Analyzing Sumo-2/3 Modification In Regulation Of Breast Cancer Progression And Mitotic Chromosome Segregation

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This PhD thesis is not just the product of my work in the laboratory and hours at the keyboard, but it is also a representation of my complete experience at Wayne State University for the last six years. Without the support of each and every person in one way or the other this research thesis would not have been possible. The lessons I learnt from my research work are presented here as a thesis and the lessons learnt in the form of experience will be with me throughout my life. Hence I take this PhD thesis as an opportunity to thank each and every soul that has made my PhD a fruitful and delightful experience.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... ii

LIST OF FIGURES ........................................................................................................................ vi

LIST OF TABLES ........................................................................................................................... viii

CHAPTER 1 INTRODUCTION ........................................................................................................ 1
  1.1 The SUMO pathway .............................................................................................................. 2
  1.2 History of SUMO ................................................................................................................ 6
  1.3 The known functions of SUMO modification ..................................................................... 7
  1.4 Role of SUMO conjugation in regulation of biological processes ................................. 9
  1.5 The association of the SUMOylation pathway with human diseases .......................... 24
  1.6 Project outline .................................................................................................................. 30

CHAPTER 2 ANALYSIS OF SUMO-2/3 MODIFICATION DURING BREAST CANCER PROGRESSION AND METASTASIS ............................................................... 33
  2.1 Abstract ............................................................................................................................ 34
  2.2 Introduction ...................................................................................................................... 34
  2.3 Materials and Methods .................................................................................................... 38
  2.4 Results ............................................................................................................................. 44
  2.5 Discussion ........................................................................................................................ 65

CHAPTER 3 POLYMERIC SUMO-2/3 CHAINS AT KINETOCHORES IS ESSENTIAL FOR CHROMOSOME ALIGNMENT BY RECRUITING CENP-E TO KINETOCHORES ........................................................................ 72
  3.1 Abstract ............................................................................................................................ 73
  3.2 Introduction ...................................................................................................................... 73
  3.3 Materials and Methods .................................................................................................... 76
  3.4 Results ............................................................................................................................. 80
  3.5 Discussion ........................................................................................................................ 94

CHAPTER 4 FUTURE DIRECTIONS .......................................................................................... 98
4.1 Which PIAS protein stimulates the SUMO-2 modification of Nuf2? ..................98

4.2 Do multiple SIM motifs at CENP-E tail domain mediate the interaction of CENP-E with polymeric SUMO-2/3 modified Nuf2? ........................................101

4.3 Will longer chains of SUMO-2 fused to Nuf2 boost the rescue of mitotic defects caused by SENP2 overexpression? ......................................................105

APPENDIX A .........................................................................................................................109

APPENDIX B .......................................................................................................................112

REFERENCES ......................................................................................................................114

ABSTRACT ..........................................................................................................................126

AUTOBIOGRAPHICAL STATEMENT ..................................................................................128
LIST OF FIGURES

Figure 1.1. The SUMO Pathway.................................................................................................................. 3
Figure 1.2. Functions of SUMO-modified targets ..................................................................................... 9
Figure 1.3. SUMO modification plays a role in transcription repression ............................................... 11
Figure 1.4. Both SUMO signals and known SUMO substrates are associated with the mitotic centromere and kinetochore of a condensed chromosome ......................................................... 15
Figure 1.5. Dysregulation of SUMOylation in tumorigenesis ................................................................. 27
Figure 2.1. Dysregulation of SUMOylation during breast cancer progression and metastasis .............................................................................................................................................................................. 45
Figure 2.2. The subcellular distributions of SUMO-conjugates among four mouse breast cancer cell lines derived from the same primary tumor .............................................................................. 47
Figure 2.3. A method for purification and identification of the proteins that are differentially modified by endogenous SUMO-2/3 between metastatic and non-metastatic breast cancer cells ..................................................................................... 48
Figure 2.4. Mapping of the epitope peptide sequences of mouse mAbs specific to SUMO-1 (21C7) and SUMO-2/3 (8A2) .......................................................................................................................................................................................... 49
Figure 2.5. Identification of the SUMO-1 and SUMO-2 epitope peptide sequences recognized by anti-SUMO-1 (21C7) and anti-SUMO-2/3 (8A2) mAbs .................................................................................... 50
Figure 2.6. The alignment of SUMO-epitope-peptide sequences among SUMO sequences of difference species and the application of SUMO-2/3-ePIPE for purification of endogenous SUMO-conjugates in Drosophila S2 cells .................................................................................................................................................................................. 52
Figure 2.7. Affinity purification of endogenous SUMO-2/3-conjugates in human glioblastoma (GBM) tumor tissues using SUMO-2/3-ePIPE .................................................................................................................. 53
Figure 2.8. SUMO-2/3 epitope peptide elution ............................................................................................ 54
Figure 2.9. Functional classification and interaction network analysis of the SUMO-2/3-conjugated protein identified in metastatic and non-metastatic breast cancer cells .................................... 56
Figure 2.10. Validation of SUMO substrates by in vitro SUMOylation assays .......................................... 59
Figure 2.11. Verifying the changes of SUMO-2/3 modification of individual substrates in metastatic cells and investigating the effects of SUMO-23 modification on PML-NB assembly and 3-D cell migration .............................................................................................................................. 62
Figure 2.12. Overexpression of GFP-tagged SUMO-2/3 wild-type or non-conjugatable mutants increases 3D cell migration .................................................................................................................. 64
Figure 2.13. A model elucidates that dysregulation of SUMOylation might contribute to breast cancer progression and metastasis.................................................................65

Figure 3.1. Nuf2 is required for CENP-E localization to kinetochores ........................................81

Figure 3.2. SUMO modification at kinetochores is required for chromosome segregation by recruiting CENP-E to kinetochores .................................................................83

Figure 3.3. Nuf2 is modified by polymeric SUMO-2/3 chains .....................................................86

Figure 3.4. Polymeric SUMO-2 chain fused to Nuf2 is critical for CENP-E localization to kinetochores and chromosome alignment to metaphase plates ........................................88

Figure 3.5. A minimum of trimeric SUMO-2 chain fused to Nuf2 is required for targeting CENP-E to kinetochores in cells overexpressing SENP2 ............................................90

Figure 3.6. Nuf2-3xSUMO-2 can localize more CENP-E compared to Nuf2 in cells depleted with Ubc9 .................................................................................................................91

Figure 3.7. Nuf2-3xSUMO-2 has higher binding affinity to CENP-E than Nuf2 ................................93

Figure 3.8. Nuf2 is SUMO-2 modified at several lysine residues ................................................94

Figure 4.1. What is the E3 ligase that stimulates Nuf2 SUMOylation? ......................................100

Figure 4.2. Diagram showing the putative SIM motifs in the tail domain of CENP-E using the software GPS-SUMO .........................................................................................102

Figure 4.3. SIM 2 and SIM 4 motifs of CENP-E are not required for its kinetochore localization .............................................................................................................................103

Figure 4.4. Diagram showing interaction between a multi-SIM protein and a substrate ..........104

Figure 4.5. Cartoon showing the two fusions constructs: GST-Nuf2-3xSUMO-2 and GST-Nuf2-COMP-SUMO-2 ..........................................................105

Figure 4.6. Diagram showing the interaction between a poly SUMO-modified protein and its interacting partner ..........................................................106

Figure 4.7. Construction of FLAG-Nuf2-4xSUMO-2 and FLAG-Nuf2-5xSUMO-2 vectors .......................106
LIST OF TABLES

Table 1.1. The known SUMO targets at centromeres and kinetochores in vertebrates..................17
Table 2.1. Endogenous SUMO-2/3-Conjugated Proteins Identified with SILAC Heavy / Light (H/L) Ratios by Quantitative Mass Spectrometry ...............................................................5
CHAPTER 1 INTRODUCTION

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1.1 THE SUMO PATHWAY

The small ubiquitin-related modifiers (SUMOs) are small proteins that are reversibly conjugated to hundreds of different proteins and therefore regulate many cellular processes including cell cycle, nucleocytoplasmic transport, DNA repair, gene expression, and protein stability. Currently, the fundamental molecular mechanisms for SUMO conjugation and deconjugation have been relatively well established. At the same time, not only many SUMO substrates have been identified but also the effects of SUMOylation on these substrates have been elucidated in multiple model systems and organisms, including budding yeast, fission yeast, mammalian culture cells, *Xenopus* and *Arabidopsis*.

1.1.1 SUMO as a post-translational modifier:

Although a single SUMO protein is expressed in yeast and invertebrates, vertebrates express three SUMO proteins: SUMO-1, SUMO-2 and SUMO-3 (Geiss-Friedlander and Melchior, 2007). In human, SUMO-2 and SUMO-3 are ~96% homologous to other and are thus denoted as SUMO-2/3, whereas the identity between SUMO-2/3 and SUMO-1 is less than 45%. All SUMOs are expressed as the SUMO precursor proteins that must be post-translationally processed to expose their C-terminal double-glycine (GG) motifs to generate the mature SUMOs for their conjugation to protein targets (Dasso, 2008; Geiss-Friedlander and Melchior, 2007; Johnson, 2004). This processing step is catalyzed by the SUMO isopeptidases, called ubiquitin like protein proteases (Ulps) in yeast (Li and Hochstrasser, 1999) and Sentrin-specific proteases (SENPs) in vertebrates (Gong et al., 2000). Like ubiquittination, SUMOylation requires a similar enzymatic cascade that involves three classes of enzymes. In the first step, which is ATP driven, the SUMO
E1 activating enzyme (Aos1-Uba2) activates the mature SUMO when a thioester bond is formed between the C-terminal glycine residue of SUMO and the cysteine (C) residue of the E1 enzyme (Figure 1.1) (Johnson et al., 1997). The second step involves the transfer of SUMO from E1 to the catalytic cysteine (C) residue of the SUMO E2 conjugating enzyme (Ubc9) (Figure 1.1) (Johnson and Blobel, 1997; Lee et al., 1998). The last step is the transfer of SUMO from Ubc9 to the target protein where an isopeptide bond is formed between the C-terminal glycine residue (G) of SUMO and a lysine (K) residue of the target (Figure 1.1). This is usually facilitated by an E3 ligase that promotes SUMO conjugation to specific proteins (Figure 1.1) (Johnson, 2004; Melchior et al., 2003). SUMO isopeptidases (SENPs) catalyze the reverse process of deconjugating SUMO from the substrate.

Figure 1.1. The SUMO pathway. The precursor SUMO is processed by SUMO isopeptidases to generate the mature SUMO exposing the terminal di-glycine motif. The mature SUMO is first activated and then conjugated to the substrate by means of three enzymes E1 activating enzyme, E2 conjugating enzyme and E3 ligases. This dynamic modification is reversible, where SENPs deconjugate SUMOs from the substrates.

1.1.2 SUMO consensus sequence:

Many SUMO substrates contain the SUMOylation consensus sequence Ψ-K-x-[E/D], where Ψ is a hydrophobic amino acid residue, K is the lysine residue for SUMO conjugation, x is any amino acid, and E/D represents either a glutamic acid (E) or an aspartic acid (D). This motif is recognized by Ubc9, unlike the E2 conjugating enzymes for ubiquitination (Bernier-Villamor et
Interestingly, recent studies have shown that some SUMOylation sites contain an inverted SUMOylation consensus sequence \([E/D]-x-K-\Psi\) (Matic et al., 2010). In many cases, the SUMOylation consensus sequence \(\Psi-K-x-[E/D]\) also contains an adjacent motif to enhance its SUMO conjugation. The extended SUMOylation consensus sequence motifs include the negatively charged amino acid-dependent SUMOylation motif (NDSM) with extra negatively charged amino acids in close proximity to its core SUMOylation consensus sequence, the hydrophobic cluster SUMOylation motif (HCSM) with a cluster of hydrophobic amino acids, and the phosphorylation-dependent SUMOylation motif (PDSM) with an adjacent serine (S) residue for phosphorylation (Blomster et al., 2009; Matic et al., 2010). On the other hand, many SUMO conjugations occur at the non-consensus sequences, whereas many non-SUMOylated proteins also contain the SUMOylation consensus sequences. Therefore, SUMOylation sites must be directly determined and confirmed by \textit{in vitro} and/or \textit{in vivo} experiments.

1.1.3 SUMO E3 ligases:

Although SUMO E1 enzyme and Ubc9 alone are sufficient for \textit{in vitro} SUMO modification of many known substrates, SUMO E3 ligases play an important regulatory role \textit{in vivo} by increasing the SUMOylation efficiency and also by determining the substrate specificity. Based upon their evolutionary conservation, the current known SUMO E3 ligases can be classified into two main groups. A conservative group of E3 ligases has been found in all eukaryotes and contains a RING-finger like domain called SP-RING domain, which is responsible for recruiting Ubc9 (Geiss-Friedlander and Melchior, 2007; Johnson, 2004; Yunus and Lima, 2009). The SP-RING E3 ligases include the PIAS (protein inhibitor of activated STAT) family proteins (PIAS1, PIAS3, PIAS\(x_\alpha\), PIAS\(x_\beta\) and PIASy) in vertebrates and the Siz family proteins (Siz1 and Siz2) in \textit{Saccharomyces cerevisiae} (Dasso, 2008; Johnson, 2004; Melchior et al., 2003). The Siz1 and Siz2
are required for most SUMO conjugation in budding yeast (Johnson and Gupta, 2001; Takahashi et al., 2001). In yeast and human, the SUMO E3 ligase Mms21 also contains a SP-RING domain and plays an essential role in DNA repair (Johnson and Gupta, 2001; Potts and Yu, 2005). Furthermore, the yeast SP-RING E3 ligase Zip3 regulates the assembly of synaptonemal complex during meiosis (Cheng et al., 2006a). On the other hand, the human PIAS-like E3 ligase hZimp10 is capable to stimulate the SUMOylation of androgen receptor (AR), leading to an increase of transcription activity of AR (Sharma et al., 2003). In contrast to the conservative group of SP-RING E3 ligases, the non-conservative group of E3 ligases is vertebrate-specific and has no obvious yeast homologues. These vertebrate-specific E3 ligases include the nucleoporin Nup358/RanBP2 (Pichler et al., 2002), the polycomb-group protein Pc2 (Kagey et al., 2003), the histone deacetylases including HDAC4 (Zhao et al., 2005) and HDAC7 (Gao et al., 2008), and the topoisomerase I-binding protein Topors (Pungaliya et al., 2007; Weger et al., 2005).

1.1.4 SUMO isopeptidases:

DeSUMOylation is essential to ensure the reversible nature of SUMO conjugation (Hay, 2007; Mukhopadhyay and Dasso, 2007). SUMO isopeptidases (Ulps/SENPs) are responsible for both processing the SUMO precursors and deconjugating the SUMOs from their protein targets (Figure 1.1) (Mukhopadhyay and Dasso, 2007). Budding yeast has two SUMO isopeptidases (Ulp1 and Ulp2/Smt4). While Ulp1 is associated with the nuclear pore complex (NPC) (Panse et al., 2003), Ulp2 has a distribution throughout the nucleus (Li and Hochstrasser, 2000). Ulp1 is responsible for processing the SUMO precursors and also essential for the cell cycle progression through the G2/M phase (Li and Hochstrasser, 1999). Although Ulp2 is not essential for vegetative growth, it is crucial for meiosis (Li and Hochstrasser, 2000). During mitosis, the Ulp2 is preferentially required for sister chromatid cohesion at centromere regions, and the defects in Ulp2
leads to the precocious loss of centromeric cohesion (Bachant et al., 2002). Interestingly, Ulp2 is also specifically required for disassembly of polymeric SUMO chains (Bylebyl et al., 2003). The distinct subcellular localizations of Ulp1 and Ulp2 are important to determine their substrate specificities (Li and Hochstrasser, 2003). On the other hand, there are six different isopeptidases (SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7) in human cells. The six SENPs share a common C-terminal catalytic domain but have distinct N-terminal domains, which are critical for their subcellular localizations and substrate specificities (Hay, 2007; Mukhopadhyay and Dasso, 2007). The sequence alignment of the human SENPs and the budding yeast Ulps suggests that SENP1, 2, 3 and 5 belong to the Ulp1-like branch, whereas SENP6 and 7 fall into the Ulp2-like branch (Mukhopadhyay and Dasso, 2007). SENP1 and SENP2 are most closely related to each other and catalyze both processing and deconjugation of SUMO-1 and SUMO-2/3 (Shen et al., 2006a). In addition, both SENP1 and SENP2 are associated with the nuclear pore complex (NPC) and have a distribution throughout the nucleus (Bailey and O’Hare, 2004; Hang and Dasso, 2002). Among the six SENPs, SENP3 and SENP5 are most closely related with each other and localize to the nucleolus with a preference for SUMO-2/3 processing and deconjugation (Yun et al., 2008). Similar to Ulp2, SENP6 and SENP7 localize throughout the nucleoplasm and have a strong preference for disassembly of SUMO-2/3 polymeric chains (Lima and Reverter, 2008). The different sub-cellular localization of SUMO isopeptidases may determine their accessibilities to discrete SUMO targets and therefore their substrate specificities.

1.2 HISTORY OF SUMO

About two decades ago, SUMO-1 modification was originally identified in mammalian cells while investigating nuclear transport. Ran-GTPase-activating protein RanGAP1 is the first discovered SUMO-1 substrate (Matunis et al., 1996). RanGAP1 hydrolysis Ran from its GTP
bound state to GDP bound state; whereas RCC1 which is mostly localized to the nucleus converts RanGDP to RanGTP. This results in a concentration gradient of RanGTP between the nucleus and cytosol which is important for nuclear-cytoplasmic transport. Unmodified RanGAP1 was found in the cytosol whereas the SUMO-1 modified form was found localized to the cytoplasmic fibers of the nuclear pore complex (Mahajan et al., 1998) where it forms a complex with Ran Binding Protein 2 (RanBP2) (Mahajan et al., 1997) and Ubc9. RanGAP1 is SUMO modified at a conserved lysine residue towards the C-terminus of the protein (Mahajan et al., 1998).

1.3 THE KNOWN FUNCTIONS OF SUMO MODIFICATION

1.3.1 Regulation of protein-protein interaction

SUMO modification on a protein can either enhance (Figure 1.2A) or disrupt its contact with an interacting partner. Here are a few examples where SUMO modification promotes protein-protein interaction (i) SUMO modified PCNA recruits the helicase Srs2 (Pfander et al., 2005), (ii) SUMO modification of Promyelocytic leukemia (PML) recruits several other proteins and is important for the formation of PML nuclear bodies (Shen et al., 2006b), (iii) SUMO-modified Elk2 (transcription factor) localizes HDAC2. This is possible because of the non-covalent contact between the SUMO on the modified protein and the SUMO Interacting Motif (SIM) on its interacting partner (Minty et al., 2000). SIMs generally have a hydrophobic stretch (V/I-X-V/I-V/I) which neighbors either a few acidic residues or phosphorylated serine/threonine residues. It has also been shown that non-covalent interactions occur between these negatively charged residues on SIM and the basic residues on SUMO (Lys39 in SUMO-1 and Lys 35 in SUMO-2). The SIMs form a β strand which interacts either in parallel or anti-parallel orientation to the β2 strand on SUMO (Hecker et al., 2006). Sometimes SIMs exhibit paralogue specific binding; one such example is DAXX. DAXX is a transcriptional co-repressor that binds to different SUMO-
modified transcription factors near the promoter region (Chang et al., 2011). The hydrophobic residues in the SIM in DAXX are flanked by serine residues which gets phosphorylated facilitating binding to SUMO-1 and not to SUMO-2/3 (Chang et al., 2011).

### 1.3.2 Affects protein stability

SUMO modification of proteins has been shown to either stimulate (Figure 1.2B) or antagonize ubiquitin-mediated degradation indirectly. Examples for the former are PML and CENP-I (Mukhopadhyay et al., 2010; Tatham et al., 2008). In the presence of Arsenic trioxide, PML gets polymeric SUMO-2/3 modified. RNF4 which is a SUMO targeted ubiquitin E3 ligase (STUbL) has four SIMs in close proximity that recognize the polymeric SUMO-2/3 chains on PML and gets recruited. This leads to poly ubiquitin moieties being added on PML thereby leading to its degradation via the 26S proteasome (Tatham et al., 2008). In certain other cases like CDK6 and IκBα, SUMOylation can act as an inhibitor of ubiquitination. IκBα, inhibitor of NF-κB, retains it to the cytosol. IκBα can get both ubiquitinated and SUMOylated at the same lysine residue. SUMO modification of IκBα prevents its ubiquitination leading to the stabilization of the protein. Stable IκBα retains NF-κB to the cytosol thereby inhibiting the transcription of its downstream genes (Desterro et al., 1998).

### 1.3.3 Modulates enzyme activity

In some cases, SUMO modification of an enzyme can regulate the activity of the enzyme (Figure 1.2C). Thymine DNA Glycosylase (TDG) is a DNA mismatch repair enzyme and a bona fide SUMO target. When a G-U or G-A mismatch occurs, TDG binds to this site and removes the mutated base. TDG has high affinity to this site and SUMO modification of TDG is required to bring about a conformation change, so that it can be released from the site. Once released, SENPs quickly remove SUMO from the enzyme, so that it can bind to another mismatched site (Hardeland...
et al., 2002). This is yet another example of SUMOylation being a highly dynamic process as the deconjugation takes place rapidly when compared to the entire reaction cycle.

Figure 1.2. Functions of SUMO-modified targets. SUMO modification of a substrate can modulate the protein in one or more ways. (A) SUMO-modification of a protein might increase the binding site for interaction. (B) Poly SUMO-2/3 modification recruits RNF4 (Ubiquitin E3 ligase) via its SIM leading to ubiquitination and degradation via the proteasome. (C) In certain other cases, SUMO modification regulate the enzyme activity.

1.4 ROLES OF SUMOYLATION IN REGULATION OF BIOLOGICAL PROCESSES

1.4.1 DNA replication and repair:

Each cell takes the responsibility of maintaining its own genome. When the genome integrity is altered, the cell has to quickly repair the damage which will otherwise affect gene expression leading to adverse effects. SUMOylation and other post translational modifications can reversibly change the localization, stability and other properties of DNA repair enzymes which regulate the DNA repair pathways. Some of the DNA repair proteins modified by SUMO are PCNA (Hoege et al., 2002), Rad52 (Sacher et al., 2006), Thymine DNA Glycosylase (TDG)
Proliferative Cell Nuclear Antigen (PCNA) is a homotrimeric clamp that surrounds DNA acting as a scaffold to localize proteins involved in DNA replication and repair. PCNA is modified by SUMO at two lysine residues (Lys 164 and to a lesser extent Lys 127) during S phase. Conjugation to Lys 164 results in the recruitment of Srs2 (helicase-like enzyme). This displaces Rad51, a recombinase, from the chromatin preventing undesirable recombination between sister chromatids during S phase (Pfander et al., 2005). SUMO modification of PCNA at Lys 127 prevents the binding of Eco1, a protein required for sister chromatid cohesion. The authors speculate that this modification is probably required to keep certain heavily transcribed regions free of cohesion (Moldovan et al., 2006).

Rad52 is a protein involved in the repair of DNA double strand breaks through homologous recombination by binding to single stranded DNA ends. During double strand breaks, Rad52 gets SUMO modified and this leads to protection from ubiquitin mediated proteasomal degradation probably by isolating Rad52 into DNA repair foci (Sacher et al., 2006).

### 1.4.2 Transcription:

Gene expression is initiated after transcription factors binds to the promoters which then recruits chromatin remodelers, transcription activators or repressors. Many of the SUMO targets are transcription factors. Although there are examples of SUMOylation leading to transcription activation, it is generally associated with transcription repression (Gill, 2004). SUMO modification of heat shock transcription factors HSF1 and HSF2 results in transcription activation. Conjugation of SUMO to these proteins results in enhanced DNA binding. It has been shown that SUMO deficient mutant of HSF1 leads to decreased transcription of its downstream genes.
al., 2001; Hong et al., 2001). There are also reports showing that SUMO modification of p53 and Nuclear Factor of Activated T-cells (NFAT) results in transcriptional activation (Gostissa et al., 1999; Rodriguez et al., 1999; Terui et al., 2004).

SUMO modification leading to transcription repression can be brought about by one or both of the following mechanisms. In both cases SUMO modification may only be required for a short period of time resulting in repressed chromatin state. Transcription factors that are conjugated to SUMO can recruit chromatin remodelers with repressive activity (Figure 1.3) (Hay, 2005). Protein300 (p300) and Elk1 are both transcription regulators that can be SUMO modified. p300 can be SUMO modified at two sites in tandem that recruits HDAC6 leading to transcription repression (Girdwood et al., 2003). Similarly, SUMO modified Elk1 recruits HDAC2, which is again a corepressor complex (Yang and Sharrocks, 2004). Histone H4 is another bona fide SUMO target that localizes HDAC1 and Heterochromatin Protein 1-γ (HP1-γ) (Shiio and Eisenman, 2003). In all these cases, SUMO modification of transcription factors localizes repressor complexes to the promoters leading to transcription repression of downstream genes.

![Figure 1.3. SUMO modification plays a role in transcription repression.](image-url)

**Figure 1.3. SUMO modification plays a role in transcription repression.** Many SUMO substrates are transcription factors. SUMOylation of a transcription factor can recruit a repressor protein leading to transcription repression of the downstream genes.

In certain other cases, SUMO modification of transcription factors can localize them to a repressive nuclear domain. Polycomb group (PcG) bodies are known for transcription repression and one of its component is SOP-2. SOP-2 can be SUMO modified, which is required for its
recruitment to the PcG nuclear bodies. It has been shown that SUMO modification of SOP-2 leads to transcriptional repression of Hox genes (Zhang et al., 2004). Various SUMO isopeptidases remove SUMO from these transcription factors to derepress transcription (Girdwood et al., 2003).

**1.4.3 Nuclear transport:**

One of the major cellular processes that SUMOylation plays a role is in the regulation of proteins in nucleo-cytoplasmic transport. In addition to SUMO-1 modification of RanGAP1 translocating it to the nuclear pore complex, SUMO modification affects the nuclear localization of several substrates including p53, PTEN, CtBP.

p53 is a transcription factor that can be both SUMOylated and ubiquitinated (Rodriguez et al., 1999). MDM2 is the ubiquitin E3 ligase responsible for the ubiquitination of p53 (Haupt et al., 1997). Depending on MDM2 levels, p53 can either be mono or polyubiquitinated. Monoubiquitination of p53 (i) results in exposing the nuclear export signal ensuing the cytoplasmic localization of p53 and (ii) promotes its SUMO modification by PIASy (Carter et al., 2007). In addition to this model, another theory proposes that SUMO-1 modification of p53 at NPC disrupts its interaction with CRM1 (nuclear export receptor) thereby releasing the cargo in the cytoplasm (Santiago et al., 2013). Polyubiquitination of p53 leads to its degradation via the 26S proteasome (Kubbutat et al., 1997).

PTEN which is a tumor suppressor has a well characterized role as a phosphatase in the PI3K-AKT pathway in the cytosol (Cantley and Neel, 1999). In addition, PTEN also has important functions in the nucleus including apoptosis and chromosome stability (Planchon et al., 2008). SUMO modification of PTEN has been shown to play an important part in the nucleo-cytoplasmic shuttling. PTEN gets SUMO modified at two lysine residues K254 and K266. SUMOylation at
residue 266 results in the retention of PTEN near the plasma membrane, however K254 SUMOylation retains PTEN in the nucleus (Huang et al., 2012).

C-terminal Binding Protein (CtBP) functions as a transcriptional corepressor and has been identified as a SUMO target. SUMO modification of CtBP grips it in the nucleus and the SUMO deficient mutant of CtBP has cytoplasmic localization (Lin et al., 2003).

1.4.4 Cell cycle progression through mitosis:

1.4.4.1 SUMO signals at centromere and kinetochores:

The subcellular localization of SUMO at mitotic centromeres and kinetochores has been extensively analyzed by fluorescence microscopy in different organisms including human, *Xenopus* and *Drosophila* (Azuma et al., 2005; Nie et al., 2009; Zhang et al., 2008) (Figure 1.4). The mitotic centromere and kinetochore can be structurally divided into four distinct regions including inner centromere, inner kinetochore, outer kinetochore and fibrous corona (McEwen and Dong, 2010) (Figure 1.4). Here we briefly summarize the key findings about the SUMO signals at centromeres and kinetochores during mitosis (Azuma et al., 2005; Nie et al., 2009; Zhang et al., 2008) (Figure 1.4). 1) Immunofluorescence microscopy analysis using antibodies specific to SUMO-2/3 showed that SUMO-2/3 signals are concentrated to many distinct foci on the condensed chromosomes from prophase to metaphase and eventually coat the entire chromosomes during anaphase and telophase in mammalian cells (Zhang et al., 2008). During metaphase, the SUMO-2/3 foci on the chromosomes are significantly co-localized with the inner centromere protein CENP-B and partially overlapped with the inner kinetochore protein CENP-C (Zhang et al., 2008) (Figure 1.4). In contrast, the SUMO-1 signals are localized to the mitotic spindle during metaphase and later concentrated to the spindle midzone during anaphase and telophase (Zhang et al., 2008). These results support a model that SUMO-1 and SUMO-2/3 paralogs are conjugated to
different subsets of proteins at distinct subcellular localizations and therefore regulate discrete mitotic processes in vertebrates (Zhang et al., 2008). 2) In Xenopus egg extracts, the EGFP-SUMO-2 signals are co-localized with Aurora B at inner centromeres of condensed chromosomes (Azuma et al., 2005) (Figure 1.4). It has been shown that in the mitotic Xenopus egg extracts, topoisomerase IIα (Topo IIα) localized to the inner centromere region, is the major SUMO-2/3 substrate (Azuma et al., 2005; Azuma et al., 2003). 3) In Drosophila cultured cells, SUMO conjugates are mainly localized to inner centromeres and outer kinetochore plates during prometaphase and are also targeted to the spindle midzone during anaphase. This result suggests that the single SUMO in invertebrates, such as Drosophila, plays the roles of both vertebrate SUMO-1 and SUMO-2/3 during mitosis (Nie et al., 2009) (Figure 1.4). In all the above organisms ranging from invertebrates to mammals, the SUMO signals have been observed at centromeres and kinetochores during the early stages of mitosis including prophase, prometaphase and metaphase (Figure 1.4).

Three types of posttranslational modifications, including phosphorylation, ubiquitination and SUMOylation, have been demonstrated to play the essential roles in chromosome segregation during mitosis (Dasso, 2008). Interestingly, only “SUMOylation” signals have been reported to be directly detected at mitotic centromeres and kinetochores in both invertebrate and vertebrate cells (Azuma et al., 2005; Nie et al., 2009; Zhang et al., 2008) (Figure 1.4). These evolutionally conserved “SUMOylation” signals at mitotic centromeres and kinetochores are consistent with a model that SUMOylation functions as a master regulator of centromere and kinetochore activities during mitosis.
Figure 1.4. Both SUMO signals and known SUMO substrates are associated with the mitotic centromere and kinetochores of a condensed chromosome. The enlarged region of the mitotic chromosome represents inner centromere, inner kinetochore, outer kinetochore, and fibrous corona. The exact localizations of SUMO signals at centromeres and kinetochores in human HeLa cells, Xenopus egg extracts and Drosophila culture cells are determined by fluorescence or immunofluorescence microscopy (on the left). The known vertebrate SUMO targets, which have been identified and confirmed in vivo, are schematically represented here (on the right) and also described in Table 1.1.

Although the “SUMOylation” signals have not been directly detected in yeast, many centromere and kinetochore proteins have been identified as SUMO substrates in yeast, supporting a conserved role of SUMOylation in regulation of mitosis in all eukaryotes (Dasso, 2008). Consistent with the conserved role of SUMOylation in regulation of the centromere/kinetochore activities, SUMOs have been identified as suppressors of the temperature-sensitive mutants of the centromeric protein CENP-C in both yeast and chicken cells by genetic screenings (Fukagawa et al., 2001; Meluh and Koshland, 1995).

1.4.4.2 Role of SUMO modification at centromeres and kinetochores:

Consistent with the observed SUMO signals at centromeres and kinetochores in both invertebrates and vertebrates (Figure 1.4), many centromere and kinetochore proteins have been identified as SUMO targets in yeast and vertebrates [8]. Some of the vertebrate SUMO targets
associated with centromeres and kinetochores and also the roles of their SUMOylation in control of chromosome segregation will be discussed. The precise localizations of these vertebrate SUMO targets at the centromere and kinetochore region are elucidated in Figure 1.4 and Table 1.1. Furthermore, the other information and properties of these SUMO targets, including their protein GI numbers, SUMOylation sites, SUMO-1 or SUMO-2/3-preferential modification, SUMOylation time during the cell cycle, associated protein complexes, and corresponding reference(s), are summarized in Table 1.1.

- **Topoisomerase IIα**

  Topoisomerase IIα (Topo IIα) has been identified as one of the first mitotic SUMO targets in budding yeast and vertebrates (Table 1.1 and Figure 1.4) (Azuma et al., 2003; Bachant et al., 2002). During mitosis, Topo IIα is re-localized from chromosome arms to the centromeres of sister-chromatids (Christensen et al., 2002; Tavormina et al., 2002). One of the main functions of Topo IIα at the centromere is to decatenate the last major site of attachment between sister chromatids for chromosome segregation (Lee and Bachant, 2009). Accumulating evidence has indicated that SUMOylation plays a critical role in regulation of Topo IIα-mediated decatenation of centromeric DNA during mitosis (Ryu et al., 2010). PIASy has been found to be required for SUMO-2/3 modification of Topo IIα and also the accumulation of SUMO-2/3 conjugates at the inner centromere region of mitotic chromosomes in *Xenopus* extracts (Figure 1.4) (Azuma et al., 2005).
<table>
<thead>
<tr>
<th>Locations</th>
<th>SUMO Targets</th>
<th>Organisms (GI number)</th>
<th>SUMOylation Sites</th>
<th>SUMO-1 or SUMO-2/3</th>
<th>SUMOylation Time</th>
<th>In Protein Complexes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centromere</td>
<td>Aurora B</td>
<td>Human (83776600)</td>
<td>K202</td>
<td>SUMO-2/3</td>
<td>Early Mitosis</td>
<td>CPC</td>
<td>(Ban et al., 2011; Fernandez-Miranda et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Borealin</td>
<td>Human (8922438)</td>
<td>Unknown</td>
<td>SUMO-2/3</td>
<td>Early Mitosis</td>
<td>CPC</td>
<td>(Klein et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Topo IIα</td>
<td>Xenopus (148222806)</td>
<td>K660</td>
<td>SUMO-2/3</td>
<td>Early Mitosis</td>
<td>None</td>
<td>(Azuma et al., 2003; Ryu et al., 2010)</td>
</tr>
<tr>
<td>Inner Kinetocho re</td>
<td>CENP-H</td>
<td>Human (12597655)</td>
<td>Unknown</td>
<td>SUMO-2/3</td>
<td>S Phase</td>
<td>CENP-H/I/K</td>
<td>(Mukhopadhyay et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>CENP-I</td>
<td>Human (41352697)</td>
<td>Unknown</td>
<td>SUMO-2/3</td>
<td>S Phase</td>
<td>CENP-H/I/K</td>
<td>(Mukhopadhyay et al., 2010)</td>
</tr>
<tr>
<td>Outer Kinetocho re</td>
<td>Nuf2</td>
<td>Human (117968420)</td>
<td>Unknown</td>
<td>SUMO-2/3</td>
<td>Unknown</td>
<td>Ndc80/Hec1</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>BubR1</td>
<td>Human (59814247)</td>
<td>K250</td>
<td>SUMO-2/3</td>
<td>Late Mitosis</td>
<td>None</td>
<td>(Yang et al., 2011; Zhang et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>CENP-E</td>
<td>Human (71061468)</td>
<td>Unknown</td>
<td>SUMO-2/3</td>
<td>Unknown</td>
<td>None</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>Fibrous Corona</td>
<td>RanGAP1</td>
<td>Mouse (341941806)</td>
<td>K526</td>
<td>SUMO-1</td>
<td>Constitutive</td>
<td>RRSU</td>
<td>(Joseph et al., 2004; Mahajan et al., 1997; Mahajan et al., 1998; Matunis et al., 1996; Matunis et al., 1998)</td>
</tr>
</tbody>
</table>
The RRSU complex consists of RanBP2/Nup358, RanGAP1-SUMO1 and Ubc9. The chromosomal passenger complex (CPC) contains Aurora B kinase, INCENP, Survivin and Borealin.

Recent studies by Ryu and Azuma have shown that the N-terminus of PIASy is responsible for the kinetochore localization of PIASy by interacting with Rod and Zw10, components of the RZZ complex at the kinetochore fibrous corona (Ryu and Azuma, 2010). Furthermore, the kinetochore localization of PIASy is essential for the centromeric SUMO-2/3 modification of Topo IIα and other chromosome-associated proteins during mitosis (Ryu and Azuma, 2010). Interestingly, PIASy-dependent SUMO-2/3 modification of Topo IIα significantly inhibits the decatenation activity of Topo IIα in Xenopus egg extracts (Ryu et al., 2010). Furthermore, Topo IIα is modified by SUMO-2/3 at the lysine 660 (K660) within the DNA gate domain involved in the DNA cleavage and re-ligation. The SUMO-2/3 modification of Topo IIα at the K660 is responsible for SUMOylation-mediated inhibition of Topo IIα, supporting a model that PIASy-dependent SUMOylation of Topo IIα functions in temporally preventing the resolution of centromeric DNA until the onset of anaphase (Ryu et al., 2010).

- **The chromosome passenger complex at inner centromere**

  The chromosomal passenger complex (CPC) consists of the Aurora B kinase and three non-enzymatic subunits INCENP, Survivin and Borealin (Ruchaud et al., 2007). The CPC has a dynamic localization during mitosis. At the entry of mitosis, the CPC is initially localized to both chromosome arms and inner centromeres (Figure 1.4). As the cell cycle progresses to prometaphase and metaphase, the CPC is mainly concentrated to the inner centromeres. Upon the sister-chromatid separation at the onset of anaphase, the CPC is re-localized from the inner centromeres to the spindle midzone (Ruchaud et al., 2007). The CPC plays a central role in correcting erroneous kinetochore-microtubule attachments during prometaphase, SAC, and cytokinesis (Carmena and Earnshaw, 2003; Ruchaud et al., 2007).
Recent studies have revealed that the human Aurora B kinase is preferentially modified by SUMO-2/3 at lysine 202 (K202) within its kinase domain (Table 1.1 and Figure 1.4) (Ban et al., 2011; Fernandez-Miranda et al., 2010). As the major SUMOylation site of human Aurora B, the K202 is located within a highly conserved sequence region (IHRDIKPE:N), which is identical among the Aurora B proteins of different species ranging from yeast to human and also contains the SUMOylation consensus motif (IKPE) (Ban et al., 2011; Fernandez-Miranda et al., 2010). PIAS3 efficiently stimulates the SUMO-2/3-specific modification of Aurora B in vivo (Ban et al., 2011). Consistent with the PIAS3-specific stimulation of Aurora B SUMOylation, the GFP-tagged PIAS3 proteins have been found to be associated with kinetochores as paired foci on both sides of Aurora B during prophase and prometaphase (Ban et al., 2011). The authors then showed that in HeLa cells, deSUMOylation of Aurora B occurs only by overexpression of FLAG-SENP2 but not by any of the other FLAG-tagged mammalian SENPs (SENP1, SENP2, SENP5 and mouse SENP2 isotype Smt3IP2/Axam2), (Ban et al., 2011). Consistent with the previous finding (Zhang et al., 2008), overexpression of FLAG-SENP2 does not affect Aurora B localization to inner centromeres during mitosis, indicating that SUMO-2/3 modification of Aurora B is not required for its localization to inner centromeres (Ban et al., 2011). Moreover, it has been shown recently that SUMO-2/3 modification of Aurora B can greatly enhance its autophosphorylation in vivo, which is essential for its activation during mitosis (Ban et al., 2011). This result supports a model that SUMOylation of Aurora B is a novel mechanism to regulate its kinase activity during mitosis (Ban et al., 2011).

Analysis of SUMOylation of the CPC in mammalian cells has revealed that its non-enzymatic subunit, Borealin, is preferentially modified by SUMO-2/3, and that the level of its SUMOylation in metaphase is higher than that in anaphase (Table 1.1 and Figure 1.4) (Klein et
al., 2009). Interestingly, Nup358/RanBP2 has been identified as the SUMO E3 ligase for Borealin both in vitro and in vivo (Klein et al., 2009). Furthermore, the SUMO isopeptidase SENP3 has a specific interaction with Borealin in vivo and is responsible for deSUMOylation of Borealin (Klein et al., 2009). However, SUMOylation of Borealin does not affect the assembly of the CPC as well as its localization at centromeres and spindle midzone (Klein et al., 2009). Interestingly, the yeast Survivin homolog Bir1 has also been identified as a SUMO target, but the role of its SUMOylation is currently unknown (Montpetit et al., 2006).

- **CENP-H/I/K complex at the inner kinetochore**

Studies of the mammalian SUMO isopeptidase SENP6 have shown that the inner kinetochore proteins CENP-H/I are specifically modified by polymeric SUMO-2/3 chains (Table 1.1 and Figure 1.4) (Mukhopadhyay et al., 2010). RNAi-depletion of SENP6 results in an accumulation of the polymeric SUMO-2/3 chain-modified CENP-H and CENP-I during S phase, rather than in mitosis (Mukhopadhyay et al., 2010; Mukhopadhyay and Dasso, 2010). Because the CENP-H/I/K complex is recruited to the constitutive inner kinetochore structures during S phase, it has been hypothesized that SUMOylation of CENP-H/I promotes the assembly of inner kinetochores (Mukhopadhyay et al., 2010; Mukhopadhyay and Dasso, 2010). In SENP6-depleted cells, the poly-SUMO-2/3 chains on CENP-H/I are recognized by RNF4, a SUMO targeted ubiquitin ligase (STUbL), leading to polyubiquitination and proteasome-mediated degradation of CENP-H/I (Mukhopadhyay et al., 2010). Furthermore, SENP6 depletion leads to the chromosome congression defect, the prolonged mitotic arrest, and the chromosome missegregation in mammalian cells (Mukhopadhyay et al., 2010). The mitotic defects in SENP6-depleted cells are comparable to those in the CENP-H/I/K-depleted cells, suggesting that SENP6 is a key regulator of inner kinetochore assembly by preventing the polymeric SUMO-2/3 chain modification of the
CENP-H/I/K complex and thereby protecting CENP-H/I/K from RNF4-mediated degradation during S phase (Mukhopadhyay et al., 2010; Mukhopadhyay and Dasso, 2010).

- **Nuf2, BubR1 and CENP-E at outer kinetochore and fibrous corona**

  Studies of mitotic SUMOylation in mammalian cells have shown that the outer kinetochore protein Nuf2 and the fibrous corona-associated proteins BubR1 and CENP-E are specifically modified by SUMO-2/3 (Table 1.1 and Figure 1.4) (Zhang et al., 2008). Recent studies have shown that SUMOylation of BubR1 is essential for its function during mitosis (Table 1.1 and Figure 1.4) (Yang et al., 2011). As a key component of the spindle assembly checkpoint (SAC), BubR1 is localized to unattached kinetochores during early prophase and then disassociates from microtubule-attached kinetochores following chromosome congression to the metaphase plate (Cheeseman and Desai, 2008). BubR1 is predominantly modified by SUMO at Lysine 250 (K250), and its SUMOylation is strongly stimulated after a prolonged mitotic arrest caused by the treatment of either nocodazole or taxol (Yang et al., 2011). Interestingly, SUMOylation of BubR1 does not regulate its kinase activity and kinetochore localization (Yang et al., 2011). However, ectopic expression of the SUMOylation-deficient BubR1 mutant causes a defect in the timely removal of the BubR1 mutant from the kinetochore during metaphase for SAC inactivation, which leads to a delay of progression through mitosis, and also an increase of lagging chromosomes during anaphase (Yang et al., 2011). These results indicate that BubR1 SUMOylation plays a critical role in its disassociation from kinetochores, the checkpoint inactivation for timely entry of anaphase, and therefore accurate chromosome segregation.

  The highly conserved KMN network present in the outer kinetochore consists of three complexes, Knl1, Mis12 and Ndc80 (Cheeseman et al., 2004). The components of the KMN network are essential for viability and are important for kinetochore-microtubule attachments
The Knl1 complex comprises KNL1 and ZWINT and Mis12 complex consists of MIS12, NSL1, NNF1 and DSN1 (Varma and Salmon, 2012). The Ndc80/Hec1 complex, including Ndc80/Hec1, Nuf2, Spc24 and Spc25 subunits, is found at the outer kinetochore plate and plays a major role in kinetochore-microtubule attachments (Ciferri et al., 2007; Tooley and Stukenberg, 2011). It has been reported that Nuf2 can directly interact with CENP-E in vitro and is also required for targeting CENP-E to kinetochores in mammalian cells (Liu et al., 2007). Although Ndc80 has been identified as a SUMO substrate in budding yeast, the function significance of its SUMOylation is still unknown (Montpetit et al., 2006). It would be very interesting to know whether the mammalian Ndc80 homolog, Hec1, is also a SUMO target in vivo. As a SUMO-2/3 substrate (Zhang et al., 2008), the BubR1 kinase is a key component of SAC (Musacchio and Salmon, 2007) and a CENP-E interacting protein at the fibrous corona (Chan et al., 1998; Yao et al., 2000). In addition, BubR1 and CENP-E can be co-immunoprecipitated from HeLa cells (Chan et al., 1998; Yao et al., 2000). Interestingly, the tail domain of CENP-E is specifically modified by SUMO-2/3, suggesting that this modification might be important for its kinetochore localization (Zhang et al., 2008). Because the SUMOylation sites at CENP-E tail domain have not been identified, the effect of CENP-E SUMOylation on its kinetochore localization cannot be directly tested by using the SUMOylation-deficient CENP-E mutant (Zhang et al., 2008).

The chromosome congression defect in SENP2 overexpressing cells is almost the same as the defect caused by depletion or inhibition of CENP-E (Putkey et al., 2002; Wood et al., 1997; Yao et al., 2000; Yucel et al., 2000). The chromosome congression defect is a failure in complete chromosome alignment to the metaphase plate with some chromosomes detected at the spindle pores. CENP-E is a plus end directed, kinesin-7 family motor protein and is found mainly
associated with the kinetochore fibrous corona (Kapoor et al., 2006; Kim et al., 2008). CENP-E functions in the kinetochore-microtubule attachment and plays a major role in chromosome congression from the spindle poles to the metaphase plate (Schaar et al., 1997; Wood et al., 1997). It has been shown that the loss of CENP-E at kinetochores in the SENP2 overexpressing cells is caused by the defect in targeting CENP-E to kinetochores other than by the degradation of CENP-E (Zhang et al., 2008). Unlike the RNAi-depletion of SENP6, SENP2 overexpression does not affect in the overall structure and function of kinetochores, because the centromere and kinetochore proteins (including Aurora B, Survivin, CENP-B, CENP-C, Hec1, CENP-F, Nup96 and Nup107) exhibit correctly localization on mitotic chromosomes in SENP2 overexpressing cells (Mukhopadhyay et al., 2010; Zhang et al., 2008). In SENP2 overexpressing cells, the signals of spindle assembly checkpoint (SAC) proteins, including Bub1, BubR1 and Mad2, are largely undetectable on aligned chromosomes at the metaphase plate but accumulated on unaligned chromosomes at the spindle poles, indicating that the mitotic arrest caused by SENP2 overexpression is due to the activation of SAC.

- **RanGAP1 at kinetochore fibrous corona**

  In vertebrates, SUMO-1 modification of RanGAP1 is required for the assembly of a highly stable multiprotein complex, called the RRSU complex, which consists of RanBP2/Nup358, RanGAP1-SUMO1 (SUMO-1-modified RanGAP1) and Ubc9 (Dasso, 2008; Mahajan et al., 1997; Matunis et al., 1998; Reverter and Lima, 2005; Saitoh et al., 1997; Zhang et al., 2002). In interphase cells, the unmodified RanGAP1 is localized to the cytoplasm, whereas SUMO-1 modification of RanGAP1 facilitates RanGAP1 interaction with RanBP2/Nup358 and Ubc9 at the cytoplasmic filaments of the NPC, leading to the RRSU complex assembly (Mahajan et al., 1997; Matunis et al., 1998; Reverter and Lima, 2005; Saitoh et al., 1997; Zhang et al., 2002). During
mitosis, the RRSU complex is localized to kinetochore fibrous corona (Table 1.1 and Figure 1.4) and mitotic spindles (Dasso, 2008; Joseph et al., 2002; Matunis et al., 1996). The association of the RRSU complex with kinetochores appears immediately after nuclear envelope breakdown and persists until the late anaphase (Joseph et al., 2002). As the key component of the RRSU complex, RanBP2 is required for targeting RanGAP1 to the NPC during interphase and to the kinetochore during mitosis (Joseph et al., 2004; Joseph et al., 2002; Mahajan et al., 1998; Matunis et al., 1998).

1.5 THE ASSOCIATION OF THE SUMOYLATION PATHWAY WITH HUMAN DISEASES

Consistent with the essential roles of the SUMO pathway in many biological processes, perturbations of SUMOylation have been implicated to human diseases including various types of cancer and multiple neurodegenerative diseases. Any imbalance caused either in the conjugation or deconjugation of SUMO to proteins involved in cancer metastasis can lead to tumorigenesis. Many tumor suppressors and oncoproteins have been identified as SUMO targets. The role of SUMO in both neurodegenerative diseases and tumorigenesis will be discussed below.

1.5.1 SUMOYLATION in neurodegenerative diseases

Neurodegenerative diseases are characterized by the loss of specific neurons in certain parts of the brain due to the aggregation of misfolded proteins. Similar to other post-translational modifications like phosphorylation and ubiquitination, SUMOylation is also important for neuronal development and appropriate functioning of the central nervous system (Krumova and Weishaupt, 2013). Although initially SUMOylation have been studied using non-neuronal cell lines, increasing lines of evidence indicate that SUMOylation is vital for the development of the nervous system (Dorval and Fraser, 2007) and its disruption or imbalance is associated with several
neurodegenerative diseases including Huntington’s disease (Steffan et al., 2004), Alzheimer’s disease (Zhang and Sarge, 2008) and Parkinson’s disease (Krumova et al., 2011).

1.5.1.1 Huntington’s disease (HD)

HD is one of the neurodegenerative diseases, where the Huntington (Htt) gene is mutated having long CAG (cytosine-adenine-guanine) trinucleotide repeats. The normal gene has about 10-35 repeats of CAG, whereas the mutated version can have anywhere between 36 and 120 repeats resulting in long stretches of glutamine residues. These long mutant proteins are fragmented into smaller segments forming aggregates resulting in loss of function of neurons. Htt proteins can be modified by both SUMO-1 and ubiquitin on the same lysine residue. SUMO modification of Htt stabilizes the mutant protein aggravating neurodegeneration in Drosophila, whereas, ubiquitination of Htt leads to its degradation reducing the pathology of the disease (Steffan et al., 2004). Inhibiting SUMOylation by targeting the identified E3 ligase would be an ideal therapy for HD.

1.5.1.2 Alzheimer’s disease (AD)

AD is a type of neurocognitive disorder that is accompanied with a gradual death of brain cells frequently resulting in dementia. Amyloid Precursor Protein (APP) is cleaved by β-secretase and γ-secretase to form small peptides called Amyloid beta (Aβ). When tau proteins, most commonly found in the neurons, become defective and fail to stabilize microtubules, it results in AD. Misfolding and aggregation of both Aβ and tau are commonly observed in this neurodegenerative disease. APP is modified by both SUMO-1 and SUMO-2 at Lys587 and Lys595 which are in the SUMO consensus sequence (Zhang and Sarge, 2008). The β-secretase cleavage site is located just to the C-terminus of the SUMO modification site. It has been shown that loss
of SUMO modification of APP increases Aβ aggregates, demonstrating that the modification obstructs the formation of Aβ aggregates.

1.5.1.3 Parkinson’s disease (PD)

PD is a central nervous system disorder that progressively affects movement, expression and speech. PD is associated with abnormal accumulation of α-synuclein in the brain cells in the form of Lewy bodies. The Lys 92 and Lys 102 residues of α-synuclein were identified as the major sites for SUMO modification. It has been demonstrated that when as little as 10% of α-synuclein is SUMO modified, it can result in a dramatic decrease in the aggregation of the protein in vitro. More importantly, in vivo studies indicate that the SUMO-deficient mutant of α-synuclein has increased aggregation compared to its WT form in dopaminergic neurons (Krumova et al., 2011). This agrees with the general idea that SUMO modification of a protein increases its solubility.

1.5.2 SUMO modification in cancer progression

1.5.2.1 Pertubations of the SUMO conjugation/deconjugation machinery in cancer

Several studies have shown that one or more components of the SUMOylation machinery exhibit changes in expression levels, which may cause an imbalance in SUMO modification and subsequent tumorigenesis (Figure 1.5). For example, Ubc9, the sole E2 conjugating enzyme, has varied expression levels depending on the type of cancer. In prostate and colon cancer, Ubc9 levels are higher; whereas in lung and breast cancer, Ubc9 levels are lower compared to their normal respective tissues (Moschos et al., 2010). Moreover, one of the studies demonstrated that overexpression of Ubc9 resulted in increased cancer cell growth (Mo et al., 2005). On the other hand, the SUMO E3 ligase PIAS3 is upregulated in lung, prostate and brain tumors (Wang and Banerjee, 2004). SUMO deconjugating enzymes SENP1 and SENP3 have also been shown to be upregulated in prostate cancer cell lines (Cheng et al., 2006b). Furthermore, in a screen involving
hepatocellular carcinoma, patients with lower survival rates have increased expression of the SUMO E1 enzyme (Lee and Thorgeirsson, 2004).

![Diagram of SUMOylation process](image)

**Figure 5. Dysregulation of SUMOylation in tumorigenesis.** Altered expression in any of the components of the SUMO pathway can lead to dysregulation of SUMOylation. This imbalance in SUMO-modified substrates (oncogenes or tumor suppressors) can lead to cancer progression and tumorigenesis.

### 1.5.2.2 SUMOylation in cancer metastasis

Cancer metastasis is the process in which primary tumor cells spread to distant sites and establish secondary tumors. It is not unexpected that SUMO modification has been implicated in cancer metastasis. KAI1 is a metastatic suppressor gene whose transcriptional expression is activated and repressed by the co-activator Tip60 and the β-catenin/reptin complex, respectively (Kim et al., 2005). Reptin is SUMO modified at Lys456, and the SUMO isopeptidase SENP1 is responsible for its deSUMOylation (Kim et al., 2006). SUMO modification of reptin alters the metastatic potential of KAI1 in prostate cancer cells. Wild-type reptin has better interaction with HDAC1 compared to its SUMO-deficient variant. SUMO-modified reptin represses KAI1 expression by recruiting HDAC1, resulting in enhanced invasion of LNCaP cells. This study establishes a direct link between SUMOylation and cancer metastasis (Kim et al., 2006).
Moreover, SUMO-modified reptin levels are higher in metastatic prostate cells LNCaP compared to normal epithelial prostate cells RWPE1. This is probably because of lower levels of SENP1 and higher levels of Ubc9 in LNCaP cells compared to RWPE1 cells.

1.5.2.3 SUMOylation in tumorigenesis

Several proteins involved in cancer progression, including pRb, p53, PML, BRCA1, RhoGDI, Rac1, and Merlin, are known SUMO targets whose SUMOylation regulates cancer metastasis in different types of cancer (Kim and Baek, 2006; Seeler et al., 2007). Retinoblastoma protein (pRb) is a tumor suppressor that binds and thus inhibits the E2F family of transcription factors. As long as pRb is bound to E2F factors, the cell cannot enter S-phase and is stalled in G1 phase. Also pRb-E2F dimer can recruit HDACs thereby bringing about further repression. SUMO deficient mutant of pRb represses the E2F dependent reporter genes more efficiently than its WT counterpart. (Ledl et al., 2005). The authors therefore hypothesize that SUMO modification on pRb displaces E2F family transcription factors. It would be interesting to investigate if SUMO modification of pRb is higher in cancer cells compared to normal epithelial cells.

BRCA1 (Breast Cancer 1 predisposition protein) is mutated in different types of breast cancer. BRCA1 is recruited at DNA double strand breaks and repairs damaged DNA. Moreover, it is a ubiquitin E3 ligase whose activity is required for tumor suppression (Morris et al., 2006). SUMO modification of BRCA1 increases its E3 ligase activity (Morris et al., 2009). Hence, BRCA1 is considered as a SUMO-regulated ubiquitin E3 ligase (Morris et al., 2009).

Merlin (Moesin-Ezrin-Radixin like protein) is a cytoskeleton protein and has tumor suppressor activity in humans. It is predominantly located in the nervous tissue, whose mutations result in the development of tumors in the nervous system and neurofibromatosis (Ruttledge and Rouleau, 2005). It regulates various signaling pathways controlling cell proliferation and
migration. Merlin is generally found in the plasma membrane, whereas SUMO deficient mutant of Merlin was found in the cytosol. SUMOylation of Merlin is required for its stability and in a xenograft mouse model, merlin sumoylation was found to be important for tumor suppressing activity (Qi et al., 2014).

Rac1 belongs to the Rho family of GTPases and has been shown to play a role in cell motility and adhesion by making the cytoskeletal rearrangements and the formation of lamellipodia. Mutations of Rac1 are found in several cancers including breast cancer and non-small cell lung cancer which leads to enhanced cell migration leading to cancer metastasis (Schnelezter et al., 2000). This is one of the examples of SUMO-1 modification taking place in a non-consensus motif of Rac1. Overexpression of PIAS3 upregulates SUMO modification of Rac1 in response to hepatocyte growth factor (HGF) and this leads to increased lamellipodia formation and cell migration compared to controls (Castillo-Lluva et al., 2010). This clearly indicates the role of SUMOylation in cell migration and cancer metastasis.

The Rho-GDP dissociation inhibitor RhoGDI binds to the GDP bound form of RhoGTPase (inactive form). The RhoGTPases, including RhoA, Rac1 and Cdc42, play an important role in cell migration, invasion, lamellipodia formation through actin cytoskeleton reorganizations, and their functions are inhibited by RhoGDI binding. Recently, RhoGDI was identified as a SUMO target. The cells transfected with RhoGDI WT had decreased rates of cell invasion compared to cells transfected with RhoGDI K138R, the SUMOylation-deficient mutant. Therefore, SUMO modification of RhoGDI increases its inhibitory effects on cell migration (Yu et al., 2012).

1.5.2.4 Cancer therapy through the SUMO pathway

Myc is a proto-oncogene, whose product is a transcription factor. Myc is known to be mutated and over-expressed in several cancers (Gurel et al., 2008; Palaskas et al., 2011), leading
to upregulation of its downstream genes some of which are involved in cell proliferation. On the other hand, KRAS is a small GTPase playing a role in signal transduction pathways and its mutated version is often found in cancers (Bos et al., 1987). Direct targeting and inhibition of these two oncoproteins have been shown to be highly difficult, suggesting that it is necessary for developing alternative approaches.

A genome-wide RNAi screen was performed to identify the genes that show synthetic lethality with Myc hyperactivation (Kessler et al., 2012). It was found that loss of the SUMO E1-activating enzyme subunit SAE2 is synthetic lethal with overexpression of Myc. Furthermore, the authors found that RNAi-depletion of SAE2 in cells with Myc overexpression causes mitotic aberrations with spindle defects. In addition, the metastasis-free survival rate of human breast cancer patients with high-Myc expression and low-SAE2 levels are much higher than those with high-Myc expression and high-SAE2 levels (Kessler et al., 2012). Together these data suggest that targeting SAE2 can be an approach to treat cancer with hyperactivated Myc.

Another genome-wide screen in KRAS mutant cells identified both SAE1 and Ubc9 as its synthetic lethal partners (Luo et al., 2009). A following study demonstrated that Ubc9 is important for both anchorage dependent and anchorage independent clonogenic growth of KRAS mutant colorectal tumor cells and it aids KRAS-mediated transformation (Yu et al., 2015). These data suggest that the components of the SUMO pathway could be potential therapeutic targets for treating cancers in which KRAS is mutated.

1.6 PROJECT OUTLINE

At the end of a cell cycle in a somatic eukaryotic cell, each cell divides into two. A loss of control of cell-cycle progression often leads to uncontrolled cell proliferation and cancer development. Several lines of evidence suggest that SUMOylation of various proteins involved in
cell cycle regulation are critical for accurate cell cycle progression through mitosis. As mentioned above there are also a number of tumor suppressor and oncogenes that are bona fide SUMO targets.

In order to understand the role of SUMOylation in cancer progression, I developed a technique called SUMO-epitope-peptide elution (SUMO-ePIPE), which in combination with mass spectrometry was employed for the purification and identification of SUMO-2/3 targets in two different mouse breast cancer cell lines, which is described in Chapter 2. About 66 SUMO-modified protein targets have been identified, and they are involved in different biological processes including cell migration, stress response, gene expression, cell cycle and metabolism. Out of the 66 targets, about 10 of them were confirmed as bona fide SUMO-2/3 substrates using either in vitro or in vivo approaches. Furthermore, we showed that global SUMO-2/3 upregulation stimulates 3D cell migration.

Uncontrolled cell division is a hallmark of human cancers. In Chapter 3, I therefore concentrated on the importance of SUMOylation in cell division by primarily focusing on the regulation of cell-cycle progression through mitosis. We found that polymeric SUMO-2 modification but not polymeric SUMO-1 modification at the centromere/kinetochore region is required for CENP-E targeting to kinetochores and chromosome alignment to the metaphase plates in cells with inhibition of SUMOylation by overexpressing the isopeptidase SENP2 or by depleting the sole E2 enzyme Ubc9. Also, using biochemical assays, we found that a kinetochore protein Nuf2 which has three SUMO-2 moieties fused to its C-terminus has a higher binding-affinity to CENP-E compared to Nuf2 alone, suggesting that polymeric SUMO-2 chain modification of Nuf2 recruits CENP-E to kinetochores by enhancing the interaction of Nuf2 with CENP-E.

We found that a minimum of trimeric SUMO-2 chain fused to Nuf2 is essential for chromosome alignment and CENP-E recruitment to kinetochores in cells overexpressing SENP2.
However, it is not known if a longer length of polymeric SUMO-2 chain (4xSUMO-2 and 5xSUMO-2) fused with Nuf2 can achieve a greater rescue in cells with overexpression of SENP2. This can be tested by co-transfecting cells with SENP2 along with either Nuf2-4xSUMO-2 or Nuf2-5xSUMO-2. Another area to explore would be to test if other putative SIMs on CENP-E have SUMO-2 binding activity and if they are required for CENP-E localization to the kinetochores. In an effort to identify the E3 ligase for the regulation of Nuf2 SUMOylation, an in vitro binding assay between Nuf2 and the different PIAS proteins indicated that both PIASxα and PIASy interacted with Nuf2. It would be interesting to test if PIASxα or PIASy regulate the SUMO-2 modification of Nuf2. These future directions are discussed in Chapter 4.
CHAPTER 2 ANALYSIS OF CHANGES IN SUMO-2/3 MODIFICATION DURING BREAST CANCER PROGRESSION AND METASTASIS

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2.1 ABSTRACT

SUMOylation is an essential posttranslational modification and regulates many cellular processes. Dysregulation of SUMOylation plays a critical role in metastasis, yet how its perturbation affects this lethal process of cancer is not well understood. We found that SUMO-2/3 modification is greatly up-regulated in metastatic breast cancer cells compared to non-metastatic control cells. To identify proteins differentially modified by SUMO-2/3 between metastatic and non-metastatic cells, we established a method in which endogenous SUMO-2/3-conjugates are labeled by stable isotope labeling by amino acids in cell culture (SILAC), immunopurified by SUMO-2/3 monoclonal antibodies and epitope-peptide elution, and analyzed by quantitative mass spectrometry. We identified 66 putative SUMO-2/3-conjugated proteins, of which 15 proteins show a significant increase/decrease of SUMO-2/3 modification in metastatic cells. Targets with altered SUMOylation are involved in cell cycle, migration, inflammation, glycolysis, gene expression and SUMO/ubiquitin pathways, suggesting that perturbations of SUMO-2/3 modification might contribute to metastasis by affecting these processes. Consistent with this, up-regulation of PML SUMO-2/3 modification corresponds to an increased number of PML nuclear bodies (PML-NBs) in metastatic cells, whereas up-regulation of global SUMO-2/3 modification promotes three-dimensional (3D) cell migration. Our findings provide a foundation for further investigating the effects of SUMOylation on breast cancer progression and metastasis.
2.2 INTRODUCTION

SUMOylation is an essential posttranslational modification characterized by covalent conjugation of SUMO proteins to many different proteins in eukaryotes. By tightly regulated cycles of conjugation and deconjugation, SUMOs act as molecular switches for controlling protein activity, localization, stability, and/or protein-protein interaction (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Hay, 2005; Wang and Dasso, 2009; Yeh, 2009). SUMOylation has emerged as a major regulatory mechanism for a variety of cellular processes including cell cycle control, cell migration, gene expression, DNA repair, signal transduction and stress response (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Hay, 2005; Wang and Dasso, 2009; Yeh, 2009). SUMO is conjugated to a substrate using E1-activating enzyme (SAE1/SAE2), an E2-conjugating enzyme (Ubc9), and multiple E3 ligases (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Hay, 2005; Wang and Dasso, 2009; Yeh, 2009). Furthermore, SUMOs are deconjugated from their substrates by SUMO-specific isopeptidases (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Hay, 2005; Wang and Dasso, 2009; Yeh, 2009). Proteomic analyses have revealed that SUMO-1 and SUMO-2/3 are conjugated to distinct and overlapping sets of proteins, indicating that SUMO-1 and SUMO-2/3 modifications may regulate both unique and redundant biological processes (Vertegaal et al., 2006). During mitosis, SUMO-2/3-modified proteins are uniquely localized on mitotic chromosomes including centromeres and kinetochores, whereas SUMO-1-modified proteins are associated with the mitotic spindle and spindle midzone, indicating that SUMO-1 and SUMO-2/3 are conjugated to different target proteins and thereby play distinct mitotic functions (Wan et al., 2012a; Zhang et al., 2008). Both SUMO-2/3, but not SUMO-1, contain a SUMOylation consensus motif (ΨKxE/D)(Ψ is a large hydrophobic amino acid, K is the lysine residue that is modified, x is any residue, and E or
D is an acidic residue), which is responsible for the formation of polymeric SUMO-2/3 chains (Ulrich, 2008; Vertegaal). However, the molecular mechanisms underlying these paralog-specific modifications are still not well understood.

Accumulating lines of evidence support the idea that balanced levels of SUMO-modified and unmodified proteins are required for normal cellular processes, whereas an imbalance in SUMOylation often results in human diseases including cancer (Bawa-Khalfe et al., 2010; Bawa-Khalfe et al., 2012; Bawa-Khalfe and Yeh, 2010; Driscoll et al., 2009; Kessler et al., 2012; Kim et al., 2006; Kim and Baek, 2006; Mo et al., 2005; Wang and Banerjee, 2004; Yang et al., 2013). Consistent with this model, dysregulation of SUMOylation caused by overexpression of SUMO-conjugating and/or deconjugating enzymes is frequently associated with advanced stages of cancer and low survival rates (Bawa-Khalfe and Yeh, 2010; Kessler et al., 2012). Furthermore, many proteins, including promyelocytic leukemia (PML), BRCA1, estrogen receptor, p53, Rac1 and RhoGDI, which play a critical role in tumorigenesis and metastasis, have been identified as SUMO-modified substrates (Boddy et al., 1996; Castillo-Lluva et al., 2010; Gostissa et al., 1999; Kamitani et al., 1998; Morris et al., 2009; Muller et al., 1998; Rodriguez et al., 1999; Sentis et al., 2005; Sternsdorf et al., 1997; Yu et al., 2012). Moreover, human genome-wide RNAi screens of Myc or Ras synthetic lethal genes have revealed that SUMOylation is required for tumorigenesis driven by Myc hyperactivation or Ras mutations (Kessler et al., 2012; Luo et al., 2009). Because Ras and Myc oncoproteins are difficult to inhibit pharmacologically, targeting the SUMO pathway may have therapeutic efficacy for cancers driven by Myc and/or Ras oncogenes (Kessler et al., 2012; Luo et al., 2009). Recent studies revealed that dysregulation of SUMOylation promotes cancer cell proliferation, migration, invasion and metastasis, (Bawa-Khalfe et al., 2012; Kessler et al., 2012; Kim and Baek, 2006; Luo et al., 2009) yet how an imbalance in SUMOylation
contributes to the important processes of cancer is not well understood.

Using a well-established mouse breast cancer progression and metastasis model, (Aslakson and Miller, 1992) we found that SUMO-2/3 modification is greatly up-regulated in metastatic cells compared to non-metastatic control cells. To specifically purify endogenous SUMO-conjugates in these breast cancer cells, we first developed an affinity-purification technique called “SUMO-epitope-peptide elution” (SUMO-ePIPE). Recently, Melchior and colleagues (Becker et al., 2013) have described a technique that is similar to our SUMO-ePIPE approach. However, a proteomic method has not been established or applied for the identification of proteins that are differentially modified by endogenous SUMOs between two biological samples, such as metastatic and non-metastatic cells. To identify the target proteins differentially modified by SUMOs between metastatic and non-metastatic cells, we developed a proteomic strategy by coupling SUMO-ePIPE with SILAC (stable isotope labeling by amino acids in cell culture) and quantitative mass spectrometry analysis (Olsen and Mann, 2013). Using this strategy, we resolved a network of proteins that are differentially modified by SUMO-2/3 in metastatic breast cancer cells when compared to non-metastatic control cells. The identified proteins are involved in multiple cellular processes including cell cycle, cell migration, gene expression, glycolysis, inflammation, stress response, and SUMO/ubiquitin pathways. Furthermore, we verified a list of SUMO targets by in vitro SUMOylation assays and also confirmed that the SUMO-2/3 modification of PML, Macrophage Migration Inhibitory Factor (MIF), α-Enolase, Pyruvate kinase M2 (PKM2) and hnRNPU is significantly enhanced in metastatic cells compared to non-metastatic cells. Moreover, we found that up-regulation of PML SUMO-2/3 modification is associated with an increased assembly of PML-NBs in metastatic cells when compared to non-metastatic cells. Lastly, in support of our proteomic results suggesting a link between SUMO-2/3 modification and cell
migration, we demonstrated that up-regulation of global SUMO-2/3 modification by overexpressing GFP-SUMO-2/3 in breast cancer cells significantly increased the linear speed of 3D migration.

2.3 MATERIALS AND METHODS

Primers, Plasmids, Antibodies and Peptides

PCR primers were used to amplify different fragments of human SUMO-1 and SUMO-2 and also to generate non-conjugatable SUMO-2 and SUMO-3 mutants by site-directed mutagenesis (Appendix A Table A.1, A.2 and A.3). Gateway entry plasmids were purchased from DNA Plasmid Repository at Arizona State University (DNASU), control and epitope peptides for SUMO-ePIPE were chemically synthesized (Bio-Synthesis), and antibodies for Western blotting (WB) and immunofluorescence microscopy (IF) were listed (Appendix A Table A.4, A.5, A.6).

Cell Culture and SILAC Method

All the mouse breast cancer cell lines including 67NR, 168FARN, 4TO7 and 66cl4 (a gift from Dr. Fred Miller, Karmanos Cancer Institute, Wayne State University) were grown in DMEM (HyClone) media supplemented with 10% FBS (Invitrogen) and 1% Pencillin-Streptomycin (Invitrogen) and maintained at 37°C. For immunopurification of SUMO-2/3 conjugates using SUMO-2/3-ePIPE, 168FARN and 66cl4 cells were SILAC labeled in light (Arg0 and Lys 0) and heavy (Arg10 and Lys8) media (Cambridge Isotope Laboratories), respectively (Tatham et al., 2011). The final concentration of all the isotopes was 0.26 mM. The cells were grown for eight passages on the SILAC media before SUMO-2/3-ePIPE. In addition, mass spectrometry was used to confirm that the proteins in 66cl4 cells were incorporated with the heavy isotopes.
Purification of Endogenous SUMO-Conjugates by SUMO-ePIPE

Cells were lysed under a stringent denaturing condition and used for immunopurification of endogenous SUMO-1 and SUMO-2/3 conjugates using anti-SUMO-1 (21C7) (Matunis et al., 1996) and anti-SUMO-2/3 (8A2) (Zhang et al., 2008) mAbs (Appendix A Table A.6) that were crosslinked to Protein-G beads using Disuccinimidyl suberate (DSS) (Thermo Fisher Scientific). SUMO-1-ePIPE and SUMO-2/3-ePIPE were used to elute endogenous SUMO-1 and SUMO-2/3 conjugates from the beads using SUMO-1 and SUMO-2/3 epitope peptides, respectively (Appendix A Table A.5). For quantitative proteomic analyses of endogenous SUMO-2/3 conjugates, 50 plates (150 mm) of SILAC-labeled 168FARN (Light) or 66cl4 cells (Heavy) were respectively lysed in 1× Lysis Buffer along with protease inhibitors and 20 mM NEM as described (Zhang et al., 2008). The 1× Lysis Buffer was obtained by diluting 2× SSB (5% SDS, 150 mM Tris-HCl pH 6.7, 20% glycerol) with 1× RIPA buffer (20 mM HEPES pH 8.0, 300 mM NaCl, 2 mM EDTA, 0.5% Sodium deoxycholate, 1% Triton X-100, and 6% glycerol) in 1:4 ratio. For every four plates, 3 ml of 1× Lysis Buffer was used. The whole cell lysates were sonicated and ultra-centrifuged at 100,000×g for 1 h at 4°C. After centrifugation, the supernatant was passed through a 0.22 μm filter. The filtered lysates from the two cell lines were mixed in an equal ratio (1:1) of total proteins, diluted with 1× RIPA buffer to reduce the SDS concentration to 0.1%, and loaded onto a column containing 2 ml of Protein G beads crosslinked with 8A2 mAbs. After binding, the beads were washed with 100 ml of wash buffer (20 mM HEPES pH 8.0, 750 mM NaCl, 2 mM EDTA, 0.5% Sodium deoxycholate, 1% Triton X-100, 6% glycerol, 0.1% SDS, and 10 mM NEM). Elution was performed with either the control or SUMO-2/3-epitope peptides ((0.2 mg/ml) (Bio-Synthesis) (Appendix A Table A.5) in 1× Elution Buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 5% glycerol, and 2 mM EDTA). The eluted proteins were precipitated by
trichloroacetic acid (TCA) and dissolved in 2× SSB for immunoblotting, silver staining, Coomassie blue staining and quantitative mass spectrometry.

**Proteomic Analysis**

The following was performed by our collaborators (Dr. Jesper Oslen’s laboraotry) at University of Copenhagen, Denmark. To identify the proteins that are differentially modified by endogenous SUMO-2/3 between SILAC-labeled metastatic and non-metastatic cells, the gel slices in Figure 2.8D were in-gel digested with trypsin as described (Lundby and Olsen, 2011). Briefly, the excised gel slices were minced and destained (50% 25 mM ammonium bicarbonate, 50% acetonitrile for 3 times 20 min, 800 rpm, room temperature (RT)) and dehydrated (acetonitrile, 10 min, 800 rpm) followed by reduction of disulfide bonds (10 mM dithiothreitol in 25 mM ammonium bicarbonate, 45 min, RT, 800 rpm) and alkylation of cysteines (55 mM chloroacetamide in 25 mM ammonium bicarbonate, 30 min, 24 °C in darkness, 800 rpm). After washing in 25 mM ammonium bicarbonate the gel plugs were dehydrated in acetonitrile and proteins were digested with trypsin (50 μL 12.5 ng/μL sequencing grade trypsin from Promega in 25 mM ammonium bicarbonate overnight at 37 °C). Trypsin activity was quenched by acidification with a 10% trifluoroacetic acid (TFA) solution to pH~2 and peptides were extracted from the gel plugs with 30% acetonitrile in 3% trifluoroacetic acid (30 min, 800 rpm) followed by 80% acetonitrile in 0.5% acetic acid (30 min, 800 rpm) and finally in 100% acetonitrile. Organic solvents were removed by evaporation in a vacuum centrifuge. Extracted peptides were desalted and concentrated on STAGE-tips with reversed-phase C18 filters. The Peptide fractions were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) on an LTQ Orbitrap Velos instrument essentially as described (Olsen et al., 2009). Peptides were eluted from the STAGE tips into 96 well microtiterplates with 2×20 μL 40% acetonitrile in 0.5% acetic
acid and the acetonitrile was evaporated using a vacuum centrifuge reducing the sample volume to 4 μL. Online peptide separation was performed by reversed-phase C\textsubscript{18} HPLC on an Easy nLC system (Thermo Fisher Scientific) in a 15 cm long analytical column packed with 3 μm C\textsubscript{18} beads, and eluting peptides using a 45 min segmented gradient of increasing (5%-80%) buffer B (80% acetonitrile in 0.5% acetic acid) at a constant flow of 250 nl/min. The effluent from the HPLC was directly electrosprayed into an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) through a nano-spray ion source. The peptide mixture was analyzed by full-scan MS spectra (m/z 300-2000, resolution 30,000) in the Orbitrap analyzer after accumulation of 1,000,000 ions in the Orbitrap within a maximum fill-time of 1.000 ms. For every full-scan the ten most intense peptide ions were sequentially isolated and fragmented by higher energy collisional dissociation (HCD) and fragments were recorded by the orbitrap mass analyzer after accumulation of 50,000 ions with a maximum fill-time of 250 ms and using a normalized collision energy of 40%. The acquired MS raw data was processed by MaxQuant version 1.2.7.5 (www.maxquant.org), where peptides and proteins are identified by the Andromeda search algorithm via matching of all MS and MS/MS spectra against a target/decoy-version of the Uniprot human and mouse databases supplemented with reversed copies of all sequences as well as frequently observed contaminants. Maximal MS/MS tolerance was 20 ppm, a maximum of 2 missed cleavages was allowed. Carbamidomethylated cysteines were set as a fixed modification, whereas N-pyroglutamine, oxidation of methionine and N-terminal acetylation were searched as variable modifications. Minimum peptide length was set at 7 amino acids. Peptide quantitation, statistical evaluation and filtering of the resulting peptide datasets were performed in MaxQuant to achieve a FDR<0.01. Protein ratios were calculated as the mean of the SILAC ratios determined for all individual peptides for each protein.
Gene Ontology and Functional Networks

The STRING (Search Tool for Retrieval of Interacting Genes/Proteins) database (Version 9.1) (Franceschini et al., 2013) was used for analyzing gene ontology and functional networks of the putative SUMO substrates identified by SUMO-2/3-ePIPE and mass spectrometry. The known direct and indirect protein-protein interactions within these substrates were identified with a high confidence level of 0.7.

In vitro SUMOylation Assays

The DNA inserts encoding SUMO substrates were transferred from the entry plasmids (DNASU) (Appendix A Table A.4) to the destination vector pDEST15 using Gateway cloning technology according to the manufacturer’s instructions (Invitrogen). The generated destination plasmids were transformed into E. coli BL21 (DE3) cells alone or co-transformed with the pT-E1E2S2 plasmid encoding SUMO E1, E2 and SUMO-2 as described (Uchimura et al., 2004). After induction with IPTG at a final concentration of 0.1 mM for 4 h, the GST tagged proteins were pulled down using Glutathione Sepharose beads (GE Healthcare). The eluted proteins were analyzed by immunoblotting with anti-GST and anti-SUMO-2/3 antibodies. In addition, in vitro SUMO-1 and SUMO-2 modifications of GST-tagged Vimentin and FLAG-tagged Myosin-12 motor domain were analyzed by incubating with recombinant SUMO-E1 and Ubc9 in the presence or absence of SUMO-1/SUMO-2 for 2 h at 37 °C and by immunoblotting with anti-GST and anti-FLAG antibodies as described (Zhang et al., 2008).

3D Cell Migration Assays

Three dimensional (3D) collagen migration assays were performed as previously described (Indra et al., 2011). 168FARN cells were either transfected with GFP empty vector or co-transfected with GFP-SUMO-2 and GFP-SUMO-3 wild-type (WT) or non-conjugatable mutant
using Lipofectamine-Plus reagent according to the manufacturer’s instructions (Invitrogen). After 24-36 hours of transfection, cells were suspended in buffered liquid collagen type I (1.5 mg/ml, pH 7.4) (Advanced Biomatrix) and seeded on glass chambers for polymerization at 37 °C. The 168FARN cells expressing GFP or GFP-SUMO-2/3 were identified by fluorescence microscopy using the Olympus IX81 ZDC inverted microscope. The migration of a single transfected cell was monitored at 37 °C by time-lapse phase-contrast video microscopy with a 40×/0.75 NA Plan-Neofluar lens. The phase-contrast images of each cell were captured at 2 min intervals over a 120 min period. 10 sets of time-lapse microscopy videos of 168FARN cells expressing either GFP or GFP-SUMO-2/3 WT or mutant were used to calculate the linear speed of cell migration (μm/min) with each bar representing the mean value ± SEM.

**Immunofluorescence Microscopy**

To accurately compare the morphologies of the four mouse breast cancer cells, the adhesion of these cells on coverslips was enhanced by incubating the coverslips in PBS containing 50 μg/ml collagen (BD Biosciences), 50 μg/ml fibronectin (Sigma) and 10 μg/ml BSA (bovine serum albumin) (Sigma) at RT for 2 h. These coated coverslips were then used for growing cells. Once the cells were fixed with 3.5% paraformaldehyde for 10 min, they were permeabilized with 0.2% Triton X-100 for 10 min, incubated with mouse anti-SUMO-1 (21C7) or SUMO-2/3 (8A2) primary antibodies at RT for 1 h in PBS containing 2% BSA, washed three times in PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibodies (Invitrogen) at RT for 30 min in PBS containing 2% BSA. To compare the assembly of PML nuclear bodies (PML-NBs) between 168FARN and 66cl4 cells, cells were fixed at RT for 20 minutes in PBS containing 2% formaldehyde, and then permeabilized at RT for 10 min in PBS containing 0.5% Triton X-100. Cells were incubated with rabbit anti-PML antibodies (Santa Cruz)
and mouse anti-SUMO-2/3 mAbs (8A2). Olympus inverted IX81 fluorescence microscope was used to take images. To specifically analyze the cytoplasmic distribution of SUMO-1 and SUMO-2/3 in breast cancer cells, non-metastatic 168FARN and metastatic 66cl4 cells were fixed with 3.5% paraformaldehyde at RT for 30 min, incubated with 0.04% digitonin at RT for 10 min to selectively permeabilize cell membrane but not nuclear envelope, double labeled with rabbit anti-RanGAP1 antibodies and mouse anti-SUMO-1 (21C7) or anti-SUMO-2/3 (8A2) mAb, and analyzed by indirect immunofluorescence microscopy.

2.4 RESULTS

2.4.1 Dysregulation of SUMOylation during Breast Cancer Progression and Metastasis

To test if SUMOylation is differentially regulated during breast cancer development, I performed immunoblot analysis to compare both SUMO-1 and SUMO-2/3 modifications among three non-metastatic (67NR, 168FARN, and 4TO7) and one metastatic (66cl4) isogenic mouse breast cancer cell line (Aslakson and Miller, 1992). These cells are derived from a single mammary tumor that spontaneously formed in a wild-type BALB/c mouse, and exhibit sequentially increasing metastatic capacity after injection of these cells into the mouse mammary fatpads (Aslakson and Miller, 1992). The different behaviors of these cell lines reflect the distinct steps of the metastatic process from the primary mammary tumor to the secondary lung metastases (Figure 2.2A) (Aslakson and Miller, 1992). The metastatic process of carcinoma, such as breast cancers, has been proposed to be classified into the following distinct steps: (1) Invasion - Primary tumor cells lose cell-cell adhesion, gain motility and invade surrounding tissue; (2) Intravasation - Tumor cells enter the systemic circulation by penetrating through the blood and/or lymphatic vessels, (3) Extravasation - Some of these tumor cells that survive during the circulation
extravasate through the capillary vessels at distant sites/organs; and (4) Metastatic growth - A small subset of these metastasizing cells are able to survive, proliferate and establish secondary tumors (Aslakson and Miller, 1992; Yang et al., 2004). Among the three non-metastatic cells, which are defined by their failure to establish secondary tumors, 67NR cells are primary tumor cells incapable of local invasion, whereas both 168FARN and 4TO7 cells are capable for local invasion and also intravasation but only 4TO7 cells can extravasate the circulatory system. In contrast, 66cl4 cells can establish secondary tumors in lung and are thus metastatic. Interestingly, we found that levels of SUMO-2/3 modification in metastatic 66cl4 cells were significantly up-regulated compared to the three non-metastatic cells (Figure 2.1). In addition, levels of free SUMO-2/3 were also notably elevated in metastatic cells. On the other hand, levels of SUMO-1 modification were down-regulated in 66cl4 cells relative to 168FARN, 4TO7 and 67NR cells. Consistent with previous findings, (Indra et al., 2011) N-Cadherin was specifically expressed in 67NR and 66cl4 cells (Figure 2.2B).

Figure 2.1. Dysregulation of SUMOylation during breast cancer progression and metastasis. Total cell lysates of four indicated breast cancer cell lines derived from the same primary tumor and with increasing metastatic capacity were analyzed by immunoblotting with antibodies specific to SUMO-1, SUMO-2/3, Ubc9 and α-Tubulin. Notes: -, non-metastatic cells; +, metastatic cells.

Furthermore, we found that cells from each cell line showed unique morphologies under
phase-contrast microscopy (Figure 2.2C and D). We first fixed the cells with paraformaldehyde and then permeabilized both cell membrane and nuclear envelope with 0.2% Triton X-100 before indirect immunofluorescence analysis. Although both SUMO-1 and SUMO-2/3 were mainly localized in the nucleus for each cell line, their cytoplasmic staining was obvious as well. The relatively weak cytoplasmic staining of SUMO-1 and SUMO-2/3 might be overshadowed or interfered by their dominant nuclear staining. In order to better detect and also to compare the cytoplasmic SUMO-1 and SUMO-2/3 staining between non-metastatic 168FARN and metastatic 66cl4 cells, both cells were first fixed with paraformaldehyde but then incubated with a low concentration of digitonin, a detergent that preferentially permeabilizes cell membrane but not nuclear envelope (Matunis et al., 1996). Using this approach, we were able to detect the relatively strong cytoplasmic staining of SUMO-1 and SUMO-2/3 in both cells that were double labeled by rabbit anti-RanGAP1 and mouse anti-SUMO-1 or anti-SUMO-2/3 mAbs and analyzed by indirect immunofluorescence microscopy (Figure 2.2E and F). Because it has been shown previously that RanGAP1 is localized at the cytoplasmic filaments of nuclear pore complexes (Mahajan et al., 1997; Matunis et al., 1996) and also at the cytoplasmic pore complexes of annulate lamellae, their immunofluorescence staining at the nuclear envelope and the annulate lamellae can be used as a marker for the permeabilization of cell membrane (Figure 2.2E and F). We found that the cytoplasmic staining of SUMO-1 and SUMO-2/3 is overall slightly stronger in 66cl4 cells than in 168FARN cells.
Figure 2.2. The subcellular distributions of SUMO-conjugates among four mouse breast cancer cell lines derived from the same primary tumor. (A) The four mouse breast cancer cell lines, including 67NR, 168FARN, 4TO7 and 66cl4, are listed here with increasing metastatic capacities. While 67NR, 168FARN and 4TO7 are non-metastatic, 66cl4 cells are metastatic with the capacity to form secondary tumors in lung. (B) Whole cell lysates of the four breast cancer cells were used for immunoblot analysis with antibodies specific to N-Cadherin and α-Tubulin. (C and D) The four breast cancer cells were fixed with formaldehyde, permeabilized with Triton X-100, stained with mAbs specific to SUMO-1 (21C7) (C) or SUMO-2/3 (8A2) (D), and analyzed by immunofluorescence microscopy. Distinct morphologies were observed among these cells using phase-contrast microscopy. Nuclear DNA was stained with DAPI (4’,6-diamidino-2-phenylindole). (E and F) Non-metastatic 168FARN and metastatic 66cl4 cells were fixed with paraformaldehyde, incubated with a low concentration of digitonin to preferentially permeabilize cell membrane but not nuclear envelope, double labeled with rabbit anti-RanGAP1 antibodies and mouse anti-SUMO-1 (21C7) (E) or anti-SUMO-2/3 (8A2) mAb (F), and analyzed by indirect immunofluorescence microscopy. Bar, 10 μm.
To identify proteins differentially modified by endogenous SUMOs between metastatic and non-metastatic cells, we established an affinity purification technique that we refer to as “SUMO-epitope-peptide elution” (SUMO-ePIPE) (Figure 2.3A). This technique, similar to the one recently developed by Melchior and colleagues (Becker et al., 2013), utilizes two widely used and commercially available mAbs specific to SUMO-1 (21C7) (Matunis et al., 1996) (Invitrogen) and SUMO-2/3 (8A2) (Zhang et al., 2008) (Abcam). Consistent with their findings (Becker et al., 2013), we found that the SUMO-1 epitope recognized by mAb 21C7 lies between amino acids 55-67, and the SUMO-2 epitope for mAb 8A2 locates between amino acids 58-69 (Figure 2.4 and 2.5). We also established that the SUMO-1 and SUMO-2 epitope peptides could be used to effectively and specifically elute endogenous SUMO-1 and SUMO-2/3 conjugates that were immunoprecipitated from human 293T cell lysates using 21C7 and 8A2 mAbs cross-linked to Protein-G beads, respectively (Figure 2.3B).

Figure 2.3. A method for purification and identification of the proteins that are differentially modified by endogenous SUMO-2/3 between metastatic and non-metastatic breast cancer cells. (A) A model elucidates how
SUMO-ePIPE can be used for purification of endogenous SUMO-conjugates. Endogenous SUMO-conjugates are immunoprecipitated from cell lysates using SUMO-specific mAbs immobilized on the beads. A buffer with low/high pH or SDS detergent elutes both SUMO-conjugates and contaminant proteins (X, Y and Z), whereas an excess amount of SUMO-epitope peptides specifically elute SUMO-conjugates. (B) The SUMO-1- and SUMO-2/3-epitope-peptides specifically eluted SUMO-1- and SUMO-2/3-conjugates immunoprecipitated from 293T cell lysates, whereas 1× SDS sample buffer (1× SSB) non-specifically eluted both SUMO-conjugates and contaminant proteins including heavy chains (H.C.) and light chains (L.C.) of mAbs. Notes: **, free SUMO; *, SUMO-epitope peptide.

Figure 2.4. Mapping of the epitope peptide sequences of mouse mAbs specific to SUMO-1 (21C7) and SUMO-2/3 (8A2). (A and C) The diagrams show the procedures for mapping the epitope peptide sequences of mAbs 21C7 (A) and 8A2 (C). The overlapping fragments of GST-tagged SUMO-1 (A) and SUMO-2 (C) were cloned using pGEX-4T-1 vector, expressed in E. coli BL21 (DE3) cells, purified using Glutathione Sepharose 4B (GE Healthcare), and analyzed by immunoblotting with mAbs 21C7 and 8A2 to identify the smallest fragment containing SUMO-1 or SUMO-2 epitope peptides, respectively. The fragments containing the SUMO-1 or SUMO-2 epitope peptide are highlighted in red or blue boxes. Following three rounds of subcloning and immunoblotting analysis, the SUMO-1 epitope peptide sequences were mapped to two overlapping fragments including B34 (13 aa) and B35 (12 aa), whereas the SUMO-2 epitope peptide sequences were mapped to C14 (12 aa) and C15 (13 aa). (B and D) Purified GST-SUMO-1 or GST-SUMO-2 fragments were analyzed by SDS-PAGE and Coomassie blue staining. After each round
of sub-cloning, the epitope-containing region was determined by immunoblot analysis using mAbs 21C7 (B) or 8A2 (D). After the third round, the epitope region was mapped to ~12 amino acids in length. The DNA primers for PCR amplification of each SUMO-1 and SUMO-2 fragment are listed in Appendix A Table A.1 and A.2.

Figure 2.5. Identification of the SUMO-1 and SUMO-2 epitope peptide sequences recognized by anti-SUMO-1 (21C7) and anti-SUMO-2/3 (8A2) mAbs. (A and E) The diagrams show the different sizes of SUMO-1 (A) and SUMO-2 (E) fragments that can be detected by 21C7 and 8A2 mAbs in immunoblotting analyses, respectively. The epitope peptide sequences of SUMO-1 (B34) and SUMO-2 (C14) fragments are framed by open boxes. (B and F) The locations of SUMO-1 (B) and SUMO-2 (F) epitope peptide sequences on their corresponding 3D structures are highlighted by the pink arrows. (C and D) The GST-SUMO-1 full-length (97 aa), B3 (21 aa), B34 (13 aa), B35 (12 aa) and B36 (10 aa) fragments were analyzed by immunoblotting with mAbs specific to SUMO-1 (21C7) and GST (Santa Cruz) (C). The immunoblotting signals of SUMO-1 were quantified using Image J software (NIH) and then normalized with the corresponding signals of GST (D). Compared to the other two short fragments including B35 and B36, B34 showed a relatively higher SUMO-1/GST signal ratio and thus was considered as the SUMO-1-epitope peptide for 21C7 mAbs. (G and H) The GST-SUMO-2 full-length (93 aa), C1 (22 aa), C13 (12 aa), C14 (12 aa) and
C16 (10 aa) fragments were analyzed by immunoblotting with mAbs specific to SUMO-2 (8A2) and GST (G). The immunoblotting signals of SUMO-2 were normalized with those of GST (H). Compared to the other two short fragments including C13 and C16, C14 showed a relatively higher SUMO-2/GST signal ratio and thereby was considered as the SUMO-2-epitope peptide for 8A2 mAbs.

Notably, our sequence analyses using Jalview software (Waterhouse et al., 2009) revealed that the SUMO-1 and SUMO-2/3 epitope peptides recognized by mAbs 21C7 and 8A2 are widely conserved across all vertebrate species (Figure 2.6A and B). In addition, the single SUMO in D. melanogaster contains a nearly identical sequence with just a valine (V) different from the conserved isoleucine (I) of the SUMO-2/3-epitope peptides (Figure 2.6B). Consistent with the result reported by Melchior and colleagues (Becker et al., 2013), 8A2 mAbs can detect endogenous SUMO-conjugates in Drosophila S2 cells. Interestingly, we found that levels of SUMO-conjugates in Drosophila S2 cells are at least three-fold higher than those of SUMO-2/3-conjugates in human HeLa cells after normalized with α-Tubulin as a loading control for total proteins (Figure 2.6C). Furthermore, we demonstrated that the SUMO-2/3-epitope peptides can be used to elute the endogenous SUMO-conjugates immunoprecipitated from Drosophila S2 cells by 8A2 mAbs with high specificity and efficiency (Figure 2.6D). Therefore, these results indicated that SUMO-2/3-ePIPE is broadly applicable for affinity purification of endogenous SUMO-2/3-conjugates in all the vertebrate species as well as the single SUMO-modified proteins in certain invertebrate species including D. melanogaster.
Figure 2.6. The alignment of SUMO-epitope-peptide sequences among SUMO sequences of different species and the application of SUMO-2/3-ePIPE for purification of endogenous SUMO-conjugates in Drosophila S2 cells. (A) The SUMO-1-epitope-peptide sequence for 21C7 is conserved among vertebrate species. The SUMO-1 proteins from human (H. sapiens), mouse (M. musculus), chicken (G. gallus) and Xenopus (X. laevis) contain the identical SUMO-1-epitope-peptide sequence, whereas the SUMO-1 protein from zebrafish (D. rerio) contains a nearly identical sequence with only a valine (V) different from the methionine (M) conserved in other vertebrate species. This result suggests that SUMO-1-ePIPE should be applicable for purification of endogenous SUMO-1-conjugates in most vertebrate species. (B) The SUMO-2/3-epitope-peptide sequence for 8A2 is highly conserved among a variety of species ranging from human to Drosophila. The SUMO-2/3 proteins from all the analyzed vertebrate species contain the identical SUMO-2/3-epitope-peptide sequence, whereas the sole SUMO protein from the invertebrate species D. melanogaster contains an almost identical sequence with just a valine (V) residue different from the isoleucine (I) residue conserved among vertebrate species. This result suggests that SUMO-2/3-ePIPE should be applicable for purification of endogenous SUMO-2/3-conjugates in most vertebrate species and certain invertebrate species including D. melanogaster. (C) The total cell lysates from human HeLa and Drosophila S2 cells were immunoblotted using mAbs specific to α-Tubulin and SUMO-2/3 (8A2). (D) Endogenous SUMO-conjugates in Drosophila S2 cells were immunoprecipitated by 8A2, eluted by SUMO-2/3-epitope-peptides, and then blotted with 8A2. Arrow head indicates the heavy chains of 8A2 eluted by 2× SSB.

Lastly, we showed that SUMO-2/3-epitope peptides can be used for purification of endogenous SUMO-2/3-conjugates in human brain tumor samples. Glioblastoma multiforme (GBM) is the most common and lethal type of human primary brain tumor (Yang et al., 2013). Levels of SUMO-2/3-conjugates are up-regulated about 29.0-fold in GBM samples compared to control samples (Yang et al., 2013). Total proteins were extracted from ~100 mg of GBM samples as described (Yang et al., 2013) and used for immunoprecipitation of SUMO-2/3-conjugates using 8A2 mAbs. Immunoprecipitated SUMO-2/3-conjugates could be specifically eluted by SUMO-2/3-epitope peptides when compared to control peptide elution (Figure 2.7).
Figure 2.7. Affinity purification of endogenous SUMO-2/3-conjugates in human glioblastoma (GBM) tumor tissues using SUMO-2/3-ePIPE. About 100 mg of GBM tumor tissue samples were used for immunopurification of endogenous SUMO-2/3-conjugates using anti-SUMO-2/3 mAbs (8A2) immobilized on Protein-G beads. The immunoprecipitated SUMO-2/3-conjugates were eluted by either control or SUMO-2/3-epitope peptides. Different percentages of control- or epitope-peptide elution products were separated by SDS-PAGE and analyzed by immunoblotting using 8A2 mAbs (3%) (A), silver staining (15%) (B) and Coomassie blue staining (82%) (C).

### 2.4.3 Analysis of Changes in SUMO-2/3 Modification in Metastatic Breast Cancer Cells

Having established SUMO-ePIPE as an efficient method for purifying endogenous SUMO-conjugates, we next applied this technique to characterize the relationships between the metastatic progression of breast cancer and the changes of SUMO-2/3 modification. We focused on identifying the proteins differentially modified by SUMO-2/3 in metastatic 66cl4 cells compared to non-metastatic 1F68FARN cells. To achieve this goal, we coupled SUMO-2/3-ePIPE with SILAC-based quantitative mass spectrometry (Andersen et al., 2009; Schimmel et al., 2008; Tatham et al., 2011; Vertegaal et al., 2006; Westman et al., 2010; Yang et al., 2012). We first cultured 168FARN cells in light (L) medium (Arg0, Lys0) and 66cl4 cells in heavy (H) medium (Arg10, Lys8) (Figure 2.8A). The SILAC-labeled 168FARN and 66cl4 cells (~ 3x10^10 total) were then lysed under stringent denaturing conditions including 10 mM NEM (N-ethylmaleimide) to block de-SUMOylation mediated by SUMO-isopeptidases. The cell lysates were mixed in a 1:1
ratio of total proteins and then passed through a 2 ml column of Protein G beads cross-linked to 8A2 mAbs. The immunoprecipitated SUMO-2/3 conjugates were eluted by either control- or SUMO-2/3-epitope-peptides, enriched by TCA precipitation, and analyzed by immunoblotting, silver staining and Coomassie-blue staining (Figure 2.8B-D). Using 100 ng of His<sub>6</sub>-SUMO-2 as a loading control, we estimated that about 2-3 μg of SUMO-2/3-conjugates were purified by SUMO-2/3-ePIPE. Notably, few proteins were eluted by control peptides, indicating the high specificity of SUMO-2/3-ePIPE in eluting SUMO-2/3 conjugates. The gel lanes corresponding to control- and epitope-peptide elution were sliced and used for quantitative mass spectrometry (Figure 2.8D).

Figure 2.8. SUMO-2/3 epitope peptide elution. (A) A diagram shows that SUMO-2/3-ePIPE can be coupled with SILAC-based quantitative mass spectrometry for identifying the proteins differentially modified by endogenous SUMO-2/3 between non-metastatic 168FARN and metastatic 66cl4 cells. (B-D) SUMO-2/3-ePIPE was used to purify a large quantity of endogenous SUMO-2/3-conjugates from SILAC-labeled 168FARN and 66cl4 cells for quantitative proteomic analysis. The immunoprecipitated SUMO-2/3 conjugates were eluted using control or SUMO-2/3-epitope peptides, analyzed by immunoblotting (B), silver staining (C), and Coomassie Blue staining (D). His<sub>6</sub>-SUMO-2 proteins (100 ng) were used as a loading control for quantification of immunopurified SUMO-2/3-conjugates (B and C).

The nanoflow liquid chromatography-tandem mass spectrometry analyses (LC-MS/MS) have identified 66 putative SUMO-2/3 substrates after subtracting the background from a total of 151 unique proteins (Supplementary dataset 1). Proteins identified in both SUMO-2/3-ePIPE (SE)
and control-peptide elution (CL) samples with a SE/CL ratio less than two were eliminated. Among the 66 putative substrates, 47 proteins were detected with a SILAC H/L ratio that represents the relative levels of SUMO-2/3 modification on each protein between 66cl4 and 168FARN cells (Dataset 1). We analyzed the gene ontology of these 66 substrates using the STRING program (Franceschini et al., 2013) and grouped them into seven categories based on their primary biological functions, including gene expression (35%), metabolism (26%), cell migration (14%), stress response (8%), cell cycle (8%), SUMO/ubiquitin pathways (6%), and inflammation (5%) (Figure 2.9A). Using the STRING program (Franceschini et al., 2013), we also identified six major high-confidence interaction networks within the 66 substrates, including cell migration, translation, glycolysis, SUMO/ubiquitin pathways, cell cycle, and stress response (Figure 2.9B).

To evaluate the quality of our proteomic analyses, we compared the above 66 SUMO-2/3 substrates with those identified by three well-recognized proteomic studies of SUMO-2/3 modification using human HeLa cells (Bruderer et al., 2011; Schimmel et al., 2008; Tatham et al., 2011) and also with those whose SUMOylation was validated previously (Figure 2.9A and Supplementary dataset 1 including references). We found that 50 of the 66 substrates (76%) overlap with those identified by the three proteomic studies, whereas 22 proteins (33%) have been formerly validated. Together, 51 substrates (77%) are classified as “Known SUMO Substrates” based on the three previous proteomic studies and also the studies for validation of individual substrates. Furthermore, 45 proteins (68%) have been previously implicated in cancer and thus categorized as “Linked to Cancer”, whereas 37 proteins (56%) represent an overlap between “Known SUMO Substrates” and “Linked to Cancer”. Lastly, we compared to the SUMO-2/3 substrates identified in this study with those identified by Melchior and colleagues using human
HeLa cells and also a technique nearly identical to our SUMO-2/3-ePIPE (Becker et al., 2013). We found an overlap of eight substrates between these two studies, including PML, SAE2, SUMO-2/3, Trim28, H2B, RPS9, RPS16, and NUMA1. This limited overlap between these two proteomic studies was at least partially caused by using different cells, human cervical cancer cells (HeLa) and mouse breast cancer cells (168FARN and 66c14).

Figure 2.9. Functional classification and interaction network analysis of the SUMO-2/3-conjugated protein identified in metastatic and non-metastatic breast cancer cells. (A) Among the 66 SUMO-2/3 substrates, the percentage of unique proteins in each functional category is indicated in parentheses. These proteins were classified...
into the seven functional groups. Alternatively, they were classified into three different categories, including 51 “Known SUMO Substrates”, 45 proteins “Linked to Cancer”, and 7 proteins as “Other” that have not been previously identified as either “Known SUMO Substrates” or “Linked to Cancer”. (B) The high-confidence interaction networks within the putative SUMO-2/3 substrates identified by SUMO-2/3-ePIPE and mass spectrometry. The above SUMO substrates are indicated by their official gene names. The protein-protein interaction networks were obtained using the STRING database (version 9.1) with a high-confidence level (0.700). The major interaction networks include the SUMO-2/3-modified proteins known to play a role in cell migration, glycolysis, protein translation, cell cycle, stress response, and SUMO/Ubiquitin pathways. The “Known SUMO Substrates” are highlighted by black circle. The proteins with at least a 50% increase (H/L≥1.5) or decrease (H/L≤0.5) of SUMO-2/3 modification in metastatic cells compared to non-metastatic cells were labeled in red or blue color, respectively, whereas the rest proteins are labeled in light gray color.

Consistent with up-regulation of global SUMO-2/3 modification in 66cl4 cells compared to 168FARN cells (Figure 2.1), our quantitative proteomic analysis revealed that the SILAC H/L ratio of SUMO-2/3 is 2.24 between 66cl4 (H) and 168FARN (L) cells (Table 2.1 and Supplementary dataset 1). Because free SUMO-2/3 was not detected in our SUMO-2/3-ePIPE sample (Figure 2.8B), this H/L ratio likely represents the relative levels of SUMO-2/3-conjugates purified from 66cl4 and 168FARN cells. In addition to the increased levels of SUMO-2/3 detected in the immunopurified SUMO-2/3-conjugates in 66cl4 cells compared to 168FARN cells, our quantitative proteomic analyses have also identified five proteins with more than 50% increase of SUMO-2/3 conjugation in 66cl4 cells (H/L≥1.5), including PML (6.15-fold), MIF (2.42-fold), α-Enolase (2.01-fold), Fructose-1,6-bisphosphate aldolase (Aldolase A) (1.79-fold), and eEF2 (Elongation Factor 2) (1.54-fold). These proteins are known to play a critical role in cell cycle (PML), inflammation (MIF), glycolysis (α-Enolase and Aldolase A) and translation (eEF2) (Table 2.1). On the other hand, we also identified about ten proteins with a significant decrease of SUMO-2/3 modification in 66cl4 cells compared to 168FARN cells (SILAC H/L≤0.5) (Table 2.1 and Supplementary dataset 1). These proteins of particular interest, including Vimentin (0.34-fold), Plectin (0.33-fold), Gelsolin (0.19-fold), and Fibronectin (0.18-fold), are involved in cell migration.
Table 2.1. Endogenous SUMO-2/3-Conjugated Proteins Identified with SILAC Heavy / Light (H/L) Ratios by Quantitative Mass Spectrometry

<table>
<thead>
<tr>
<th>Inflammation</th>
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<tr>
<td>MIF* Kininogen-1*</td>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>PML*, α-Tubulin, β-Tubulin</td>
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<tr>
<td>SUMO / Ub Pathways</td>
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<tr>
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<tr>
<td>Stress Response</td>
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<tr>
<td>Murinoglobulin*, Hsp70, Hsp90β, Cyclophilin A, SOD1,</td>
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<tr>
<td>Cell Migration</td>
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<tr>
<td>Metabolism</td>
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<tr>
<td>α-Enolase*, Aldolase A*, PKM2, LDH</td>
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<tr>
<td>ATP synthase α and β subunits</td>
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*The proteins with a significant increased or decreased SUMO-2/3 modification in metastatic cells (H) compared to non-metastatic cells (L) are marked by “+” (H/L ≥ 1.50) or “−” (H/L ≤ 0.50).

2.4.4 Validating Putative SUMO-2/3 Substrates by in vitro SUMOylation Assays and Confirming Changes of SUMO-2/3 Modification of Individual Substrates in Metastatic Cells

To verify the proteomic analysis, we first used the well-established bacterial SUMOylation assays (Uchimura et al., 2004) to test whether the selected SUMO-2/3 substrates, including MIF, α-Enolase, Actinin-4, RhoGDI and Gelsolin, can efficiently modified in vitro. These proteins were expressed as GST-tagged fusion proteins in the presence or absence of SUMO-2, SUMO-E1 and Ubc9 in E. coli BL21 cells (Figure 2.10C-G). The GST and GST-tagged PML (a well-established SUMO substrate) were used as negative and positive control, respectively (Figure 2.10A and B). We found that all six GST-tagged fusion proteins but not GST were efficiently modified by
Further, we demonstrated that Vimentin as well as the conserved motor domain of Myosin-12 were efficiently modified by SUMO-1 and SUMO-2 in vitro by using recombinant E1, Ubc9 and SUMO-1 or SUMO-2 (Figure 2.10H and J). Because the motor domain is highly conserved among the Myosin family members (Mermall et al., 1998), this result suggests that various Myosin members, including Myosin-9, which was identified as a putative SUMO-2/3-conjugated substrate in this study, might be modified by SUMOs within their conserved motor domains. Consistent with this idea, both Myosin-9 and Myosin-12 contain at least one SUMOylation consensus motif with a high score (≥0.85) predicted by the SUMOplot Analysis Program (Abgent) (Figure 2.10I).

Figure 2.10. Validation of SUMO substrates by in vitro SUMOylation assays. (A-G) Bacterial SUMOylation assays were used to validate five SUMO-2/3 substrates including GST-tagged MIF (C), α-Enolase (D), Actinin-4 (E), RhoGDI (F) and Gelsolin (G), while GST (A) and GST-tagged PML (B) were used as negative and positive controls, respectively. The purified GST or GST-tagged proteins were analyzed by immunoblotting with antibodies specific to GST and SUMO-2. (H) GST-tagged Vimentin was incubated with recombinant SUMO-E1 and Ubc9 in the presence or absence of SUMO-1 or SUMO-2, and analyzed by immunoblotting with anti-GST antibodies. (I) The SUMOplot
The Analysis Program (Abgent) was used to predict the SUMOylation consensus motif (ΨKxE/D) within the conserved motor domains of human Myosin family members, including Myosin-9 (1960 aa) (UniProt: P35579) and Myosin-12 (1855 aa) (UniProt: Q9Y4I1) (Mermall et al., 1998). The three lysine residues (K228, K289 and K355) within the Myosin-9 motor domain (77-775 aa) as well as the lysine residue (K471) within the Myosin-12 motor domain have the highest SUMOplot scores and are thereby marked in bold and also underlined. (F) The purified recombinant FLAG-tagged Myosin-12 motor domain was incubated with recombinant SUMO-E1 and Ubc9 in the presence or absence of SUMO-1 or SUMO-2 and analyzed by immunoblotting with mAbs specific to FLAG.

Our quantitative proteomic analysis revealed that SUMO-2/3 modification of PML (6.15-fold), MIF (2.42-fold), α-Enolase (2.01-fold), Pyruvate kinase M2 (PKM2) (1.37-fold), hnRNP U (0.49-fold) was significantly up- or down-regulated in 66cl4 cells compared to 168FARN cells. To confirm the changes of SUMO-2/3 modification of these substrates in 66cl4 cells, we immunopurified SUMO-2/3 conjugates from 168FARN and 66cl4 cells, respectively, and then immunoblotted with antibodies specific to α-Tubulin, SUMO-2/3, MIF, α-Enolase, PKM2, hnRNP U and PML (Figure 2.11A-F). Consistent with our quantitative mass spectroemtry result, immunoblotting analysis demonstrated that SUMO-2/3 modification of MIF, α-Enolase, PKM2 and PML was at least two to six times higher in 66cl4 cells than in 168FARN cells (Figure 2.11B-D and F). Although our quantitative mass spectroemtry result suggested a decrease of SUMO-2/3 modification of hnRNP U in 66cl4 cells, immunoblotting analysis of two independently immunopurified SUMO-2/3 conjugates has reproducibly shown that SUMO-2/3 modification of hnRNP U was about three times higher in 66cl4 cells than in 168FARN cells (Figure 2.11E). Unmodified MIF was observed as a 15 kDa band, whereas SUMO-2/3-modified MIF was detected as a smear from 30 to 45 kDa, indicating that MIF could be modified by one to three SUMO-2/3 moieties (Figure 2.11B). Furthermore, unmodified α-Enolase and PKM2 were found with the corresponding sizes of 45 and 58 kDa, while their SUMO-2/3-modified forms were observed as a high-molecular-weight smear and also as distinct bands ranging from 60 to 200 kDa and from 72 to 180 kDa respectively, suggesting that both substrates were modified at multiple sites and/or by poly-SUMO-2/3-chains (Figure 2.11C and D). Moreover, unmodified hnRNP U was detected as
a ~120 kDa band, whereas SUMO-2/3-modified hnRNP U was observed as a major band of ~200 kDa, suggesting that hnRNP U could be simultaneously modified by a fixed number of SUMO-2/3 moieties. Consistent with this, mouse hnRNP U (NP_058085.2) contains seven SUMOylation consensus motifs with high scores (0.61 to 0.91) predicted by the SUMOplot Analysis Program (Abgent). Lastly, three isoforms of unmodified PML were found to be around 65, 75 and 90 kDa in 66cl4 cells, whereas its SUMO-2/3-modified forms were detected as a high-molecular-weight smear from ~117 to >201 kDa, suggesting that multiple SUMO-2/3 moieties were conjugated to PML at different sites and/or as poly-SUMO-2/3-chains (Figure 2.11F). Consistent with this idea, endogenous poly-SUMO-chain-modified PML was also detected in HeLa cells (Bruderer et al., 2011).
Figure 2.11. Verifying the changes of SUMO-2/3 modification of individual substrates in metastatic cells and investigating the effects of SUMO-23 modification on PML-NB assembly and 3-D cell migration. (A-F) SUMO-2/3 conjugates were immunoprecipitated from 168FARN and 66cl4 cells, eluted by either control or SUMO-2/3-epitope peptides, and analyzed by immunoblotting using antibodies specific to SUMO-2/3 (A), MIF (B), α-Enolase (C), PKM2 (D), hnRNP U (E), and PML (F). The arrows indicate unmodified proteins (B-F). (G) Cells were double labeled with rabbit anti-PML (red) and mouse anti-SUMO-2/3 (green) antibodies and analyzed by indirect immunofluorescence microscopy. Bar equals 5 μm. (H) The number of PML-NBs was significantly higher in 66cl4 cells (5.45/cell) than in 168FARN cells (2.75/cell). We randomly selected 100 cells from each of the two lines, 168FARN and 66cl4, and counted all the PML-NBs in each cell under Olympus inverted IX81 fluorescence microscope using Z-stacks. The number of PML-NBs in each cell for all the analyzed cells was included in Supplementary dataset 1. Each column represents the mean value ± SEM (N = 100, *P = 6.5x10^{-24}, Student’s t test). (I) Overexpression of GFP-SUMO-2/3 in 168FARN cells increases the linear speed (μm/min) of 3D cell migration within a collagen gel. Each bar represents the mean value ± SEM from 10 sets of time-lapse microscopy videos of 168FARN cells expressing GFP or GFP-SUMO-2/3 proteins (N = 10, *P = 3.0 × 10^{-6}, Student’s t test).
2.4.5 Functional Analyses of Enhanced PML and Global SUMO-2/3 Modification on PML-NB Assembly and 3D Cell Migration

It has been shown previously that both SUMO-modification of PML and its SUMO-binding activities are required for the assembly of PML nuclear bodies (PML-NBs) (Shen et al., 2006b). To test if up-regulation of SUMO-2/3 modification of PML increases the formation of PML-NBs in 66cl4 cells, we compared the number of PML-NBs between 168FARN and 66cl4 cells by immunofluorescence microscopy using antibodies specific to PML and SUMO-2/3 (Figure 2.11G). Consistent with our hypothesis, the average number of PML-NBs was significantly higher in 66cl4 cells (5.45/cell) than in 168FARN cells (2.75/cell) (Figure 2.11H).

A previous study has shown that the linear speed of 3D cell migration of metastatic 66cl4 cells is higher than that of non-metastatic 168FARN cells (Indra et al., 2011), which is positively correlated to the up-regulation of SUMO-23 modification in 66cl4 cells compared to 168FARN cells (Figure 2.1). Furthermore, our proteomic analysis of changes in SUMO-2/3 modification between 66cl4 and 168FARN cells has revealed a list of putative SUMO substrates involved in cell migration (Table 2.1), suggesting that perturbations of SUMO-2/3 modification may affect cell migration. To test whether up-regulation of global SUMO-2/3 modification contributes to breast cancer metastasis by promoting cell migration (Indra et al., 2011), we overexpressed both GFP-tagged SUMO-2 and SUMO-3 fusion proteins in non-metastatic 168FARN cells. Overexpression of GFP-SUMO-2/3 is known to increase global SUMO-2/3 modification in human HeLa cells (Ayaydin and Dasso, 2004). Consistent with our hypothesis, we found that overexpression of GFP-SUMO-2/3 in 168FARN cells leads to a two-fold increase of 3D migration speed compared to control cells overexpressing GFP (Figure 2.11I).
Figure 2.12 Overexpression of GFP-tagged SUMO-2/3 wild-type or non-conjugatable mutants increases 3D cell migration. (A) 293T cells were transfected with either GFP empty vector or plasmids encoding GFP-SUMO-2, GFP-SUMO-3 or GFP-SUMO-2/3 wild-type (WT) and also their corresponding non-conjugatable mutants. Site-directed mutagenesis was used to mutate the DNA sequences encoding the glycine-glycine (GG) residues at the C-terminal end of mature SUMO-2 and SUMO-3 WT to those encoding alanine-alanine (AA) residues of non-conjugatable mutants. The transfected cells were lysed in 2× SSB and analyzed by immunoblotting with antibodies specific to GFP and α-Tubulin. (B) Overexpression of GFP-SUMO-2/3 (GFP-S2/3) WT or their non-conjugatable mutants in 168FARN cells increases the linear speed (μm/min) of 3D cell migration within a collagen gel. Each bar represents the mean value ± SEM from 10 sets of time-lapse microscopy videos of 168FARN cells expressing GFP, GFP-S2/3 WT, or GFP-S2/3 Mutant (N = 10, P < 0.0001, Student’s t test).

Futhermore, we tested whether the increase of 3D cell migration mediated by overexpression of GFP-SUMO-2/3 is definitely dependent on the conjugation activity of GFP-SUMO-2/3. Interestingly, we found that overexpression of either GFP-SUMO-2/3 wild-type (WT) or non-conjugatable mutant significantly increased 3D cell migration (Figure 2.12), suggesting that free SUMO-2/3 somehow could also promote cell migration. Consistent with this idea, levels of both high-molecular weight SUMO-2/3 conjugates and unconjugated SUMO-2/3 in 66cl4 are higher than in 168FARN cells (Figure 2.1).
Figure 2.13 A model elucidates that dysregulation of SUMOylation might contribute to breast cancer progression and metastasis by affecting multiple cellular processes, including cell migration, cell cycle, inflammation, glycolysis, SUMO/ubiquitin pathway, and gene expression.

2.5 DISCUSSION

Most recent proteomic studies of SUMOylation often involve the development of stably transfected cells expressing affinity-tagged-SUMO variants (Schimmel et al., 2008; Tatham et al., 2011; Vertegaal et al., 2004). The expression of tagged-SUMO over the background of endogenous SUMOs likely affects many processes critical for cancer. Clearly, the lack of an effective strategy to identify the targets differentially modified by endogenous SUMO between cells at different stages of cancer has obstructed the progression toward elucidating how SUMOylation affects cancer progression and metastasis. In this study, we established a proteomic method by coupling SUMO-2/3-ePIPE with SILAC-based quantitative mass spectrometry for analysis of the changes in SUMO-2/3 modification between metastatic and non-metastatic cells. Using this method, we identified 66 putative SUMO-2/3 targets, in which at least 15 proteins exhibit a significant increase/decrease of SUMO-2/3 modification in metastatic 66cl4 cells when
compared non-metastatic 168FARN cells. Furthermore, we validated the increase of SUMO-2/3 modification of PML, MIF, α-Enolase and PKM2 in 66cl4 cells compared to 168FARN cells (Figure 2.11). Clearly, our results demonstrated that this proteomic method is a very useful tool for identifying the proteins whose SUMO-modification is different or altered between two biological samples of particular interest. These samples could be originated from various sources ranging from human to *Drosophila* and from cultured cells to tissue samples. Moreover, our immunofluorescence microscopy analysis indicated that up-regulation of SUMO-2/3 modification of PML is associated with an increased number of PML-NBs in 66cl4 cells compared to 168FARN cells (Figure 2.11G and H), suggesting that the increase of SUMO-2/3 modification of PML may promote the assembly of PML-NBs in 66cl4 cells. Consistent with this idea, the massive increase of SUMOylation triggered by hypothermia stress is known to elevate the assembly of PML-NBs in both number and size (Lee et al., 2007). Although PML has been regarded as a tumor suppressor, recent studies have revealed that PML can also play a pro-survival role and its overexpression is associated with a subset of triple-negative breast cancers (Carracedo et al., 2012). Hence, it is possible that up-regulation of PML SUMO-2/3-modification and PML-NB assembly may contribute to the increased metastatic capacity of 66cl4 cells compared to 168FARN cells.

Notably, we have identified and confirmed MIF as a novel SUMO substrate both *in vitro* and *in vivo*. As a key pro-inflammatory cytokine and breast cancer marker, MIF represents a direct link between inflammation and cancer by contributing to a microenvironment that favors cancer growth and progression (Conroy et al., 2010; Grieb et al., 2010; Simpson et al., 2012; Verjans et al., 2009). Therefore, it would be very interesting to explore whether up-regulation of SUMO-2/3 modification of MIF plays a role in breast cancer progression and metastasis.
Similar to ubiquitination, SUMO-2/3 modification is up-regulated in response to various types of stress and thus serves as an important mechanism to protect cells from stress-induced cell death or apoptosis (Guo et al., 2013; Saitoh and Hinchey, 2000). Furthermore, up-regulation of SUMOylation has been frequently observed in cancers with poor prognosis (Bawa-Khalfe and Yeh, 2010; Kessler et al., 2012; Kim and Baek, 2006). Hence, it is possible that compared to non-metastatic cells, up-regulation of SUMO-2/3 modification in metastatic cells may presumably increase the capacity of these cells in response to various types of stress and protect them from stress-induced death so that they are capable to migrate, survive, proliferate and form secondary tumors at distal organ(s). Consistent with this idea, we found that SUMO-2/3 modification of eEF2, an essential factor for protein synthesis, was up-regulated more than 50% in 66cl4 cells than in 168FARN cells. SUMOylation of eEF2 is not only involved in drug resistance in lung carcinoma cells but also critical for its stability and anti-apoptotic activity (Chen et al., 2011). In addition, we found that levels of ubiquitin (1.5-fold) covalently associated with the immunopurified SUMO-2/3-conjugates in 66cl4 cells are 50% higher than those in 168FARN cells, which is positively correlated to the increase of SUMO-2/3 modification in 66cl4 cells. This positive correlation likely reflects the tight link between ubiquitination and polymeric SUMO-2/3 modification, since it is known that poly-SUMO-2/3-chains can be recognized by the SUMO-targeted ubiquitin ligase (STUbL), RNF4, leading to ubiquitination of poly-SUMO-2/3-chain-modified proteins (Tatham et al., 2008; Tatham et al., 2011).

Although up-regulation of Ubc9 expression is known to increase global SUMOylation and also promote cancer cell proliferation, invasion and metastasis, (Kim et al., 2006; Mo et al., 2005) we did not detect any obvious change of Ubc9 expression among these four breast cancer cell lines (Figure 2.1). On the other hand, our quantitative proteomic analysis has shown that SUMO-2/3
modification of SAE2, one of the SUMO-E1 subunits, is ~43% lower in 66cl4 cells than in 168FARN cells (Supplementary Dataset 1). Since SUMOylation of SAE2 can significantly reduce SUMOylation by inhibiting the transfer of SUMO from E1 to Ubc9 (Truong et al., 2012), the decrease of SUMO-2/3 conjugation to SAE2 may partially contribute to the increase of global SUMO-2/3 modification in 66cl4 cells.

Interestingly, our proteomic analyses have revealed a network of nine SUMO-2/3 substrates critical for cell migration (Table 2.1). We have successfully validated five of them, including α-Actinin-4, RhoGDI, Vimentin, Myosin-9 and Gelsolin by in vitro SUMOylation assays. Our proteomic analysis revealed that SUMO-2/3 modification of RhoGDI is about 32% lower in 66cl4 cells than in 168FARN cells (Supplementary Dataset 1). Because SUMOylation of RhoGDI is known to inhibit cell migration (Yu et al., 2012), the decreased SUMO-2/3 modification of RhoGDI in 66cl4 cells may partially contribute to the increased speed of 3D cell migration of these cells compared to 168FARN cells (Indra et al., 2011). Clearly, the decrease of SUMO-2/3 modification of several proteins in 66cl4 cells when compared to 168FARN cells, including RhoGDI, Gelsolin and Vimentin, is in contrast to the increase of global SUMO-2/3 modification in 66cl4 cells. This indicated that up-regulation of global SUMO-2/3 modification reflects a combination of both increase and decrease of SUMO-2/3 modification of all individual substrates in 66cl4 cells. Consistent with the previous finding that the increase of global SUMOylation promotes cell migration and invasion (Kim et al., 2006), here we showed that up-regulation of global SUMO-2/3 modification by overexpressing GFP-SUMO-2/3 can also lead to a significant increase of 3D cell migration (Figure 2.11I). Since SUMOylation of Rac1 can increase its ability to stimulate lamellipodia formation, cell migration and invasion (Castillo-Lluva et al., 2010), it would be very interesting to investigate if SUMO-2/3 modification of Rac1 is up-
regulated in 66cl4 cells when compared to 168FARN cells. Interestingly, we found that overexpression of non-conjugatable GFP-SUMO-2/3 mutant also significantly increased 3D cell migration (Figure 2.12), suggesting that free SUMO-2/3 can also promote cell migration through unknown mechanism(s) independent of their conjugation activities. Overexpression of non-conjugatable GFP-SUMO-2/3 mutants might indirectly affect SUMOylation of certain substrates critical for cell migration and/or competitively inhibit the interaction of these SUMOylated substrates with their binding proteins containing SUMO-interacting motif (SIM), leading to an increase of 3D migration.

A nearly-universal property of cancer cells is up-regulation of glycolysis even under aerobic conditions, a phenomenon known as Warburg effect, which promotes both proliferation and invasion (Gatenby and Gillies, 2004). We have identified a group of enzymes involved in glycolysis as SUMO-2/3 substrates including α-Enolase, PKM2, Aldolase A, and Lactate dehydrogenase (LDH) (Table 2.1 and Figure 2.9B). All the enzymes were also found as SUMO-2/3 targets by the three proteomic studies of SUMO-2/3 modification in HeLa cells (Bruderer et al., 2011; Schimmel et al., 2008; Tatham et al., 2011), and their activity and expression are significantly higher in breast cancer metastases compared to the primary tumors (Fantin et al., 2006; Hennipman et al., 1988; Radenkovic et al., 2012). Intriguingly, levels of SUMO-2/3 modification on all the enzymes are up-regulated in 66cl4 cells compared to 168FARN cells (Supplementary Dataset 1). Studies in yeast revealed that hypoxia stimulates SUMOylation of several key glycolytic enzymes, including α-Enolase, Pyruvate kinase, and Aldolase A (Agbor et al., 2010). Furthermore, up-regulation of global SUMOylation by overexpressing His-SUMO-1 in mouse embryonic fibroblast cells greatly increases glycolysis under normoxic conditions (Agbor et al., 2010). Hence, the increase of SUMO-2/3 modification on multiple glycolytic
enzymes could stimulate glycolysis in 66cl4 cells and thus contribute to the increased metastatic capacity of these cells compared to 168FARN cells.

Compared to the metastatic 66cl4 cells, the non-metastatic 168FARN cells do not have the capacities to exit the lymphatic vessels as well as to establish the secondary tumors. Hence, the changes in SUMO-2/3 modification of these identified target proteins in 66cl4 cells compared to 168FARN cells might contribute to the metastatic capacity of 66cl4 cells by promoting their extravasation and metastatic growth in lung. Given the expected effect of host tissue environment on SUMOylation profiles of tumor cells, it would be ideal to perform quantitative proteomic analyses of SUMO-2/3 modification between the two mammary tumors formed by injection of 66cl4 and 168FARN cells into the mammary glands of BALB/c mice, respectively, instead of using these two cell lines cultured in vitro. Since SUMO-2/3-ePIPE can be used for affinity purification of endogenous SUMO-2/3 conjugates in human GBM tumor samples, this approach can be combined with the isobaric tag for relative and absolute quantification (iTRAQ) method and quantitative mass spectrometry analysis (Casado-Vela et al., 2010; Kocher et al., 2009; Miller et al., 2013) to identify the proteins differentially modified by SUMO-2/3 between two mammary tumor samples with different metastatic capacities.

Our findings not only provide important information but also establish a very useful method for further investigation of how perturbations of SUMOylation affect cancer progression and metastasis. Up-regulation of global SUMO-2/3 modification in breast cancer cells may increase the metastatic capacities of these cells by altering multiple cellular processes including glycolysis, cell migration, cell cycle, stress response, inflammation, gene expression, and SUMO/ubiquitin pathways (Figure 2.13). Interestingly, levels of SUMO-1 modification in metastatic 66cl4 cells are significantly lower than those in the three non-metastatic cells, including
67NR, 168FARN and 4TO7 (Figure 2.1). The decrease of SUMO-1 modification in metastatic 66cl4 cells may also contribute to their increased metastatic capacity. To understand the roles of SUMO-1 modification in breast cancer metastasis, we can apply a similar proteomic approach by coupling SUMO-1-ePIPE with SILAC-based quantitative mass spectrometry to identify the substrates with changes of SUMO-1 modification between metastatic and non-metastatic cells. Although the molecular details of how SUMOylation affects cancer progression and metastasis are still not well understood, accumulating lines of evidence have revealed that targeting the SUMO pathway may have therapeutic efficacy for human cancers, especially for types of cancers driven by Myc or Ras oncogene (Kessler et al., 2012; Luo et al., 2009).
CHAPTER 3 POLYMERIC SUMO-2/3 CHAINS AT KINETOCHORES IS ESSENTIAL FOR CHROMOSOME ALIGNMENT BY RECRUITING CENP-E TO KINETOCHORES

3.1 ABSTRACT

Cell cycle progression through mitosis involves several post-translational modifications including phosphorylation, acetylation, ubiquitination, SUMOylation and detyrosination. A number of evidences have indicated the direct role of SUMO modification in precise chromosome alignment and segregation. Inhibition of global SUMOylation either by over-expression of SENP2 or by depletion of Ubc9 results in a prometaphase arrest and disappearance of Centromere Protein E (CENP-E) from kinetochores in mammalian cells. This led us to propose that CENP-E is recruited to kinetochores by binding to SUMO-modified kinetochore interactors (Nuf2) through CENP-E’s SUMO-Interacting Motif. We have shown that Nuf2 is crucial for CENP-E localization to kinetochores and is modified by polymeric SUMO-2/3 chains at multiple lysine residues. In support of the above proposition, we have demonstrated that trimeric SUMO-2 chain fused to the C-terminus of Nuf2 rescues the prometaphase arrest and CENP-E mislocalization caused by SENP2 overexpression. However, monomeric or dimeric SUMO-2 or trimeric SUMO-1 chain fused to Nuf2 does not rescue the mitotic defects caused by inhibition of SUMOylation. Furthermore, we demonstrate that compared to Nuf2 alone, the trimeric SUMO-2 and Nuf2 fusion protein has a higher binding affinity to CENP-E wild-type but not its SUMO Interacting Motif (SIM) mutant.

3.2 INTRODUCTION

SUMOylation is an essential post translational modification involved in many processes, such as DNA repair, nuclear transport, cell cycle progression, and stress response (Flotho and
SUMO-2/3 can form polymeric chains \textit{in vivo} (Tatham et al., 2001), but SUMO-1 appears to be a chain terminator on SUMO-2/3 chains \textit{in vivo} (Matic et al., 2008). Although about 75% of targets get SUMO modified within the SUMO consensus site ($\Psi$-K-X-D/E) (Sampson et al., 2001), SUMO modification can also occur at non-consensus site (Yang et al., 2011).

Several studies have indicated the role of SUMO modification in chromosome alignment and segregation during mitosis (Dasso, 2008; Wan et al., 2012b). Interestingly, it has been reported that SUMO-1, concentrated in the spindle pole and spindle midzone and SUMO-2/3, contained to the mitotic chromosome, clearly have unique localization during mitosis. Smt3 (yeast SUMO) polymeric chains are shown to be essential for chromosome segregation (Srikumar et al., 2013). There are at least ten proteins in the centromere/kinetochore region that are identified as SUMO targets during mitosis in various organisms ranging from yeast to humans, many of them being specifically modified by SUMO-2/3 (Wan et al., 2012b). Furthermore, Ubc9 and the E3 ligases PIAS3 and PIASy are localized on the kinetochores during mitosis (Agostinho et al., 2008; Ban et al., 2011; Joseph et al., 2004). Earlier studies have shown that depletion of yeast Ubc9 results in chromosome segregation defects (Seufert et al., 1995). In mammalian cells, depletion of SENP6 lead to mitotic defects and degradation of Centromere Protein I (Mukhopadhyay et al., 2010).

One of the important proteins for chromosome congression to the metaphase plates, CENP-E, is SUMO-2/3 modified both \textit{in vitro} and \textit{in vivo} (Zhang et al., 2008). CENP-E is a plus end directed kinesin-7 family protein with an N-terminal microtubule binding motor domain, a coiled-coil domain and a C-terminal kinetochore binding domain. During G2 phase of the cell cycle, this kinesin like protein is accumulated and found in the cytosol (Yen et al., 1992). However, during mitosis it is found on the outer kinetochores from prophase through metaphase, and is localized to
the spindle midzone during anaphase (Cooke et al., 1997; Yen et al., 1991). As CENP-E is essential for stable kinetochore microtubule attachments, human cells with depletion of CENP-E are arrested in prometaphase (Putkey et al., 2002; Schaar et al., 1997). Furthermore, anti-CENP-E small molecule inhibitors like UA62784 and GSK923295A exhibit anti-tumor activity (Henderson et al., 2009; Wood et al., 2010). The SUMO Interacting Motif found on CENP-E is required for its kinetochore localization and CENP-E specifically binds to polymeric SUMO-2/3 chains (Zhang et al., 2008).

Of the various kinetochore proteins, Nuf2 (Liu et al., 2007), BubR1 and CENP-F (Chan et al., 1998) are known to interact with CENP-E. Nuf2 is part of a dumb-bell shaped tetrameric complex along with Hec1/Ndc80, Spc24 and Spc25. The C-termini of Spc24 and Spc25 are towards the kinetochore, whereas the N-termini of Spc24 and Spc25 interact with the C-termini of Nuf2 and Hec1, respectively. The N-termini of Nuf2 and Hec1 face the plus ends of the microtubules. Nuf2, localized to the outer kinetochore during all phases of mitosis (Hori et al., 2003), is crucial for stable microtubule-kinetochore attachments and chromosome congression on to metaphase plates (Chan et al., 1998; McCleland et al., 2003). Depletion of Nuf2 leads to prolonged mitotic arrest and eventually apoptosis (DeLuca et al., 2002). Additionally, Nuf2 is specifically modified by SUMO-2/3 in vivo (Zhang et al., 2008).

Recent studies in mammalian cells have shown that depletion of SUMO-2/3 from the centromere by either over-expressing SENP2 or by depleting Ubc9 resulted in a prometaphase arrest and a mislocalization of CENP-E from the kinetochores (Zhang et al., 2008). How does inhibition of SUMOylation lead to disappearance of CENP-E from kinetochores remains an open question. We hypothesized that SUMO modification of one/more of the CENP-E interacting protein/s (Nuf2 and/or BubR1) is/are SUMO modified which is important for recruiting CENP-E
through its SIM. In order to test this hypothesis, we generated various FLAG-tagged Nuf2 constructs encoding FLAG-Nuf2 (negative control), FLAG-Nuf2-SUMO-2, FLAG-Nuf2-2xSUMO-2, FLAG-Nuf2-3xSUMO-2, FLAG-Nuf2-Ubc9 (WT), FLAG-Nuf2-Ubc9 (C93A) and FLAG-Nuf2-3xSUMO-1 fusion constructs. Consistent with our hypothesis, overexpression of FLAG-Nuf2-3xSUMO-2 and FLAG-Nuf2-Ubc9 (WT) but not the other FLAG-Nuf2 fusion proteins rescued the defect in chromosome alignment to metaphase plates by recruiting CENP-E to kinetochores in cells with SENP2 overexpression. Furthermore, we observed that the Nuf2-3xSUMO-2 has higher binding affinity to CENP-E compared to Nuf2. In this chapter, I have demonstrated the molecular mechanism by which CENP-E is recruited to kinetochores by means of SUMO modification of CENP-E-interacting proteins in the kinetochore region.

### 3.3 MATERIALS AND METHODS

**Plasmids, Antibodies and siRNA oligos:**

The pFLAG-Nuf2 construct was generated by cloning Nuf2 in between ClaI and BamHI sites of pFLAG-CMV vector. The pFLAG-Nuf2-3xSUMO-1, pFLAG-Nuf2-3xSUMO-2 and pFLAG-Nuf2-Ubc9(WT) plasmids were created by sub-cloning 3xSUMO-1, 3xSUMO-2 and Ubc9(WT) between BamHI and SmaI to the C-terminus of Nuf2 in pFLAG-Nuf2. Constructs containing 3xSUMO-1 and 3xSUMO-2 were a kind gift from Dr. Michael Matunis’s lab. pFLAG-Nuf2-Ubc9 (C93A) was generated by performing a site-directed mutagenesis to convert Cys93 to Ala in the pFLAG-Nuf2-Ubc9 (WT) construct. pFLAG-Nuf2-SUMO-2 was constructed by subcloning Nuf2-SUMO-2 from pFLAG-Nuf2-3xSUMO-2 vector between ClaI and XbaI in pFLAG-CMV vector. pFLAG-Nuf2-2xSUMO-2 were constructed by creating a stop codon at the end of the second SUMO-2 of pFLAG-Nuf2-3xSUMO-2 by site-directed mutagenesis. For *in vitro* binding assay, Nuf2 and Nuf2-3xSUMO-2 were cloned in to pGEX6p-1 between SmaI and SalI.
sites. pGEX6p-1-Nuf2 (6K/R) used for in vitro SUMOylation assays was obtained by performing site directed mutagenesis using pGEX6p-1-Nuf2 (WT) as template. The primers used for cloning for all the above constructs are listed in Appendix B Table B.1. Primers used for mutagenesis are listed in Appendix B Table B.2. Antibodies used in the chapter are recorded in Appendix B Table B.3. Finally, Appendix B Table B.4 contains the siRNA oligos used in this study.

**Cell culture and transfections:**

Human cervical cancer HeLa cells and Human Embryonic Kidney (HEK) 293T cells were cultured in DMEM media (HyClone) with 1% Penicillin-Streptomycin and enhanced with 10% Fetal Bovine Serum. HeLa cells were transfected with oligos and plasmids using oligofectamine and lipofectamine (Invitrogen) respectively; whereas HEK 293T cells were transfected with plasmids using CaCl₂.

**Immunofluorescence microscopy:**

HeLa cells were grown to 70% confluency on coverslips. After transfection, cells were fixed with 3.5% paraformaldehyde for 7 min and permeabilized with 0.02% Triton-X-100 for 20 min. The fixed cells were incubated with primary antibodies (Appendix B Table B.3) for one hour, secondary antibodies for half an hour, mounted on glass slides and observed using Olympus inverted 1X81 florescence microscope.

**Quantification:**

For experiments involving knockdown of Nuf2 (Figure 3.1), mitotic cells without CENP-E were manually counted (Figure 3.1C). Mitotic cells were classified into Pro (Prophase and Prometaphase), Met (Metaphase) and Ana/Tel (Anaphase/Telophase) based on DAPI staining. Mitotic index is the number of mitotic cells over the total number of cells (Figure 3.1D). To quantify cells for chromosome alignment (Figures 3.2E, 3.4D and 3.5D), mitotic cells transfected
with both Myc-SENP2 and one of the FLAG-Nuf2 constructs were counted manually and was classified under one of the mitotic stages depending on their DAPI stain. For CENP-E localization (Figures 3.2G, 3.4F and 3.5F), mitotic cells transfected with Myc-SENP2 were manually counted. The mitotic stages of the cells were determined by DAPI stain of chromosome DNA. These experiments were repeated at least three times by counting 100 mitotic cells each time.

For cells treated with Ubc9 siRNA oligos and then transfected with FLAG-Nuf2 or FLAG-Nuf2-3xSUMO-2, quantification for CENP-E localization was done as follows. Percentage of mitotic cells with obvious CENP-E staining without FLAG signals were subtracted from the percentage of mitotic cells with CENP-E staining with FLAG signals. This difference in the percentage was plotted in the Y-axis.

**In vitro binding assay:**

GST-Nuf2 and GST-Nuf2-3xSUMO-2 coding sequences were cloned into pGEX-6P-1 and transformed into BL21 DE3 cells. The cells were grown till they reached OD 0.6 and then induced with IPTG at a final concentration of 400 µM overnight at 4°C. The induced GST-tagged proteins were then purified using Glutathione sepharose beads (GE healthcare). FLAG-CENP-E tail domain WT and its SIM mutant were transfected into HEK293T cells. The transfected cells were then lysed in lysis buffer (50 mM Tris HCl pH-7.5, 0.2% NP-40, 10% Glycerol, 2 mM EDTA, 25 mM NaF, 50 mM NaCl, 10 mM NEM, 1 mM Aprotonin, 1 mM LAP and 1 mM PMSF). The whole cell lysates were then incubated with either GST-Nuf2 or GST-Nuf2-3xSUMO-2 immobilized to Glutathione beads in binding buffer, which is the same as lysis buffer except that the concentration of NP-40 is 0.02%. After five hours, the beads were washed six times with wash buffer (50 mM Tris HCl pH-7.5, 0.02% NP-40, 10% Glycerol, 2 mM EDTA and 50 mM NaCl). The bound CENP-E was eluted with 2x SDS Sample Buffer (2x SSB) and analyzed by
immunoblotting with anti-GST and anti-FLAG antibodies. The quantification was done using Image J software.

**In vitro SUMOylation:**

GST-Nuf2 was purified as described in the previous section. Nuf2 was then cleaved from the GST tag using PreScission Protease. Purified Nuf2 or GST-RanGAP1 (NΔ419) was incubated with E1 activating enzyme (0.5µg), E2 conjugating enzyme (0.3µg), along with the ATP regenerating system in the presence or absence of SUMO-1 (WT), SUMO-1 (K-less) or SUMO-2 (1 µg) for the indicated times at 37°C. The samples were run on an SDS-PAGE, and a Western blot analysis was performed using anti-Nuf2 to detect SUMO-modified Nuf2 or anti-GST antibodies to detect SUMO-modified-GST-RanGAP1 (NΔ419).

**Immunoprecipitation:**

HEK293T cells were transfected with FLAG-Nuf2 using CaCl₂. 48 hours after transfection, cells were treated with either DMSO or nocodazole (100ng/ml) for five hours, after which they were lysed in 1× lysis buffer along with protease inhibitors (PMSF, Aprotinin and LAP) and 10 mM NEM. The 1× lysis buffer was obtained by diluting 2× SSB (5% SDS, 150 mM Tris-HCl pH 6.7, 20% glycerol) with 1× RIPA buffer (20mM HEPES pH 8.0, 300 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, and 6% glycerol) in 1:4 ratio. The cell lysates were then sonicated and centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was bound to anti-FLAG M2 beads (Sigma) for five hours at 4°C. The beads were then washed for five times in wash buffer (20 mM HEPES pH 8.0, 750 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 6% glycerol, 0.1% SDS, and 10 mM NEM). The immunopurified proteins were eluted in 2x SDS Sample Buffer and run on an SDS-PAGE followed by immunoblot analysis using anti-FLAG and anti-SUMO-2/3 antibodies.
3.4 RESULTS

3.4.1 Nuf2 is important for recruiting CENP-E to kinetochores and mitotic progression

Nuf2 is an outer kinetochore protein required for stable microtubule-kinetochore attachments and chromosome congression to the metaphase plates (McCleland et al., 2003). Nuf2 is localized to the outer kinetochores throughout mitosis (DeLuca et al., 2002). There are two contradicting studies about if Nuf2 is required for CENP-E localization to kinetochores during mitosis. One study shows that Nuf2 is critical for the kinetochore localization of CENP-E in HeLa cells (Liu et al., 2007), whereas the other one, which was also performed in HeLa cells using the same siRNA oligos to knockdown Nuf2, showed that Nuf2 is dispensable for CENP-E targeting to kinetochores (DeLuca et al., 2002). Therefore, I first investigated the role of Nuf2 in localizing CENP-E to the kinetochores. HeLa cells were transfected with either scrambled RNAi oligos or with one of the two RNAi oligos, siRNA 1 (DeLuca et al., 2002; Liu et al., 2007) and siRNA 2, against Nuf2. After 72 hours, the cells were lysed and the efficiency of knockdown was tested by Western blot analysis using antibodies against Nuf2 (Figure 3.1A). A significant knockdown in the levels of Nuf2 were observed when either of the oligos specific to Nuf2 was used compared to the scrambled RNAi oligos. Next, in order to test if Nuf2 is required for CENP-E localization to the kinetochores, HeLa cells were transfected with either scrambled siRNA oligos or siRNA oligos specific to Nuf2. 72 hours after transfection, cells were fixed and stained with anti-Nuf2 and anti-CENP-E antibodies. It was found that control cells had obvious CENP-E staining on the mitotic chromosomes, whereas most of the cells treated with Nuf2 siRNA oligos had no detectable
Figure 3.1. Nuf2 is required for CENP-E localization to kinetochores. (A to E) HeLa cells were transfected with either scrambled oligos or with two siRNA oligos (1 and 2) against Nuf2. After 72 hours, (A) cells were lysed and a WB analysis was performed against anti-Nuf2 and anti-tubulin antibodies, (B) cells were fixed and immunofluorescence was performed with anti-CREST and anti-CENP-E antibodies, (C) % of mitotic cells (Pro: Prophase, Met: Metaphase, A&T: Anaphase and Telophase) with no obvious CENP-E staining was counted, (D) mitotic index for each transfection was calculated, (E) % of mitotic cells in each phase of mitosis was calculated.

CENP-E staining on the chromosomes (Figure 3.1B). This result firmly establishes that Nuf2 is required for kinetochore localization of CENP-E. We found that more than 75% of mitotic cells transfected with either of the oligos against Nuf2 had no CENP-E on the kinetochores (Figure 3.1C) whereas, in cells transfected with scrambled RNAi, only about 5% of mitotic cells had lost CENP-E staining from the kinetochores. Consistent with previous studies (DeLuca et al., 2002; Hori et al., 2003), depleting Nuf2 resulted in a mitotic index of 20-27% depending on the oligos.
Moreover, about 95% of the mitotic cells were arrested in prometaphase when cells were transfected with either of the siRNA oligos against Nuf2 with only less than 5% of cells in the other three phases of mitosis. Whereas in the control cells there was almost an equal distribution of cells in different phases of mitosis (Figure 3.1E).

**3.4.2 SUMO modification at kinetochores is required for chromosome segregation by recruiting CENP-E to kinetochores**

Inhibition of SUMOylation by over expression of Myc-SENP2 causes a prometaphase arrest and mislocalization of CENP-E from the kinetochores (Zhang et al., 2008). In addition to being a SUMO-2/3 target, CENP-E has a bona fide SUMO Interacting Motif (2307-2319aa) in its C-terminal domain which is required for its localization to the kinetochores. Two of the CENP-E interacting outer kinetochore proteins, BubR1 and Nuf2, are specifically modified by SUMO-2/3 in vivo (Zhang et al., 2008). Based on these observations, we hypothesize that SUMO modification of one or more kinetochore proteins including Nuf2 recruits CENP-E to the kinetochores via its SIM facilitating the chromosomes to align on the metaphase plates. A potential way to test this would be to enhance the SUMOylation of Nuf2 by increasing the local concentration of Ubc9 in the centromere region (Jakobs et al., 2007). In order to accomplish this, we made a fusion construct, FLAG-Nuf2-Ubc9, where Ubc9 was fused to the C-terminus of Nuf2. We also fused the catalytic mutant of Ubc9 (Azuma et al., 2003; Banerjee et al., 1995) to the C-terminus of Nuf2, FLAG-Nuf2-Ubc9 (C93A) in order to understand if SUMOylation caused by Ubc9 contributes to cell cycle progression through mitosis. The construct FLAG-Nuf2 was used as a control. A schematic of these constructs is shown in Figure 3.2A. These constructs were transfected in to 293T cells and a western blot analysis of the cell lysates indicated that the different FLAG-tagged Nuf2 fusion proteins were expressed with the expected size (Figure 3.2B). In order to test if fusing Ubc9 to Nuf2 enhances SUMO-2/3 modification of Nuf2, 293T cells were transfected with either
FLAG-Nuf2 or FLAG-Nuf2-Ubc9 (WT). 48 hours after transfection, immunoprecipitation using anti-FLAG beads were performed. The immunopurified proteins were run on an SDS-PAGE and a WB analysis using anti-SUMO-2/3 antibodies indicated the SUMO-modified forms of Nuf2. Although there was no difference between higher molecular weight conjugates of Nuf2 when transfected with either of the two constructs, there were three specific bands when transfected with FLAG-Nuf2-Ubc9 (WT) but not in FLAG-Nuf2, which are indicated with asterisk.

Figure 3.2. SUMO modification at kinetochores is required for chromosome segregation by recruiting CENP-E to kinetochores. (A) Diagram showing the different FLAG-Nuf2 constructs. (B) 293T cells expressing each of the FLAG-Nuf2 constructs were lysed and blotted with anti-FLAG antibodies. (C) 293T cells were transfected with either
FLAG-Nuf2 or FLAG-Nuf2-Ubc9. 48 hours after transfection, IP using anti-FLAG beads was performed followed by WB using anti-FLAG and anti-SUMO-2/3 antibodies. (D-G) HeLa cells were co-transfected with Myc-SENP2 along with one of the FLAG-Nuf2 constructs for 48 hours, after which the cells were fixed and stained with anti-FLAG and anti-Myc antibodies (D) or stained with anti-Myc and anti-CENP-E antibodies (F). Percentages (%) of cells in different stages of mitosis in each of these transfections were counted and quantified (E and G).

To test if enhancing SUMO modification of Nuf2 can rescue the phenotypes caused by Myc-SENP2 overexpression, we transfected HeLa cells with Myc-SENP2 along with a construct expressing FLAG-Nuf2 (a negative control), FLAG-Nuf2-Ubc9 (WT), or FLAG-Nuf2-Ubc9 (C93A) (a negative control). 48 hours after transfection, the cells were fixed and stained with both anti-FLAG and anti-Myc antibodies. Immunofluorescence microscopy analysis showed the percentage of transfected cells in each phase of mitosis. While only about 2 or 4% of cells with over-expression of Myc-SENP2 had their chromosomes aligned on the metaphase plates when transfected with either FLAG-Nuf2 or FLAG-Nuf2-Ubc9 (C93A), cells transfected with FLAG-Nuf2-Ubc9 (WT) had about 9% of Myc-SENP2 transfected cells in any of the three phases of mitosis including metaphase and anaphase/telophase (Figure 3.2D and E). Next, in order to check whether the chromosome congression to metaphase plates were due to CENP-E targeting to the kinetochores in these cells, we performed similar transfections as mentioned above (Myc-SENP2 along with either FLAG-Nuf2, FLAG-Nuf2-Ubc9 (WT) or FLAG-Nuf2-Ubc9 (C93A)) and stained the cells with anti-Myc and anti-CENP-E antibodies. Consistent with our model that the SUMO modification of one or more kinetochore proteins recruits CENP-E to kinetochores, we found that there were about 2.5 to 3 times increase in the number of mitotic cells that had rescued CENP-E mislocalization caused by inhibition of SUMOylation in cells transfected with FLAG-Nuf2-Ubc9 (WT) compared to cells transfected with FLAG-Nuf2-Ubc9 (C93A) or FLAG-Nuf2 (Figure 3.2F and G). These results therefore indicate that fusing Ubc9 (WT) to Nuf2 leads to CENP-E targeting to kinetochores in cells overexpressing SENP2.
3.4.3 Nuf2 is modified by polymeric SUMO-2/3 chains both in vivo and in vitro

Previous studies have indicated that Nuf2 is specifically modified by SUMO-2/3 in vivo (Zhang et al., 2008). However, an in vitro SUMO modification assay using recombinant Nuf2, E1 activating enzyme, Ubc9 and along with either SUMO-1 or SUMO-2 indicated that Nuf2 can be robustly modified by either SUMO with equal efficiency (Figure 3.3B). Since the function of Nuf2 is well established during mitosis, we wanted to test if the SUMO-2 modification of Nuf2 is enhanced when cells are enriched in mitotic phase compared to asynchronous cells. In order to examine that, 293T cells were transfected with FLAG-Nuf2 for 48 hours after which the cells were treated with either DMSO (control) or nocodazole (to arrest cells in prometaphase) for five hours. Immunoprecipitation using anti-FLAG beads followed by WB using anti-FLAG and anti-SUMO-2/3 antibodies was performed. The levels of SUMO-2/3 modification on Nuf2 were slightly higher in cells treated with nocodazole, indicating that SUMO-2/3 modification of Nuf2 increases to some extent during mitosis (Figure 3.3A). The higher molecular weight smear of SUMO-2/3 modification on Nuf2 (Figure 3.3A) could either indicate polymeric SUMO-2/3 chain modification of Nuf2 on one/more lysine residues or monomeric SUMO-2/3 modification of Nuf2 on several lysine residues. In order to distinguish these two possibilities, we turned to the in vitro SUMO modification assay using recombinant proteins with either WT or chain deficient mutant of SUMO-1. Purified Nuf2 was incubated with recombinant SUMO E1 and Ubc9 in the presence of SUMO-1 WT or K-less mutant for the indicated times (Figure 3.3D). Although we could observe a few modified bands of Nuf2 using the K-less mutant, the higher molecular weight bands of SUMO-1 modified Nuf2 was obvious only when SUMO-1 WT was used, suggesting that Nuf2 is preferentially modified by polymeric SUMO-1 chains. In order to confirm that the efficiency of the first modification on a substrate is similar between WT and K-less mutant of SUMO-1, we
Figure 3.3. Nuf2 is modified by polymeric SUMO-2/3 chains. (A) 293T cells were transfected with FLAG-Nuf2 for 48 hours after which cells were treated with DMSO as control or with nocodazole for five hours. IP with anti-FLAG beads followed by WB with anti-FLAG and anti-SUMO-2/3 antibodies was performed. (B) *In vitro* SUMOylation assay for two hours with Nuf2, SUMO-1 or SUMO-2, recombinant E1 and E2, followed by WB analysis using the specified antibodies. (C) Similar *in vitro* SUMOylation as in (B) but with purified RanGAP1(NΔ419) as substrate using either SUMO-1 WT or K-less mutant. (D) Similar *in vitro* SUMOylation assay as in (B) with purified Nuf2 along with either SUMO-1 WT or K-less mutant. A similar *in vitro* SUMOylation assay was performed for the indicated times which showed that the first modification on RanGAP1 was similar irrespective of whether WT or K-less mutant was used.

### 3.4.4 Polymeric SUMO-2 chain fused to Nuf2 is critical for CENP-E localization to kinetochores and chromosome alignment to metaphase plates

As shown in Figure 3.2, we found that FLAG-Nuf2-Ubc9 (WT) can rescue the mitotic defects caused by SENP2 overexpression. However, Ubc9 fused to Nuf2 can potentially enhance both SUMO-1 and SUMO-2/3 modification of kinetochore proteins including Nuf2. In addition, our results strongly suggested that Nuf2 is primarily modified by polymeric SUMO chains *in vitro* (Figure 3.3). We therefore asked if polymeric SUMO-1 or SUMO-2/3 modification caused by
FLAG-Nuf2-Ubc9 rescues the phenotype caused by SENP2 overexpression. To address this question, three constructs encoding FLAG-Nuf2, FLAG-Nuf2-3xSUMO-1 and FLAG-Nuf2-3xSUMO-2 were generated, where three SUMO-1 or SUMO-2 moieties in tandem were cloned to the C-terminus of Nuf2. These SUMO-1 and SUMO-2 chains are non-cleavable as the terminal GG motifs of SUMO-1 and SUMO-2 have been mutated to AA (Figure 3.4A). The protein encoded by these constructs show the expected size when transfected into 293T cells, lysed and blotted with anti-FLAG antibodies (Figure 3.4B). Our WB results confirmed that the fusion proteins expressed were not cleaved by endogenous SENP2 as expected. In order to understand if polymeric SUMO-1 or SUMO-2 modification of kinetochore proteins is essential for cell cycle progression through mitosis, we transfected HeLa cells with Myc-SENP2 (to inhibit SUMOylation) along with the plasmid encoding FLAG-Nuf2, FLAG-Nuf2-3xSUMO-1 or FLAG-Nuf2-3xSUMO-2. Using indirect immunofluorescence microscopy, we found that the mitotic cells expressing Myc-SENP2 and FLAG-Nuf2-3xSUMO-2 had about four fold higher percentage of metaphase cells compared to those expressing Myc-SENP2 and FLAG-Nuf2 or FLAG-Nuf2-3xSUMO-1 (Figures 3.4C and D). Next, we asked if the rescue of chromosome congression defect by FLAG-Nuf2-3xSUMO-2 in Myc-SENP2 overexpressing cells is due to CENP-E targeting to the kinetochores. To answer this question, we transfected cells with the plasmid encoding Myc-SENP2 along with either the construct expressing FLAG-Nuf2, FLAG-Nuf2-3xSUMO-1 or FLAG-Nuf2-3xSUMO-2, fixed the cells and stained with anti-FLAG and anti-CENP-E antibodies. Interestingly, we found that about 13% cells have CENP-E localization in the kinetochores when cells were transfected with FLAG-Nuf2-3xSUMO-2, while only about 5% cells had CENP-E localized to the kinetochores when cells were transfected with FLAG-Nuf2 or FLAG-Nuf2-3xSUMO-1(Figures 3.4E and F). These results clearly indicate that polymeric SUMO-2 chains at the kinetochores play an important role in
localizing CENP-E to the kinetochores, thereby allowing cells to align their chromosomes on metaphase plates. This is consistent with the result that Nuf2 is specifically polymeric SUMO-2/3 modified in vivo and that CENP-E binds to SUMO-2 polymeric chains and not SUMO-1 chains in vitro (Zhang et al., 2008).

Figure 3.4. Polymeric SUMO-2 chain modification of Nuf2 is critical for CENP-E localization to kinetochores and therefore chromosome alignment to metaphase plates. (A) Diagram showing the different Nuf2 constructs. (B) 293T cells were transfected with the different Nuf2 fusion constructs. 48 hours after transfection, cells were lysed and blotted with anti-FLAG antibodies. (C-F) HeLa cells were cotransfected with Myc-SENP2 along with one of the FLAG-Nuf2 constructs. After 48 hours, cells were fixed and stained with anti-FLAG and anti-Myc antibodies (C) with anti-CENP-E and anti-Myc antibodies (E). The transfected cells in each stage of mitosis was counted and quantified in D (results from C) and F (results from E).
3.4.5 A minimum of trimeric SUMO-2 chain fused to Nuf2 is required for targeting CENP-E to kinetochores in cells overexpressing SENP2

Recent studies have shown that the yeast strains incapable of forming SUMO chains have defects in chromosome segregation during mitosis (Srikumar et al., 2013). These yeast strains were expressing smt3<sup>allR</sup> proteins, in which all the lysine residues were mutated to arginine. Although several studies have indicated that SUMOylation plays an important role in mitotic progression (Ban et al., 2011; Fernandez-Miranda et al., 2010; Mukhopadhyay et al., 2010; Yang et al., 2011), it is still not clear about the exact role of monomeric or polymeric SUMO modification during mitotic progression especially in vertebrate cells. To get a better understanding on the role of polymeric SUMO-2/3 chain in chromosome congression and CENP-E localization to the kinetochores, we generated the constructs expressing a monomeric (FLAG-Nuf2-SUMO-2), dimeric (FLAG-Nuf2-2xSUMO-2) or trimeric (FLAG-Nuf2-3xSUMO-2) SUMO-2 fused to the C-terminus of Nuf2 (Figure 3.5A). Our immunoblot analysis showed that the proteins expressed by these constructs are of the expected size (Figure 3.5B). HeLa cells were transfected with Myc-SENP2 along with FLAG-Nuf2 (as a negative control), FLAG-Nuf2-SUMO-2, FLAG-Nuf2-2xSUMO-2 or FLAG-Nuf2-3xSUMO-2 and stained with anti-FLAG and anti-Myc antibodies. Using indirect immunofluorescence microscopy, the percentage of transfected mitotic cells in each case was analyzed. Only 1.5% mitotic cells transfected with FLAG-Nuf2, FLAG-Nuf2-SUMO-2, or FLAG-Nuf2-2xSUMO-2 had their chromosomes aligned on the metaphase plates compared to 7.5% mitotic cells expressing FLAG-Nuf2-3xSUMO-2 at metaphase (Figure 3.5C and D). Consistent with our previous results of CENP-E localization (Figure 3.4E and F), FLAG-Nuf2-3xSUMO-2 overexpression significantly increased the percentage of SENP2-overexpressing cells with CENP-E localization at the mitotic chromosomes (11.5%) in comparison to FLAG-Nuf2 (5%), FLAG-Nuf2-SUMO-2 (4%) and FLAG-Nuf2-2xSUMO-2 (5%) (Figures 3.5E and F).
result indicates that a minimum of three SUMO-2 fused to the C-terminus of Nuf2 is required to rescue the defects in CENP-E localization to kinetochore and chromosome congression in cells overexpressing SENP2.

Figure 3.5. A minimum of trimeric SUMO-2 chain fused to Nuf2 is required for targeting CENP-E to kinetochores in cells overexpressing SENP2. (A) Diagram showing the Nuf2 constructs with different SUMO-2 chain lengths. (B) The FLAG-Nuf2 constructs with varying chain lengths of SUMO-2 fused to its C-terminal was transfected into 293T cells. The cell lysates were collected 48 hours post transfection and were run on an SDS-PAGE gel and a WB using anti-FLAG antibodies was performed. (C and E) HeLa cells were cotransfected with Myc-SENP2 along with one of the FLAG-Nuf2 fusion constructs shown in A. 48 hours after transfection, cells were fixed and stained with anti-FLAG and anti-Myc antibodies in (C) and anti-CENP-E and anti-Myc antibodies in (E). (D and F) Transfected cells were counted for chromosome alignment in (D) and CENP-E localization in (F).
3.4.6 Nuf2-3xSUMO-2 can localize more CENP-E compared to Nuf2 in cells depleted with Ubc9

It has been shown previously that inhibition of SUMOylation by either SENP2 overexpression or RNAi-depletion of Ubc9 causes a loss of CENP-E from kinetochores (Zhang et al., 2008). In Figures 3.4 and 3.5, I have shown that FLAG-Nuf2-3xSUMO-2 can recruit more CENP-E compared to FLAG-Nuf2 in cells with overexpression of SENP2. In order to confirm the above finding using another approach to inhibit SUMOylation (depletion of Ubc9), HeLa cells were first transfected with either siRNA oligos against Ubc9 or with control siRNA oligos for 72 hours, lysed, and analyzed by immunoblotting with anti-tubulin and anti-Ubc9 antibodies. The results indicate that the RNAi-knockdown of Ubc9 was very efficient (Figure 3.6A). Further, to test the above hypothesis, Ubc9 siRNA treated cells were transfected with either FLAG-Nuf2 or FLAG-Nuf2-3xSUMO-2 for 48 hours followed by fixing the cells with 3.5% paraformaldehyde. The fixed cells were then stained with anti-FLAG and anti-CENP-E antibodies.

Figure 3.6. Nuf2-3xSUMO-2 can localize more CENP-E compared to Nuf2 in cells depleted with Ubc9. (A) HeLa cells were transfected with control or Ubc9 specific siRNA oligos for 72 hours and analyzed by WB with anti-tubulin (loading control) and anti-Ubc9 antibodies. (B) HeLa cells were transfected with Ubc9 siRNA oligos. 24 hours after transfection, cells were transfected with either FLAG-Nuf2 or FLAG-Nuf2-3xSUMO-2. After 48 hours, cells were fixed and stained with anti-CENP-E antibodies. Using immunofluorescence microscopy, transfected cells with CENP-E staining were counted and quantified.
About 37% of cells expressing FLAG-Nuf2-3xSUMO-2 had CENP-E staining on mitotic chromosomes compared to only about 10% of FLAG-Nuf2-expressing cells with CENP-E staining. The percentage of cells with CENP-E staining in both FLAG-Nuf2 and FLAG-Nuf2-3xSUMO-2 cells (Figure 3.6) was calculated by subtracting the percentage of non-transfected cells from transfected cells. This indicates that polymeric SUMO-2 chains at the kinetochore recruits CENP-E in cells with global inhibition of SUMOylation using two independent approaches - overexpression of SENP2 (Figure 3.4) and depletion of Ubc9 (Figure 3.6).

### 3.4.7 Nuf2-3xSUMO-2 has a higher binding to CENP-E than Nuf2

Since FLAG-Nuf2-3xSUMO-2 can recruit CENP-E to centromeres better than FLAG-Nuf2 in cells with SENP2 overexpression or Ubc9 depletion, we hypothesized that Nuf2-3xSUMO-2 has better affinity to CENP-E compared to Nuf2. To test this idea, we performed an \textit{in vitro} binding assay, where purified GST, GST-Nuf2 or GST-Nuf2-3xSUMO-2 was immobilized on Glutathione beads. The tail domain of FLAG tagged CENP-E was transiently expressed in 293T cells, and the cell lysates were passed through the glutathione beads containing the above mentioned GST or GST-tagged fusion proteins. The tail domain of CENP-E contains the SIM, kinetochore targeting domain and is about 750 aa in length. After binding for five hours at 4°C, the beads were washed and the proteins were eluted in 2x SSB followed by immunoblot analysis using anti-FLAG antibodies. Levels of FLAG-tagged CENP-E were quantified using ImageJ and normalized with GST levels (Figures 3.7A and 3.7B top panel). We found that the binding affinity between GST-Nuf2-3xSUMO-2 and FLAG-CENP-E is about three fold higher when compared to the binding affinity between GST-Nuf2 and FLAG-CENP-E (Figure 3.7C left panel).
Given that Nuf2 can interact with CENP-E (Liu et al., 2007), our results suggested that CENP-E binds better to Nuf2-3xSUMO-2 than Nuf2 through the interaction between the SIM motifs of CENP-E and the SUMO-2 moieties of Nuf2-3xSUMO-2. If this were true, the binding affinity between FLAG-CENP-E SIM mutant and GST-Nuf2-3xSUMO-2 should be comparable to that between FLAG-CENP-E and GST-Nuf2. To test this, we performed a similar binding assay with FLAG-CENP-E SIM mutant. We found that FLAG-CENP-E SIM mutant bound to both GST-Nuf2 and GST-Nuf2-3xSUMO-2 with the similar binding affinity (Figures 3.7B bottom panel and 3.7C right panel). These results indicate that the trimeric SUMO-2 chains of GST-Nuf2-3xSUMO-2 interact with the SIM(s) of CENP-E and enhances the interaction between CENP-E and Nuf2-3xSUMO-2.
3.4.8 Nuf2 is SUMO-2 modified at several lysine residues

From previous studies and from Figure 3.3, we know that Nuf2 is modified by SUMO-2 both in vitro and in vivo (Zhang et al., 2008). We next set out to identify the major SUMOylation sites on Nuf2. We made use of bioinformatics tools, GPS-SUMO 1.0 and SUMOplot, to identify the top six putative SUMOylation sites on Nuf2 (Figure 3.8A).

![Diagram showing top six putative SUMO modification consensus motifs of Nuf2 with the lysine residues in red.](image)

**Figure 3.8. Nuf2 is SUMO-2 modified at several lysine residues.** (A) The diagram shows the top six putative SUMO modification consensus motifs of Nuf2 with the lysine residues in red. (B) In vitro SUMO-2 modification assay with either purified Nuf2 (WT) or Nuf2 with all the six lysine residues mutated to arginine (6x K/R) simultaneously was performed along with the SUMO conjugation machinery for the indicated times followed by WB analysis using anti-Nuf2 antibodies.

After mutating the six lysine residues to arginine simultaneously (6K/R), an in vitro SUMO-2 modification assay was performed, using Nuf2 as the substrate along with recombinant E1, E2 and ATP regeneration system (Figure 3.8B). The SUMO-2 modified chain of Nuf2 were comparable irrespective of whether WT or 6K/R mutant form of Nuf2 was used. This suggests that other lysine residues on Nuf2 could be modified by SUMO-2.

3.5 DISCUSSION

Previously, polymeric SUMO-2/3 modification of substrates like PML and CENP-I have been linked to degradation via the proteasome (Lallemand-Breitenbach et al., 2008; Mukhopadhyay et al., 2010). Polymeric SUMO-2/3 chain modification of PML recruit RNF4, a
SUMO targeted E3 ubiquitin ligase containing four SIMs in tandem. Studies in budding yeast have shown that ZIP1 interacts with Smt3 chains but not its monomers (Cheng et al., 2006a). Despite previous studies indicating the importance of polymeric SUMO chains, our study provided the first direct evidence that polymeric SUMO-2/3 chains play a critical role during mitosis by targeting CENP-E to kinetochores and subsequent chromosome alignment to metaphase plates. It is very interesting to reveal that the trimeric SUMO-2/3 chain modification can act as a specific signal distinct from the trimeric SUMO-1 modification and the monomeric and dimeric SUMO-2/3 modifications, in this case, for targeting the polymeric SUMO-2/3 chain-binding protein, CENP-E, to kinetochores.

Our results strongly suggest that polymeric SUMO-2/3 chains at the kinetochore region are required for CENP-E recruitment, chromosome alignment and segregation. There is a significant rescue in both chromosome alignment and CENP-E localization when cells are transfected with FLAG-Nuf2-3xSUMO-2 or FLAG-Nuf2-Ubc9 compared to FLAG-Nuf2 in cells with overexpression of SENP2. If we could assume that mitotic cells are equally distributed among the three stages: prophase/prometaphase, metaphase and anaphase/telophase, we would expect about 60% of mitotic cells in metaphase and anaphase/telophase together if there was a complete rescue of chromosome segregation in cells with overexpression of SENP2 along with FLAG-Nuf2-3xSUMO-2 or FLAG-Nuf2-Ubc9. Nevertheless, as expected, we were not able to achieve complete rescue for both chromosome segregation and CENP-E localization when SUMOylation is inhibited by SENP2 overexpression using either FLAG-Nuf2-3xSUMO-2 or FLAG-Nuf2-Ubc9 constructs and there could be several possibilities for partial rescue. First, trimeric SUMO-2 chain is probably insufficient for effective CENP-E recruitment and thereby chromosome alignment. A longer length of SUMO-2 chain fused to Nuf2 would probably achieve a higher percentage of
rescue of these mitotic defects than Nuf2-3xSUMO-2. Second, polymeric SUMO-2/3 modification of the other CENP-E interacting kinetochore proteins other than Nuf2 (for example, BubR1 and CENP-F), may also contribute to CENP-E recruitment and chromosome segregation. Transfecting cells with a construct consisting of SUMO-2 chains fused to either BubR1 or CENP-F might improve the percentage of CENP-E rescue. Third, though the FLAG-Nuf2-3xSUMO-2 fusion construct was intended to mimic endogenous Nuf2 SUMOylation, it might not truly replicate endogenous Nuf2 SUMO-2/3 modification. This is because the orientation of the linear SUMO-2 chains within the Nuf2-3xSUMO-2 fusion protein is different from the natural polymeric SUMO-2/3 modification of Nuf2.

Compared to FLAG-Nuf2-3xSUMO-2, FLAG-Nuf2-Ubc9 is able to rescue the defects in chromosome segregation and CENP-E localization to kinetochores more efficiently in cells with inhibition of SUMOylation by SENP2 overexpression. One explanation is that SUMO modification on Nuf2 brought by FLAG-Nuf2-Ubc9 is cleavable and this deSUMOylation at the centromeres is required for CENP-E to exit the chromosomes and localize to the spindle midzone during anaphase/telophase. However, the trimeric SUMO-2 chain in FLAG-Nuf2-3xSUMO-2 is non-cleavable and therefore might probably prevent CENP-E from exiting the chromosomes and inhibiting the cells from passing through metaphase.

Using the GPS SUMO1.0 and JASSA software, we found that there are at least five other putative SUMO Interacting Motifs in the tail domain of CENP-E. They are from amino acid residues 2034-2037, 2209-2212, 2439-2442, 2464-2467, 2495-2498. Since we show that a minimum of trimeric SUMO-2 chain fused to Nuf2 is essential to rescue CENP-E mislocalization caused by SENP2 overexpression, it is possible that one or more of these putative SIM(s) binds to
polymeric SUMO modified Nuf2 and hence plays an important role in CENP-E localization to kinetochores.

CENP-E first appears on the centromeres during prometaphase (Brown et al., 1994; Yen et al., 1991; Yen et al., 1992). Nuf2 is found in the cytosol during interphase and is targeted to the centromeres throughout mitosis (Hori et al., 2003). Since we have shown that SUMOylation of kinetochore proteins including Nuf2 is critical for CENP-E localization to the kinetochores it would be interesting to examine if SUMOylation of Nuf2 occurs prior to CENP-E targeting to kinetochores.

In this study, we focused on investigating if SUMOylation of Nuf2, one of the known CENP-E interacting proteins, plays a critical role in targeting CENP-E to kinetochores. Further, it would be interesting to understand how its SUMO-2/3 modification is regulated. The E3 ligase identified can be used as a tool to regulate SUMO-2/3 modification of Nuf2, thereby gaining more insight into the mechanism of CENP-E localization to the kinetochores.
CHAPTER 4 FUTURE DIRECTIONS

4.1 Which PIAS protein stimulates the SUMO-2 modification of Nuf2?

SUMOylation of quite a few mitotic targets are regulated by PIAS family E3 ligases (Azuma et al., 2005; Ban et al., 2011; Fernandez-Miranda et al., 2010; Sridharan et al., 2015). PIAS proteins contain an N-terminal SAP domain that is involved in DNA binding, PINIT domain required for their sub-cellular localization, SP-RING domain that contains the SUMO E3 ligase activity, SIM for non-covalent SUMO interactions, and S/T domain (the serine threonine rich domain) (Rytinki et al., 2009). In mammalian cells, PIAS3-mediated SUMO-2/3 modification of Aurora-B enhances its autophosphorylation that is required for its activation (Ban et al., 2011). In *Xenopus*, one of the major SUMO-2/3 substrates associated with mitotic centromeres is Topo IIα (Azuma et al., 2003). SUMO-2/3 modification of Topo IIα is spatially and temporally regulated by PIASy (Azuma et al., 2005). SUMOylation of Topo IIα by PIASy is required for the recruitment of Topo IIα to the centromere (Diaz-Martinez et al., 2006). Polo-like kinase 1-interacting checkpoint helicase (PICH) is a protein essential for mitotic progression, especially during chromosome segregation (Kurasawa and Yu-Lee, 2010). SUMOylation of PICH is regulated by PIASy and decreases its binding to mitotic chromosomes *in vitro* (Sridharan et al., 2015).

Based on these data, we hypothesized that one of the PIAS proteins is critical for Nuf2 SUMOylation. Identifying the E3 ligase could be another potential way to test if Nuf2 SUMOylation is important for CENP-E targeting to the kinetochore. We performed a preliminary *in vitro* binding assay to test if Nuf2 binds to any of the proteins from the PIAS family. GST-Nuf2 was purified from *E.coli* using Glutathione beads. 293T whole cell lysates over expressing each of the FLAG-PIAS proteins was passed through Glutathione beads containing GST-Nuf2. The beads were washed and the bound proteins were eluted. The eluted proteins were run on an SDS-PAGE
and a Western blot analysis using anti-FLAG antibodies was performed (Figure 4.1A). The results indicated that both PIASxα and PIASy specifically interact with Nuf2. Of the different PIAS proteins, PIAS3 and PIASy are known to be localized to the kinetochores during mitosis (Agostinho et al., 2008; Ban et al., 2011). We then performed an *in vivo* co-immunoprecipitation assay between Nuf2 and two of the PIAS proteins, PIAS3 and PIASy. PIAS3 was used as a negative control. 293T cells were co-transfected with FLAG-Nuf2 along with either GFP-PIAS3 or GFP-PIASy. 24 hours post transfection, anti-FLAG beads were used to pull down Nuf2 under non-denaturing conditions. The proteins bound to the beads were eluted and run on an SDS-PAGE gel followed by WB analysis using anti-FLAG and anti-GFP antibodies. Nuf2 interacted more efficiently with PIASy compared to PIAS3 (Figure 4.1B). We then performed an *in vitro* SUMOylation assay to test if PIASy stimulates Nuf2 SUMOylation (Figure 4.1C). Nuf2 along with E1, E2, with the ATP regenerating system was present in all lanes. The first lane without SUMO-2 was used as a negative control. The second lane with SUMO-2 showed higher molecular weight SUMO conjugates of Nuf2. The third lane was another negative control which had SUMO-2 and the buffer in which purified PIASy was dissolved. The fourth lane had SUMO-2 along with purified recombinant PIASy. There was no increase in the SUMO-modified forms of Nuf2 with addition of PIASy by comparing lanes 3 and 4 (Figure 4.1C). We then performed an *in vivo* experiment to test if PIASy stimulates Nuf2 SUMOylation. 293T cells were transfected with FLAG-Nuf2 along with one of the following constructs - the empty vector, GFP-PIAS3, and GFP-PIASy, respectively. FLAG-Nuf2 was pulled down under denaturing conditions using anti-FLAG beads and the SUMOylation of Nuf2 under each of these conditions was analyzed by immunoblotting with antibodies against SUMO-1, SUMO-2/3, GFP, FLAG and tubulin. We found that the SUMOylation of Nuf2 was the highest when empty vector was transfected. However,
SUMOylation of Nuf2 was more abundant when GFP-PIASy was used compared to GFP-PIAS3 (Figure 4.1D). From Figures 4.1C and D, we cannot conclude if PIASy stimulates Nuf2 SUMOylation.

Figure 4.1. What is the E3 ligase that stimulates Nuf2 SUMOylation? (A) 293T cells over expressing one of the five FLAG-PIAS proteins were passed over Glutathione beads containing GST-Nuf2 proteins. The bound proteins were eluted with 2xSSB and the eluted proteins were run on an SDS-PAGE following which WB analysis against α-FLAG antibodies was performed. (B) 293T cells were transfected with FLAG-Nuf2 along with either GFP-PIAS3 or GFP-PIASy. Under non-denaturing conditions, the cell lysates were purified using anti-FLAG beads. The bound proteins were eluted and a WB analysis against anti-FLAG and anti-GFP was performed. (C) An in vitro SUMOylation assay was performed with purified Nuf2, E1 activating enzyme, E2 conjugating enzyme in all the lanes, along with SUMO-2, PIASy as indicated. (D) 293T cells were transfected with FLAG-Nuf2 along with one of the following as indicated-empty vector, GFP-PIAS3 or GFP-PIASy. Under denaturing conditions, FLAG-Nuf2 was purified using anti-FLAG beads and a WB analysis was performed against anti-FLAG antibodies and anti-SUMO-2/3 antibodies to detect SUMO-2/3 modified forms of Nuf2.
Further experiments should be performed in order to identify the E3 ligase for Nuf2. To make sure the purified PIASy is catalytically active, the *in vitro* SUMOylation reaction in Figure 4.1C can be repeated using a positive control (Topo IIα whose SUMOylation is known to be stimulated by PIASy (Azuma et al., 2005)). Another possibility is that PIASxα stimulates SUMO-2/3 modification of Nuf2. To test this, *in vitro* SUMOylation reaction similar to the one in Figure 4.1C can be performed using purified PIASxα instead of PIASy.

4.2 Do multiple SIM motifs at CENP-E tail domain mediate the interaction of CENP-E with polymeric SUMO-2/3 modified Nuf2?

The known polymeric SUMO chain interacting proteins, including RNF4, Arcadia, and ZMYM2, have at least three SIMs that contribute to their binding to SUMO-2 chains (Aguilar-Martinez et al., 2015; Erker et al., 2013; Sun and Hunter, 2012; Tatham et al., 2008). However, so far only one SIM (SIM3 in Figure 4.2) has been identified in CENP-E that is important for binding to polymeric SUMO-2 chains and for its kinetochore localization (Zhang et al., 2008). Moreover, *in vitro* CENP-E binds to polymeric SUMO-2 chains rather than monomeric SUMO similar to RNF4 (Xu et al., 2014). Also, from Chapter 3, we know that Nuf2-3xSUMO-2 targets CENP-E to the kinetochores better than Nuf2-SUMO-2 or Nuf2-2xSUMO-2 in cells with overexpression of SENP2. Based on these observations, we hypothesized that there are multiple SIMs in CENP-E that regulate its binding to SUMO-2 chains. Using GPS-SUMO, a bioinformatics tool, we identified five other putative SIMs in the tail domain of CENP-E.
In order to investigate if these putative SIMs are important for binding to SUMO-2 chains, three of the four hydrophobic core residues within SIM2 and SIM4 were simultaneously mutated to alanine using site directed mutagenesis. As a preliminary experiment, I tested if these mutants had defect in localizing to the kinetochores similar to SIM3 mutant. HeLa cells were transfected with either WT or one of the SIM mutants (SIM2, 3 or 4) of FLAG-CENP-E (tail domain). 48 hours after transfection, cells were fixed with 3.5% paraformaldehyde for 7 min and permeabilized with 0.2% Triton-X-100 for 20 min. The cells were stained with anti-FLAG and anti-CREST primary antibodies for one hour. After washing the coverslips, secondary antibodies were added and mounted on glass slides. The slides were observed using Olympus inverted IX81 fluorescence microscope. Similar to WT FLAG-CENP-E transfected cells, both SIM2 and SIM4 mutants of FLAG-CENP-E co-localized with CREST, suggesting that each of the two putative SIMs is not required for CENP-E kinetochore localization. Consistent with the published data, SIM3 mutant of FLAG-CENP-E did not co-localize with CREST (Zhang et al., 2008)(Figure 4.3).
Figure 4.3. SIM 2 and SIM 4 motifs of CENP-E are not required for its kinetochore localization. HeLa cells were transfected with either WT or one of the SIM mutants of the tail domain of CENP-E as indicated. 48 hours after transfection, cells were fixed and an immunofluorescence was performed using anti-FLAG and anti-CREST antibodies.

Further, I would test if the other SIM mutants, SIM1, SIM5 and SIM6 have defect in kinetochore localization by performing similar experiments as in Figure 4.3.

Both RNF4 and ZMYM2 mutants where only a single SIM is mutated have decreased binding capacities to polymeric SUMO-2 chains compared to their respective WTs. However, when more than one SIM motifs are mutated, the defect in binding to poly-SUMO-2 chains is more pronounced (Aguilar-Martinez et al., 2015; Xu et al., 2014). Therefore, it would be interesting to
test if combinations of the several SIM mutants (simultaneous mutation of SIM2 and SIM4 and likewise) in CENP-E has defect in its localization to the kinetochores because of deficiency in binding to polymeric SUMO-2 chains.

ZMYM2 (a multi-SIM protein) binds better to multiSUMOylated protein rather than to polySUMOylated protein (Aguilar-Martinez et al., 2015) (Figure 4.4). PolySUMOylation is a linear chain of SUMO whereas, multiSUMOylation is either mono SUMO moieties on the same protein or on different proteins in a complex. Based on the results from Figure 3.4 in Chapter 3, Nuf2-3xSUMO-2 (a linear chain of SUMO-2) can rescue chromosome segregation defects and CENP-E mislocalization better than Nuf2, in cells with overexpression of SENP2. However, the rescue is only partial. One reason as mentioned before could be due to the orientation of the SUMO-2 chains. In this case, we assume that CENP-E through its SIM/s binds to linear poly SUMO-2 chains. However, there is a possibility that CENP-E binds with better affinity to multiSUMOylated protein (Nuf2) than with polySUMOylated protein (Nuf2) similar to ZMYM2 (Aguilar-Martinez et al., 2015).

Figure 4.4. Diagram showing interaction between a multi-SIM protein and a substrate which has SUMO chains from a single lysine residue (polySUMOylation) versus a substrate which has single SUMO moieties at multiple lysine residues (multiSUMOylation).

In order to test this, a fusion construct GST-Nuf2-COMP-SUMO-2 can be utilized. The linker region COMP corresponds to the coiled coil pentamerization domain of Cartilage
Oligomeric Matrix Protein (Aguilar-Martinez et al., 2015) (Figure 4.5). An in vitro binding assay can be performed to check if CENP-E (tail domain) binds to GST-Nuf2-COMP-SUMO-2 with better affinity than to GST-Nuf2-3xSUMO-2. If this is true, then we can test if FLAG-Nuf2-COMP-SUMO-2 can rescue CENP-E mislocalization caused by SENP2 overexpression better than FLAG-Nuf2-SUMO-2.

Figure 4.5. Cartoon showing the two fusions constructs: GST-Nuf2-3xSUMO-2 and GST-Nuf2-COMP-SUMO-2.

4.3 Will longer chains of SUMO-2 fused to Nuf2 boost the rescue of mitotic defects caused by SENP2 overexpression?

RNF4 binds SUMO-2/3 chains through its four SIMs found in the N-terminal and leads to ubiquitination of polymeric SUMO-2/3 modified substrates and proteasome-mediated degradation. Interestingly, it is found that RNF4 interacts with SUMO-2 chains with at least three moieties or higher (Xu et al., 2014). A large number of SUMO targets are being identified rapidly and as mentioned above SUMO modification of many of these proteins regulate their localization, function or activity. Although it is known that polymeric SUMO-2/3 chains are conjugated to many substrates in vivo, how these chains regulate the protein function or activity is largely unknown. Similar to a single SUMO moiety conjugated to a substrate (target protein) can serve as a hindrance for its interaction with its partner (interacting protein), one can imagine that a polymeric SUMO chain can have the same effect as shown in the cartoon below in Figure 4.6A. In certain other cases, a polymeric SUMO chain can recruit proteins containing a SIM Figure 4.6B.
Figure 4.6. **Diagram showing the interaction between a poly SUMO-modified protein and its interacting partner.** (A) Sometimes, the interaction between two proteins is disrupted when the poly-SUMO chains on one protein blocks the interacting motif on it. (B) In certain other cases, poly-SUMO chains can enhance the interaction with its binding partner because of the presence of SUMO Interacting Motif (SIM).

The latter is consistent with the data that CENP-E binds to Nuf2-3xSUMO-2 with better affinity than with Nuf2 (Zhang et al., 2008). Besides, an earlier study in budding yeast showed that a protein ZIP1 interacts with chains of Smt3 (Cheng et al., 2006a). ZIP1, a part of the synaptonemal complex, is essential for meiotic chromosome synapsis. However, only a single SIM is recorded on ZIP1 so far, as against to multiple SIMs in close proximity, as one would expect.

In the study in Chapter 3, I have demonstrated that trimeric SUMO-2 chain fused to Nuf2 acts as the minimum signal for the recruitment of CENP-E to the kinetochores. However, it would be interesting to test if a longer polymeric chain of Nuf2 can serve as a better signal for localizing CENP-E to the kinetochores during overexpression of SENP2. In order to investigate this possibility, fusion constructs with either four or five SUMO-2 moieties fused to the C-terminus of Nuf2 was created. Below is the diagram of the constructs (Figure 4.7A). These constructs were confirmed by restriction digestion as shown below (Figure 4.7B and C).
Figure 4.7. Construction of FLAG-Nuf2-4xSUMO-2 and FLAG-Nuf2-5xSUMO-2 vectors. (A) Diagram showing the FLAG-Nuf2 construct with either four or five SUMO moieties fused to its C-terminus. (B and C) Conformation of the vectors using restriction digestion to cleave the four and five SUMO moieties.

After confirming these constructs for expression of the expected proteins, HeLa cells will be co-transfected with Myc-SENP2 and one of the four constructs, including FLAG-Nuf2 (control), FLAG-Nuf2-3xSUMO-2 (positive control), FLAG-Nuf2-4xSUMO-2, and FLAG-Nuf2-5xSUMO-2. Similar to experiments performed in Figures 3.2, 3.4 and 3.5 in Chapter 3, 48 hours after transfection cells will be fixed and stained with anti-FLAG and anti-Myc. The transfected mitotic cells in different phases of cell cycle will be counted and quantified using immunofluorescence microscopy. A second set of transfected cells will be stained with anti-CENP-E and anti-Myc antibodies. Transfected mitotic cells will then be analyzed by
immunofluorescence microscopy for chromosome alignment to metaphase plates and CENP-E localization to kinetochores. These results will address the question whether the longer polymeric SUMO-2 chain modification of Nuf2 can further enhance CENP-E targeting to kinetochores and chromosome congression in cells with inhibition of SUMOylation.
## APPENDIX A

### Table A.1 PCR primers for subcloning human SUMO-1

<table>
<thead>
<tr>
<th>SUMO-1</th>
<th>Size (aa)</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>38</td>
<td>GCGGATCCATGTCTGACCAGGAG</td>
<td>GCCTCGAGCACTTTGAGAGTGAATCTC</td>
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<tr>
<td>B</td>
<td>42</td>
<td>GCGGATCCCGACAGGATAGCGGT</td>
<td>GCCTCGAGCTGACCCCTCAAGAG</td>
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<td>C</td>
<td>38</td>
<td>CGCGGATCCAAATTCACTCAGGT</td>
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<td>B1</td>
<td>21</td>
<td>CGGGATCCCGACAGGATAGCGGT</td>
<td>GCCTCGAGTTTTAGCTTTCTTTGATG</td>
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<tr>
<td>B2</td>
<td>20</td>
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<td>B3</td>
<td>21</td>
<td>CGGGATCCCGAAATCATACTGTCAAAGAGAG</td>
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<td>B31</td>
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### Table A.2 PCR primers for subcloning human SUMO-2

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<tr>
<td>C</td>
<td>43</td>
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<td>21</td>
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        |      |  GCCTCGAGACCTCCCGTCTGC

C11 |  13  |  CGGGATCCCAGGGATTTGCTCA
       |      |  GCCTCGAGGTCAAATCGGAATCT

C12 |  12  |  CGGGATCCTCAATGAGGCAGATC
       |      |  GCCTCGAGGATGGTTGCCC

C13 |  12  |  CGGGATCCAGGCAGATCAGA
       |      |  GCCTCGAGGATTGGTTGCCC

C14 |  12  |  CGGGATCCATCAGATTCCGA
       |      |  GCCTCGAGTTCATTGATTTG

C15 |  13  |  CGGGATCTCTCCGATTTGAC
       |      |  GCCTCGAGTGTGTCTTTCATTG

C16 |  10  |  CGGGATCCCATCAGATTCCGA
       |      |  GCCTCGAGGATGGTTGCCC

### Table A.3 PCR primers for site-directed mutagenesis to generate non-conjugatable SUMO-2/3 mutants

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### Table A.4 Plasmids used in Chapter 2

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<td>Gelsolin</td>
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<tr>
<td>HsCD00434238</td>
<td>Vimentin</td>
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### Table A.5 Peptides used in Chapter 2 for SUMO-1-ePIPE and SUMO-2/3-ePIPE

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<td>SUMO-1-ePIPE</td>
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<td>SUMO-1-ePIPE</td>
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<td>51-QGLSVRQIRFRFD-63</td>
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<tr>
<td>SUMO-2 Epitope (C14)</td>
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### Table A.6 Antibodies used in Chapter 2

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<td>Santa Cruz, Santa Cruz, CA, USA</td>
<td>WB / IF</td>
<td>1:500 / 1:50</td>
</tr>
<tr>
<td>MIF</td>
<td>Rabbit</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
<td>WB</td>
<td>1:2000</td>
</tr>
<tr>
<td>FLAG (M2)</td>
<td>Mouse</td>
<td>Sigma -Aldrich, St. Louis, MO, USA</td>
<td>WB</td>
<td>1:800</td>
</tr>
<tr>
<td>RanGAP1</td>
<td>Rabbit</td>
<td>Dasso Laboratory, NIH</td>
<td>IF</td>
<td>1:2000</td>
</tr>
<tr>
<td>hnRNP U (3G6)</td>
<td>Mouse</td>
<td>Dreyfuss Lab (HHMI, U of Penn) / Abcam</td>
<td>WB</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-Enolase</td>
<td>Rabbit</td>
<td>Abcam, Cambridge, MA, USA</td>
<td>WB</td>
<td>1:5000</td>
</tr>
<tr>
<td>Pyruvate Kinase M2</td>
<td>Rabbit</td>
<td>GeneTex, Irvine, CA, USA</td>
<td>WB</td>
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### APPENDIX B

#### Table B.1 Primers used for pFLAG-Nuf2 constructs in Chapter 3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>pFLAG-Nuf2</td>
<td>CCATCGATAATGGAAACTTTGTCTTTTCCCCAG</td>
<td>GCGGATCCTCAGGTGACATTTGTAACATCTTCC</td>
</tr>
<tr>
<td>pFLAG-Nuf2 (mutated stop codon)</td>
<td>CCATCGATAATGGAAACTTTGTCTTTTCCCCAG</td>
<td>GCGGATCCTCAGGTGACATTTGTAACATCTTCC</td>
</tr>
<tr>
<td>3xSUMO-1 to generate pFLAG-Nuf2-3xSUMO-1</td>
<td>CGAGGATCCATGTCTTGACCTTGTCTTTTCCCCAG</td>
<td>TAATTTTAACCCGGGAAGCTCGAGTCACACC</td>
</tr>
<tr>
<td>pFLAG-Nuf2-3xSUMO-2</td>
<td>CCATCGATAATGGAAACTTTGTCTTTTCCCCAG</td>
<td>GCTCTAGAGAAATTCAGCTCAGCTCAC</td>
</tr>
<tr>
<td>3xSUMO-2 to generate pFLAG-Nuf2-3xSUMO-2</td>
<td>CGGGATCCATGGCGCCAGCTTTGTCTTTTCCCCAG</td>
<td>TCCCCCAGGGCCCTCTAGATGCA TGCTCGAG</td>
</tr>
<tr>
<td>Ubc9 to generate pFLAG-Nuf2-Ubc9(WT)</td>
<td>TGGGATCCATAATGTCGAAGTCTTGCGTCTTCTGTCCGAG</td>
<td>ATCCCCGGGTAAAAAGCAGGAGGCA AACCTTCTT</td>
</tr>
<tr>
<td>pGEX6p-1 GST-Nuf2</td>
<td>GACCCCGGGAATGGAACTTTGTCTTTTCCCCAG</td>
<td>GGCCTGCTCGACTCTCAGTCTGAGGTCAGCT</td>
</tr>
<tr>
<td>pGEX6p-1 GST-Nuf2-3xSUMO-2</td>
<td>GACCCCGGGAATGGAACTTTGTCTTTTCCCCAG</td>
<td>CGCAGCTGCTCGACTCTCAGTCTGAGGTCAGCTA</td>
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#### Table B.2 Primers used for mutagenesis in Chapter 3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFLAG-Nuf2-2xSUMO-2</td>
<td>CAGACGTGAGCTCTGTGAGCTCAG</td>
<td>AGGAGCTTCTGAGAGCTCTGA</td>
</tr>
<tr>
<td>pFLAG-Nuf2-2xSUMO-2 (C93A)</td>
<td>TCGGGGACAGTGCCCTCTGAGCTCAG</td>
<td>GGTGGAGCTCAGTGCCCTCCTGA</td>
</tr>
<tr>
<td>Nuf2 K41R</td>
<td>CTTTATCCCAAATCGCCCGTCTGTCCTGAGCTCAG</td>
<td>GGTGGAGCTCAGTGCCCTCCTGA</td>
</tr>
<tr>
<td>Nuf2 K165R</td>
<td>GAGGATTAATGAGACTGAGAGACTGAGACTTCTGCTGAGCTCAG</td>
<td>GATTTGGAATGCTCTGCAGCATTAATGCGCTCA</td>
</tr>
<tr>
<td>Nuf2 K402R</td>
<td>CAAGAAATCCCAAATCGCCCGTCTGTCCTGAGCTCAG</td>
<td>GATTTGGAATGCTCTGCAGCATTAATGCGCTCA</td>
</tr>
<tr>
<td>Nuf2 K447R</td>
<td>GAGGACCTCCTCATGACTAGAGATGAGAGACTGAGACTTCTGCTGAGCTCAG</td>
<td>GATTTGGAATGCTCTGCAGCATTAATGCGCTCA</td>
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</table>
Table B.3 Antibodies used in Chapter 3

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Species</th>
<th>Sources</th>
<th>Applications</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuf2</td>
<td>Rabbit</td>
<td>Bethyl Laboratories, Montgomery, TX, USA</td>
<td>WB / IF</td>
<td>1:1000 / 1:200</td>
</tr>
<tr>
<td>SUMO-2/3 (8A2)</td>
<td>Mouse</td>
<td>Zhang Lab / Abcam / DSBH</td>
<td>WB / IF</td>
<td>1:800 / 1:300</td>
</tr>
<tr>
<td>α-Tubulin (DM1A)</td>
<td>Mouse</td>
<td>Sigma - Aldrich, St. Louis, MO, USA</td>
<td>WB</td>
<td>1:5000</td>
</tr>
<tr>
<td>Ubc9</td>
<td>Rabbit</td>
<td>GeneTex, Irvine, CA, USA</td>
<td>WB</td>
<td>1:5000</td>
</tr>
<tr>
<td>Myc</td>
<td>Rabbit</td>
<td>Cell Signaling, Danvers, MA, USA</td>
<td>IF</td>
<td>1:300</td>
</tr>
<tr>
<td>FLAG</td>
<td>Rabbit</td>
<td>Bethyl Laboratories, Montgomery, TX, USA</td>
<td>IF</td>
<td>1:200</td>
</tr>
<tr>
<td>GST</td>
<td>Mouse</td>
<td>Santa Cruz, Santa Cruz, CA, USA</td>
<td>WB</td>
<td>1:150</td>
</tr>
<tr>
<td>CREST</td>
<td>Human</td>
<td>Brinkley lab, Baylor College of Medicine</td>
<td>IF</td>
<td>1:10000</td>
</tr>
<tr>
<td>CENP-E</td>
<td>Mouse</td>
<td>Yen lab, Fox Chase Cancer Center</td>
<td>IF</td>
<td>1:500</td>
</tr>
<tr>
<td>FLAG (M2)</td>
<td>Mouse</td>
<td>Sigma - Aldrich, St. Louis, MO, USA</td>
<td>WB</td>
<td>1:800</td>
</tr>
<tr>
<td>RanGAP1</td>
<td>Rabbit</td>
<td>Dasso lab, NIH</td>
<td>IF</td>
<td>1:2000</td>
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Table B.4 siRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Targeting region (nt)</th>
<th>siRNA sense sequence</th>
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<tbody>
<tr>
<td>Control siRNA</td>
<td>Non-targeting</td>
<td>UUCUCCGAACGUGUCACGU</td>
</tr>
<tr>
<td>RNAi 1</td>
<td>Nuf2 (397-415)</td>
<td>GCAUGCCGUGAAACGUAUA</td>
</tr>
<tr>
<td>RNAi 2</td>
<td>Nuf2 (241-259)</td>
<td>GGCUUCUUAACAUUCAGCA</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ubc9 (86-104)</td>
<td>CAAAAAATCCCGATGGCAC</td>
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</tbody>
</table>
REFERENCES


ABSTRACT

ANALYZING SUMO-2/3 MODIFICATION IN
REGULATION OF BREAST CANCER PROGRESSION
AND MITOTIC CHROMOSOME SEGREGATION
by

DIVYA SUBRAMONIAN

May 2016

Advisor: Dr. Xiang-Dong Zhang
Major: Biological Sciences
Degree: Doctor of Philosophy

Roles of SUMOylation in cancer metastasis and mitotic progression has been elucidated in this thesis. This vital and dynamic post-translational modification has been known to be dysregulated in several kinds of cancer. Global SUMO-2/3 conjugation is upregulated in a mouse metastatic breast cancer cell line, 66cl4, compared to a non-metastatic cell line 168FARN from the same genetic background. In order to identify the SUMO-2/3 substrates, SILAC labeled lysates from the two cell lines were immunopurified using SUMO-2/3 monoclonal antibodies. By using SUMO-2/3 epitope peptide elution (SUMO-2/3-ePIPE) and quantitative mass spectrometry, 66 SUMO-2/3 targets were identified, of which 15 targets are upregulated/downregulated in 66cl4 compared to 168FARN and 45 substrates are linked to cancer. About ten of these substrates were validated using in vitro and in vivo SUMOylation assays. Further, overexpression of GFP-tagged SUMO-2/3 in 168FARN cells increased in 3D cell migration compared to control cells overexpressing GFP. These results clearly show a link between SUMO and cancer metastasis. As we know, uncontrolled cell division is a hallmark of cancer and therefore I focused on
understanding the role of SUMO-2/3 modification in regulating cell cycle progression through mitosis in the second half of my thesis. Inhibition of global SUMOylation resulted in a prometaphase arrest and mislocalization of CENP-E from kinetochores. CENP-E has a SUMO interacting motif required for its kinetochore localization. We found that these mitotic defects in cells with inhibition of SUMOylation can be rescued by overexpressing Nuf2-SUMO2-SUMO2-SUMO2 fusion proteins, but not by Nuf2-SUMO2, Nuf2-SUMO2-SUMO2, and Nuf2-SUMO1-SUMO1-SUMO1 fusion proteins. Notably, we used these Nuf2 fusion proteins to mimic the various types of Nuf2 SUMOylation at kinetochores. We also demonstrated that Nuf2 is critical for CENP-E localization to kinetochores and also modified by polymeric SUMO chains both in vitro and in vivo. Importantly, we show that Nuf2-SUMO2-SUMO2-SUMO2 fusion protein has better binding affinity to CENP-E compared to Nuf2. These results suggest that trimeric SUMO-2/3 modification of Nuf2 represent the minimum and distinct signal for targeting CENP-E to kinetochores and mitotic progression in mammalian cells.

Supplementary dataset 1 is included along with this thesis.
AUTOBIOGRAPHICAL STATEMENT

DIVYA SUBRAMONIAN

EDUCATION

2009-2015 PhD in Biological Sciences, Wayne State University, Detroit, MI, USA.

PUBLICATIONS


HONOURS AND AWARDS

2015 First place poster award-Annual Graduate Exhibition-Wayne State University
2014-2015 Thomas C. Rumble University Graduate Fellowship-Graduate School-College of Liberal Arts and Sciences-Wayne State University