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Studies Of Sumoylation In Regulating Mif Stability And Rangap1 Nucleo-Cytoplasmic Shuttling In Controlling Its Sumo Modification

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STUDIES OF SUMOYLATION IN REGULATING MIF STABILITY AND RANGAP1 NUCLEO-COTYPLASMIC SHUTTLING IN CONTROLLING ITS SUMO MODIFICATION

by

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DEDICATION

To my dearest Maa

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CHAPTER 1: INTRODUCTION

Post-translational modification (PTM) of proteins significantly contributes to the complexity and diversity of proteome (Walsh, Garneau-Tsodikova et al. 2005). PTMs alter function of target proteins by altering their subcellular localization, activity, stability or interaction with other proteins. A protein can be modified at multiple residues simultaneously. PTMs may be dynamic and reversible in nature. More than 200 different types of PTMs are known so far (Prabakaran, Lippens et al. 2012). There are two major forms of post-translational modification (Figure 1.1):

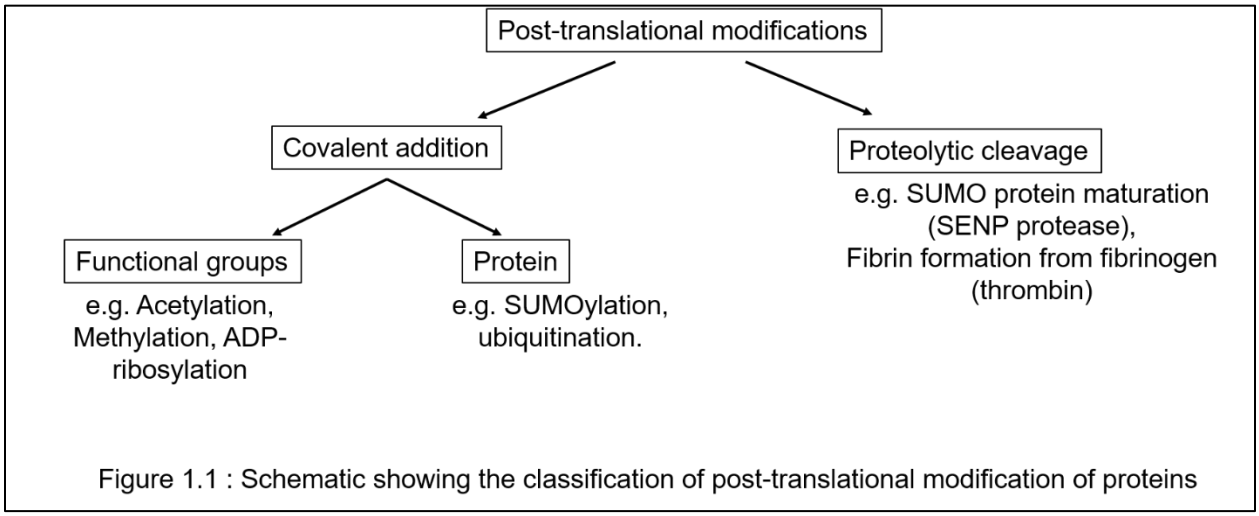

Figure 1.1 : Schematic showing the classification of post-translational modification of proteins

a) Covalent addition of functional groups or other proteins or peptides to target proteins.

b) Proteolytic processing of proteins.

<u>Covalent addition of functional groups/proteins/peptides to substrate proteins</u>

Post-translational processing can modify the N-terminus, C-terminus or any specific internal residue of a protein (Wold 1981). Functional groups such as glycosyl-, acetyl-, methyl-, phosphoryl-, ADP-ribosyl- can be added to the target proteins. Metabolic donors like ATP, Acetyl-CoA, NAD carry the functional groups, and the forward and

reverse modifications involve single enzymes respectively. Polypeptide modifications such as ubiquitination, SUMOylation and neddylation also occur. For polypeptide conjugation, the forward reactions require a set of enzymes, whereas the removal of modifiers need single enzymes.

Proteolytic processing of proteins

Highly regulated proteolytic cleavage of proteins is an irreversible yet ubiquitous form of post translational modification. Proteolysis gives rise to neo- N- and C- termini of proteins (Neurath and Walsh 1976). An example of proteolysis is the absence of the initial methionine residue in a matured, newly synthesized protein. Specific families of proteases perform proteolytic processing of proteins, these proteases are named after the amino acid residues they target for proteolysis. These include cysteine proteases, metalloproteases, aspartic acid proteases, mixed proteases, serine proteases and threonine proteases (Lopez-Otin and Bond 2008). There are myriad of examples where proteases and peptidases help in maturation of precursor proteins. For examples, newly synthesized SUMO is cleaved at its C-terminus by a family of cysteine proteases, known as Sentrin like proteases (SENPs) in vertebrates, to form a mature and functional modifying protein (Xu and Au 2005). Various hormones, enzymes, and blood complement factors are synthesized as precursors, they are activated by proteolytic cleavage of their polypeptide structures, etc (Brinkhous and Scarborough 1969, Orci, Ravazzola et al. 1987, Terada and Nakanuma 1995).

However, there other post-translational modifications whose deconjugation machinery is not known so far. These modifications fall in the category of irreversible enzymatic PTMs. One such example is alkylation; there is no known modification enzyme

for this PTM so far, however, in 2005 an oxidative route for removal of the N-alkyl bonds has been reported (Walsh, Garneau-Tsodikova et al. 2005, Li, Chordia et al. 2007).

Furthermore, there are non-enzymatic modifications of proteins which occur under various environmental conditions, such as glycation and carbonylation. In general, glycation affects the normal functions of target proteins in an adverse way, and carbonylation is a result of oxidative stress (especially metal catalyzed) on proteins (Harding 1985).

The SUMO pathway

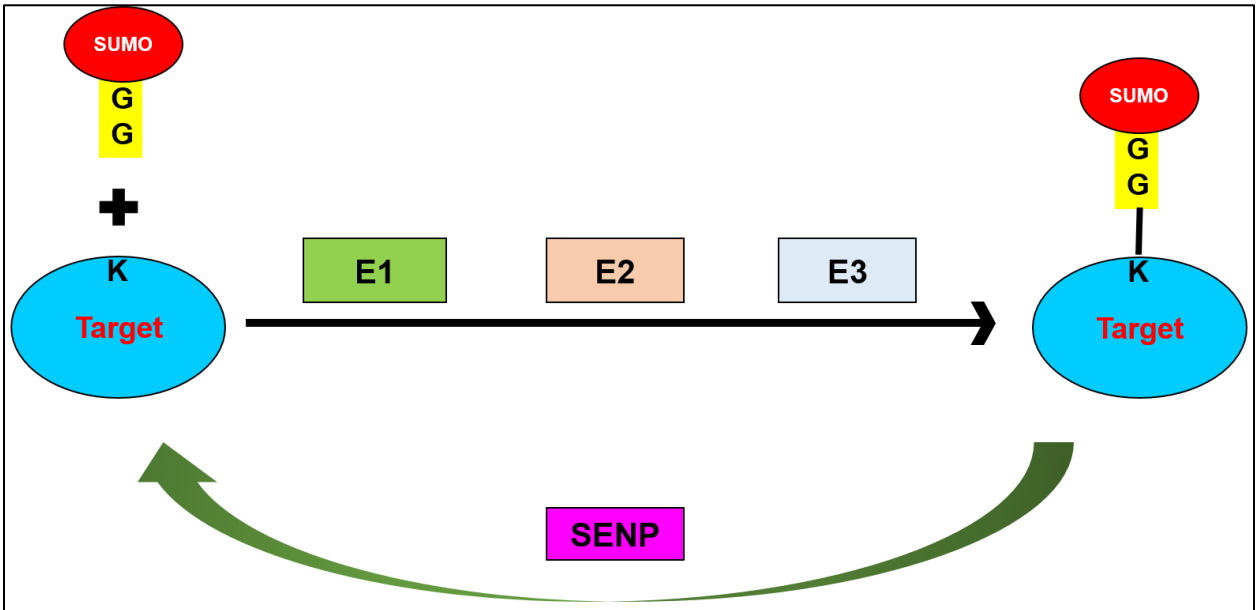

Figure 1.2 : The SUMOylation pathway. SUMOylation is an enzymatic reversible post-translational modification of proteins. The conjugation involves the E1 activating enzyme, E2 conjugating enzyme and E3 ligase enzyme, and the deconjugation is mediated by the SUMO deSUMOylase, Sentrin-like protease (SENP).

Small ubiquitin-like modifiers (SUMOs) are around 100 amino acid residue-containing proteins (about 10kDa), which can modify hundreds of specific target proteins at their lysine residues in a reversible manner (Figure 1.2). This modification affects several aspects of cell physiology including nucleo-cytoplasmic shuttling, cell migration,

DNA repair, transcription, cell cycle and protein stability. First discovered by Michael Matunis, SUMO-1 was found to be essential for localization of the first discovered SUMO target, RanGAP1, at the nuclear pore complex (Matunis, Coutavas et al. 1996). RanGAP1 regulates nulceo-cytoplasmic transport of proteins, through activating the hydrolysis of the Ran GTPase-bound GTP to GDP (Matunis, Wu et al. 1998).

SUMO proteins are present in all eukaryotic organisms from yeasts to humans. In yeast, *C. elegans* and *D. melanogaster*, there is a single gene for SUMO, whereas in plants and vertebrates there are multiple SUMO genes (Muller, Hoege et al. 2001, Park, Kim et al. 2011). In humans, there are three SUMO paralogs, SUMO1, SUMO2 and SUMO3. SUMO2 and 3 are 96% identical with each other, whereas SUMO1 is only 45% identical to SUMO2/3 (Hay 2005). The SUMO proteins are synthesized as precursors, and they have an extra 2-11 amino acid residues after an invariant di-glycine (GG) motif at their C-terminal ends (of mature SUMOs). SENPs in vertebrates and its homolog Ubl-specific protein protease in yeast serve for removing the extra residues after the GG motif to generate the mature SUMOs.

The SUMOylation machinery

SUMOylation is a post-translational protein modification like ubiquitination. The SUMOylation of proteins involves three steps involving different classes of enzymes, E1 activating enzyme, E2 conjugating enzyme and E3 ligating enzyme (Hay 2005, Geiss-Friedlander and Melchior 2007, Wang and Dasso 2009, Gareau and Lima 2010). SUMO E1 heterodimer SAE1/SAE2 (SUMO activating enzyme subunit 1/SUMO activating subunit 2) activate the SUMO by formation of a thioester bond between the catalytic cysteine residue of SAE2 and the C-terminal glycine carboxyl group of SUMO. During the

second step, SUMO transfers to the E2 conjugating enzyme Ubc9. The catalytic cysteine residue of Ubc9 forms a thioester bond with the C-terminal carboxy group of SUMO. The third step involves the transfer of SUMO from Ubc9 to the substrate protein, resulting in the formation of an isopeptide bond between the terminal glycine of SUMO and the ε-chain of a lysine residue in the substrate protein. At present, only a single E1 heterodimer and a universal E2 enzyme (SAE1/SAE2 and Ubc9 respectively) are known for the SUMOylation pathway; the presence of multiple SUMO E3 ligases determines target specificity *in vivo* (Geiss-Friedlander and Melchior 2007). Several SUMO isopeptidases, known as Sentrin-like proteases (SENPs), de-conjugate SUMO from its targets (Li and Hochstrasser 1999, Mukhopadhyay and Dasso 2007).

SUMO E3 ligases

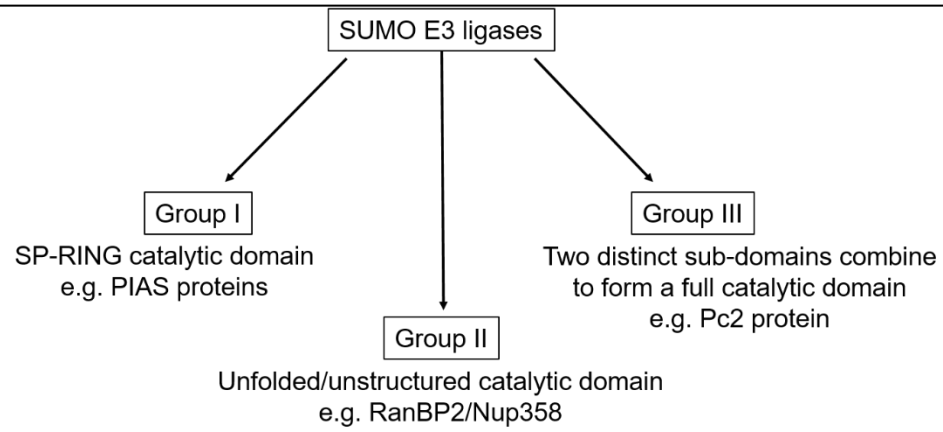

Figure 1.3 : Classification of the SUMO E3 ligases. They constitute three major groups, out of which the group I category E3 ligases are present in all eukaryotes ranging from yeast to human. The groups II and II, however, are restricted to the vertebrates.

The specificity of the SUMO modification is attributed to SUMO-specific E3 ligases that designate specific targets or distinct group of substrate proteins for their conjugation. There are three major categories of SUMO E3 ligases: the group I E3 ligases has a characteristic SP-RING catalytic domain, the group II has a highly unfolded E3 catalytic

domain, and the group III E3 ligases. The group III E3 ligases contain two distinct domains with no similarity in sequence to the other two classes of SUMO E3 enzymes (Figure 1.3). The group I E3 ligases include PIAS (protein inhibitor of activated STAT) proteins that comprise of PIAS1, PIASxα, PIASxβ, PIAS3, PIASy and Topors (Topoisomerase I binding protein) in vertebrates. Mms1 is present both in vertebrates and yeast (Jackson 2001, Kotaja, Karvonen et al. 2002, Weger, Hammer et al. 2003, Duan, Sarangi et al. 2009). These E3 ligases have an N-terminal scaffold attachment factor-A/B (SAP) domain, a PIAS motif, a PINIT domain and the C-terminal serine/threonine rich region, in addition to a highly-conserved SP-RING domain (Schmidt and Muller 2003, Weger, Hammer et al. 2005, Sharrocks 2006). RanBP2 (Ran binding protein 2) or Nup 358 represents the group II SUMO E3 ligases, it is a vertebrate specific SUMO E3 ligase. The catalytic domain of RanBP2 binds the SUMO E2 enzyme, Ubc9 and the SUMO protein, and positions the charged Ubc9 with the bound SUMO for a favorable interaction with an acceptor lysine residue in a target protein. RanBP2 is known to stimulate the SUMOylation of Sp100, HDAC4 and PML (Kirsh, Seeler et al. 2002, Pichler, Gast et al. 2002, Pichler, Knipscheer et al. 2004). The group III is represented by the Pc2/polycomb group member 2 SUMO E3 ligase (Kagey, Melhuish et al. 2003). The Pc2 proteins are present only in vertebrates. Transcription regulators, such as the deacetylases HDAC4 and HDAC7 also function as highly specific SUMO E3 ligases. However, detailed analyses are needed to demonstrate if they fall in one of the groups mentioned above, or they form a separate category of SUMO E3 ligases (Zhao, Sternsdorf et al. 2005, Gao, Ho et al. 2008).

SUMO isopeptidases

SUMO isopeptidase	Sub-cellular localization	SUMO maturation	SUMO paralog specificity
SENP1	Nuclear envelope, nuclear foci (PML nuclear bodies)	SUMO 1/2/3	SUMO 1/2/3
SENP2	Nuclear envelope, nuclear foci (PML nuclear bodies)	SUMO 1/2/3	SUMO 1/2/3
SENP3	Nucleolus	SUMO 2/3	SUMO 2/3
SENP5	Nucleolus	SUMO 2/3	SUMO 2/3
SENP6	Nucleoplasm	-	SUMO 2/3 chain editing
SENP7	Nucleoplasm	-	SUMO 2/3 chain editing
USPL1	Cajal bodies (colocalizes with coilin)		SUMO 1/2/3
DeSI-1	Cytoplasm	-	SUMO 1/2/3, SUMO 2/3 chain editting
DeSI-2	Cytoplasm	-	SUMO 1/2/3, SUMO 2/3 chain editing

Table 1.1 : The major categories of SUMO isopeptidases (SENPs). They are classified depending on their SUMO paralog specificity and subcellular localization.

SUMOylation can be reversed by a group of de-SUMOylases that remove the SUMO moieties from target proteins, rendering this PTM dynamic. The SUMO isopeptidases, also called the Sentrin like proteases or SENPs, are cysteine proteases with a conserved set of around 200 amino acid residues near their C-terminal ends which contain the catalytic triad. The N-terminal regions of the SENPs are distinct and determine their specific subcellular distribution and substrate specificities (Nayak and Muller 2014). There are six SUMO isopeptidases in human namely SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7; all of them have distinct subcellular localization. There are different families of cysteine proteases and the SENPs lie in the C48 cysteine protease family. Also, each of the SENPs have distinct subcellular distribution. SENP1 shuttles between cytoplasm and nucleus, SENP2 is highly enriched at the nuclear pore complexes, SENP3 and SENP5 are present in the nucleolus, and the SENP6 and SENP7 are mainly present

in the nucleoplasm (Nayak and Muller 2014) (Table 1.1). There is another group of SUMO isopeptidases found only in the mammals with no homologues in other eukaryotes, DeSi-1 and DeSi-2 (Shin, Shin et al. 2012). DeSi-1 is distributed in both cytoplasm and nucleus whereas the DeSi-2 is found only in the cytoplasm. The catalytic domains of DeSi-1 and -2 lie near their N-terminal regions, in contrast to the C-terminal catalytic domains of the other SENPs. The most recent addition to this growing list of SUMO isopeptidases is USPL1/ubiquitin specific protease-like 1. USPL1 colocalizes with coilin in Cajal bodies and is not very abundant in cells (Schulz, Chachami et al. 2012).

Target site selection and the SUMOylation motif

There are three different but not mutually exclusive ways by which SUMO can modify an acceptor lysine in a target protein (Flotho and Melchior 2013).

1) The lysine in the target protein is present in a small signature motif, known as SUMOylation consensus site, which is recognized by the SUMO E2 enzyme Ubc9 for conjugation.
2) The target protein has a SUMO interaction motif (SIM) which recruits the Ubc9~SUMO thioester through its interaction with the SUMO moiety and results in the SUMO conjugation of a nearby lysine residue. This process is called SIM-mediated SUMOylation.
3) The SUMO E3 ligases can simultaneously bind both the charged Ubc9 and the target protein, and thus orients the specific lysine residue of the substrate protein for modification. The process is known as the E3 ligase-dependent SUMOylation.

Name	Motif
Classical SUMOylation consensus motif	ψKx(E/D)
Inverted SUMOylation motif	(E/D)xKψ
Hydrophobic consensus motif	ψψψKxE
Phosphorylated SUMOylation motif(pSuM)	ψKx(pS)
Extended pSuM	ψKx(pS)(pS)xx(pS)
Phosphorylation dependent SUMOylation motif	ψKxE(pS)

Table 1.2 : The known SUMOylation motifs. In addition to these canonical sites, some proteins are SUMOylated at non-canonical sequences.

SUMOylation occurs at the lysine residues present within the consensus sequence- ψKx(E/D); where ψ is a hydrophobic residue, x is any residue, and E/D represents glutamic acid (E) and aspartic acid (D) respectively (Rodriguez, Dargemont et al. 2001). Interestingly, recent studies have shed light on some variations to the consensus SUMO motif. These motifs include an inverted motif (E/D)xKψ, hydrophobic consensus motif ψψψKxE, phosphorylated SUMOylation motif (pSuM) ψKx(pS) (pS represents phosphorylated Serine residue) and extended pSuM with the sequence ψKx(pS)(pS)XXX(pS)P (Matic, Schimmel et al. 2010). Furthermore, studies show that the SUMO consensus motif in certain proteins also contain additional residues and motifs that facilitate SUMOylation. One such example is phosphorylation-dependent SUMOylation at ψKxExx(pS) (Hietakangas, Anckar et al. 2006, Yang and Gregoire 2006) (Table 1.2). However, some proteins are SUMOylated at lysines that do not lie in any of the signature sequences mentioned above (Hoege, Pfander et al. 2002, Pichler, Knipscheer et al. 2005, Figueroa-Romero, Iniguez-Lluhi et al. 2009). One such example is the *S. cerevisiae* protein PCNA, SUMOylated at a non-canonical site lysine 164. Human E2-25K modified at lysine 14, also lacks the canonical SUMOylation sequence (Hoege,

Pfander et al. 2002, Pichler, Knipscheer et al. 2005, Gali, Juhasz et al. 2012). The occurrence of the non-canonical sites has widened the repertoire of the SUMO targets.

SUMO interaction motif

First reported during a study on PML protein in yeast, non-covalent interaction of SUMO with target proteins is important for formation of multiprotein complexes (Boddy, Howe et al. 1996). Numerous studies suggest that these interactions are central to various cellular processes, such as, protein stability and chromosome segregation during mitosis (Poulsen, Hansen et al. 2013, Sridharan and Azuma 2016). SUMO binding motif (SBM) was first reported in yeast (Minty, Dumont et al. 2000). In this study, analysis of the SBM of the SUMO1-interacting partners revealed a common sequence of 11- amino acid region (probable SBM) that constituted the core SXS motif (two serine residues with a central amino acid). Acidic residues (D/E) flank the C-terminal side of the motif and the N-terminal region flanked by hydrophobic residues. However, a study done by Jing Song et al in 2004 showed that the SUMO-binding motif is essentially a hydrophobic amino acid rich region with the sequence V/I-X-V/I-V/I. The second position in the sequence can be occupied by a polar or acidic residue (Song, Durrin et al. 2004). Further studies on yeast identified a similar sequence with a hydrophobic core and flanking acidic residues (Hannich, Lewis et al. 2005, Hecker, Rabiller et al. 2006). In RanBP2, the hydrophobic core is preceded by the acidic stretch; whereas in PIASxα, the acidic stretch follows the hydrophobic SIM motif. TTRAP (TRAF and TNF receptor associated protein) interacts with the TNF-R family of receptors and preferentially binds SUMO2. The SIM motif of TTRAP lacks any acidic amino acid stretch (Hecker, Rabiller et al. 2006). Another study performed with PIAS1, PIAS2 and PIAS3 shows that the presence of phosphorylated

residues along with the hydrophobic core is important for binding for both SUMO1 and SUMO2 (Stehmeier and Muller 2009). The SIM binds the unstructured region of the SUMO protein (Song, Zhang et al. 2005).

There are numerous examples of SIM motif functions in proteins. A classic example is nuclear transport involving the nucleoporin RanBP2/Nup358; RanGAP1, when modified by SUMO1, binds Nup358/RanBP2 and forms a stable complex at the nuclear pore (Song, Durrin et al. 2004, Song, Zhang et al. 2005). In the PML complex, the PML protein recruits Sp100, DAXX and CBP. These proteins, including PML, have SUMO-binding motifs (Lin, Huang et al. 2006, Shen, Lin et al. 2006). The transcription repressors, HDAC2 and HDAC6, are recruited by SUMO-modified transcription coactivator p300 and transcription factor Elk-1, via SUMO interaction motifs (Girdwood, Bumpass et al. 2003, Yang and Sharrocks 2004). Hence, several cellular processes such as nuclear transport, transcription and protein degradation require SIM for SUMOylation-dependent regulation (Geoffroy, Jaffray et al. 2010, Kolesar, Sarangi et al. 2012, Gartner and Muller 2014).

Mechanism of SUMOylation activity

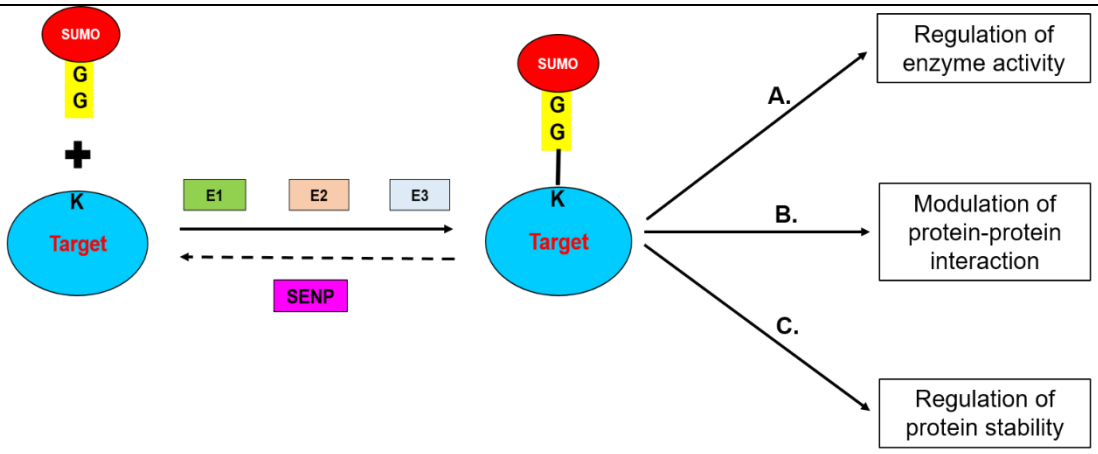

Figure 1.4 : Mechanism of SUMOylation activity. A. SUMOylation of target protein may result in altering its structure, hence, affecting its function. B. Modulation by either enhancing or inhibiting protein-protein interactions is the most predominant mechanism by which SUMOylation functions. C. SUMOylation can effectively regulate protein stability by either competing with ubiquitination and preventing degradation or by stimulating polyubiquitination-mediated protein degradation.

SUMOylation influences cellular pathways via one of the three mechanisms to function, which include protein-protein interaction, subcellular localization and protein stability (Figure 1.4).

Regulation of protein-protein interaction

One of the major mechanisms by which SUMOylation regulates protein function is by modulating interactions with other proteins. In most cases, SUMOylation enhances such interactions and facilitates in formation or maintenance of large protein complexes. Alternatively, SUMOylation can hinder binding of a protein and disrupt complex formation. These two possible outcomes can regulate downstream signaling pathways. One of the most well-studied examples is the formation of PML complex in the nucleus (Zhong, Muller et al. 2000, Lin, Huang et al. 2006). Another example is the modulation of transcription factor Elk-1. Elk-1 SUMOylation leads to the interaction with histone deacetylase protein HDAC2, and this binding results in a decreased histone deactylation

and inhibition of Elk-1 target gene transcription (Yang and Sharrocks 2004). SUMO1 modification of RanGAP1 mediates the formation of a stable nuclear pore complex consisting of SUMO1-modified RanGAP1, RanBP2/Nup358 and Ubc9. This complex is essential for nuclear transport (Matunis, Wu et al. 1998, Zhang, Saitoh et al. 2002).

Modulation of enzymatic activity of substrate protein

SUMOylation of a target enzyme can alter the enzyme structure and modulate its activity. One of the classical examples is the SUMO-modified enzyme, thymine DNA glycosylase (TDG), which is a mismatch repair enzyme. SUMO modifies TDG at its C-terminal region, and SUMOylation decreases its binding to DNA dramatically (Hardeland, Steinacher et al. 2002, Baba, Maita et al. 2005). As soon as the TDG leaves the repair site of the DNA, SENPs remove the SUMO from TDG swiftly and the enzyme is readily available for a next round of repair. This example of TDG illustrates that at any given time, a small population of protein is SUMOylated, yet SUMOylation has an enormous effect on a cellular pathway.

Regulation of protein stability

SUMOylation has been shown to work in conjunction with other post-translational modifications, such as ubiquitination, acetylation (Stankovic-Valentin, Deltour et al. 2007) and phosphorylation (Khan, Rozhon et al. 2014). There are various studies that reveal crosstalk between ubiquitination and SUMOylation. SUMOylation can either stimulate or prevent ubiquitin mediated degradation of substrate proteins. For example, PML, in the presence of arsenic trioxide, is modified by polymeric SUMO2/3 chains. RNF4, a poly-SUMO-dependent ubiquitin E3 ligase (STUbL), binds the poly-SUMO2/3 chains with its four SIMs and polyubiquitinates the SUMOylated PML, leading to proteasomal

degradation (Tatham, Geoffroy et al. 2008, Maroui, Kheddache-Atmane et al. 2012). Alternatively, SUMOylation can prevent ubiquitination of proteins by either modifying the same lysine residue designated for polyubiquitination, or causing stearic hindrance by modification of a nearby lysine residue, hence preventing the degradation of the substrate protein. For instance, cyclin dependent kinase CDK6 is modified by SUMO1 at lysine 216 during the progression of glioblastoma compared to normal cells. The SUMOylation of CDK6 inhibits polyubiquitination at lysine 147 by preventing access to this lysine (Bellail, Olson et al. 2014). Furthermore, protein IkBα, a negative regulator of the NFκB pathway, is tightly regulated by SUMOylation and ubiquitination, both competing for the same lysine residue of lysine 21 (Rodriguez, Wright et al. 1996, Desterro, Rodriguez et al. 1998). The stabilized IkBα retains NFκB in the cytosol and inhibits the activation of the NFκB pathway, thus preventing the transcription of its target genes.

Biological functions of SUMOylation

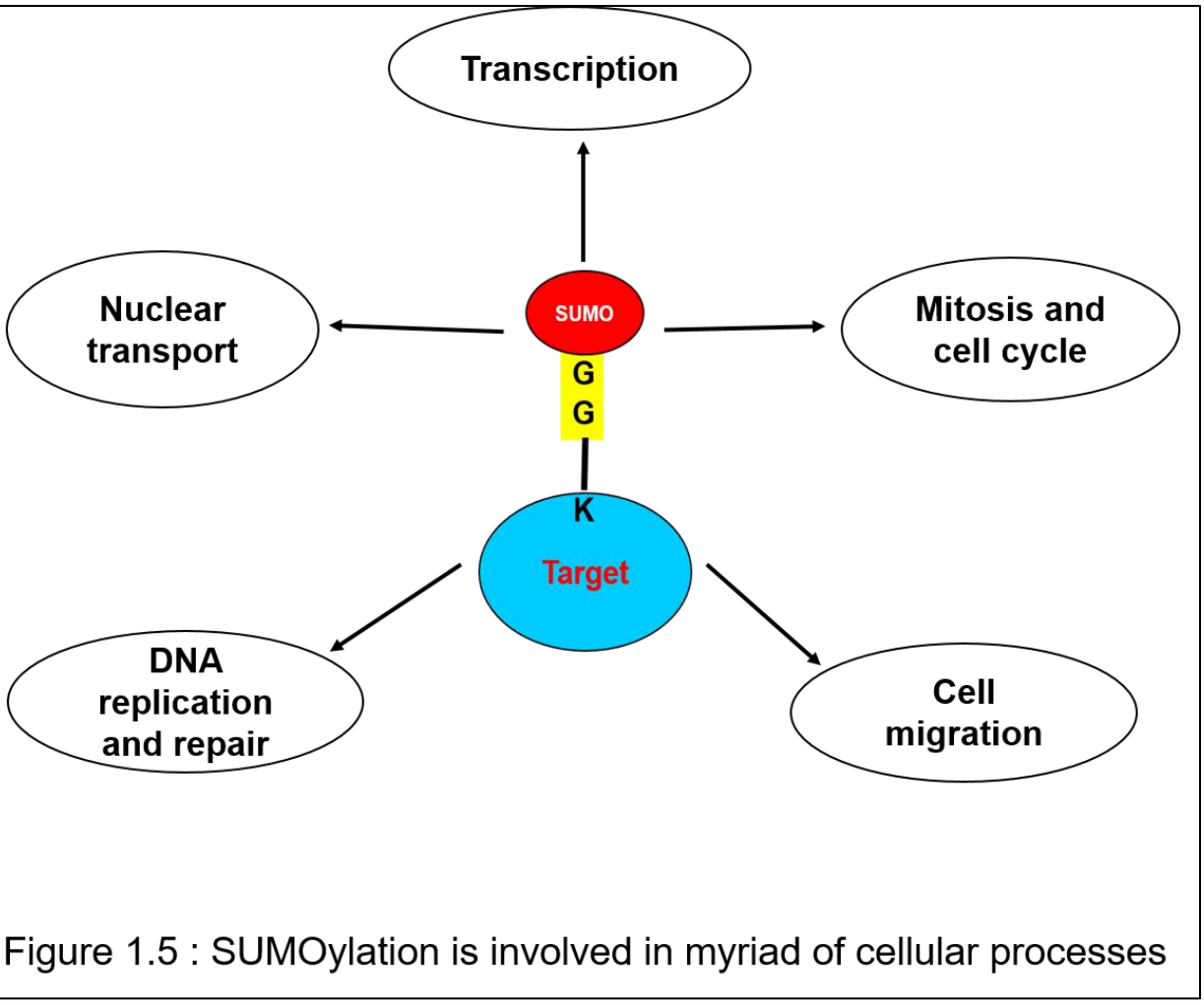

Figure 1.5 : SUMOylation is involved in myriad of cellular processes

Proteomic studies have identified over 3000 target proteins modified by SUMOs, supporting the general idea that SUMOylation is a common protein modification, similar to phosphorylation and ubiquitination, (Hendriks and Vertegaal 2016). The following diagram (Figure 1.5) shows the most important functions of SUMOylation.

Nucleo-cytoplasmic transport of substrate proteins

Thousands of nuclear pore complexes (NPCs) are present in the nuclear membrane, as the sole channels for transporting numerous proteins between the nuclear and cytoplasmic compartments (Wente and Rout 2010). The NPC consists of two groups of proteins, the nucleoporins (Nups) that permanently associate with the core NPC

structure, and the other transiently interacting proteins that cycle on and off the NPC. Studies performed on vertebrates and budding yeast have shown that the core structure of NPC consists of 30 unique proteins; however, due to the eight-fold symmetry of the nuclear pore, each interacting protein is present in multiple copies (at least eight copies). Around 400-500 proteins are present at each NPC, forming rings on the nucleoplasmic and cytoplasmic sides (Kabachinski and Schwartz 2015). It has a thickness of 50nm and an inner diameter of 40nm. Moreover, recent electron tomographic visualization reveals the nuclear basket-like structure and the cytoplasmic filaments (Bui, von Appen et al. 2013). The Nup proteins organize into four major sub-complexes at the NPC. The Nup62 complex proteins have FG repeats and are present in the central pore. The Nup214 complex is present on the cytoplasmic filaments of the NPC. The two other sub-complexes, the Nup107 complex and the Nup93 complex, provide essential structural scaffold. Nup 107 and Nup 93 complexes operate as adaptors to attach the FG-repeat containing Nups with the nuclear membrane (Hu, Guan et al. 1996, Fornerod, van Deursen et al. 1997, Kampmann and Blobel 2009, Bui, von Appen et al. 2013, Vollmer and Antonin 2014). Cargo proteins, with molecular weight below 40kDa, diffuse in and out of the nuclear pore freely. However, the larger proteins require transport factors. Large proteins that shuttle between the nuclear and cytoplasmic compartments of the cell have distinct motifs recognized by the transport proteins, the nuclear localization signal (NLS) and the nuclear export signal (NES). The largest family of transport proteins are the karyopherins. Budding yeast has 14 and higher eukaryotes have 20 karyopherins respectively (Chook and Blobel 2001).

In addition to NLS/NES and the karyopherins, the GTPase Ran is necessary for association and dissociation of cargo proteins with the corresponding nuclear transport receptor known as karyopherin. There is a steep gradient of RanGTP across the nuclear pore (Becskei and Mattaj 2003), high in the nucleus due to Ran gunanine exchange factor (RanGEF) but low in the cytoplasm due to the presence of RanGTPase activating protein-1 (RanGAP1). RanGTP binding to an importin-cargo complex leads to the release of the cargo from its carrier protein in the nucleus, whereas, the export complex (containing RanGTP, cargo and exportin) is assembled only in the presence of high concentration of RabGTP in the nucleus. As the first discovered SUMO target, unmodified RanGAP1 is predominantly cytoplasmic, its modification by SUMO1 targets RanGAP1 to the NPCs (Matunis, Wu et al. 1998, Zhu, Goeres et al. 2009). SUMO1-modified RanGAP1 (SUMO1*RanGAP1) shows a very stable nuclear pore complex localization along with SUMO E2 Ubc9 and the SUMO E3 ligase RanBP2/Nup358 (Flotho and Werner 2012), resulting in the formation of the RanBP2/RanGAP1*SUMO1/Ubc9 complex at the cytoplasmic side of the NPCs. SUMOylation-mediated formation of the above-mentioned stable complex at the NPC provides a strong evidence of coupling of two major processes in the cell, SUMOylation and nuclear transport. CRM1 (chromosome region maintenance 1)/Xpo1 (exportin 1) is a major exportin, involved in the transport of over 80% of cargoes from nucleus to the cytoplasm (Hutten and Kehlenbach 2007). For example, the Crm1-mediated cargoes include transcription factors, RNA, translation factors and many other proteins. Crm1 has also been reported from a recent study from our laboratory to be essential for the nucleo-cytoplasmic shuttling of RanGAP1 (Cha, Sen et al. 2015). In this study, we observed that the localization of the SUMO1-modified RanGAP1 at the NPC is

highly stable in comparison to the unmodified RanGAP1 in the cytoplasm. In chapter 3, I describe my published studies on nucleo-cytoplasmic shuttling of RanGAP1 in details.

The presence of the SUMO E3 ligase (RanBP2/Nup358) and the isopeptidases (SENP1 and SENP2) at the NPCs suggests that levels of SUMOylation on target proteins may be altered during their shuttling between the nucleus and the cytoplasm (Zhang, Saitoh et al. 2002, Melchior, Schergaut et al. 2003, Goeres, Chan et al. 2011). Also, accumulating lines of evidence support such a model that there is a close functional relationship between SUMOylation and nuclear transport (Pichler and Melchior 2002, Du, Bialkowska et al. 2008). There are proteins whose SUMOylation depends on the presence of a functional nuclear localization sequence (NLS) (Matunis, Wu et al. 1998). On the other hand, there are examples where SUMOylation of protein substrates is the prerequisite for their nuclear localization or retention (Du, Bialkowska et al. 2008, Hofmann, Arduini et al. 2009). For example, one of the SUMO targets, Sp100, which is a transcription factor and a component of the PML nuclear body, is modified by SUMO only when it contains a functional NLS (Pichler, Gast et al. 2002). The NLS mutant of the PML protein has a significant reduction in its SUMOylation compared to the corresponding wild-type protein (Muller, Matunis et al. 1998, Duprez, Saurin et al. 1999). Furthermore, nuclear localization of protein Ataxin-1 is crucial for SUMOylation at all its lysine residues (Riley, Zoghbi et al. 2005). Moreover, several studies on the tumor suppressor p53 show that SUMOylation at lysine 386 promotes its nuclear export (Carter, Bischof et al. 2007), and the SUMO modification helps release p53 from the nuclear export receptor Crm1 (Santiago, Li et al. 2013). Lastly, actin is another protein whose SUMOylation by SUMO2/3 at lysines 68 and 284 dictates its nuclear localization. Actin is primarily a

cytoskeletal protein, and the nuclear actin has been reported to be involved in transcription, nuclear export, chromatin remodeling and intranuclear transport of mRNA molecules (Hofmann, Arduini et al. 2009, Louvet and Percipalle 2009).

Transcription

SUMOylation is associated with gene expression, especially transcription. Several transcription factors are SUMO targets and in most cases SUMOylation either enhances or hinders their interaction with other transcription factors, chromatin remodelers, co-repressors or co-activators (Gill 2003, Verger, Perdomo et al. 2003, Hay 2005). There are a handful of examples of a positive regulatory role of SUMOylation on transcription, most studies elucidate that SUMOylation negatively affects the transcription of various genes. For instance, the heat shock factors HSF1 and HSF2 illustrate the former scenario, in which modification by SUMO1 enhances their DNA binding activity under stress and stimulates their function (Goodson, Hong et al. 2001, Hong, Rogers et al. 2001). Additionally, SUMOylation of the transcription regulators GRIP1 (glucocorticoid receptor interacting protein-1) and viral protein IE2-p86 augments their activities in regulation of gene expression (Hofmann, Floss et al. 2000, Kotaja, Karvonen et al. 2002).

Accumulating lines of evidence have revealed that the SUMOylation site(/s) of various transcription factors are often present within their negative regulatory domains. Such domains are found in c-Myb, CCAAT/ enhancer binding protein (C/EBP), steroid receptors and Sp3 (Poukka, Karvonen et al. 2000, Subramanian, Benson et al. 2003). All these proteins contain the synergy control (SC) motifs and also the SUMOylation consensus sequence ψ-K-x-E/D in the SC motifs (Poukka, Karvonen et al. 2000, Subramanian, Benson et al. 2003). For example, the SUMOylation site of Sp3 lies in its

SC motif, and mutation of this specific K residue strongly stimulates its transcriptional activity (Sapetschnig, Rischitor et al. 2002). Similarly, CBP/p300 and Elk-1 get SUMOylated in their negative regulatory domains, and mutations preventing the binding of Ubc9 or covalent SUMO binding, eliminate transcriptional repression (Girdwood, Bumpass et al. 2003, Yang, Jaffray et al. 2003). On the other hand, SUMOylation of certain transcription factors, such as Smad4, can either stimulate or inhibit their activity dependent on the target genes (Long, Wang et al. 2004).

There are two general mechanisms by which SUMOylation can affect transcription. Modification of a transcription factor by SUMOs can recruit chromatin modifiers to alter the chromatin structures. The p300 SUMOylation at its CRD domain recruits the SIM-containing histone deacetylases HDAC6, which deacetylates histone and thus leads to transcriptional repression (Girdwood, Bumpass et al. 2003). In addition, transcription factor Elk-1 is SUMOylated at its R motif and recruits the histone deacetylase HDAC2 at specific promoters, leading to a decrease in histone acetylation and thus a repression of transcription (Yang and Sharrocks 2004). Alternatively, SUMOylation can target its substrate proteins to certain repressive domains, such as the PML nuclear body (PML-NB). PML protein is not only a SUMO target but also contain SUMO-interacting motifs, which are critical for recruiting various proteins, including DAXX, Sp100, CBP and ISG20 (Zhong, Muller et al. 2000). PML-NBs are the storage domains for many different transcription factors that are SUMO targets or contain SUMO-interacting motifs. For example, SUMO modification of Sp100 enhances its interaction with the heterochromatin protein 1 (HP1) and leads to transcriptional repression (Seeler, Marchio et al. 1998). Another protein complex, the Polycomb group (PcG) body, is a center of transcriptional

repression, and SUMOylation of SOP-2, one of the PcG body components, causes inhibition of the Hox genes (Zhang, Smolen et al. 2004).

DNA replication and repair

A dynamic and highly efficient system of DNA repair enzymes protect the DNA from exogenous and endogenous damaging agents to maintain genome integrity. SUMOylation, along with several other PTMs, play critical role in this process mostly by altering DNA-protein and protein-protein interactions. Some of the most relevant proteins whose modification by SUMOs significant alters their activity include PCNA, Rad52, BRCA1 and TDG.

PCNA (proliferating cell nuclear antigen) is a ring-shaped, homo-trimeric clamp protein which maintains the processivity of replicative DNA polymerases (Choe and Moldovan 2017). It can also interact with a variety of other factors and plays a central role in nucleotide and base excision repair, as well as mismatch repair. PCNA has two principal lysines for SUMOylation, K164 and K127, and SUMOylation leads to the recruitment of helicase Srs2 during S-phase. Srs2 then dismantles the recombination filaments formed by Rad51 and prevents homologous recombination (Papouli, Chen et al. 2005, Colby, Matthai et al. 2006, Watts 2006, Parker, Bucceri et al. 2008, Gazy and Kupiec 2012). Egl1 also interacts with SUMOylated PCNA, and it is responsible for removing PCNA from DNA (Pfander, Moldovan et al. 2005). Both Egl1 and Srs2 have SIM that bind SUMOylated PCNA (Ulrich, Vogel et al. 2005). On the contrary, SUMOylation of PCNA at K127, during S-phase, blocks interaction with Eco1, a sister chromatid cohesion protein (Moldovan, Pfander et al. 2006).

Moreover, SUMOylation of Rad52, a protein involved in homologous recombination, prevents proteasome-mediated degradation and sustains its activity at the DNA damage site (Sacher, Pfander et al. 2006). Moreover, a recent report reveals that SUMOylation of Rad52 leads to its dissociation from DNA and attenuates single strand annealing function (Altmannova, Eckert-Boulet et al. 2010).

Mitosis and cell cycle

The link between the SUMO pathway and mitosis preceeded the discovery of the SUMO proteins. In budding yeast, Ubc9 is critical for B-type cyclin degradation (Seufert, Futcher et al. 1995). Moreover, specific SUMO E3 ligases have been reported to be essential; chromosome segregation in *Xenopus* and mammalian system requires PIASy (Azuma, Arnaoutov et al. 2005). Besides the SUMOylation machinery, several proteins that play roles during mitosis and cell cycle are modified by SUMO. The SUMOylation modulates their activities during cell cycle (Dasso 2008).

Topoisomerase IIα, an enzyme that alters DNA topology, is needed during transcription (Dawlaty, Malureanu et al. 2008, Zhang, Wang et al. 2014, Edgerton, Johansson et al. 2016, Yoshida, Ting et al. 2016). It is selectively modified by SUMO2/3 during mitosis in vertebrates. Treating *Xenopus* egg extracts with dominant negative Ubc9 (dn Ubc9) does not alter its activity, but increased unmodified Topoisomerase IIα on the chromosomes (Yoshida, Ting et al. 2016). The study reported a defect in the segregation of chromosome at anaphase-telophase junction. Hence, SUMOylation of topoisomerase IIα is essential for its removal from mitotic chromosomes and the progression through anaphase and telophase (Azuma, Arnaoutov et al. 2003).

The kinesin-like motor protein CENP-E (centromere-associated protein E) is not only a known SUMO2/3 target but also contains a functional SIM motif (Zhang, Goeres et al. 2008). It has been shown previously that global inhibition of SUMOylation by overexpression of SUMO isopeptidase SENP2 or the RNAi-mediated depletion of the SUMO E2 enzyme Ubc9 leads to CENP-E mislocalization and a prometaphase arrest (Zhang, Goeres et al. 2008). In addition, the localization of CENP-E at the outer kinetochore of mitotic chromosomes has been suggested to be mediated through its non-covalent interaction with other SUMO2/3-modified kinetochore proteins via their SIM domain.

Furthermore, SUMO1 modification of RanGAP1 is essential for the formation of the highly stable RanBP2/RanGAP1*SUMO1/Ubc9 (RRSU) complex at the NPC (Matunis, Wu et al. 1998, Reverter and Lima 2005, Dasso 2008). The RRSU complex is stable through cell cycle. Intriguingly, the RRSU complex also localizes at the outer kinetochore or fibrous corona (Joseph, Liu et al. 2004). The kinetochore localization of the RRSU complex, which is mediated by both CRM1 and Ran-GTP, plays an important role for the kinetochore-fiber assembly and chromosome segregation at anaphase (Arnaoutov, Azuma et al. 2005).

Lastly, the transcription factor FoxM1 (Forkhead box protein M1), a key regulator in cell cycle progression, has been recently identified as a SUMO target. SUMOylation of FoxM1 is enhanced greatly during G2 and M phases, during which FoxM1 plays a critical role. SUMOylation of FoxM1 increases its transcriptional activity by preventing its dimerization that is known to abolish its function in regulation of its gene expression (Schimmel, Eifler et al. 2014).

SUMOylation and human diseases

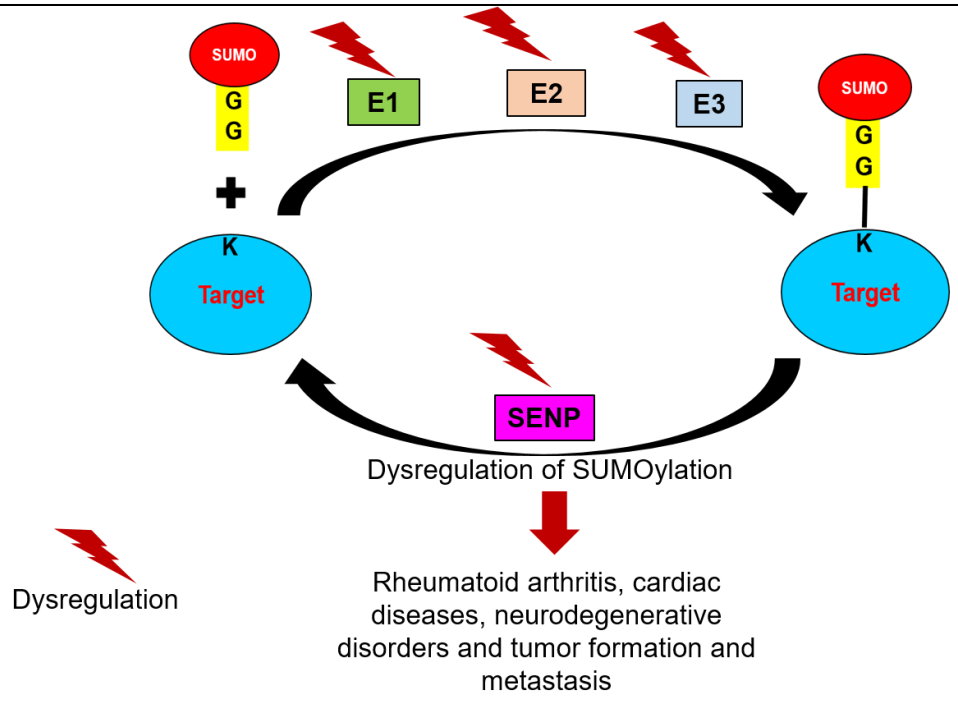

Figure 1.6 : Up- or down-regulation of the SUMOylation pathway components can lead to dysregulated SUMOylation of several target proteins. This can further dismantle pathways requiring the SUMOylation dependent regulation of specific targets; major disorders including cancer, neurodegenerative diseases and cardiac ailments have been characterized by dysregulated SUMOylation of substrates.

As a balance in SUMOylation and deSUMOylation is critical for controlling a variety of cellular pathways in normal cells, perturbation in this post-translational modification pathway is implicated in various human diseases including neurodegenerative disorders, cardiac diseases, tumorigenesis and metastasis (Figure 1.6). Here I briefly summarize and discuss our current understanding of how an imbalance in SUMO modification affects these diseases.

SUMOylation in neurodegenerative disorders

SUMOylation, similar to phosphorylation and ubiquitination, is essential for the proper development and functions of the central nervous system (Wilkinson, Konopacki et al. 2012). Evidently, various neurodegenerative disorders, including Alzheimer's

disease, Parkinson's disease and Huntington's disease, are characterized by a correlated disruption in SUMO pathway.

Alzheimer's disease

As an age-related neurodegenerative disorder, Alzheimer's disease is characterized by gradual loss of neurons, accumulation of amyloid plaques in the brain and progressive dementia. The amyloid β (Aβ) plaque deposition and neurofibrillary tangles (NFTs) are the most prominent factors responsible for the disease (Anderson, Wilkinson et al. 2009, Lee, Sakurai et al. 2013). Amyloid precursor protein (APP) generates Aβ peptides through cleavages mediated by β-secretase and γ-secretase. As the SUMOylation sites of APP at lysine residues 587 and 595, are juxtaposed with the β-secretase cleavage site of the protein, the modification of APP by SUMOs negatively regulates the formation of Aβ aggregates in mammalian cells (Zhang and Sarge 2008). Also, another study has shown that the modification of APP by polymeric SUMO3 chains reduces Aβ formation, as observed in mammalian cells in culture and human brain tissue (Li, Wang et al. 2003).

The accumulation of hyperphosphorylated, microtubule-associated Tau protein leads to NFTs. Tau's phosphorylation state negatively regulates Tau's function. Previously it has been shown that SUMO1 colocalizes with phosphorylated Tau in transgenic AD mice that carry the APP mutations (Takahashi, Ishida et al. 2008). However, the same group observed that another model of transgenic mouse, containing the mutated hyperphosphorylated Tau, lacks colocalized SUMO1 staining (Takahashi, Ishida et al. 2008). This work signifies that only SUMO1-associated hyperphosphorylated Tau is associated with AD, though the functional implications still need to be investigated.

Polyglutamine diseases

Polyglutamine diseases are a family of neurodegenerative disorders are caused by aberrant proteins with a toxic stretch of polyglutamine (polyQ) repeats, that range from 36 to over 300. These diseases include Huntington's disease (HD), dentatorubral pallidoluysian atrophy (DRPLA), spinocerebellar ataxias (SCAs) and spinobulbular muscular atrophy (SBMA).

In Huntington's disease, the Huntingtin (Htt) protein contains a polyQ stretch of 36-120 glutamine repeats, in comparison to that of less than 35 glutamine in the normal Htt protein (Landles and Bates 2004). The pathogenic fragment of Htt (truncated Htt or Httex1p) is modified by SUMO and ubiquitin at lysines K6 and K9, respectively (Steffan, Agrawal et al. 2004). In a *Drosophila* model, SUMOylation of the Httex1p stimulates the aggregate formation and the neurodegeneration, whereas, its ubiquitination has an opposite effect. Furthermore, mutations inhibiting both SUMOylation and ubiquitination reduce the pathogenesis of this disease, suggesting the dominant roles of SUMOylation in the disease progression (Steffan, Agrawal et al. 2004).

Atrophy of the cerebellar Purkinje layer cause SCAs. An extended polyQ stretch in the ataxin-1 protein causes SCA type 1. Importantly, the increase of the polyQ stretch of the mutant ataxin-1 protein reduces its SUMOylation in comparison to the wild-type protein. On the other hand, phosphorylation mutation (S776A) on the ataxin-1 mutant with 82Q restores the levels of SUMOylation to those of its wild-type (Riley, Zoghbi et al. 2005). Hence, there is an interplay between phosphorylation and SUMOylation of ataxin-1 that influence the progression of SCAs.

Parkinson's disease

As a chronic and progressive movement disorder, Parkinson's disease is primarily caused by a loss of dopaminergic neurons in the substantia nigra of the brain. The hallmark of this disease is the accumulation of Lewy bodies, consisting of α-synuclein. α-synuclein is modified by SUMOs at the lysine residues 96 and 102 (Krumova, Meulmeester et al. 2011). Notably, the presence of less than 10% SUMOylated form of α-synuclein is sufficient to defer the formation of aggregates under *in vitro* conditions. *In vivo* studies indicate that a SUMOylation-deficient mutant of α-synuclein causes an increased tendency of aggregate formation as well as an enhanced cytotoxicity in dopaminergic neurons.

SUMOylation in cardiac diseases

Like its critical functions in many other cellular pathways, SUMOylation plays an important role in cardiac function and heart development. The transcription factors, which are essential for normal heart development, have been identified as SUMO targets, including GATA4, myocardin and PPARγ. Additionally, SUMOylation is associated with cardiovascular disorders including familial dilated cardiomyopathy (Wang and Schwartz 2010, Wang 2011).

Known as a key regulator in cardiomyocyte differentiation and cardiogenesis, the zinc-finger containing transcription factor GATA4 is SUMOylated at K366, within its transactivation domain (Wang, Feng et al. 2004). Blocking the SUMOylation at this residue by site-directed mutagenesis prevents nuclear localization and hinders target gene expression (Wang, Feng et al. 2004).

Myocardin is a cardiac and smooth muscle-specific transcriptional coactivator, and SUMOylation regulates its function during heart development. Modification of myocardin

by SUMOs at K445 enhances its target gene expression in cardiac muscle cells, including α-actin and α-myosin (Wang, Li et al. 2007).

Being a structural component of the nuclear membrane, lamin A is associated with familial dilated cardiomyopathy. Missense mutations within its SUMO consensus motif (MKEE), E203G and E203K, are associated with this disease (Wang and Schwartz 2010). As observed in fibroblast cells isolated from patients, each of these lamin A mutant proteins have altered subcellular localization compared to its wild-type protein (Wang and Schwartz 2010).

TRPM4 is a Ca^{2+}-activated non-selective cation (CAN) channel protein with a high expression in cardiac tissue, and it also associates with the heart disease known as progressive familial heart block type I (PFHBI) (Kruse, Schulze-Bahr et al. 2009). A study has shown that a missense mutation in the *TRPM4* gene ($TRPM4^{E7K}$), causes decreased cardiac conduction. This mutation leads to a constitutively SUMOylated TRPM4 protein that shows a greatly increased distribution in the plasma membrane.

SUMOylation in tumor formation and metastasis

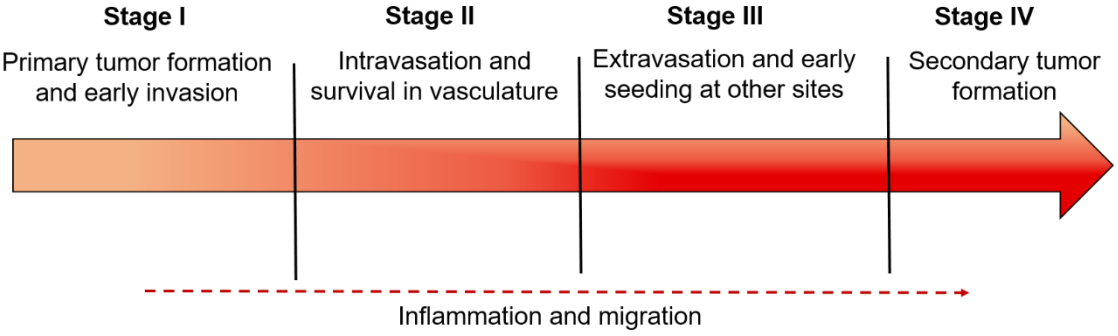

Figure 1.7 : Schematic representation of the critical steps of tumor progression towards metastasis. Cancer progression is a very complicated process involving the interplay of numerous factors and pathways. A large number of SUMO substrates are transcription factors, tumor suppressors, cell migration proteins, nuclear transport factors (MIF, Merlin, Rac1, RhoGDI1, p53, BubR1), and studies have reported dysregulation in their SUMOylation during cancer progression.

Accumulating lines of evidence have shown that disruption of the SUMOylation pathway is associated with tumorigenesis and metastasis (Figure 1.7). The disruption may result from increased/decreased expression of enzymes in SUMOylation or deSUMOylation as well as in levels of the numerous target proteins during cancer progression and metastasis.

Role of SUMO conjugation enzymes in carcinoma

Several reports have found that the SUMO activating enzyme (E1) subunit SAE2 plays a pivotal role in cancer progression (He, Riceberg et al. 2015, Shao, Wang et al. 2015). SAE1 is important for growth and maintenance of tumor stem cells, and inhibition of its expression by RNAi leads to sensitization of the tumor cells to chemotherapy for suppression of cancer malignancy. Besides, the sole SUMO E2 enzyme Ubc9 is also a major player in tumor progression towards metastasis as indicated by accumulating evidence from studies on colon, lung, prostate and breast carcinomas (Moschos, Jukic et al. 2010). A study of melanoma has demonstrated that Ubc9 has an anti-apoptotic function and its siRNA-mediated knockdown leads to a significant decrease in melanoma cell proliferation (Moschos, Smith et al. 2007). Furthermore, a drastic increase in levels of Ubc9 proteins has been observed in certain patients with acute myeloid leukemia (AML) (Geletu, Balkhi et al. 2007). Additionally, the SUMO E3 ligases are known in association with specific cancer types. For examples, PIAS3 is upregulated in different types of cancers, including prostate, breast, lung and brain cancers (Wang and Banerjee 2004, Wang and Schwartz 2010). Moreover, PIAS1 and PIAS3 are responsible for

androgen receptor (AR) -mediated target gene expression in prostate cancer cells (Gross, Liu et al. 2001) (Table 1.3).

SUMO conjugating enzyme	Cancer types
SAE1	Upregulated in breast cancer
SAE2	Upregulated in gastric cancer, breast cancer, hepatocellular carcinoma
Ubc9	Upregulation in breast cancer, lung, colon and prostate carcinoma, myeloid leukemia
PIAS1	Upregulation in breast cancer, prostate cancer
PIAS3	Upregulated in breast cancer, prostate cancer, lung and brain tumorigenesis

Table 1.3 : The SUMOylation conjugation machinery components are associated with several different types of tumorigenesis and metastases

Role of SUMO isopeptidases in cancer progression

Several lines of evidence suggest that dysregulation of the SUMO isopeptidases associates with different types of tumors including prostate cancer and thyroid oncocytic adenocarcinoma. Studies have shown that SENP1 expression is much higher in neoplastic prostate cells in comparison to normal control cells, which promotes the prostate cell transformation and the cancer progression and metastasis. Also, SENP1 activates of AR-mediated gene expression in prostate cancer cells, and the AR, in turn, potentiates SENP1 expression (Bawa-Khalfe and Yeh 2010). The positive feedback loop is disrupted in cells with siRNA-mediated knockdown of SENP1, leading to a decrease in androgen-driven prostate cell proliferation (Bawa-Khalfe and Yeh 2010). Moreover, SENP3 is upregulated in prostate, colon, ovarian and lung carcinomas (Han, Huang et al. 2010). SENP3 overexpression is associated with mild oxidative stress, a signature in cancers (Lim, Sun et al. 2005). Lastly, levels of SENP6 mRNAs are decreased in human

breast cancer tissues in comparison to healthy control tissues (Mooney, Grande et al. 2010).

Effect of SUMOylation on its target proteins during tumorigenesis and metastasis

There is a myriad of SUMO substrates associated with cancer progression, including tumor suppressor proteins, proto-oncogene products, cell migration factors, signaling proteins, and transcription factors, such as p53, BRCA1, Reptin, Rac1, RhoGDI, Vimentin, MIF, actin, PML and pRB. SUMOylation of the above proteins is tightly associated with tumorigenesis and metastasis (Kim and Baek 2006, Bettermann, Benesch et al. 2012).

The tumor suppressor protein BRCA1 (breast cancer 1) is involved in DNA damage repair. Through its N-terminal domain, BRCA1 interacts with BRCA1-associated RING domain-1 protein (BARD1) to form a functional ubiquitin E3 ligase heterodimer. SUMO modification of BRCA1 is facilitated by the SUMO E3 ligases PIAS1 and PIASy (Morris, Boutell et al. 2009). Moreover, the two E3 ligases are co-localized with the E2 enzyme Ubc9 and the SUMOylated BRCA1 protein at the DNA damage sites. In addition, BRCA1 SUMOylation enhances its E3 ligase activity for ubiquitination (Morris, Boutell et al. 2009) Furthermore, RNAi-mediated knockdown of the PIAS proteins prevents the localization of BRCA1 to the DNA damage sites. Numerous reports indicate that several missense mutations of BRCA1 predispose cells to breast cancer and ovarian cancer, and these mutations prevent its dimerization and SUMOylation (Morris, Pangon et al. 2006).

The tumor repressor protein Reptin is a member of the AAA+ family ATPases and a component of the large protein complexes involved in chromatin remodeling and transcription. Reptin is overexpressed in different types of cancer, including

hepatocellular carcinoma, breast cancer, and acute leukemia. Reptin is responsible for repressing β-catenin-TCF transcriptional activity, which represses the expression of the tumor suppressor KAI-1 protein (Kim, Choi et al. 2006, Grigoletto, Lestienne et al. 2011). The study by Kim et al (in 2006) showed an enhanced effect of Reptin SUMOylation (lysine 456) in suppressing KAI-1 expression and its function in human prostate carcinoma cell line LNCaP. This effect is due to a predominant subcellular distribution of SUMOylated Reptin in nucleus in comparison to a prominent cytoplasmic localization of the SUMOylation-deficient Reptin. Its repressive function is abrogated by its deSUMOylation enzyme SENP1 as SENP1 overexpression leads to an activation of Reptin target genes, such as KAI-1.

The Rho-GDP dissociation inhibitor, RhoGDI, reduces the cell migration by binding to Rho and Rho-like GTPases and keeping them in the inactive GDP-bound forms. Therefore, RhoGDI adversely affects the formation of actin filaments, cell migration and invasion, which are important for cancer progression and metastasis. SUMOylated RhoGDI has higher affinity towards GDP-bound Rho and Rho-like GTPases (Yu, Zhang et al. 2012). SUMOylation of RhoGDI is tightly regulated by the SUMO E3 ligase PIAS3 (Schou, Kelstrup et al. 2014) and the SUMO-specific isopeptidase SENP1 (my unpublished results). Moreover, a significant decrease in RhoGDI SUMOylation in metastatic breast cancer cell line in comparison to a non-metastatic control cell line (Subramonian, Raghunayakula et al. 2014).

Macrophage migration inhibitory factor (MIF), also known as glycosylation-inhibiting factor (GIF), is a pro-inflammatory cytokine. MIF plays a critical role during cancer progression and metastasis by stimulating inflammation, angiogenesis, and

cancer cell migration and invasion (Mitchell 2004, Conroy, Mawhinney et al. 2010, Simpson and Cross 2013). The tumor-derived MIF protein can exert its effects on various cell types both locally and distally during tumor progression by functioning as either autocrine or paracrine. Studies on various types of cancer have identified a close correlation between an increase in levels of MIF protein and a poor prognosis of cancer patients. In fact, MIF is a known biomarker of breast cancer (Xu, Wang et al. 2008, Verjans, Noetzel et al. 2009). Our recent proteomic study has revealed a drastic upregulation in levels of MIF SUMO2/3 modification in metastatic mouse breast cancer cell line compared non-metastatic control cell line (Subramonian, Raghunayakula et al. 2014). Based on this observation, I have further investigated the role of SUMOylation in regulation of MIF stability as shown in Chapter 2.

CHAPTER 2: AN INVESTIGATION INTO THE ROLE OF SUMOYLATION ON MIF STABILITY

Abstract

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in various types of human cancers, and represents a direct link between inflammation and cancers. Our recently published proteomic studies have shown that levels of MIF SUMO2/3 modification increase in metastatic breast cancer cells compared to non-metastatic control cells. In this study, we found that the increased levels of MIF SUMO2/3 modification in metastatic breast cancer cells positively correlate with levels of unmodified MIF proteins compared to non-metastatic control cells. Based on the observation and the known role of SUMOylation in regulation of protein stability, we asked if MIF SUMOylation affects its stability. Our results provide several lines of evidence that SUMOylation of MIF increases its stability by preventing its ubiquitination and proteasome-mediated degradation in cells. Furthermore, MIF gets modified at a single lysine residue, K78, which is present within a non-consensus SUMOylation motif. Therefore, our study supports the idea that global inhibition of SUMO modification may be useful in treatment of various cancers by specifically destabilizing MIF proteins.

Introduction

MIF is expressed in and secreted by both immune cells and non-immune cells (Bucala 1996, Bernhagen, Calandra et al. 1998). It was first isolated from the sensitized peritoneal lymphocytes of guinea pig and showed the capacity to inhibit the migration of macrophages and monocytes (Bloom and Bennett 1966, David 1966, Weiser, Temple et al. 1989). It has a molecular weight of 12.5 kDa and possesses two distinct enzymatic activities of phenylpyruvate tautomerase and disulfide reductase (Kleemann, Kapurniotu

et al. 1998, Matsunaga, Sinha et al. 1999). MIF has both autocrine and paracrine functions (Figure 2.1), and glucocorticoids induce its expression and secretion.

MIF in diseases

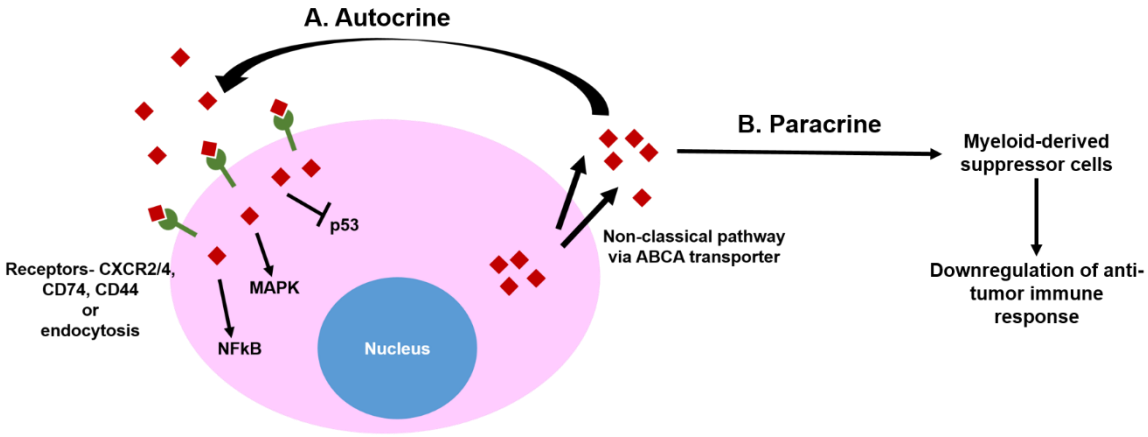

Figure 2.1 : Macrophage migration inhibitory factor (MIF) is a mitogenic and pro-inflammatory cytokine. It functions utilizing both autocrine as well as paracrine signaling pathways. A. Autocrine pathway- secreted MIF influence the same cell by affecting several intracellular targets that include p53, Jab1/Csn5, MAPK and NFkB. B. Paracrine pathway- the released extracellular MIF can influence different types of cells in the tumor microenvironment including the myeloid-derived suppressor cells (MDSCs), which in turn, inhibits anti-tumor functions of immune cells.

Unlike other cytokines, MIF expression and secretion is induced by glucocorticoids. As a pro-inflammatory factor, MIF protein level in patient serum greatly increases during inflammation, stress and infection. Several studies on arthritis, glomerulonephritis, septic shock due to infections, cardiac diseases and cancer, report this increase in MIF level, as shown in Figure 2.2 (Lue, Kleemann et al. 2002). MIF is an important factor involved in endotoxemia caused by bacterial infections (Bernhagen, Calandra et al. 1993). Earlier studies showed that mice with an exposure to bacterial endotoxin, also known as lipopolysaccharide (LPS), leads to an increase in MIF expression and secretion (Bucala 1996). Additionally, MIF has been implicated in rheumatoid arthritis; experiments using MIF antibodies exhibited a significant decrease in the inflammatory response in mouse model for type II collagen-induced arthritis

(Ichiyama, Onodera et al. 2004). Similar results were observed in adjuvant-induced and

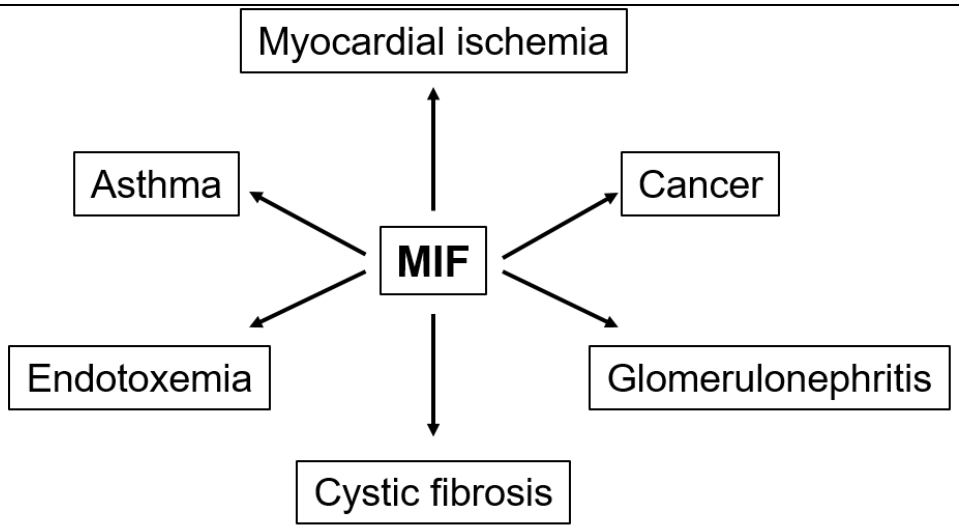

Figure 2.2 : MIF is associated with almost every inflammatory disease

antigen-stimulated arthritis in mice (Santos, Hall et al. 2001, Morand, Leech et al. 2006). MIF has also been observed to be an important mediator in glomerulonephritis; studies with human patients have elucidated a positive correlation between an upregulation of renal MIF expression and urinary MIF levels. Levels of MIF proteins in urine can be used to determine the degree of renal dysfunction that associates with macrophage and T cell infiltration in proliferative glomerulonephritis (Brown, Nikolic-Paterson et al. 2002, Bruchfeld, Wendt et al. 2016). Additionally, MIF plays a major role in cardiac diseases. The release of MIF from cardiomyocytes after myocardial ischemia and infarction is protective in nature, whereas the prolonged high levels of MIF in plasma due to the infiltrated macrophages and other immune cells leads to irreparable damage of heart tissue (Dayawansa, Gao et al. 2014). Furthermore, MIF enacts a critical role in other diseases, including cystic fibrosis, asthma and lupus, a chronic autoimmune disease.

MIF and cancer

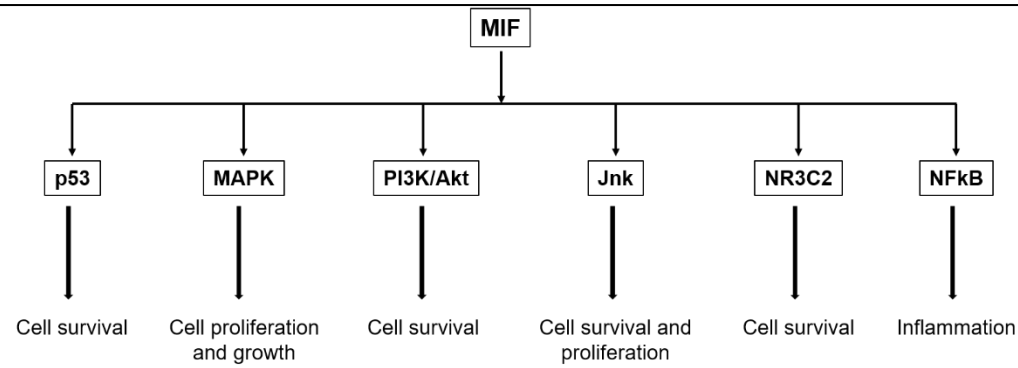

Figure 2.3 : Chart showing the specific target proteins regulated by MIF during tumor progression towards metastasis

Tumor initiation, progression and metastasis represent a series of processes. Those processes include unregulated cell proliferation, primary tumor formation, angiogenesis via various pro-inflammatory factors (such as MIF, TNFα, NFκB, IL-1 and IL-6), migration of tumor cells through intravasation and extravasation, and successful survival and proliferation of tumor cells at secondary sites leading to the formation of secondary tumors. Importantly, MIF has been considered as a key driver of inflammation to create a tumor microenvironment that promotes tumorigenesis and metastasis (Conroy, Mawhinney et al. 2010).

Besides triggering its own synthesis and release by both autocrine and paracrine pathways, MIF also stimulates the release of various other cytokines, including VEGF, TNFα, IL-6, IL-8 and IL-12, (Chesney and Mitchell 2015). Through its effects on a variety of downstream protein targets, MIF can promote tumor growth, inflammation and angiogenesis (Figure 2.3).

<u>Inhibition of p53 function</u>

Under normal physiological condition, the p53 protein is maintained at very low levels as it is degraded via the ubiquitin E3 ligase Mdm2-mediated ubiquitination (Haupt,

Maya et al. 1997, Nag, Qin et al. 2013). However, in response to various extracellular and intracellular signals, such as oxidative stress, DNA damage, hypoxia, and oncogene overexpression, p53 is phosphorylated and thus stabilized, since this modification blocks the interaction between p53 and Mdm2 (Shieh, Ikeda et al. 1997). The increase in levels of p53 leads to the transcription of its downstream genes, including the cell-cycle inhibitor p21 (Yu, Zhang et al. 1999, He, Siddik et al. 2005). Importantly, MIF promotes the degradation of p53 by stabilizing the interaction between p53 and Mdm2 and hence decreases the expression of p21 (Hudson, Shoaibi et al. 1999). Moreover, MIF can also inhibit p53 by suppressing its function as a transcriptional activator of several p53 dependent target genes, including p21 (Fingerle-Rowson, Petrenko et al. 2003).

MAPK activation

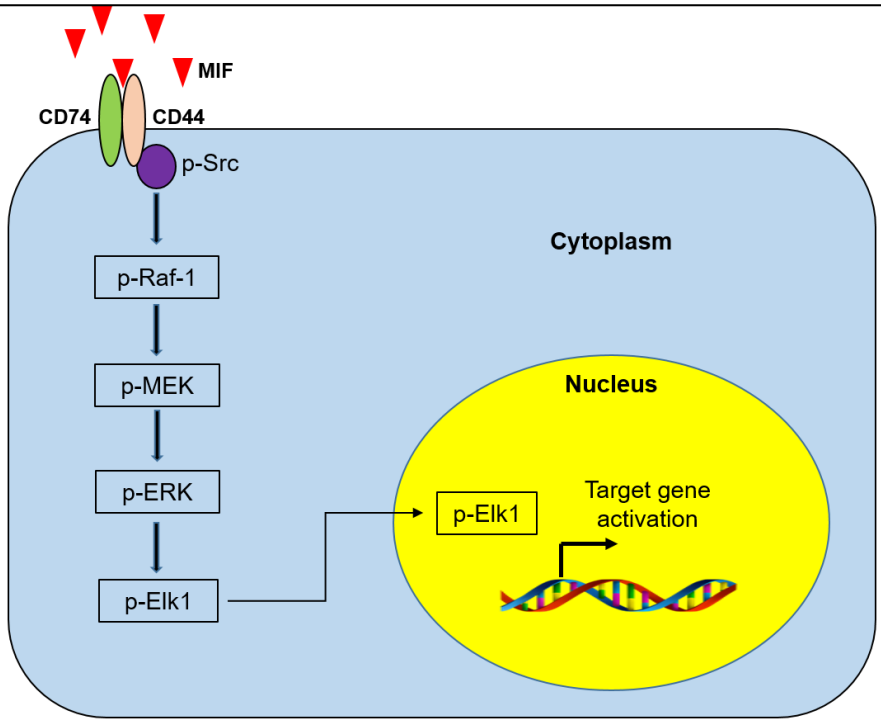

Figure 2.4: MIF regulates cell proliferation and growth by enhancing the MAPK pathway.

MIF promotes cell proliferation by activating the MAPK pathway (Figure 2.4). Through a direct interaction with its cell surface receptor CD74 along with its co-receptor

CD44, the extracellular MIF induces the activation of the Src-family tyrosine kinase (Lue, Kapurniotu et al. 2006). The Src-kinase subsequently activates the MAPK/ERK signaling pathway through the cascade of the Raf-MEK-ERK kinases. The phosphorylated ERK (p-ERK) then activates the transcription factor Elk-1, which induces the expression of several genes required for cell proliferation and growth.

Inhibition of NR3C2

A recent study revealed a novel MIF-mediated signaling pathway, in which MIF inhibits the expression of the tumor suppressor protein NR3C2 in pancreatic ductal adenocarcinoma (PDAC) by increasing levels of the miR-301b microRNA, a negative regulator of NR3C2 expression (Yang, He et al. 2016). NR3C2 inhibits the cell proliferation, colony formation, and invasive capacities of the PDAC cells (Yang, He et al. 2016). Moreover, MIF upregulates levels of miR-301b through activation of the PI3K/Akt pathway.

Activation of the PI3K/Akt pathway

Besides inducing cell proliferation and inflammation in a tumor microenvironment, MIF also increases the cell survival important for tumor formation, progression, and metastasis. Studies have shown that MIF binds the CXCR7-CXCR4 receptors and activates the phosphatidyl-inositol kinase PI3K by inducing its phosphorylation (Lue, Thiele et al. 2007). The p-PI3K then activates Akt (also known as protein kinase B) generating p-Akt (phosphorylated Akt), leading to an increase in cell survival. The MIF-stimulated cell survival is reported in fibroblast cells, HeLa cervical carcinoma cells, and PTEN- null and p53-null breast cancer cells.

Activation of the Jnk pathway

There are contradictory roles of MIF in activation of the Jnk kinases pathway. Cell exposure to recombinant MIF proteins leads to the phosphorylation and activation of both Src and PI3K kinases. They, in turn, phosphorylate and activate Jnk via activation of the above mentioned Akt pathway. Furthermore, this activated p-Jnk phosphorylates and activates c-Jun (Lue, Dewor et al. 2011). Conversely, there are other studies which report an antagonistic role of MIF on the Jnk pathway (Kleemann, Hausser et al. 2000).

Regulation of NFkB pathway

Activation of NFκB pathway has long been associated with inflammation and has complex outcomes (Lawrence 2009). One study showed that treating CD4+T cells with NFκB inhibitors, lead to a marked increase in MIF synthesis and secretion via the production of reactive oxygen species (Cho, Moon et al. 2009). Another study presented that MIF promoter has four NFκB binding sites, and IL-1b-dependent stimulation of MIF synthesis requires NFκB function (Veillat, Lavoie et al. 2009). Therefore, the functions of the cytokines and other signaling molecules, including NFκB, in regulation of MIF expression are context dependent.

Evidently, a large number of reports on different types of cancers, including breast carcinoma, prostate cancer, ovarian tumor, colon cancer, gastric cancer and lung cancer, described MIF as a marker of poor prognosis (Meyer-Siegler, Bellino et al. 2002, Mor, Visintin et al. 2005, Verjans, Noetzel et al. 2009, Xia, Yang et al. 2009, Grieb, Merk et al. 2010). MIF co-ordinates with many different signaling molecules/proteins for its diverse functions; MIF's specific and highly dynamic post-translational modifications that include acetylation, phosphorylation, SUMOylation and ubiquitination, influence MIF activity too. Proteomic studies using human cell lines revealed that MIF is modified by both acetylation

(K78) and phosphorylation (Y37)) (Choudhary, Kumar et al. 2009, Moritz, Li et al. 2010). Similarly, proteomic studies also demonstrated that MIF is modified by ubiquitin (K78) (Kim, Bennett et al. 2011, Wagner, Beli et al. 2011).

Various studies have shown that perturbation of SUMOylation correlates with human cancers (Kim and Baek 2006). Dysregulation of SUMOylation could be due to an increase or a decrease in expression of SUMO conjugating enzymes or de-conjugating enzymes. A previous study from our laboratory has identified MIF as a novel SUMO substrate; moreover, its SUMO-2/3 modification increases significantly in a metastatic mouse breast cancer cell line in comparison to a non-metastatic cell line (Subramonian, Raghunayakula et al. 2014). This finding is consistent with the established role of MIF as a key biomarker for breast cancer. MIF displays an increase in its synthesis and stability in cancer cells compared to normal control cells (Meyer-Siegler 2000, Meyer-Siegler, Iczkowski et al. 2005). However, it is not known how SUMOylation can affect MIF expression or functions during tumorigenesis and metastasis. Here we investigated whether global SUMOylation or MIF SUMOylation by itself is responsible for enhancing MIF stability in cells thus leading to its increased activity during tumor progression and metastasis.

Materials and methods

Plasmid extraction and purification

Plasmids were extracted from *E. coli* DH5α and XL1-Blue cells using the Qiagen Miniprep kit or midiPrep protocol. The manual midiprep protocol included the following reagents: Solution I (50 mM glucose, 25 mM Tris-Hcl pH 8.0, 10 mM EDTA), freshly prepared Solution II (1% SDS, 0.2 N NaOH), solution III (potassium acetate and glacial

acetic acid). The purified plasmid DNAs were resuspended in TE buffer (10mM Tris-Hcl pH 8.0, 1mM EDTA).

Mammalian cell culture and transfection

Human embryonic kidney (HEK) 293T cells were cultured in HyClone Dulbecco's Modified Eagles Medium with High Glucose (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin-amphotericin B (Gibco). The cells were maintained within a 5% CO_2 incubator at 37^{o}C. The 293T cells were cultured at a confluency of 70-75% and then transfected using the calcium phosphate transfection method as described previously (Jordan, Schallhorn et al. 1996). The cells were collected and lysed in SDS lysis buffer after transfection for 24 to 48 hours.

Western blot analysis

The SDS-PAGE analyses were performed using 10%, 12.5% or 15% gels. Proteins were blotted onto PVDF membranes (Bio-Rad) from the SDS-PAGE gels followed by blocking with 5% non-fat dry milk and by incubation with primary and secondary antibodies. The Amersham ECL-Prime Western Blotting Detection Reagent Kit (GE Healthcare Life Sciences) was used for detection and analysis of proteins. The X-Ray films were developed using the OptiMax X-Ray Film Processor after the films were exposed to the Western blots. Levels of proteins were quantified with ImageJ software and statistical significance determined by the Student's *t*-test.

Antibodies

The primary antibodies used in this work include rabbit anti-MIF (polyclonal, FL-115, SantaCruz Biotechnology), mouse anti-FLAG (monoclonal, M2, Sigma), mouse anti-tubulin (monoclonal, DMIA, Sigma), mouse anti-GST (monoclonal, B-14, SantaCruz

Biotechnology), mouse anti-GFP (monoclonal, GF28R, UBPBio), mouse anti-SUMO1 (monoclonal, 21C7, Zhang lab and Life Technologies), mouse anti-SUMO2/3 (monoclonal, 8A2, Zhang lab and Abcam), mouse anti-Hsp90α/β (monoclonal, F-8, SantaCruz Biotechnology), rabbit anti-PIAS3 (polyclonal, H-169, SantaCruz Biotechnology), mouse anti-HA (monoclonal, F-7, a kind gift from Dr. Sokol Todi). The following secondary antibodies were used in this study: sheep anti-mouse HRP-linked antibody (GE Healthcare Life Sciences), and donkey anti-rabbit HRP-linked antibody (GE Healthcare Life Sciences).

Plasmids

The pDEST15-GST-MIF construct was used for the bacterial expression of GST-tagged MIF fusion proteins followed by affinity purification of the recombinant proteins for *in vitro* SUMOylation and protein binding assays. The pDONR221-MIF entry clone (DNASU) was used to shuttle the MIF gene into the Gateway destination vector for mammalian expression, pMSCV-N-FLAG-HA-IRES-PURO, by performing the Gateway LR recombination reaction as described by the manufacturer (Invitrogen). Additionally, the pDEST15-GST-MIF plasmid was used as a template to clone MIF in the pcDNA3-HA-N vector using the primers 5'- CGCACGGATCCATGCCGATGTTCATCGTA -3' (forward) and 5'- AGACAGAATTCTTAGGCGAAGGTGGAG -3' (reverse). The additional plasmids used in this study include (Zhang laboratory): pEYFP-C1-SUMO1, pEGFP-C1-SUMO2, pEGFP-C1-SENP1, pEGFP-C1-SENP2, pEGFP-C1-SENP3, pEGFP-C1-SENP6, pEGFP-C1, pC1-FLAG-PIAS1, pCMV-FLAG-PIASxα, pC1-FLAG-PIAS3, pCMV-FLAG-PIASy.

In vitro SUMOylation assay

The purified GST-MIF proteins (1.5 μg total protein) were incubated with the purified His-SUMO-1/SUMO-2 (1 μg), ATP (20 μM), the SUMO-activating enzyme SAE1/SAE2 (250 ng), the SUMO-conjugating enzyme Ubc9 (225 ng), and an ATP regenerating system in a reaction solution containing HEPES (20 mM, pH 7.3), potassium acetate (110 mM), magnesium acetate (2 mM), EGTA (1 mM) and DTT (4 mM). The SUMOylation assays were performed at 37 ^{o}C for 2 hours, reactions were terminated with SDS sample buffer containing Tris-HCl (pH 6.8, 125mM), SDS (4%), β-mercaptoethanol (10%), glycerol (20%) and bromophenol blue (20mg). Western blot was performed and the modifications detected by blotting the membranes with antibodies against GST (Santa Cruz Biotechnology), MIF (Santa Cruz Biotechnology), SUMO1 (Life Technology, Zhang laboratory) and SUMO2/3 (Abcam, Zhang laboratory), respectively.

In-vitro protein binding assay

In each protein binding assay, 25 μg (total protein) of GST-tagged recombinant proteins were first immobilized on glutathione beads. The 293T cells, transiently expressing FLAG-tagged SUMO E3 ligases, were lyzed in a lysis buffer containing Tris-HCl (pH 7.5, 50 mM), NaCl (150 mM), Triton X-100 (1%), glycerol (10%), EDTA (2 mM), sodium fluoride (25 mM) and protease inhibitors (LAP, PMSF, and aprotinin). The glutathione beads, immobilized with GST-tagged proteins, were incubated with the 293T cell lysate for 4 hours at 4 ^{o}C, and then washed with the wash buffer containing Tris-HCl (pH 7.5, 50mM), NaCl (300mM), Triton X-100 (1%), glycerol (10%), EDTA (2mM) and 25mM sodium fluoride. The bound proteins were eluted with the SDS sample buffer and analyzed by Western blot with anti-FLAG antibody.

Site-directed mutagenesis

The pDEST15-MIF (WT) construct encoding the wild-type (WT) MIF protein was used as a DNA template for site-directed mutagenesis to generate the constructs encoding several single and double lysine (K) to arginine (R) MIF mutants, respectively. The MIF mutants include the K33R, K67R, K78R single mutants as well as the K33,67R, K33,78R and K67,78R double mutants. The PCR reactions for mutagenesis were performed using the primer sets as shown in appendix A2 and the Pfu Turbo DNA Polymerase enzyme (Agilent). The PCR products were subjected to DpnI digestion for one hour at 37^{o}C and then transformed into NEB5-alpha competent cells (NEB). The transformed cells were selected on Luria Bertani (LB) agar plates with the appropriate antibiotics.

Bacterial protein expression and purification

The *E. coli* BL21 strain was used for expression and purification of GST-tagged MIF. The BL21 competent cells were first transformed with the pDEST15-GST-MIF plasmids via electroporation and then selected on the LB agar plate with Ampicillin. The transformed cells were first cultured in 2 ml of LB media overnight at 37^{o}C, and then 1 ml of the cell culture was inoculated into 250 ml of fresh LB media to grow until the O.D.600 reached 0.9. The induction reagent, isopropyl-B-D-1-thiogalactopyranoside (IPTG), was added to stimulate the overexpression of the tagged protein for 4 hours at 37^{o}C. The cells were lysed with the lysis buffer (1x phosphate buffer saline solution (PBS), 0.1% (w/v) lysozyme, 1% detergent Triton X-100 and 0.1% (v/v) Benzonase, and the protease inhibitors (leupeptin, antipain, pepstatin, aprotinin and PMSF). The cell lysates were centrifuged at 19,000 rpm at 4^{o}C, and the supernatant with the soluble GST-tagged MIF protein. The glutathione beads were washed twice with PBS and once with the lysis buffer

for equilibration before incubation with the supernatant at 4^{o}C for an hour. The beads were washed with PBS for five times, and the bound GST-tagged protein were eluted from the beads at room temperature with the elution buffer (50mM Tris-HCl pH 8.0 with 10 mM glutathione). The purified proteins, along with bovine serum albumin (BSA) proteins with known concentrations, were separated on the SDS-PAGE gels followed by Coomassie blue staining to determine the concentration of these recombinant proteins. Additionally, the Bio-Rad protein assays were also applied to measure their concentration.

Co-immunoprecipitation (Co-IP)

The cells, expressing FLAG-tagged proteins, were lysed in the lysis buffer containing 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 10% glycerol, 2 mM EDTA pH 8.0, 25 mM sodium fluoride, 150 mM NaCl and protease inhibitors, incubated with the FLAG-M2 beads (Sigma) for three hours at 4^{o}C, and washed five times with the lysis buffer with 300 mM NaCl (final concentration). The interacting proteins pulled down by the FLAG-M2 beads were eluted with 2X SDS sample buffer (4% SDS and 10% β-mercaptoethanol) and analyzed by Western blot.

Protein stability assays

The 293T cells with 70-75% confluency were transfected with the construct encoding FLAG-tagged MIF protein for 36 hours and treated with 150 μg/ml cycloheximide (CHX) for 0, 3, and 6 hours. The cycloheximide solution was freshly prepared in double distilled water before treatment of the cells. The cells were treated with CHX in the presence or absence of 15 μM of the proteasome inhibitor MG132 (a kind

gift from Dr. Sokol Todi) and then lysed with 2X SDS sample buffer followed by Western blot analysis of both endogenous and tagged MIF proteins.

Results

Inhibition of global SUMOylation leads to a decrease in MIF protein levels

A recent proteomic study in our laboratory for analysis of SUMO2/3 modification during breast cancer progression and metastasis has identified MIF as a novel SUMO2/3

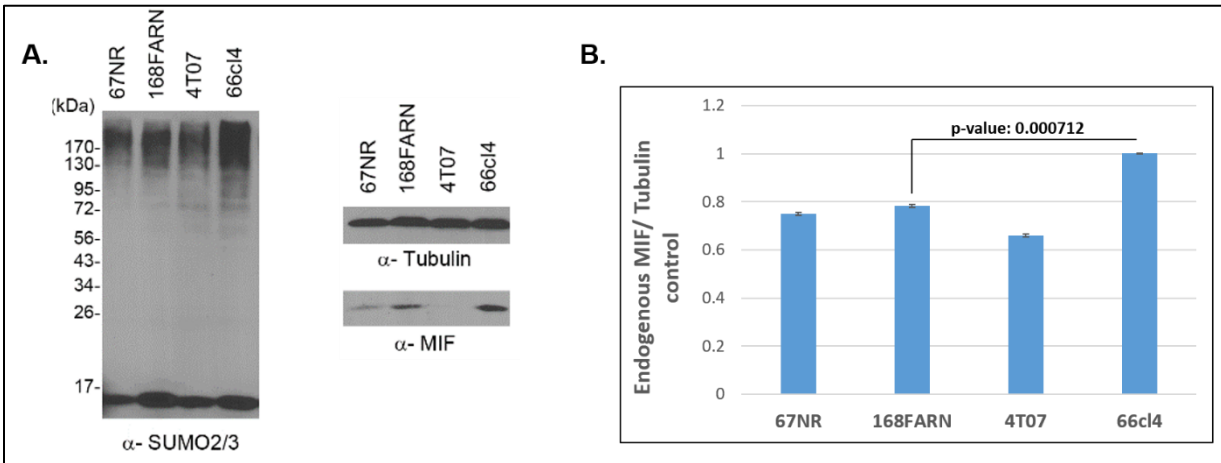

Figure 2.5 : Endogenous MIF protein levels are lower in non-metastatic 168FARN than in isogenic 66cl4 metastatic mouse breast cancer lines. A. Global SUMO2/3 modification in isogenic mouse breast cancer cell lines. There is a significant increase in the level of unmodified endogenous MIF in metastatic 66cl4. B. Endogenous MIF levels were normalized to tubulin. The mean ± S.D is depicted.

target (Subramonian, Raghunayakula et al. 2014). By comparing levels of MIF proteins among four breast cancer cell lines with different metastatic capacity, we observed a steep increase of endogenous unmodified MIF in the metastatic mouse breast cancer cell line compared to the three non-metastatic cell lines (Figure 2.5 A and B). The increased level of MIF proteins in the metastatic cell line positively correlated to the elevated levels of global SUMO2/3 modification. The positive correlation led us to hypothesize that SUMOylation plays important role in regulating levels of MIF proteins *in vivo*.

To test this hypothesis, we co-transfected the 293 cells with the plasmids encoding HA-tagged MIF and one of the SUMO-specific isopeptidases, SENP1, SENP2, SENP3

and SENP6. We noticed that global inhibition of SUMO modification by overexpressing each isopeptidase reduced the level of MIF protein (Figure 2.6 A and B). It has been shown previously that among all the SUMO isopeptidases, SENP1 and SENP2 play a major role in deconjugating SUMOs from their substrates (Mikolajczyk, Drag et al. 2007). Therefore, we further examined the effect of SENP1 and SENP2 on levels of endogenous MIF proteins, and found that overexpression of SENP2, but not SENP1, resulted in a significant decrease in levels of endogenous MIF (Figure 2.6 C and D). Since SENP2 downregulate levels of both tagged and endogenous MIF proteins, we also tested whether its catalytic activity is necessary for this function. The catalytic site of SENP2 is at their C-terminal regions and consists of a catalytic triad - C548-H478-Asp495. We used the plasmid encoding the Cys548Ser catalytic mutant protein (SENP2C548) for our assay. As shown in Figure 2.6 A and B, we found that the decrease in levels of HA-tagged MIF proteins was independent of the catalytic activity of the isopeptidases SENP2. As the SUMOylation machinery enzymes have been shown to affect the assembly and disassembly of protein complexes by enhancing and disrupting protein-protein interactions (Werner, Flotho et al. 2012), one explanation of this intriguing result is that overexpression of SENP2 catalytic mutant might affect the protein complexes containing MIF and thus indirectly decreases its stability.

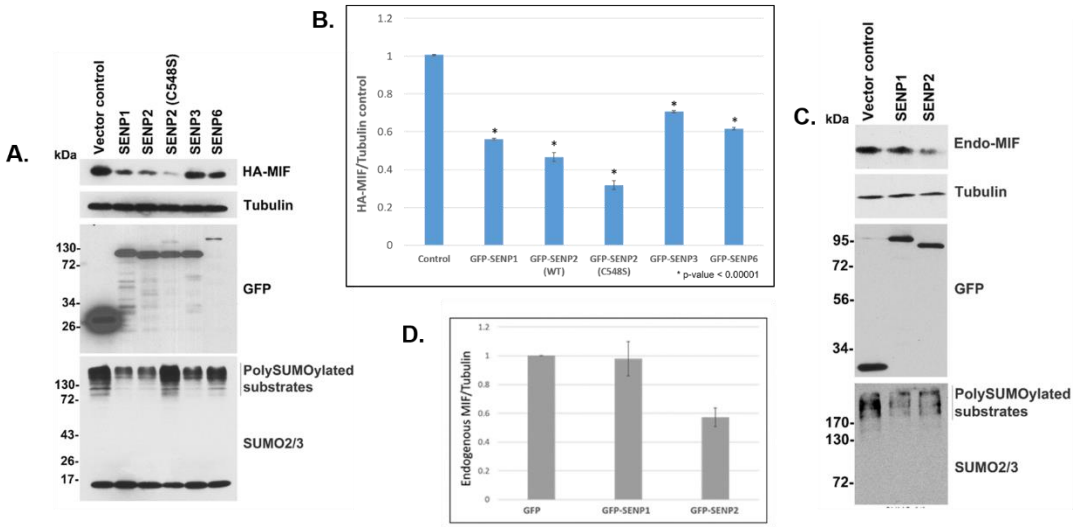

Figure 2.6 : MIF protein levels decline in the presence of SUMO isopeptidases. A. SENPs lead to a steep decline in HA-MIF. This effect was observed to be independent of the catalytic function of the SENPs. B. HA-MIF was quantified from three independent experiments, Tubulin was the normalizing protein. Graphs depict mean MIF levels ± S.D. C. SENP1 and SENP2 were overexpressed in HEK293T cells, producing a sharp decline in the level of endogenous MIF level in the presence of SENP2. D. Quantification was done as in B.

Analysis of MIF stability

MIF is a well-known cancer marker and has higher protein levels in cancer cells in comparison to normal tissue cells. In this study, we investigated if the elevated levels of MIF proteins in metastatic cells compared to non-metastatic cells are due to its increased stability that is enhanced by its SUMOylation. Treatment of 293T cells with cycloheximide showed that the half-life of the FLAG-tagged MIF is approximately three hours, and that its degradation is inhibited in the presence of the proteasome inhibitor, MG132 (Figure 2.7 A). Together with the quantified analysis the result revealed that MIF stability is dependent on its degradation mediated by the proteasome pathway (Figure 2.7 A and B), which is consistent with other cell lines (Schulz, Marchenko et al. 2012). Moreover, we also analyzed the half-life of endogenous MIF protein in 293T cells, our immunoblotting results showed that compared to the stability of FLAG-tagged MIF (Figure 2.7 A and B), endogenous MIF has a similar half-life of about three hours (Figure 2.7 C and D).

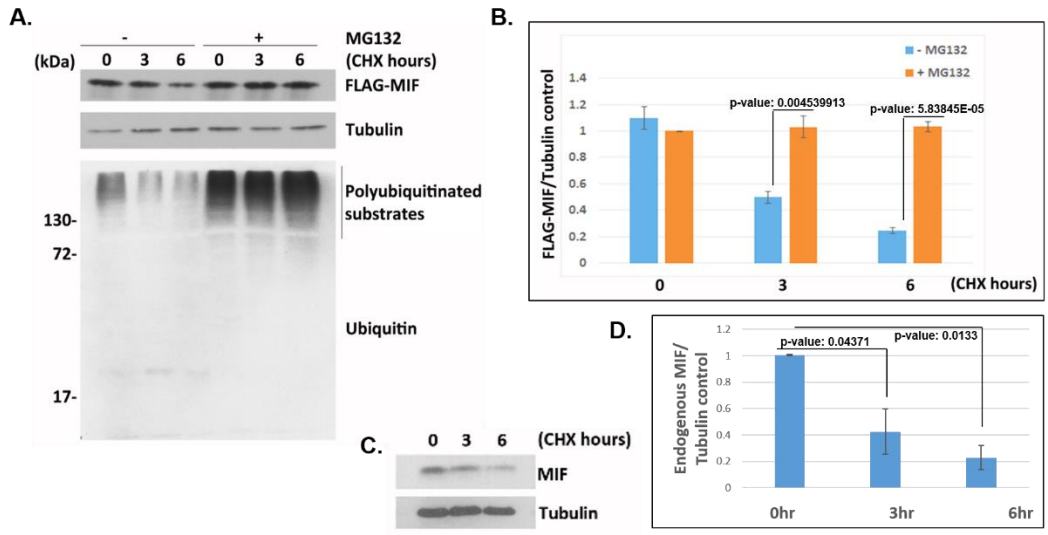

Figure 2.7 : MIF protein stability is regulated via the proteasomal pathway. A and B. FLAG-MIF was overexpressed in HEK293T cells, followed by CHX treatment (150ug/ml) for 0, 3 and 6 hrs respectively. Western blot shows a distinct decline in FLAG-MIF level, as also evident from the quantification in figure B from three replicates. The protein level is restored in the presence of MG132 (15uM). Tubulin was used for FLAG-MIF normalization and graph is represented as mean ± S.D. C and D. HEK293T cells were treated with CHX, and lysed, Western blot analysis revealed a clear decrease in endogenous MIF level by 3 hrs and further by 6 hrs. The result was consolidated with a quantification from three individual experiments, utilizing Tubulin for normalization, showed as mean ± S.D.

Analysis of MIF SUMOylation and identification of its SUMOylation site (s)

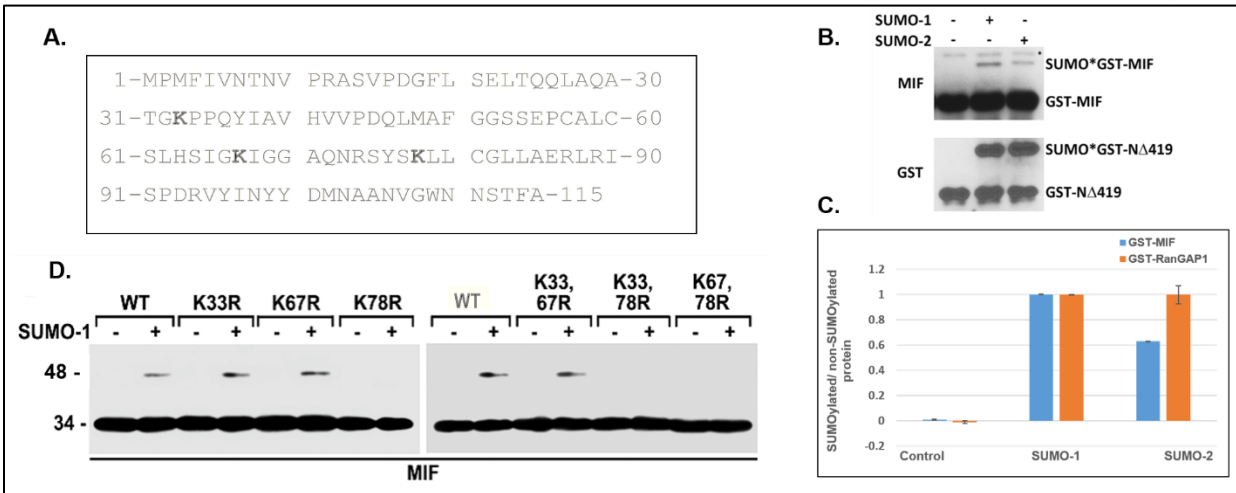

Figure 2.8 : Lysine 78 is the sole site of SUMOylation in MIF. A. MIF has three lysines, K33, K67 and K78 (bold). B and C. *In vitro* SUMOylation with purified SUMO E1, E2, SUMO1/2, ATP and GST-MIF (WT). GST-NΔ419 (RanGAP1) was the positive control. MIF is more strongly SUMOylated by SUMO1. D. Purified GST-tagged MIF (WT; K33R; K67R; K78R; K33,67R; K33,78R and K67,78R) proteins were tested for SUMOylation by SUMO1.

To determine the effect of SUMOylation on MIF stability, it was important to identify the lysine residue (s) responsible for its SUMOylation. SUMOylation occurs on a lysine residue within a SUMOylation consensus motif established as ψKXE/D or its inverted

motif (Matic, Schimmel et al. 2010). However, SUMOylation at lysine residues present in non-consensus motifs do occur (Hoege, Pfander et al. 2002, Pichler, Knipscheer et al. 2005, Figueroa-Romero, Iniguez-Lluhi et al. 2009). All the three lysine residues in MIF (K33, K67 and K78) are present at non-consensus motifs. Thus, to identify the specific SUMOylation site (Figure 2.8 A), we performed site-directed mutagenesis to create lysine to arginine point mutant proteins including K33R, K67R, K78R, K33,67R, K67,78R and K33,78R. Before identifying the MIF SUMOylation site(s), we analyzed and compared levels of MIF modification by SUMO1 and SUMO2 in vitro (Figure 2.8 B and C). We set up the in vitro SUMOylation assays using the purified SUMO E1 and E2 enzymes and ATP in the presence or absence of SUMO1 or SUMO2 proteins. As expected, MIF was modified by both SUMO1 and SUMO2; however, its modification was consistently higher by SUMO1. Based on this result, we performed the *in vitro* SUMOylation assay with SUMO1 to identify the site(s) of MIF SUMOylation followed by Western blot analysis. We demonstrated that the lysine residue 78 (K78) of MIF is its SUMOylation site *in vitro* (Figure 2.8 D).

SENP2-mediated reduction of MIF is dependent on the proteasomal pathway

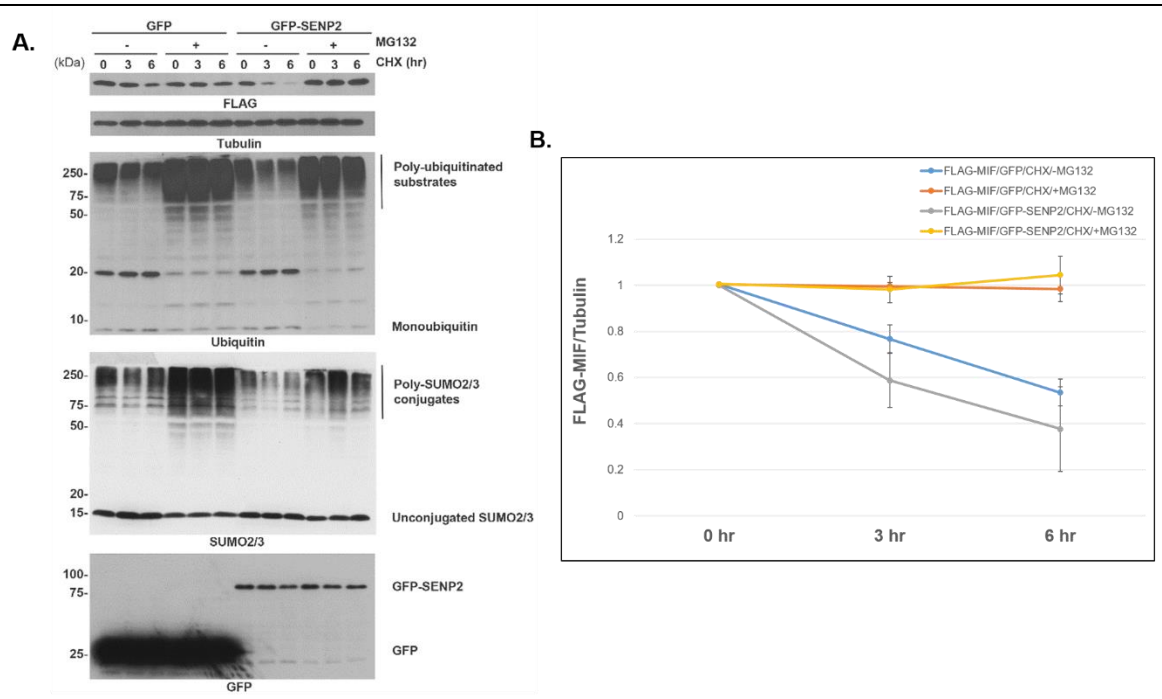

Figure 2.9 : SENP2 dependent reduction in MIF level is mediated by a regulated degradation by the proteasomal pathway. A. HEK293T cells expressing FLAG-MIF and GFP/GFP-SENP2 were treated with CHX and MG132. B. Quantification was performed on three independent experiments, using Tubulin for normalization. Graph shows mean ± S.D.

We next tested if the decrease in levels of MIF proteins in cells with SENP2 overexpression is due to reduction in MIF stability instead of an inhibition of its expression. We analyzed the MIF stability in the presence or absence of SENP2 overexpression and found that overexpression of SENP2 further enhances the decline in levels of MIF proteins in cells treated with CHX, which blocked the protein synthesis (Figure 2.9 A and B). Notably, this effect is reversed by incubating cells with the proteasome inhibitor MG132. Hence, our results demonstrated that SENP2 overexpression decreases the stability of MIF and that the increased degradation of MIF is via the proteasomal pathway in these cells.

Identification of the SUMO E3 ligase that interacts with MIF protein

We also examined if one or more members of the PIAS family of the major SUMO E3 ligases, including PIAS1, PIAS3, PIASxα and PIASy, interact with MIF specifically. Our *in vitro* protein binding assay showed a stable interaction of PIAS3 with GST-tagged MIF (WT), when compared with PIAS1, PIASxα and PIASy (Figure 2.10 A). Interestingly, PIAS3 is upregulated in different types of cancer, including breast cancer and lung cancer (Wang and Banerjee 2004). Therefore, it would be worthy to test further if PIAS3 can enhance MIF SUMOylation both *in vitro* and *in vivo*.

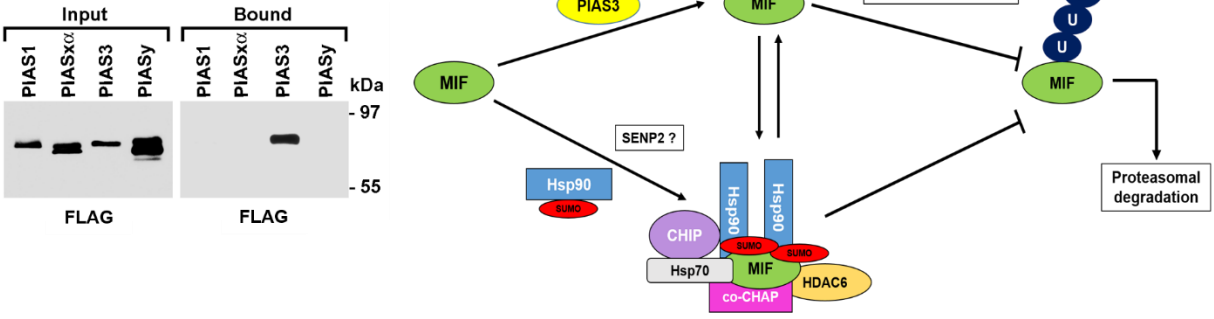

Figure 2.10 : The SUMOylation pathway regulates MIF stability by inhibiting ubiquitin-dependent proteasomal degradation. A. Mammalian SUMO E3 ligase PIAS3 interacts with MIF. B. A model proposing a possible mechanism by which MIF stability is regulated. SUMOylation blocks ubiquitination by preventing MIF interaction with the ubiquitin ligase CHIP, preventing MIF degradation.

Discussion

The previously published proteomic analysis in our laboratory revealed that levels of both global SUMO2/3 modification and MIF SUMO2/3 modification are markedly higher in the metastatic cells 66cl4 compared to the non-metastatic cells 168FARN (Subramonian, Raghunayakula et al. 2014). Moreover, there was a consistent increase in levels of the unmodified endogenous MIF proteins in 66cl4 cells when compared with those in 168FARN levels (Figure 2.4 A and B). This result lead us to determine whether the SUMOylation pathway is critical for regulating the stability of MIF *in vivo*. We first showed that overexpression of the SUMO-specific isopeptidase, SENP1, SENP2, SENP3

or SENP6, results in a drastic decrease in levels of MIF proteins. We observed that SENP2, among the above SUMO isopeptidases, exhibited the highest negative effect on levels of MIF proteins. In the same experiment, the catalytic C548S SENP2 mutant protein had an even higher adverse effect on MIF. This result was very interesting to us as there has not been any report of a similar phenomenon. It can be speculated that the SENP2 is working in a complex, a mechanism of action known for certain DUBs (deubiquitinating enzymes) such as the JAMM/MPN+ DUBs (Komander, Clague et al. 2009), an allosteric effect. Another possibility is that a different catalytic site in the protein is created in the presence of the interacting partners; it is an interesting feature observed in the multisubunit SUMO E3 ligase complex consisting of the Ubc9/RanGAP1*SUMO1/RanBP2 (Werner, Flotho et al. 2012). In this study, Werner et al. observed the masking of the actual RanBP2-IR1 (internal repeat 1) based catalytic domain in the entire complex; surprisingly, the IR-2 (internal repeat 2) domain of RanBP2 was found to be essential for Ubc9-SUMO1 binding that leads to SUMOylation of the chromosome passenger complex protein Borealin. Mutations at this IR-2 domain prevented this successful modification. Therefore, these two possibilities present two potential mechanisms in which SENP2 might be affecting MIF protein level, independent of its known catalytic residues/domain.

Our analysis of MIF stability indicated that MIF has a half-life of around 3 hours and is degraded via the proteasomal pathway. We further showed that overexpression of the SUMO isopeptidase SENP2 decreases the stability of the MIF protein, and that the adverse effect of SENP2 overexpression can be reversed by treatment of cells with the proteasome inhibitor, MG132. Therefore, SENP2-mediated decline in MIF protein stability

is dependent upon the proteasomal pathway. As a novel SUMO target, the SUMOylation site (/s) on MIF is unknown. Site-directed mutagenesis was performed of all three lysine residues (K33, K67, K78) to arginine and the *in vitro* SUMOylation assay. We demonstrated that K78 is the only site for the SUMO conjugation of MIF proteins *in vitro*. Notably, the K78 residue of MIF is not present within a SUMOylation consensus motif, which is consistent with previous reports that many protein substrates are modified by SUMOs at lysine residues within non-consensus sequences. It would be significant in future to investigate the role of MIF SUMOylation in regulating its stability and activity especially during tumor progression and metastasis. Furthermore, we found that among the SUMO E3 ligases, PIAS1, PIASxα, PIAS3 and PIASy, only PIAS3 has a highly specific interaction with MIF. This result is very intriguing as PIAS3 is implicated in various human cancers, including breast cancer, lung carcinoma, and prostate cancer, and its high levels of expression correlate with poor prognosis (Wang and Banerjee 2004, Eifler and Vertegaal 2015). Furthermore, PIAS3 is the SUMO E3 ligase for Vimentin and Rac1 (Castillo-Lluva, Tatham et al. 2010, Wang, Zhang et al. 2010), both of which are involved in cell migration, a necessary process for tumor cell invasion and metastasis. Therefore, it would be very interesting to determine if PIAS3 regulates MIF SUMOylation. Importantly, this study might provide a novel mechanism by which the SUMOylation pathway affects MIF stability and activity in various types of cells especially in tumor cells undergoing the process of metastasis.

Our results lead us to propose a model where either one or both the following mechanisms could play a significant role in regulation of MIF protein stability (Figure 2.9 B). One possible mechanism is that the PIAS3-mediated MIF SUMOylation competes

with its ubiquitination at the same (K78) or other lysine residues (K33/K67) and thereby prevents its degradation by proteasomes. The other potential mechanism is that, SUMOylation of MIF or the heat shock protein 90 (Hsp90) might facilitate the assembly of a large protein complex. The protein complex might include MIF, Hsp90, the heat shock protein 70 (Hsp70), the ubiquitin E3 ligase CHIP, HDAC6 and other protein factors, and therefore inhibits the ubiquitination and degradation of MIF. It has been shown earlier that SENP2 is the SUMO isopeptidase for Hsp90 (Preuss, Pfreundschuh et al. 2015). Importantly, the large protein complex, including MIF, Hsp90, Hsp70, CHIP, HDAC6 and other protein factors are known to stabilize MIF by blocking its CHIP-mediated ubiquitination and degradation, leading to an increase in MIF stability (Schulz, Marchenko et al. 2012). SENP2-mediated deSUMOylation of Hsp90 or MIF may hinder the assembly of the MIF-Hsp90 complex, and may thereby lead to MIF degradation by CHIP-mediated ubiquitination. Both mechanisms are promising and worthy of further investigation.

Future directions

We found that the SUMOylation pathway greatly influences cellular MIF protein levels. More specifically, the overexpression of SUMO isopeptidases leads to a drastic decrease in MIF protein level. Further studies showed us that this effect is not dependent on the catalytic function of the SUMO isopeptidases, as we observed that overexpression of the catalytic mutant (C548S) of SENP2 lead to an even greater decrease in the protein level of MIF in comparison to wild-type SENP2. We further investigated the effect of a SENP2 catalytic double mutant (C548A, W457A) on levels of MIF proteins and observed a similar result when compared to the single mutant (C548S) of SENP2.

<u>Investigating the mechanisms by which SENP2 overexpression decreases MIF stability</u>

One explanation would be that both SENP2 wild-type and its catalytic mutants interact with MIF, prevent MIF from forming the stable MIF-Hsp90 complex, and therefore reduce the MIF stability. To test this hypothesis, we could perform the following experiments. The different truncation mutation constructs of SENP2 can be generated and then transfected into 293T cells to determine which of them results in a decrease in levels of MIF proteins compared to the SENP2 wild-type or catalytic mutant. Next, co-immunoprecipitation assays can be performed with the wild-type, the catalytic mutant or the truncation mutants of SENP2 to determine whether they differ in the co-immunoprecipitation of endogenous MIF proteins. The experiment will provide the critical evidence to support the above hypothesis.

Crosstalk between SUMOylation and ubiquitination pathways in controlling MIF stability

The next important question would be to determine if SUMOylation and ubiquitination are competing for maintaining MIF protein levels in cells. The reason for this hypothesis is that previous proteomic studies indicate the K78 residue of MIF is a ubiquitination site, and that our analysis here revealed that the same lysine residue is also the sole SUMOylation site in vitro. Therefore, it would be interesting to find if the same residue is important for both modifications in vivo. Moreover, it has previously been shown that SUMOylation of CDK6 at one lysine residue prevents its ubiquitination at a nearby lysine residue within its 3-D structure (Bellail, Olson et al. 2014). The following experiments could be used to test this idea. The *in vitro* ubiquitination assays with the wild-type and the single lysine residue mutants (K33R, K67R, K78R) of MIF can be carried out to test if the K78 residue is its sole ubiquitination site, as suggested in the previous proteomic analyses, or only one of the lysine residues for MIF ubiquitination. Moreover,

we could further examine whether the in vitro ubiquitination site(s) of MIF is/are responsible for its ubiquitination in vivo by performing transfection of a series of MIF lysine to arginine mutant constructs followed by immunoprecipitation and immunoblot analysis.

Identification of the specific E3 ligase for MIF SUMOylation

Our protein binding assays revealed that the SUMO E3 ligase PIAS3 interacts specifically with MIF in vitro after examining multiple members of the PIAS family. The PIAS3 protein level increases in various types of human cancer, including breast tumor, colorectal cancer and prostate cancer. Therefore, it would be very interesting to test if PIAS3 stimulates MIF SUMOylation both *in vitro* and *in vivo*.

CHAPTER 3: THE CELLULAR DISTRIBUTION AND SUMOylation OF RanGAP1 IS REGULATED BY ITS CRM1-DEPENDENT NUCLEAR EXPORT IN MAMMALIAN CELLS

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*These authors contributed equally to this work.

Abstract

RanGAP1 is the Ran GTPase activating protein required for RanGTP hydrolysis in the cytoplasm and therefore plays a critical role in nuclear transport. With a predominant localization in the cytoplasm, SUMO1 modification of RanGAP1 results in its redistribution at the cytoplasmic filaments of the nuclear pore complex (NPC). RanGAP1 contains nine nuclear export signal sequences (NESs) and one putative nuclear localization signal sequence (NLS). However, it was unclear whether RanGAP1 shuttles between nuclear and cytoplasmic compartments of mammalian cells and how its predominant cytoplasmic localization is regulated. This chapter primarily describes my contribution to the paper recently published in PLoS ONE (Cha, Sen et al. 2015), in which I am one of the two co-first authors and have contributed equally to this work. In this study, we showed that treatment of mammalian cells leptomycin B (LMB), a highly specific inhibitor of the nuclear export receptor CRM1, leads to a drastic redistribution of the cytosolic and the NPC-associated RanGAP1 into the nucleoplasm. A time-course analysis demonstrated that the NPC-associated RanGAP1 relocates to the nucleoplasm at much lower rate than the cytoplasmic RanGAP1. Moreover, the LMB-induced accumulation of RanGAP1 in the nucleoplasm correlates with a significant increase in the SUMOylation of RanGAP1. This result demonstrated that the RanGAP1 SUMOylation occurs mainly in the nucleus. We

also determined that the C-terminal region of the mammalian RanGAP1 protein contains a functional NLS (residues 541- 566), this NLS is for the nuclear accumulation of RanGAP1 in response to LMB treatment. Therefore, this study clearly demonstrated that RanGAP1 shuttles between the nuclear and cytoplasmic compartments, and that CRM1 mediates the RanGAP1 nuclear export.

Introduction

Ran GTPase is required for nuclear transport, mitotic spindle assembly, and nuclear envelope formation (Dasso 2001, Guttinger, Laurell et al. 2009). The nuclear transport mediated by Ran is dependent on a large family of nuclear transport receptors, also known as karyopherins, which include both importins and exportins (Pemberton and Paschal 2005). Importin binds to the nuclear localization signal (NLS) of a cargo protein and imports it to the nuclear compartment where the interaction of importin with RanGTP leads to dissociation of the cargo from the importin. The importin-RanGTP complex then comes out of the nucleus. In the cytoplasm, RanGTP hydrolysis aided by the RanGAP1 and the RanBP1 or RanBP2 leads to the release of the importin from the RanGDP. Similarly, exportin binds the nuclear export signal (NES) on a cargo protein along with RanGTP in the nucleus and releases the cargo in the cytoplasm when RanGAP1 hydrolyzes the RanGTP. Under both the conditions, the steep gradient of the RanGTP across the nuclear envelope, which is high in the nucleus but low in the cytoplasm, is maintained by the presence of the RanGAP1 in the cytoplasm and the Ran guanine nucleotide exchange factor (RanGEF), also known as Rcc1, in the nucleus. Ran, by itself, does not have sufficient activity to alternate between its GTP and GDP bound states

under physiological conditions, whereas Rcc1 and RanGAP1 increase the activity by 10^5-fold (Klebe, Bischoff et al. 1995).

RanGAP1 is present in all eukaryotes, ranging from yeast to humans with a conserved N-terminal leucine-rich repeat domain (LRR domain; 330-350 amino acid residues) and the acidic region (40 amino acid residues) (Bischoff, Krebber et al. 1995). Unlike the yeast RanGAP (Rna1p), the vertebrate RanGAP1 protein has a C-terminal 230 amino acid residue stretch which is not present in the yeast Rna1p; moreover, the mammalian RanGAP1 protein is modified by SUMO1. The unmodified RanGAP1 is localized in the cytoplasm whereas the SUMO1-modified RanGAP1 is targeted to the nucleoplasm and the cytoplasmic filaments of the NPC by formation of a highly stable complex with RanBP2 and Ubc9.

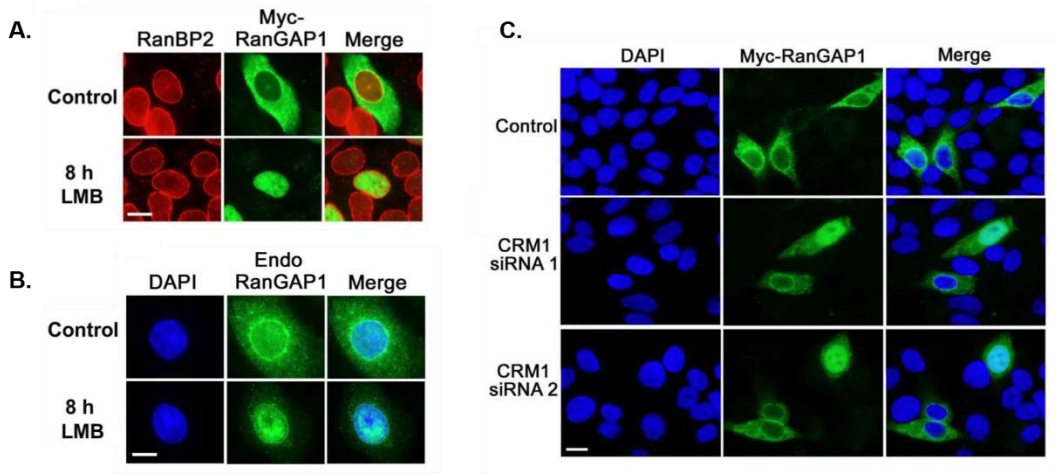


Figure 3.1 : Mammalian RanGAP1 shuttles between nucleus and cytoplasm and its nuclear export is mediated by Crm1. A. HeLa cells were overexpressed with Myc-RanGAP1 for 24 hrs, followed by treatment with Leptomycin B (LMB). Immunofluorescence analysis was performed using anti-Myc and RanBP2 antibodies. B. BRL cells were treated with either control DMSO or LMB (20 nM) for 8 hrs, followed by immunofluorescence using anti-RanGAP1 and RanBP2 antibodies. C. Endogenous Crm1 was knocked down using two different siRNA oligonucleotides in HeLa cells for 24 hrs, followed by transfection of Myc-tagged RanGAP1 for another 24 hrs. Immunofluorescence analysis was performed with anti-Myc antibody.

RanGAP1 contains nine putative NESs in its LRR region (conserved from yeast to human), whereas, the C-terminal region of the RanGAP1 contains a probable non-classical NLS (Matunis, Wu et al. 1998, Feng, Benko et al. 1999). The NESs are

recognized by the major exportin CRM1, also known as Exportin1 or Xpo1, which is

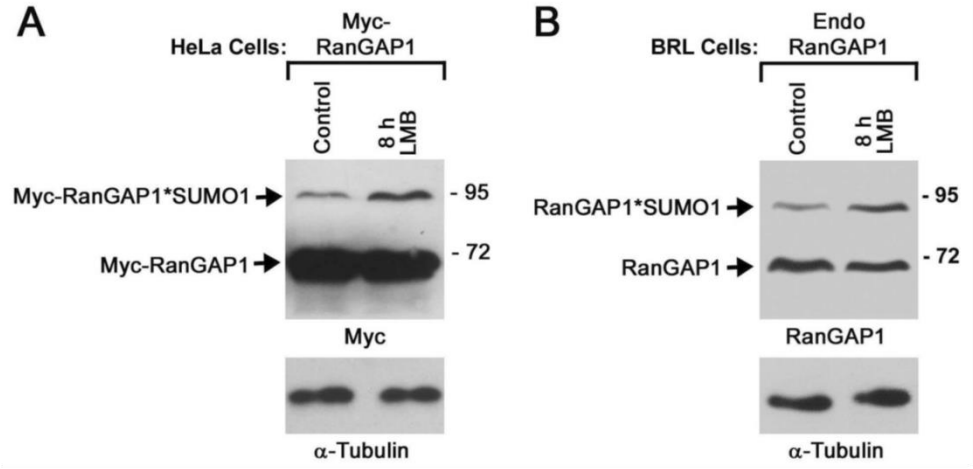


Figure 3.2 : LMB treatment leads to an enhanced RanGAP1 SUMOylation. A. HeLa cells were transfected with Myc-tagged RanGAP1 for 24 hrs, followed by 8 hr treatment with 20 nM LMB or control DMSO solution, Western blot was performed using anti-Myc and tubulin antibodies. B. BRL cells were incubated with LMB or control solution for 8 hrs, followed by Western blot using anti-RanGAP1 and Tubulin antibodies.

responsible for exporting over 80% of cargos (Fornerod, Ohno et al. 1997, Kudo, Khochbin et al. 1997, Fung and Chook 2014). Despite having this promising information regarding RanGAP1, it was unknown whether the mammalian RanGAP1 shuttles between the nucleus and the cytoplasm, also if CRM1 is its specific exportin. Our studies first showed that RanGAP1 shuttles between the nucleus and the cytosol in mammalian cells using the nuclear export inhibitor, leptomycin B (LMB) (Figures 3.1 A and B). LMB has been widely used to identify the cargo proteins exported by CRM1. Furthermore, we observed that knockdown of endogenous CRM1 protein (around 70% knockdown) using two different siRNA oligonucleotides, leads to a drastic increase in nuclear localization of Myc-tagged RanGAP1 (Figure 3.1 C). Therefore, these results indicated that the mammalian RanGAP1 translocates between the nucleus and the cytosol, and that CRM1 is mainly responsible for its nucleocytoplasmic shuttling.

Earlier findings in this study also revealed that LMB treatment of mammalian cells resulted in an increase in SUMOylation of both Myc-tagged and endogenous RanGAP1 (Figure 3.2 A and B). This result provided us with a hypothesis that this nuclear redistribution of the RanGAP1 has a significant role in orchestrating its SUMOylation. The hypothesis is based on the prior knowledge that the SUMOylation machinery components are predominantly localized in nucleus of all eukaryotes (Azuma, Tan et al. 2001, Saitoh, Pizzi et al. 2002, Zhang, Goeres et al. 2008).

However, the above findings raise several important questions. First, 8-hour treatment of mammalian cells by LMB lead to a predominant accumulation of endogenous RanGAP1 in the nucleoplasm along with its decreased but still prominent distribution at the NPC. I wanted to see if a prolonged treatment of the mammalian cells lead to a significantly robust redistribution of the NPC-associated RanGAP1 (endogenous) into the nucleoplasm. Second, there have been speculations about whether RanBP2 is the SUMO E3 ligase for RanGAP1 (Zhu, Goeres et al. 2009, Hamada, Haeger et al. 2011). It has been shown in earlier studies that RanBP2 forms a highly stable complex with SUMO1-modified RanGAP1 and Ubc9 at the NPC and therefore prevents the SUMO1-modified RanGAP1 from deSUMOylation mediated by the SUMO isopeptidases (Zhu, Goeres et al. 2009, Hamada, Haeger et al. 2011). On the other hand, the RanBP2/RanGAP1*SUMO1/Ubc9 complex is known to function as a multisubunit SUMO E3 ligase (Flotho and Werner 2012). Here I aimed to determine whether RanBP2 is the E3 ligase responsible for RanGAP1 SUMOylation. Third, I wanted to determine whether the C-terminal region of the mammalian RanGAP1 contains a functional NLS, and whether the nuclear localization of RanGAP1 depends on its SUMOylation. The latter

question is based on the observation that both the SUMO E1 enzyme (SAE1/SAE2) and the E2 enzyme (Ubc9) are predominantly localized in the nucleus, and that the nuclear localization signals of several tested SUMO target proteins are necessary for their SUMOylation(Kishi, Nakamura et al. 2003, Besnault-Mascard, Leprince et al. 2005, Hofmann, Arduini et al. 2009).

Materials and methods

Plasmids and siRNAs

The pcDNA3-Myc-RanGAP1 plasmid was used for transient expression of Myc-tagged full-length mouse RanGAP1 (1-589) wild-type (WT) in mammalian cells. The C-terminal deletion mutants of Myc-RanGAP1, including CΔ23 (1-566) and CΔ49 (1-540), were constructed using the pcDNA3-Myc-RanGAP1 plasmid as the template DNA for PCR amplification. The forward PCR primer (ATTGGTACCGAGCTCGGATCCACTAG) was paired with the reverse PCR primer (GCTCTAGACTATGTCACAAATGCCAA) for amplification of the Myc-RanGAP1-CΔ23 deletion mutant and the reverse primer (GCTCTAGACTAGGGGCCATGCAGGCT) for amplification of the Myc-RanGAP1-CΔ49deletion mutant. To knockdown the expression of RanBP2 by RNA interference, control siRNA (UUCUCCGAA CGUGUCACGU) (Sekhri, Tao et al. 2015) and RanBP2-specific siRNA (CACAGAC AAAGCCG UUGAA) (Hutten, Flotho et al. 2008) were purchased from Dharmacon.

Antibodies

The following antibodies were used in my study: anti-RanGAP1 (Dr. Michael Matunis), anti-RanBP2 (Abcam), anti-tubulin (Sigma), anti-Myc (Santa Cruz Biotechnology), anti-SUMO1 (Zhang laboratory, Life Technologies). The detailed

information about the sources, catalog numbers and dilutions of these antibodies is listed in the Appendix B.

Cell culture, transfection and LMB treatment

Human cervical cancer (HeLa) cells and Buffalo rat liver (BRL) cells were cultured in HyClone Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% HyClone fetal bovine serum (FBS) and 1% penicllin-streptomycin-ampotericin B (Gibco). The cells were maintained within a 5% CO_2 incubator at 37^{o}C. For CRM1 inhibition, 20 μM LMB stock solution was prepared in the PBS (phosphate-buffered saline) buffer with 0.3% DMSO. A final concentration of 20 nM LMB was used for treatment of cells to inhibit CRM1-mediated nuclear export (Kudo et al., 1999).

For siRNA transfection, Oligofectamine (Invitrogen) was used following the manufacturer's protocol. RNAi-mediated knockdown of RanBP2 was performed for 72 hours, followed by control/LMB treatment for 8 hours. Western blot analysis was performed using anti-RanGAP1, anti-RanBP2 and anti-tubulin antibodies. Calcium phosphate-mediated transfection method (Jordan, Schallhorn et al. 1996) was applied for transfecting the DNA constructs encoding the RanGAP1 WT and its truncation mutants in HeLa cells, followed by immunofluorescence microscopy using anti-Myc, anti-RanBP2 and anti-SUMO2/3 antibodies. Additionally, Lipofectamine-Plus reagent (Invitrogen) was also used for transfection followed by Western blot analysis.

Immunofluorescence microscopy

BRL and HeLa cells were fixed with 3.5% paraformaldehyde for 30 mins, washed with 1x PBS buffer and permeabilized with the 1x PBS buffer containing 0.5% Triton X-100 for five mins. The cells were then incubated for 1 hour using anti-RanGAP1, anti-

RanBP2 and anti-Myc primary antibodies followed by staining with Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen) for 30 mins. After incubation with the mounting solution containing DAPI for staining of nuclear DNA for five mins, the cells were visualized under the inverted Olympus IX81 fluorescence microscope with U-Plan S-Apo 60×/1.35 NA oil immersion objective. The MicroSuite acquisition software (Olympus) was used to acquire the immunofluorescence images.

Western blot analysis

The 10% and 12.5% denaturing SDS-PAGE gels were used for Western blot analysis. The proteins were separated on the SDS-PAGE gels and then transferred to the PVDF membranes (Bio-Rad). After blocking with 5% non-fat dry milk, the membranes were incubated with the primary antibodies, the secondary HRP-conjugated antibodies (GE Healthcare Life Sciences), and then the Amersham ECL-Prime Western blotting detection reagent (GE Healthcare Life Sciences). Following exposure with the membranes, the films (Denville Scientific) were developed using the OptiMax X-Ray Film Processor.

Results

Prolonged Crm1 inhibition leads to a significant redistribution of the stably associated, NPC localized, endogenous RanGAP1 into the nucleus

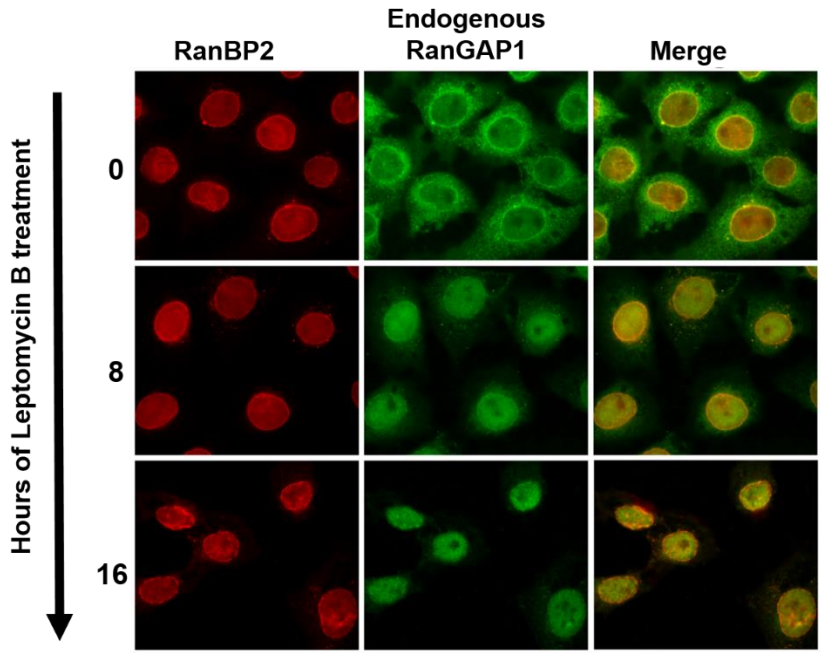


Figure 3.3 : Prolonged LMB treatment leads to redistribution of highly stable NPC-associated RanGAP1. A. BRL cells were treated with 20 nM LMB for 8 and 16 hrs respectively. The cells were then immuno-stained with anti-RanGAP1 and RanBP2 antibodies for immunofluorescence analysis.

The studies performed by my co-authors showed that the inhibition of CRM1-mediated nuclear export by LMB causes the redistribution of the Myc-tagged RanGAP1 from the cytoplasm and the NPC to the nucleoplasm following an 8-hour of LMB treatment. On the contrary, the endogenous RanGAP1, after a similar LMB treatment, shows a relatively strong localization at the NPC. This result suggested that compared to the Myc-tagged SUMO1-modified RanGAP1, the endogenous SUMO1-modified RanGAP1 is more stably associated in the NPC by forming the complex with RanBP2 and Ubc9 (Matunis, Coutavas et al. 1996, Matunis, Wu et al. 1998, Ritterhoff, Das et al. 2016). A prolonged 16-hour treatment (along with 8-hour treatment) of the BRL cells with LMB showed a complete disappearance of the RanGAP1 from the NPC (almost 100%) and an obvious predominant nucleoplasmic localization (Figure 3.3). This result

suggested a time-dependent redistribution of the protein; it also indicated that the Myc-tagged RanGAP1 is less stably bound at the NPC in comparison to the endogenous RanGAP1.

The increase of RanGAP1 SUMOylation in cells treated with LMB positively correlates with its nuclear accumulation and independent of the SUMO E3 ligase RanBP2

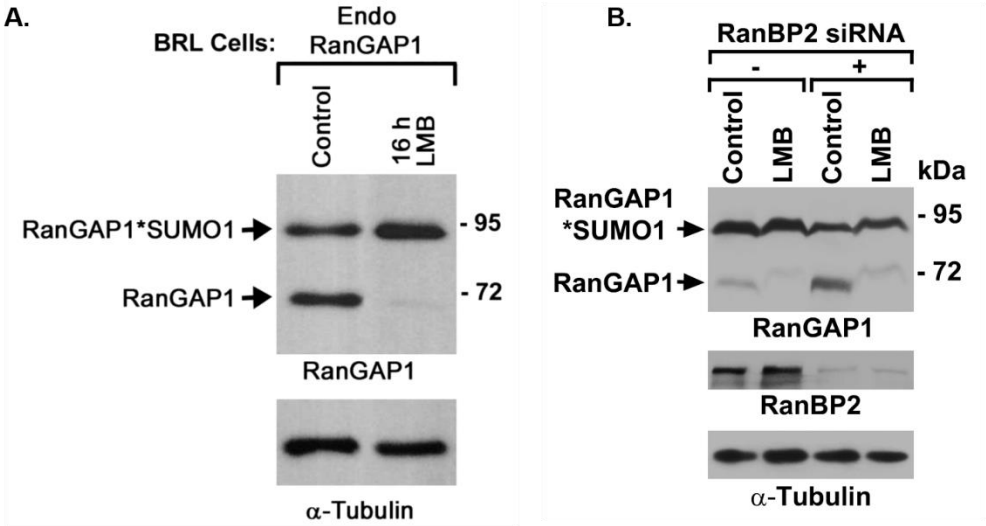


Figure 3.4 : LMB treatment results in a drastic increase in RanGAP1 SUMOylation, independent of the SUMO E3 ligase function of RanBP2/Nup358. A. BRL cells were treated with 20 nM LMB for 16 hrs. Cells were lysed and Western blot was performed using anti-RanGAP1 and Tubulin antibodies. B. HeLa cells were transfected with control or RanBP2 specific siRNA oligonucleotides for 72 hrs. The cells were then treated with 20 nM LMB for 8 hrs, followed by Western blot with anti-RanBP2, RanGAP1 and tubulin antibodies.

The SUMO E1 enzyme dimer (SAE1/SAE2), the SUMO E2 enzyme (Ubc9) and the SUMO E3 ligases are all principally present in the nucleus (Azuma, Tan et al. 2001, Rodriguez, Dargemont et al. 2001, Zhang, Saitoh et al. 2002, Hay 2005, Yeh 2009, Gareau and Lima 2010, Sekhri, Tao et al. 2015), suggesting that SUMOylation might mainly occur in the nucleus. Previous experiments in this study, suggested that RanGAP1 likely shuttles between the nucleus and the cytoplasm in mammalian cells. Despite being discovered as the first SUMO substrate (Matunis, Coutavas et al. 1996), it was still unclear where in the cell RanGAP1 gets modified in vertebrate cells. Therefore, we wanted to

determine whether the LMB-induced nuclear accumulation of RanGAP1 causes an increase in its SUMOylation. As observed by my co-authors, an 8-hour LMB treatment only resulted in a modest increase in levels of SUMOylation on endogenous RanGAP1 (Cha, Sen et al. 2015). Based on my immunofluorescence microscopy results revealing a complete redistribution of endogenous RanGAP1 from the cytoplasm and the NPC to the nucleoplasm in cells treated with LMB for 16 hours, we asked whether this same treatment will result in a more obvious and robust increase in levels of RanGAP1 SUMOylation. Consistent with our prediction, we found that levels of RanGAP1 SUMOylation drastically increase after 16-hour LMB treatment, which is accompanied with a nearly disappearance of unmodified RanGAP1 when compared to control cells (Figure 3.4 A). Evidently, the nuclear distribution of RanGAP1 leads to a remarkable enhancement in its SUMOylation.

RanBP2 is present in a highly stable complex with SUMO1-modified RanGAP1 and Ubc9 at the NPC, which blocks the deSUMOylation mediated by the SUMO isopeptidases (Zhang, Saitoh et al. 2002, Zhu, Goeres et al. 2009, Werner, Flotho et al. 2012, Ritterhoff, Das et al. 2016). Consistent with this model, RNAi mediated knockdown or conditional knockout in mouse embryonic fibroblasts (MEFs) of endogenous RanBP2, causes a steady decline in SUMO1 modified RanGAP1 with a correlated increase in its unmodified form (Hutten, Flotho et al. 2008, Hamada, Haeger et al. 2011). When tested in vitro using the internal repeat (IR) region that contains its SUMO E3 ligase domain, RanBP2 can stimulate SUMOylation of several target proteins such as Sp100, but RanGAP1 (Kirsh, Seeler et al. 2002, Pichler, Gast et al. 2002, Zhu, Goeres et al. 2009). One possibility is that only the full-length RanBP2 but not its IR fragment might function

as the SUMO E3 ligase for RanGAP1. Therefore, it was unclear whether the increase in levels of RanGAP1 SUMOylation in cells treated with LMB is dependent on the E3 ligase activity of RanBP2 *in vivo*. To address this question, HeLa cells were transfected with either control siRNAs or siRNAs specific for RanBP2 for 72 hours followed by 8-hour treatment with LMB. Consistent with previously published results (Zhu, Goeres et al. 2009, Hamada, Haeger et al. 2011), RNAi-knock down of RanBP2 caused a marked increase in levels of unmodified RanGAP1 compared to control RNAi. Importantly, an 8-hour LMB treatment resulted in a complete disappearance of the unmodified RanGAP1 in cells transfected with either control or RanBP2-specific siRNAs (Figure 3.4 B). Hence, our results demonstrated that RanGAP1 SUMOylation is greatly enhanced by its accumulation in the nucleus and independent of the E3 ligase activity of RanBP2.

RanGAP1 contains a functionally active NLS at its C-terminal region

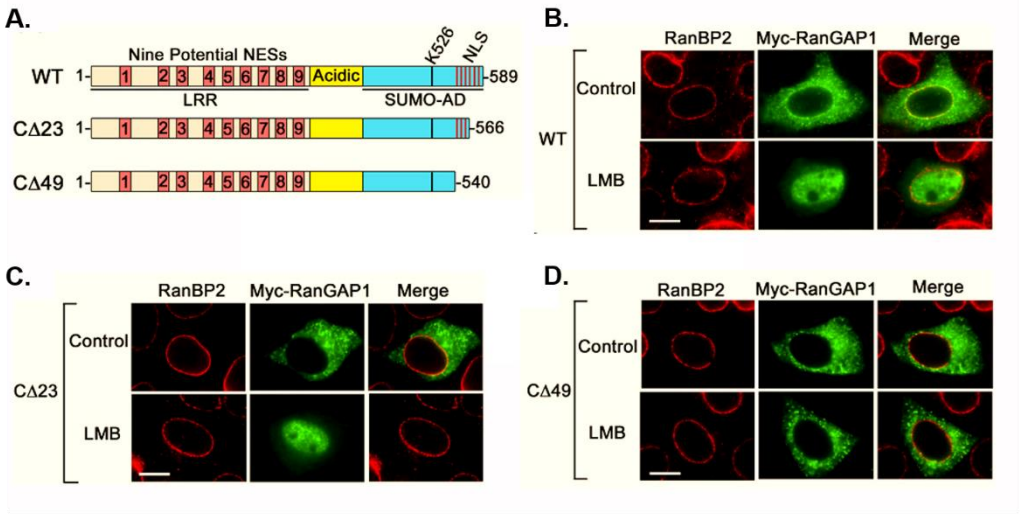


Figure 3.5 : The C-terminal region of mouse RanGAP1 has a functional NLS. A. The diagram is a schematic representation of the WT (1-589), CΔ23 deletion (1-566) and the CΔ49 (1-541) deletion constructs of RanGAP1. K526 is the SUMOylation site, present in all the constructs, the CΔ23 and CΔ49 constructs lack the putative NLS at the C-terminal region. B-D. HeLa cells were transfected with Myc-tagged RanGAP1 constructs (WT, CΔ23 and CΔ49) for 24 hrs, followed by LMB treatment (20 nM) for 8 hrs, and the cells were subjected to immunofluorescence analysis using anti-Myc and RanBP2 antibodies. The 26 amino acid stretch from 541-566 contains a functional NLS, as evident from the anti-Myc immuno-staining result.

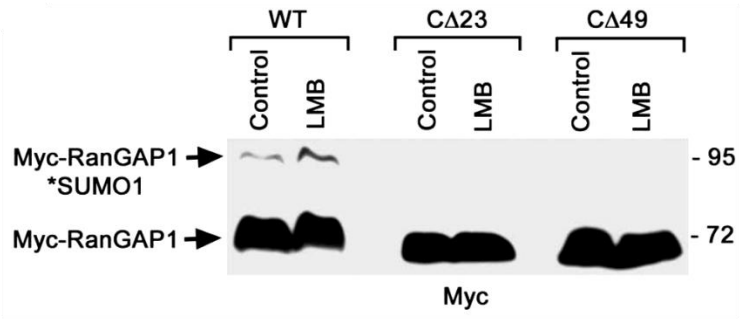
In mammalian cells, RanGAP1 is present as a homodimer of 150 kDa, a size at which it is impossible for RanGAP1 to diffuse into the nucleus passively. It is highly probable that RanGAP1 is transported into the nucleus by importins, and that RanGAP1 may have a functional NLS recognized by the importins. It has been shown previously that the C-terminal fragment of RanGAP1 (541-589 amino acids) may function as an NLS when the fusion of this fragment with the cytoplasmic pyruvate kinase (PK) protein, leading to the nuclear localization of the fusion protein (Matunis, Wu et al. 1998). Besides, the 541–589 sequence of the mouse RanGAP1 has about 69% homology with those of the *Xenopus* and the human RanGAP1, suggesting that this C-terminal region is relatively conserved amongst the vertebrates. However, it is still unclear whether this C-terminal NLS is functional or critical for mediating the nuclear import of RanGAP1.

To address this question, we transfected HeLa cells with the constructs encoding Myc-tagged RanGAP1 full length, CΔ23 and CΔ49, the latter two constructs lacking the C-terminal 23 and 49 amino acid residues, respectively (Figure 3.5 A). The cells were then treated with 20 nM LMB or control solution for 8 hours, followed by immunofluorescence staining with anti-Myc and anti-RanBP2 antibodies (Figure 3.5 B, C and D). In the control cells, the full-length RanGAP1 localized in the cytosol and at the NPC, whereas its CΔ23 and CΔ49 mutants were exclusively cytosolic. LMB treatment caused a nucleoplasmic distribution of both the full-length RanGAP1 and its CΔ23 mutant (Figure 3.5 B and C), but did not alter the cytosolic localization of its CΔ49 mutant (Figure 3.5 D). This result strongly suggested that the 26-residue region (541-566) of RanGAP1 near its C-terminal region constitutes a functional NLS required for RanGAP1 nuclear import.

To compare SUMOylation of the Myc-RanGAP1 full-length and its mutants in the presence and absence of LMB treatment, we performed immunoblot analysis with anti-Myc antibody (Figure 3.6 A). As anticipated, the full-length RanGAP1 showed an enhancement in its SUMO1 modification following LMB treatment. On the contrary, both the CΔ23 and CΔ49 RanGAP1 failed to get SUMOylated in both control and LMB-treated cells (Figure 3.6 A and B). This result is consistent with a previous finding that the CΔ23 RanGAP1 does not get SUMOylated (using *in vitro* SUMOylation assays), which might be caused by the inability of this mutant to interact with the sole E2 enzyme Ubc9 (Matunis, Wu et al. 1998, Sampson, Wang et al. 2001).

Discussion

A.



B.

	SUMO Modificaton	*LMB (-) Localization*	*LMB (+) Localization*
WT	+	C / NPC	N
CΔ23	-	C / -	N
CΔ49	-	C / -	C

Figure 3.6 : The putative NLS at the C-terminal region of RanGAP1 is required for its nuclear accumulation and SUMOylation. A. HeLa cells were overexpressed with Myc-tagged WT, CD23 and CD49 mouse RanGAP1 for 24 hrs. They were treated with 20 nM LMB for 8 hrs, immunoblot was done using anti-Myc antibodies. B. Summary table representing the subcellular localization and SUMOylation status of the WT, CD23 and CD49 RanGAP1 proteins, in response to LMB treatment. C- cytoplasm; N- nucleoplasm; NPC- nuclear pore complex.

Using both RNAi-mediated CRM1 knockdown and LMB-dependent inhibition of CRM1 function, we determined that CRM1 is the nuclear exporter for RanGAP1 and required for a predominant distribution of RanGAP1 in the cytoplasm and at the nuclear envelope. Also, the LMB treatment leads to a significant increase in RanGAP1 SUMOylation in mammalian cells, which is an interesting finding and consistent with the model that the major components of the SUMOylation machinery are predominantly present in the nucleus. Moreover, we demonstrated that this enhancement in RanGAP1 SUMOylation by LMB treatment is independent of the SUMO E3 ligase activity of RanBP2, suggesting that RanBP2 is not the E3 ligase for RanGAP1 SUMOylation. The result supported the idea that by forming a stable complex with Ubc9 and RanBP2 at the NPC, SUMO1 modified RanGAP1, is protected from de-SUMOylation by the isopeptidases (Zhu, Goeres et al. 2009). Furthermore, we also elucidated that the 26-residue region (541-566) of RanGAP1 is essential for nuclear localization of the protein and represents a functional NLS. Our results support a model that the NPC-associated SUMO1-modified RanGAP1 is highly stable and gets de-SUMOylated at a very low rate to join the pool of unmodified RanGAP1 in the cytoplasm. This population of the unmodified RanGAP1 is imported into the nucleus by the specific importin, modified by SUMO, and then exported by CRM1. The SUMO1-modified RanGAP1 associates with Ubc9 and RanBP2 to form a highly stable complex at the cytoplasmic filaments of the NPC.

Unlike the Myc-tagged RanGAP1 with a complete redistribution from the NPC to the nucleus in response to the LMB treatment for 8 hours, the same treatment only leads to a partial loss of endogenous RanGAP1 at the NPC. This result prompted us to propose

that the Myc-tagged RanGAP1 is less stably associated at the NPC with Ubc9 and RanBP2 when compared to the endogenous RanGAP1. A prolonged treatment with LMB for 16 hours resulted in a complete disappearance of the NPC-localized RanGAP1, which indicates that the endogenous RanGAP1 has a much higher stability at the NPC in comparison to the Myc-tagged RanGAP1. Another explanation could be only a small fraction of the Myc-tagged RanGAP1 is SUMOylated; on the contrary, a much higher proportion of the endogenous RanGAP1 is SUMOylated and localized at NPC in BRL cells (Matunis, Wu et al. 1998, Zhu, Goeres et al. 2009). The 16-hour LMB treatment of BRL cells resulted not only in a uniform nucleoplasmic distribution of RanGAP1 but also a great increase in its SUMOylation level, almost 100% SUMO1 modified RanGAP1 in LMB treated cells in comparison to 50% SUMOylated RanGAP1 in control cells.

Previous studies have speculated the role of RanBP2 as the SUMO E3 ligase for RanGAP1. This study revealed that RanBP2, even though acts a SUMO E3 ligase for targets such as Sp100 and HDAC4 (Kirsh, Seeler et al. 2002, Pichler, Gast et al. 2002), is not the E3 ligase for RanGAP1; HeLa cells treated with LMB still showed significantly high levels of SUMO1-modified RanGAP1 in the absence of endogenous RanBP2.

The C-terminal amino acid stretch from 541-589 has been observed to redistribute the cytoplasmic pyruvate kinase into the nucleus (Matunis, Wu et al. 1998), However, it unknown how the RanGAP1 shuttling across the nuclear membrane is regulated. The NLS sequence at the C-terminal region of mammalian RanGAP1, which we found to be functional, seems to be conserved in various organisms ranging from yeast to human. Therefore, our studies of mammalian cells along with previous analysis of yeast cells

strongly suggested that the nucleocytoplasmic shuttling of RanGAP1 may represent a highly-conserved process in eukaryotes.

In cancer cells, rapid cell proliferation requires a higher rate of nuclear transport, in comparison to normal cells; (Kau, Way et al. 2004). In fact, the most essential protein factors for nuclear transport, including RanGAP1, RanGTP, CRM1 and RanBP1, exhibit an increased expression in metastatic melanoma compared to primary melanoma (Pathria, Wagner et al. 2012). Also, RanGAP1 has been reported to be a known target in diffuse large B-cell lymphoma (Chang, Chang et al. 2013). It seems to be highly possible that an efficient nuclear transport, which requires the predominant localization of RanGAP1 in the cytosol and at the NPC, is critical for maintaining an elevated cell proliferation in tumor cells. For therapeutic purpose, a disruption of the normal distribution of RanGAP1 in the cytosol and at the NPC by inhibiting its exportin CRM1 might be helpful to treat various types of human cancer.

CHAPTER 4: SUMMARY AND CONCLUSIONS

MIF is a known mitogenic cytokine. The expression and stability of MIF increase during tumor progression and metastasis. Elevated levels of MIF protein during cancer progression is associated with poor prognosis. In normal cells, immunostaining analyses revealed a predominant cytoplasmic staining of MIF. It is very evident that MIF actively shuttles between the two cellular compartments; although, being a small protein of 12.5kDa, MIF can diffuse from one compartment to another. However, earlier studies, regarding the MIF structure, have determined that in cells MIF can be found as a monomer, homodimer or homotrimer (Sun, Bernhagen et al. 1996, Pantouris, Syed et al. 2015). There has been speculation regarding the activity of the three forms of MIF.

Several important studies suggest that a highly active nuclear transport is key to uncontrolled cell proliferation, the first critical step of tumorigenesis (Kau, Way et al. 2004). Nuclear transport is an important avenue of research, depending on the growing number of studies that demonstrate an increased level of expression of the principal nuclear exportin CRM1, RanGAP1, RanGTP as well as the nuclear importin karyopherin β1 during cancer progression towards metastasis (van der Watt, Maske et al. 2009, Pathria, Wagner et al. 2012, Chang, Chang et al. 2013). In chapter 3, I described that RanGAP1, the sole Ran GTPase activating protein, shuttles between the nucleus and the cytoplasm, and CRM1 mediates its nuclear export. Recent studies have also found RanGAP1 as a potential therapeutic target for diffuse large B-cell lymphoma (Chang, Chang et al. 2013).

Given all these facts, it would be interesting to determine how the MIF protein is transported in and out of the nucleus using these proteins, mentioned above. Studies of

pancreatic carcinoma, cervical cancer, ovarian cancer and glioma showed that these cancers are associated with upregulation of CRM1 (Noske, Weichert et al. 2008, Huang, Yue et al. 2009, Shen, Wang et al. 2009). As CRM1 is responsible for exporting over 80% of cargos, it would be interesting and also important to determine if CRM1 mediates the nuclear export of MIF.

APPENDIX A1: ANTIBODIES USED IN THE CHAPTER 2

Antibody	Species	Source	Dilution
MIF (FL-115)	Rabbit	SantaCruz Biotechnology	1:1000
FLAG (M2)	Mouse	Sigma	1:800
Tubulin (DM1A)	Mouse	Sigma	1:20000
GFP (GF28R)	Mouse	UBPBio	1:1000
Hsp90 a/b (F-8)	Mouse	SantaCruz Biotechnology	1:800
GST (B-14)	Mouse	SantaCruz Biotechnology	1:150
SUMO1 (21C7)	Mouse	Zhang laboratory, Life Technologies	1:1000
SUMO2/3 (8A2)	Mouse	Zhang laboratory, Abcam	1:800
PIAS3 (H-169)	Rabbit	SantaCruz Biotechnology	1:200
HA	Mouse	Todi Laboratory (Dr. Sokol Todi)	1:200

APPENDIX A2: SITE-DIRECTED MUTAGENESIS PRIMERS OF MIF IN THE CHAPTER 2

Name	Sequence
MIF-K33R-F	5’- CAG GCC ACC GGC AGG CCC CCC CAG TAC -3’
MIF-K33R-R	5’- GTA CTG GGG GGG CCT GCC GGT GGC CTG -3’
MIF-K67R-F	5’- CAC AGC ATC GGC AGG ATC GGC GGC GCG -3’
MIF-K67R-F	5’- CGC GCC GCC GAT CCT GCC GAT GCT GTG -3’
MIF-K78R-F	5’- CGC TCC TAC AGC AGG CTG CTG TGC GGC -3’
MIF-K78R-R	5’- GCC GCA CAG CAG CCT GCT GTA GGA GCG -3’

APPENDIX B: ANTIBODIES USED IN THE CHAPTER 3

Antibody	Species	Source	Dilution
RanGAP1 (19C7)	Mouse	Dr. Michael Matunis	1:1000
RanBP2 (ab64276)	Rabbit	Abcam	1:2000
Tubulin (DM1A)	Mouse	Sigma	1:20000
Myc (9E10)	Mouse	SantaCruz Biotechnology	1:1000
SUMO1 (21C7)	Mouse	Zhang laboratory, Life Technologies	1:1000

APPENDIX C1: REAGENTS FOR BACTERIAL PASMID DNA PURIFICATION

Name	Conc./Percentage/Ingredients
SolutionI	50mM glucose, 25mM Tric-Hcl (pH 8.0), 10mM EDTA (pH 8.0)
Solution II	0.2N sodium hydroxide, 1% (w/v) SDS
Solution III	Potassium acetate, glacial acetic acid (3M for potassium, 5M for acetate)
Isopropanol	50%
Ammonium acetate	10mM
PEG8000	15% in 1.6M sodium chloride
TE	10mM Tris-Hcl (pH 8.0), 1mM EDTA (pH 8.0)
Ethanol	70%
RNase A	20μg/ml

APPENDIX C2: REAGENTS AND BUFFERS FOR BACTERIAL PROTEIN EXPRESSION AND PURIFICATION

Name	Ingredients/percentage/conc.
Lysis buffer	1X PBS, 0.1% (w/v) lysozyme, 0.1% Benzonase, 1% Triton X-100, supplemented with LAP (leupeptin, pepstatin, antipain) and PMSF (phenylmethyl sulfonyl fluoride)
Wash buffer	1X PBS, 0.1% lysozyme, 0.1% Benzonase, 1% Triton X-100
1X Phosphate buffer saline	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4
Glutathione sepharose beads 4B	100ul slurry/50ml culture
Glutathione elution buffer	50mM Tris-Hcl (pH 8.0), 10mM glutathione
IPTG (isopropyl-B-D-1-thiogalactopyranoside)	0.1mM/1.0mM
BSA (protein control)	Fixed concentrations (0.5μg/1.0μg/2.0μg/4.0μg/5.0μg)

APPENDIX C3: BUFFERS AND SOLUTIONS FOR THE *IN VITRO* SUMOylation ASSAY

Name	Ingredients/Percentage/Conc.
Transport buffer	20mM HEPES pH 7.3, 110mM potassium acetate, 2mM magnesium acetate, 1mM EGTA, 4mM DTT
SDS sample buffer	125mM Tris-Hcl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, bromophenol blue

APPENDIX C4: BUFFERS AND SOLUTIONS FOR *IN VITRO* PROTEIN BINDING ASSAY

Name	Ingredients/Percentage/conc.
Lysis buffer	50mM Tris-Hcl (pH 7.5), 150mM sodium chloride, 1% Triton X-100, 10% glycerol, 2mM EDTA, 25mM sodium fluoride, protease inhibitors (LAP, aprotinin, PMSF)
Wash buffer	50mM Tris-Hcl (pH 7.5), 300mM sodium chloride, 1% Triton X-100, 10% glycerol, 2mM EDTA, 25mM sodium fluoride

APPENDIX C5: BUFFERS AND SOLUTIONS FOR WESTERN BLOT ANALYSIS

Name	Ingredients/Stock Percentage/ Stock Conc.
SDS PAGE tank buffer	Tris base, Glycine, SDS (4X stock)
Transfer buffer	Tris base, glycine, methanol, SDS (10X stock)
SDS PAGE resolving gel	Acrylamide-bis acrylamide solution, 1M Tris-Hcl (pH 9.1), 10% SDS, 3% ammonium persulfate, TEMED
SDS PAGE stacking gel (4%)	Acrylamide-bis acrylamide solution (30%-0.44%), 0.5M Tris-Hcl (pH 6.8), 10% SDS, 3% ammonium persulfate, TEMED
TS wash buffer	Tris base, sodium chloride, 12.1N hydrogen chloride (20X)
TS Tween-20 wash buffer	Tris base, sodium chloride, 12.1N hydrogen chloride (20X) + 0.02% Tween-20 (added fresh)
Primary antibody solution	2% bovine serum albumin in 1X PBS + 0.02% sodium azide
Secondary antibody solution	5% milk in 1X TS wash buffer

APPENDIX C6: REAGENTS FOR IMMUNOFLUORESCENCE ANALYSIS

Name	Ingredients/percentage/conc.
Paraformaldehyde	3.5% in 1X PBS
Triton X-100	0.2% in 1X PBS
Primary antibody solution	1X PBS with 1% goat normal serum + 0.1% Triton X-100
Secondary antibody solution	1X PBS with 1% goat normal serum + 0.1% Triton X-100
Mounting solution	DAPI in solution containing 50mM Tris-Hcl (pH 8.0), 0.1% *p*-phenylenediamine, 80% glycerol

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ABSTRACT

STUDIES OF SUMOYLATION IN REGULATING MIF STABILITY AND RANGAP1 NUCLEO-COTYPLASMIC SHUTTLING IN CONTROLLING ITS SUMO MODIFICATION

by

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SUMOylation is an essential post-translational modification that regulates a variety of critical cellular pathways ranging from nuclear transport to protein stability. Accumulating lines of evidence have shown that a perturbation of the SUMOylation pathway is associated with human diseases, especially various types of cancer. Our recent proteomic studies revealed a drastic increase in levels of SUMO2/3 modification on the proinflammatory cytokine MIF in the metastatic breast cancer cell line compared to the non-metastatic control cell line. Interestingly, the increase in levels of both MIF and global SUMO-2/3 modification in the metastatic cells are positively correlated to that of unmodified MIF proteins when compared to the non-metastatic control cells. Furthermore, global inhibition of SUMOylation by overexpression of the SUMO-specific isopeptidase SENP2 greatly decreases levels of MIF proteins. In addition, we found that endogenous MIF has a half-life of about three hours and is degraded through the proteasomal pathway. Moreover, global inhibition of SUMOylation by SENP2 overexpression significantly reduce the stability of MIF proteins. Furthermore, the lysine 78 of MIF is required for its SUMOylation in vitro. Importantly, we showed that MIF has a specific

interaction with the SUMO E3 ligase PIAS3, which has been previously known to be upregulated in various types of cancer. In addition, my graduate studies had also focused on elucidating how SUMOylation of the Ran GTPase activating protein RanGAP1 is regulated. As a key regulator of nuclear transport, RanGAP1 along with other important players in this process are often upregulated in various types of cancer. We demonstrated that RanGAP1 shuttles between the nucleus and the cytoplasm in mammalian cells, and that the exportin CRM1 mediates its nuclear export. Additionally, the NPC-associated SUMO1-modified RanGAP1 is stably associated with the cytoplasmic filaments of the nuclear pore complex (NPC) and requires longer hours to redistribute into the nucleoplasm in cells with inhibition of the CRM1-mediated export when compared to the cytoplasmic unmodified RanGAP1. The C-terminal 541-589 amino acid region of RanGAP1 is crucial for its nuclear import and the 26-residue region (541-566) within this C-terminus contains a functional nuclear localization signal. We also demonstrated that SUMOylation of RanGAP1 is independent of SUMO E3 ligase RanBP2. Our studies have focused on SUMOylation of these two important proteins MIF and RanGAP1, both of which are intricately associated with tumorigenesis and metastasis. As the relative distribution of MIF between the nucleus and the cytoplasm is an important prognostic determinant in cancer progression, it would be interesting to investigate the role of RanGAP1 in the nucleocytoplasmic transport of MIF proteins. The further studies of these two proteins and their SUMOylation in the future may provide the important information that may lead to novel therapeutic treatment of human cancers.

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PUBLICATION

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2. *Cha, K., ***Sen, P**., Raghunayakula, S., and Zhang, X.D. (2015). "The Cellular Distribution of RanGAP1 Is Regulated by CRM1-Mediated Nuclear Export in Mammalian Cells." PLoS One **10**(10): e0141309.
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