ROLE OF MEL-18 IN REGULATING PROTEIN SUMOYLATION AND IDENTIFICATION OF A NEW POLYMORPHISM IN BMI-1

Jie Zhang
University of Kentucky

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ABSTRACT OF DISSERTATION

Jie Zhang

Graduate Center of Toxicology
College of Medicine
The Graduate School
University of Kentucky
2009
ROLE OF MEL-18 IN REGULATING PROTEIN SUMOYLATION AND IDENTIFICATION OF A NEW POLYMORPHISM IN BMI-1

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School at the University of Kentucky

By
Jie Zhang
Lexington, Kentucky

Director: Dr. Kevin D. Sarge, Professor of Molecular and Cellular Biochemistry
Lexington, Kentucky
2009

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ABSTRACT OF DISSERTATION

ROLE OF MEL-18 IN REGULATING PROTEIN SUMOYLATION AND IDENTIFICATION OF A NEW POLYMORPHISM IN BMI-1

Small ubiquitin-like modifier (SUMO) regulates numerous biological functions. In a previous study we found that sumoylation of HSF2 is involved in regulating HSF2 bookmarking function, but the mechanism that mediates this regulation was unknown. The results in my work support the intriguing hypothesis that polycomb protein, Mel-18, actually functions as an anti-SUMO E3 protein, interacting both with HSF2 and the SUMO E2 Ubc9, but acting to inhibit Ubc9 activity and thereby decrease sumoylation of the HSF2.

This study also suggested that Mel-18 negatively regulates the sumoylation of other cellular proteins, and we extend its targets to RanGAP1 protein. The results also show that RanGAP1 sumoylation is decreased during mitosis, and that this is associated with increased interaction between RanGAP1 and Mel-18.

Previous studies showed little evidence of anti-SUMO E3 proteins, however, my study, taken together, found Mel-18 actually functions as a novel anti-SUMO E3 protein, interacting both with substrates and the SUMO E2 Ubc9 but acting to inhibit Ubc9 activity to decrease sumoylation of target proteins and also provide an explanation for how mitotic HSF2/RanGAP1 sumoylation is regulated. This finding also gives a clue for a future study direction in Mel-18 as a tumor suppressor: the anti-SUMO E3 function.

Additionally, we identify a single-nucleotide polymorphism in another human PcG protein, Bmi-1, that changes a cysteine residue within its RING domain, cysteine 18, to a tyrosine. This C18Y polymorphism is associated with a significant decrease in levels of the Bmi-1 protein. Furthermore, the C18Y Bmi-1 protein exhibits a very high level of ubiquitination compared to wild-type Bmi-1, suggesting that that the low levels of this form of Bmi-1 are due to its destruction by the ubiquitin-proteasome system. Consistent with this hypothesis, treatment of cells with the proteasome inhibitor MG-132 results in a significant increase in levels of C18Y Bmi-1. This is the first example of a polymorphism in human Bmi-1 that reduces levels of this important protein.
KEYWORDS: Mel-18, small ubiquitin-like modifier (SUMO), heat shock factor 2 (HSF2), RanGAP1, Bmi-1

Jie Zhang

January 7, 2009
ROLE OF MEL-18 IN REGULATING PROTEIN SUMOYLATION AND IDENTIFICATION OF A NEW MUTATION IN BMI-1

By

Jie Zhang

Kevin D. Sarge
Director of Dissertation

David K. Orren
Director of Graduate Studies

January 7, 2009
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DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School at the University of Kentucky

By

Jie Zhang

Lexington, Kentucky

Director: Dr. Kevin D. Sarge, Professor of Molecular and Cellular Biochemistry

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CHAPTER ONE:
INTRODUCTION

I. SUMO

I A. SUMO biological functions

Small ubiquitin-like modifier (SUMO) is a protein containing 97 amino acids and has similar structure with ubiquitin. The modification of proteins by SUMO regulates numerous biological functions, such as subcellular localizations, protein–protein interactions and enzymatic activities. For example, SUMO conjugation was determined to be essential for Progressive multifocal leukoencephalopathy (PML) protein localization in nuclear bodies (PML NBs), and subcellular localizations of many other important proteins, such as heat shock factor 2 (HSF2), Ran GTPase-activating protein 1 (RanGAP1), P53, etc, are also dependent on the SUMO modifications (22). Meanwhile, interaction partners of these proteins will be also altered after SUMO conjugations (22,24). Additionally, although sumoylation results in repression of most transcription factors, SUMO modification appears to have a positive effect on transcriptional activation by the heat shock factors 1 and 2 (25,26).

I B. SUMO pathway

There are four different ubiquitous SUMO-related proteins identified in mammalian cells, SUMO-1, -2, -3 and –4. SUMO-2 and SUMO-3 have greater sequence relatedness with each other and contain an internal consensus site for SUMO polymerization, which is missing in SUMO-1. SUMO-4, homologous to SUMO-2/3, has also been identified in human kidney as tissue-specific SUMO-4, indicating that some SUMO proteins could possibly have tissue-specific functions. Like ubiquitin, SUMO has been found to be covalently attached to certain lysine residues of specific target proteins containing the consensus sequence ΨKXE (Ψrepresents hydrophobic amino acids) by utilizing a multistep enzymatic pathway (Figure 1.1): SUMO protease Ulp1 cleaves SUMO into its mature form. Then in the activation step, SUMO is conjugated to the Uba2 subunit of the E1-activating heterodimer Aos1/Uba2 in an ATP-dependent manner. In the following conjugation step, SUMO is transferred to the E2-conjugating enzyme Ubc9. And in the final step, SUMO is transferred and ligated to substrate proteins by forming a bond between the terminal glycine on SUMO and the lysine in the target protein. As with other post-translational modifications, the pattern of SUMO conjugates is also dynamic, SUMO groups can be removed by SUMO-specific proteases, such as SUMO1/sentrin specific peptidase 1 (SENP) (27).
I B.1. Ubc-9

In the conjugation step, SUMO-1 is transferred to the SUMO E2 enzyme Ubc9 by conjugation of its C-terminal Gly with the -SH group of the active site Cys93 residue on Ubc9 (28). Ubc9 structure is similar to other ubiquitin E2 family members: an N-terminal α helix, five- to seven-stranded β sheet, the loop containing the catalytic cysteine (cys 93), and three C-terminal α helices (Fig 1.2). Mutation studies showed that residues Asp100 and Lys101 are involved in substrate recognition, possibly due to close to the active site Cys in the tertiary structure of Ubc9 (29). It was also demonstrated that the binding sites of target proteins on Ubc9 are around residue 129, which is near to the Cys93 in the 3-D structure of Ubc9. Studies also indicated that the region of first α-helix, first β-strand and the loop between them interacts with SUMO protein, and mutations in this region significantly decrease the efficiency in the transfer of SUMO-1 from E1 to E2, but they will not affect substrate recognition or transfer of SUMO-1 from E2 to the target protein (30).

I B.2. E3 ligases

For the final ligation step in the SUMO conjugation pathway, the Ubc9-substrate interaction can also be facilitated by SUMO E3 ligases in a substrate-specific manner. These proteins meet the definition of an E3 in that they can bind both the E2 and substrate, and also promote transfer of SUMO from the E2 to the substrate in vitro (22). Three different general types of SUMO E3 ligases have been described. The first E3 group is the protein inhibitor of activated STAT (PIAS) family of proteins, which mediate the sumoylation of c-jun, p53, etc. These E3 proteins share a common RING domain and bind directly to the Ubc9 E2 enzyme and some SUMO protein targets. The RING finger motif is required for the E3 activity of PIAS proteins, and has also been identified in some of the ubiquitin E3 ligases (32). A second type of SUMO E3 protein is RAN binding protein 2 (RanBP2), a component of the nuclear pore complex (33). The functional E3 domain of RanBP2 is the internal repeat (IR) domain and shares no sequence similarity with any ubiquitin E3 proteins. RanBP2 promotes sumoylation of several proteins, including Histone deacetylase 4(HDAC4), Sp100, and RanGAP1. The final E3 protein type is polycomb 2 protein (Pc2), a member of Poly-comb protein family that stimulates sumoylation of the transcriptional corepressor, C-terminal Binding Protein (CtBP). Pc2 has no obvious sequence similarity to any other known E3s, and its E3 function depends on a C-terminal substrate binding domain and an N-terminal portion containing the chromodomain (34).
I B.3. RanGAP1

RanGAP1, a Ran GTPase-activating protein that plays a critical role in nuclear transport, was the first identified substrate for SUMO modification (35,36). Unsumoylated RanGAP1 localizes predominantly in the cytoplasm, and SUMO conjugation directs RanGAP1 to the cytoplasmic fibers of the NPC (35,37) in the interphase and mitotic spindles during mitosis. SUMO modification promotes association of RanGAP1 with the NPC by an interaction with RanBP2/Nup358 (38). Meanwhile RanBP2 also colocalizes with RanGAP1 on spindles, and this two protein interaction may be related to mitotic targeting of RanGAP1 to the spindles. Additionally, RanGAP1 can also be phosphorylated during mitosis, but phosphorylation will not affect SUMO1 modification or association with RanBP2 (39).

I C. Regulation of SUMO process

The sumoylation process can itself be regulated in a more global way or in a substrate-specific manner. H$_2$O$_2$ inactivates Ubc9 and causes a global decrease in sumoylation levels (40). Conversely, many cellular stresses, including heat shock, cause global increases in sumoylation (41). MAPK pathways can promote de-sumoylation or sumoylation under different circumstances (22). Meanwhile, other post-translational modifications can also regulate the SUMO modification of a protein. For example, phosphorylation negatively regulates the sumoylation of several substrate proteins, including c-Jun, PML and P53 (42), but phosphorylation of some proteins, such as HSF1, can also stimulate their sumoylation (43,44).

II. PcG

The Polycomb (PcG) gene was discovered as a mutation inducing a posterior transformation phenotype in *Drosophila*. Following research showed that Polycomb is a general repressor of homeotic genes, while trithorax group (TrxG) genes were shown to counteract PcG-mediated repression of homeotic genes (45).

II A. PcG complexes

PcG proteins have been categorized into two families on the basis of their physical associations in various multiprotein complexes named as Polycomb repressor complexes (PRCs)- PRC1 and PRC2. PRC1 were first isolated from *Drosophila* embryos. It is about 2 MDa in size and consists of PcG proteins PC, PH, PSC and SCM. It is also associated with more than 30 other polypeptides, including several TBP-associated factors (TAF). PRC1 is primarily responsible for the maintenance of repression of gene expression (46-49), meanwhile PRC2 initiates repression. Transcription can be repressed by establishing a chromatin structure that blocks access of the transcription machinery directly, or indirectly, by inhibiting
chromatin remodeling that might be required for access. For example, the isolated core PRC1 complex, containing PC, PH, PSC and dRING1, is sufficient to inhibit chromatin remodeling by the SWI/SNF complex \textit{in vitro} (50), and this complex can also induce \textit{in vitro} compaction of polynucleosomal chromatin templates (51). The presence of TAFs in the PRC1 complex indicates the repression may also relate to inhibit the assembly or function of the transcription machinery. Additionally, the PRC1 complex also could promote gene silencing by possessing H2A-K119 ubiquitin E3 ligase activity (52).

The function of PRC2 is recognized as the ‘code of silence’-to initiate repression (50,53-55). PRC2 is about 600 kDa, in addition to ESC and E (Z), this complex also contains the SU(Z)12 and associates with the histone deacetylase RPD3. The E(z) could methylate histone H3 at lysine 27 (H3-K27) both \textit{in vivo} and \textit{in vitro}. Because many Polycomb Response Elements (PREs) and other PcG-silenced regions are associated with methylation of H3-K27, it has been suggested that this methylation mark is essential for PcG-mediated silencing. In fact, H3-K27 methylated histones interact specifically with the chromodomain of Polycomb (Pc), a member of PRC1, and the lack of H3-K27 methylation caused by the disruption of the PRC2 complex is associated with a loss of Pc binding and derepression (46,47,49). Thus, PRC2 could help to target PRC1 to specific genomic loci by making the H3-K27 methylated mark.

II B. Mel-18

Mel-18 is a member of the polycomb group of proteins that play a vital role in development and differentiation by controlling patterns of gene expression. Mel-18 knockout mice shows severe combined immunodeficiency, growth retardation, and skeletal malformations (56). It has also been suggested Mel-18 could be a tumor suppressor: Mel-18 expression is decreased in human breast cancer cells (57). Additionally, inhibition of Mel-18 expression enabled immortal NIH 3T3 to cells form tumors in nude mice (58). Mel-18 anti-tumor activity is related with negative regulations of cell cycle progression by several ways: (a) Mel-18 can negatively regulate the activation of Cyclin-dependent kinases (CDK) by binding the c-Myc promoter, repressing its expression and then inhibiting c-myc/cdc25a cascade (59); (b) Bmi-1, another PcG protein, is an oncogene protein, and Mel-18–mediated repression of Bmi-1 is also associated with down regulation of c-myc, which will bind on the Bmi-1 promoter and promote its activities (60); (c) Direct Mei-18 interactions with cyclin D2 promote the inhibition of cyclin D2 activity (61); (d) Mel-18 could induce the inhibition of phosphatidylinositol 3-kinase/Akt signaling pathway, which is required for cytoplasm localization of p27(Kip1) and then activation of Cdk4 and Cdk2 activities (62).

Mel-18 contains a conserved Really Interesting New Gene (RING) domain at the NH2 terminus. RING finger domain is a type of zinc finger containing a
Cys3HisCys4 amino acid motif and this domain plays a very important role in Mel-18 functions: (a) the formation of Mel-18 homodimers requires the N-terminal domain containing RING finger motifs (63); (b) the RING finger domain shows DNA binding activities such as binding the c-myc promoter (59); (c) Mel-18 RING domain also participates in the Mel-18 recognition of the nucleosome (64).

II C. Bmi-1

Bmi-1 was first cloned as a c-myc cooperating oncogene in murine lymphomas (65,66), and subsequently shown to be a transcriptional repressor belonging to the PcG class of proteins (67). Previous studies have identified several ways in which Bmi-1 mediates its effects on cell proliferation, including inhibiting expression of the Ink4A/ARF locus (68), modulating the p21-Rb pathway (69), and inducing telomerase activity (70). In addition to its function as an oncogene, Bmi-1 also plays important roles in determination of cell fate and stem cell renewal of the neural, hematopoietic, and other cell lineages (67,71-77).

Bmi-1 contains a conserved RING finger domain at the NH2 terminus, which plays a very important role in Bmi-1 function. RING finger domain is required for the subnuclear localization of Bmi-1 to the nuclear rim and is associated with its ability to transform (78). Deletion analysis of the Bmi-1 protein indicates that this domain, as well as another conserved domain, are also required to induce telomerase activity and immortalize human Mammary Epithelial Cells (70). A previous study also showed the regulation of TH2 cell differentiation by Bmi-1 is in a RING finger-dependent manner by controlling GATA binding protein 3 (GATA3) stability (79). An intact Bmi-1 RING finger is also necessary but not sufficient for dinG protein binding (80). Meanwhile, Bmi-1 has been found to be predominantly localized to the nucleus, which is mediated by a nuclear localization sequence (NLS) located in the C-terminal region of this protein (70,78).

II D. Distinct and overlapping functions for Mel-18 and Bmi-1

II D.1. Similar structures, synergetic and overlapping functions

Bmi-1 and Mel-18, both belonging to the PRC1 complex, exhibit high homology to each other. They have 65% amino acid identity and also contain the similar structures: a RING finger domain in the N terminus, a helix-turn-helix domain in the middle and proline-rich sequences in the C-terminus.

Animal studies showed \( Bmi-1^{-/-} \) and \( Mel-18^{-/-} \) mice have similar phenotypes, such as severe combined immunodeficiency, growth retardation, and skeletal malformations (67,81). Mice with a double knockout of these two proteins display a more serious exacerbation of the single \( Bmi-1 \) or \( Mel-18 \) protein knockout phenotype (82).

Bmi-1 and Mel-18 also play significant roles in the regulation of lymphocyte differentiation: (a) For B cell development, Bmi-1 is very important (67);
meanwhile, without Mel-18, B cell maturation is also arrested between the pro- and pre-B cell stages (83). Meanwhile, in mature resting B cells, Mel-18 inhibits B cell receptor-induced proliferation by transcriptional repression of c-myc protein and then down regulation of c-myc/cdc25a cascade (59); (b) In thymocyte development, Mel-18 is indispensable for the expansion of adult and fetal early T progenitors by regulation of Hes-1 gene expression (84), Bmi-1 is also important for thymocyte development at an immature stage (85); (c) Both of Bmi-1 and Mel-18 regulate TH2 cell differentiation by controlling GATA3 (79); (d) Severe combined immunodeficiency seen in Mel-18 and Bmi-1 mutants is associated with impairment in the IL-7–dependent proliferation of lymphocyte precursors (83).

II D.2. Complicated roles in tumor cell growth

Bmi-1 was first cloned as a c-myc cooperating oncogene in murine lymphoma cells (65,66). Previous studies have identified several ways in which Bmi-1 mediates its effects on promotion of cell proliferation (68,85) and inducing telomerase activity (70). Unlike Bmi-1, the highly homologous Mel-18 was shown to have tumor-suppressive effects and also down regulate cell progression by several ways (59-62).

Recent studies suggest that Bmi-1 and Mel-18 may have overlapping functions in cancer cell growth (56): both Bmi-1 and Mel-18 can increase the proliferation of rodent fibroblasts (78,86); knockdown of either Bmi-1 or Mel-18 results in the inhibition of proliferation in human medulloblastoma DAOY cells, but not in normal human WI38 fibroblasts. Finally, gene expression analysis demonstrated Bmi-1 and Mel-18 share many target proteins in a number of cancer-relevant pathways (56).

III. Heat Shock Factors (HSFs)

HSFs proteins are characterized by binding to the heat shock element (HSE), nGAAAn, on Heat shock protein (Hsp) genes, which are typically silent at normal growth temperatures but are expressed at extremely high levels at elevated temperatures or other kinds of stress. All HSFs share structurally conserved domains, including the N-terminal looped helix-turn-helix DNA-binding domain (DBD). HSF1 is activated and binds to HSE by heat shock and other forms of stress, whereas HSF2 is activated during hemin-induced differentiation of human K562 erythroleukemia cells, but it has also shown that HSF2, in common with HSF1, participates in the activation of the hsp70 promoter by heat shock (9). The regulation of HSF2 has been linked to certain development- and differentiation-related processes, such as gametogenesis and pre- and postimplantation development of mouse embryos (10-14).

Previous works in our lab showed that HSF2 plays a crucial role in bookmarking the hsp70 promoter during mitosis. Most sequence-specific DNA-
binding proteins are released, and chromatin undergoes condensation (15,16) during mitosis, however, the promoters of some genes still remain accessible (15,17-19), which is referred to as “bookmarking”. HSF2 mediates bookmarking of the stress-inducible \textit{hsp70} gene by binding to heat shock elements (HSEs) in its promoter, recruiting the phosphatase PP2A, and interacting with the CAP-G subunit of the condensin complex to promote dephosphorylation of the nearby condensin complexes, thereby preventing compaction of this region of chromosomal DNA in mitotic cells (20).

This study also found that sumoylation of HSF2 protein is upregulated in mitotic cells compared with asynchronous cells and is crucial for the ability of HSF2 to interact with the CAP-G, indicating that this modification could be a regulator of the HSF2 bookmarking function (Figure 1.3). However, how the increase in HSF2 sumoylation during mitosis is regulated was not known. In order to further understanding of the regulation and function of HSF2 in cells, our former labmate Dr. Goodson performed a yeast two-hybrid screen using the HSF2 protein as a bait. One of the HSF2-interacting clones obtained from this screen represented a region of the polycomb group protein Mel-18, then the following characterization and function studies of this interaction were the focus of my doctoral work. As described above in the introduction, previous studies showed that RING finger proteins could function as an E3 protein to stimulate sumoylation of specific target proteins, so we sought to determine whether this RING finger protein Mel-18 may play a role in regulating sumoylation of HSF2. Here my research shows that Mel-18 inhibits HSF2 sumoylation by interacting with both HSF2 and ubc9, and inhibiting the ability of ubc9 to transfer the SUMO protein to HSF2. This study also suggests that Mel-18 regulates the sumoylation of other cellular proteins, but the identities of these other proteins were unknown. RanGAP1 is a very important cellular SUMO substrate protein, in fact the first identified substrate for SUMO-1 conjugation, however the regulation of its sumoylation was also unclear. Therefore, in the following study we sought to determine whether the sumoylation of RanGAP1 is also regulated by Mel-18, and if so whether this involves interaction between these two proteins. My study suggests that Mel-18 interacts with RanGAP1 and inhibits its sumoylation, and that these activities do not require the RING domain of Mel-18. The results also show that RanGAP1 sumoylation is decreased during mitosis, and that this is associated with increased interaction between RanGAP1 and Mel-18. Previous studies showed little evidence of anti-SUMO E3 proteins, but taken together, my doctoral work suggests that Mel-18 actually functions as a novel anti-SUMO E3 protein, interacting both with HSF2/RanGAP1 and the SUMO E2 Ubc9 but acting to inhibit Ubc9 activity to decrease sumoylation of target proteins and also provide an explanation for how mitotic HSF2/RanGAP1
sumoylation is regulated. This finding also gives a clue for a future study direction in Mel-18 as a tumor suppressor: the anti-SUMO E3 function.

Additionally, searching in SNP database, we identify a single-nucleotide polymorphism in human Bmi-1 that changes a cysteine residue within its RING domain, cysteine 18, to a tyrosine. Because its RING finger has been found to be related with its subnuclear localization, therefore, we hypothesized that this C18Y polymorphism could affect its pattern of localization within the cell. However results of the fluorescence microscopy experiment did not indicate a difference in localization, but they did show that a less intensity of the signal from the C18Y GFP-Bmi-1. So we hypothesized that this may be associated with a decrease in levels of C18Y Bmi-1 protein. Consistent with this hypothesis we find that the low levels of this form of Bmi-1 are due to its destruction by the ubiquitin-proteasome system. This is the first example of a polymorphism in human Bmi-1 that reduces levels of this important protein.
SUMO protease Ulp1 cleaves SUMO into its mature form. Then in the activation step, SUMO is conjugated to the Uba2 subunit of the E1-activating heterodimer Aos1/Uba2 in an ATP-dependent manner. In the following conjugation step, SUMO is transferred to the E2-conjugating enzyme Ubc9. And in the final step, SUMO is transferred and ligated to substrate proteins by forming a bond between the terminal glycine on SUMO and the lysine in the target protein. SUMO groups are removed by SUMO-specific proteases, such as specific peptidase, SENPs.
Fig 1.2 Ubc9 ribbon diagram and secondary structure assignment (Taken from Ref. 31)

Ubc9 structure is similar to other ubiquitin E2 family members: an N-terminal $\alpha$ helix, five- to seven-stranded $\beta$ sheet, the loop containing the catalytic cysteine (cys 93), and three C-terminal $\alpha$ helices.
Figure 1.3 Model of events leading to ‘bookarking’ of the hsp70 promoter during mitosis. (Taken from Ref. 21)

HSF2 mediates bookmarking of the stress-inducible hsp70 gene. HSF2 binds to heat shock elements (HSEs) in its promoter after sumoylation and trimization, recruits the phosphatase PP2A, and interacts with the CAP-G subunit of the condensin complex to promote dephosphorylation of the nearby condensin complexes, thereby prevents compaction of this region of chromosomal DNA in mitotic cells.
CHAPTER TWO:  
MEL-18 INTERACTS WITH HSF2 AND THE SUMO E2 UBC9 TO INHIBIT HSF2

BACKGROUND

Covalent attachment of Small Ubiquitin-like Modifier (SUMO) proteins to lysine residues in target proteins, or sumoylation, is an important regulator of protein functional properties (23,40,87-90). SUMO proteins are covalently attached to target lysine residues by the SUMO E2 enzyme, Ubc9, and these substrate lysines are typically found within the consensus sequence ΨKXE (Ψ represents hydrophobic amino acids) (42,91-93). SUMO E3 proteins have been identified that enhance the efficiency of sumoylation by interacting with both Ubc9 (SUMO E2) and the target protein, thereby acting as bridging factors to increase the rate of the sumoylation reaction (94,95).

Previous studies in our and another laboratory revealed that a DNA-binding protein called heat shock factor 2 (HSF2) is a target of sumoylation in vivo (96,97). Our prior work indicated that one of the functions of HSF2 is to bind during mitosis to heat shock elements (HSEs) in the promoters of the hsp70 and other heat shock protein genes to mediate an epigenetic function called gene bookmarking on these promoters (20,21). The results of this study also indicated that sumoylation of HSF2 is up-regulated during mitosis and is important for this factor’s interaction with a subunit of the condensin complex during the bookmarking process, suggesting that this modification is involved in regulating HSF2 bookmarking function (20). However, how the increase in HSF2 sumoylation during mitosis is regulated was not known.

Mel-18 is a member of the polycomb group of proteins that play a vital role in development and differentiation by controlling patterns of gene expression (98-102). One important development with respect to the functional roles of polycomb proteins was the discovery that at least one of them, Pc2 (Cbx4), functions as a SUMO E3 to stimulate the sumoylation of specific target proteins (102,103).

The results presented in this chapter now identify the existence of an interaction between HSF2 and the polycomb group protein Mel-18, and suggest that cell-cycle-dependent interaction between Mel-18 and HSF2 functions as a mechanism for the previously observed up-regulation of HSF2 sumoylation during mitosis. The results also support the intriguing hypothesis that Mel-18, in contrast to the polycomb protein Pc2/Cbx4 whose SUMO E3 activity stimulates sumoylation of certain proteins, actually functions like an anti-SUMO E3 protein, interacting both with HSF2 and the SUMO E2 Ubc9, but acting to inhibit Ubc9 activity and thereby decrease sumoylation of a target protein, in this case that of HSF2.
MATERIALS AND METHODS

Cell culture: Hela-ATCC cells and HEK 293T cells were grown at 37°C in DMEM supplemented with 10% FBS.

Generation of antibodies against Mel-18: Affinity purified goat polyclonal antibody to Mel-18 was prepared by Bethyl Laboratories (Montgomery, TX) and was raised against the synthetic peptide STSRGRKMTVNGAPVPLT, which corresponds to the C-terminal sequence of the human Mel-18 polypeptide.

Plasmid construction: pEGFP-Mel-18 plasmid was generated by using PCR to amplify from the plasmid pSG5-Mel-18 cDNA a coding fragment of Mel-18 having KpnI and BamHI sites at the ends using the following primers: 5′-GCG GGT ACC TCC ATG CAT CGG ACT ACA CGG-3′ and 5′-CGC GGA TCC AGA GGG TGG TCC TCA AGG-3′. PCR amplifications were performed using the following program: 95°C for 5 min, 30× (95°C for 1 min, 59°C for 1 min, 72°C for 2 min), 72°C for 5 min. This PCR product was then cloned into pEGFP-C1 vector (Clontech, Mountain View, CA) at the KpnI and BamHI sites to form the pEGFP-Mel-18 plasmid. This plasmid was confirmed by DNA sequencing.

GST-pulldown assays: For in vitro binding between Mel-18 and HSF2, GST-HSF2 was expressed in E. coli from the pGEX-HSF2 plasmid and GST was expressed from the pGEX plasmid, and then bacteria were resuspend and sonicated 5 times in 1×PBS plus 1.5% sarkosyl, 1mM PMSF and 1× protease inhibitor (1×PI), finally lysates of these bacteria were incubated with glutathione-agarose beads for about 2 hours at 4°C with rotation followed by washes. The GST and GST-HSF2 bound to beads were then incubated with [35S]methionine-labeled Mel-18 created by coupled in vitro transcription and translation system in rabbit reticulocyte lysates (TNT, Promega, Madison, WI) overnight at 4°C with rotation in Buffer D (20 mM HEPES, (pH 7.9), 0.2 mM EDTA, 0.1 M KCl, and 20% v/v glycerol), in a total volume of 650 μl. After washing four times with Buffer D, the amount of [35S]-methionine-labeled Mel-18 bound to the GST or GST-HSF2 was determined by boiling the beads in SDS-PAGE buffer followed by SDS-PAGE and autoradiography. For in vitro binding between Mel-18 and Ubc9, similar amounts of purified GST or GST-Ubc9 were bound to glutathione–agarose beads and then incubated with extracts of HeLa cells made using NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). After washing six times with NP-40 buffer, bound protein complexes were separated by SDS–PAGE and analyzed by Western blot using the anti-Mel-18 goat polyclonal antibodies (Bethyl) raised as described above.

Immunoprecipitation analysis: For co-immunoprecipitation experiments, asynchronous HeLa cells or HeLa cells blocked in mitosis (treated with 400 ng/ml nocodazole for 16 hours) were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, and complete
protease inhibitor cocktail (Roche Applied Science)) for 20 minutes. Lysates were then cleared by centrifugation at 16,438 g for 10 minutes at 4°C. Supernatants were precleared by incubation with rabbit (control) IgG and protein G-sepharose beads for 2 hours at 4°C with gentle rotation. Precleared extracts were then incubated with primary rabbit polyclonal HSF2 antibody or control IgG and 50% slurry of protein G-sepharose for 4 hours at 4°C with rotation. After washing beads 6 times for 5 minutes each at 4°C with NP-40 buffer, bound proteins were released by boiling in SDS PAGE sample dye and analyzed by Western blot using the anti-Mel-18 goat polyclonal antibody (Bethyl) or anti-HSF2 goat polyclonal antibody (Bethyl). For immunoprecipitation analysis of HSF2 sumoylation, HEK 293T cells were transfected with pEGFP-Mel-18 or pEGFP-C1 along with myc-sumo-1 expression plasmid using Effectene transfection reagent according to the manufacturer’s instructions (Qiagen, Valencia, CA). After 48 hours cell extracts were prepared in NP-40 Buffer (50 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche Applied Science), 10 mM N-ethylmaleimide) before adding HSF2 polyclonal antibodies or non-specific IgG to proceed as described above, followed by Western blot assay using anti-myc monoclonal antibody (Invitrogen, Carlsbad, CA). For immunoprecipitation analysis of HSF1 sumoylation, HEK 293T cells were transient transfected with pEGFP-Mel-18/pEGFP-C1 and myc-sumo-1 using Effectene transfection reagent according to the manufacturer’s instructions (Qiagen). After 48 hours, the cells were heat-treated at 42°C for 1 hour and then harvested. Cell extracts were prepared in NP-40 Buffer before adding HSF1 polyclonal antibodies to proceed as above, followed by Western blot using anti-myc monoclonal antibody (Invitrogen). This assay was normalized with cell number.

**In vitro sumoylation assay:** Full length HSF2 was *in vitro* translated in a rabbit reticulocyte lysates using the TNT-coupled transcription-translation system (Promega) with 35S methionine at 30°C for 2 h. Then in vitro translated product was incubated with Ubc9 and GST-SUMO-1 in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl2, 2mM ATP, 1 mM dithiothreitol) at 30°C for 1 h (44) in the presence or absence of purified recombinant GST-Mel-18 or GST. Samples were resolved out on SDS-PAGE gels.

**Mel-18 RNAi:** Mel-18 short-hairpin RNA (shRNA) was designed by Oligoengine (Seattle, Washington) and cloned in the pSUPER-EGFP vector at the *Bgl*II and *Hind*III sites. The sequence of the shRNA used was as follows: 5′ CGACGCCACCACUAUCGUG 3′. The pSuper-Scramble plasmid (GATCCCTTTCTCCGAACGTGTCACGTTCAGAGAAGACGTGACACGTTCCGA GAATTTTTA), generously provided by Doug Andres (University of Kentucky), was used as a control for this experiment. The pSUPER-shRNA-Mel-18 and pSUPER-scrambled were transiently transfected into HeLa cells using Jet-PEI
RESULTS

In order to further understand the regulation and function of HSF2 in cells, our former labmate Dr. Goodson performed a yeast two-hybrid screen using the HSF2 protein as a bait. One of the HSF2-interacting clones obtained from this screen represented a region of the polycomb group protein Mel-18. This clone did not interact with a bait containing HSF1, a protein with high sequence-relatedness to HSF2, indicating the specificity of this interaction. The location of the region in Mel-18 found in the interacting yeast two-hybrid clone, which comprises amino acids 144-271 of the protein, is shown in the schematic in Figure 2.1A. Then my doctoral work focuses on the following characterization and function analysis for this interaction.

As an independent test of the interaction between HSF2 and Mel-18, and to determine whether the interaction is direct, an in vitro binding experiment was performed in which 35S-labelled in vitro translated Mel-18 was incubated with GST-HSF2 or GST-HSF1 bound to glutathione-agarose beads. The results of this experiment demonstrate the ability of HSF2 to interact with Mel-18, and indicate that the interaction is direct (Fig 2.1B).

To determine whether endogenous HSF2 and Mel-18 proteins interact, immunoprecipitation analysis was performed. Because our previous studies revealed that HSF2 function is regulated in a mitosis-dependent manner (20), this analysis was performed using extracts of asynchronous cells as well as those of cells blocked in mitosis by nocodazole treatment. The results of this experiment indicate that endogenous HSF2 and Mel-18 proteins do associate, and that lower levels of HSF2-Mel-18 complex are observed in extracts of mitotic cells compared to those of asynchronous cells (Fig 2.2).

As described above in the Introduction, previous studies showed that the polycomb protein Pc2/Cbx4 functions as an E3 protein to stimulate sumoylation of specific target proteins (103,104). Based on these findings, we sought to determine whether this polycomb protein Mel-18 may play a role in regulating sumoylation of HSF2. Specifically, our previous finding of increased sumoylation of HSF2 during mitosis (20), coupled with our new results in Figure 2.2 indicating that interaction between HSF2 and Mel-18 is decreased during mitosis, suggested the intriguing hypothesis that Mel-18 may actually function as a negative regulator of HSF2 sumoylation. This would be in contrast to the sumoylation-stimulatory function of the polycomb protein Pc2/Cbx4. As a first test of this hypothesis, we determined whether adding purified recombinant GST-Mel-
18 to an in vitro sumoylation assay would affect the SUMO modification of HSF2 in this system. As shown in Figure 2.3, addition of purified GST-Mel-18 is associated with decreased sumoylation of HSF2 in the in vitro modification assay.

Next, we wanted to test whether Mel-18 can inhibit the sumoylation of HSF2 expressed in cells. To test this, GFP-Mel-18 or GFP expression constructs, along with myc-SUMO-1 expression plasmid, were transfected into cells and then extracts of the cells were subjected to immunoprecipitation with anti-HSF2 antibodies or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of the HSF2 protein. The results of this experiment, shown in Figure 2.4A, indicate that expression of GFP-Mel-18, but not GFP, is associated with decreased HSF2 sumoylation. To probe the specificity of this effect of GFP-Mel-18 in inhibiting HSF2 sumoylation, we performed a similar experiment to analyze the related HSF1 protein, which we and others have previously shown to be sumoylated in response to stress (25,26). The results show that HSF1 sumoylation is not significantly affected by expression of GFP-Mel-18 (Figure 2.4B), indicating the selectivity of the inhibitory effect of Mel-18 overexpression on sumoylation of HSF2.

As a reverse, complementary approach for testing this hypothesis that Mel-18 may be a negative regulator of HSF2 sumoylation, we determined the effect of reducing cellular levels of Mel-18, using the RNAi methodology, on HSF2 sumoylation. According to our hypothesis and the results in Figure 2.3 and 2.4, we predicted that knockdown of Mel-18 should result in an increase in sumoylation of HSF2. To test this, cells were transfected with Mel-18 shRNA or scrambled shRNA, along with myc-SUMO-1 expression plasmid, and then extracts of the cells were subjected to immunoprecipitation with anti-HSF2 antibodies or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of HSF2. As shown in Figure 2.5, the results of this experiment indicate that, consistent with our hypothesis, a reduction in Mel-18 protein levels is associated with an increase in HSF2 sumoylation. These results, together with those shown in Figure 2.3 and 2.4 above, support the hypothesis that Mel-18 functions as an inhibitor of HSF2 sumoylation.

Next, we sought to probe the underlying mechanism by which Mel-18 inhibits the sumoylation of HSF2. A previous study showed that the polycomb protein Pc2 mediates its effects on sumoylation of its target proteins by interacting with Ubc9, the SUMO E2 enzyme (104). Based on this finding, we hypothesized that Mel-18 may also interact with Ubc9 as part of its mechanism for inhibiting HSF2 sumoylation. To test this hypothesis, we performed an in vitro binding experiment to determine whether Mel-18 protein present in whole cell extracts can interact with purified recombinant GST-Ubc9 bound to glutathione-agarose beads. In this experiment, after incubating the GST-Ubc9 or GST bound to glutathione-agarose with HeLa cell extracts and washing the beads to remove
unbound proteins, the amount of Mel-18 bound was determined by boiling the beads in SDS-PAGE buffer followed by Western blot using anti-Mel-18 antibodies. The results of this experiment indicate that purified recombinant Ubc9 is able to interact with Mel-18 present in cell extracts (Figure 2.6A).

To test for interaction between endogenous Mel-18 and Ubc9 proteins, we subjected cell extracts to immunoprecipitation using Mel-18 antibodies, followed by Western blot of the immunoprecipitates using Ubc9 antibodies. The results, shown in Figure 2.6B, indicate that endogenous Mel-18 and Ubc9 do interact. The results also suggest that there is more complex between Mel-18 and the form of Ubc9 that is covalently charged with SUMO (39 kDa form), relative to the non-SUMO-carrying form of Ubc9 (18 kDa form).

Based on this finding of interaction between Mel-18 and Ubc9, we envisioned a mechanism in which Mel-18 bound to HSF2 inhibits its sumoylation by binding to and inhibiting the activity of Ubc9 enzymes that approach HSF2. One way of inhibiting Ubc9 activity would be to block its ability to transfer the SUMO group from its active site to the target protein. Therefore, as a means for testing this proposed mechanism, we determined whether increasing the level of Mel-18 in cells results in increased amounts of the form of Ubc9 that has SUMO remaining covalently bound to it. GFP-Mel-18 or GFP alone were expressed in HEK 293 cells by transfection and then extracts of the transfected cells were subjected to Western blot with anti-Ubc9 antibodies, which will detect both the 18 kDa non-SUMO-containing form of Ubc9 and the 39 kDa SUMO-containing form of Ubc9. The results of this experiment, shown in Figure 2.6C, indicate that expression of the GFP-Mel-18 results in increased levels of the SUMO-containing form of Ubc9 compared to cells expressing the GFP alone construct. This result supports the hypothesis that Mel-18 inhibits Ubc9 activity by decreasing its ability to transfer SUMO groups to target proteins.

Based on the results shown in Figure 2.6 indicating that Mel-18 interacts with Ubc9 and may inhibit its ability to attach SUMO groups to proteins, we hypothesized that Mel-18 may inhibit the sumoylation of other proteins in addition to HSF2, perhaps even acting to down-regulate cellular sumoylation globally. To test this hypothesis, extracts of HEK 293 cells transfected with GFP-Mel-18 or GFP expression constructs along with the myc-SUMO-1 expression plasmid from the experiment shown in Figure 2.4 were subjected to Western blot using anti-myc and anti-SUMO-1 antibodies to detect sumoylated forms of cellular proteins. Consistent with this hypothesis, the results of this analysis indicate that increased expression of Mel-18 is associated with a detectable decrease in conjugation of SUMO-1 to cellular proteins (Figure 2.7).
CONCLUSION

The results presented in this chapter support the hypothesis that Mel-18 bound to HSF2 inhibits its sumoylation by binding to and inhibiting the activity of SUMO E2 (Ubc9) enzymes in the vicinity of the HSF2 protein. Further, our results showing that the interaction between HSF2 and Mel-18 is decreased during mitosis would provide a mechanism to explain the previously observed finding that HSF2 sumoylation is increased during this stage of the cell cycle (20).

The results described in this paper also suggest that Mel-18 actually functions like an anti-SUMO E3 protein, because like a traditional SUMO E3 protein it interacts both with the SUMO E2 enzyme Ubc9 and a sumoylation substrate protein (HSF2), but instead of stimulating SUMO modification of HSF2 it inhibits it. This is in contrast to the sumoylation-stimulating activities of another polycomb protein, Pc2, a traditional SUMO E3, indicating that members of the polycomb group of proteins are involved in both the positive and negative regulation of protein sumoylation through their interactions with Ubc9 and substrate proteins. The results also indicate that Mel-18 is able to detectably inhibit conjugation of SUMO-1 to proteins, suggesting that Mel-18 likely inhibits the sumoylation of other proteins in addition to HSF2, perhaps even acting to down-regulate sumoylation in cells globally.

Future studies investigating whether additional polycomb proteins act as positive or negative regulators of the sumoylation of proteins in cells, and identifying other SUMO substrate proteins whose modification is regulated by these polycomb proteins, would likely provide important insights into both the regulation of protein sumoylation and the mechanisms by which polycomb proteins mediate their important biological functions.
Figure 2.1 HSF2 interacts with Mel-18

(A) Schematic depicting the location within Mel-18 of the segment (amino acids 144-271) identified as an HSF2-interacting region in the yeast two-hybrid assay. (B) $^{35}$S-labeled *in vitro* translated Mel-18 was incubated with GST-HSF2, GST-HSF1 that were bound to glutathione-agarose beads. After washing, the amount of bound $^{35}$S-labeled Mel-18 was determined by SDS-PAGE and autoradiography (upper panel). The amounts of GST-HSF2 and GST-HSF1 bound to the beads were determined by performing anti-GST Western blot (lower panel). This figure is representative of this experiment performed minimally 3 times.
Figure 2.2 Interaction between HSF2 and Mel-18 is decreased during mitosis

Extracts of asynchronous or mitotic HeLa cells were immunoprecipitated using anti-HSF2 antibodies or non-specific IgG and the immunoprecipitates subjected to Western blot using anti-Mel-18 antibodies. The amounts of HSF2 in the Input and anti-HSF2 immunoprecipitate samples were measured by subjecting these samples to Western blot using goat polyclonal anti-HSF2 antibodies. This figure is representative of this experiment performed minimally 3 times.
Figure 2.3 Purified Mel-18 inhibits *in vitro* sumoylation of HSF2

$^{35}$S-labeled *in vitro* translated HSF2 was subjected to *in vitro* sumoylation in the absence of any additional purified proteins (lane 2), or in the presence of purified GST-Mel-18 (lane 3) or GST (lane 4). Samples were then analyzed by SDS-PAGE and autoradiography (upper panel). The anti-GST Western blot (lower panel) shows the relative amounts of GST-Mel-18 or GST that were added to the reactions in lanes 3 and 4 of the upper panel, respectively. This figure is representative of this experiment performed minimally 3 times.
Figure 2.4 Mel-18 inhibits sumoylation of HSF2 in vivo

(A) HEK 293 cells were transfected with GFP-Mel-18 or GFP expression constructs along with myc-SUMO-1 expression plasmid, and then extracts of the cells were subjected to immunoprecipitation with anti-HSF2 antibodies (rabbit polyclonal) or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of HSF2, and to Western blot using goat polyclonal HSF2 antibodies (Bethyl Inc.). The cell lysates were subjected to anti-GFP Western blot to analyze expression levels of GFP-Mel-18 and GFP, and to anti-β-actin Western blot as a loading control. (B) A similar experiment to that described in panel A was performed, except that here the cells were subjected to a 42°C heat treatment for 60 minutes prior to harvesting them for immunoprecipitation analysis (to allow stress-induced HSF1 sumoylation) and that anti-HSF1 antibodies were used for the immunoprecipitation step, so that sumoylated forms of HSF1 would be detected by the subsequent anti-myc (detecting myc-SUMO-1) Western blot. This figure is representative of this experiment performed minimally 3 times.
Figure 2.5 Knockdown of Mel-18 protein levels is associated with increased HSF2 sumoylation

HeLa cells were transfected with Mel-18 shRNA or scrambled shRNA along with the myc-SUMO-1 expression plasmid, and then extracts of the cells were subjected to immunoprecipitation with anti-HSF2 antibodies (rabbit polyclonal) or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of HSF2, and to Western blot using goat polyclonal HSF2 antibodies (Bethyl Inc.). The cell lysates were subjected to anti-Mel-18 Western blot to confirm reduction of Mel-18 protein levels by Mel-18 shRNA treatment, and to anti-β-actin Western blot as a loading control. This figure is representative of this experiment performed 2 times.
Figure 2.6 Mel-18 interacts with the SUMO E2 enzyme, Ubc9

(A) GST or GST-Ubc9 proteins bound to glutathione-agarose beads were incubated with extracts of HeLa cells, and then after washing the amount of bound Mel-18 was determined by Western blot using anti-Mel-18 antibodies. The anti-GST Western blot (lower panel) shows the relative amounts of GST-Ubc9 or GST that were used in the binding reactions, respectively. (B) Extracts of HeLa cells were subjected to immunoprecipitation using anti-Mel-18 antibodies, followed by Western blot of the immunoprecipitates using Ubc9 antibodies. (C) HEK 293 cells were transfected with the GFP or GFP-Mel-18 expression constructs, and then extracts of the transfected cells were subjected to Western blot assay using anti-Ubc9 antibodies to detect the non-SUMO-conjugated (18 kDa) and SUMO-containing (39 kDa) forms of Ubc9. The cell lysates were also subjected to anti-β-actin Western blot as a loading control. This figure is representative of this experiment performed minimally 3 times.
Figure 2.7 Mel-18 inhibits general protein sumoylation in cells
Extracts of HEK 293 cells transfected with GFP-Mel-18 or GFP expression constructs along with the myc-SUMO-1 expression plasmid from the experiment shown in Figure 2.4 were subjected to Western blot using anti-myc and anti-SUMO-1 antibodies to detect sumoylated forms of cellular proteins, and to anti-β-actin Western blot as a loading control. This figure is representative of this experiment performed minimally 3 times.
CHAPTER THREE: MEL-18 INTERACTS WITH RANGAP1 AND INHIBITS ITS SUMOYLATION

BACKGROUND

Covalent attachment of Small Ubiquitin-like Modifier (SUMO) proteins to lysine residues in target proteins, or sumoylation is an important regulator of protein functional properties (88-90,105-106). SUMO proteins are covalently attached to target lysine residues by the SUMO E2 enzyme, Ubc9, and these substrate lysines are typically found within the consensus sequence $\Psi$KXE ($\Psi$ represents hydrophobic amino acids) (42,91-93).

SUMO E3 proteins have been identified that enhance the efficiency of sumoylation by interacting with both Ubc9 (SUMO E2) and the target protein, thereby acting as bridging factors to increase the rate of the sumoylation reaction (33,88,90,94,95,104,105,107). However, the results in the previous chapter revealed the surprising finding that a member of the polycomb group of proteins, called Mel-18, functions like an anti-SUMO E3 protein (108). In this study it was found that Mel-18 binds to and inhibits the sumoylation of a protein called HSF2 by interacting with and inhibiting the activity of Ubc9 enzymes in the vicinity of HSF2. These results also indicated that Mel-18 may function to inhibit the sumoylation of other cellular proteins, but the identities of these other proteins were unknown.

RanGAP1, a Ran GTPase-activating protein that plays a critical role in nuclear transport, was the first identified substrate for SUMO modification (37,38). The results presented in this chapter reveal that Mel-18 interacts with RanGAP1 and inhibits its sumoylation, and that these effects do not require the RING domain of Mel-18. The results also show that RanGAP1 sumoylation decreases during mitosis and that this is correlated with increased interaction between RanGAP1 and Mel-18 during this stage of the cell cycle, which is the reverse of the regulatory relationship between Mel-18 and HSF2 sumoylation with respect to mitosis. These results strengthen support for the function of the polycomb protein Mel-18 as an anti-SUMO-E3 factor, and indicate that it is an important regulator of the sumoylation of a number of vital proteins in the cell.

MATERIALS AND METHODS

Cell culture: HEK 293T cells were grown at 37°C in DMEM supplemented with 10% FBS.

Mutagenesis of plasmids: pEGFP-Mel-18 plasmid mutations, including the Δ17–56 mutant of Mel-18 (ring finger deletion referred to as RINGΔ) and the C53G and C56G substitution mutants, were generated using the QuickChange mutagenesis method (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. PCR amplifications were performed using the following program: 95°C
for 30 s, 25× (95°C for 30 s, 55°C for 1 min, 68°C for 12 min). Mutations were confirmed by DNA sequencing.

**Immunoprecipitation analysis:** For co-immunoprecipitation experiments, HEK 293 cells were transfected with GFP-Mel-18 expression constructs (wild-type, ring finger deletion (RINGΔ), C53G, or C56G mutations) using jetPEI reagent according to the manufacturer’s instructions (polylip-transfection, New York, NY), and then blocked in mitosis by treatment with 400 ng/ml nocodazole for 16 h. After 48 h transfected cells were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche Applied Science)) for 20 min. Lysates were then cleared by centrifugation at 16,438 g for 10 min at 4 °C. Supernatants were precleared by incubation with goat (control) IgG and protein G-sepharose beads for 2 h at 4 °C with gentle rotation. Precleared extracts were then incubated with primary goat polyclonal RanGAP1 antibody or control IgG and 50% slurry of protein G-sepharose for 4 h at 4 °C with rotation. After washing beads six times for 5 min each at 4 °C with NP-40 buffer, bound proteins were released by boiling in SDS–PAGE sample dye and analyzed by Western blot using the GFP mouse monoclonal antibody (JL-8 clone, Invitrogen). For immunoprecipitation analysis of RanGAP1 sumoylation, HEK 293T cells were transfected with pEGFP-Mel-18 (wild-type or mutants) or pEGFP-C1 along with the myc-sumo-1 expression plasmid using jetPEI reagent according to the manufacturer’s instructions (polylip-transfection). After 48 hours cell extracts were prepared using NP-40 Buffer (50 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche Applied Science), 10 mM N-ethylmaleimide) before adding RanGAP1 polyclonal antibodies or non-specific IgG to proceed as described above, followed by Western blot assay using anti-myc monoclonal antibody (Invitrogen) or anti-SUMO-1 antibodies (Bethyl). This assay was normalized with cell number.

**In vitro sumoylation assay:** T7-RanGAP1 Δ419 (gift of Mike Matunis) was in vitro translated in a rabbit reticulocyte lysates using the TNT-coupled transcription-translation system (Promega) with 35S methionine at 30°C for 2 h. Then in vitro translated product was incubated with Ubc9 and GST-SUMO-1 in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl2, 2mM ATP, 1 mM dithiothreitol) at 30°C for 1 h (44) in the presence or absence of purified recombinant GST-Mel-18 or GST. Samples were resolved out on SDS-PAGE gels.

**RESULTS**

Our previous study showed that Mel-18 interacts with HSF2 and inhibits its sumoylation by binding to and inhibiting the activity of Ubc9 enzymes in the vicinity of HSF2 (108). The results also suggested that Mel-18 inhibits the
sumoylation of other cellular proteins. RanGAP1 is a very important cellular SUMO substrate protein, in fact the first identified substrate for SUMO-1 conjugation (37,38). Therefore, in the present study we sought to determine whether the sumoylation of RanGAP1 is regulated by Mel-18, and if so whether this involves interaction between these two proteins.

As a first test of this hypothesis, we determined whether adding purified recombinant GST-Mel-18 would affect the SUMO modification of RanGAP1 in an in vitro sumoylation assay. The results of this experiment, shown in Figure 3.1A, indicate that the addition of purified GST-Mel-18 is indeed associated with decreased sumoylation of RanGAP1. Next, we wanted to examine whether Mel-18 can inhibit the sumoylation of RanGAP1 expressed in cells. To test this, GFP-Mel-18 or GFP expression constructs, along with a myc-SUMO-1 expression plasmid, were transfected into cells and then extracts of these cells were subjected to immunoprecipitation using anti-RanGAP1 antibodies or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of the RanGAP1 protein. The immunoprecipitates were also subjected to Western blot using anti-RanGAP1 antibodies in order to provide an additional way to visualize the sumoylated form of RanGAP1. The results of this experiment, shown in Figure 3.1B, indicate that expression of GFP-Mel-18, but not GFP, is associated with decreased RanGAP1 sumoylation. The results of the experiments shown in Figure 3.1 demonstrate the ability of Mel-18 to inhibit RanGAP1 sumoylation.

As described above, our previous work demonstrated that Mel-18 binds to HSF2 and inhibit its sumoylation (108). Therefore, we next investigated whether the inhibition of RanGAP1 sumoylation demonstrated by the data in Figure 3.1 above could also involve interaction between RanGAP1 and Mel-18. To examine this, HEK 293 cells were transfected with GFP-Mel-18 expression constructs and then extracts of the cells were subjected to immunoprecipitation using anti-RanGAP1 antibodies or non-specific IgG (negative control), followed by anti-GFP antibody Western blot (Figure 3.2A). As a complementary approach, we preformed the reverse approach of subjecting extracts of these transfected cells to immunoprecipitation with anti-GFP antibodies or non-specific IgG (negative control), followed by Western blot using anti-RanGAP1 antibodies (Figure 3.2B). The results of these experiments both indicate that RanGAP1 and Mel-18 proteins expressed in cells do interact.

Although it has been described that RanGAP1 remains SUMO modified during mitosis (110), it is still unclear whether the SUMO level is changed during this part of the cell cycle. Our previous finding showed that increased sumoylation of HSF2 in mitosis is coupled with decreased interaction between HSF2 and Mel-18, so we hypothesized that RanGAP1 may also exhibit mitotic-dependent regulation of its sumoylation, which might be associated with different
interaction level with Mel-18 during this stage of the cell cycle. As a first test of this hypothesis, extracts of asynchronous or mitotic HEK 293 cells were immunoprecipitated using anti-RanGAP1 antibodies or non-specific IgG and the immunoprecipitates were subjected to Western blot using anti-SUMO-1 antibodies (top panel) or anti-RanGAP1 antibodies (middle panel) (Figure 3.3A). The results of this experiment indicate that sumoylation of RanGAP1 is decreased during mitosis.

Next, we wanted to determine whether this mitotic-dependent decrease in RanGAP1 sumoylation is associated with increased interaction between Mel-18 and RanGAP1 during this part of the cell cycle, as predicted by our hypothesis. To test this, HEK 293 cells were transfected with GFP-Mel-18 expression constructs, and then extracts of asynchronous or mitotic transfected HEK 293 cells were immunoprecipitated using anti-RanGAP1 antibodies and the immunoprecipitates subjected to Western blot using anti-GFP antibodies. The results of this experiment indicate that higher levels of interaction between RanGAP1 and Mel-18 are indeed observed in extracts of mitotic cells compared to those of asynchronous cells (Fig 3.3B).

The Mel-18 protein contains a RING finger domain in its N-terminal region (111-113). To test whether this RING domain is important for the ability of Mel-18 to interact with RanGAP1 or inhibit RanGAP1 sumoylation, we made mutant GFP-Mel-18 plasmids with a RING finger deletion (RINGΔ) or cysteine-to-glycine substitutions at key cysteine residues 53 and 56 of the RING domain (C53G, C56G). First, to test the importance of the RING finger for Mel-18 interaction with RanGAP1, HEK 293 cells were transfected with the wild-type or mutant (RINGΔ, C53G, or C56G) GFP-Mel-18 constructs along with the myc-SUMO-1 expression plasmid, and then extracts of the transfected cells were subjected to immunoprecipitation using anti-RanGAP1 antibodies or non-specific IgG followed by Western blot of the immunoprecipitates with anti-GFP antibodies (Figure 3.4A). The results indicate that none of the RING finger mutations appear to affect Mel-18 interaction with RanGAP1. To examine their effect on RanGAP1 sumoylation, HEK 293 cells transfected with these constructs along with the myc-SUMO-1 expression plasmid were subjected to immunoprecipitation using anti-RanGAP1 antibodies or non-specific IgG, followed by Western blot of the immunoprecipitates with anti-myc antibodies to detect sumoylated RanGAP1. The results, shown in Figure 3.4B, indicate that deletion of the RING domain or mutation of cysteines 53 or 56 also has no effect on RanGAP1 sumoylation.

CONCLUSION

The results presented in this chapter show that Mel-18 not only acts as an anti-SUMO E3 factor for the HSF2 protein, but for RanGAP1 as well. This finding increases the likelihood that Mel-18 regulates the sumoylation of other, as-yet-
undiocovered cellular proteins. Indeed, our previous results indicated that there are many sumoylated bands on SDS–PAGE gels whose amounts change significantly in response to changes in Mel-18 level, indicating that Mel-18 may in fact regulate the sumoylation of a large number of proteins. Thus, one important goal of future studies is to identify these other targets whose SUMO modification is modulated by Mel-18. Mel-18 is known to act as a tumor suppressor, and so it would be particularly exciting if it was found to regulate the sumoylation of cellular proteins involved in the control of cell proliferation.

Another intriguing finding of this paper is that the cell cycle-dependence of the interaction between Mel-18 and HSF2 vs. RanGAP1 are exactly the opposite of each other: HSF2 interaction with Mel-18 decreases during mitosis, resulting in elevated HSF2 sumoylation, in contrast to higher interaction of RanGAP1 and Mel-18 and less RanGAP1 sumoylation during this stage of the cell cycle. This suggests that there is a mechanism or mechanisms for differentially controlling Mel-18 interaction with partners in a mitosis-dependent manner. Future studies into this area would likely reveal valuable insight into the regulation of the anti-SUMO E3 function of Mel-18.

Finally, our results indicate that the conserved RING finger motif of Mel-18, found in its N-terminal region (111-113), is not required for its interaction with RanGAP1 or its ability to inhibit RanGAP1 sumoylation. Since the results of our previous study suggested that Mel-18 interaction with the SUMO E2 enzyme Ubc9 is likely important for the sumoylation-inhibitory function of Mel-18 (108), this suggests that other regions of the Mel-18 protein are involved in binding RanGAP1 and Ubc9. Future studies to identify these regions would reveal important new functional domains of the Mel-18 protein.
A WCE (E1) - + + +
Ubc9 - + + +
SUMO-1 - + + +
GST - - + -
GST-mel-18 - - - +

-ΔRanGAP1-SUMO
-ΔRanGAP1
-GST-mel-18
-GST

B

<table>
<thead>
<tr>
<th>IP: α-RanGAP1</th>
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<td>-</td>
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<tr>
<td>GFP-mel-18</td>
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<td>myc-SUMO-1</td>
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anti-myc WB

-RanGAP1-SUMO
-RanGAP1
-GFP-mel-18

anti-GFP WB of lysates

-GFP

anti-β-actin WB of lysates
Figure 3.1 Mel-18 inhibits RanGAP1 sumoylation

(A) Purified Mel-18 inhibits *in vitro* sumoylation of RanGAP1. $^{35}$S-labeled *in vitro* translated T7-RanGAP1 Δ419 RanGAP1 fragment was subjected to *in vitro* sumoylation in the absence of any additional purified proteins (lane 2), or in the presence of purified GST (lane 3) or GST-Mel-18 (lane 4). Samples were then analyzed by SDS–PAGE and autoradiography (top panel). The anti-GST Western blot (lower panel) shows the relative amounts of GST-Mel-18 or GST that were added to the reactions in lanes 3 and 4 of the top panel, respectively.

(B) Mel-18 inhibits sumoylation of RanGAP1 *in vivo*. HEK 293 cells were transfected with GFP-Mel-18 or GFP expression constructs along with myc-SUMO-1 expression plasmid, and then extracts of the cells were subjected to immunoprecipitation with anti-RanGAP1 antibodies or non-specific IgG (negative control), followed by anti-myc Western blot to detect the sumoylated forms of RanGAP1, and to Western blot using anti-RanGAP1 antibodies. The cell lysates were subjected to anti-GFP Western blot to analyze expression levels of GFP-Mel-18 and GFP, and to anti-b-actin Western as a loading control. This figure is representative of this experiment performed minimally 3 times.
Figure 3.2 RanGAP1 interacts with Mel-18.

(A) HEK 293 cells were transfected with GFP-Mel-18 expression constructs, and then extracts of the cells were subjected to immunoprecipitation with anti-RanGAP1 antibodies or non-specific IgG (negative control), followed by anti-GFP Western blot to detect the interaction between Mel-18 and RanGAP1. (B) A similar experiment to that described in (A) was performed, except that here the cell extracts were subjected to immunoprecipitation with anti-GFP antibodies or non-specific IgG (negative control), followed by anti-RanGAP1 Western blot. This figure is representative of this experiment performed minimally 3 times.
Figure 3.3 Decreased RanGAP1 sumoylation and increased interaction between RanGAP1 and Mel-18 during mitosis.

(A) Sumoylation of RanGAP1 decreases during mitosis. Extracts of asynchronous or mitotic HEK 293 cells were immunoprecipitated using anti-RanGAP1 (goat polyclonal) antibodies and the immunoprecipitates subjected to Western blot using anti-SUMO-1 antibodies (top panel) or anti-RanGAP1 antibodies (middle panel). The cell lysates were subjected to anti-b-actin Western blot as a loading control (bottom panel). (B) Interaction between RanGAP1 and Mel-18 increases during mitosis. HEK 293 cells were transfected with GFP-Mel-18 expression constructs, and then extracts of asynchronous or mitotic transfected cells were immunoprecipitated using anti-RanGAP1 antibodies or non-specific IgG and the immunoprecipitates subjected to Western blot using anti-GFP antibodies (top panel). The cell lysates were subjected to anti-b-actin Western blot as a loading control (bottom panel). This figure is representative of this experiment performed 2 times.
Figure 3.4 RING finger domain of Mel-18 is not required for its interaction with RanGAP1 or inhibition of RanGAP1 sumoylation.

(A) HEK 293 cells were transfected with GFP-Mel-18 expression constructs (wild-type, ring finger deletion, C53G, or C56G mutations), along with the myc-SUMO-1 expression plasmid. Extracts of the transfected cells were immunoprecipitated using anti-RanGAP1 antibodies or nonspecific IgG and the immunoprecipitates subjected to Western blot using anti-GFP antibodies (top panel). (B) As in (A), except that here the anti-RanGAP1 or nonspecific IgG immunoprecipitates were subjected to Western blot using anti-myc antibodies to examine the levels of sumoylated RanGAP1. The anti-RanGAP1 immunoprecipitates were also subjected to anti-RanGAP1 Western blot to normalize for levels of RanGAP1. This figure is representative of this experiment performed minimally 3 times.
CHAPTER FOUR:
IDENTIFICATION OF A RING FINGER POLYMORPHISM OF HUMAN BMI-1
THAT CAUSES ITS DEGRADATION BY THE UBIQUITIN-PROTEASOME
SYSTEM

BACKGROUND

Bmi-1 is a member of the polycomb group family of proteins, which function
as negative regulators of the transcription of a number of important target genes
(114). Bmi-1 was first cloned as a c-myc cooperating oncogene in murine
lymphomas (65,66), and found to have significant sequence relatedness to
Drosophila polycomb proteins(66). Previous studies have identified several ways
in which Bmi-1 mediates its effects on cell proliferation, including inhibiting
expression of the Ink4A/ARF locus(68,85), modulating the p21-Rb pathway (69),
and inducing telomerase activity (70).

In addition to its function as an oncogene, Bmi-1 also plays important roles
in determination of cell fate and stem cell renewal of the neural, hematopoietic,
and other cell lineages (67,71-77). Bmi-1 contains a conserved RING finger
domain near the NH2 terminus, which is important for the function of this protein
(65,70,78-80,82). Bmi-1 has been found to be predominantly localized to the
nucleus, which is mediated by a nuclear localization sequence (NLS) located in
the C-terminal region of this protein (70,78,115).

In this paper we characterize a polymorphism in the human Bmi-1 protein
that changes a cysteine in the RING finger domain (cysteine 18) to tyrosine. The
results show that the C18Y polymorphism results in a significant reduction in
levels of the Bmi-1 protein by leading to its ubiquitination and destruction by the
proteasome. In light of the important functions of Bmi-1 in stem cell renewal and
determination of cellular identity, these results suggest that this C18Y
polymorphism could have deleterious effects in the people that have it.

MATERIALS AND METHODS

SNP database searching: To identify potential polymorphisms within the
Bmi-1 gene, we searched the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html).
This search identified a SNP polymorphism (rs1042059) that is predicted to change the cysteine at amino acid
18 of Bmi-1 to tyrosine. This Bmi-1 C18Y polymorphism was found in both the
CEU (Utah residents with ancestry from northern and western Europe) and YRI
(Yoruba in Ibadan, Nigeria) populations that are part of the International HapMap
Project.

Cell culture and plasmids: HeLa-ATCC cells and HEK 293T cells were
grown at 37°C in DMEM supplemented with 10% FBS and 100x antibiotic-
antimycotic (Invitrogen) in 5% CO2. pEGFP-Bmi-1 plasmid was generated by
using PCR to amplify from the plasmid pOTB7-hBmi-1 cDNA (Accession number BC011652, Open Biosystems, Huntsville, AL) a coding fragment of Bmi-1 having KpnI and BamHI sites at the ends using the following primers: 5′-GCG GGT ACC ATG CAT CGA ACA ACG AGA-3′ and 5′- CGC GGA TCC TCA ACC AGA AGA AGT TGC TGA-3′. PCR amplifications were performed using the following program: 95°C for 5 min, 30× (95°C for 1 min, 59°C for 1 min, 72°C for 2 min), 72°C for 5 min. This PCR product was then cloned into pEGFP-C1 vector (Clontech, Mountain View, CA) at the KpnI and BamHI sites to make the pEGFP-Bmi-1 plasmid. This plasmid was confirmed by DNA sequencing. pEGFP-C18Y-Bmi-1 plasmid was generated using the QuickChange mutagenesis method (Stratagene) according to the manufacturer’s protocol. PCR amplifications were performed using the following program: 95°C for 30 s, 18× (95°C for 30 s, 55°C for 1 min, 68°C for 12 min). The mutation was confirmed by DNA sequencing.

Fluorescence microscopy: HEK293T cells were seeded onto coverslips that were acid-washed and flamed, and then coated with laminin (5 µg/ml) (Sigma-Aldrich, St. Louis, MO). 48 h after transfection with the wild-type or C18Y GFP-Bmi-1 fusion proteins, cells were washed twice in ice-cold 1× PBS, followed by fixation in 3.7% paraformaldehyde for 20 minutes at room temperature. After a final wash with PBS, coverslips were mounted on a slide with Vectashield mounting medium plus 1.5 µg/ml DAPI (4′, 6 diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA). Fluorescence of the GFP-Bmi-1 proteins was visualized using a Nikon fluorescent microscope with a 100x oil immersion objective and a Nikon Spotcam digital-imaging camera.

Extract preparation and Western blot assay: HEK293 cells were transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids using Jet-PEI reagent according to the manufacturer’s instructions. At 48 h post-transfection, cells were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche Applied Science) and 20 mM N-ethylmaleimide (added fresh)) for 20 minutes. After centrifugation at 16,438 g at 4°C for 10 minutes, the supernatant was transferred to a fresh tube, and then the whole cell lysate was used for the following assays: SDS-PAGE and Western blot were performed according to standard protocols. The antibodies and dilutions used to probe the Western blots were as follows. Goat anti-GFP antibody (Bethyl Laboratories, Inc.) was used at 1:2,000. For the immunoprecipitation assay of ubiquitinated Bmi-1, mouse monoclonal anti-ubiquitin antibody (gift of Dr. Haining Zhu, University of Kentucky, Lexington, KY) was used at 1:1,000. This assay was normalized with cell number.

Proteasome Inhibition Assay: HEK293T cells were transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids as described above, except that at 44 h post-transfection 10 µM MG132 proteasome inhibitor (Calbiochem,
Gibbstown, NJ, gift of Dr. Dan Noonan, University of Kentucky, Lexington, KY) was added and incubated for 4 hours. This assay was normalized with cell number.

**Bmi-1 Ubiquitination Assay:** HEK293T cells were transfected with pEGFP-Bmi-1 or pEGFP-C18Y-Bmi-1 as described above. At 48 hours post-transfection, cells were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche Applied Science) and 20 mM N-ethylmaleimide (added fresh)) for 20 minutes. Lysates were then cleared by centrifugation at 16,438 g for 10 minutes at 4°C. Supernatants were precleared by incubation with goat (control) IgG and protein G-sepharose beads for 2 hours at 4°C with gentle rotation. Precleared extracts were then incubated with primary goat polyclonal anti-GFP antibody or control IgG and 50% slurry of protein G-sepharose for 4 hours at 4°C with rotation. After washing beads 6 times for 5 minutes each at 4°C with NP-40 buffer, bound proteins were released by boiling in SDS-PAGE sample dye and analyzed by Western blot assay using the anti-ubiquitin mouse monoclonal antibody or anti-GFP goat polyclonal antibody. This assay was normalized with cell number.

**RESULTS**

As depicted in Figure 4.1A, the Bmi-1 protein contains a RING finger domain in its N-terminal region (65,70,78-80,82). Examination of the dbSNP database revealed the existence of a polymorphism in human Bmi-1 that changes amino acid 18, a cysteine in the RING finger domain, to tyrosine (Figure 4.1B). Further analysis revealed that this polymorphism is found heterozygous in individuals of both the CEU (Utah residents with ancestry from northern and western Europe) and YRI (Yoruba in Ibadan, Nigeria) populations that are part of the International HapMap Project.

Because the cysteine that is changed to tyrosine in this polymorphism is a residue of the RING finger domain, we hypothesized that this alteration could lead to alteration in the functional properties of the Bmi-1 protein. Bmi-1 has been found to be a predominantly nuclear-localized protein (70,78,115), mediated by a nuclear localization sequence (NLS) in the C-terminal region (Figure 4.1A) (70,78). Therefore, one functional property of Bmi-1 we hypothesized could be affected by the C18Y polymorphism is its pattern of localization within the cell. To test this hypothesis we transfected wild-type and C18Y GFP-Bmi-1 expression plasmids into HEK293 cells and then examined the sub-cellular localization of the transfected proteins using fluorescence microscopy analysis. The results of this experiment, shown in Figure 4.2, revealed that the wild-type and C18Y Bmi-1 proteins both exhibit predominant
nuclear localization, indicating that the C18Y polymorphism does not appear to significantly alter the subcellular localization of the Bmi-1 protein.

Although the results of the fluorescence microscopy experiment shown in Figure 4.2 did not indicate a difference in localization, they did show that the intensity of the signal from the C18Y GFP-Bmi-1 was markedly less than that exhibited by wild-type Bmi-1. This suggested that the C18Y polymorphism may be associated with a decrease in levels of the Bmi-1 protein. To test this we subjected extracts of HEK293 cells transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids to Western blot assay with anti-GFP antibodies to detect the transfected Bmi-1 proteins, or with β-actin antibodies as a loading control. The results of this experiment show that the levels of the C18Y GFP-Bmi-1 protein are indeed significantly lower than that of the wild-type GFP-Bmi-1 (Figure 4.3).

We hypothesized that the C18Y polymorphism could be resulting in decreased levels of Bmi-1 by leading to increased destruction of this protein by the ubiquitin-proteasome degradation pathway. To test this hypothesis, we subjected extracts of HEK293 cells transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids to immunoprecipitation using anti-GFP antibodies or non-specific IgG control antibodies, followed Western blot assay with anti-ubiquitin antibodies to compare the amounts of ubiquitinated forms of the transfected wild-type vs. C18Y Bmi-1 proteins. The results of this analysis, shown in Figure 4.4A, indicate that C18Y Bmi-1 exhibits significantly higher levels of ubiquitination than wild-type Bmi-1. Next, to test the involvement of the proteasome in the lower protein levels of C18Y Bmi-1, we tested whether levels of this form of Bmi-1 increase in cells that have been treated with the proteasome inhibitor MG-132. The results of this experiment show that MG-132 treatment does indeed result in the presence of significantly higher levels of the C18Y Bmi-1 protein (Figure 4.4B). The results shown in Figure 4 support the hypothesis that the C18Y polymorphism of Bmi-1 leads to lower levels of this protein by causing its turnover by the ubiquitin-proteasome degradation pathway.

CONCLUSION

The results described in this part characterize a polymorphism in the human Bmi-1 protein that changes the cysteine at amino acid position 18 to tyrosine (C18Y). The results also show that the C18Y substitution causes a significant reduction in Bmi-1 protein levels and that this is associated with increased ubiquitination leading to degradation by the proteasome. This is the first demonstration of a polymorphism in human Bmi-1 that leads to alteration in levels of this important protein.
Figure 4.1 Identification of a SNP resulting in a C18Y polymorphism of Bmi-1.

(A) Schematic showing the location of the RING finger and NLS in the Bmi-1 protein, as well as its nuclear localization sequence (NLS). (B) Schematic showing the amino acid sequence of the RING finger domain and location of the C18Y polymorphism of Bmi-1.
Figure 4.2 Fluorescence microscopy analysis of wild-type and C18Y Bmi-1 localization in cells.
(A) Wild-type and C18Y GFP-Bmi-1 expression plasmids were transfected into HEK 293T cells, and the subcellular localization of the GFP–Bmi-1 proteins examined by fluorescence microscopy. GFP images shown are 1.25 second exposures. DNA was visualized by staining with DAPI. (B) Fluorescence microscopy analysis of wild-type GFP-Bmi-1 taken at shorter exposure time for the GFP channel (0.45 second) than that shown in panel A. This figure is representative of this experiment performed minimally 3 times.
Figure 4.3 C18Y polymorphism is associated with a significant decrease in Bmi-1 protein level.

Wild-type or C18Y GFP-Bmi-1 expression plasmids were transfected into HEK293 cells, and then extracts prepared from the transfected cells were subjected to Western blot assay using anti-GFP antibodies (upper panel), or anti-β-actin antibodies as a loading control (lower panel). This figure is representative of this experiment performed minimally 3 times.
Figure 4.4 C18Y Bmi-1 protein is degraded by the ubiquitin-proteasome system.

(A) HEK293 cells were transfected with wild-type or C18Y GFP-Bmi-1 expression plasmids, and then extracts of the cells were subjected to immunoprecipitation...
with anti-GFP antibodies (goat polyclonal) or non-specific IgG (negative control), followed by anti-ubiquitin Western blot to detect the ubiquitinated forms of Bmi-1, and to Western blot using goat polyclonal anti-GFP antibodies. (B) Wild-type or C18Y GFP-Bmi-1 expression plasmids were transfected into HEK293 cells, in the absence or presence of 10 µM MG132 proteasome inhibitor (inhibitor added at 44 h post-transfection and incubated for 4 hours). Extracts prepared from the transfected cells were then subjected to Western blot assay using anti-GFP antibodies (upper panel), or anti-β-actin antibodies as a loading control (lower panel). This figure is representative of this experiment performed minimally 3 times.
CHAPTER FIVE:
DISCUSSION and Future Directions

I. Mel-18 is a novel anti-SUMO E3 protein.

Previous studies showed little evidence of anti-SUMO E3 proteins. Our research is the first that suggests that Mel-18 actually functions as an anti-SUMO E3 protein, because like a traditional SUMO E3 protein it interacts both with the SUMO E2 enzyme Ubc9 and sumoylation substrate proteins (HSF2/RanGAP1), but instead of stimulating SUMO modifications of HSF2/RanGAP1, it inhibits them by binding to and inhibiting the activity of SUMO E2 (Ubc9) enzymes in the vicinity of the HSF2/RanGAP1 proteins. This is in contrast to the sumoylation-stimulating activities of another polycomb protein, Pc2, a traditional SUMO E3, indicating that members of the polycomb group of proteins are involved in both the positive and negative regulation of protein sumoylation through their interactions with Ubc9 and substrate proteins. Future studies investigating whether additional polycomb proteins act as positive or negative regulators of the sumoylation of proteins in cells would likely provide important insights into both the regulation of protein sumoylation and the mechanisms by which polycomb proteins mediate their important biological functions.

Mel-18 has a well conserved RING finger motif at its N-terminal region, which is shown to be important in DNA binding and homodimerization (63); however our results indicate that this domain is not required for its interaction with RanGAP1 or its ability to inhibit RanGAP1 sumoylation. Since these results also demonstrated that Mel-18 interaction with the SUMO E2 enzyme Ubc9 is likely important for the sumoylation-inhibitory function of Mel-18, this suggests that other regions of the Mel-18 protein are involved in binding RanGAP1 and Ubc9. Future studies to identify these regions would reveal important new functional domains of the Mel-18 protein. This would also support our hypothesis that Mel-18, like Pc2, has multiple domains contributing to its anti-SUMO E3 activities. A carboxyl-terminal fragment of Pc2 can form a scaffold by recruiting E2 (Ubc9) and substrate (CtBP), and this region can also interact with the amino-terminal domain, which will facilitate the transfer of SUMO from E2 to substrate. Besides the conserved RING domain at the N-terminal region (Cys18 ~ Cys56), Mel-18 also has other two important domains: well conserved HTH structure in the middle region (Lys158 ~ Val229), that is important in homodimerization (63), and a Proline/serine-rich domain (P/S domain) in the C-terminal region (Gln230 ~ Thr344), that is important in protein-protein interactions, such as binding with cyclin D2 (61). Our study of yeast two-hybrid screening showed that the interaction region of Mel-18 with HSF2 localizes to amino acids 144-270, so we can perform in vitro binding or yeast two hybrid assays to identify the interaction region of Mel-18 with RanGAP-1, as well as Ubc-9. We can also do the Ubc9
preloading assay to test which domain(s) will contribute to the anti-SUMO E3 activities of Mel-18 (34).

We propose two possible models for Mel-18 acting as an anti-SUMO E3 protein: I. Keeping Ubc9 away from substrates, such as HSF2/RanGAP1. This hypothesis is based on the adaptor model of most SUMO E3 proteins, such as Pc2. The carboxyl-terminal region of Pc2 functions as a scaffold, recruits E2 (Ubc9) and substrate (CtBP), and facilitates its sumoylation. II. Allosterical conformation change of Ubc-9 and/or substrates by Mel-18: This hypothesis of decreased affinity between Ubc-9 and substrates by conformation change is based on the RanBP2 study reports: RanBP2, SUMO-1 E3 ligase, can allosterically change Ubc-9 conformation and induce increased affinity for specific substrates (33).

Mel-18 can work in one of these two models or both of them. To test whether Mel-18 keeps Ubc-9 from substrates, we can utilize the cross-linking technique. Cells will be transfected with GFP-Mel-18 or GFP constructs, and then cell extracts will be subjected to immunoprecipitation assay after cross-linking treatments. By comparison of the complexes levels of Ubc-9 and substrates, we can know whether Ubc-9 is kept away from substrates by Mel-18. Allosterical conformation change of Ubc-9 and substrates by Mel-18 can be detected by the change of infrared (IR), UV, or CD spectra when associated with Mel-18 binding (116).

Another intriguing finding is that the cell cycle dependence of the interaction between Mel-18 and HSF2 vs. Ran-GAP1 are exactly the opposite of each other: HSF2 interaction with Mel-18 decreases during mitosis, resulting in elevated HSF2 sumoylation, in contrast to higher interaction of RanGAP1 and Mel-18 and less RanGAP1 sumoylation during this stage of the cell cycle. This suggests that there is a mechanism or mechanisms for differentially controlling Mel-18 interaction with partners in a mitosis-dependent manner. We propose a potential mechanism for regulating the anti-SUMO E3 activities of Mel-18 by different phosphorylation levels during different cell cycle phases. Previous studies showed that the dephosphorylated form of Mel-18 dissociates from chromatin during mitosis (64). Meanwhile, it was also reported that many PcG proteins will dissociate from the chromatin during mitosis, and disperse into the cytoplasm (117). Consistent with those findings, our studies also indicate that Mel-18 will mainly localize to the cytoplasm during mitotic phase (results not shown). Therefore, we hypothesize that different interaction levels between Mel-18 and HSF2/RanGAP1 in the interphase and mitosis are regulated by the phosphorylation states of Mel-18. During interphase, Mel-18 is phosphorylated and colocalizes with HSF2 in the nucleus, but not with RanGAP1 which localizes in the cytoplasm. In the mitosis, Mel-18 is dephosphorylated and dispersed from nucleus to cytoplasm, where RanGAP1 localizes, but HSF2 still localizes in
nucleus, so Mel-18 interaction with HSF2 decreases, but increases for RanGAP1 during mitosis (Figure 5.1).

My results show that Mel-18 not only acts as an anti-SUMO E3 factor for the HSF2 protein, but for RanGAP1 as well. These results also indicate that Mel-18 is able to inhibit conjugation of SUMO-1 to proteins, suggesting that Mel-18 likely inhibits the sumoylation of other proteins in addition to HSF2/RanGAP1, perhaps even acting to down-regulate sumoylation in cells globally. This finding increases the likelihood that Mel-18 regulates the sumoylation of other, as-yet-undiscovered cellular proteins. Thus, one important goal of future studies would be to identify these other targets whose SUMO modification is modulated by Mel-18. Meanwhile, Mel-18 is shown to have tumor suppressor activities, which are associated with c-myc/cdc25 pathway or regulation of cyclin D2, so we propose its anti-SUMO E3 activities could contribute to another novel mechanism for Mel-18 as a tumor suppressor. In support of this hypothesis, studies showed that many oncogenic proteins or tumor suppressor, such as P-53, PML, MDM2 and c-jun (118-121), are sumo modified, and their sumoylation levels change in tumor cells. It would be particularly exciting if Mel-18 is found to regulate the sumoylation of cellular proteins involved in the control of cell proliferation. To test this hypothesis, we could compare the protein sumoylation levels in cells overexpressing Mel-18 with those in control cells and then use Mass Spectrometry to identify candidate proteins with different sumoylation levels in both groups. This study would also provide insights that could make Mel-18 as a potential target site for pharmaceutics cancer treatments.

In summary, the results of our study, show that Mel-18 functions as an anti-SUMO E3 protein, interacting both with HSF2/RanGAP1 and the SUMO E2 Ubc9, but acting to inhibit Ubc9 activity in order to decrease sumoylation of target proteins, such as HSF2 and RanGAP1, and that these activities do not require the RING domain of Mel-18. The results also show that RanGAP1 sumoylation is decreased during mitosis, and that this is associated with increased interaction between RanGAP1 and Mel-18 during this stage of the cell cycle. Intriguingly, this regulatory relationship is the opposite of that found for Mel-18 and HSF2, in which the interaction between these two proteins decreases during mitosis, resulting in elevated HSF2 sumoylation.

II. C18Y polymorphism of Bmi-1 is associated with increased ubiquitination leading to increased degradation by the proteasome.

The results described in chapter 4 characterize a polymorphism in the human Bmi-1 protein that changes the cysteine at amino acid position 18 to a tyrosine (C18Y). The results also show that the C18Y substitution causes a significant reduction in steady state Bmi-1 protein levels and that this is associated with increased ubiquitination, presumably leading to degradation by
the proteasome. This is the first demonstration of a polymorphism in human Bmi-1 that leads to alteration in levels of this important protein. Cysteine 18 is part of the RING finger motif responsible for binding zinc. Thus, we hypothesize that the most likely explanation for the decreased level of C18Y Bmi-1 is that changing this cysteine to tyrosine disrupts the structure of the RING domain, leading to its recognition as a mal-folded protein by the ubiquitination machinery, covalent ubiquitin attachment, and subsequent degradation by the proteasome.

Bmi-1 is important for the determination of cell fate and the renewal of adult stem cells of a number of cell lineages, including neural, hematopoietic, and intestinal lineages (67,71-77,122). Since the C18Y Bmi-1 polymorphism causes a significant decrease in Bmi-1 levels, we hypothesize that people with the C18Y form of Bmi-1 may be affected by problems such as decreased ability to renew stem cells. The individuals identified in the SNP database as having this polymorphism, who belong to the CEU (Utah residents with ancestry from northern and western Europe) and YRI (Yoruba in Ibadan, Nigeria) populations of the International HapMap Project, are heterozygous for it, and thus would be expected to have half the normal levels of Bmi-1. The results of mouse studies showing that Bmi-1+- heterozygote mice, although they are not as severe as those observed for Bmi-1-/- mice, do exhibit phenotypic differences in stem cell characteristics compared to wild-type mice (74,75,123). Therefore, we hypothesize that these people that are heterozygous for C18Y Bmi-1 could possibly have been affected in some way in terms of their health. However, because the health records of these individuals are not available we can not know this, and in any event it is possible that the effects may not be strong enough to cause significant symptoms. Our results suggest that individuals that are homozygous for C18Y Bmi-1 would have extremely low levels of Bmi-1 protein. Therefore, based on results showing that Bmi-1-/- mice exhibit significant problems resulting in death either days after birth or maximally by approximately 2 months of age (67,71-77), we would expect that homozygous C18Y individuals could experience significant deleterious health effects.

In addition to its importance for stem cell renewal and cell identity, Bmi-1 also has oncogenic functions (65,66,68-70,85). Thus, it is conceivable that the decreased levels of Bmi-1 expected in people with the C18Y polymorphism could be beneficial to these people in terms of decreasing their risk of cancer. In support of this, mice lacking Bmi-1 showed a decreased incidence of tumor cells (124). Intriguingly, even cells from Bmi-1+- heterozygous mice showed a significant decrease in tumor formation(85), suggesting that individuals with the C18Y Bmi-1 polymorphism may have a lower susceptibility to developing tumors.

In summary, the results of our study, coupled with the results of previous studies, suggest that individuals that have the C18Y polymorphism of Bmi-1 could have health problems related to an inability to renew adult stem cells
and/or establishing cell identities. This is likely to be particularly true in the case of an individual is homozygous for this polymorphism, as this person would likely have very low levels of Bmi-1, but is not clear if heterozygote C18Y individuals would experience significant problems. On the other hand, the decreased levels of Bmi-1 protein caused by the C18Y polymorphism may also provide the advantage of decreased susceptibility to developing cancer, even in people that are only heterozygous for the polymorphism. Future studies are warranted to test these hypotheses related to the C18Y Bmi-1 polymorphism.

III. PcG proteins have opposing roles.

PcG proteins have been shown to work together as complexes to silence genes (46-49). Our studies, coupled with other research data, suggest that proteins in PcG complexes could also have opposing roles: (a) SUMO E3 and anti-SUMO E3 functions: Mel-18 functions like an anti-SUMO E3 protein, interacting both with substrates and the SUMO E2 Ubc9 but acting to inhibit Ubc9 activity to decrease sumoylation of target proteins (108); Pc2, another Polycomb protein stimulates sumoylation of transcriptional corepressor, CtBP (34,104); (b) regulation of both SUMO and Ubiquitin pathways: These two modifications can have the same target proteins, but cause distinct consequences (125), and PcG proteins can regulate both of these two pathways. PRC1 complex containing Ring1B protein have H2A-K119 ubiquitin E3 ligase activity (52); however, Mel-18 and Pc2 are shown to be anti-SUMO and SUMO E3 protein, respectively (34,104,108); (c) tumor suppressor(s) and oncogene protein(s): Bmi-1 promotes cell proliferation and functions as an oncogene protein (68,85,70). Unlike Bmi-1, the highly homologous Mel-18 was shown to have tumor-suppressive effects and down regulate cell progression (59-62); meanwhile, Hpc-2, another PcG protein, can also arrest cells in G2-M phase (126).

Therefore the amounts of each protein in PcG complexes will determine the eventual activities of the whole complex. Quantitative changes of proteins will break the internal balance and then affect the quantity or quality of protein complexes, and then finally result in the alteration of a set of downstream genes and maybe different cell fates.
Fig 5.1 Different interactions between Mel-18 and HSF2 during different cell cycle phases.

In interphase, Mel-18 is phosphorylated and colocalizes with HSF2 in nucleus, thereby inhibits HSF2 sumoylation and binding on HSE. In mitosis, Mel-18 is dephosphorylated and disperses from nucleus to cytoplasm, but HSF2 remains in nucleus, therefore, Mel-18 interaction with HSF2 decreases, HSF2 sumoylation increases and HSF2 binds on HSE.
APPENDIX

Appendix: List of Abbreviations

CDK, Cyclin-dependent kinases
CtBP, C-terminal Binding Protein
DAPI, 4',6-diamidino-2-phenylindole
DNA, deoxyribonucleic acid
DTT, dithiothreitol,
EDTA, ethylene diamine triacetic acid
FITC, fluorescein isothiocyanate
FCS, fetal calf serum
GATA3, GATA binding protein 3
GFP, green fluorescent protein
GST, glutathione-s-transferase
GTP, guanosine 5'-triphosphate
HDAC4, Histone deacetylase 4
HEPES, n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid
HSF1, heat shock factor 1
HSF2, heat shock factor 2
IgG, immunoglobulin G
IR, internal repeat
kDa, kilodalton
MDa, megadalton
mRNA, messenger ribonucleic acid
NLS, nuclear localization sequence
nM, nanometer
ORF, open reading frame
PBS, phosphate buffered saline
Pc, chromodomain of Polycomb,
Pc2, polycomb 2 protein
PcG, Polycomb
PCR, polymerase chain reaction
PIAS, protein inhibitor of activated STAT
PML, Progressive multifocal leukoencephalopathy
PMSF, phenylmethanesulfonyl fluoride
PRCs, Polycomb repressor complexes
PREs, Polycomb Response Elements
RanBP2, RAN binding protein 2
RanGAP1, Ran GTPase-activating protein
RING, Really Interesting New Gene
RNA, ribonucleic acid
SDS, sodium dodecyl sulfate
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP, SUMO1/sentrin specific peptidase 1
SUMO, Small ubiquitin-like modifier
TAF, TBP-associated factors
TrxG, trithorax group
REFERENCE

VITA

Jie Zhang
Birthplace: Lanzhou, China
Feb 28, 1978

EDUCATION
2001-2004 M.S. Toxicology, Beijing University, China
1996-2001 M.D. Beijing University, China

PROFESSIONAL POSITIONS
2005-2008 Graduate Research Assistant, University of Kentucky
2001-2004 Graduate Research Assistant, Beijing University
1998-2000 Hospital Internship at Beijing Railway Hospital

SCHOLASTIC AND PROFESSIONAL HONORS
2008-present: Society of Toxicology (SOT)
2007-present: American Association for the Advancement of Science (AAAS)
2009: Society of Toxicology (SOT) graduate student travel award
2004-2005: Research Challenge Trust Fellowship, University of Kentucky
1996-2001: Full Academic Scholarship, Beijing University

PROFESSIONAL PUBLICATIONS

PROFESSIONAL POSTER PRESENTATIONS:
Jie Zhang, Michael L. Goodson, Yiling Hong and Kevin D. Sarge. Tumor Suppressor Mel-18 is a novel global sumoylation inhibitor. Baltimore, Maryland. SOT. 2009. (to be presented)
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