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Regulation Of Nuclear Localization Of The Sole Sumo-Conjugating Enzyme, Ubc9

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**REGULATION OF NUCLEAR LOCALIZATION OF THE SOLE
SUMO-CONJUGATING ENZYME, UBC9**

by

PALAK SEKHRI

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

In partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2013

MAJOR: BIOLOGICAL SCIENCES

Approved By:

Advisor

Date

DEDICATION

I dedicate this thesis to

My parents, Minu and Anil Sekhri

And my brother, Agrim Sekhri

Who have always supported and encouraged me to do best in life.

ACKNOWLEDGMENTS

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Words cannot suffice to express my appreciation to my family members for all their love and concern. Although thousands of miles away, the feeling of their spiritual existence is enough to drive me further towards my goals and their wishes shall forever remain an immeasurable source of strength for me. I also am thankful to all my friends for their affection

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CHAPTER 1 - INTRODUCTION

The SUMOylation pathway

Small ubiquitin-related modifier proteins (SUMOs) contain approximately 100 amino acids and are covalently attached to hundreds of different proteins in eukaryotic cells. SUMOylation is an essential posttranslational modification in eukaryotes and regulates a wide range of biological processes, including cell cycle progression, DNA repair, gene expression, nucleocytoplasmic transport, protein degradation/stability, and stress response (Pichler and Melchior, 2002; Johnson, 2004). Three different SUMO isoforms, including SUMO-1, SUMO-2 and SUMO-3, are expressed in mammals, whereas only one SUMO called Smt3 is expressed in budding yeast. The human SUMO-2 and SUMO-3 are about 95% identical to each other and therefore referred to as SUMO-2/3, whereas they share only 45% identity to SUMO-1. The N-terminal extension of SUMO-2/3 acts as a site for the formation of polymeric SUMO-2/3 chains.

The enzymatic cascade of SUMOylation is similar to that of ubiquitination. In eukaryotic cells, SUMO is expressed as a precursor protein whose C-terminal region needs to be processed by a family of SUMO-specific isopeptidases to expose its double glycine motif, leading to the generation of mature SUMO (Figure 1). This mature SUMO is then activated by a SUMO-E1 activating enzyme (SAE1/SAE2, also known as Aos1/Uba2) by forming a thioester bond between the catalytic cysteine of Uba2 and the C-terminal glycine of SUMO. This is followed by the transfer of SUMO from the E1 to the sole SUMO-E2 conjugating enzyme (Ubc9), leading to the formation of a thioester bond between the catalytic cysteine of Ubc9 and the C-terminal glycine of SUMO (Friedlander and Melchior, 2007). Subsequently, the charged Ubc9 recognizes the SUMOylation consensus motif Ψ KxD/E (Ψ represents a hydrophobic amino acid residue, K is a lysine residue, x can be any amino acid residue, and D/E represents aspartic acid

or glutamic acid) which are present in many SUMO targets (Villamor *et al.*, 2002; Tatham *et al.*, 2003). SUMO is then transferred from the charged Ubc9 to the substrate by forming an isopeptide bond between the C-terminal glycine of SUMO and the lysine residue of the target protein (Johnson, 2004). Several E3 ligases, including different PIAS family proteins and Nup358/RanBP2, facilitate the transfer of SUMO from Ubc9 to the target proteins. As a reversible process of SUMOylation, SUMO is deconjugated from its target by a family of SUMO-specific isopeptidases, including SENP1, SENP2, SENP3, SENP5, SENP6, SENP7, DeSI-1, DeSI-2, and USPL1 in mammals. However, only two SUMO-specific isopeptidases called Ulp1 and Ulp2 have been identified in budding yeast (Hickey *et al.*, 2012).

Interestingly, all the protein components essential for SUMOylation, including SUMO E1 and E2 (Ubc9) enzymes as well as SUMO-1 and SUMO-2/3, are mainly localized in the nucleus in vertebrate cells (Azuma *et al.*, 2001; Saitoh *et al.*, 2001; Lee *et al.*, 1998; Zhang *et al.*, 2002). In HeLa cells, SUMO E1 enzyme (Aos1/Uba2) is primarily nuclear (Azuma *et al.*, 2001), whereas Ubc9 is predominantly localized in the nucleus as well as at the nuclear pore complex (Saitoh *et al.*, 2001; Lee *et al.*, 1998; Zhang *et al.*, 2002) (Figure 2). In addition, both SUMO-1 and SUMO-2/3 are majorly concentrated in the nucleus under immunofluorescence microscopy using antibodies specific to SUMO-1 and SUMO-2/3 respectively (Zhang *et al.*, 2008) (Figure 2). These observations are consistent with the observations that most of the SUMOylated substrates are nuclear proteins. It has been hypothesized that SUMO-modification predominantly occurs in the nucleus and thus the nuclear localization of SUMO E1 and E2 enzymes in the nucleus is critical for efficient SUMOylation. Consistent with this hypothesis, it has been shown recently that the inhibition of Ubc9 nuclear localization in cells expressing a mutant form of lamin A responsible for the premature aging disease called Hutchinson-Gilford

progeria syndrome is correlated with a decrease of SUMO-2/3 modification in the nucleus (Kelly *et al.*, 2011). Furthermore, the artificial fusion of the SUMOylation consensus motif to Pyruvate Kinase (PK), a cytoplasmic protein, has been found to be efficient for its SUMOylation *in vitro*, yet its modification *in vivo* requires the additional presence of a nuclear localization signal (NLS) for its nuclear localization, suggesting that most SUMO substrates need to be imported into the nucleus for their sufficient sumoylation (Rodriguez *et al.*, 2001).

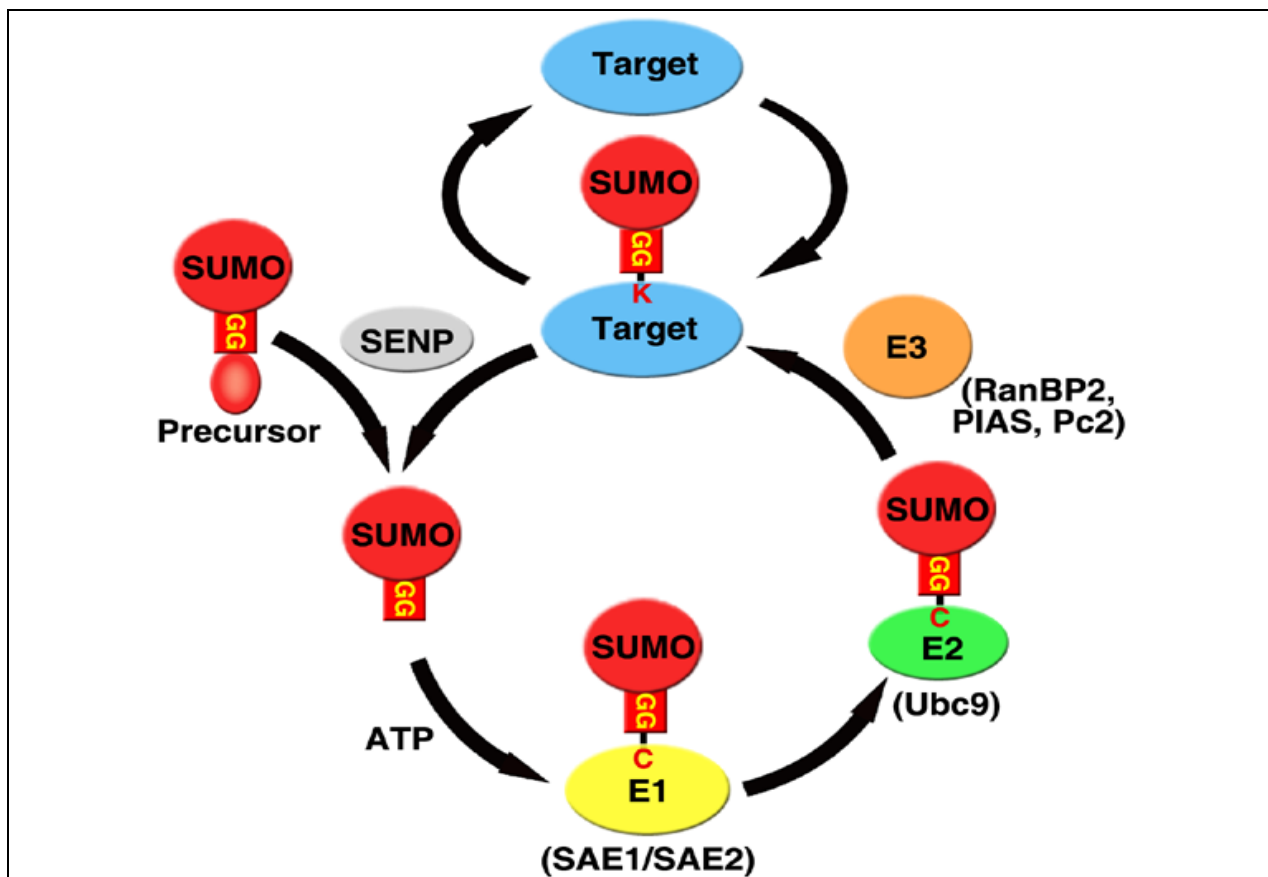
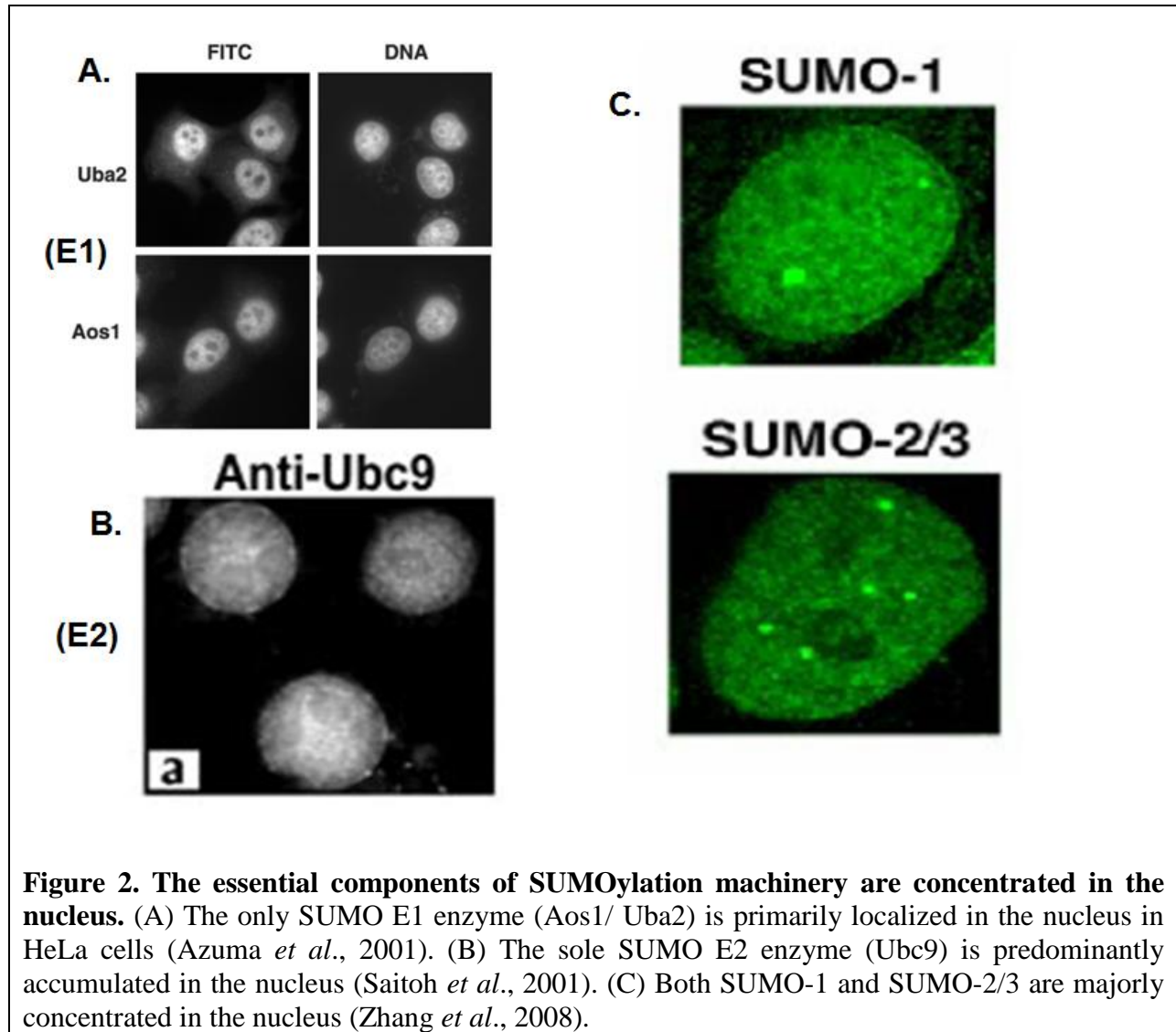


Figure 1. The SUMO pathway. The SUMO precursor is processed by SUMO isopeptidases (SENPs) in vertebrates to expose its C-terminal double-glycine (GG) motif. The mature SUMO is activated by the E1 activating enzyme to form a thioester bond between the C-terminal glycine of SUMO and the catalytic cysteine (C) of the E1. SUMO is then transferred to the catalytic cysteine (C) of the E2 conjugating enzyme Ubc9. Finally, SUMO is transferred from Ubc9 to a substrate by forming an isopeptide bond between SUMO and a lysine (K) of the substrate. The last step is often facilitated by an E3 ligase. SUMO is deconjugated by SENP isopeptidases. This figure is adapted from Wan *et al.*, 2012.



The sole SUMO-E1 activating enzyme consists of two subunits, Aos1 and Uba2, and is known to be imported from the cytoplasm to the nucleus by Importin α/β . Importin α can bind to each of the E1 subunits either individually or to the Uba2 subunit in the cytoplasm and actively transports them to the nucleus (Moutty *et al.*, 2011). However, the mechanism underlying the nuclear localization of Ubc9 is still unclear. *In vitro* study has shown that Importin 13 (Imp13), one of the members of the karyopherin β family, translocates Ubc9 from the cytoplasm to the nucleus (Mingot *et al.*, 2001). In this study, *in vitro* nuclear import assays were performed using fluorescently labeled recombinant Ubc9. Different importin receptors, including Imp α/β , Imp5,

Imp7, Imp13 and transportin, were respectively added to the permeabilized cells in the presence of Ran and ATP as the energy source. The nuclear import of Ubc9 was stimulated only by Imp13, and this Imp13-Ubc9 interaction was disrupted in the presence of RanGTP *in vitro*, indicating that Ubc9 is released from the import complex by the high concentration of RanGTP in the nucleus (Mingot *et al.*, 2001).

As the only SUMO-E2 enzyme, Ubc9 plays an essential role in SUMOylation and thus regulates many critical cellular processes. In yeast, Ubc9 is required for cell cycle progression through mitosis as its deletion causes the cells to arrest at G2/M phase (Seufert *et al.*, 1995). Ubc9 has been implicated in tumorigenesis since Ubc9 is upregulated in various types of human cancer, including ovarian and lung cancers, as compared to the corresponding normal control samples (Mo *et al.*, 2005; Li *et al.*, 2013). Upregulation of Ubc9 expression has been found to promote the migration, invasion and metastasis of lung cancer cells (Li *et al.*, 2013). Ubc9 forms a complex at the nuclear pore complex with proteins including SUMO-1 modified RanGAP1 and Nup358/RanBP2 (Zhang *et al.*, 2002). One of the interesting feature of Ubc9 is that it can also be SUMOylated, leading to the formation of SUMO-modified Ubc9 (Ubc9*SUMO). The SUMO modification site of Ubc9 in mammals and budding yeast is Lys14 and Lys153 respectively. The SUMOylated form of Ubc9 governs several functions. In mammalian cell, SUMOylation of Ubc9 can alter its target specificity by repressing RanGAP1 modification but activating SUMOylation of a transcriptional regulator called Sp100 (Knipscheer *et al.*, 2008). In budding yeast, a SUMOylation-deficient mutant of Ubc9 exhibits a defect in synaptonemal complex formation during meiosis (Klug *et al.*, 2013).

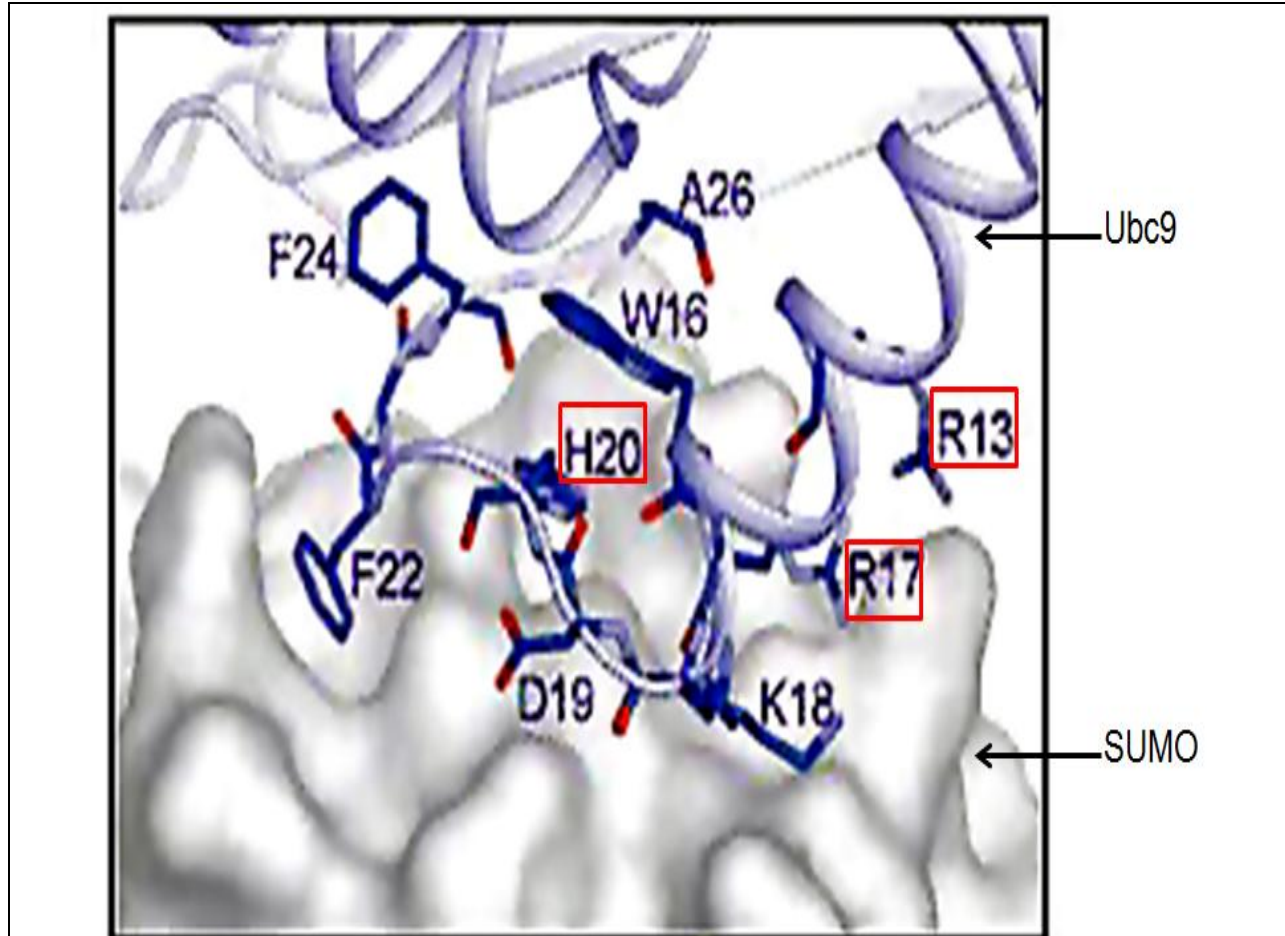


Figure 3. Non-covalent interactions between Ubc9 and SUMO. Picture shows amino acid residues of Ubc9 that are involved in Ubc9 interaction with SUMO. R13, R17 and H20 are critical sites on Ubc9 which are crucial for Ubc9-SUMO complex formation (Knipscheer *et al.*, 2007).

During the process of SUMO-conjugation, Ubc9 is known to interact with the E1 enzyme, SUMO, a SUMO-E3 ligase, and a protein substrate. Structural studies in combination with mutational and biochemical analyses have demonstrated that Ubc9 directly interacts with the SUMOylation consensus motif (Ψ KxD/E) on SUMO substrates. Ubc9 can also form a complex with a Nup358/RanBP2, a SUMO E3 enzyme, and also the SUMO-1-modified RanGAP1 at the nuclear pore complex (NPC) (Reverter and Lima, 2005; Matunis *et al.*, 1996). The residues that are crucial for Ubc9 binding to the substrate and also required for SUMO-modification have been identified previously (Bernier-Villamor *et al.*, 2002). As shown in

Figure 3, multiple amino acid residues of Ubc9 are critical for its interaction with SUMO. Several Ubc9 mutants such as R17E, R13A and H20D are deficient in forming a complex with SUMO. Biochemical analyses revealed that the Ubc9-R17E and Ubc9-R13A mutants with a defect in SUMO-binding also have defects in the formation of Ubc9~SUMO thioester bond as well as the assembly of free poly-SUMO chain *in vitro*. On the other hand, the Ubc9-H20D mutant with a defect in SUMO-binding doesn't show a deficiency in the formation of thioester bond between Ubc9 and SUMO (Ubc9~SUMO) (Tatham *et al.*, 2003; Capili and Lima, 2007; Knipscheer *et al.*, 2007). These studies suggest that the non-covalent interaction of Ubc9 with SUMO and/or the SUMO-consensus motifs of nuclear proteins might be crucial for Ubc9 nuclear localization.

Nucleocytoplasmic transport

NPCs are the gates at nuclear envelope for transport of macromolecules between the cytoplasm and the nucleus. Passive diffusion through NPCs is a common mode of transport for molecules with sizes up to ~20-40 kDa, while other larger molecules require active transport to pass through the NPCs (Gorlich and Kutay, 1999; Mohr *et al.*, 2009). This active transport of cargoes is mainly facilitated by a family of nuclear transport receptors called karyopherin- β which are broadly classified into two categories including importins and exportins. Importin binds the nuclear localization signal (NLS) of an import cargo in the cytoplasm and then translocates it into the nucleus, where the cargo is released from the importin-cargo complex upon RanGTP binding (Rexach and Blobel, 1995; Pemberton and Paschal, 2005). On the other hand, exportin forms the exportin-RanGTP-cargo complex in the nucleus, translocates through the NPC, and releases its cargo in the cytoplasm when RanGTP is hydrolyzed to RanGDP (Cook and Conti, 2010). Both nuclear import and export cycles are illustrated in Figure 4.

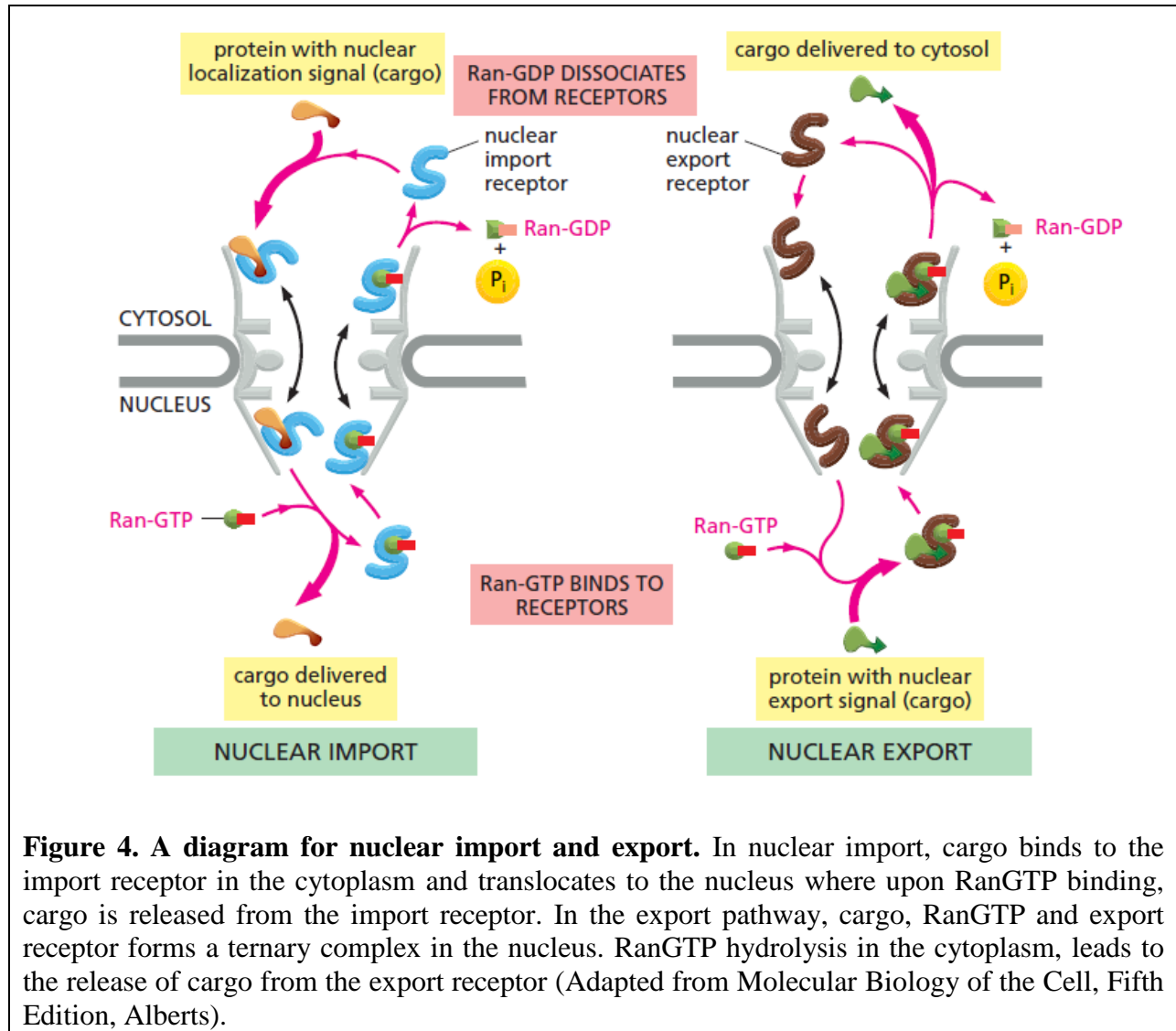


Figure 4. A diagram for nuclear import and export. In nuclear import, cargo binds to the import receptor in the cytoplasm and translocates to the nucleus where upon RanGTP binding, cargo is released from the import receptor. In the export pathway, cargo, RanGTP and export receptor forms a ternary complex in the nucleus. RanGTP hydrolysis in the cytoplasm, leads to the release of cargo from the export receptor (Adapted from Molecular Biology of the Cell, Fifth Edition, Alberts).

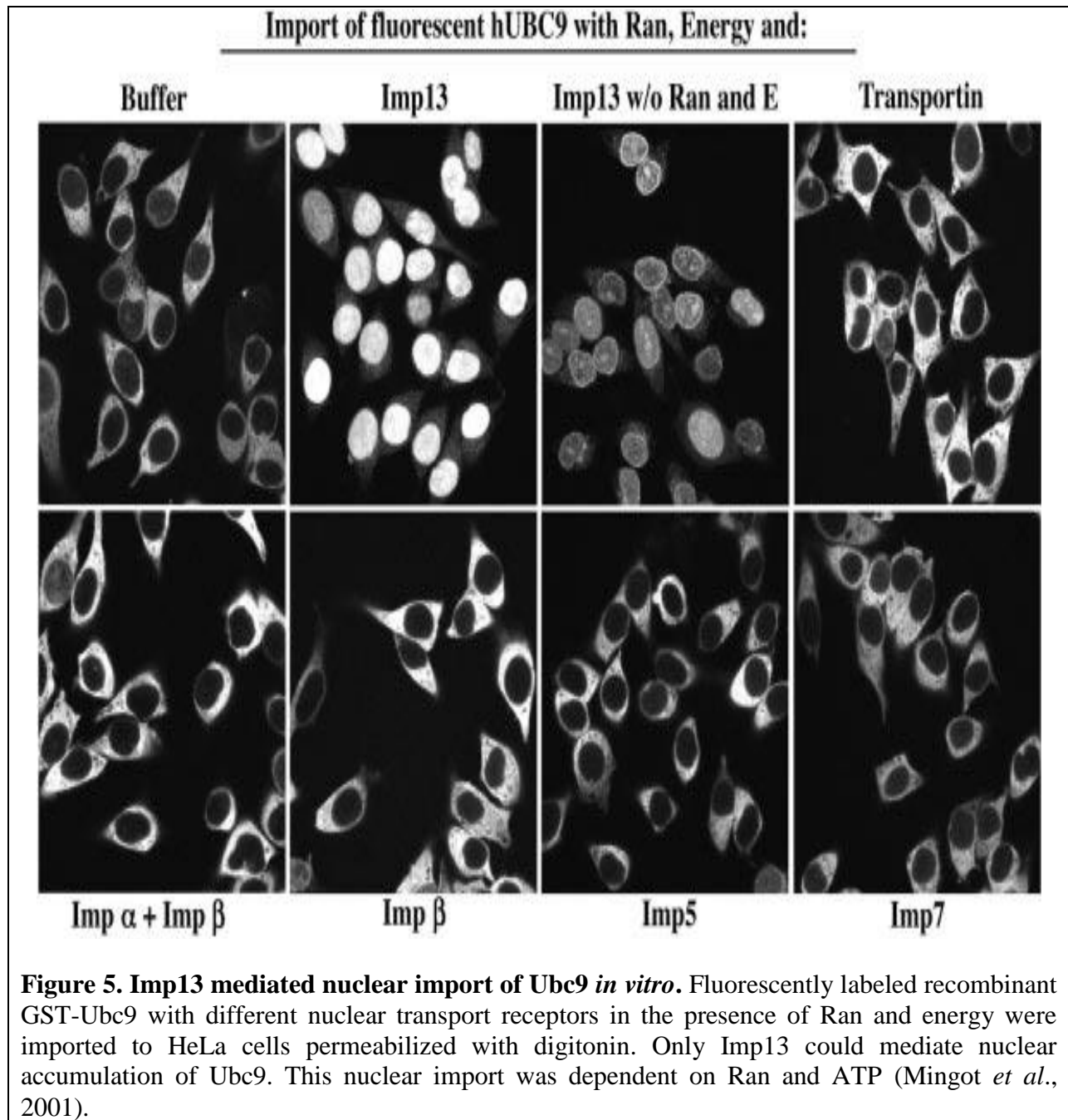
A few karyopherins such as Imp13, Msn5 and Exportin 4 have been identified as a bidirectional transport receptors that can both import and export cargoes in and out of the nucleus, respectively (Mingot *et al.*, 2001; Yoshida and Blobel, 2001; Gontan *et al.*, 2009). One of the directions that my study has focused on is Imp13-mediated nuclear import of Ubc9.

Imp13: Bi-directional transport receptor

Several studies have shown that Imp13 mediates both nuclear import and export of multiple cargoes. In addition to Ubc9 as the first identified import cargo of Imp13 (Mingot *et al.*, 2001), the other Imp13 import cargoes include the paired-type homeodomain transcription

factors (Pax6, Pax3 and Crx) (Ploski *et al.*, 2004), glucocorticoid receptor (Tao *et al.*, 2006), the actin bundling protein called myopodin (Liang *et al.*, 2008), two histone fold heterodimeric proteins (CHRAC-15/CHRAC-17 and p12/CHRAC-17) (Walker *et al.*, 2009), and the core components of exon junction complex (EJC) including Mago and Y14 (Bono *et al.*, 2010) in mammalian cells. So far, the eukaryotic translation initiation factor called eIF1A is the only known export cargo of Imp13 (Mingot *et al.*, 2001).

Apart from being a bi-directional transport receptor, Imp13 has been identified as a potential marker for corneal epithelial progenitor cells (Wang *et al.*, 2009). Furthermore, recent studies have shown that Imp13 is significantly upregulated in endometriosis and endometrial carcinoma when compared to secretory phase endometrium and can be used as an endometrial progenitor/stem cell marker (Zeng *et al.*, 2012). Moreover, Imp13 is highly expressed in the epithelial cells of pterygium and plays a critical role in enhancing cell proliferation (Xu *et al.*, 2013). By analyzing the expression of Imp13 among different stages of embryonic and adult brain tissues, levels of Imp13 mRNAs and proteins were consistently decreased during mouse brain development (You *et al.*, 2013). Intriguingly, Imp13 localized in the cytoplasm at the early stages and then accumulated in the nucleus at the late stages of brain development (You *et al.*, 2013).



As previously mentioned, Ubc9 nuclear import is mediated by Imp13 *in vitro* (Mingot *et al.*, 2001). In this study, a nuclear import assay was performed where fluorescently labeled GST tagged Ubc9 was used. Cells were permeabilized with digitonin and then incubated with different importins including Imp α/β , Imp5, Imp7, Imp13 and transportin respectively in the presence of Ran GTPase and ATP. As shown in Figure 5, only Imp13 facilitated the transport of

Ubc9 to the nucleus. Structural study of the Imp13-Ubc9 complex has revealed that Ubc9 binds to the N-terminus of Imp13 (Grünwald and Bono, 2011). *In vitro* binding assay indicated that the interaction between Ubc9 and Imp13 was disrupted when the R17 residue on Ubc9 was mutated to E or when the D426 residue on Imp13 was mutated to R. This study revealed the residues on both Imp13 and Ubc9 that are critical for their interaction (Grünwald and Bono, 2011).

Although *in vitro* nuclear import assays have revealed that Imp13 can mediate nuclear import of Ubc9, little is known about whether Imp13 is the major nuclear import receptor for Ubc9 and whether Imp13-mediated nuclear import of Ubc9 is important for efficient SUMOylation *in vivo*. In this study, we investigated how the nuclear localization of Ubc9 is regulated in mammalian cells and also addressed the functional significance of Ubc9 nuclear localization in control of efficient SUMOylation *in vivo*. We first focused on Imp13-mediated nuclear import of Ubc9 in regulation of Ubc9 nuclear localization and also the efficient SUMOylation *in vivo*. We then tested whether the non-covalent interaction between Ubc9 and SUMO also plays a role in control of Ubc9 nuclear distribution.

CHAPTER 2 - METHODS

Cell culture and transfection

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; HyClone) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Pencillin-Streptomycin (Invitrogen) and cultured at 37°C with 5% CO₂. Cells were transfected with the indicated plasmids using Lipofectamine and Plus reagents (Invitrogen) following the manufacturer's protocols. The transfected cells were analyzed by Western blot and/or immunofluorescence microscopy.

Immunofluorescence microscopy

HeLa cells were grown on coverslips in 6-well plates. After 24-48 h of transfection, cells were washed twice with 1xPBS (137 mM NaCl, 2.7 mM KCl, 1.5mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.36) fixed with 3.5% paraformaldehyde in 1xPBS for 30 min. Cells were then permeabilized with ice-cold acetone for 5 min, incubated with rabbit anti-HA primary antibody (Santa Cruz) (1:50 dilution in 1xPBS containing 2%BSA and 0.02% sodium azide) for 1 h followed by washing with 1xPBS for three times. Cells were further incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) (1:200 dilution) for 30 min at room temperature (RT) and then washed three times with 1xPBS. The coverslips with cell side down were slowly placed onto the mounting solution (100 mM Tris pH8.8, 50% Glycerol, 2.5% DABCO and 0.2 µg/ml DAPI for DNA staining) on the microscope glass slide, incubated for 3-5 min, and then sealed with colorless nail polisher. Images were collected using Olympus inverted IX81 fluorescence microscope.

Plasmid constructs

Mammalian Imp13 vector (pCMV-SPORT6) was purchased from Invitrogen. Complete ORF was subcloned into pEGFP-C1 vector between Xho I and BamH I sites. The Imp13

fragment (1-488 amino acids) was subcloned between BamH I and Xho I sites in pGEX-4T-1 vector. The mouse C-terminal HA-tagged Imp13 was obtained from Dr. Tao Tao (Tao *et al.*, 2006). The Ubc9-WT (wild type) and Ubc9-R17E mutant were subcloned into pEGFP-C1 vector between EcoR I and BamH I sites. For protein expression and purification, Ubc9-WT or Ubc9-R17E was subcloned into pGEX-6P-1 vector between BamH I and Xho I restriction sites. All clones were verified by restriction digestions and DNA sequencing. Primers for PCR amplifications and subclonings are listed in Appendix A.

Site-directed mutagenesis

Ubc9 and Imp13 mutants were generated using site-directed mutagenesis. To generate the Ubc9-R17E mutant, the arginine (R) 17 residue of human Ubc9 was mutated to glutamic acid (E). To study the non-covalent interaction between Ubc9 and SUMO, the arginine 13 of Ubc9 was mutated to alanine for the generation of the Ubc9-R13A mutant, whereas the histidine 20 was mutated to aspartic acid for generating the Ubc9-H20D mutant. To generate the mouse Imp13-D426R mutant with a defect in Ubc9 interaction, the aspartic acid (D) 426 of Imp13 was mutated to R. The PCR amplifications were performed using Pfu Turbo DNA polymerase (Agilent) and the corresponding mutagenesis primers listed in Appendix B. The PCR products were treated with 1 μ l of Dpn I restriction enzyme (NEB) for 1 h at 37° C and transformed to XL1-Blue electro-competent cells (lab made). The transformed cells were plated on Luria Bertani (LB) plates containing the respective antibiotics.

Protein expression and purification

The GST-tagged human Imp13 and Ubc9 were expressed in *E.coli* strain BL21. The expression of GST-Imp13 was induced with 0.5 mM IPTG at 18°C overnight. Induction of GST-Ubc9-WT, GST-Ubc9-R17E and GST were carried out for 4 h at 37°C with 1 mM IPTG.

After induction, cells were lysed in 1x Lysis buffer comprising of 25 units/ μl Benzonase, 0.2% TritonX-100, 1 mg/ml Lysozyme, and protease inhibitors (including 5 $\mu\text{g/ml}$ LAP (Leupeptin, Antipain and Pepstatin), 1 mM PMSF and 20 $\mu\text{g/ml}$ Aprotinin) in 1x PBS. The GST or GST-tagged proteins were then bound to the Glutathione Sepharose beads (GE Healthcare).

The GST tag of GST-Ubc9 was cleaved using GST-tagged PreScission Protease. The GST-tagged Ubc9 fusion proteins were first bound to the glutathione sepharose beads and then incubated with the PreScission Protease at 4°C overnight. The supernatant containing the untagged Ubc9 was collected and then used for *in vitro* Imp13-Ubc9 binding assays. All the protein concentrations were measured using Bradford protein assays (Bio-Rad). The purified proteins were also analyzed using Coomassie Blue staining and immunoblot analyses.

***In vitro* binding assays**

To characterize the interaction between Imp13 and Ubc9, 24 μg of GST-Imp13 and 10 μg of GST alone were immobilized to the glutathione sepharose beads (GE Healthcare), respectively, and then blocked with 200 μl of Binding buffer (20mM HEPES pH 7.5, 50 mM NaCl, 1 mM DTT, 10% glycerol, and 0.01% NP-40) containing 2% BSA and 0.02% Sodium Azide for 30 min at 4°C. After blocking, the beads were washed twice with Binding Buffer. 5 μg of Ubc9-WT or Ubc9-R17E were incubated with the beads immobilized with either GST-Imp13 or GST for 2 h at 4°C. The beads were washed three times with Washing Buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 1 mM DTT, 10% glycerol and 0.1% NP-40) at 4°C. Samples were eluted in 15 μl of 2x SDS sample buffer by incubation at 95°C for 5-10 min. One-third of the sample was loaded on a 15% SDS-PAGE gel for Western Blot analysis using anti-Ubc9 antibodies. Inputs were analyzed by Coomassie Blue staining.

RNA interference

HeLa cells were grown in a 6-well plate to 30-50% confluency and then transfected with Imp13 siRNA oligonucleotides using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Control and Imp13 specific siRNA double stranded oligonucleotides were purchased from Dharmacon. The sequences of siRNA oligos are listed in Appendix C. 72 h post-transfection, cells were lysed in 2x SDS sample buffer and analyzed by Western Blot using antibodies specific to Imp13, SUMO-1, SUMO-2/3, Tubulin, Ubc9 and Actin.

To investigate the effect of Imp13 RNAi on Ubc9 nuclear localization, HeLa cells were transfected with GFP-Ubc9-WT using Lipofectamine-Plus reagents (Invitrogen) after 48 h of Imp13 RNAi and grown for additional 24 h. The transfected cells were fixed with 3.5% paraformaldehyde for 30 min and directly analyzed by fluorescence microscopy.

Western blot analysis

Proteins were first separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membrane for 2 h. After transfer, the membrane was blocked with 5% milk in 1x TS buffer (50 mM Tris and 135mM NaCl) for 30 min at RT. Membrane was incubated with the antibodies listed in Appendix D. All primary antibodies were diluted in 1x PBS containing 2% BSA and 0.02% sodium azide. After incubation for 1 h at RT, the membrane was washed several times with 1x TS buffer containing 0.02% Tween-20 and then blotted with HRP-conjugated secondary antibody (GE Healthcare) (1:5000 dilution in 1xTS buffer with 5% milk) for 30 min at RT. After several washes, proteins were detected using ECL Prime (Western blotting detection reagent kit) (GE Healthcare).

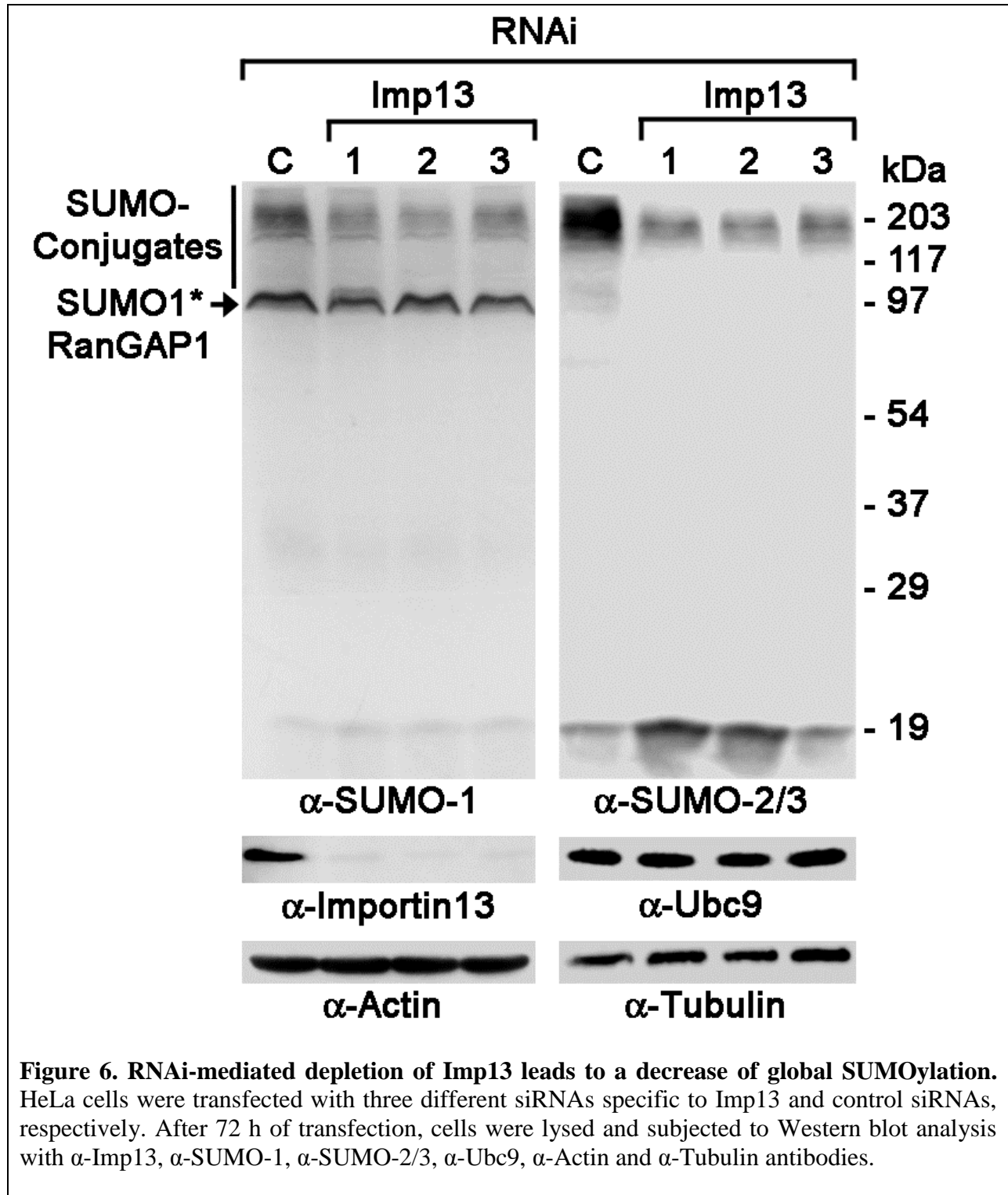
Data quantification

For statistical analysis, fluorescent images were taken at 60x magnification. The fluorescence intensities of Ubc9 in the nucleus and the cytoplasm were measured using Image J software (<http://rsbweb.nih.gov/ij/>). About 60 cells for each treatment were analyzed to calculate the nuclear/cytoplasmic ratio of Ubc9.

CHAPTER 3 - RESULTS

Imp13 is required for efficient global SUMOylation

Although previous studies have shown that Imp13 can mediate the nuclear import of Ubc9 using an *in vitro* nuclear import assays, little is known about the functional significance of Imp13 in regulating Ubc9 nuclear localization *in vivo*. Here we hypothesized that Imp13 is required for both Ubc9 nuclear localization and efficient SUMOylation *in vivo*. To this hypothesis, Imp13 was depleted in HeLa cells by RNA interference (RNAi). Cells were transfected with either one of the three different siRNAs specific to Imp13 or control siRNAs. After 72 h of transfection, cells were analyzed by immunoblotting using rabbit antibodies specific to Imp13. As shown in Figure 6, Imp13 expression was efficiently inhibited in cells transfected with one of the three different Imp13-specific siRNAs when compared to cells transfected with control siRNAs. Next, we investigated if knockdown of Imp13 affects global SUMOylation. Interestingly, we found a significant decrease of high molecular-weight SUMO-1 and SUMO-2/3 conjugates upon RNAi-depletion of Imp13 as compared to control RNAi (Figure 6). Furthermore, levels of Ubc9 expression remained the same upon RNAi-depletion of Imp13 (Figure 6), indicating that the decrease of global SUMOylation upon Imp13 RNAi is not caused by reduced levels of Ubc9 expression. Immunoblotting analyses of whole cell lysates using anti-Tubulin and anti-Actin antibodies indicated that equal amounts of total proteins were present among different samples (Figure 6).



Imp13 is crucial for Ubc9 nuclear import *in vivo*

The decrease of SUMOylation in cells with RNAi-depletion of Imp13 might be caused by a defect in Ubc9 nuclear localization. Because Imp13 is a known nuclear import receptor for Ubc9 using *in vitro* nuclear import assays (Mingot *et al.*, 2001), we hypothesized that Imp13 is required for the efficient nuclear import of Ubc9 *in vivo*. To test this hypothesis, we depleted endogenous Imp13 in HeLa cells using siRNAs specific for Imp13. 48 h after RNAi-depletion of Imp13, cells were transfected with plasmids encoding GFP-Ubc9 for 24 h. Fluorescence microscopy showed that Ubc9 was predominantly concentrated in the nucleus with a very low level of cytoplasmic distribution in cells transfected with control siRNAs, whereas Imp13 depletion caused a dramatic increase of the cytoplasmic distribution of Ubc9 (Figure 7A). To accurately measure the effect of Imp13-depletion on Ubc9 distribution between the nucleus and the cytoplasm, we used Image J software to calculate the nuclear to cytoplasmic signal ratio (N/C) of GFP-tagged Ubc9 in cells transfected with Imp13-specific siRNAs and then compared to cells transfected with control siRNAs. We found that around 82% of control-RNAi cells had an N/C ratio of larger than 4 (>4), whereas only 25% of Imp13-RNAi cells had the N/C ratio of >4 (Figure 7B). This result clearly indicated that Imp13 depletion causes a great increase of cytoplasmic distribution of Ubc9 and that Imp13 is critical for Ubc9 nuclear localization *in vivo*.

Interaction between Imp13 and Ubc9 is important for the nuclear localization of Ubc9

To test if the interaction between Imp13 and Ubc9 is important for Ubc9 nuclear localization, the Ubc9-R17E mutant was generated with a defect in Imp13 binding (Grünwald and Bono, 2011).

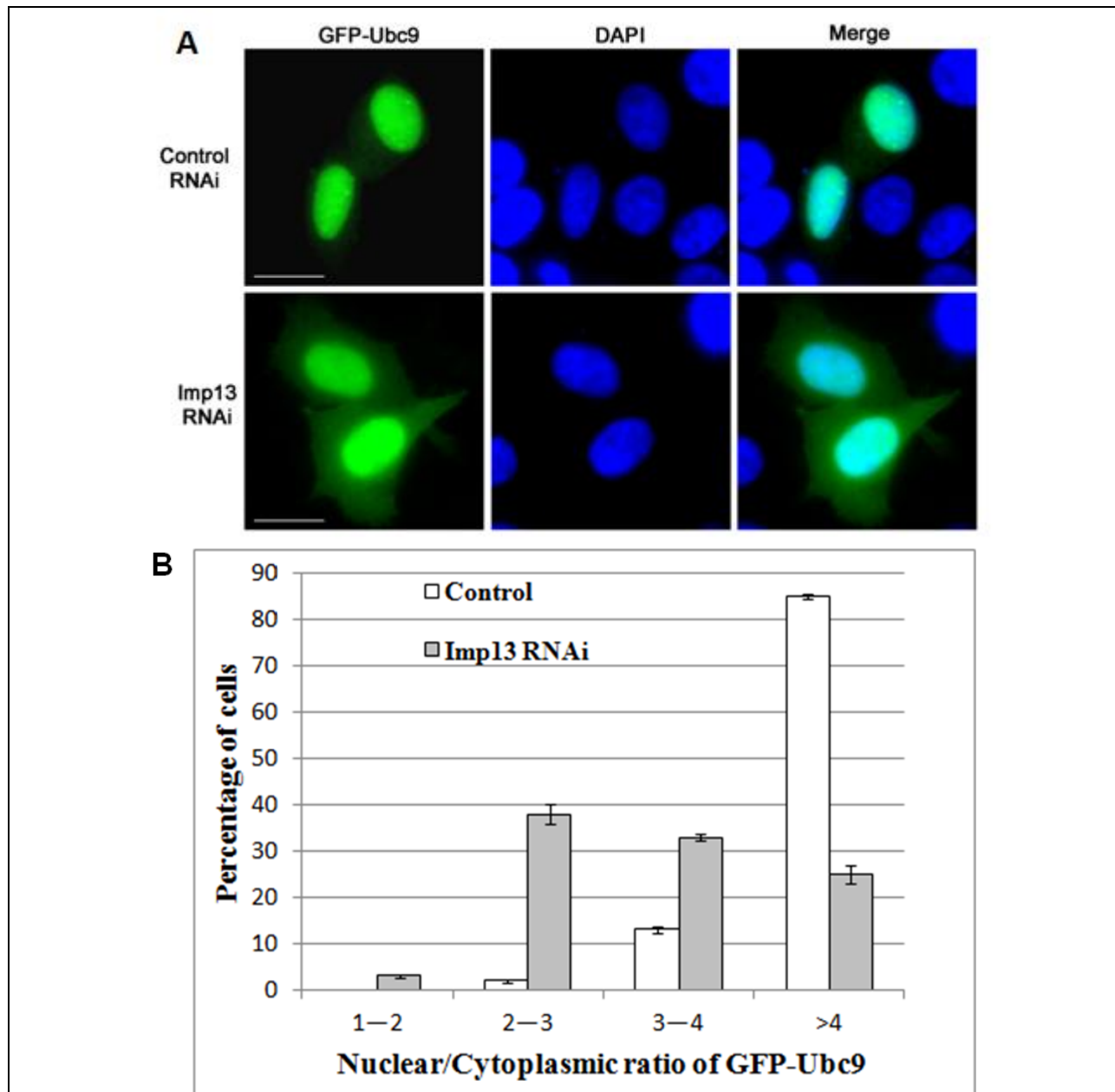
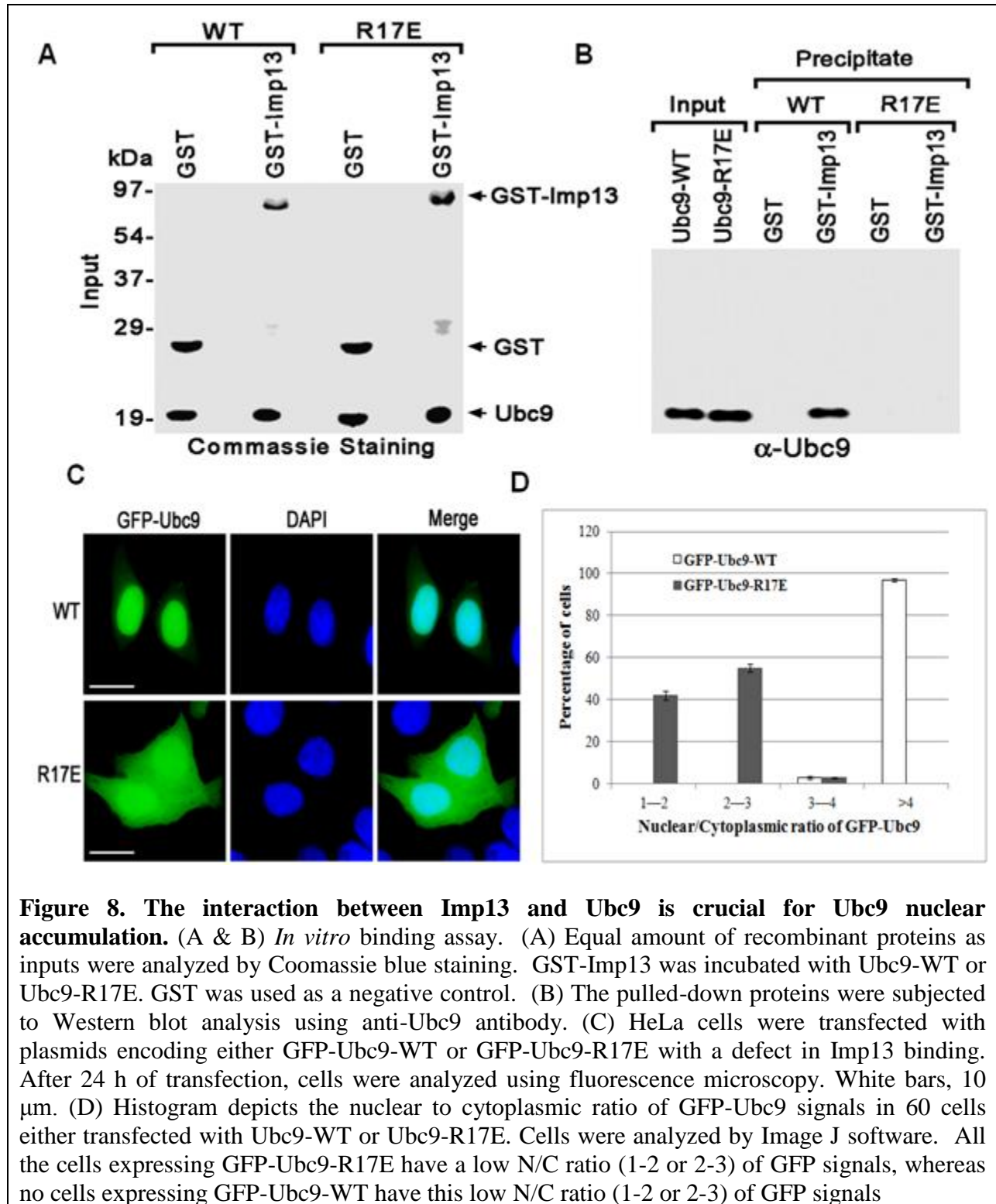


Figure 7. Imp13 depletion causes an increase in the cytoplasmic distribution of Ubc9. (A) HeLa cells were transfected with Imp13 siRNAs. After 48 h, cells were further transfected with plasmids encoding GFP-Ubc9-WT (wild-type). Fluorescence microscopy was used to analyze the result. White bars, 10 μ m. (B) A histogram depicts the nuclear to cytoplasmic ratio of GFP-Ubc9 signals in cells either transfected with the control or Imp13 siRNAs. The GFP-Ubc9 signals were quantified using Image J software. The percentage of cells with relatively low nuclear to cytoplasmic ratios of GFP-Ubc9 signals was significantly increased in the Imp13-RNAi cells when compared to the control-RNAi cells, indicating that Imp13 is crucial for the nuclear import of Ubc9 *in vivo*. 60 cells were analyzed for each sample.

This R17E mutation on Ubc9 is known to disrupt its interaction with Imp13 using *in vitro* binding assays (Grünwald and Bono, 2011). We performed a similar *in vitro* binding assay using

recombinant proteins. The purified Ubc9-WT and Ubc9-R17E proteins were incubated with the glutathione beads containing immobilized GST-Imp13 and GST proteins, respectively. Equal amounts of recombinant proteins were used for the *in vitro* binding assays as indicated by Commassie blue staining, whereas the GST proteins were used as a negative control (Figure 8A). The Ubc9-WT and Ubc9-R17E proteins bound to the glutathione beads were subjected to Western blot analysis using anti-Ubc9 antibodies (Figure 8B). The GST-Imp13 proteins efficiently pulled down Ubc9-WT, but not Ubc9-R17E (Figure 8B). These results thereby confirm that the R17 residue on Ubc9 is crucial for its interaction with Imp13.

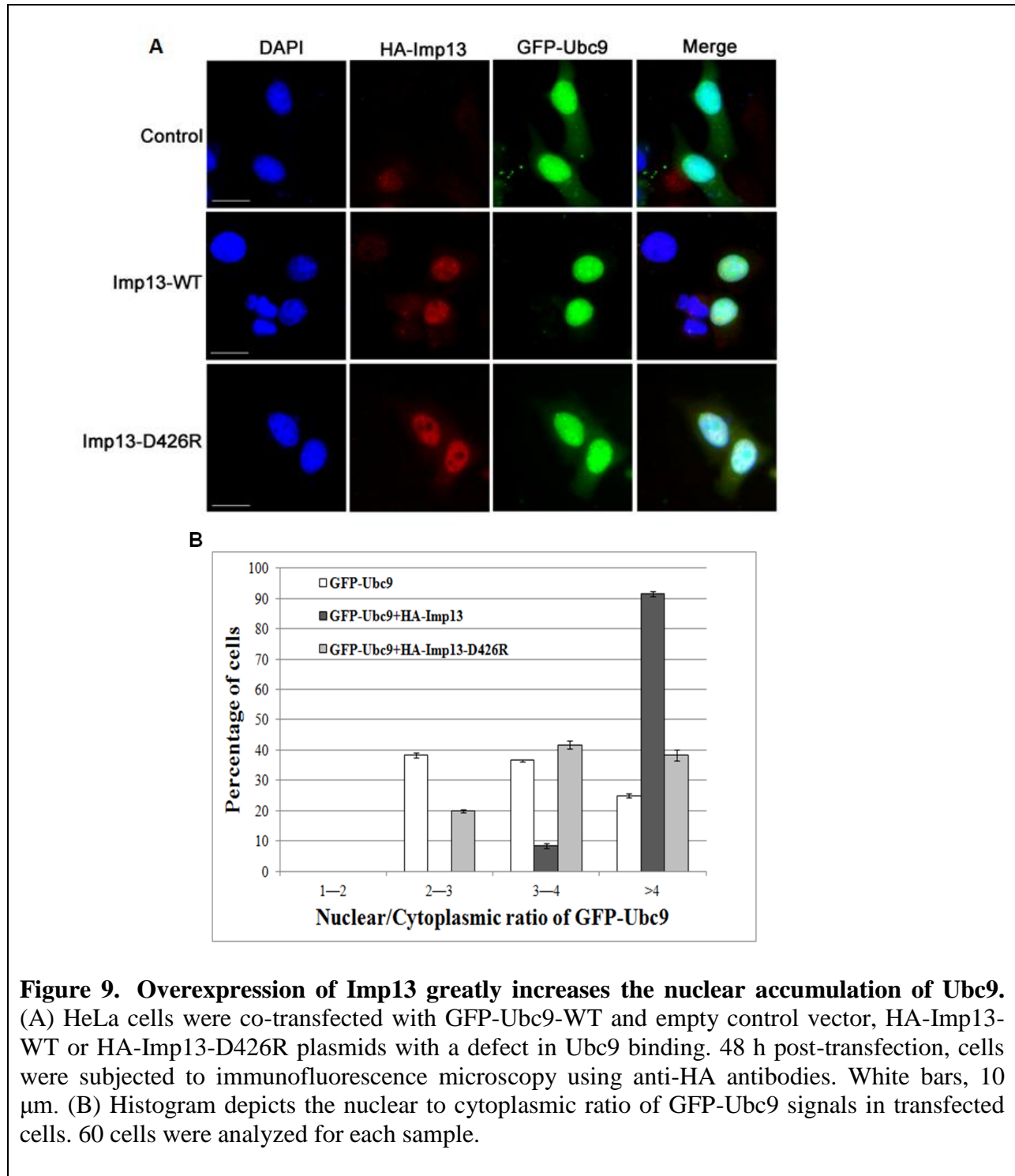
To further test whether Imp13 is important for Ubc9 nuclear localization, we compare the nuclear localization between GFP-Ubc9-WT and GFP-Ubc9-R17E. Plasmids encoding GFP-Ubc9-WT or GFP-Ubc9-R17E were transiently transfected into HeLa cells for 24 h and the localization of GFP-Ubc9 was determined by fluorescence microscopy. We found that GFP-Ubc9-WT was primarily localized to the nucleus, whereas GFP-Ubc9-R17E showed an increased cytoplasmic staining, indicating that Imp13 is an important import receptor for the nuclear accumulation of Ubc9 in mammalian cells (Figure 8C). By measuring the nuclear/cytoplasmic signal ratio of GFP-Ubc9-WT or GFP-Ubc9-R17E fusion proteins, we found that nearly all the cells expressing GFP-Ubc9-R17E have a low N/C ratio (1-2 or 2-3) of GFP signals compared to none of the cells of GFP-Ubc9-WT in this ratio (Figure 8D). Hence, a defect in Ubc9 interaction with Imp13 resulted in a dramatic increase of cytoplasmic distribution of Ubc9.



Overexpression of Imp13 enhances the nuclear accumulation of Ubc9

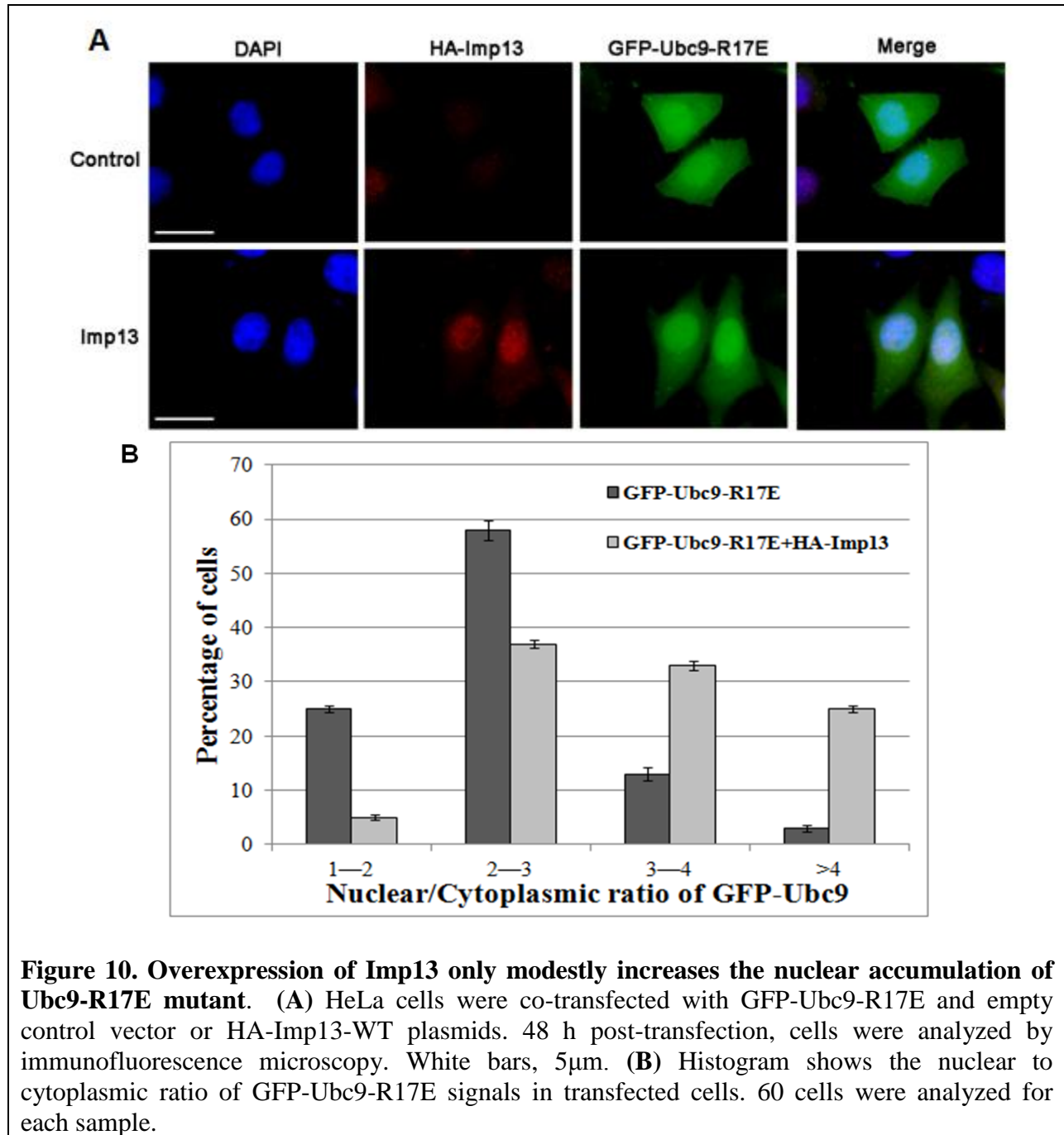
To further test the hypothesis that Imp13 is critical for the nuclear localization of Ubc9, HeLa cells were co-transfected with GFP-Ubc9-WT and HA-Imp13-WT, HA-Imp13-D426R or HA empty vector plasmids. Cells transfected with GFP-Ubc9-WT plasmids and the corresponding empty vector were used as a negative control. 48 h post-transfection, immunofluorescence microscopy was performed using anti-HA antibodies. The Ubc9 nuclear distribution was enhanced in cells co-transfected with GFP-Ubc9-WT and HA-Imp13 when compared to cells co-transfected with GFP-Ubc9-WT and empty control vector (Figure 9A, Panel 1 and 2). Furthermore, we co-transfected cells with plasmids encoding GFP-Ubc9-WT and HA-Imp13-D426R, the known Ubc9-interacting deficient mutant. Interestingly, we observed that the cells co-expressing HA-Imp13-D426R and GFP-Ubc9-WT did not show an increase in Ubc9 nuclear distribution as compared to cells co-transfected with GFP-Ubc9-WT and HA-Imp13-WT or empty control constructs (Figure 9A). Quantitative analysis was performed by calculating the percentage of cells with an indicated N/C ratio. Overexpression of HA-Imp13-WT greatly increased the percentage of cells (92%) with the N/C ratio of >4 for GFP-Ubc9-WT signals, whereas only 38% of cells co-expressing HA-Imp13-D426R and GFP-Ubc9 were found with this high N/C ratio (>4) for GFP-Ubc9-WT signals (Figure 9B).

We also performed a similar experiment by co-transfecting HeLa cells with GFP-Ubc9-R17E and HA-Imp13 or empty vector plasmids. Cells that co-expressed HA-Imp13 and GFP-Ubc9-R17E only slightly increased the nuclear accumulation of Ubc9 as compared to cells co-transfected with GFP-Ubc9-R17E and control plasmids (Figure 10A). However, this increase of Ubc9 nuclear distribution in these cells was much lower than that in cells co-transfected with

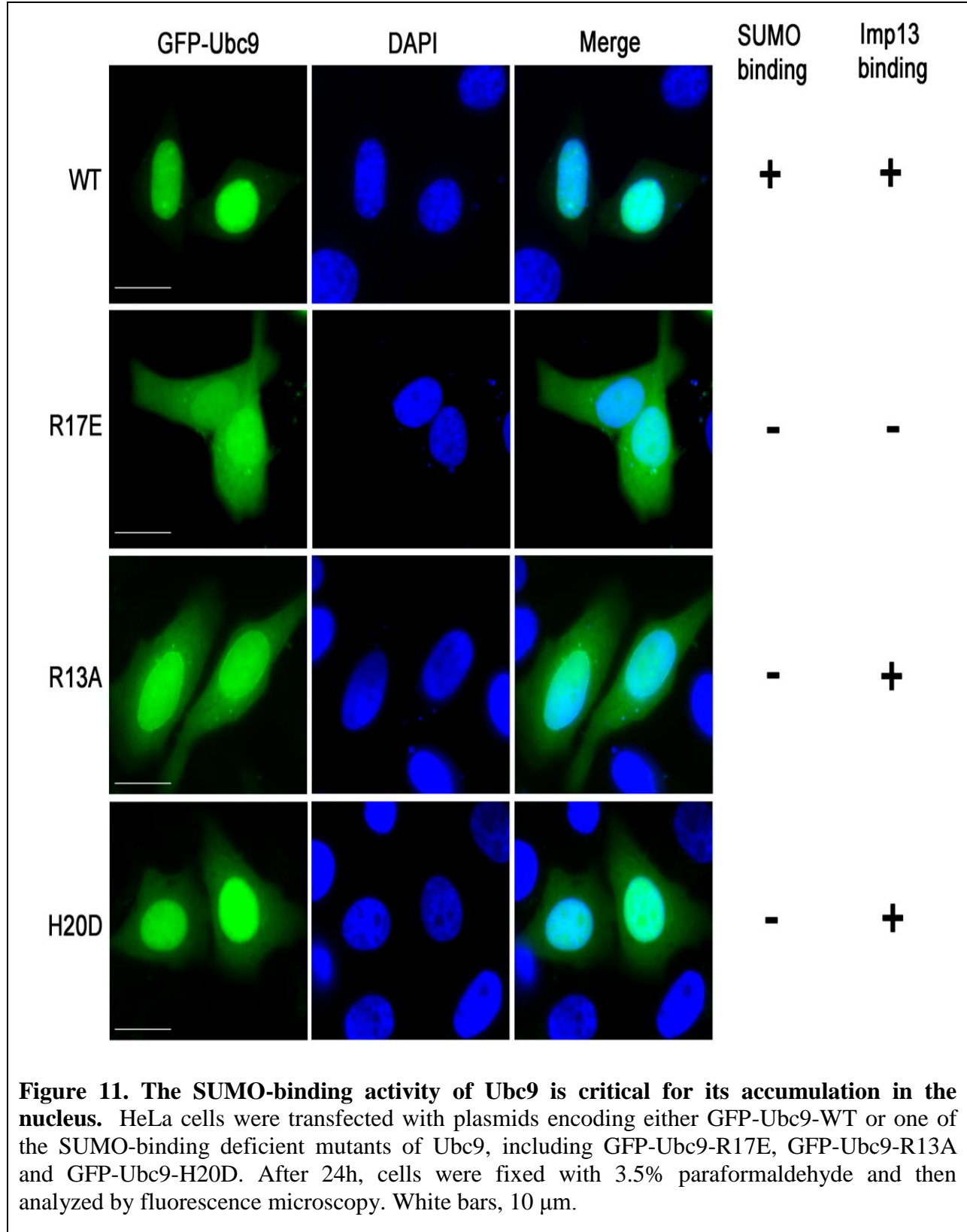


HA-Imp13 and GFP-Ubc9-WT constructs (Figures 9A and 10A). Quantitative analysis indicated that cells co-transfected with HA-Imp13 and GFP-Ubc9-R17E only modestly increased the percentage of cells with a high N/C ratio of GFP-Ubc9-R17E when compared to cells co-

transfected with GFP-Ubc9-R17E and empty vector plasmids. These results further demonstrated that Imp13 can efficiently transport Ubc9 from the cytoplasm to the nucleus and that the Imp13-Ubc9 interaction is critical for the nuclear accumulation of Ubc9 *in vivo*.



Non-covalent interaction of Ubc9 and SUMO is necessary for nuclear localization of Ubc9



The predominant nuclear distribution of both SUMO-1 and SUMO-2/3 leads us to explore whether the interaction between Ubc9 and SUMOs also contributes to the nuclear localization of Ubc9. It has been shown previously that several amino acid residues on Ubc9 are essential for its interaction with SUMOs. These known Ubc9 mutants, including R13A, R17E and H20D, have a defect in forming the Ubc9-SUMO complex (Capili and Lima, 2007; Knipscheer *et al.*, 2007). Based on these findings, we tested whether these Ubc9 mutants with a defect in their interaction with SUMO exhibit a decreased nuclear distribution along with an increase in their cytoplasmic localization.

HeLa cells were transfected with GFP-Ubc9-WT or one of the SUMO-binding mutants of Ubc9, including GFP-Ubc9-R17E, GFP-Ubc9-R13A and GFP-Ubc9-H20D. 24 h post-transfection, cells were fixed with 3.5% para-formaldehyde and analyzed by fluorescence microscopy. GFP-Ubc9-R17E, GFP-Ubc9-R13A and GFP-Ubc9-H20D mutants exhibited a decreased nuclear accumulation and also an increased cytoplasmic distribution when compared to GFP-Ubc9-WT. This result indicated that the Ubc9-SUMO interaction is also important for Ubc9 nuclear localization.

CHAPTER 4 - DISCUSSION

SUMOylation is catalyzed by a cascade of enzymes including a SUMO-E1 activating enzyme (SAE1/SAE2), a SUMO-E2 conjugating enzyme (Ubc9) and several E3 ligases. SUMO-E1, SUMO-E2 and SUMOs are known to be majorly localized in the nucleus (Azuma *et al.*, 2001; Saitoh *et al.*, 2001; Zhang *et al.*, 2008). It has been hypothesized that SUMOylation occur majorly in the nucleus since most of the SUMOylated proteins along with the SUMOylation machinery are nuclear (Rodriguez *et al.*, 2001). Therefore, it is important to understand the mechanisms regulating the nuclear localization of these enzymes. Studies have shown that SUMO-E1 enzyme is imported to the nucleus by Importin α/β (Moutty *et al.*, 2011). In our study, we elucidated the mechanisms responsible for the nuclear localization of Ubc9 and found that nuclear localization of Ubc9 is important for efficient SUMOylation. A study by Mingot *et al.*, (2001) has shown that Imp13 can mediate the nuclear import of Ubc9 *in vitro*. Based on this study, we explored whether Imp13 is critical for Ubc9 nuclear localization in mammalian cells. Consistent with our hypothesis, we demonstrated that inhibition of Imp13 causes a significant reduction of Ubc9 nuclear accumulation and also a dramatic decrease of SUMO-1 and SUMO-2/3 modification *in vivo* (Figure 6 and 7).

Structural studies have shown the R17 residue of Ubc9 is critical for its interaction with Imp13 *in vitro* (Grunwald and Bono, 2011). Our immunofluorescence microscopy revealed an increase of the cytoplasmic distribution of GFP-Ubc9-R17E as compared to GFP-Ubc9-WT. A recent study has also shown that GFP-Ubc9-R17E has an increased cytoplasmic distribution as compared to GFP-Ubc9-WT with a predominant nuclear localization (Grunwald *et al.*, 2013). To further test the function significance of the Imp13-Ubc9 interaction in control of Ubc9 nuclear localization, we compared the capacities of Imp13-WT and Imp13-D426R mutant with a

defect in Ubc9 interaction on enhancing the nuclear accumulation of GFP-Ubc9-WT. We found that overexpression of HA-Imp13-WT greatly increased the nuclear accumulation of GFP-Ubc9-WT with 92% of the cells with a high N/C ratio of >4 but only modestly elevated the nuclear distribution of GFP-Ubc9-R17E with 25% of the cells having a N/C ratio of >4 . Consistent with the above result, overexpression of Imp13-D426R mutant had very little effect on the nuclear accumulation of GFP-Ubc9-WT (Figure 9B). These results clearly indicate that interaction between Imp13 and Ubc9 is important for nuclear accumulation of Ubc9.

We also test if the SUMO-binding activity of Ubc9 plays a role in Ubc9 nuclear accumulation. This idea was supported by the findings that SUMO and Ubc9 can form a complex *in vitro* and that this complex formation is destabilized using Ubc9 mutants (Ubc9-R17E, Ubc9-R13A and Ubc9-H20D) with a defect in their SUMO binding (Capili and Lima, 2007; Knipscheer *et al.*, 2007). Consistent with our hypothesis, we found that these Ubc9 mutants displayed an increased cytoplasmic distribution when compared to Ubc9-WT. Interestingly, Ubc9-R17E showed a higher cytoplasmic distribution than Ubc9-R13A and Ubc9-H20D. Previous studies have shown that the R17 residue of Ubc9 is known to be important for its binding to both Imp13 and SUMO (Grunwald and Bono, 2010; Capili and Lima, 2007). Clearly, these results have demonstrated that both Imp13-mediate nuclear import of Ubc9 and the SUMO-binding activity of Ubc9 are important for Ubc9 nuclear localization. The Ubc9-SUMO interaction may function as an anchorage to retain Ubc9 at the nucleus.

Based on our findings, we propose a model in which two mechanisms are responsible for the nuclear localization of Ubc9 and efficient SUMOylation (Figure 12). Imp13 binds to the Ubc9 in the cytoplasm and translocates it to the nucleus where Ubc9 is then released from the Imp13-Ubc9 complex upon RanGTP binding. Ubc9 is the sole SUMO-E2 conjugating enzyme

participating in the SUMO-conjugation process. Upon depletion of Imp13 in the cells, Ubc9 nuclear import is blocked, leading to a decrease of the global SUMOylation. Notably, the Ubc9-SUMO interaction can occur between Ubc9 and free-SUMOs or SUMO-conjugates. In our study, we described three critical residues of Ubc9 (R17, R13 and H20) that are important for its SUMO-binding as well as its nuclear localization.

We are currently testing whether another possible mechanism is important for Ubc9 nuclear accumulation. As previously discussed, during the SUMO conjugation pathway, Ubc9 recognizes the SUMOylation consensus site of the target for SUMO-conjugation. Furthermore, structural studies have shown residues on Ubc9 which are crucial for its recognition of the SUMOylation consensus motif on the target proteins (Reverter and Lima, 2005; Villamor *et al.*, 2002). SUMO-conjugation assay using Ubc9 mutants with a defect in binding to the SUMO consensus motif exhibited reduced SUMO conjugation for the substrates including RanGAP1, p53 and I κ B α (Villamor *et al.*, 2002). This evidence provides a clue that Ubc9 interaction with the SUMO consensus sequence might also regulate the nuclear localization of Ubc9. To address this question, we will generate a series of Ubc9 mutants with a defect in interaction with the SUMOylation consensus site and investigate whether this interaction is important for Ubc9 nuclear localization.

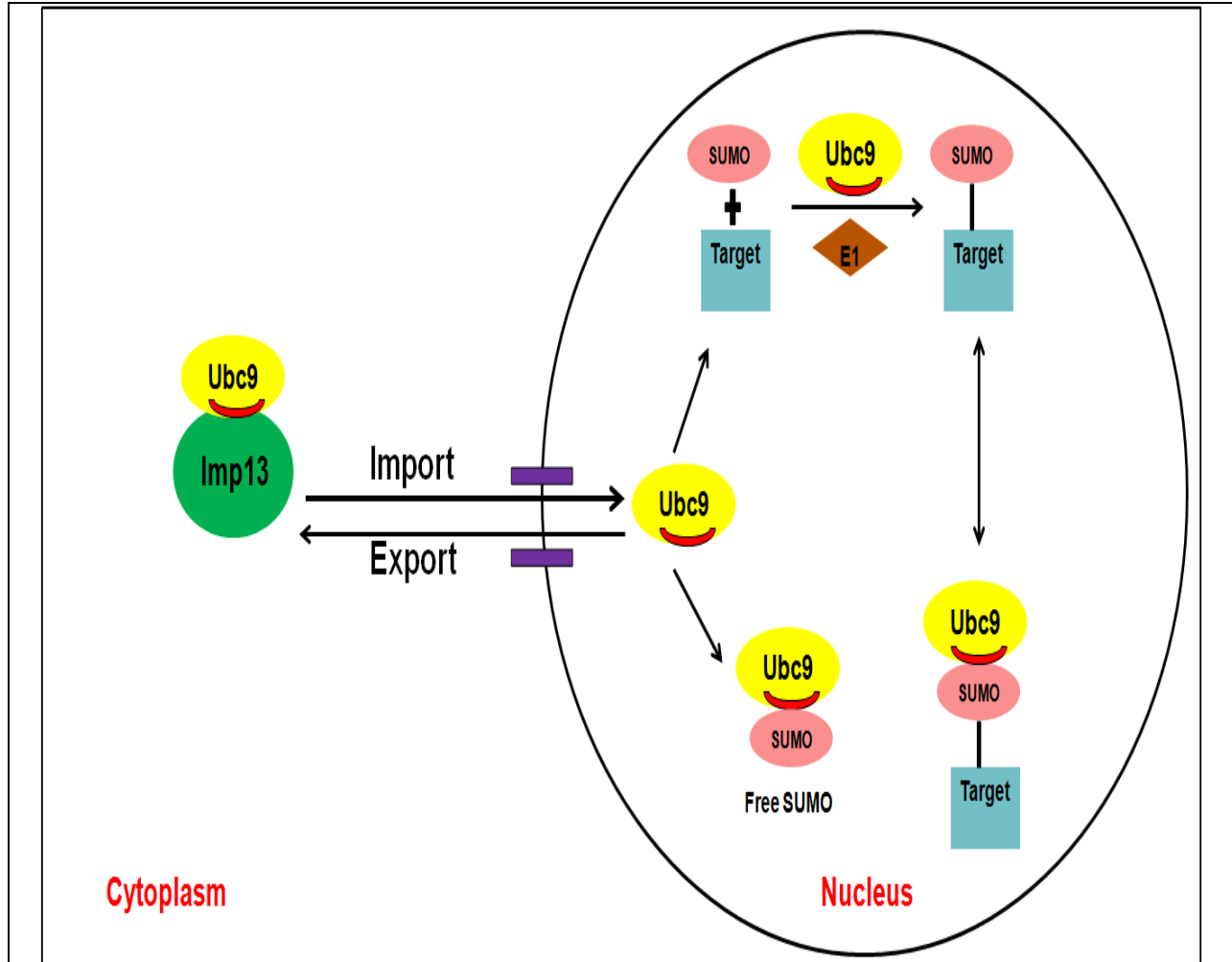


Figure 12. A model illustrates the mechanisms in regulating Ubc9 nuclear localization. Imp13 mediates the nuclear import of Ubc9, which is important for efficient global SUMOylation. As the sole E2 conjugating enzyme, Ubc9 is essential for SUMO conjugation. We found that both Imp13-mediated nuclear import of Ubc9 and the SUMO binding activity of Ubc9 are essential for Ubc9 accumulation in the nucleus. The red hinge on Ubc9 denotes its SUMO binding sites.

Appendix A: Primers used for subclonings

Primer name	Primer
GFP-Imp13-forward	GCCTCGAGCTATGGAGCGGCGG
GFP-Imp13-reverse	CGGGATCCTCAGTAGTCAGCTGTGTAAT
GST-Imp13-forward	GCGGATCCATGGAGCGGCGGG
GST-Imp13-reverse	CGCTCGAGCACACATCAGAATAG
GFP-Ubc9-forward	GGAATTCTATGTTCGGGGATCGC
GFP-Ubc9-reverse	CGGGATCCTTATGAGGGCGC
GST-Ubc9-forward	GCGGATCCATGTTCGGGGATC
GST-Ubc9-reverse	CGCTCGAGTTATGAGGGCGC

Appendix B: Mutagenesis primers

Primer name	Primer
Ubc9-R17E-forward	CAGGAGAGGAAAGCATGGGAGAAAGACCACCC
Ubc9-R17E-reverse	AAATGGGTGGTCTTTCTCCCATGCTTTCCTCTCC
Ubc9-R13A-forward	CTCGCCCAGGAGGCGAAAGCATGGAGG
Ubc9-R13A-reverse	CCTCCATGCTTTCGCCTCCTGGGCGAG
Ubc9-H20D-forward	GCATGGAGGAAAGACGACCCATTTGGTTTC
Ubc9-H20D-reverse	GAAACCAAATGGGTTCGTCTTTCCTCCATGC
Imp13-D426R-forward	CGTATCTACAGGGTGCATCTCAGACACTC
Imp13-D426R-reverse	GAGTGTGTCTGAGATGCGCACCCCTGTAGATACG

Appendix C: siRNA oligos

siRNA oligo name	Primer
Imp13 1588-forward	UGCCAUCUCACAGCCUGA
Imp13 1588-reverse	AUCAGGCUGUGAGAUGGCA
Imp13 1014-forward	CAUGAUUAUGUUCUGCACA
Imp13 1014-reverse	UGUGCAGAACAUAUAUCAUG
Imp13 114-forward	CAUUGAGAAUAAGAACCUG
Imp13 114-reverse	CAGGUUCUUAUUCUCA AUG
Control-forward	UUCUCCGAACGUGUCACGU
Control-reverse	ACGUGACACGUUCGGAGAA

Appendix D: Antibodies

Antibodies	Species	Sources	Dilution
SUMO-1 (21C7)	Mouse	Invitrogen	1:1000
SUMO-2/3 (8A2)	Mouse	Abcam	1:800
Tubulin	Mouse	Sigma	1:5000
Ubc9	Rabbit	Genetex	1:5000
Actin	Mouse	Genscript	1:3000
Importin13	Rabbit	Dr.Feige Kaplan	1:500

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ABSTRACT**REGULATION OF NUCLEAR LOCALIZATION OF THE SOLE
SUMO-CONJUGATING ENZYME, UBC9**

by

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The covalent and reversible conjugation of small ubiquitin-like modifier (SUMO) proteins to hundreds of different cellular proteins is catalyzed by a cascade of enzymes including an E1-activating enzyme (SAE1/SAE2), an E2-conjugating enzyme (Ubc9) and multiple E3 ligases. As the only E2 enzyme for SUMO-conjugation, Ubc9 localizes mainly in the nucleus and plays an essential role in regulation of many cellular processes including cell cycle progression through mitosis, cell migration, genome stability, stress response, transcription, and nuclear transport in eukaryotic cells. It is hypothesized that the nuclear localization of Ubc9 is required for efficient sumoylation inside the nucleus because both the sole SUMO E1 enzyme and SUMO-conjugates are mainly in the nucleus. However, we still have a poor understanding of how Ubc9 is accumulated in the nucleus. Although the nuclear import receptor Importin 13 (Imp13) can mediate the nuclear import of Ubc9 using *in vitro* nuclear import assays, little is known about how Ubc9 nuclear localization is regulated *in vivo*. Here, we hypothesize that Imp13 is the major nuclear import receptor for Ubc9 and thus required for efficient global sumoylation *in vivo*. Consistent with this hypothesis, we found that knockdown of Imp13 by

RNA interference (RNAi) causes a decrease of global sumoylation and also an increased cytoplasmic distribution of Ubc9. Furthermore, the Ubc9 mutant (R17E) with a defect in Imp13-interaction showed a significant increase of cytoplasmic distribution when compared to Ubc9 wild-type (WT). Moreover, overexpression of Imp13 greatly enhanced the nuclear localization of Ubc9-WT but not Ubc9-R17E mutant, whereas overexpression of Imp13 mutant (D426R) with a defect in Ubc9 binding could not promote the nuclear accumulation of Ubc9-WT. Lastly, we demonstrated that the Ubc9 mutants (R17E, R13A and H20D) with a defect in SUMO-binding have an elevated cytoplasmic distribution when compared to Ubc9-WT, suggesting that the non-covalent interaction between Ubc9 and SUMO is also important for Ubc9 nuclear accumulation. Hence, our results support a model that both Imp13-mediated nuclear import and the SUMO-binding activity of Ubc9 are critical for Ubc9 nuclear localization and efficient global sumoylation in mammalian cells.

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- Palak Sekhri and Xiang-Dong Zhang, The nuclear localization of Ubc9 is determined by both Importin 13-mediated nuclear import and the SUMO-binding activity of Ubc9, 2013 Annual Retreat, Department of Biological Sciences, Wayne State University
- Palak Sekhri and Xiang-Dong Zhang, The nuclear localization of Ubc9 is determined by both Importin 13-mediated nuclear import and the SUMO-binding activity of Ubc9, December 14-18, 2013, 53rd ASCB annual meeting, New Orleans, LA