolation from E1 to one of the many (~40) E2 conjugating enzymes. Lastly, an E3 ligase (one of thousands) binds to a substrate and ubiquitin-E2 complex and facilitates the transfer of the ubiquitin to a target lysine on the substrate (van Wijk and Timmers, 2010; Bedford *et al.* 2011b).

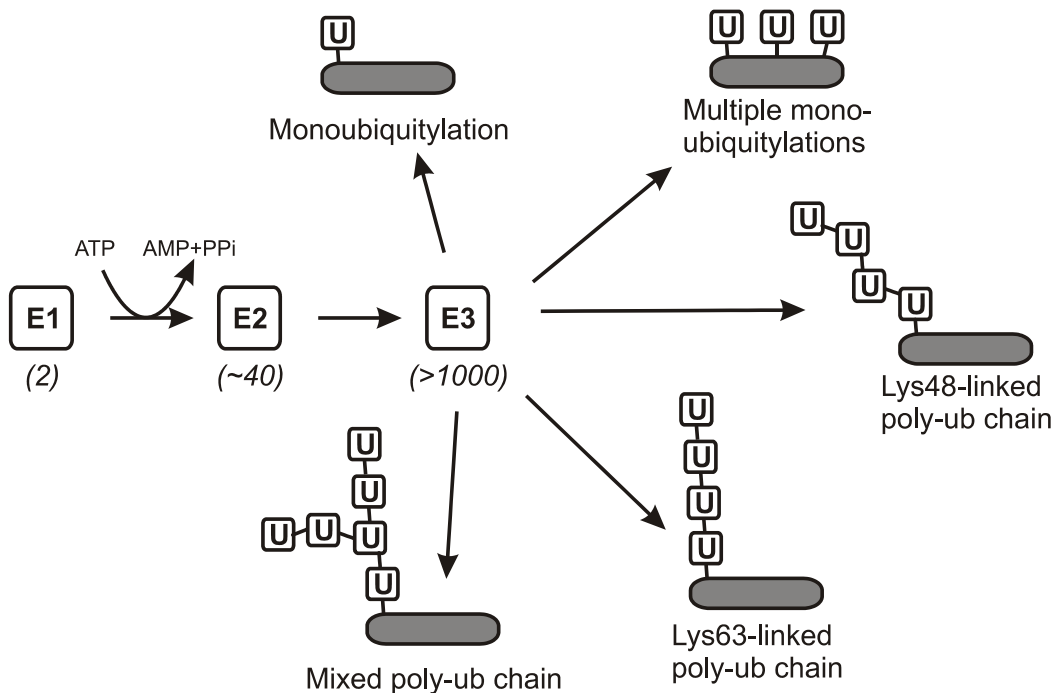*Figure 2*. Different types of chains that can result from the ubiquitylation pathway. The conjugation of ubiquitin (U) requires three steps (E1-E3); activation, conjugation, and ligation. Numbers in parenthesis indicate the amount of human proteins with the particular activity. In addition, examples of possible combinations of mono- and polyubiquitin (poly-ub) chains formed in eukaryotes are illustrated.

The family members of E2 conjugating enzymes contain a conserved ubiquitin-conjugating domain of ~150 amino acids. Inside the catalytic domain is the active-site cysteine that accepts the ubiquitin and transfers it further to substrates (Wenzel *et al.* 2010; van Wijk and Timmers, 2010). The large number of E3 ubiquitin ligases is divided into two major groups; the HECT (homologous to the E6-AP carboxyl terminus) and the RING (really interesting new gene) type of ligases. These groups differ from each other in that the HECT ligases form a thioester bond with ubiquitin before transferring it to a substrate, whereas RING ligases do not form a covalent bond, but merely bring the ubiquitin-bound E2 and substrate together for conjugation. The RING finger motif is involved in both protein-protein and protein-DNA interactions. The motif coordinates two zinc ions with the cysteine and histidine residues in a specific order: $C\text{-}x_{(2)}\text{-}C\text{-}x_{(9\text{-}39)}\text{-}C\text{-}x_{(1\text{-}3)}\text{-}H\text{-}x_{(2\text{-}3)}\text{-}C/H\text{-}x_{(2)}\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}x_{(2)}\text{-}C$ where x represents any amino acid (Nagy and Dikic, 2010; Deshaies and Joazeiro, 2009; Chasapis and Spyroulias, 2009).

Deconjugation of ubiquitin is carried out by deubiquitylating enzymes (DUBs). The DUBs are responsible for generating free ubiquitin from precursors, removing of ubiquitin chains from substrates, and trimming of ubiquitin chains. The humans have ~80 genes encoding DUBs. They can be divided into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Josephin domain proteins (MJDs), and JAB1/MPN/MOV34 metalloenzymes (JAMMs). The majority of the DUBs (UCH, USP, OUT, MJD) are cysteine proteases, except for JAMMs that are metalloproteases (Katz *et al.* 2010; Komander *et al.* 2009).

2.1.3.1 Signaling with ubiquitin-tags

Ubiquitin regulates various processes in cells, including proteolysis, gene transcription, cell-cycle progression, DNA repair, and apoptosis (Ikeda and Dikic, 2008). Ubiquitin can be linked to substrates as monoubiquitin or as polyubiquitin chains with variable lengths and compositions. Ubiquitin has seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) available for ubiquitylation to form different combinations of polyubiquitin chains (Figure 2) (Phu *et al.* 2010). The E3 ligases are crucial in the targeting and in the determination of which type of polyubiquitin chain will be formed. Moreover, together with E2 conjugating enzymes the E3 ligases are able to catalyze various combinations of polyubiquitin chains (Sadowski and Sarcevic, 2010). In addition, the DUBs can process the polyubiquitin chains (Newton *et al.* 2008).

The different ubiquitin signals are recognized by ubiquitin-binding domain (UBD)-containing proteins. According to estimates, at present UBD is found in over 150 various cellular proteins. Depending on the linkage type, the ubiquitin chains can have different conformations; Lys48-linked chains are more compact than Lys63-linked polyubiquitin chains (Varadan *et al.* 2002; Varadan *et al.* 2004). The various UBD-containing proteins have different specificities for linkage types. The UBDs are divided into groups binding to Lys48-link, Lys63-link, or a promiscuous group without preferences (Zhang *et al.* 2008; Haririnia *et al.* 2007; Varadan *et al.* 2005).

2.1.3.2 Role of ubiquitylation in protein degradation

Ubiquitin is linked to all the main pathways of degradation in cells: the proteasomal, lysosomal, and autophagosomal pathways (Figure 3). The lysosome is responsible for the degradation of many plasma membrane proteins. From the endocytosed proteins some

are selected for recycling, whereas others are targeted for lysosomal degradation. The endosomal sorting complex required for transport (ESCRT) recognizes ubiquitylated proteins for degradation. The human ESCRT-0 can recognize various ubiquitin marks. It binds to Lys63-linked tetraubiquitin chain 50-fold stronger than monoubiquitin. However, only two-fold stronger interactions with Lys63-linked than Lys48-linked tetraubiquitin chains are observed (Ren and Hurley, 2010). The autophagosomal degradation takes care of the intracellular organelles and protein aggregates that cannot be processed by the proteasome. An autophagy receptor p62/sequestosome 1 contains both a UBD and a LC3-interacting region (LC3 family include e.g. the ATG12 UBL). Hence, the p62 combines the ubiquitylation to autophagosomal system by simultaneously interacting with both ubiquitin and LC3-family proteins (Kirkin *et al.* 2009).
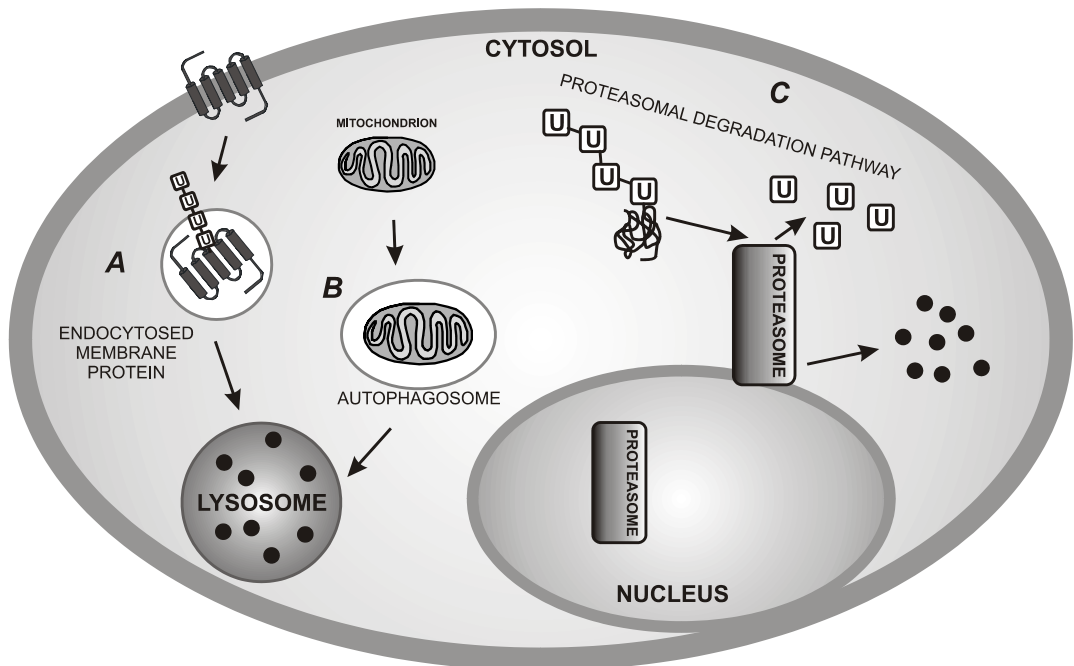
Figure 3. Overview of protein degradation pathways. A. Plasma membrane proteins are endocytosed from the membrane, the vesicles with the proteins are fused to the lysosome for degradation. B. Organelles and protein aggregates are autophagocytosed and membrane fusion with lysosome forms phagolysosome that degrades the large protein complexes. C. Proteins with tetra-ubiquitin-tags are recognized by the proteasomal proteins, the ubiquitins (U) are released and the proteins are degraded by the proteasomes.

The proteasomal pathway is the best described ubiquitin-dependent degradation system. The ubiquitin-proteasome system (UPS) is a strictly regulated main pathway of destructing proteins in the cytosol, nucleus, and endoplasmic reticulum (Coux *et al.* 1996; Finley 2009). The 26S proteasome is a 2000-kDa protein complex consisting of a 20S core particle and two 19S regulatory particles. The core forms a barrel-structure in which the proteolysis can occur. The two regulatory subunits are located at each end of the core subunit, functioning as gate-keepers, guiding and unfolding ubiquitin-tagged proteins for degradation inside the core (Unno *et al.* 2002; Coux *et al.* 1996). The regulatory particle

contains specific ubiquitin-recognizing proteins, Rpn10 and Rpn13 (Ferrell *et al.* 1996; Husnjak *et al.* 2008). Moreover, shuttling factors, such as Rad23, interacting with ubiquitylated proteins can bring substrates to the proteasomes (Watkins *et al.* 1993). Upon binding, deubiquitylating enzymes, e.g. Rpn11, remove the ubiquitin-chain (Verma *et al.* 2002). All ubiquitin-tags do not doom the protein for degradation. The 26S proteasome preferentially binds and degrades substrates with tetraubiquitin chain tags (Thrower *et al.* 2000). Traditionally, the Lys48-linked chains have been considered as the signal for degradation. At present, all the different lysine linked chains are associated with degradation through the proteasome (Xu *et al.* 2009; Jacobson *et al.* 2009; Bedford *et al.* 2011a; Dammer *et al.* 2011).

In addition to degradation, cells can remove proteins by diluting the proteome through cell division. Differentiated mammalian cells are most often slowly dividing cells and preferentially use degradation to remove proteins. Intriguing new aspects on the protein dynamics is reported by Eden and colleagues. They demonstrate that inhibition of cell division prolonged the half-life of long-lived proteins, while the half-life of the short-lived proteins remained the same. This suggests that drugs affecting cell division may have consequences on various protein levels without affecting the protein degradation pathways, especially in rapidly dividing cells (Eden *et al.* 2011).

2.1.4 Small ubiquitin-like modifiers

In the fifteen years that SUMOs have been known, the small ~100-amino acids long proteins have emerged as a modification as important as other modifications. The SUMO modification is important in many cellular processes, ranging from cellular and nuclear localization of proteins, transcriptional regulation, chromosome segregation, to DNA repair. The SUMOs are large modifications compared to e.g. phosphorylation and acetylation. Conjugation of SUMOs can therefore block protein-protein interactions. Conversely, the SUMOylation can also provide new interaction surfaces for proteins. The altered interactions and conformational changes can alter the functions and/or localizations of the substrate proteins (Wilkinson and Henley, 2010).

Humans express four SUMO paralogs, named SUMO-1, -2, -3, and -4 (Figure 4). The amino acid sequence of human SUMO-1 is approximately 50% identical with SUMO-2 or -3, whereas the sequence of SUMO-2 and -3 are close to identical (~97%). Therefore, these are most often depicted as SUMO-2/3. Although SUMO-1 and -2/3 are expressed ubiquitously, their expression levels are different. Also, the SUMO-2/3 protein levels in cells are higher than SUMO-1, and conjugation of SUMO-2/3 is dramatically increased in response to cellular stress (Saitoh and Hinchey, 2000; Su and Li, 2002; Golebiowski *et al.* 2009). SUMOs are conserved proteins, although only one gene is known to encode for SUMO in yeast and invertebrates. In contrast, plant *Arabidopsis thaliana* genome encodes eight paralogs of SUMO (Miura and Hasegawa, 2010).

A

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HsSUMO-2    MADEKPKEG----VKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMR 56
HsSUMO-3    MSEEKPKEG----VKTEN-DHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMR 55
HsSUMO-4    MANEKPTEE----VKTENNNHINLKVAGQDGSVVQFKIKRQTPLSKLMKAYCEPRGLSVK 56
HsSUMO-1    MSDQEAKPSTEDLGDKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMN 60
CeSMO-1     MADDAAQA-----GDN--AEYIKIKVVGQDSNEVHFRVKYGTSMAKLKKSYADRTGVAVN 53
            *::: .           ..   ::*::** ***.. ::*::*  *  : ** ::*.:  *:..

HsSUMO-2    QIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVY--------- 95
HsSUMO-3    QIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVPESSLAGHSF 103
HsSUMO-4    QIRFRFGGQPISGTDKPAQLEMEDEDTIDVFQQPTGGVY--------- 95
HsSUMO-1    SLRFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGGHSTV------- 101
CeSMO-1     SLRFLFDGRRINDDDTPKTLEMEDDDVIEVYQEQLGGF--------- 91
            .:** * *: *   ..*  * **::*.*:*:*:  **
```

B

Figure 4. Sequence comparison of SUMO orthologs. A. Amino acid sequence alignment of human (Hs) SUMO-1, -2, -3, -4, and *C. elegans* SMO-1 (CeSMO-1). Grey highlights the C-terminal diglycine residues. B. Phylogram tree of a selection of orthologs of SUMOs. The sequences are from National Center for Biotechnology Information (NCBI) with accession numbers for human SUMOs NP_001005781.1 (HsSUMO-1), NP_008868.3 (HsSUMO-2), NP_008867.2 (HsSUMO-3), NP_001002255.1 (HsSUMO-4), for *C. elegans* SMO-1 NP_490842 (CeSMO-1), for *Drosophila melanogaster* Smt3 NP_477411.1 (DmSmt3), for *Saccharomyces cerevisiae* Smt3p NP_010798.1 (ScSmt3p), and for *Arabidopsis thaliana* SUMOs NP_194414.1 (AtSUMO-1), NP_001154779.1 (AtSUMO-2), and NP_200328.1 (AtSUMO-3). The ClustalW2 (hosted by the European Molecular Biology Laboratory and European Bioinformatics institute (EMBL-EBI)) web based tool was used for the multiple sequence alignment and the phylogram (Chenna *et al.* 2003). *, identical; :, conserved; ., semi-conserved.

The SUMO-4 differs from the other SUMOs. To begin with, the intronless *SUMO-4* gene is only expressed in the kidney, lymph nodes, and spleen, while the other SUMOs are ubiquitously expressed (Bohren *et al.* 2004; Guo *et al.* 2004). Furthermore, there are contradicting results in whether SUMO-4 can be conjugated to substrates or not (Owerbach *et al.* 2005; Wei *et al.* 2008; Guo *et al.* 2005).

The tertiary structure and surface charge distribution of SUMOs resemble that of ubiquitin (Figure 5). The compact ubiquitin $\beta\beta\alpha\beta\beta\alpha\beta$ fold is also found in other UBLs (Huang *et al.* 2011). Overall, the SUMO-1 has a similar ubiquitin-fold, but in addition it has a long flexible N-terminal part (Bayer *et al.* 1998). The structure of SUMO-3 is closer to ubiquitin than SUMO-1 (Ding *et al.* 2005). Furthermore, the charge distribution of SUMO-2 differs from that of SUMO-1 (Huang *et al.* 2004). In addition to the differences in the tertiary structure, the main difference between SUMOs lies in the ability to form polymers. The SUMO-1 lacks the possibility to form polymeric chains, since it does not have any lysine residue (Lys11) within a SUMOylation consensus sequence as SUMO-2/3 does

(Tatham *et al.* 2001). The SUMO-1 has, however, been suggested to form polymers at non-consensus Lys *in vitro* (Yang *et al.* 2006).
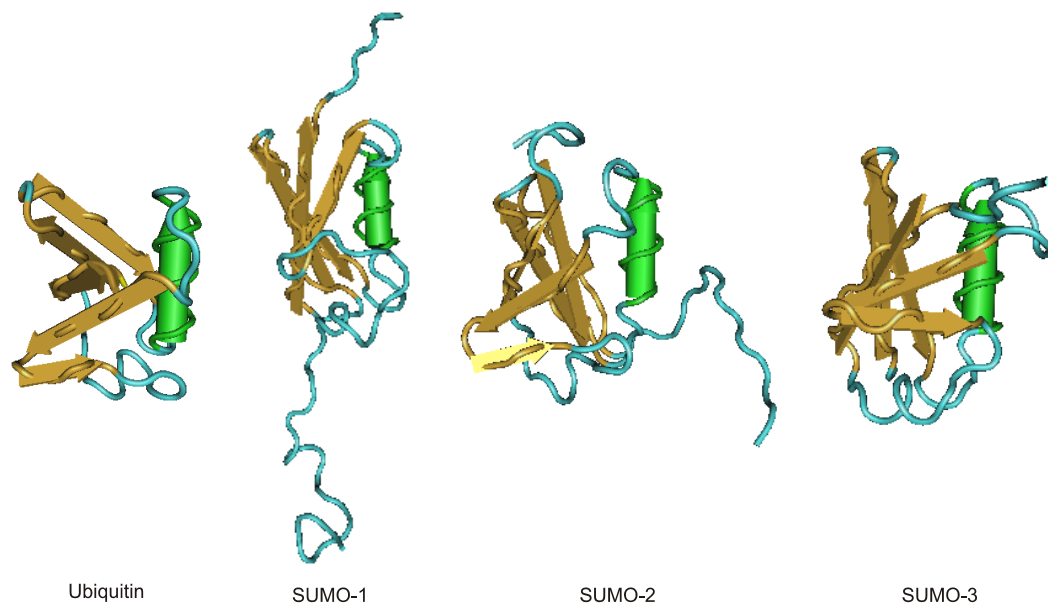
Figure 5. Three dimensional structures of ubiquitin, SUMO-1, -2, and -3. Yellow arrows indicate β-sheets, and green arrow is an α-helix. The modified images originate from Molecular Modeling Database (MMDB) maintained by the NCBI (Wang *et al.* 2007a, Chen *et al.* 2003). The MMDB identification numbers follow: 88832 (Ub), 55054 (SUMO-1), 41906 (SUMO-2), and 32257 (SUMO-3).

2.1.4.1 SUMO pathway

The SUMO conjugation (SUMOylation) pathway is highly similar to the ubiquitylation; but the enzymes are specific for the SUMOylation (Figure 6) (Kamitani *et al.* 1997). The SUMOs are conjugated to lysine residues within a consensus sequence defined as ψKxE (ψ depicts a large hydrophobic isoleucine, leucine, or valine and x any residue) (Rodriguez *et al.* 2001). In addition to the tetrameric SUMOylation consensus motifs, extended consensus sequences have been described. A phosphorylation dependent SUMOylation motif (ψKxExxSP) has a proline-directed phosphorylation site close to the lysine (Hietakangas *et al.* 2006). Moreover, hydrophobic, negatively-, and positively-charged amino acids surrounding the four amino acid SUMO consensus motif can enhance or direct SUMO-1 or SUMO-2 to the acceptor lysine (Matic *et al.* 2010; Schimmel *et al.* 2010; Yang *et al.* 2006). In addition, many lysines without a surrounding consensus sequence are targeted by SUMOylation (Blomster *et al.* 2009).

All SUMO proteins are translated as precursors. To be activated, the C-terminal tails are processed by SUMO specific proteases (SENPs; sentrin-specific protease) to reveal the diglycine residues and to generate the conjugation competent form. The mature SUMO forms a thioester bond with cysteine in the activating enzyme heterodimer SAE1/2 (SUMO activating enzyme 1 and 2) (Gong *et al.* 1999). Thereafter, SUMO is transferred from the SAE2 subunit to a conserved cysteine in conjugating enzyme UBC9 (Desterro *et al.* 1997; Gong *et al.* 1997). The UBC9 is able to conjugate SUMOs directly to substrate

lysines (Sampson *et al.* 2001; Karaca *et al.* 2011). However, E3 ligases often facilitate the conjugation by recruiting the substrate and UBC9 into same complex or by enhancing the release of SUMOs from UBC9 to substrates. The deconjugation of SUMOs is catalyzed by the SENPs.
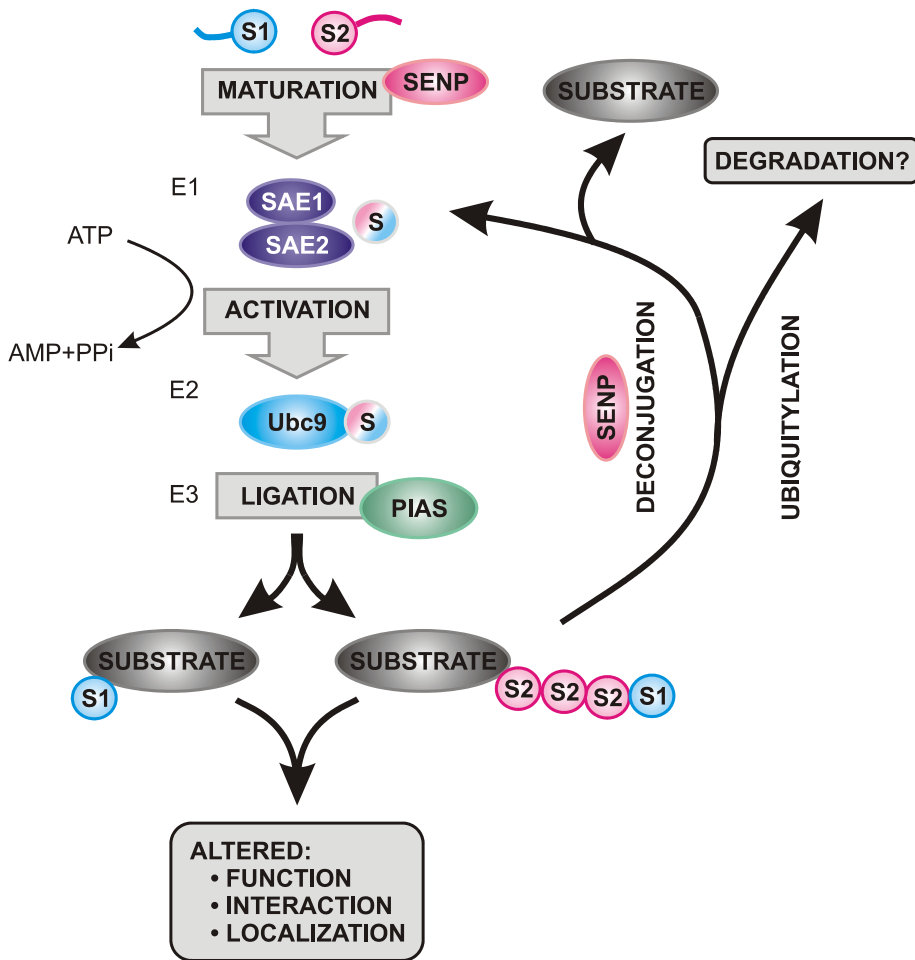
Figure 6. Schematic illustration of the SUMOylation pathway. Before conjugation the new SUMO proteins, SUMO-1 (S1) and SUMO-2 (S2), are C-terminally processed by SENPs to reveal the diglycine motif in a process called maturation. Thereafter, the SUMO is activated (E1) by SAE1/2, and conjugated (E2) by UBC9. The conjugation is enhanced by E3 ligases (PIAS). The SUMOylation results in altered function, interaction, or localization of the substrate. Subsequently, the SUMOs are deconjugated from the substrate by SENPs. The substrate can also be targeted for the ubiquitin-proteasomal degradation pathway. S: SUMO-1 or SUMO-2.

The substrate specificity of SUMOylation is still unclear in many cases. In contrast to ubiquitylation, SUMOylation has only one E1 and E2, and a limited number of E3 ligases have been identified thus far. Although SUMO-1 and SUMO-2/3 differ, the SUMO E1 and E2 enzymes do not show any paralog-specificity (Tatham *et al.* 2003). The E2 UBC9 may control on part the substrate specificity. The autoSUMOylation of UBC9 at Lys14 is one possible mechanism which can contribute to the target selection (Knipscheer *et al.*

2008; Subramaniam *et al.* 2010). Moreover, the substrate specificity may also arise from subcellular and subnuclear localization, in addition to the interaction with E3 ligases. Substrates with SUMO-interacting motifs (SIMs) can associate with SUMO-bound UBC9 without the help of E3 ligases and consequently become SUMOylated. For instance, in the case of zinc finger protein 451, disruption of the SIM reduces the SUMOylation (Karvonen *et al.* 2008).

2.1.4.2 SUMO E3 ligases

Thus far, a small number (~12) of E3 ligases for SUMOylation has been described. A family of PIAS (protein inhibitor of activated STAT) proteins are connected to large part of the SUMOylation enhancing properties (Johnson and Gupta, 2001; Kahyo *et al.* 2001; Kotaja *et al.* 2002; Rytinki *et al.* 2009; Rytinki and Palvimo, 2011). Other proteins harboring SUMOylation enhancing properties have a limited number of targets. For instance, the nucleporin RanBp2 (Pichler *et al.* 2002), the polycomb group protein Pc2 (Kagey *et al.* 2003), HDAC4 and HDAC7 (Zhao *et al.* 2005; Gao *et al.* 2008), topoisomerase I-binding, arginine/serine rich (TOPORS) (Weger *et al.* 2005), mitochondrial-anchored protein ligase (Braschi *et al.* 2009), methyl methanesulphonate-sensitivity protein 21 (Potts and Yu, 2005), tripartite motif proteins (Chu and Yang, 2011), and stanniocalcin-1 (dos Santos *et al.* 2011) are implicated as having SUMOylation enhancing properties at least for one substrate.

The human PIAS protein gene family consist of four members, PIAS1, PIAS2 (PIASx), PIAS3, and PIAS4 (PIASy). The PIAS proteins show sequence homology and contain five distinct conserved domains or motifs (Figure 7). First, in the N-terminus resides the most conserved region, the SAP (scaffold attachment factor-A/B, acinus and PIAS) domain involved in sequence or structure-specific binding to DNA (Aravind and Koonin, 2000). Second, the PINIT motif is located adjacent to the SAP domain. It is involved in the cellular localization (Duval *et al.* 2003). Third, the Siz/PIAS RING (SP-RING) motif is located centrally in the PIAS proteins. This region contains six cysteines that possibly coordinate zinc-atoms to conform a similar structure as classical RING-type ubiquitin ligases (Hochstrasser 2001). Fourth, C-terminal to SP-RING there is a region interacting non-covalently with SUMOs, a SUMO-interacting motif (Minty *et al.* 2000; Song *et al.* 2004). Lastly, outermost in the C-terminus is the least conserved and highly variable serine/threonine-rich (S/T) region.
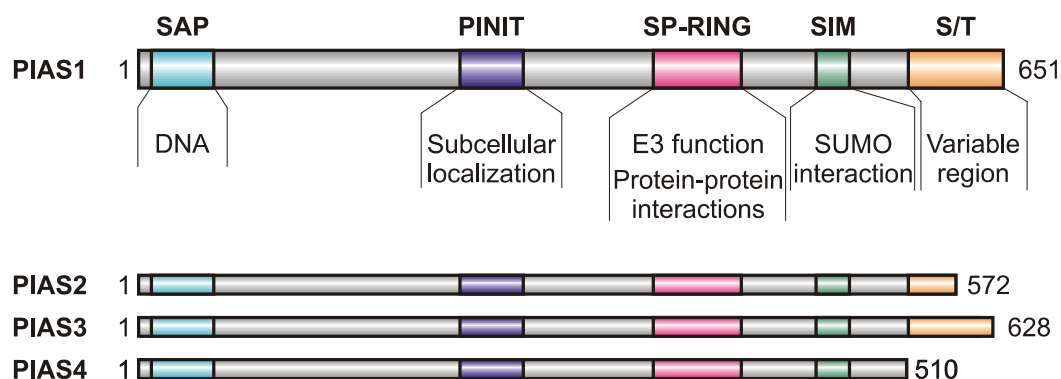
Figure 7. The domain structures of PIAS proteins. Numbers indicate amino acid residues.

2.1.4.3 SUMO specific proteases

SENPs mediate the deSUMOylation of SUMO modified proteins. Human cells express six different SUMO-specific proteases: SENP1 (Gong *et al.* 2000), SENP2 (Nishida *et al.* 2001), SENP3 (Nishida *et al.* 2000), SENP5 (Gong and Yeh, 2006), SENP6 (Kim *et al.* 2000), and SENP7 (Lima and Reverter, 2008). The seventh, SENP8 (NEDP1) has specificity against another UBL, the NEDD8 (Mendoza *et al.* 2003). The SENPs are cysteine proteases that harbor both iso- and endopeptidase activity but are distinct from the DUBs. The N-terminal parts regulate the subcellular localization, whereas the catalytic domain resides in the C-terminal region (Figure 8) (Li and Hochstrasser, 2003). The mechanism of the SENP's substrate specificity is still unclear. It is believed that the subcellular localization is one determinant. Moreover, the paralog-specificity of the SENPs differs: SENP1 and SENP2 are the most promiscuous proteases able to process both SUMO-1 and SUMO-2/3. Endogenous SENPs (SENP2, -3, -5, -6, and -7) preferentially select SUMO-2/3 over SUMO-1 (Kolli *et al.* 2010). Furthermore, all SENPs also have the capability to process polySUMO chains, therein maintaining the balance of conjugation and deconjugation (Békés *et al.* 2011).
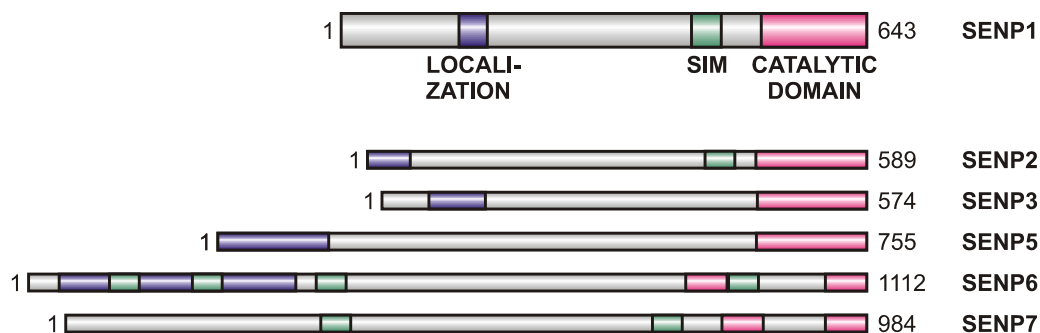
Figure 8. The domain structures of SUMO-specific proteases, SENPs. Numbers indicate amino acid residues.

2.1.4.4 SUMO-interacting motif

The SUMOs are not only effective through covalent conjugation, but it is also able to affect through non-covalent SUMO interactions. Proteins can interact with SUMOs by SUMO-interacting motifs (SIMs), although they are not always targets of SUMOylation themselves. The SIM region has a less stringent consensus sequence than the covalent conjugation sequence. It is often recognized as having a hydrophobic core $\psi x\psi\psi$ or $\psi\psi x\psi$ (ψ mostly Ile or Val) close to a cluster of negatively charged amino acids (Song *et al.* 2004). The SIM binds to a hydrophobic groove in SUMOs located between a β_2-sheet and the α_1-helix (Song *et al.* 2005; Sekiyama *et al.* 2008). In some cases, SUMO paralog-selective binding of some SIMs has been indicated *in vitro*, but how the selectivity is achieved *in vivo* needs to be determined (Hecker *et al.* 2006). Recently, phosphorylation by Ser/Thr kinase CK2 was linked to SIM functions. The phosphorylation of residues adjacent to SIM region in PIAS proteins results in enhanced binding to both SUMO paralogs. It does not affect the E3 ligase function but affects the transcriptional coregulation function of PIAS proteins (Stehmeier and Muller, 2009). Nuclear proteins in particular contain SIMs. The

SIMs appear to be important in the SUMOylation and deSUMOylation processes, but even more vital in regulation of protein-protein interactions and ultimately influences processes such as transcription regulation and DNA repair (Du *et al.* 2010; Li *et al.* 2010).

2.1.5 SUMO in action with other modifications

The crosstalk of SUMOs with other modifications has lately become evident. The "histone code" of various modifications has been known for a while. As several other proteins are targeted with many modifications, it is increasingly important and interesting, but also challenging, to evaluate the modifications in relation to each other. The methylation, acetylation, ubiquitylation and SUMOylation, can compete for the same lysine residues. However, it is not necessary for the modification to be targeted to the same residue for it to have an effect on other modifications in the same substrate.

The heat shock factor 1 (HSF1) is a well known regulator in stress response. It is a transcription factor that induces the expression of heat shock proteins in cellular stress situations. HSF1 phosphorylation and SUMOylation are both induced by stress. As a matter of fact, the SUMOylation at Lys298 of HSF1 requires the phosphorylation at Ser303 residue (Hong *et al.* 2001; Hietakangas *et al.* 2003). Later, the phosphorylation-dependent SUMOylation consensus motif was described. Proline-directed phosphorylation sites close to SUMO consensus sites were found on many transcription factors and co-regulators (Hietakangas *et al.* 2006). Moreover, phosphorylation of residues not adjacent to SUMO consensus site can also enhance or reduce the substrate SUMOylation. For instance, the phosphorylation of keratins supports the SUMOylation (Snider *et al.* 2011). On the other hand, phosphorylation of the special AT-rich sequence-binding protein 1 (SATB1) inhibits interaction with PIAS1 and therefore also reduces the SUMOylation (Tan *et al.* 2010).

The overall SUMOylation of proteins increases when the proteasome functions or the ubiquitin E1 enzymes are inhibited (Bailey and O'hare, 2005; Schimmel *et al.* 2008; Yang *et al.* 2007). One evolutionarily conserved protein is connected with both ubiquitin and SUMOs, namely ubiquitin E3 ligase RING finger protein 4 (RNF4). It was originally identified as a co-regulator for a steroid hormone receptor (Moilanen *et al.* 1998). In two separate studies, RNF4 was first described as a ubiquitin E3 ligase and later shown to non-covalently interact with SUMO-1 (Häkli *et al.* 2004; Häkli *et al.* 2005). Later on, these two properties were connected when SUMO-targeted ubiquitin-ligases (STUbLs) were discovered. The STUbLs are evolutionarily conserved proteins, orthologs of which are identified in *Schizosaccharomyces pombe* Rfp1/-2-Slx8, *Saccharomyces cerevisiae* Slx5-Slx8, and *Drosophila melanogaster* Degringolade (Sun *et al.* 2007; Prudden *et al.* 2007; Abed *et al.* 2011). Subsequently, RNF4 was implicated in arsenic-induced degradation of promyelocytic leukemia protein (PML). RNF4 can recognize and ubiquitylate polySUMO chain carrying protein, PML (Tatham *et al.* 2008; Lallemand-Breitenbach *et al.* 2008). In addition, SUMOs can induce other ubiquitylation pathways. For example, the ubiquitin E3 ligase breast cancer 1 (BRCA1) co-localizes with SUMOs, PIAS1, and PIAS4 at DNA damage sites. The SUMOs together with PIAS1 and PIAS4 activate BRCA1's ubiquitin ligase activity and support the recruitment of factors important in the double-stranded DNA damage-repair (Galanty *et al.* 2009; Morris *et al.* 2009).

2.2 *CAENORHABDITIS ELEGANS* (*C. ELEGANS*)

The large part of knowledge in SUMO field is acquired from cell culture experiments. Usage of experimental organisms, such as Ubc9-knock-out mice, has clarified the importance of the SUMOylation in context of a whole organism. The study in mice showed that deletion of the *Ubc-9* gene results in embryonic lethality before the embryonic day 7.5 due to abnormalities in chromosomal condensation and segregation, and nuclear organization (Nacerddine *et al.* 2005). Down-regulation of Ubc9 in zebrafish resulted in similar defects (Nowak and Hammerschmidt, 2006). Moreover, down-regulation of the UBC9 orthologs in *C. elegans* and *Drosophila melanogaster* leads to developmental arrest (Jones *et al.* 2002; Apionishev *et al.* 2001). These severe complications indicate the importance of Ubc9 function and SUMO conjugation in the early development. However, deletion of *Sumo-1* in mice did not result in developmental complications. Probably the presence *Sumo-2/3* in mice compensates for the lack of *Sumo-1* (Zhang *et al.* 2008). The deletions of all SUMO paralogs in zebrafish have a similar consequence as the removal of Ubc9, whereas only one of the SUMOs supported the normal development of the zebrafish (Yuan *et al.* 2010). The results suggest functional redundancy among the different SUMOs. Furthermore, *C. elegans* has only one SUMO-encoding gene, the *smo-1* and homozygous deletion of the *smo-1* gene leads to severe abnormalities in the reproductive organs and ultimately to sterility (Broday *et al.* 2004). Moreover, the use of whole organisms can reveal molecular effects of SUMOylation that are connected to higher order behaviors such as movement, reproduction and aging.

2.2.1 *C. elegans* as an experimental organism

C. elegans is a soil living nematode that is used in biological research. It is an excellent tool for research since it is a small (~1 mm long) transparent worm with a short lifecycle, and it is easy to maintain. The temperature controls the growth; at lower temperature (15°C) the growth is slower than at higher temperatures (25°C). The population of *C. elegans* consists of self fertilizing hermaphrodites (XX) and males (X0). Although the nematode can be a very potent tool for investigation, there are also limits in the usage (Herman, 2006). In the field of post-translational modifications, the specific anti-bodies against various proteins are crucial. Today, most of the commercially available antibodies do not recognize *C. elegans* proteins. However, a few highly conserved proteins, such as ubiquitin and tubulin in *C. elegans*, have epitopes that can be detected by antibodies for mammalian orthologs. Despite of the small size, *C. elegans* contains many different cell types and organs, such as neurons, muscles, intestine, and reproductive organs. The different tissues together with the information of every cell lineage give a powerful tool to investigate the development of the worm and disease models (Altun and Hall, 2009).

    C. elegans is the first multicellular model organism having its whole genome sequenced by the year 1998. The genome is divided into 6 or 7 holocentric chromosomes, including one or two X chromosomes depending on if it is a male or a hermaphrodite, respectively. The nematode genome is quite small, 100 Mb, although it is predicted to contain almost 22 000 genes (Gerstein *et al.* 2010). As a comparison the human genome consists of 3000 Mb with approximately 21 000 genes (Clamp *et al.* 2007). The large

difference in size resides in the amount of repeat sequences; the *C. elegans* has about 7% and human around 50% repeat sequences in their genomes.

2.2.2 Lifecycle of *C. elegans*

The lifecycle of *C. elegans* can be divided into an embryonic stage, four larval stages, and adulthood (Figure 9). The molting of the cuticle separates the four larval stages, named L1, L2, L3, and L4. The lifecycle is completed in three days when the hermaphrodite is ready to lay its first eggs. In general, the lifespan is around 20 days, during which the mature hermaphrodite can produce ~300 progeny limited by the amount of sperm produced. However, mating with a male can increase the progeny to ~1000. During unfavorable living conditions, the worms can go into an arrested dauer-larvae state. Environmental factors, such as high temperature, lack of food, or a pheromone indicating crowding in the population, can induce the dauer-arrest. In this state, the feeding and locomotion is reduced, and the worm can survive in this condition for up to several months. When the living conditions improve the worms can exit the dauer-state and molt to L4 larval stage (Altun and Hall, 2008).
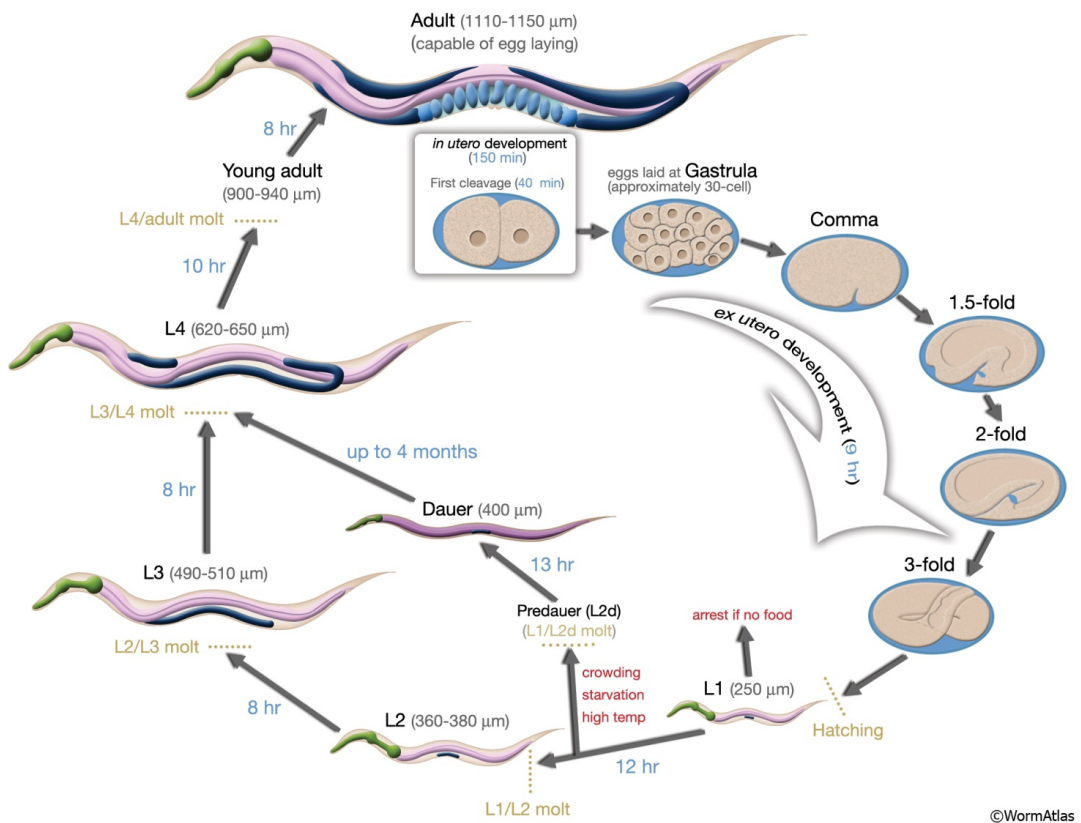

Figure 9. The *C. elegans* lifecycle at 22°C. The illustration shows the development from a fertilized egg to an adult in ~2.5 days. To reach adulthood, the nematode goes through four separate larval stages depicted L1, L2, L3, and L4 in the figure. The figure is reproduced with permission from http://www.wormatlas.org.

2.2.3 SUMO pathway in *C. elegans*

The SUMOs and SUMO pathway proteins are evolutionary well conserved in yeast, plants and mammals. In *C. elegans* the SMO-1 protein conjugation is catalyzed by AOS-1/UBA-2 and UBC-9. Thus far, GEI-17 is the only protein showing E3 ligase activity in *C. elegans* (Holway *et al.* 2005). *C. elegans* has five ubiquitin-like proteases (ULP-1–5) which have sequences similar to human SENPs.

In a recent proteomics study, 250 potential targets of SUMOylation were identified in *C. elegans* (Kaminsky *et al* 2009). A few proteins have been characterized more closely. Many of the targets of SUMOylation are important in the normal development of the nematode and especially the reproductive organ, the vulva (Zhang *et al.* 2004; Broday *et al.* 2004; Leight *et al.* 2005; Poulin *et al.* 2005; Roy Chowdhuri *et al.* 2006). In addition, two targets of SUMOylation, MUS-101 and POLH-1, are important for the DNA replication and in the DNA-damage response (Holway *et al.* 2005; Kim and Michael, 2008).

2.3 TRANSCRIPTIONAL REGULATION

Proteins are encoded from the information on mRNAs transcribed from genes in the DNA. The expression of genes is highly controlled by many factors. SUMOylation is also involved in the transcriptional regulation, since many transcription factors and co-regulators are targets of SUMOylation. To start with, the DNA is packed in chromatin that is built up by nucleosomes. The human nucleosomes contain 147 base pairs of DNA wrapped around four pairs of core histones H2A, H2B, H3 and H4 (Luger *et al.* 1997). The human DNA is divided into 46 chromosomes that have specific territories in the cell nucleus. In addition, the untranscribed regions of the chromatin are densely packed into heterochromatin, whereas the less condensed euchromatin contains the more active gene regions (Cremer and Cremer, 2010). In order to transcribe a gene, the DNA has to be made available for the transcription machinery. For that purpose, the chromatin remodeling complexes are required for the nucleosomal reorganization. Proteins in the chromatin remodeling complexes have enzymatic activities to catalyze PTMs on histones such as acetylation by the human SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (Rodriguez-Navarro, 2009). Moreover, the evolutionary conserved SWI/SNF (switching/sucrose non-fermenting) and RSC (remodeling the structure of chromatin) chromatin remodeling complexes can restructure the nucleosomes by ATP-dependent mechanisms (Tang *et al.* 2010).

Transcription factors that recognize and bind to specific sequences in the genome direct the gene transcription. Transcription factors, such as nuclear receptors, cooperate with co-activators, chromatin remodeling complexes, and mediator complexes (Figure 10). This cooperation provokes the assembly of the preinitiation-complex containing the general transcription factors (GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) and RNA polymerase II at the transcription start site (Tang *et al.* 2010; Weake and Workman, 2010). The traditional view of recruitment of factors to DNA has been recently questioned, as evidence suggests that nuclei can be composed of specific transcription factories (Chakalova and Fraser, 2010).
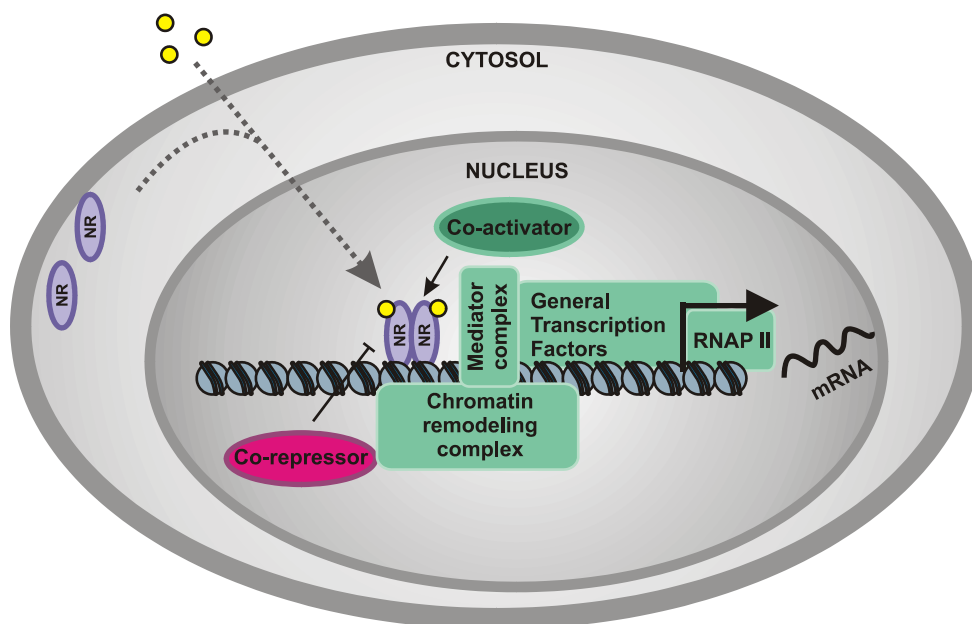
Figure 10. Overview of transcriptional activation by a nuclear receptor (NR) dimer. Ligand (yellow circle) binding and dimerization induces the NR to bind to specific response elements on DNA. The nuclear receptor dimer cooperates with co-activators, chromatin remodeling complexes, mediator complexes, general transcription factors, and RNA polymerase II (RNAP II), which results in increased transcription of the target gene.

2.3.1 Transcription factors

It is predicted that human genome encodes for more than a thousand transcription factors. The transcription factors are characterized as having sequence-specific DNA binding capacity without enzymatic activities. The transcription factors can be divided into groups according to the structure of their DNA-binding domain. The three largest groups are the C_2H_2 zinc finger-, homeodomain-, and helix-loop-helix-domain-containing transcription factors (Vaquerizas *et al.* 2009).

The nuclear receptors are a family of transcription factors with 48 members. They belong to the zinc finger group. Most of the nuclear receptors have a conserved protein structure with N-terminal activation domain, a central DNA-binding domain, and a C-terminal ligand-binding domain (Aranda and Pascual, 2001). Furthermore, the nuclear receptors can be subdivided to steroid hormone receptors such as androgen, estrogen, and vitamin D receptors, adopted orphan receptors e.g. PPARγ, liver X, and estrogen receptor related receptor, and orphan receptors e.g. small heterodimeric partner (SHP) and dosage-sensitive sex reversal (DAX-1). The nuclear receptors respond to ligands that can be, for example, steroid hormones, vitamin D, prostaglandins, and fatty acids. For the orphan receptors, the ligands are still unknown. The specific binding to DNA mostly occurs as dimers, either homodimers (steroid receptors) or heterodimers with retinoid acid receptor X (RXR) (e.g. PPARγ) (Perissi and Rosenfeld, 2005; Margolis and Christakos, 2010).

2.3.2 Co-regulators

Co-regulators often reside in multiprotein complexes and can roughly be divided into co-activators or co-repressors. Many co-regulators have originally been identified as regulators for a specific transcription factor. However, today it is known that most of the co-regulators are capable of regulating various transcription factors. Moreover, the activator vs. repressor function is not as a clear-cut division either, since the function can be dependent of different cell types and protein contexts (Rosenfeld *et al.* 2006). For instance, the co-regulator RIP140 has been reported both as a co-activator and co-repressor in different situations, although most often RIP140 acts as a repressor. RIP140 differently regulates the target genes of Liver X Receptor in hepatocytes, activating genes associated with lipogenesis and down-regulating genes associated with gluconeogenesis (Herzog *et al.* 2007).

The co-regulators bind directly to the transcription factors and enhance or reduce gene transcription by communicating with other factors in the transcription machinery. Many co-regulators can also possess enzymatic functions like acetylation and methylation by E1A binding protein p300/CREB-binding protein (p300/CBP) and co-activator-associated arginine methyltransferase 1 (CARM1), respectively. In fact, the co-regulators often reside in large complexes with proteins that possess a variety of enzymatic functions. Furthermore, the co-regulators themselves can be modified by PTMs, which regulates their function (Lonard and O'Malley, 2007).

Today, the PIAS proteins are described as SUMO E3 ligases, although the PIAS proteins possess other activities as well. The PIAS1 and PIAS3 were originally isolated as inhibitors of STAT1 and STAT3 (Chung *et al.* 1997; Liu *et al.* 1998). In many cases, the PIAS function is exerted without an increase in SUMOylation of the interaction partner. The different motifs of PIAS have been linked to transcriptional co-regulation through various mechanisms. The interaction of PIAS often alters the localization of interacting partners in a way that the function is altered. For example, PIAS1 interacts with cytokine-induced STAT1 dimer, inhibiting the DNA-binding and STAT1-induced gene transcription (Liao *et al.* 2000; Diao *et al.* 2009). Similarly, PIAS3 interacts with phosphorylated STAT3 and inhibits the transcriptional activation function by hindering DNA-binding of STAT3 (Dabir *et al.* 2009).

2.3.2.1 RIP140 and PGC-1α

In the middle of 1990s RIP140 was isolated from cDNA expression library from human breast cancer cells and characterized as a nuclear receptor interacting protein (Cavaillès *et al.* 1995). A few years later, the transcriptional co-activator PGC-1α was cloned from a brown fat cDNA library (Puigserver *et al.* 1998). The RIP140 and PGC-1α are important co-regulators especially in metabolic tissues such as adipose, muscle, and liver. Both co-regulators contain LxxLL motifs that they use to interact with many members of the nuclear receptor family, including estrogen receptor α, estrogen-related receptors, glucocorticoid receptor, thyroid receptors, liver X receptor and PPARγ.

RIP140 is a co-regulator that supports increase in fat accumulation and energy storage in liver, white adipose tissue and muscles (Fritah *et al.* 2010). Depletion of RIP140 in mice indicates that the co-repressor is necessary in release of oocytes at ovulation (White *et al.* 2000). In addition, RIP140 null mice are lean and are resistant to high-fat diet-induced fat accumulation (Leonardsson *et al.* 2004). The cold-induced PGC-1α increases

the mitochondrial function and is important in thermogenesis (Koppen and Kalkhoven, 2010). In mice lacking PGC-1α, the normal metabolism is defective with reduced mitochondrial function, high fat accumulation and problems in thermogenic response (Lin *et al.* 2004; Leone *et al.* 2005).

Both co-regulators regulate genes in metabolism and energy homeostasis, such as the uncoupling protein-1 (UCP1) involved in the non-shivering thermogenesis. Many of the regulated genes in the metabolic tissues are the same. However, RIP140 and PGC-1α show opposite effects on the genes that are regulated (Fritah *et al.* 2010; Koppen and Kalkhoven, 2010). In addition to opposing effects on various transcription factors, these two co-regulators interact directly with each other. The co-activation mediated by PGC-1α was repressed by RIP140 (Hallberg *et al.* 2008, III).

3 Aims of the Study

Most studies of SUMO proteins have been done in cultured cells under ectopic overexpression conditions. Therefore, we constructed transgenic *C. elegans* strains by introducing SUMO-1 and SUMO-2 in the genome. The generated SUMO-expressing *C. elegans* strains were utilized in the studies of SUMOs in a multicellular organism. Furthermore, SUMO conjugation to transcriptional co-regulators is increasingly detected as a regulatory mechanism. The two important co-regulators RIP140 and PGC-1α are targets of many PTMs and have lysines residing in the SUMO consensus motif. Thus, we were interested in exploring the possible role of SUMOs in the regulation of these co-regulators.

The more detailed aims of this study were to:

- Examine the effects of the overexpression of SUMO-1 and SUMO-2 in the nematode.

- Study modification of RIP140 by SUMOs and the role of the modification for the function of the co-repressor RIP140.

- Study the role of SUMOylation in the function of the co-activator PGC-1α.

4 Materials and Methods

Table 1 summarizes the methods used in the original publications used in this thesis work.

Table 1. Overview of methods used in this thesis.

Methods	Original Publication
Construction of *C. elegans* strains	I
C. elegans mRNA expression profiling	I
Quantitative RT-PCR	I
Differential-Interface contrast (Nomarski) microscopy of *C. elegans*	I
Ni^{2+}-nitriloacetic acid pulldown to isolate 6xHistidine-tagged proteins	I, II
Mammalian cell culture for ectopic expression assays	I, II, III
Construction of DNA plasmids and site-directed mutagenesis	I, II, III
Immunoprecipitation and western blotting	I, II, III
In vitro SUMOylation assay	II, III
Immunofluorescence and confocal microscopy	II, III
Reporter gene assays	II, III

5 Results and Discussion

5.1 INCREASED SUMO EXPRESSION DISTURBS GROWTH AND DEVELOPMENT OF *C. ELEGANS* (I)

5.1.1 Alterations in muscle biology by SUMOs in *C. elegans*

There are implications of the role of SUMOs in the muscle development. The overall SUMOylation levels were decreased during the progression of myogenesis of mammalian C2C12 myoblasts, and the myogenesis was also Ubc9-dependent (Riquelme *et al.* 2006). In addition, SUMO-1 was involved in the induction of cardiac muscle gene expression in pluripotent 10T1/2 fibroblasts (Wang *et al.* 2007b). SUMO conjugation also positively regulated the assembly and function of IFB-1, a cytoplasmic intermediate filament structural protein in *C. elegans* (Kaminsky *et al.* 2009). In the first section of the thesis, SUMO-1 and SUMO-2 were overexpressed in the *C. elegans* neurons and muscle cells. The expression of SUMOs in the neuronal and muscular tissues was chosen because these two are the main tissues across the whole body of the nematode.

The human *SUMO-1* and *SUMO-2* were introduced in the *C. elegans* under the regulation of pan-muscular (*myo-4*) and pan-neuronal (*aex-3*) promoters. The neuronal expression of SUMO-1 did not lead to any obvious phenotypic changes, although molecular alterations were observed in gene expression analysis (Fig. 3 and Fig. 4 in I). However, the increased amounts of SUMO-1 and SUMO-2 disrupted the normal development of muscle cells in the nematodes and resulted in an abnormal posterior part (Figure 11). Downregulation of a variety of ubiquitin-conjugating enzymes, including *ubc-9*, in *C. elegans* resulted in abnormal tail phenotypes (Jones *et al.* 2002). Although, the deletion of *ubc-9* possibly results in altered levels of free vs. conjugated SUMO-proteins, in a way similar to overexpression of SUMOs, the phenotypically abnormal tails were not identical to the phenotype in SUMO overexpressing *C. elegans*. In addition to the tail abnormalities, egg-laying, movement and survival of the SUMO overexpressing nematodes were impaired (Fig. 3 in I). Of note, these effects were not simply a result of the expression of foreign SUMOs in nematodes, since the overexpression of SMO-1 also led to similar disabilities (Fig. 3 in I). The overexpressed SUMO-1 and SUMO-2 are conjugated to target proteins in the nematodes (Fig. 1 in I). To determine whether the conjugation levels were high or low, a comparison to the normal levels of conjugation by SMO-1 would be necessary. Unfortunately, the levels of SMO-1 conjugation could not be determined due to the lack of a suitable antibody. The abnormalities were possibly a result of a misbalance in the conjugation and deconjugation of SUMOs. The expression of the conjugation-negative form of SUMOs in the nematodes was less harmful (I), at least when it was calculated as the percentage of worms with the abnormal tail. It is conceivable that both the free and conjugated SUMOs possibly disrupted the normal SUMO-regulated protein-protein interactions in the nematode.

The amino acid sequence identity of nematode SMO-1 was not as high with SUMO-2 (46%) as with SUMO-1 (63%). However, the overall function and surface properties of SMO-1 were found to resemble more that of SUMO-2 than SUMO-1 (Fig. 2

in I). Moreover, the SMO-1 conjugation pattern in mammalian cells was more similar to SUMO-2 than to SUMO-1 (Fig. 1 in I). Despite of the dissimilarity, the expression of either of the human SUMO-1 or SUMO-2 (I) partially rescued the severe *smo-1* deletion phenotype in *C. elegans*.
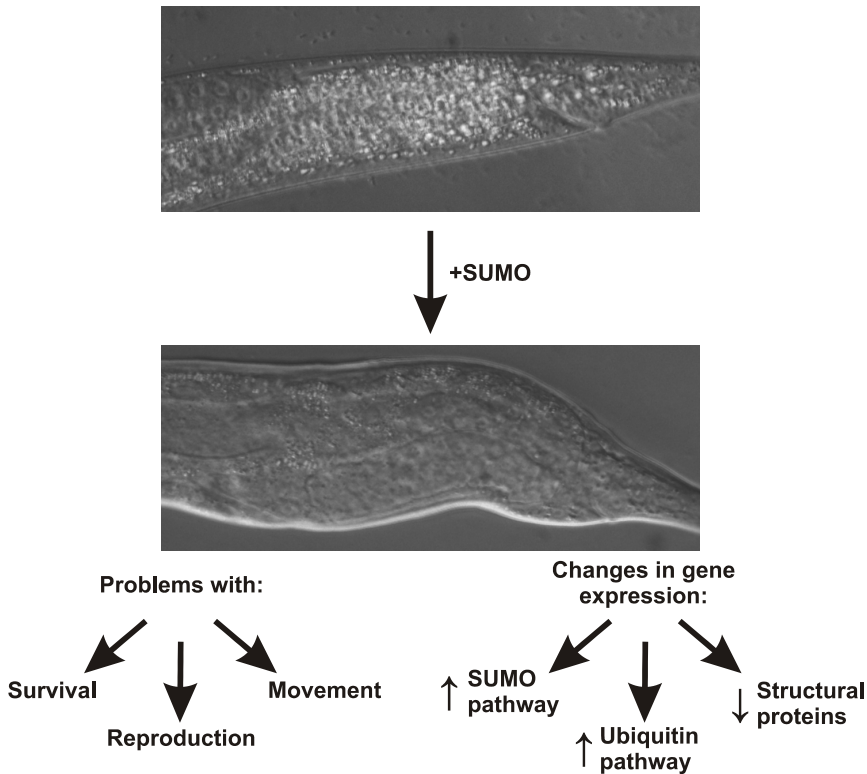
Figure 11. A summary of the biological changes in *C. elegans* due to increased SUMO expression. The pictures present the posterior part of the *C. elegans*.

5.1.2 SUMO-induced changes in gene expression in *C. elegans*

It is known that SUMOs can regulate transcriptional activity of genes through the modification of transcription factors and co-regulators. The potential alterations in gene expressions in pan-neuronal and pan-muscular SUMO-1 expressing nematodes were assessed by microarray-based profiling. Although, SUMOs are often associated with transcriptional repression, the overall pattern of the genes which had significantly different fold changes compared to wild type nematode did not however show any general repressive effect (45% repressed; 55% induced). However, the microarray analyses revealed that the expression of genes in different developmental pathways was altered, particularly the pathways in the reproductive organs (Fig. 4 in I). In addition, several nucleus/chromatin-associated and transcription factor genes were up- or downregulated, respectively.

Strikingly, several of the SUMO and ubiquitin-proteasome pathway genes were upregulated (Table 2 in I). The increased expression of these genes can be an indication of a stress response, albeit we did not see any significant increase in the expression of stress

responsive heat shock protein encoding genes. Neither did we notice marked differences in the general ubiquitylation levels of proteins. The absence of increased heat shock protein expression and ubiquitylation of proteins does not rule out the possibility that the increased SUMO pathway gene expression is related to the imbalance of SUMO levels in cells, which is potentially stressful for the cells.

The monoubiquitylation of histone 2B (H2Bub1) is a marker for gene activity. It has been linked to highly expressed genes as a marker needed for transcriptional elongation (Pavri *et al.* 2006; Minsky *et al.* 2008). Furthermore, H2Bub1 has been implicated in the regulation of other histone marks, mainly methylation on histone 3 lysine 4 and 79 (Zhu *et al.* 2005; Lee *et al.* 2007; Nakanishi *et al.* 2009; Takahashi *et al.* 2009). Ring finger proteins 20/40 (RNF20/40, BRE1A/B in yeast) are ubiquitin E3 ligases responsible for the ubiquitylation of the H2B at Lys120 in humans (Hwang *et al.* 2003; Wood *et al.* 2003; Zhu *et al.* 2005). Interestingly, in the SUMO overexpressing nematodes, the RNF20/40 ortholog *ring finger protein 1* (*rfp-1*) gene expression was significantly increased. In line with that, the H2Bub1 levels were also modestly increased in the SUMO overexpressing worms, which may in part contribute to the observed changes in gene expression.

Taken together, the unbalanced SUMOylation system disturbs the normal development of the nematodes. The altered gene expression profiles and the phenotypic changes in the nematodes indicate that SUMOs have versatile effects in muscle cells. This suggests that a proper control of the SUMO levels is also necessary to maintain appropriate protein-protein interactions needed for normal homeostasis in a multicellular organism.

5.2 SUMO MODULATES THE FUNCTION OF CO-REGULATORS (II, III)

The co-repressor RIP140 is an 1158-amino acids long protein that consists of four individual repression domains (RD1-RD4) (Figure 12). RIP140 contain LxxLL motifs that enable the interactions with nuclear receptors. The repression mediated by RD1 involves the recruitment of HDACs, and the RD2 mediate interaction with C-terminal binding protein (Christian *et al.* 2004; Castet *et al.* 2004). The repressive mechanisms of RD3 and RD4 have been unclear.

The co-activator PGC-1α is a 797-amino acids long protein that contains an N-terminal activation domain with three LxxLL motifs mediates interaction with nuclear receptors (Sadana and Park, 2007) (Figure 12). Next to the activation domain, there is a repression domain that mediates interaction with p160 myb-binding protein, and phosphorylation by p38 releases the repression (Fan *et al.* 2004). In the C-terminus, there is an RNA processing motif (Monsalve *et al.* 2000). The PGC-1β and PGC-1-related co-activator (PPRC1 or PRC), which have similar domain structure, also belong to the PGC family (Meirhaeghe *et al.* 2003; Scarpulla, 2002).

5.2.1 SUMO modification in RIP140 and PGC-1α
In our study, RIP140 was discovered as a novel target of SUMOylation at two distinct sites in the repression domain 3 and 4, Lys756 and Lys1154, respectively (Figure 12 and Fig. 1 in II). RIP140 is modified with both SUMO-1 and SUMO-2. The repression domains

had previously been identified to have individual repression capabilities but the mechanism was unknown. The SUMOylation on these two sites did not affect the stability of the co-repressor (Fig 3 in II). Normally, RIP140 resides in small speckles in nucleus. Interestingly however, the localization of RIP140 was more dispersed in the nucleus in the presence of SUMO-1. In contrary, RIP140 located in the small speckles when the SUMO consensus sites were disrupted and in the presence of conjugation-negative SUMO-1 (Fig. 7 in II). Unfortunately, the quality of the commercially available antibodies for RIP140 did not allow studies on the endogenous SUMOylation of RIP140. However, endogenous RIP140 was later identified as a SUMOylated protein in a mass spectrometry-based analysis of HeLa cell proteome (Golebiowski *et al.* 2009).

In the PGC-1α, the SUMOylation site Lys138 was identified at N-terminal activation domain of the co-activator (Figure 12 and Fig. 1 in III). Since the same site was reported as a target for acetylation, two different point mutations were made, Lys138 to Arg (KR) and Glu185 to Ala (EA), to disrupt the SUMO acceptor lysine and the SUMOylation consensus site, respectively. The subnuclear localization of PGC-1α was not altered by the SUMOylation (Fig.1 in III). The co-activator is degraded through the ubiquitin-proteasomal pathway (Olson *et al.* 2008; Trausch-Azar *et al.* 2010). However, the proteasomal degradation of PGC-1α was not dependent on the SUMO modification at the target lysine (Fig. 2 in III). Detection of a cysteine protease inhibitor sensitive band with anti-PGC-1α antibody from mouse kidney tissue suggests that PGC-1α is also endogenously SUMOylated (Fig. 1 in III). In keeping with the results, a member of the PGC-1 family, the PGC-1-related co-activator was found to be targeted by SUMOylation as an endogenous protein in a mass spectrometry-based analysis of HeLa cell proteome (Golebiowski *et al.* 2009).
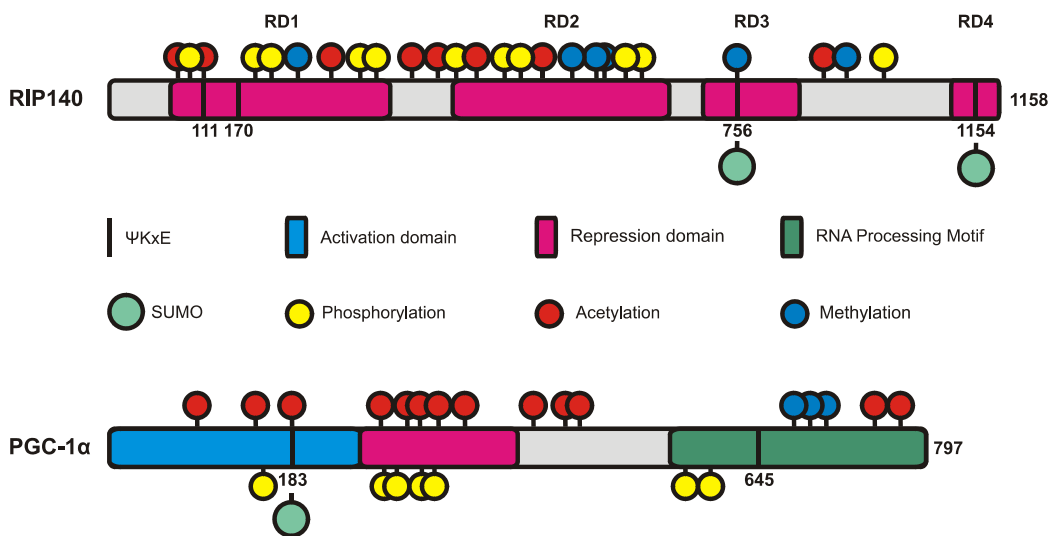

Figure 12. Schematic illustration of PTMs in RIP140 and PGC-1α. RD: repression domain.

5.2.2 Functional consequences of SUMOylation in RIP140 and PGC-1α

The RIP140 repression function was analyzed by two different reporter gene assays. The transcriptional repression by RIP140 was clearly observed in an assay, where Gal4-DNA binding domain fused RIP140, repressed the LexA-VP16-fusion-protein-activated reporter gene expression (Fig. 4 in II). The SUMOylation deficient RIP140 was significantly less repressive. Also, in the estrogen receptor α (ERα)-activated reporter gene assay, RIP140 repressed the transcriptional activation (Fig. 5 in II). The abolishment of the SUMOylation in RIP140 significantly reduced the repression activity. Similar results were also detected in the transcriptional activation mediated by the vitamin D receptor/retinoid X receptor α dimer, which suggest that the regulation was not selective only for ERα. The diminished repression was not due to a weaker interaction between RIP140 and ERα. This was ruled out by 2-hybrid interaction assays and confocal imaging of the co-localization patterns (Fig. 6 and 8 in II).

In addition to SUMOylation, the co-repressor RIP140 is also phosphorylated, acetylated, and methylated at Arg and Lys residues. Phosphorylation and acetylation increase the interaction of RIP140 with HDACs, increasing the repressive potential of the co-repressor (Gupta *et al.* 2005; Huq *et al.* 2005; Huq and Wei, 2005; Ho *et al.* 2008). Although, one study suggests that acetylation at RD2 of RIP140 would abolish the interaction with C-terminal binding protein and hence decrease the repressive function (Vo *et al.* 2001). Moreover, the methylation at Arg residues of RIP140 has been reported to decrease the repressive potential (Mostaqul Huq *et al.* 2006; Gupta *et al.* 2008), whereas the Lys-methylation was shown to increase the transcription repression function by supporting the interaction with HDAC3 (Huq *et al.* 2009). One of the methylated lysines reported by Huq et al. is the Lys756 residue that was identified as a target of SUMOylation (II). The HDAC inhibitor, trichostatin A, did not have any effect in the experimental settings used to compare the repression function of SUMOylation deficient vs. wild type RIP140 (II). This does not rule out that the possible mechanism of enhanced repression due to SUMOylation involves the recruitment of HDACs.

The transcriptional activation function of PGC-1α was first studied by a 1-hybrid assay, where the Gal4-DNA binding domain was fused to PGC-1α, activating the luciferase expression by binding to Gal4 binding sites. Activity of PGC-1α was increased when Arg-to-Lys point mutation of the target lysine blocked the SUMOylation (Fig. 4 in III). In the 1-hybrid assay, the two different mutations KR and EA showed similarly increased activation potential. However, the two different SUMOylation deficient PGC-1α forms differently modulated the transcriptional activation by PPARγ and ERRγ. The EA mutation that is predicted to only disrupt the SUMOylation, without affecting the acetylation at Lys138, was more active than the wild type PGC-1α. In contrast, the KR mutation was less active than the wild type.

In addition to the SUMOylation, PGC-1α is modified by arginine methylation, acetylation, phosphorylation, and ubiquitylation. Arginine methylation by protein arginine methyltransferase 1 (CARM1) increases the transactivation function of PGC-1α (Teyssier *et al.* 2005). In addition, phosphorylation by p38 and AMP-activated protein kinase at different sites on PGC-1α can have an increasing effect on the PGC-1α (Puigserver *et al.* 2001; Fan *et al.* 2004; Jäger *et al.* 2007). The acetylation is catalyzed by GCN5 and deacetylation is mediated by SIRT1. Acetylation of PGC-1α decreases the activation potential of the co-activator (Nemoto *et al.* 2005; Rodgers *et al.* 2005; Lerin *et al.*

2006). Many of these modifications, except for acetylation and SUMOylation, are activating the function of PGC-1α. The modifications may affect the interaction with the co-repressors and co-activators. Recently, RIP140 was reported to interact and repress the co-activation mediated by PGC-1α (Hallberg *et al.* 2008). Although differences in the interactions of RIP140 with wild type or SUMOylation deficient PGC-1α were not observed (Fig. 6 in III), when RIP140 was added to the 1-hybrid assay activated by PGC-1α, it revealed that RIP140 did not repress EA mutant of PGC-1α as potently as wild type or KR mutants of PGC-1α. The co-operation with RIP140 may explain the opposite effects of the two different SUMOylation-disrupting mutations of PGC-1α.

5.2.3 Dual modification at the same lysine residue in RIP140 and PGC-1α

Remarkably, both RIP140 and PGC-1α have SUMOylation sites that are also targeted by methylation or acetylation, respectively. Recently, the Lys756 of RIP140 was reported to be targeted by methylation (Huq *et al.* 2009). Huq and colleagues showed that mutation of the methylation-targeted lysines results in an increase in the arginine methylation. They also demonstrated that mutation of the Lys756 on RIP140 leads to decreased repression possibly due to disrupting the interaction with HDAC3 (Huq *et al.* 2009). To determine whether the methylation or SUMOylation in Lys756 is more important for the regulation of RIP140, a mutation that disrupts the SUMO consensus site but leaves the Lys756 intact would be required. To the best of my knowledge, other examples of methylation and SUMOylation targeted to a same lysine residue have not been reported.

In the case of acetylation or SUMOylation of Lys138 on PGC-1α, there were small but visible differences in the activation function (Fig. 4 and Fig. 5 in III). Nevertheless, the separate assessment of a modification in the same residue is challenging and preferentially requires the analysis by mass spectrometry. For the Krüppel-like transcription factor 8 (KLF8) a switch of acetylation and SUMOylation at the same Lys67 was described (Urvalek *et al.* 2011). The KLF8 is acetylated at Lys67, Lys93, and Lys95, and the abolishment of acetylation (by HDAC inhibitor or mutation of Lys93 and Lys95) increases SUMOylation, which is followed by repression of KLF8. In addition, p53 and hypermethylated in cancer 1 (HIC1) have a similar acetylation or SUMOylation switch at the same residue (Wu and Chiang, 2009; Stankovic-Valentin *et al.* 2007). In the case of PGC-1α, the SUMOylation was not dependent of acetylation or phosphorylation. Although the SUMOylation was decreased by increased phosphorylation catalyzed by p38, the decrease was also observed in the phosphorylation-site mutant PGC-1α (Fig. 2 in III). The attenuated SUMOylation in all p38 induced phosphorylation samples indicate that there is other targets for p38 in the SUMOylation pathway, which results in decreased SUMOylation of PGC-1α.

The dual targeting of the same residue suggests that the region is important in the regulation of the particular protein, and/or the site is highly available for the separate modifying enzymes. However in both RIP140 and PGC-1α, the effects of the modifications are similar. Methylation and SUMOylation potentiate the repressive function of RIP140, whereas acetylation and SUMOylation repress the activation function of PGC-1α. The fact that many amino acid residues are targeted in the methylation of RIP140 and, in particular in the acetylation of PGC-1α, raise a question of how important one specific modification site among the other sites can be? It is possible that certain PTMs

occur only in a particular cell cycle stage or in a subcellular compartment. Since the co-regulators function in many diverse signaling pathways, the different PTMs may direct as a "zip code" the co-regulators to separate interacting partners, pathways, and localizations.

A well known dilemma with SUMOylation is that often only a small fraction of the total pool of a specific protein is SUMOylated at a given time point. Several reasons for this phenomenon may be possible. For instance when the cells are harvested, the SENP activity cannot be inhibited quickly and sufficiently enough, which results in considerable deSUMOylation of the proteins. The SUMOylation process is probably an ongoing cycle of addition and removal of SUMOs, and therefore at a given moment the level of modification is present only in a fraction of the total pool of proteins. However, it still is a very important modification in the function of the substrate proteins.

In conclusion, in RIP140, the SUMOylation of the Lys756 in RD3 and of the Lys1154 in RD4 supports the repressive function. In the case of PGC-1α, the SUMO-tag is located in the activation domain at Lys138, which leads to repression of the activation function of the co-activator. In both cases, the bulky SUMO-tag controls the function of these important co-regulators. This adds a novel mechanism of regulation in metabolism and energy homeostasis.

6 Conclusions and Future Prospects

The research of PTMs has evolved tremendously during the last decades and the number of reports on PTMs is constantly growing. Particularly in the field of SUMOylation, the numbers of targeted proteins are increasing. At first, SUMOs were considered a modification of nuclear proteins, but this view has been revised. The role of SUMOs in a wide variety of mechanisms is intriguing. Even spatial learning of rats has been recently coupled with SUMOylation (Tai *et al.* 2011).

In this dissertation, the role of SUMO balance in a whole multicellular organism was evaluated. The approach of inducing ectopic SUMOs in to a whole organism has not previously been utilized. The importance of a balance in SUMO levels was clearly observed, as the increased SUMO levels deteriorated the normal development of nematode muscles. Moreover, one could assume that changes in the gene expression profiles would help to explain the versatile role of SUMO proteins in the development and function of the cells and tissues of a whole organism. Furthermore, the established transgenic nematodes expressing SUMO-1 and SUMO-2 provide a tool for further studies of SUMOylation in a whole organism.

Many transcription factors and co-regulators involved in various signaling pathways are regulated by SUMOylation. The activities of RIP140 and PGC-1α in the regulation of metabolism in the adipose, liver and muscle tissues are mandatory for proper energy homeostasis. This thesis shows that SUMOylation regulates the function of these important co-regulators, adding a novel pathway to the regulation of metabolism and energy homeostasis.

SUMOylation is implicated in many different disease states. SUMOs modify many oncoproteins, and thus, losing the balance in the SUMOylation and deSUMOylation may be favorable for the cancer progression. SUMOs are also present in the inclusions of many neurodegenerative diseases and many proteins involved in these diseases are targets of SUMOylation.

Paralogs of SUMOs and SUMO pathway proteins show functional redundancy in multicellular organisms. The SUMO paralogs were functionally redundant in zebrafish, and *Sumo-1*-knockout mice did not suffer severe complications. How well the deletion of the other SUMO paralogs (SUMO-2/3) will be compensated by SUMO-1 in higher order mammals remains to be determined. In the case of PIAS proteins, individual *Pias1* or *Pias4* knock-out mice have only mild defects but combined deletion of both *Pias1* and *Pias4*, results in an embryonic lethal phenotype (Tahk *et al.* 2007). In the future, the investigation of the physiological role of the SUMOylation pathway may require deletion of more than one SUMO paralog concomitantly to reveal possibly specific functions of the particular paralogs.

7 References

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Miia Rytinki

SUMOs in the Regulation of Transcriptional Co-regulators RIP140 and PGC-1α and in the Development of C. elegans

The developmentally vital conjugation of small ubiquitin-like modifier (SUMO) proteins to target proteins regulates their interactions, localizations, stabilities and activities. In this thesis, new insights into the role of SUMO in the development of a whole multicellular organism (*C. elegans*) are presented. Additionally, the SUMO conjugation is demonstrated to act as a novel mechanism in the regulation of two transcriptional co-regulators RIP140 and PGC-1α that are important for the metabolism and energy homeostasis.

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