NOVEL FUNCTIONS OF THE SUMO PROTEASES SENP1 AND SENP2 AND THE NUCLEOPORIN NUP153 AT THE NUCLEAR PORE

by

Kin-Hoe Chow

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Oncological Sciences

The University of Utah

August 2011

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The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Kin-Hoe Chow**

has been approved by the following supervisory committee members:

Name	Role	Date Approved
Katharine Ullman	, Chair	**06/08/2011**
Steve Alam	, Member	**06/08/2011**
Markus Babst	, Member	**06/08/2011**
Barbara Graves	, Member	**06/16/2011**
Jody Rosenblatt	, Member	**06/14/2011**

and by **Don Ayer (interim department chair)** , Chair of

the Department of **Oncological Sciences**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The SUMO proteases SENP1 and SENP2 are known to reside at the nuclear pore complex; however, their functional roles there remain unclear. In this study, SENP1 and SENP2 are found to regulate the nuclear pore assembly of components located at the central core and cytoplasmic fibrils of the nuclear pore complex. Interestingly, SENP1 and SENP2 are found to interact with the nuclear basket component Nup153 and regulate the SUMOylation status of Nup153. The N- and C-terminal domains of Nup153 are found to mediate a bimodal interaction with SENP1/SENP2. Nup153 N-terminal is a site of SUMO modification. This modification enhances the interaction between Nup153 and SENP1/SENP2. Its C-terminal domain, on the other hand, is found to interact with SENP1/SENP2, likely through the bridging interaction mediated by the trafficking receptor Importin α. These interactions are likely to contribute to a novel function of Nup153 in NPC biogenesis. Depletion of Nup153 or SENP1/SENP2 leads to similar defects in NPC assembly of numerous nucleoporins, suggesting Nup153 and SENP1/SENP2 are likely to work in the same pathway in mediating NPC biogenesis. These findings yielded new insights into the regulatory mechanism of NPC biogenesis in which the deSUMOylating activity of SENP1/SENP2 may be coordinated through Nup153 at the nuclear basket, to coordinate the assembly of nucleoporins to the sites of nuclear pore complex.

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CHAPTER 1

INTRODUCTION

1.1 Abstract

The SUMO modification pathway has emerged as an important post-translational modification pathway that regulates a variety of cellular events including gene expression, maintenance of genomic stability, and cell cycle progression. While recent studies have defined various regulatory complexities of this pathway, a mystery remains as to why some components of the SUMO modification pathway reside at the nuclear pore complex (NPC). A substantial number of studies using yeast as a model organism have characterized the relationship between the NPC and the associated SUMO protease Ulp1. These studies report functional relevance for this partnership in nucleocytoplasmic trafficking, DNA repair, and mRNA surveillance. However, little progress has been made in understanding the functional relevance of the NPC-residing SUMO proteases SENP1 and SENP2 in mammalian cells. In this study, I define a role for SENP1 and SENP2 in the biogenesis of NPC. In addition, I have further characterized the biochemical relationship between SENP1/SENP2 and components of the NPC and have found evidence that a partnership between SENP1/SENP2 and the nucleoporin Nup153 is required for NPC biogenesis. 1.2 The nuclear pore complex NPCs are enormous protein complexes of 40-70MDa with high architectural complexity which serve as the sole

gateways of trafficking between the nucleoplasm and cytoplasm. Despite its gigantic size, an NPC is assembled by only ~30 different nucleoporins, each in multiple copies, to constitute a highly symmetrical organization. When fully assembled, one NPC will have an estimated ~450 nucleoporin subunits (Tran and Wente, 2006). Each NPC is statically embedded in the nuclear envelope through multiple interactions with inner nuclear membrane proteins, nuclear lamina, and chromatin (Daigle et al., 2001; Rabut et al., 2004). During interphase, new NPCs are constantly assembled and inserted into the expanding nuclear envelope (Maeshima et al., 2006; Neumann and Nurse, 2007; Winey et al., 1997). However, during mitosis, the nuclear envelope is dispersed into the cytoplasm and the NPCs are disassembled into cytoplasmic subcomplexes .

The architectural structure of the NPC has close ties with its function in nucleocytoplasmic trafficking (refer to Figure 1.1 and Figure 1.2). At the nuclear envelope, transmembrane nucleoporins (Pom121, Ndc1, gp210) as well as various scaffolding (Nup107 and Nup205 complexes) and peripheral nucleoporins (Nup62 complex) constitute the pore channel spanning the cytoplasm and nucleoplasm. Numerous nucleoporins (Nup358, Nup214, Nup88, hCG1, Aladin, Rae1, and Nup98) reside on the cytoplasmic side of this pore channel and form cytoplasmic fibrils, which mediate the docking of various trafficking receptors. Another group of nucleoporins (Nup153, TPR, Nup50, Rae1, and Nup98) localize at the nucleoplasmic face of the pore channel, forming a basket-like structure that extends into the nucleoplasm and also mediates binding of trafficking receptors.

1.2 Regulation of interphase NPC biogenesis

In higher eukaryotes, both NPCs and the nuclear envelope (NE) are disassembled and dispersed throughout the cytoplasm during mitosis (open mitosis). Individual subunits or subcomplexes of nucleoporins are then sequentially recruited to chromatin and reassembled into functional NPCs in telophase, concurrent with the reformation of new nuclear envelope (Antonin et al., 2008; Kutay and Hetzer, 2008). Another pathway of NPC assembly occurs during interphase when newly synthesized nucleoporins are incorporated into the expanding NE to form NPCs *de novo* (Doucet and Hetzer, 2010). The presence of this distinct pathway is supported by the observation that the number of NPCs doubles in higher eukaryotes during S phase (Maul et al., 1972). In addition, NPC-free islands at nuclear envelope have been observed in early G1 cells which gradually disperse in G1/S, concomitant with the increase in NPC density (Maeshima et al., 2006), suggesting an active expansion of the NE in early G1 is followed by NPC incorporation through S phase. Using *in vitro* assembled nuclei from *Xenopus* egg extract, D'Angelo et al. showed that new NPCs are assembled from components on both sides of the nuclear envelope and do not contain components derived from existing NPCs, reinforcing the occurrence of *de novo* NPC assembly (D'Angelo et al., 2006). Considering the complexity of the NPC structure, the NPC assembly process is likely to include layers of regulatory steps including the availability of nucleoporins/assembly factors and post-translational modification of both nucleoporins and regulatory factors (Antonin et al., 2008). Certainly, phosphorylation has been established as a key modulator of NPC assembly status at mitosis (Macaulay et al., 1995; Laurell et al., 2011).

1.3 Nonnucleoporin mediators of interphase NPC assembly

The regulatory mechanisms controlling *de novo* NPC assembly at interphase have been partially uncovered in studies using yeast, as they do not undergo open mitosis and have to assemble NPCs *de novo* into the double bilayer of the nuclear envelope. Multiple nonnucleoporin factors have been described in yeast studies to have roles in *de novo* NPC synthesis, including Ran, RanGEF, RanGAP, NTF2 (Ran transport cofactor), Kap95 (Importin β homolog), and Kap121 (Importin 5 homolog) (Ryan et al., 2003; Ryan and Wente, 2002; Ryan et al., 2007; Lusk et al., 2002). Mutation or depletion of these factors can lead to defective incorporation of newly translated nucleoporins into NPCs, resulting in accumulation of these nucleoporins in cytoplasmic foci. It is not entirely clear how these trafficking-related factors mediate pore assembly but it is thought that RanGTP and Kap95 have antagonistic roles, with Kap95 possibly sequestering factor(s) that catalyze the fusion of nucleoporin-containing vesicles to NE. RanGTP at the NE would stimulate release of factor(s) from Kap95 to allow vesicle fusion (Ryan et al., 2007). Interestingly, a role for RanGTP and Importin β in *de novo* NPC assembly has also been found in the *Xenopus in vitro* NPC assembly system (D'Angelo et al., 2006). Proteins related to the ER, including Apq12, reticulons (RTN), and Yop1/DP1, have also revealed to have roles in *de novo* NPC synthesis (Scarcelli et al., 2007; Dawson et al., 2009), possibly through their abilities to modulate membrane form and dynamics. In the absence of Rtn1 and Yop1, NPC intermediates accumulate at the INM and ONM but failed to fuse to form a membrane spanning NPC (Dawson et al., 2009). An unspecified role for the RSC chromatin-remodeling complex in NPC assembly has also been described (Titus et al., 2010). Finally, a recent study in mammalian cells has revealed a role for Cdk1/Cdk2 in

regulating the stability and the proper targeting of nucleoporins during interphase NPC assembly (Maeshima et al., 2010).

1.4 Roles of transmembrane nups and scaffolding nups in *de novo* NPC assembly

Numerous studies in yeast have highlighted the importance of transmembrane nucleoporins and scaffolding nucleoporins in coordinating *de novo* NPC biogenesis. (For classification of yeast and mammalian nucleoporins, refer to Figure 1.2). Inactivation of the yeast transmembrane nups Pom152 and Pom34, as well as the scaffolding components Nup59/53 can lead to accumulation of newly synthesized nucleoporins in cytoplasmic foci and aberrant dilation of pore diameter (Onischenko et al., 2009). Pom152, Pom34, and Nup59/53 can interact as a complex to recruit another transmembrane nup Ndc1 and the scaffolding nups Nup170 and Nup157 (Onischenko et al., 2009). Disruption of Nup170 and Nup157 also leads to accumulation of nucleoporins normally residing at the cytoplasmic fibril and transmembrane region of NPC in cytoplasmic foci (Makio et al., 2009; Flemming et al., 2009). In addition, formation of an NPC intermediate at the INM that failed to fuse with ONM has also been detected. (Makio et al., 2009). Interestingly, these studies show a common trend that those cytoplasmic foci accumulating nucleoporins are restricted to the population normally residing at the NPC core and the cytoplasmic fibrils. The examined nucleoporins normally residing at the nuclear basket (Nup1, Nup2, Nup60, and Mlp1) in these studies appear to localize to INM, possibly at NPC intermediates that failed to fuse with the ONM (Makio et al., 2009).

The functional role of transmembrane nucleoporins and scaffolding nucleoporins in mediating *de novo* NPC assembly has also been characterized in mammalian cells. Downregulation of the conserved transmembrane nup NDC1 in mammalian cells can severely impair NPC assembly (Mansfeld et al., 2006; Stavru et al., 2006). Interestingly, complete knockout of Ndc1 homolog in *C. elegans* can also severely reduce NPC assembly, but rudimentary NPCs can still be assembled, suggesting redundancy among the three transmembrane nups Pom121, gp210, and Ndc1 in coordinating NPC assembly (Stavru et al., 2006). Indeed, depletion of Pom121 or gp210 can also lead to an NPC assembly defect (Antonin et al., 2005; Mansfeld et al., 2006; Funakoshi et al., 2007). In addition, codepletion of gp210 and Ndc1 can further enhance the severity of NPC assembly defect in mammalian cells (Mansfeld et al., 2006). A recent study reported that severe depletion of Pom121 through prolonged RNAi treatment severely inhibits *de novo* NPC assembly, probably through a failure to mediate the fusion of INM and ONM at the pore assembly site and subsequent failure to recruit the scaffolding Nup107/160 complex. The Nup107/160 complex has been demonstrated to be essential for the *de novo* NPC assembly in both mammalian cells and in the *Xenopus in vitro* NPC assembly assay (D'Angelo et al., 2006; Doucet et al., 2010). A component within the scaffolding Nup107/160 complex, Nup133, contains a membrane curvature-sensing domain that mediates the recruitment of the Nup107/160 complex to the interphase pore assembly site. Interestingly, such a Nup133 recruitment mechanism is not required for post-mitotic NPC assembly, highlighting the regulatory divergence between these pore assembly pathways (Doucet et al., 2010).

1.5 A role of a peripheral nup in NPC assembly

Most of the studies from yeast or mammalian systems have revealed functional roles for transmembrane nups and the scaffolding nups located at the core of the NPC in mediating the initial steps of *de novo* NPC assembly. Notably, however, Wu et al. reported a role of the peripheral FG nup Nup98 in mediating efficient assembly/recruitment of nups residing at the cytoplasmic fibril of NPC. Nup98 knockout cells display mislocalization of cytoplasmic fibril nups Nup358, Nup214, Nup88 into cytoplasmic annulate lamellae (nucleoporin-containing membranous structure in the cytoplasm). In addition, several transport receptors including Importin α, Importin β, and Transportin also accumulate in cytoplasmic annulate lamellae. However, the nuclear basket nups Nup153 and Nup50 displayed proper localization to the NE (Wu et al., 2001). These findings closely resemble the NPC assembly phenotypes reported upon the disruption of the scaffolding Nup107 and Nup157 in yeast (Makio et al., 2009; Flemming et al., 2009). While it is unclear whether scaffolding nups are efficiently recruited to the pore upon the disruption of Nup98, it is likely that the peripheral Nup98 can stabilize the NPC assembly process through maintaining interaction networks with multiple nups including the scaffolding nups Nup133 and Sec13, the peripheral nup Nup62, and the cytoplasmic fibril nup Nup88 and Rae1, thereby stabilizing the gigantic structure of the NPC.

While the role of several core transmembrane/scaffolding/peripheral nups in NPC biogenesis has been characterized, the assembly of the sophisticated architecture of the NPC is likely to involve additional layers of regulation. As discussed in the following sections, the yeast NPC-associated SUMO protease Ulp1 and its mammalian orthologues

SENP1 and SENP2 have been found to have intimate interactions with components of the nuclear basket as well as the core scaffolding nups, suggesting a possibility of SUMO-mediated regulation of nups by Ulp1/SENP1/SENP2.

1.6 Connection of NPC and SUMO machinery: the SUMO conjugation system

The Small Ubiquitin-like Modifier (SUMO) is an 11kD peptide that can be covalently attached to a target protein post-translationally and thereby modulate the stability, the localization, and the protein-protein interaction properties of the target protein. SUMO is first produced as a precursor that requires the SUMO deconjugation system (SUMO proteases) to post-translationally process the precursor to expose its C-terminal diglycine residues for conjugation. There are three SUMO paralogs identified in mammalian cells, SUMO1, SUMO2, and SUMO3. All these SUMO paralogs can form polySUMO chains *in vitro*. However, only SUMO2/3 poly-chains have been observed *in vitro*. SUMO is predominantly conjugated to lysine residues within a consensus context (ψKxQ/E, where ψ represents hydrophobic residue), through a series of enzymatic reactions mediated by the SUMO E1 activating enzyme (Aoa1/Uba2) and the SUMO E2 conjugating enzyme Ubc9 (Mukhopadhyay and Dasso, 2007). The specificity and efficiency of this SUMO conjugation event can be enhanced by a number of localization-specific SUMO E3 ligases. Members of the SUMO conjugation machinery have been reported to have close ties with the nuclear pore complex. It was discovered that the cytoplasmic fibril nucleoporin RanBP2/Nup358 possesses SUMO E3 ligase activity (Pichler et al., 2002). Together with the SUMO E2 conjugation enzyme Ubc9 and the RanGAP1, it is part of a ternary complex. It was later shown that the interaction between

SUMO1-RanGAP1 and Nup358 and Ubc9 protects SUMO1-RanGAP1 from being targeted by SUMO proteases (Zhu et al., 2009). Notably, this ternary complex does not get dissembled during mitosis and has regulatory function in the mitotic microtubule-kinetochore attachment (Swaminathan et al., 2004; Joseph et al., 2004). Interestingly, electron microscopy analysis from Zhang et al. have revealed that Ubc9 localizes to both the nuclear basket region as well as the cytoplasmic fibrils of the nuclear pore complex (Zhang et al., 2002); however, the binding partner Ubc9 at the basket remains unidentified.

1.7 Connection of NPC and SUMO machinery: the deconjugation system

SUMO modification can be highly dynamic as SUMO conjugation is counteracted by deconjugation mediated by a class of enzymes called SUMO proteases (SENP) (Mukhopadhyay and Dasso, 2007). Numerous SUMO proteases have been identified in mammalians and their specific subcellular localization is thought to confer specificity in SUMO deconjugation. SENP1 and SENP2 are both reported to be in both the cytoplasm and the nucleoplasm, depending on cellular context (Mukhopadhyay and Dasso, 2007). However, SENP1 and SENP2 are the only SUMO proteases that also localize to the nuclear envelope/nuclear pore. The C-terminal domain of the nuclear basket nucleoporin Nup153 has been shown to interact with the N-terminal domain of SENP2 (Zhang et al., 2002; Bailey and O’Hare, 2004; Hang and Dasso, 2002). It is unclear whether Nup153 is the major docking site of SENP2 at the NPC or if other nucleoporins or NPC-associated proteins are involved. In addition, no NPC binding partner of SENP1 has been reported.

Similar NPC localization of the yeast SUMO protease ortholog Ulp1 has also been observed. The binding determinants of Ulp1 at the yeast NPC have been much better characterized than its mammalian counterparts SENP1 and SENP2. The localization of Ulp1 to NPC is dependent on its interaction with the transport receptors Kap121 (mediates mRNA export) and Kap60-Kap95 (mediate classical NLS cargo import), and the basket nucleoporins Nup60 (ortholog of mammalian Nup153) and Mlp1-Mlp2 (ortholog of mammalian TPR) (Panse et al., 2003; Makhnevych et al., 2007; Zhao et al., 2004; Lewis et al., 2007). Ulp1 is reported to interact with Kap121 and Kap60-Kap95 at the proximal and distal regions of its N-terminal domain, respectively. Interestingly, these interactions display insensitivity to the presence of RanGTP (karyopherins normally release their cargo upon binding to RanGTP) (Panse et al., 2003). Deletion of either binding site in Ulp1 only mildly affects its localization, whereas deleting both sites leads to mislocalization of the truncated Ulp1 (the C-terminal catalytic domain) into the nucleoplasm. While exogenously expressed GFP-Ulp1 is only mildly mislocalized in a *kap95* mutant, it exhibits more defective localization in a *kap121* mutant. Interestingly, deletion of the Kap60-Kap95 binding site on Ulp1 completely mislocalizes its targeting in *kap121* mutants, highlighting the cooperative nature of the karyopherin binding sites on Ulp1 in mediating its nuclear envelope localization (Makhnevych et al., 2007). Although the localization of Ulp1's mammalian counterpart SENP2 has not been reported to be mediated through karyopherin binding, it is notable that both Ulp1 and SENP2 rely on their noncatalytic N-terminal domain for proper NPC localization (Hang and Dasso, 2002; Zhang et al., 2002).

The nuclear envelope localization of Ulp1 is thought to be mediated, in part, through components of the NPC, namely Nup60, Mlp1-Mlp2, and the Nup84 complex. Mutating Esc1, which coordinates the nuclear basket assembly of Nup60/Mlp1-Mlp2, can mislocalize Nup60, Mlp1-Mlp2, together with Ulp1, into perinuclear foci. While the Kap121-binding site deletion mutant of Ulp1 can still localize to the nuclear periphery, it fails to be recruited to those mislocalized Nup60/Mlp1-Mlp2 foci, underscoring a role for Kap121 in mediating Nup60-dependent Ulp1 localization to NPC (Lewis et al., 2007). Yet, it is clear that Nup60 and Mlp1-Mlp2 do not provide the sole docking sites for Ulp1 as it still localizes to the nuclear envelope in *nup60*Δ cells. In addition, mutations in the Nup84 complex (which do not mislocalize Nup60, Mlp1-Mlp2), also result in mislocalized Ulp1 (Palancade et al., 2007). Furthermore, overexpressed Ulp1 or its Kap121-binding domain can localize to inner nuclear envelope at regions without NPCs (Makhnevych et al., 2007; Palancade et al., 2007). These data collectively suggest that Ulp1 is likely to use multiple docking sites on NPCs and potentially has other binding partners at nuclear envelope.

1.8 SENP1/SENP2/Ulp1 functions at the nuclear pore complex

The functional roles of SUMO machinery, especially SUMO proteases, at the NPC have been characterized in yeast, *Drosophila*, and mammalian cells. In yeast, inactivation of Ulp1 has been reported to share similar phenotypes as the disruption of the SUMO activation enzyme, Uba2: defects in nuclear import of proteins containing classical nuclear import sequence (cNLS), with nuclear accumulation of Kap60 (yeast homolog of Importin α) (Stade et al., 2002). These observations suggest the possibility that a cycle of SUMO modification, regulated by Uba2 and Ulp1, on Kap60 or its

regulators could mediate the recycling of Kap60. However, the aforementioned cNLS import defect in Ulp1 mutated strain can be rescued by expressing maturated SUMO, indicating that the defect of cNLS import may be attributed to a lack of SUMO precursor processing (Ulp1 is the predominant SUMO protease in yeast that maturates SUMO precursor). As such, the mutation of Uba2 or Ulp1 would block cNLS import/ Kap60 recycling simply through disrupting the SUMOylation of a regulator(s) that mediates cNLS import (Stade et al., 2002). It has been speculated that Nup2, which functions in recycling of Kap60, could be the target of SUMOylation as Nup2 SUMOylation has been reported in a SUMO proteomic study (Hannich et al., 2005; Palancade and Doye, 2008). However, the verification of this hypothesis has yet to be reported. Interestingly, multiple trafficking factors and nucleoporins have been identified as potential SUMO substrates in SUMO proteomic studies (see Table 1.1). While further verification of the SUMOylation of these potential targets is required, it is tempting to speculate that certain aspects of nucleocytoplasmic trafficking could be regulated through transient cycles of SUMO modification on a subset of trafficking factor/nucleoporins.

A role for a NPC-associated SUMO protease in regulating nucleocytoplasmic trafficking has also been reported by Smith et al. Here, downregulation of *Drosophila* Ulp1 leads to the cytoplasmic accumulation of SUMO that is normally detected in the nucleus. SUMOylated proteins, including the SUMOylated shuttling protein EPRS, also accumulate in the cytoplasm upon the depletion of Ulp1. In this context, Ulp1 is proposed to restrict SUMO substrates to the nucleus by deSUMOylating them as they traverse through the NPC into the cytoplasm (Smith et al., 2004).

NPC association of SUMO proteases has also been reported to sequester their deSUMOylation activity away from nucleoplasm. Supporting this notion, overexpression of the catalytic domain (which lacks NPC-targeting sequence) of mammalian SENP1 and SENP2 and the yeast Ulp1 was found to induce nonspecific deSUMOylation activity and result in cell toxicity (Bailey and O'Hare, 2004; Hang and Dasso, 2002; Panse et al., 2003). Indeed, forced cytoplasmic localization of the Ulp1 catalytic domain through a cytoplasmic anchor or NES sequence can rescue the lethal phenotype of Ulp1 deletion, indicating that sequestration of Ulp1 deSUMOylating activity from nucleoplasm is a key function of Ulp1's NPC localization (Panse et al., 2003). Similar conclusions have also been derived from the observation that mutating the NPC-targeting region of Ulp1 will lead to its nucleoplasmic mislocalization and, surprisingly, rescues the lethality and hyperSUMOylation phenotypes generated from Ulp2 (a nucleoplasmic SUMO protease) deletion (Li and Hochstrasser, 2003; Lewis et al., 2007). These data suggest that compromising the NPC-targeting features of SENP1/SENP2/Ulp1 can mislocalize its deSUMOylation activity to nucleoplasm and deregulate the balance SUMO modification activity in nucleoplasm.

Ulp1 has also been revealed to have an unexpected role in mediating the repair of DNA double-stranded breaks. Mutation of Ulp1 or its NPC-binding partners Nup60/Mlp1/Mlp2 complex or Nup84 complex members leads to the accumulation of DNA double-stranded break foci (Soustelle et al., 2004; Loeillet et al., 2005; Palancade et al., 2007; Nagai et al., 2008). The accumulation of DNA damage foci in the nucleoporin mutants is attributed to the destabilization of Ulp1 upon the disruption of the nuclear basket Nup60/Mlp1/Mlp2 complex or the core scaffolding Nup84 complex (Zhao et al.,

2004; Palancade et al., 2007), which subsequently deregulates the SUMOylation of the DNA repair protein Yku70 (Palancade et al., 2007). Collectively, these data indicate the NPC binding of Ulp1 is functionally important for its stability and its downstream regulatory effect on the DNA repair pathway.

1.9 Making inroads into a role for SENP1/2 at the NPC

It remains unclear how mammalian SENP1 and SENP2 affect nucleocytoplasmic trafficking or other aspects of NPC function. In this study, I first discovered that a trafficking regulatory component RanGAP1 at the NPC cytoplasmic fibril is delocalized upon SENP1/SENP2 depletion. The disruption caused by the depletion of SENP1/SENP2 is not limited to RanGAP1 as other components of cytoplasmic fibrils, the core scaffolding nups, as well as peripheral nups also display delocalization phenotypes, indicative of a larger defect in NPC biogenesis rather than a trafficking specific defect. This assembly phenotype converged with a partnership that I have characterized for the nuclear basket component Nup153 with SENP1/SENP2, leading to insight into new players in the coordination of NPC biogenesis. In contrast with previous model of *denovo* NPC assembly in which the central scaffolding core components (Nup107 complex, Nup205 complex, and Nup62 complex) are first recruited to the nuclear pore assembly site established by the transmembrane Pom121 (Doucet et al., 2010), data from this study suggests that the nuclear basket components are recruited to the nuclear pore assembly site prior to the central scaffolding core components. In this model, SUMOylation of the newly translated central scaffolding nucleoporin(s) prevent their premature association with the transmembrane nucleoporin Pom121 at the cytoplasm. Such premature association with Pom121 may interference with Pom121 ability to mediate NE fusion.

The nuclear basket component Nup153 will be recruited first to the Pom121 NE fusion site, bringing SENP1/SENP2 to the vicinity of the nuclear pore assembly site to mediate the deSUMOylation of central scaffolding components, allowing coordinated association of these components with Pom121.

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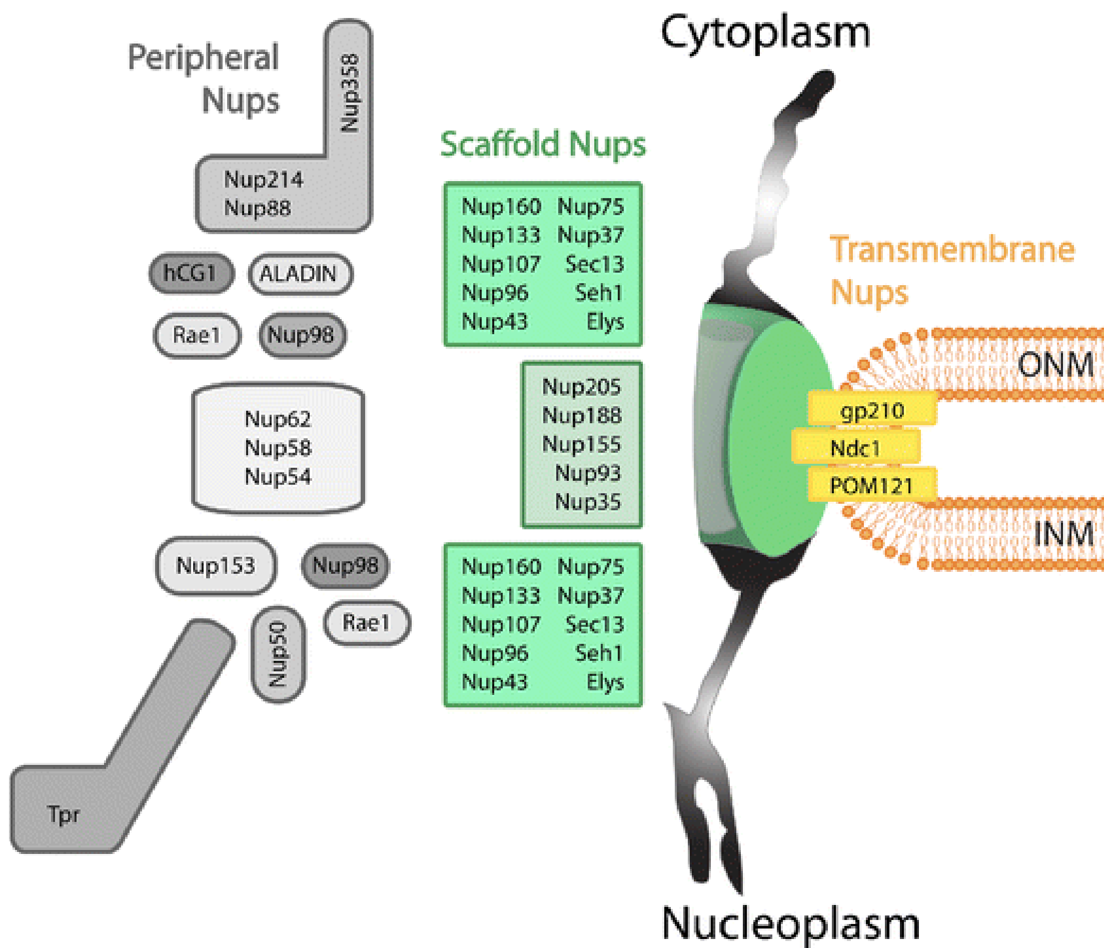
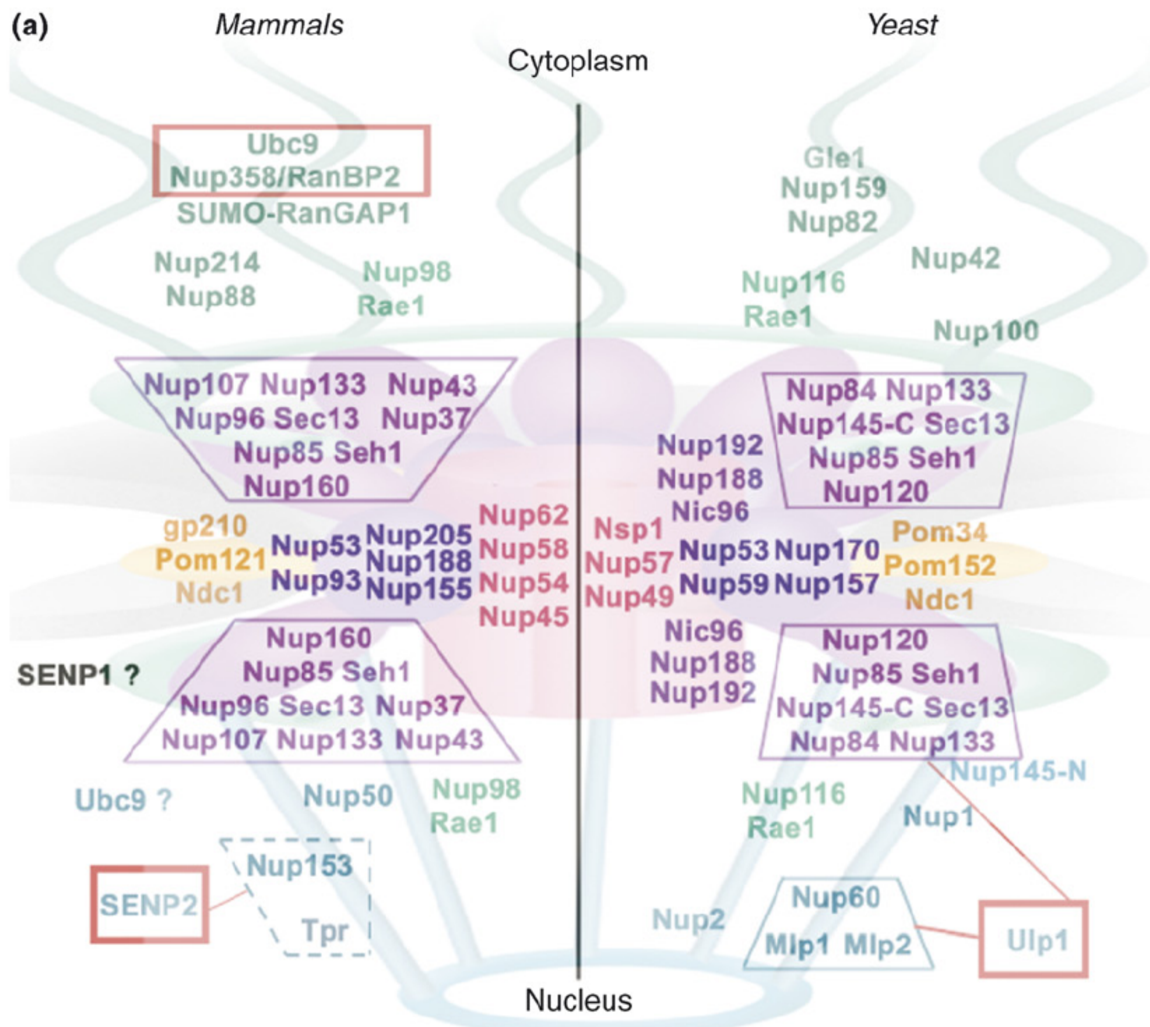


Adapted from Doucet et al., 2010

Figure 1.1. Classification of mammalian nucleoporins. The nuclear pore complex has a basic structure of a central pore channel that spans the nuclear envelope, with cytoplasmic fibrils and nuclear basket-like structures protruding from the central channel facing the cytoplasm and the nucleoplasm respectively. The various nucleoporins and their classification into transmembrane nup, scaffolding nup, or peripheral nup are highlighted in this diagram



Adapted from
Palancade and Doye., 2008

Figure 1.2. Comparison of the mammalian and yeast nucleoporins. The localization of the mammalian (left) and yeast (right) transmembrane, scaffolding, and peripheral nucleoporins and their homologous/orthologous counterparts are symmeterically arranged in this NPC diagram. The nucleoporins boxed in purple color at the central core are conserved subcomplexes of the scaffolding nups (Nup107-160 complex in mammal and Nup84 in yeast). The mammalian Nup153 at the nuclear basket has a functional ortholog Nup60 in yeast, both form a complex with the orthologous TPR (mammal) and Mlp1/Mlp2 (yeast) respectively (in blue boxes). Components of the SUMO machinery are highlighted in red boxes with red lines connecting to their binding determinants at the pore. The binding determinant(s) of the SUMO protease SENP1 and Ubc9 (in green boxes) at the nuclear basket have not been determined.

Table 1.1. Potential SUMO targets of trafficking factors and nucleoporins

Potential SUMO targets of trafficking factors and nucleoporins	Species	References
14-3-3	Drosophila	(Nie et al., 2009)
Importin-alpha re-exporter (CSE1)	Drosophila / Human	(Nie et al., 2009; Golebiowski et al., 2009)
Exportin 1 (XPO1)	Drosophila / Human	(Nie et al., 2009; Golebiowski et al., 2009)
Karyopherin beta-4 subunit	Drosophila / Yeast	(Nie et al., 2009)
Karyopherin alpha subunit	Drosophila	(Nie et al., 2009)
Ran	Human	(Rosas-Acosta et al., 2005)
Importin beta-1	Human	(Matafora et al., 2009)
Importin 7	Human	(Golebiowski et al., 2009)
Nup43 (in Nup107 complex)	Human	(Vertegaal et al., 2004)
Nup107 (in Nup107 complex)	Human	(Golebiowski et al., 2009)
Nup133 (in Nup107 complex)	Human	(Golebiowski et al., 2009)
Nup155 (in Nup205 complex)	Human	(Vertegaal et al., 2006; Golebiowski et al., 2009)
Nup205 (in Nup205 complex)	Human	(Blomster et al., 2009)
Nup93 (in Nup205 complex)	Human	(Blomster et al., 2009; Golebiowski et al., 2009)
Nup153 (nuclear basket)	Human	(Golebiowski et al., 2009)
TPR (nuclear basket)	Human	(Matafora et al., 2009; Golebiowski et al., 2009)
Mlp1/Mlp2 (TPR ortholog, nuclear basket)	Yeast	(Denison et al., 2005; Wohlschlegel et al., 2004)
Nup2 (nuclear basket)	Yeast	(Wohlschlegel et al., 2004)

Listed here are potential SUMO targets of that have been identified through various SUMO proteomic studies. Non e of the targets have been experimentally verified in these studies to have SUMO modification.

CHAPTER 2

THE SUMO PROTEASES SENP1 AND SENP2 HAVE A NOVEL FUNCTION IN NPC BIOGENESIS

2.1 Abstract

The SUMO proteases SENP1 and SENP2 are known to localize to the nuclear pore complex (NPC). However, their functional roles at this site are poorly defined. In this study, SENP1 and SENP2 are shown to have a redundant role that influences NPC biogenesis. Depletion of SENP1/SENP2 was found to result in a nuclear pore assembly defect characterized by mislocalization of nucleoporins normally found at the cytoplasmic fibrils and the central core of the NPC. Interestingly, no assembly defect was observed for components of the nuclear basket. The nuclear basket component Nup153 interacts with both SENP1 and SENP2. Here, we show that this is due to a bimodal interaction that requires discrete regions in both the N- and C-terminal domains of Nup153. In addition, depletion of Nup153 results in a similar nuclear pore assembly defect, suggesting that SENP1/SENP2 and Nup153 work in the same pathway in regulating NPC biogenesis.

2.2 Introduction

The nuclear pore complex (NPC) is a structure embedded in the nuclear envelope that regulates nucleocytoplasmic trafficking of macromolecules. Each NPC is assembled by numerous nucleoporins (Nups) with specialized architectural and trafficking roles. These Nups can be generally categorized as the transmembrane Nups, scaffolding Nups, and peripheral Nups, and are assembled into a membrane-spanning central channel with cytoplasmic fibrils and a basket-like structure protruding into the cytoplasm and nucleoplasm respectively (Tran and Wente, 2006). In higher eukaryotes, the NPC is disassembled in concert with dispersal of the nuclear envelope at mitosis. Both NPCs and the nuclear envelope reform in late anaphase telophase. The assembly of NPCs post-mitosis has been well studied in terms of the sequential recruitment of nucleoporins to the nascent nuclear envelope and the requirement for certain nups in mediating NPC assembly (Antonin et al., 2008). In interphase, new NPCs are continuously assembled into the expanding nuclear envelope; however, the process of interphase NPC assembly remains poorly understood. Recent studies in mammalian cells have highlighted several key regulatory players in pore assembly, including the transmembrane nups Pom121 and Ndc1, the scaffolding Nup133 , the peripheral Nup98, as well as Cdk1/Cdk2 (Antonin et al., 2005; Mansfeld et al., 2006; Stavru et al., 2006; Funakoshi et al., 2007; D'Angelo et al., 2006; Doucet et al., 2010; Wu et al., 2001; Maeshima et al., 2010). Similar requirements of transmembrane/scaffolding/peripheral nups in regulating NPC biogenesis have also been observed in yeast, in which NPCs are continuously assemble into an intact NE as reviewed by (Doucet and Hetzer, 2010).

The NPC has been reported as a localization site for several enzymes of the SUMO modification pathway. The mammalian SUMO E2 conjugating enzyme Ubc9 is localized at both the cytoplasmic fibrils and the nuclear basket structures of the NPC (Zhang et al., 2002). At cytoplasmic fibrils, Ubc9 forms a tight complex with the trafficking factor RanGAP1 and the nucleoporin Nup358/RanBP2 (Matunis et al., 1996; Mahajan et al., 1997; Matunis et al., 1998; Saitoh et al., 1997), which itself is reported to have SUMO E3 ligase activity (Pichler et al., 2002). On the nucleoplasmic side of the NPC, the nuclear basket is a localization site for the SUMO proteases SENP1 and SENP2 (Palancade and Doye, 2008). SENP2 is known to interact with the nuclear basket component Nup153 (Hang and Dasso, 2002; Zhang et al., 2002), while the pore-binding partner of SENP1 has yet to be identified. Such NPC association for SUMO proteases appears to be conserved as similar localization has been reported for the yeast and *Drosophila* SUMO proteases, termed Ulp1 (Li and Hochstrasser, 2003; Smith et al., 2004). In yeast, Ulp1 NPC localization is mediated by the nuclear basket Nup60-Mlp1/2 complex, the core scaffolding Nup84 complex and the transport receptors Kap121, Kap60/Kap95 (Panse et al., 2003; Zhao et al., 2004; Palancade et al., 2007).

Emerging evidence indicates that NPC-associated SUMO proteases are involved in mediating various aspects of NPC function. For example, disruption of the yeast Ulp1 results in a recycling defect of the transport receptor Kap60 and defective import of cNLS cargo (Stade et al., 2002). In addition, disruption of the *Drosophila* Ulp1 results in aberrant cytoplasmic accumulation of SUMO, suggestive of a role in restricting SUMO modification to the nucleus (Smith et al., 2004). Other noncanonical NPC functions, such

as DNA repair and DNA replication, have been found to depend on the NPC connection of yeast Ulp1 (Palancade et al., 2007; Loeillet et al., 2005).

In this study, we have characterized a role for the mammalian NPC-associated SENP1 and SENP2 in mediating NPC biogenesis. This function of SENP1/SENP2 is likely to be coordinated through Nup153. First, Nup153 is found to be a binding partner for SENP1, as previously reported for SENP2 (Zhang et al., 2002). Moreover, downregulation of SENP1/SENP2 or Nup153 leads to similar disruption of NPC biogenesis, indicating that these proteins converge in the same pathway to regulate NPC biogenesis.

2.3 Results

2.3.1 Codepletion of SENP1 and SENP2 perturbs cellular distribution of RanGAP1

Evidence from yeast and *Drosophila* studies has suggested a role of pore-associated SUMO proteases in regulating aspects of nucleocytoplasmic trafficking (Stade et al., 2002; Smith et al., 2004). Using a siRNA knockdown approach, we sought to determine if nucleocytoplasmic trafficking machinery is perturbed when SENP1 and SENP2 are downregulated. As shown in Figure 2.1A, siRNA oligos against SENP1 or SENP2 in Hela cells resulted in significant depletion of SENP1 or SENP2 48 hours post-transfection. An independent set of SENP1/SENP2 oligos was also tested, with similar knockdown efficiency (Figure 2.1A). Indirect immunofluorescence analysis of cells transfected with both sets of SENP1/SENP2 siRNA oligos revealed no gross perturbation in the distribution of the trafficking factors Exportin1, Transportin, RCC1, CAS, Importin α, and Importin β (Figure 2.1B-D). However, depletion of SENP2 resulted in

accumulation of the trafficking factor RanGAPl in cytoplasmic foci, whereas the nuclear basket component TPR was unperturbed (Figure 2.2). Interestingly, such mislocalization of RanGAP1 became more severe when SENP1 and SENP2 were codepleted. In contrast, downregulation of SENP1 alone did not result in a detectable mislocalization of RanGAP1 (Figure 2.2). These observations suggest that SENP1 may have an auxiliary role, along with SENP2, in the regulation of RanGAP1 distribution.

RanGAP1 has been previously reported as the predominant SUMO1 substrate in cells and its SUMO1 modification allows it to form a tight complex with Ubc9 and RanBP2 at cytoplasmic fibrils of the NPC (Matunis et al., 1996; Mahajan et al., 1997; Saitoh et al., 1997; Matunis et al., 1998). We therefore tested whether RanGAP1 is delocalized in cytoplasm in a SUMO1-modified form upon SENP1/SENP2 depletion. As shown in Figure 2.3, cytoplasmic foci accumulation of SUMO1 is detected upon SENP2 depletion or, more so, with SENP1/SENP2 codepletion. Consistent with the idea that SUMO1-modified RanGAP1 is delocalized in the cytoplasm, we were unable to detect any delocalization of SUMO2/3 upon SENP1/SENP2 depletion. In addition, western analysis shows no change on the SUMO status of RanGAP1 upon SENP1/SENP2 depletion (not shown). Depletion of SENP1/SENP2 using an independent set of siRNA oligos similarly resulted in delocalization of endogenous SUMO1 but not SUMO2/3 (Figure 2.3). These observations were independently verified using Hela cell lines that stably expressing either T7-tagged SUMO1 or T7-tagged SUMO3 (Figure 2.4).

2.3.2 Members of the RanGAP1-Ubc9-RanBP2 complex, Nup62, and Nup133 are delocalized upon SENP1/SENP2 codepletion

Since SUMO1-modified RanGAP1 is known to form a tight complex with Ubc9 and RanBP2 at the cytoplasmic fibril of NPC, we next examined whether Ubc9 and RanBP2 are similarly delocalized upon SENP1/SENP2 depletion. As shown in Figure 2.5, both Ubc9 and RanBP2 were detected in cytoplasmic foci upon the depletion of SENP2 or codepletion of SENP1 and SENP2. As revealed by the 414 antibody (which recognizes RanBP2/Nup358, Nup214, Nup153, and Nup62) as well as the RanGAP1 antibody, the mislocalization phenotypes of these proteins occur at high penetrance (Figure 2.6 left/central panels). Similar to the RanGAP1 delocalization phenotype, the Ubc9 and RanBP2 delocalization phenotypes are less severe with single depletion of SENP2 (Figure 2.5, Figure 2.6). To determine if these defects are specific to components of the cytoplasmic fibrils of the NPC, we next examined whether nucleoporins situated at other architectural elements of the NPC are similarly delocalized. Nup62, a symmetrical core peripheral nucleoporin (Figure 2.6 right panel), as well as Nup133, a member of the scaffolding Nup107 complex (Figure 2.14A), are found to colocalize as cytoplasmic foci upon SENP1/SENP2 codepletion. Interestingly, the transmembrane Pom121 (Figure 2.7) and the nuclear basket nucleoporins Nup153 (Figure 2.7) and TPR (Figure 2.2) exhibit normal localization upon SENP1/SENP2 codepletion, implicating regulatory specificity of SENP1 and SENP2 towards the localization of a subset of nucleoporins.

2.3.3 The nuclear basket Nup153 is a novel binding partner and target of SENP1

Nup153 has been reported to interact with SENP2, possibly serving as a SENP2 docking site at the nuclear basket (Zhang et al., 2002). However, the nuclear pore binding partner of SENP1 has not been reported. We therefore first tested whether SENP1 can interact with Nup153. To this end, GFP or GFP-SENP1 fusion protein were individually expressed in Hela cells and then trapped on an affinity matrix for binding analysis. As shown in Figure 2.8A (lane 7), Nup153 is co-recovered with GFP-SENP1. The lack of detectable Nup50 or Nup62 in association with GFP-SENP1 underscores the specificity (Figure 2.8A). The control GFP protein alone does not bind to any of the tested nucleoporins (Figure 2.8A, lane 6).

Bailey et al. previously showed that a population of SENP1 can be detected in the cytoplasm in addition to its predominant nuclear and nuclear rim localization. SENP1 seemed to have exclusive nuclear localization when it is catalytically inactivated, suggesting that the enzymatic activity of SENP1 could play a role in mediating its localization (Bailey and O’Hare, 2004). We therefore examined whether the catalytic activity of SENP1 influences its interaction with Nup153. As shown in Figure 2.8A, both wildtype and the catalytic dead (CD) GFP-SENP1 fusion proteins can interact with endogenous Nup153. However, expression of the catalytic dead SENP1 results in a modified form of Nup153 that interacts significantly more robustly.

A previous study using catalytic dead SENP1 resulted in enrichment of a SUMO target that it regulates (Witty et al., 2010). We therefore considered whether the modified form of Nup153 is SUMOylated Nup153. To verify if Nup153 is indeed a SUMO target

regulated by SENP1 and SENP2, these enzymes were individually depleted or codepleted by siRNA in a Hela cell line that stably expresses T7-tagged SUMO3. Nup153 was then immunoprecipitated from cell lysates and subjected to western analysis using anti-T7 antibody. As shown in Figure 2.8B, downregulation of SENP1 or SENP2 reveals some T7-SUMO3 modification of Nup153. Codepletion of SENP1 and SENP2 synergistically enhances detection of T7-SUMO-modified Nup153, demonstrating that Nup153 is a SUMO target coregulated by SENP1 and SENP2. Similar observation was made when overexpression of catalytic dead SENP2 resulted in the SUMO modification of Nup153 (Figure 2.8C). In this assay, the catalytic dead SENP2 was coexpressed with T7-tagged SUMO1, SUMO2, or SUMO3. Nup153 was then immunoprecipitated from cell lysates and subjected to western analysis using T7 antibody. As shown in Figure 2.8C, Nup153 is detected modified by SUMO2 and SUMO3, highlighting the role of SENP2 towards the SUMO regulation of Nup153.

2.3.4 Nup153 has dual binding platform for SENP1

To map which domains of Nup153 interact with SENP1, we coexpressed GFP-SENP1 fusion protein together with Flag-tagged fusion proteins of the three domains of Nup153. GFP alone was used as a binding control. Western analysis of GFP proteins recovered from cell lysates revealed that SENP1 bind to both the N- and C-terminal domain of Nup153 although binding to the N-terminal domain appeared considerably weaker than interaction with the C-terminal domain (Figure 2.9A). Interestingly, in this binding assay, we also detected a similar interaction between SENP2 and the N-terminal domain of Nup153, along with more robust binding to the C-terminal domain (Figure 2.9A). SENP2 is reported to interact with the Nup153 C-terminal domain, but not its N-

or zinc finger domain (Zhang et al., 2002). A difference in detection sensitivity could explain why SENP2 was not previously found interact with Nup153's N-terminal domain. Consistently, however, we do not detect binding between either SENP1or SENP2 and the zinc finger domain of Nup153 (Figure 2.9B). Collectively, these findings indicate that Nup153 has a dual binding platform for both SENP1 and SENP2.

2.3.5 Characterization of SENP1 binding to Nup153 C-terminal domain

The most prominent feature of the C-terminal domain of Nup153 is its FG rich nature, which is a property shared by several nups and facilitates interactions with transport receptors. Yet within this region, there are distinctions: the proximal end of the C-terminal domain contains FG linkers that are rich in acidic residues when compared with the FG linkers at the distal end (see Figure 2.10B). In addition, a specific binding site for Importin α has been mapped to the terminal residues (Figure 2.10B underlined) (Moroianu et al., 1997). To test the contribution of these features, we engineered various constructs, encompassing the full-length C-terminal domain (875-1475), or its proximal (875-1262) and distal (1263-1475) regions. The Importin α binding site was deleted to create CΔ (875-1457) and C-distalΔ (1263-1457). This panel of GFP fusion proteins was expressed in Hela cells and then recovered and analyzed for SENP1 binding. As shown in Figure 2.10A, the C-terminal domain and its proximal and distal ends bind to endogenous SENP1 and SENP2; however, the interaction of SENP1/SENP2 with the distal region is consistently more robust. Truncation of the specific Importin α binding site from the C-terminal domain or the C-distal region resulted in significant loss of SENP1/SENP2 binding (Figure 2.10B). These observations collectively suggest that Nup153 interaction

with SENP1/SENP2 is bridged by Importin α binding predominantly to a terminal site in the C-terminal domain.

2.3.6 SUMOylation of Nup153's N-terminal domain modulates its interaction with SENP1

As shown in Figure 2.8A, catalytic dead SENP1 or SENP2 is found to interact robust with SUMOylated Nup153, suggesting that cycles of SUMOylation on Nup153 could be a recruiting signal for SENP1/SENP2. To test this possibility, we first mapped which domain of Nup153 contains the SUMOylation site. Coexpression of catalytic dead SENP1 with Nup153's N-terminal domain leads to an accumulation of a modified form of N-terminal domain (Figure 2.11A). In contrast, coexpression with wildtype SENP1 did not result in N-terminal domain modification. Under these assay conditions, the zinc finger and C-terminal domain displayed no sign of being modified (Figure 2.11A). Based on the finding that catalytic dead SENP2 leads to the SUMOylation of Nup153 (Figure 2.8C), we conclude that the N-terminal domain of Nup153 is the SUMOylation site.

To next determine if SUMOylation of the Nup153 N-terminal domain enhances interaction with SENP1, we affinity-captured GFP-SENP1 and examined what species of Flag-tagged N-terminal domain was co-recovered. As shown in Figure 2.11B, both GFP-SENP1 wildtype and catalytic dead fusion proteins can interact with the unmodified Flag-tagged N-terminal domain of Nup153. However, the catalytic dead GFP-SENP1 fusion protein consistently forms a robust association with a modified species of the Nup153 N-terminal domain (Figure 2.11B). A similar observation is seen with respect to the interaction between SENP2 and the Nup153 N-terminal domain. No enhanced interaction is detected in the case of Nup153's C-terminal domain interaction with SENP1 or SENP2

(Figure 2.11B). These observations suggest that the N-terminal domain is SUMOylated and this modification enhances SENP1/SENP2 association. Collectively, both the ability to bind Importin α and be a target of SUMO modification may allow Nup153 to coordinate a bimodal interaction with SENP1 and SENP2.

2.3.7 Nup153 is a specific SUMO target regulated by SENP1 and SENP2

To further examine the specificity of SENP1/SENP2-SUMO regulation towards nucleoporins and NPC-associated proteins, we dominantly interfered with the deSUMOylating activity of endogenous SENP1 or SENP2 by overexpressing catalytic dead versions of SENP1 and tracked the SUMOylation status of a variety of nucleoporins/associated proteins. As shown in Figure 2.12A, Nup153 is robustly SUMOylated when catalytic dead SENP1, but not wildtype SENP1, is overexpressed. However, the nucleoporins TPR, Nup50, Nup358, Nup98, Nup62, and NPC-associated proteins LaminA, LaminB2, and Importin β are not detectably SUMOylated. Similar results are found in cells overexpressing catalytic dead SENP2 (not shown). Overexpression of the catalytic dead version of the nucleoli-specific SUMO protease SENP3, however, did not result in Nup153 SUMOylation (Figure 2.12B). Together, these observations suggest Nup153 is a specific NPC SUMO target regulated by the pore-associated SENP1 and SENP2.

2.3.8 Nup153 regulates NPC biogenesis

Since SENP1 and SENP2 bind to Nup153, it is possible that Nup153 coordinates the SENP1/SENP2 regulatory role in NPC biogenesis by recruiting them to the nuclear pore. We therefore probed for a role of Nup153 in NPC biogenesis. Nup153 has been

previously described to mediate the post-mitotic assembly of the nuclear basket, with moderate depletion of Nup153 resulting in delocalization of the nuclear basket components Nup50 and TPR exclusively in cells undergoing cytokinesis (Mackay et al., 2010). In addition, near-complete depletion of Nup153 has been reported to prevent all recruitment of TPR and Nup50 to the nuclear basket (Hase and Cordes, 2003). We have now uncovered a distinct NPC assembly defect upon moderate depletion of Nup153. As shown in Figure 2.13, downregulation of Nup153 using siRNA resulted in accumulation of RanGAP1 (Figure 2.13A), Ubc9, Nup358/RanBP2, Nup62 (Figure 2.13B), and Nup133 (Figure 2.14A) in cytoplasmic foci in cells. In contrast, the transmembrane nucleoporin Pom121 (Figure 2.14B) and the nuclear basket nucleoporin TPR (Figure 2.13A) do not exhibit signs of delocalization. Moderate downregulation of Nup153 using an independent siRNA oligo also yielded similar phenotypes (Figure 2.15). In contrast, downregulation of other nuclear basket nucleoporins Nup50 (Figure 2.13) or TPR (not shown) did not result in any localization defect of the aforementioned nucleoporins or associated proteins. The cytoplasmic foci phenotypes of select nucleoporins and associated proteins upon moderate downregulation of Nup153 are highly similar, albeit with greater phenotypic severity, to those observed upon the codepletion of SENP1 and SENP2. Coupled with the observation that SENP1/SENP2 interacts with Nup153 and regulates its SUMOylation status, it is possible that SENP1, SENP2, and Nup153 work in the same pathway to regulate NPC biogenesis.

2.4 Discussion

2.4.1 Interphase NPC biogenesis

The major distinguishing feature of interphase NPC biogenesis is that the NPC must assemble into a preexisting double lipid bilayer. Whereas Pom121 is dispensable for the earlier, post-mitotic route of NPC assembly, this transmembrane nucleoporin is implicated in facilitating INM/ONM fusion followed by the recruitment of the Nup107 scaffolding complex during interphase NPC assembly (Doucet et al., 2010). The regulation of the various steps of interphase NPC biogenesis are not well characterized, but import of the transmembrane protein Pom121 to the INM, as well as the curvature sensing domain of Nup133 within the Nup107 complex are crucial for initiating establishment of nascent pores (Doucet et al., 2010; Doucet and Hetzer, 2010). A role of Cdk1/Cdk2 in the interphase NPC biogenesis has also been established but it is unclear how Cdk1/Cdk2 is exerting its influence (Maeshima et al., 2010).

2.4.2 Novel role of Nup153, SENP1, and SENP2 in NPC biogenesis

In this study, we have uncovered the nuclear basket nucleoporin Nup153 and its binding partners SENP1 and SENP2 as additional players in the interphase NPC biogenesis pathway. Downregulation of SENP1/SENP2 or Nup153 leads to accumulation of Nup358, Ubc9, RanGAP1, Nup133, and Nup62 in cytoplasmic foci, while the localization of the Pom121 and the nuclear basket nucleoporins Nup153 and TPR remain normal. This suggests that in the absence of SENP1/SENP2, Pom121 can still be efficiently recruited to the INM/ONM but lack of deSUMOylation activity from Nup153-associated SENP1/SENP2 may block subsequent recruitment of Nup133, Nup62, and the Nup358-Ubc9-RanGAP1complex. A possible model is that some nucleoporins and/or

associated proteins at this stage are SUMO-modified, which could block their premature recruitment to the nascent interphase nuclear pore structure, possibly through interference of protein-protein interactions. Spatially-restricted SENP1/SENP2-mediated deSUMOylation may in turn allow these nucleoporins/associated proteins to establish interaction with the nascent pore structures. Such SUMO-mediated regulatory features of nucleoporins or associated proteins may coordinate precise spatiotemporal regulation of nuclear pore assembly.

2.4.3 Cytoplasmic foci of nucleoporins/associated proteins upon SENP1/SENP2 or Nup153 depletion

The nature of the cytoplasmic structures that host the delocalized population of nups/associated proteins upon SENP/Nup153 depletion remains unclear. Our analysis revealed that some of these proteins colocalize as cytoplasmic aggregates. Upon SENP/Nup153 depletion, the scaffolding nup Nup133 colocalized with the peripheral nup Nup62 in cytoplasmic foci. While no direct interaction between Nup133 and Nup62 has been reported, it is possible that they may indirectly associate through a network of protein-protein interactions among the nucleoporins. Since it is unclear whether certain nucleoporins form subcomplexes in the cytoplasm before being incorporated to the NPC assembly site, it is tantalizing to postulate that the Nup133/Nup62 cytoplasmic foci may reflect an intermediate subcomplex that failed to be incorporated into NPC in the absence of SENP/Nup153. Alternatively, these cytoplasmic foci of nucleoporins could be nucleoporin-enriched membrane structures called annulate lamellae. We did not detect Pom121 in cytoplasmic foci upon the depletion of SENP/Nup153, but the other two

transmembrane nups GP210 and Ndc1 may be present and sufficient to induce the pore-like structures found in annulate lamellae.

2.4.4 Nup153 mediates bimodal interactions with SENP1 and SENP2

We have found that Nup153 provides a dual binding platform for SENP1 and SENP2. The C-terminal domain of Nup153 is likely to coordinate an Importin α-mediated interaction with SENP1/SENP2 while SUMOylation of Nup153's N-terminal domain serves as a second SENP1/SENP2 recruitment signal. Such bimodal interactions between Nup153 and SENP1/SENP2 may allow regulated targeting of SENP1/SENP2 activity towards various proteins that bind to Nup153. The targeting of SENP1/SENP2 activity through Nup153's N- and/or C-terminal domain could be an important event in coordinating regulated deSUMOylation of SUMOylated nucleoporins or associated proteins present during assembly of intermediate structures of the interphase nuclear pore. Through the current and previous studies, Nup153, Nup358, RanGAP1, and Lamin A are the only nucleoporin/associated protein confirmed to be SUMO modified (Pichler et al., 2002; Matunis et al., 1996; Zhang and Sarge, 2008); SUMO proteomic studies have identified other relevant targets such as Nup205, Nup214, TPR, and Importin β (Matafora et al., 2009). While the SUMOylation of these targets has yet to be experimentally verified, it is possible that transient SUMOylation of certain nucleoporin/associated proteins and their subsequent deSUMOylation mediated through the Nup153-SENP1/SENP2 partnership are a part of coordinated assembly of interphase NPC.

2.4.5 Subset of nucleoporins contribute to NPC assembly

The Nup153 depletion phenotype draws striking similarity to a previously characterized feature of Nup98 knockout cells which displayed cytoplasmic accumulation of numerous nucleoporins including the peripheral Nup62 and members of cytoplasmic fibrils, RanBP2, Nup214, and Nup88 (Wu et al., 2001). However, members of the nuclear basket Nup153, Nup50, and a member of the scaffolding Nup107 complex, Nup96, did not show delocalization in Nup98 knockout cells (Wu et al., 2001). These observations suggest that a subset of nucleoporins, such as Nup153 and Nup98, may have fundamental roles in coordinating NPC biogenesis. However, it is unlikely that Nup98 shares the specific mechanistic role of coordinating SENP1/SENP2 activity since Nup98 does not seem to bind to SENP1 (not shown) and SUMO modification of Nup98 was not detected in our experiments (not shown). The reported interaction between Nup98 and both Nup133 and Nup88 (Belgareh et al., 2001; Griffis et al., 2003) suggests that Nup98 could be important in stabilizing the Nup107 complex and members of the cytoplasmic fibrils.

2.4.6 Nup153 coordinates both interphase and mitotic NPC assembly

In this study, a role for Nup153 in regulating NPC assembly has been characterized in relation to its partnership with SENP1/SENP2. Interestingly, Nup153 has recently been found to lead to a distinct phenotype in post-mitotic cells, where downregulation of Nup153 results in accumulation of the nuclear basket components Nup50 and TPR in cytoplasmic foci. This nuclear basket assembly defect has been associated with the activation of an Aurora B-mediated abscission checkpoint and results in abscission delay (Mackay et al., 2010). Here, we consistently observed that downregulation of Nup153 leads to Nup50 and TPR foci exclusively in cells undergoing

cytokinesis (not shown). A more thorough survey found, however, that upon Nup153 depletion, assembly defect of Nup62, RanBP2, RanGAP1, Ubc9, and Nup133 are observed in a large population of cells. Importantly, these latter NPC assembly defects are not restricted to cells undergoing cytokinesis and, indeed, are more prevalent in G1/S cells. The hallmark phenotypes of cytoplasmic mislocalization of nucleoporins/associated proteins upon SENP1/SENP2 or Nup153 depletion share striking similarity with the interphase NPC assembly defects reported when Cdk1/Cdk2 or scaffolding nups are disrupted (Maeshima et al., 2010; Makio et al., 2009).

The nuclear basket Nup50 and TPR appear to have no role in coordinating the pore assembly of Nup62, RanBP2, RanGAP1, and Ubc9 as depletion of TPR or Nup50 did not lead to similar phenotypes. In addition, although the levels are reduced at the nuclear rim, Nup50 and TPR are not detected to be mislocalized to cytoplasmic foci in G1/S cells upon Nup153 depletion. Furthermore, a role of Nup153 in mediating the post-mitotic nuclear basket assembly of Nup50 and TPR appears to be independent of its partnership with SENP1/SENP2 as no cytoplasmic foci accumulation of Nup50 or TPR is detected upon the depletion of SENP1/SENP2. Collectively, these observations suggest that during post-mitotic assembly, Nup153 is critical for rapid assembly of basket assembly. As cells transition from telophase into late cytokinesis/G1, Nup153 coordinates the proper incorporation of Nup62, Nup133, and components of cytoplasmic fibrils into NPCs. The similar phenotypic profiles of SENP1/SENP2 knockdown, as well as their association with Nup153, suggest that these proteins converge in a regulatory mechanism that influences NPC biogenesis.

2.5 Materials and Methods

2.5.1 siRNA depletion of SENP1, SENP2, Nup153, and Nup50

Previously validated siRNA oligos against SENP1 (Li et al., 2008; Yun et al., 2008), SENP2 (Yun et al., 2008; Zhu et al., 2009), Nup153 and Nup50 (Mackay et al., 2009) were transfected into Hela cells (in 10 μM final concentration) for 48 hr using Lipofectamine RNAi MAX as previously described in Mackay et al.

2.5.2 Indirect immunofluorescence analysis

Hela cells post RNAi treatment were rinsed with 1xPBS followed by one of the following fixation methods: for RanGAP1, Ubc9, SUMO1, and SUMO2/3 antibody staining, cells were fixed for 10 min 3% PFA followed by 2min MeOH at -20°C; for antibody against TPR, Nup153, Nup358, or Nup62 antibody staining, cells were fixed 4 min with MeOH at -20°C. Following fixation, cells were rinsed and blocked with 3% FBS in 1xPBS, 0.02% Tx100 for 30 min. Subsequent antibody treatments were performed as described in Mackay et al.

2.5.3 GFP affinity trap and co-recovery analysis

Hela cells were lysed with lysis buffer (1xPBS, 0.25% Tx100, 60mM β-Glycerolphosphate, 10mM Sodium Orthovanadate, 2x COMPLETE protease inhibitor, 40mM N-Ethylmaleimide). Following brief sonication and centrifugation, 200-500 μg of cell lysates were incubated with 10 μL of GFP-Trap agarose beads (Chromotek) for at least 30 min. Beads were later washed 3 times with lysis buffer (without the protease inhibitor/ N-Ethylmaleimide). Proteins were eluted with SDS loading buffer and subjected to western analysis.

2.5.4 Nup153 immunoprecipitation and T7-SUMO3 modification analysis

Hela cells stably expression T7-tagged SUMO3 were treated with SENP1/SENP2 siRNA oligos and lysates were generated as described above. Cell lysates were later incubated with Nup153 antibody (SA1) (Bodoor et al., 1999) preimmobilized on protein-G beads for 4 hr at 4°C. Proteins were eluted with SDS loading buffer and subjected to western analysis.

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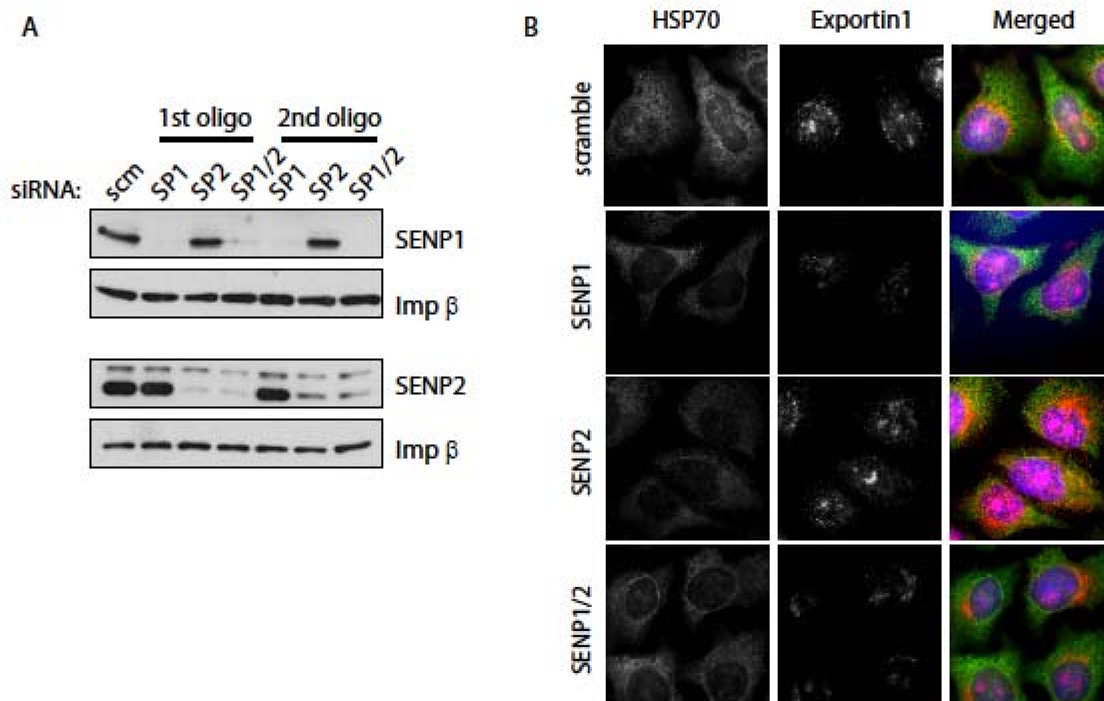
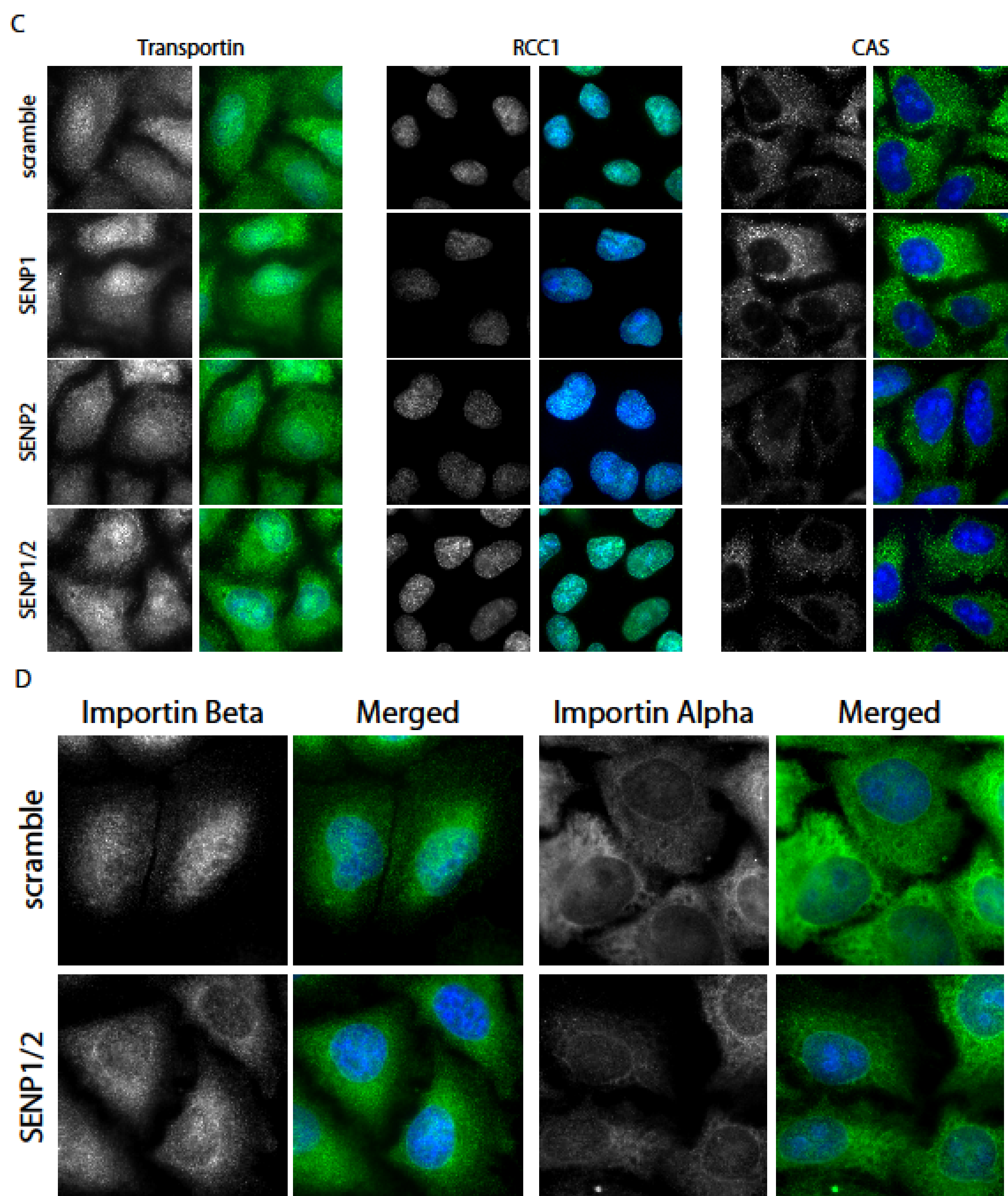


Figure 2.1. Depletion of SENP1/SENP2 does not globally perturb trafficking machinery. (A) Hela cells were treated with two independent sets of siRNA against SENP1, SENP2 or both or scramble oligo (scm) of the first SENP2 oligo for 48hr. Cell lysates were subjected western analysis with antibodies against SENP1 and SENP2. Levels of Importin b were monitored as a loading control. (B) siRNA-treated cells were subjected to indirect immunofluorescence analysis using antibodies against HSP70, Exportin1 (B), Transportin, RCC1, CAS (C), Importin alpha/beta (D)



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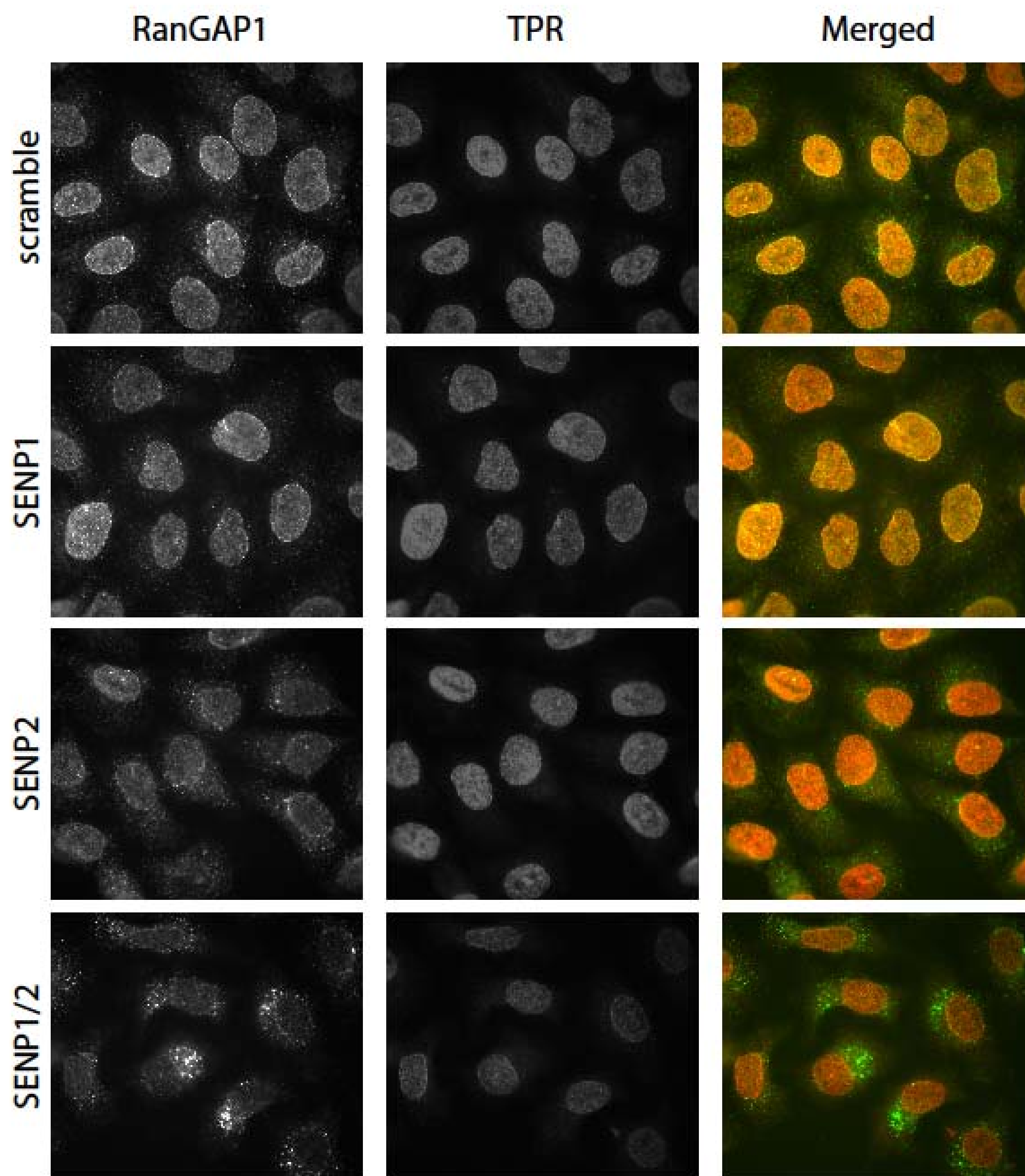


Figure 2.2. Depletion of SENP1/SENP2 perturbs the localization of RanGAP1. Hela cells were treated with siRNA against SENP1, SENP2 or both for 48hr before indirect immunofluorescence analysis using antibodies against RanGAP1 and TPR**.**

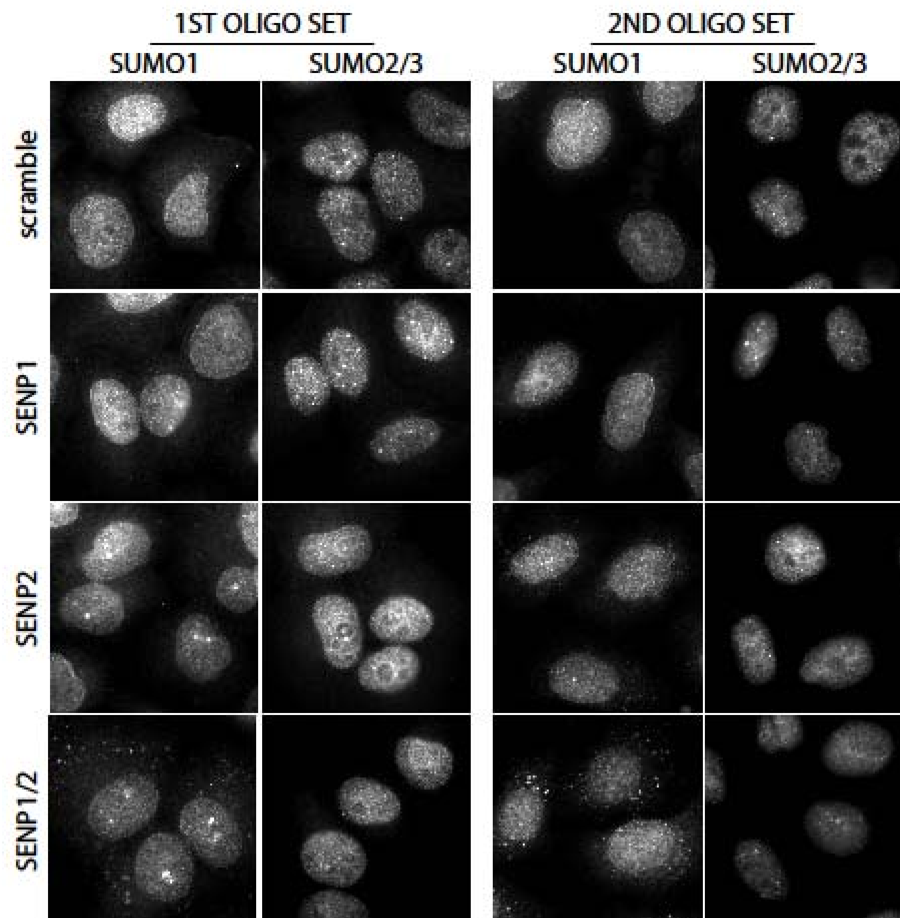


Figure 2.3. Depletion of SENP1/SENP2 perturbs localization of SUMO1. Hela cells were treated with two independent sets of siRNA against SENP1, SENP2 or both for 48hr before indirect immunofluorescence analysis to localize SUMO1 and SUMO2/3.

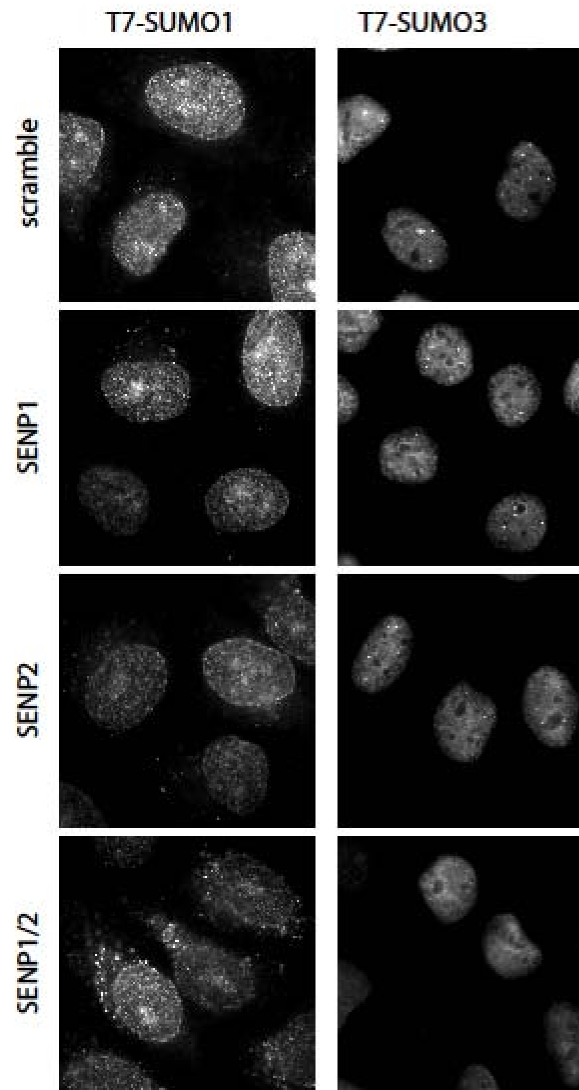


Figure 2.4. Depletion of SENP1/SENP2 perturbs localization of T7-SUMO1. Hela cells that stably expressed T7-tagged SUMO1 or SUMO3 were treated with siRNA against SENP1, SENP2 or both for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against T7.

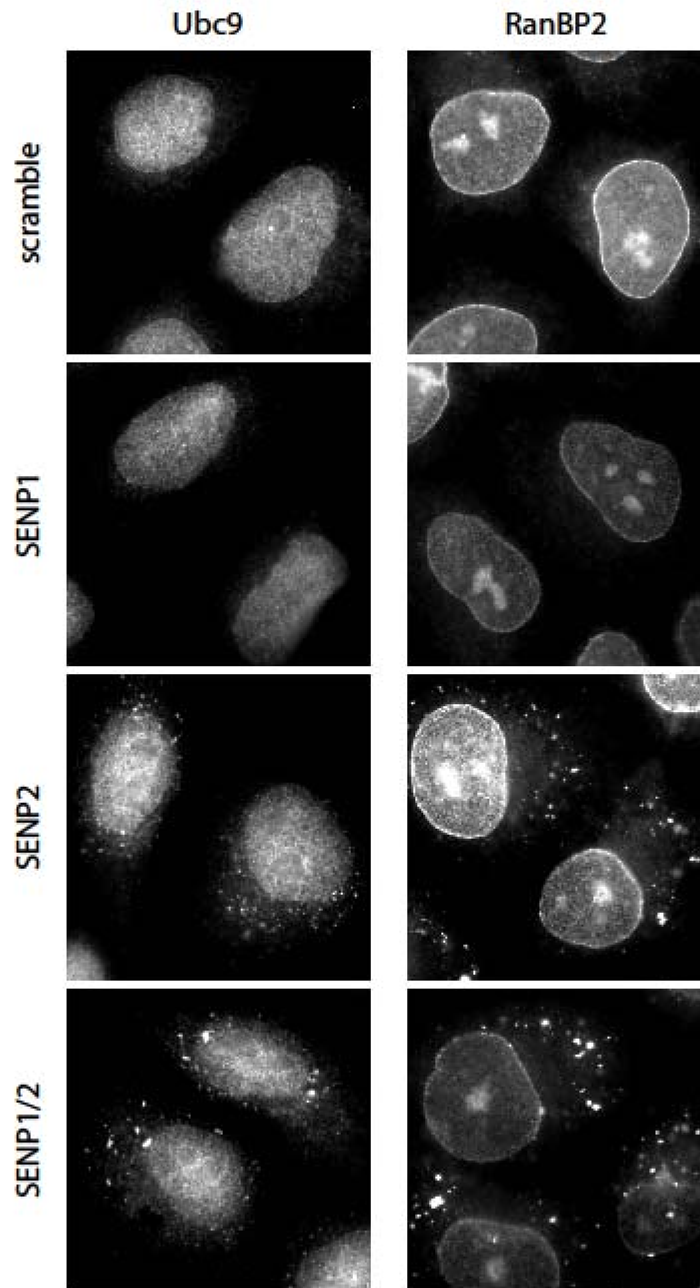


Figure 2.5. Depletion of SENP1/SENP2 perturbs the NPC targeting of Ubc9 and RanBP2. Hela cells were treated with siRNA against SENP1, SENP2 or both for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against Ubc9 and RanBP2.

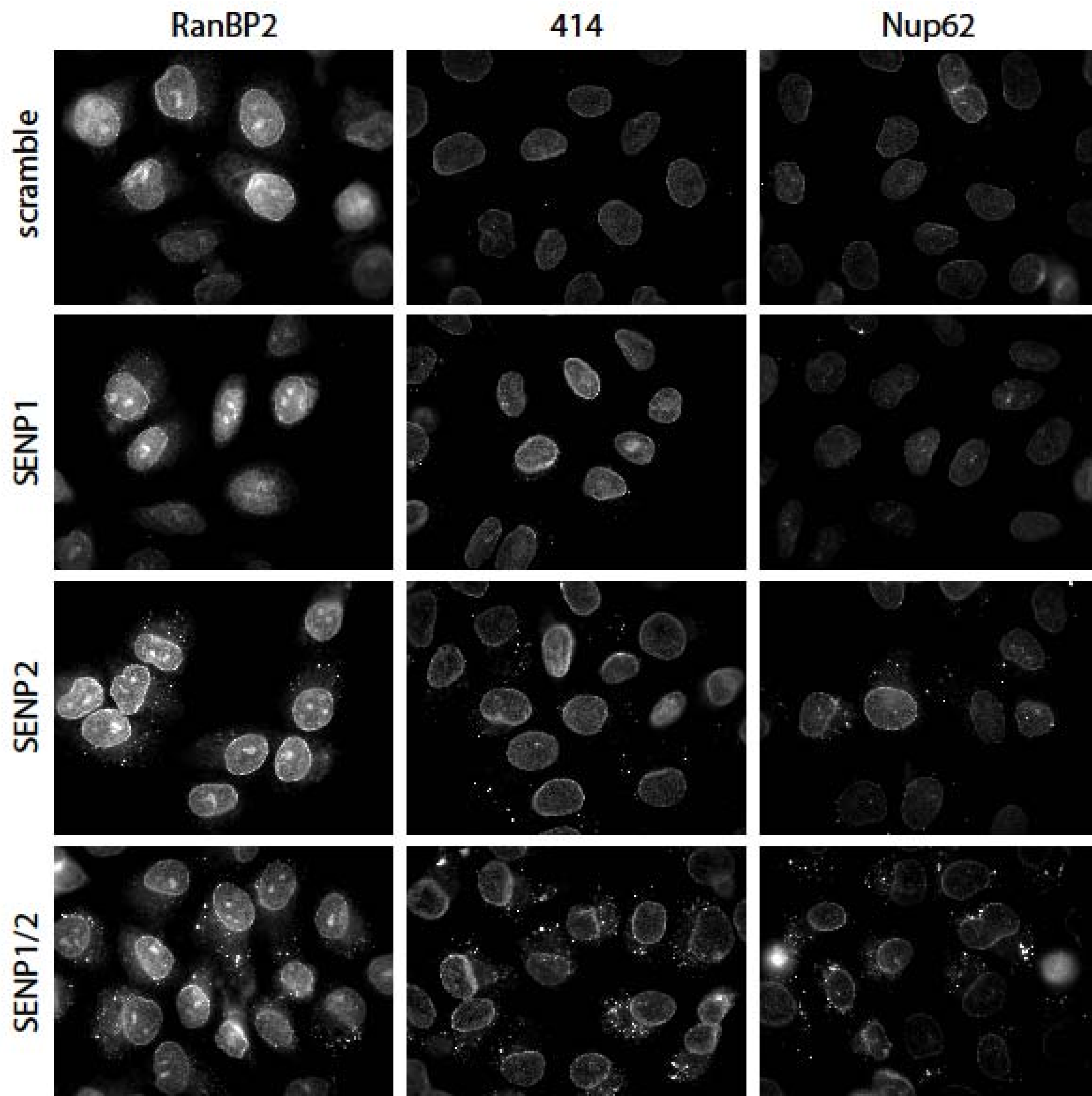


Figure 2.6. Depletion of SENP1/SENP2 perturbs the NPC targeting of RanBP2 and Nup62. Hela cells were treated with siRNA against SENP1, SENP2 or both for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against RanBP2, Nup62, and the 414 antibody (recognizes RanBP2, Nup214, Nup153, and Nup62).

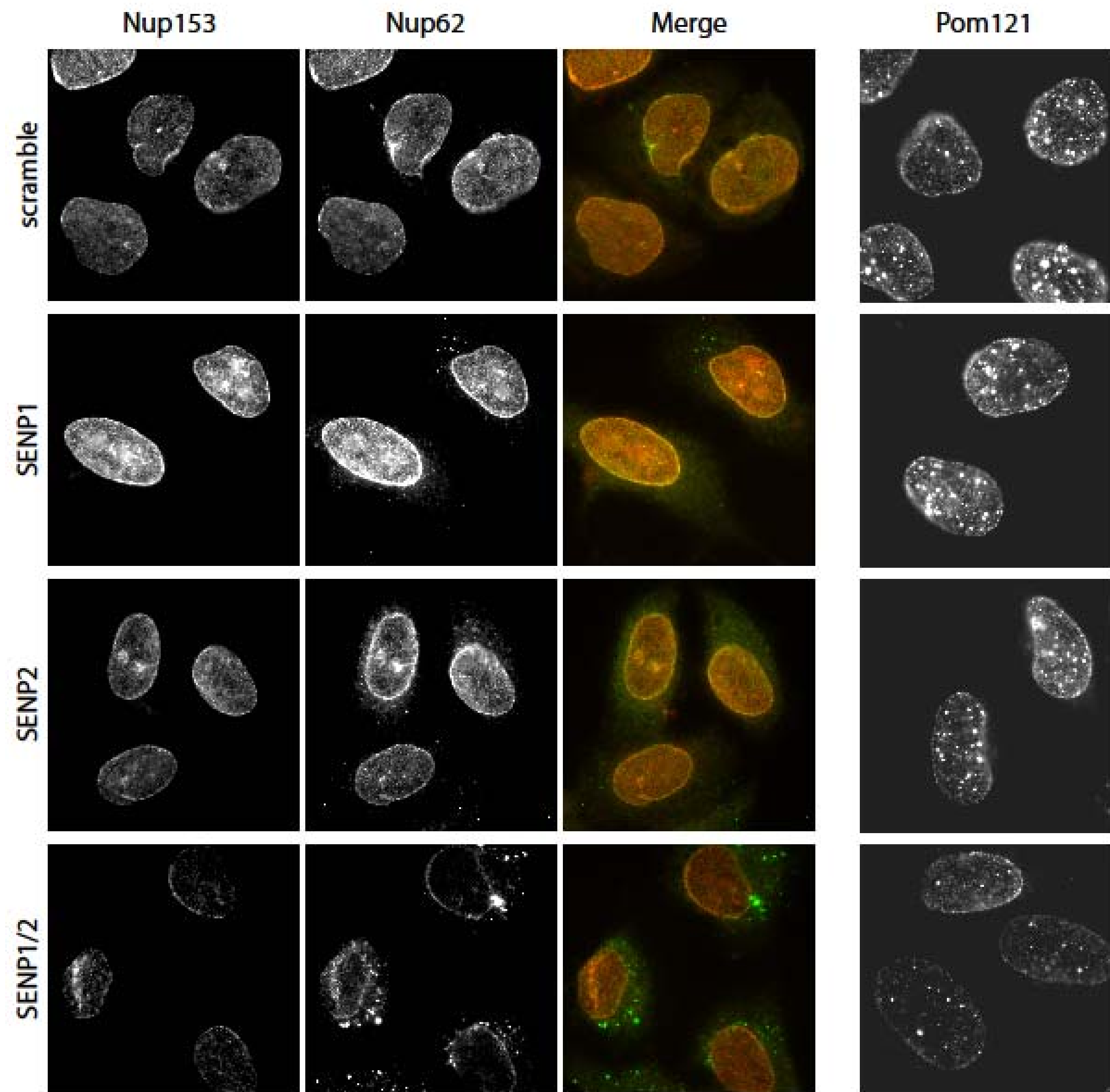


Figure 2.7. Depletion of SENP1/SENP2 perturbs the NPC targeting of Nup62 but not Nup153 and Pom121. Hela cells or Hela stably expressing GFP-Pom121 were treated with siRNA against SENP1, SENP2 or both for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against Nup153, Nup62, and GFP.

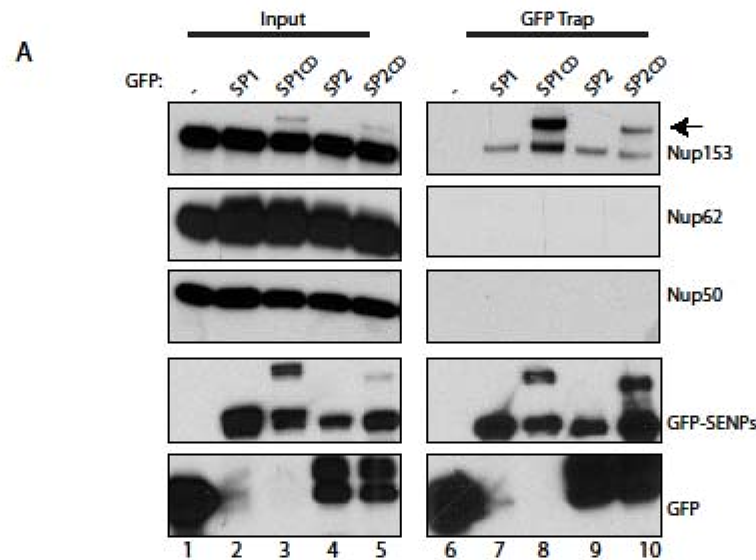
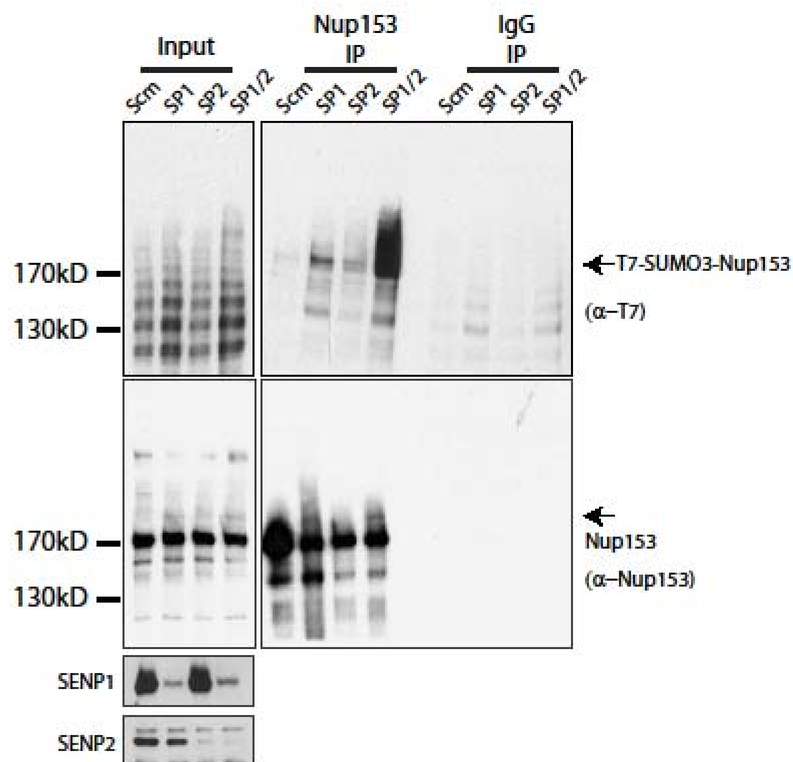


Figure 2.8. SENP1/SENP2 bind to Nup153 and regulate its SUMOylation. (A) GFP (-) or GFP fusion proteins of SENP1 (SP1) and SENP2 (SP2) and their catalytic dead counterparts (CD) were expressed in Hela cells for 24hr before being harvested for GFP recovery followed by western analysis using antibodies against Nup153, Nup50, Nup62, and GFP. (B) A Hela cells that stably express T7-tagged SUMO3 were treated with siRNA against SENP1 (SP1), SENP2 (SP2), or both (SP1/2) for 48hr before being subjected to Nup153 immunoprecipitation followed by western analysis using antibodies against T7, Nup153, SENP1, and SENP2. (C) Flag-tagged fusion protein of catalytic dead SENP2 was coexpressed with either T7-tagged SUMO1 (S1), SUMO2 (S2), or SUMO3 (S3) in Hela cells. After 24hr, Nup153 was immunoprecipitated from cell lysates and subjected to western analysis using antibodies against T7 and Nup153. Arrow indicates SUMOylated Nup153.



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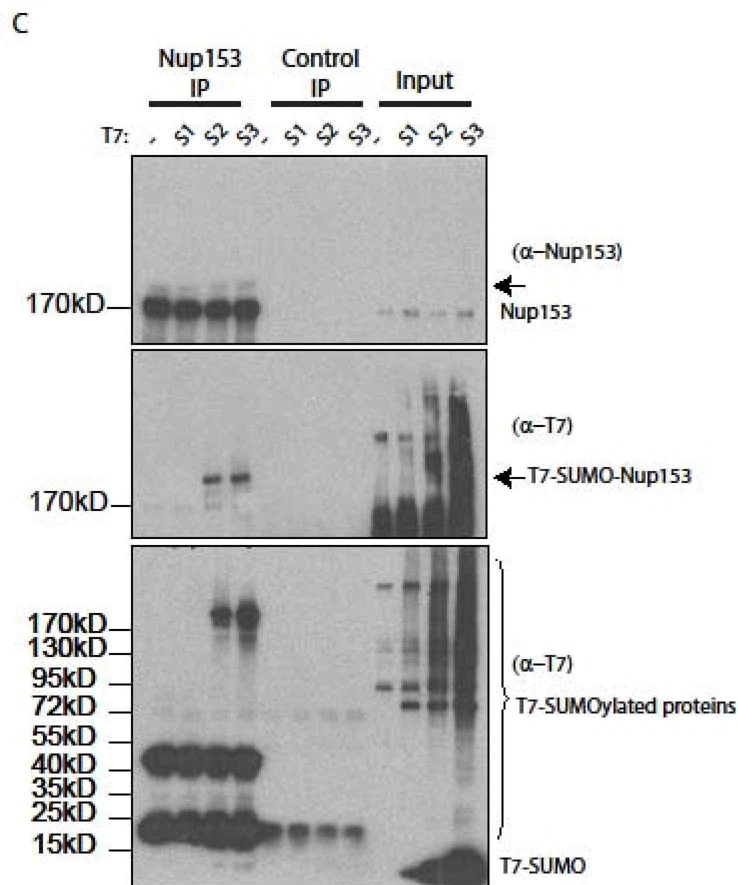

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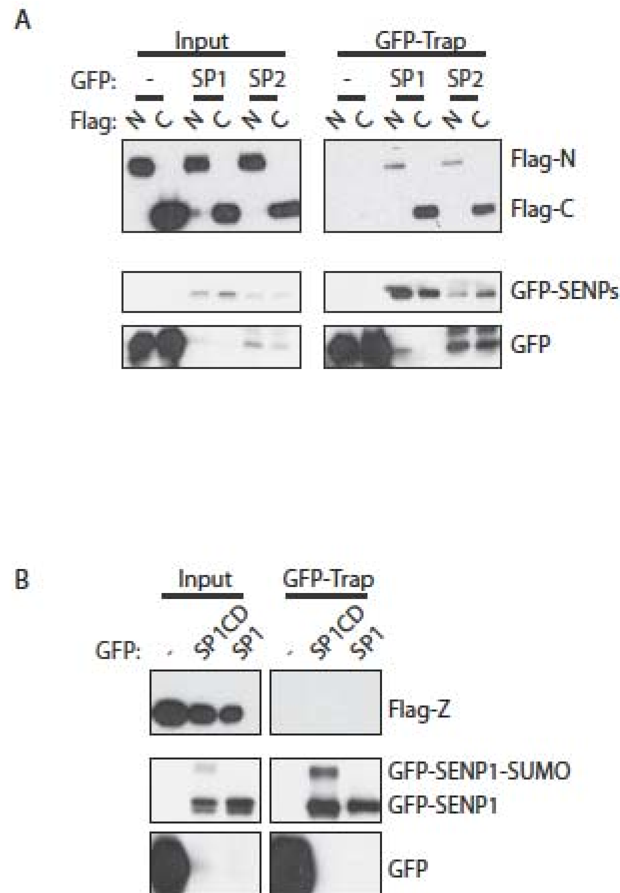

Figure 2.9. Nup153 has a bimodal interaction with SENP1 and SENP2. Flag-tagged fusion proteins of Nup153 N- and C-terminal domain (A) or zinc finger domain (B) were coexpressed with GFP (-) or GFP fusion proteins of SENP1 (SP1) or SENP2 (SP2) in Hela cells. After 24hr, GFP fusion proteins were recovered from cell lysates followed by western analysis using antibodies against Flag and GFP.

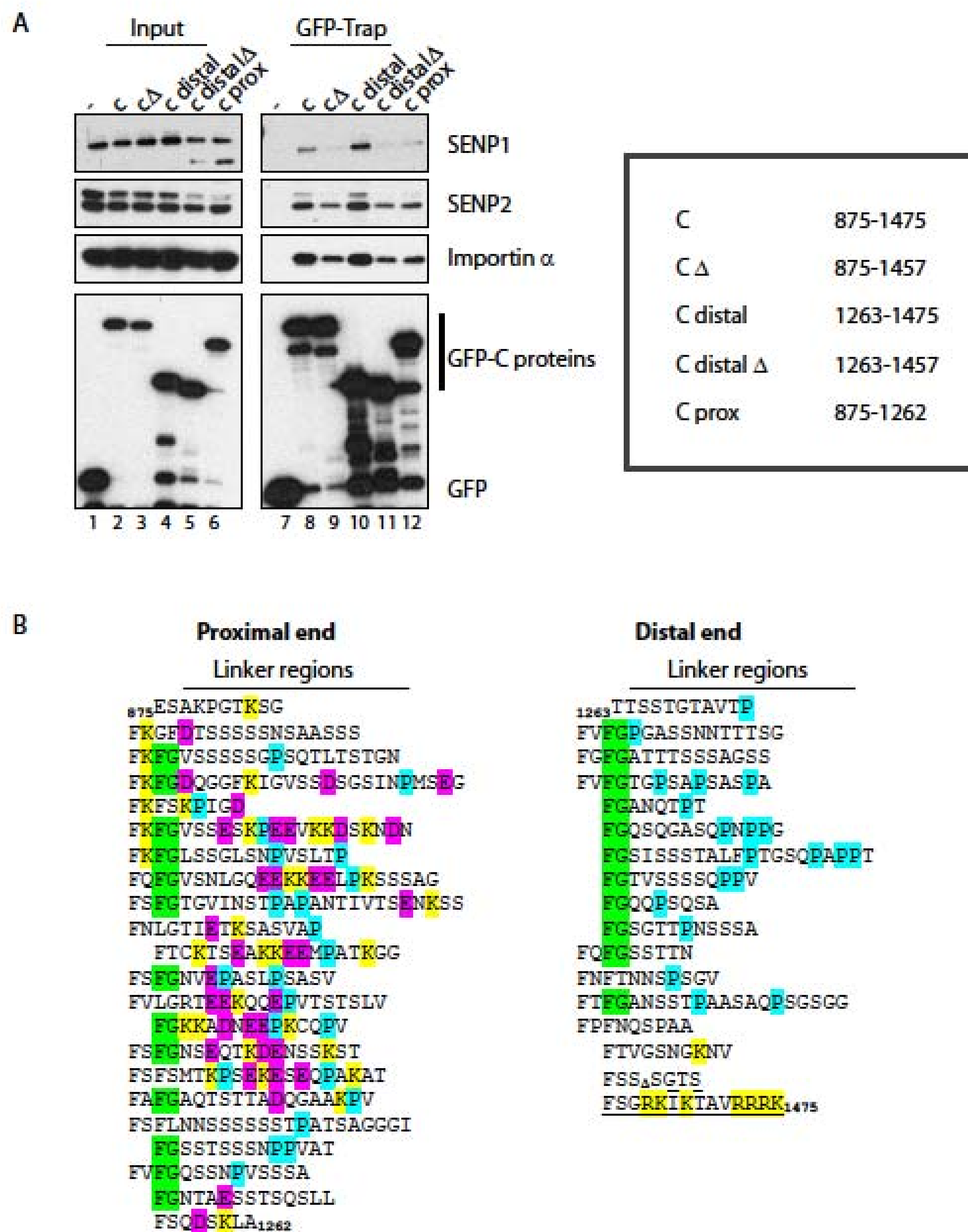

Figure 2.10 Characterization of C-terminal domain interaction with SENP1 and SENP2. (A) GFP (-) or GFP fusion proteins of various C-terminal domain constructs were expressed in Hela cells for 24hr. GFP proteins were then captured, followed by western analysis using antibodies against SENP1, SENP2, Importin a, and GFP. The amino acid localization fo the various C-terminal domain truncations is listed in the box. (B) The sequence of C-terminal domain proximal and distal ends is presented with the FG repeats aligned vertically (green). The basic residues (yellow), acidic residues (purple), and proline residues (blue) are highlighted in the linker regions. The Importin a binding site at the terminal distal end is underlined.

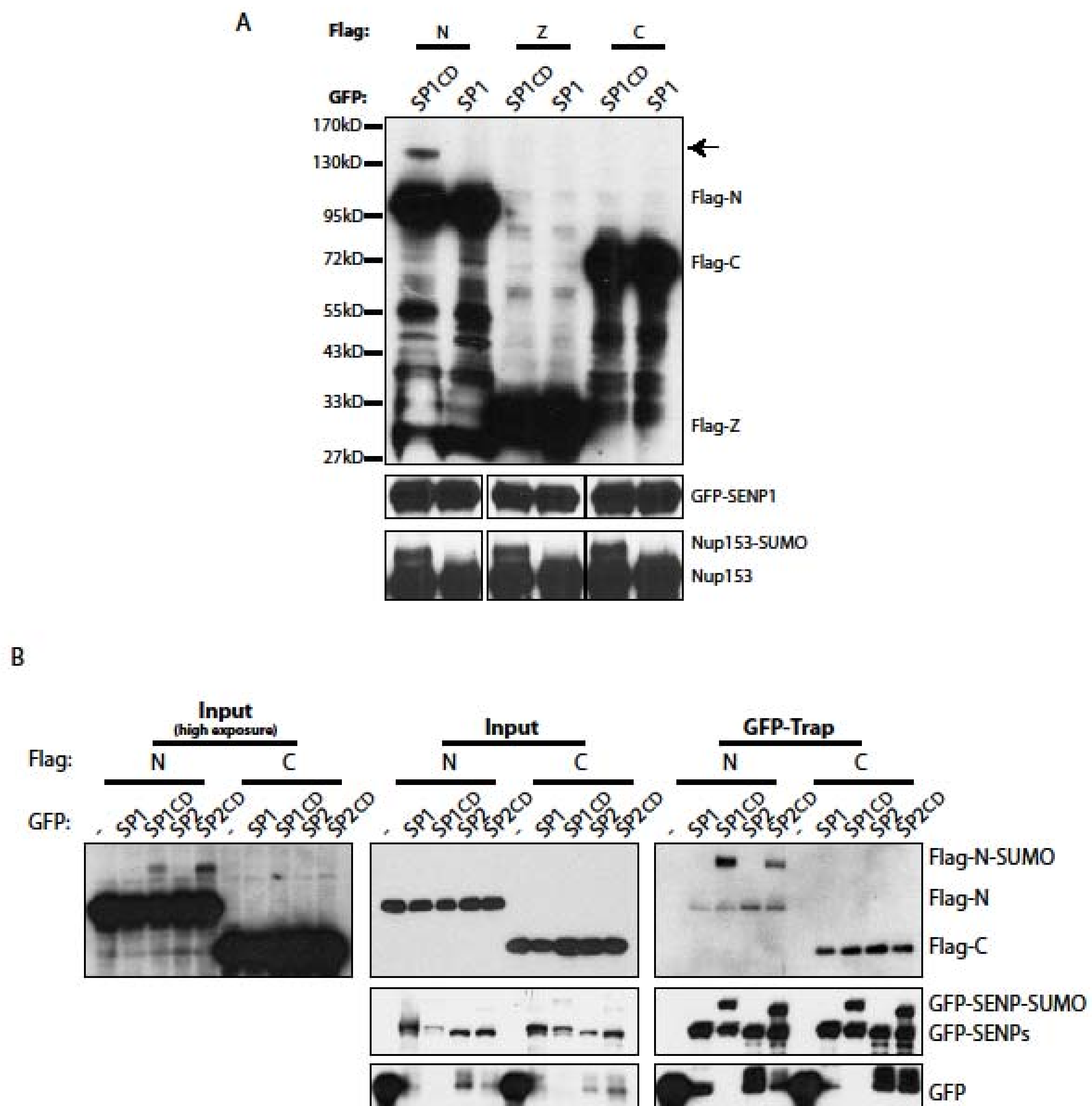

Figure 2.11. The N-terminal domain of Nup153 has a SUMO-mediated interaction with SENP1/SENP2. (A) Flag fusion proteins of N-, zinc finger, and C-terminal domain were coexpressed with GFP fusion proteins of SENP1 (SP1) or catalytic dead SENP1 (SP1 CD) in Hela cells for 24hr. Cell lysates were then subjected to western analysis using antibodies against Flag, GFP, and Nup153. Arrow indicates SUMOylated N-terminal domain. (B) Flag fusion proteins of N- and C-terminal domain were coexpressed with GFP fusion proteins of SENP1 (SP1), SENP2 (SP2) or their catalytic dead counterpart (CD) in Hela cells for 24hr. GFP proteins were then recovered from cell lysates followed by western analysis using antibodies against Flag and GFP.

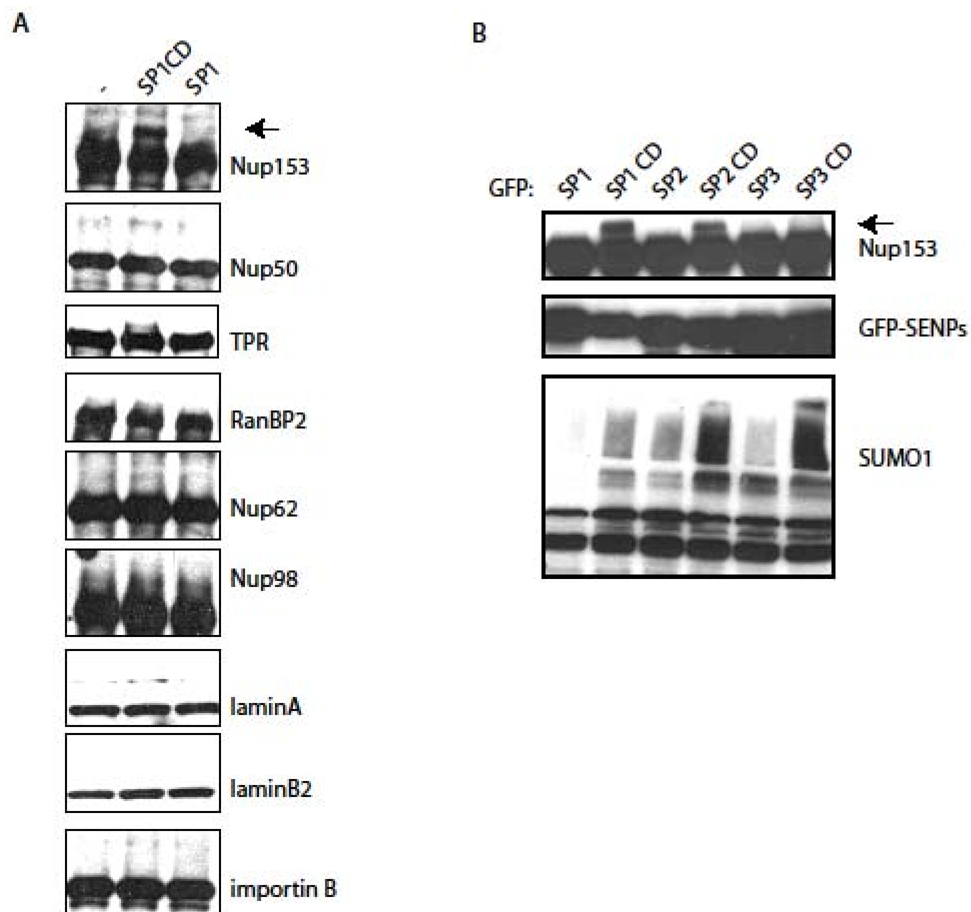

Figure 2.12. Nup153 is a unique target of SENP1 and SENP2. (A) Flag fusion proteins of SENP1 (SP1) or catalytic dead SENP1 (SP1 CD) were expressed in Hela cells for 24hr. Cell lysates were then harvested for western analysis using antibodies against indicated proteins. (B) GFP fusion proteins of SENP1 (SP1), SENP2 (SP2) or SENP3 (SP3), as well as their catalytic dead counterparts (CD) were expressed in Hela cells for 24hr. Cell lysate were then harvested for western analysis using antibodies against Nup153, GFP, and SUMO1. Arrows indicate SUMOylated Nup153.

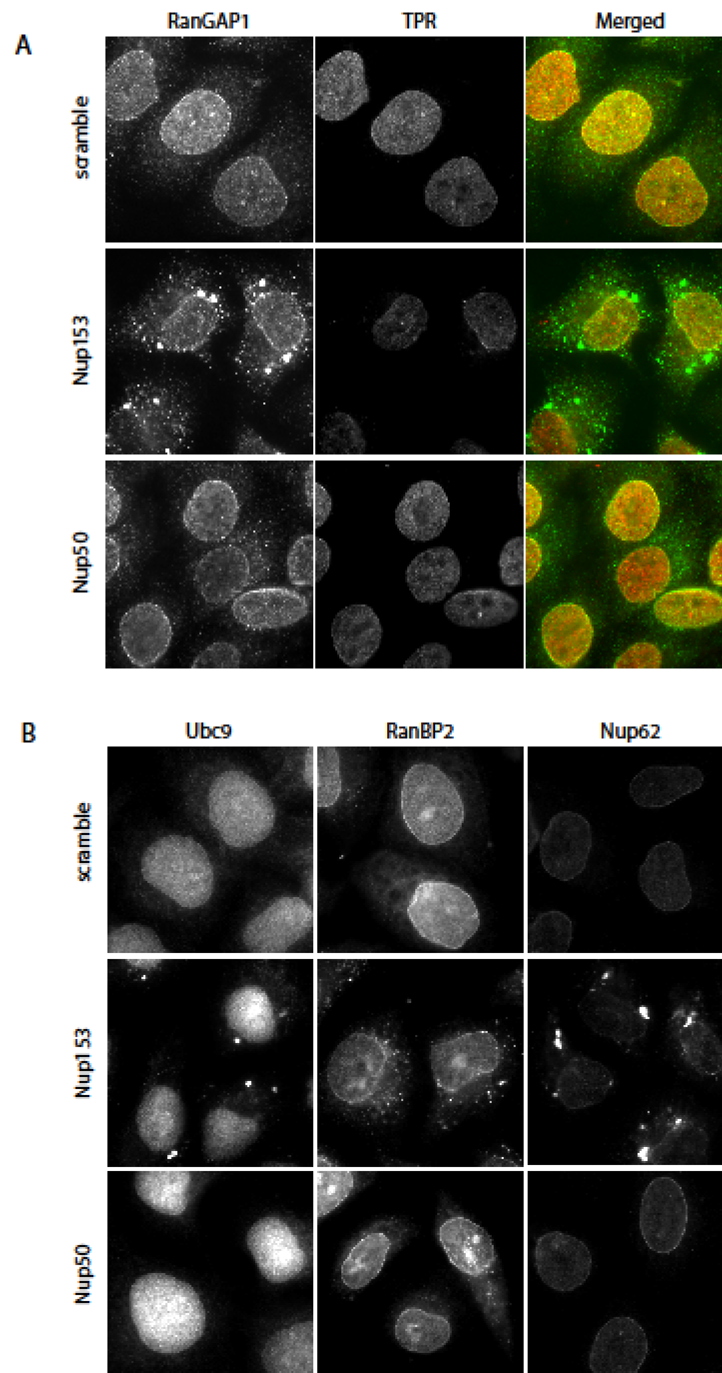

Figure 2.13. Disruption of Nup153 leads to NPC assembly defect. Hela cells were treated with siRNA against Nup153 or Nup50 for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against RanGAP1 and TPR (A), Ubc9, RanBP2and Nup62 (B).

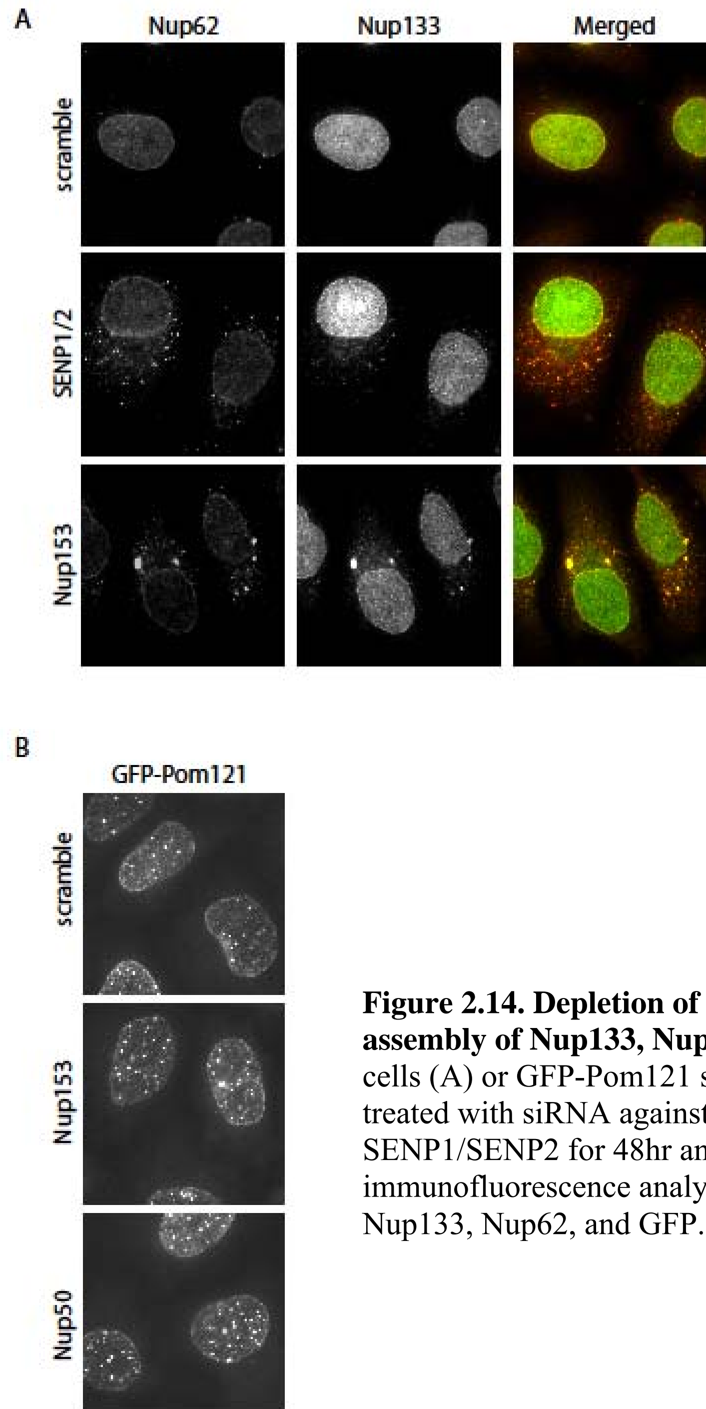

Figure 2.14. Depletion of Nup153 perturbs the assembly of Nup133, Nup62 but not Pom121. Hela cells (A) or GFP-Pom121 stable Hela line (B) were treated with siRNA against Nup153, Nup50, or SENP1/SENP2 for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against Nup133, Nup62, and GFP.

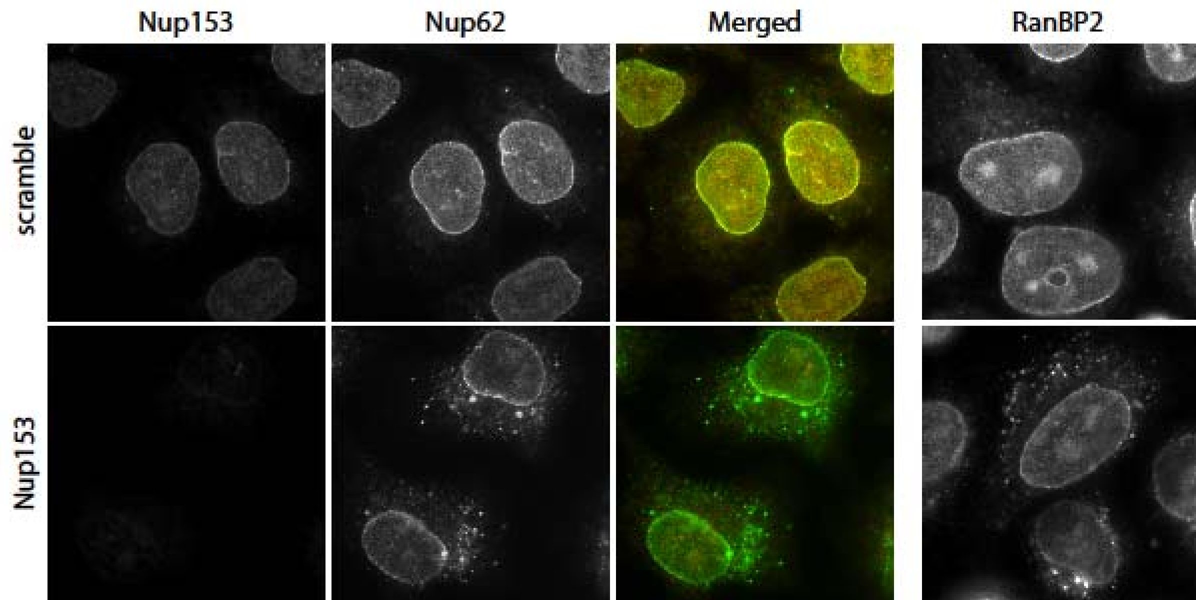

Figure 2.15. Depletion of Nup153 with an independent oligo results in NPC assembly defect. Hela cells were treated with an independent siRNA against Nup153 for 48hr and then subjected to indirect immunofluorescence analysis using antibodies against Nup153, Nup62, and RanBP2.

CHAPTER 3

FURTHER CHARACTERIZATION OF NUP153, SENP1, SENP2 SUMO REGULATION

3.1 Abstract

Nup153 and SENP1/SENP2 have been uncovered in this study to regulate NPC biogenesis; however, the molecular mechanisms behind this regulation pathway remain unclear. Nup153 interacts with and is SUMO regulated by SENP1/SENP2. Here, a SUMOylation site in Nup153 has been mapped to the lysine 353 in its N-terminal domain. Nup153 is found to interact stably with the SUMO E2 conjugating enzyme Ubc9. Such interaction is likely relevant in mediating the SUMOylation of Nup153 and may also be a determinant for Ubc9 localization to the NPC basket. While Nup153 SUMOylation is found to mediate enhanced interaction with SENP1/SENP2, this modification does not appear to affect Nup153 interaction with its binding partner TPR or its localization to the NPC or post-mitotic NPC assembly site. Interestingly, Nup153 and SENP1/SENP2 are found to have a mutual dependency for stability. In addition, proper assembly of the post-mitotic nuclear basket is critical for the recruitment of SENP1. SENP1 and SENP2 are themselves targets of SUMOylation, regulated by their own catalytic activities. The

SUMOylation site of SENP2 is mapped to lysine 48 at its N-terminal domain, where SUMOylation may regulate nuclear localization. SENP1/SENP2 specificities in SUMO paralog targeting and their role in cell cycle progression are also further characterized in this study.

3.2 Characterization of Nup153 SUMOylation

3.2.1 Nup153 SUMOylation is cell cycle specific

Nup153 has been reported to play roles throughout the cell cycle. In interphase, Nup153 is known to mediate nucleocytoplasmic trafficking through its interaction with a variety of trafficking receptors. Nup153 has also been reported in *Drosophila* to play a role in dosage compensation and transcriptional regulation (Mendjan et al., 2006; Vaquerizas et al., 2010). In addition, Nup153 has been described to play mitotic roles. Using nuclei assembled from *Xenopus* egg extract, Nup153 was shown to facilitate mitotic disassembly of the nuclear envelope, through a role in recruiting the COPI coatomer complex (Liu et al., 2003; Prunuske et al., 2006). Depletion of Nup153 in Hela cells was found to result in prolonged mitotic progression with delayed cytokinesis and multinucleated cells (Mackay et al., 2009). A link between the role for Nup153 in NPC basket assembly and an Aurora B-mediated abscission checkpoint was later established (Mackay et al., 2010). Collectively, these observations highlight the multifunctional nature of Nup153 throughout the cell cycle. A major question is that of how Nup153 transitions between its various functions.

Posttranslational modification is likely to contribute to the switch to certain roles. For instance, phosphorylation at mitosis may alter Nup-Nup interactions and downregulate Nup153's role in the architecture of the NPC basket. SUMOylation is

another posttranslational modification that is a good candidate for modulating Nup153 function. To probe for a functional role of Nup153 SUMOylation, we sought to determine the window during the cell cycle when Nup153 is SUMOylated. To this end, Hela cells that stably express T7-tagged SUMO3 were arrested at metaphase using nocodazole. Metaphase-arrested cells were harvested and released into fresh media. Cell lysates were generated from these metaphase-arrested cells, cells 3 hr post-nocodazole release, or asynchronized cells. Nup153 was then immunoprecipitated and subjected to western analysis using anti-T7 antibody to determine Nup153 SUMOylation status. As shown in Figure 3.1, Nup153 SUMOylation is detected in asynchronous cells as well as cells 3 hr post-nocodazole release. In contrast, no SUMOylation of Nup153 is detected in metaphase-arrested cells, suggesting Nup153 SUMOylation is likely to function in post-mitotic or interphase events and is prevented at the time of mitosis itself.

3.2.2 The nuclear pore SUMO E3 ligase RanBP2/Nup358 does not mediate Nup153 SUMOylation

A constituent of the NPC cytoplasmic fibrils, RanBP2/Nup358, has been reported to have SUMO E3 ligase activity (Pichler et al., 2002), raising the possibility that RanBP2 may SUMOylate components of the NPC, including Nup153. To test this possibility, a siRNA against RanBP2 was used to deplete RanBP2 and a catalytic dead SENP1 construct was overexpressed to interfere with endogenous SENP activities. As shown in Figure 3.2, dominant interference of endogenous SENP activities by the catalytic dead SENP1 reveals the SUMOylation of Nup153 (Figure 3.2, lane 4-6). Treatment of RanBP2 siRNA efficiently downregulated the level of RanBP2. Yet,

concurrent depletion of RanBP2 failed to reduce levels of Nup153 SUMOylation, indicating that the SUMO E3 ligase RanBP2 is not required for Nup153 SUMOylation.

3.2.3 Nup153 interacts with the SUMO E2 conjugating enzyme Ubc9

Ubc9 has been reported localized to both the cytoplasmic fibrils and the nuclear basket of the NPC by immuno-electronmicroscopy (Zhang et al., 2002). Ubc9 is known to form a complex with RanGAP1 and RanBP2 at the NPC cytoplasmic fibrils; however, the binding partner of Ubc9 at the NPC nuclear basket is still uncharacterized. With the finding that Nup153 is a SUMO target, it is likely that Nup153 provides at least a transient binding platform for Ubc9. To test this hypothesis, GFP or a GFP fusion protein of Ubc9 was expressed in Hela cells and GFP proteins were isolated on an affinity matrix. As expected, RanBP2 was recovered with GFP-Ubc9 (Figure 3.3). Interestingly, the nuclear basket Nup153 but not another basket component TPR, was also found in association with Ubc9 (Figure 3.3), suggesting that Nup153 may serve as a binding platform for Ubc9 at the NPC nuclear basket.

3.2.4 Perturbation of Nup153 deregulates SUMOylation

Since Nup153 can interact with SENP1, SENP2, and Ubc9, it is possible that Nup153 functions as a SUMO enzyme scaffold to regulate the SUMOylation / deSUMOylation of specific target(s) at the NPC. To test this possibility, Nup153 was depleted through siRNA treatment and overall SUMOylation activity was probed by western analysis using SUMO1 and SUMO2/3 antibodies. While the pattern of targets was largely unaffected, depletion of Nup153 but not RanBP2 led to the accumulation of a specific SUMO1 band migrating at around 140kD (Figure 3.4). Interestingly, depletion of

another nuclear basket component, Nup50, resulted in the accumulation of a similar SUMO1 band (Figure 3.4) which persisted when the SUMO E3 ligase RanBP2 was codepleted (Figure 3.4 lanes 5, 6). While Nup153 is related to the SUMO pathway through its interaction with SENP1, SENP2, and Ubc9, it is unclear how Nup50 exerts a regulatory role on the SUMO modification pathway. No significant perturbation of SUMO2/3 modification was observed under these experimental conditions (not shown).

3.2.5 Lysine 353 in the unique Nup153 N-terminal domain is a SUMOylation site

The observation that SUMOylated Nup153 can associate robustly with catalytic dead SENP1/SENP2 (see Chapter 2) suggests that SUMO modification of Nup153 may be a recruitment signal for interaction with SENP1/SENP2. SENP2 has been previously shown to interact with the C-terminal domain of Nup153. Both the N- and C-terminal domains of Nup153 harbor a predicted SUMO consensus site, specifically amino acids 352-355 and amino acids 1070-1073, but SUMOylation is detected only in the N-terminal domain of Nup153 (see Chapter 2). Mutating lysine 353 to arginine (K353R) within the predicted SUMO consensus site of Flag fusion protein of Nup153 N-terminal domain abolished its SUMOylation when coexpressed with catalytic dead SENP1 (Figure 3.5A). A similar pattern was observed with Flag-tagged fusion proteins of full-length Nup153 (Figure 3.5B) or Nup153 devoid of its C-terminal domain (Figure 3.5A), indicating that lysine 353 of Nup153 is a *bone fide* SUMOylation site and is the major site of SUMOylation in Nup153.

3.2.6 SUMOylation on lysine 353 modulates Nup153 interaction with SENP1/SENP2

To confirm that the SUMO-modified Nup153 N-terminal domain has an enhanced interaction with SENP1, we tested the sensitivity of this interaction to mutation of lysine 353. As shown in Figure 3.6A, a GFP fusion protein of catalytic dead SENP1 robustly co-isolates with the SUMOylated HA-tagged Nup153 N-terminal domain; however, the K353R mutation abrogates the SUMOylation of N-terminal domain and the enhanced interaction with SENP1. To further verify a role of lysine 353 in mediating Nup153 interaction with SENP1, GFP fusion proteins of Nup153 N-terminal domain (GFP-N), either wildtype or harboring a K353R mutation, were tested for ability to associate with Flag-tagged catalytic dead SENP1. As shown in Figure 3.6B, western analysis of the isolated GFP proteins with anti-Flag antibody revealed association between GFP-N and the catalytic dead Flag-SENP1. However, K353R mutation of GFP-N drastically reduces its interaction with catalytic dead Flag-SENP1. Collectively, these observations suggest that while SENP1/SENP2 can interact with both the N- and the C-terminal domain of Nup153, lysine 353 SUMOylation may provide an additional signal for dynamic recruitment of SENP1/SENP2 to the N-terminal domain of Nup153. Moreover, the results of Figure 3.6B, in which SENP1 does not bind to Nup153 N-terminal domain when SENP1 itself is modified, underscores another potential mechanism for regulating this interaction.

3.2.7 Nup153 SUMOylation does not modulate its interaction with TPR

The Nup153 N-terminal domain has been previously reported to have interactions with numerous proteins, as well as RNA, at regions close to lysine 353 (Ball and Ullman,

2005). Thus, one hypothesis is that SUMOylation of Nup153 modulates its repertoire of partners. To test this possibility, we determined whether the absence of Nup153 SUMOylation affects its binding with TPR, a nucleoporin previously reported to bind to the N-terminal domain of Nup153. As shown in Figure 3.7A, a GFP fusion protein of the Nup153 N-terminal domain harboring the K353R mutation still interacts with TPR, suggesting that SUMOylation is not required for TPR interaction. However, this does not rule out the possibility that Nup153 SUMOylation can block the binding of TPR. Since SUMOylation is a highly dynamic process *in vivo*, it is difficult to recover sufficient SUMOylated Nup153 for a binding assay with TPR. To circumvent this problem, a bacterial SUMOylation system (Saitoh et al., 2009) was employed to generate SUMOylated Nup153. To this end, a minimal fragment of Nup153 N-terminal domain (amino acid 245-400) that is sufficient to bind to TPR was coexpressed in *E. coli* as a GST fusion protein (GST-N) together with SUMO E1 activation enzymes, SUMO E2 conjugation enzyme, and SUMO1. As shown in Figure 3.7B, more than 50% of the affinity purified GST-N is SUMOylated in this system. In contrast, the GST fusion of Nup153 zinc finger domain (GST-Z) is not SUMOylated in this system. In addition, mutation of K353R within GST-N completely abolishes SUMOylation in this system, indicating it is specific to the *bone fide* SUMO site. To test whether SUMOylated GST-N can still interact with TPR, Hela cell lysate was incubated with these affinity purified GST fusion proteins and TPR then immunoprecipitated. As shown in Figure 3.7C (lanes 4, 5), both SUMOylated and unSUMOylated GST-N similarly recover with TPR, indicating SUMOylation does not block the interaction between GST-N and TPR.

3.2.8 Nup153 K353R localizes properly to the interphase nuclear envelope and to post-mitotic telophase NPC assembly sites

Previous studies have mapped the nuclear envelope and nuclear pore targeting motifs of Nup153 to its N-terminal domain, at aa 2-144 and aa 39-339 respectively (Bastos et al., 1996; Enarson et al., 1998). To determine if SUMOylation of Nup153 regulates pore targeting, we examined the nonSUMOylatable mutant of Nup153 N-terminal domain for its intracellular localization. A GFP fusion protein of Nup153 N-terminal domain (GFP-N) expressed in Hela cells localizes to nuclear envelope in interphase cells (not shown) and appears at post-mitotic pore assembly site (Figure 3.8). Mutating lysine 353 does not lead to any significant perturbation in localization (Figure 3.8), suggesting that Nup153 SUMOylation may not be required for pore targeting of Nup153.

3.3 Characterization of SENP1 and SENP2 SUMO regulation

3.3.1 SENP1 and SENP2 differ in affinity toward SUMO targets

SENP1 and SENP2 are the only reported SUMO proteases to localize at the vertebrate NPC. Studies from animal models and tissue culture systems have highlighted nonredundant functional roles of SENP1 and SENP2. To further understand if SENP1 and SENP2 differ in their SUMO regulation of potential NPC targets, the binding affinities of SENP1 and SENP2 towards various SUMO paralogs were characterized. The C602S and C549S mutations on SENP1 and SENP2 can, respectively, inactivate their deSUMOylation activities (catalytic dead) but do not block their interactions with SUMO targets. These mutants are therefore suitable for characterizing the SUMO paralog targeting specificity of SENP1 and SENP2. To this end, GFP fusion proteins of wildtype

and catalytic dead mutants of SENP1 and SENP2 were expressed in Hela cells. GFP fusion proteins along with associated proteins were recovered from cell lysates and subjected to western analysis using SUMO1 or SUMO2/3 antibodies. As shown in Figure 3.9 (left panel), SENP1CD interacts robustly with both SUMO1 and SUMO2/3-modified proteins, consistent with the ability of SENP1 WT to remove both SUMO1 and SUMO2/3 (see input lanes). While SENP2 CD displayed similar strong affinity towards SUMO2/3-modified proteins, it shows only weak interaction with SUMO1 targets. Consistent with preferred targeting SUMO2/3, SENP2 WT strongly deSUMOylates SUMO2/3-modified proteins and exerts weak deSUMOylating activity towards SUMO1 modification. The differences of SUMO paralog specificity of SENP1 and SENP2 is further exemplified by examining the SENP1/SENP2 targeting of a major SUMO1 target, RanGAP1. As shown in Figure 3.9 (right bottom panel), SENP1, but not SENP2, can bind and deSUMOylate SUMO1-modified RanGAP1. Collectively, these data indicate that while SENP1 and SENP2 can localize to the NPC (possibly through Nup153), they differentially discriminate among SUMO substrates.

3.3.2 SENP1 and SENP2 are both targets of SUMO modification

SENP1 and SENP2 share a similar C-terminal catalytic domain but differ within their N-terminal domains, which likely contribute to their targeting specificity towards various SUMO substrates. It is reported that SENP2 is expressed in various isoforms that include truncation of its N-terminal domain (Nishida et al., 2001; Best et al., 2002; Kadoya et al., 2000; Jiang et al., 2010). These SENP2 isoforms display changes in localization and substrate targeting. No isoform variation has been reported for SENP1, but SENP1 has been reported to be SUMO modified when its catalytic activity is

inactivated (Bailey and O'Hare, 2004), suggesting that its deSUMOylating activity may regulate its own SUMO modification status. The SUMO modification site however has never been mapped and the functional role of this SUMO modification is not known. Nonetheless, SENP1 SUMOylation offers a possible regulatory mechanism for its function. We therefore sought to determine whether the NPC-associated SUMO proteases SENP1 and SENP2 are uniquely regulated by SUMO modification. To this end, catalytic dead mutants of SENP1, SENP2, and a nucleoli-localized SENP3, as well as their wildtype counterparts, were expressed as GFP fusion proteins in Hela cells and subjected to western analysis using a GFP specific antibody. As shown in Figure 3.10A, both the catalytic dead SENP1 and SENP2 GFP fusion proteins display protein mobility shifts indicative of possible SUMOylation. In contrast, the nucleoli-associated SENP3 showed no sign of being post-translationally modified. To verify that the shifted species of catalytic dead SENP1 and SENP2 are SUMO-modified species, these GFP fusion proteins were coexpressed with T7-tagged SUMO1 or SUMO2 and recovered from cell lysates. The samples were then probed for SUMO by western analysis using T7 antibody. As shown in Figure 3.10B, catalytic dead SENP1 is found to be modified by both SUMO1 and SUMO2; however, catalytic SENP2 appeared to be modified by SUMO2 alone, further underscoring the SUMO paralog specificity of SENP1 and SENP2, and confirming that NPC-associated SUMO proteases are targets of SUMO modification.

3.3.3 Characterization of SENP2 SUMOylation

To further probe for a functional role of SENP2 SUMOylation, we sought to map the SUMOylation site of SENP2. As shown in Figure 3.11A, mutation of lysine 48 to arginine (K48R) can significantly block the SUMOylation of catalytic dead SENP2.

Interestingly, K48R mutant of SENP2 also displayed lack of stability and localized to the cytoplasm (Figure 3.11B). The cytoplasmic mislocalization of K48R SENP2 mutant suggests that SUMOylation at lysine 48 may be important for nuclear localization of SENP2. A previous study indicated that forced cytoplasmic localization of SENP2 can lead to its ubiquitylation and degradation (Itahana et al., 2006). The instability of K48R SENP2 mutant may be indicative of a cytoplasmic ubiquitin-mediated degradation. Further characterization is required to determine how lysine 48 SUMOylation regulates SENP2 nuclear localization. This study as well as a previous study has attempted to map the SUMOylation site of SENP1. Mutating lysine 568 at the predicted consensus site (Bailey and O'Hare, 2004), as well as lysines at nonSUMO consensus sites (lysines 574, 575, 577, 589, 590, 616, 632) did not abrogate SENP1 SUMOylation (not shown). Further mutagenesis analysis at other nonSUMO consensus sites is required for map the SUMOylation site of SENP1.

3.3.4 A role of SENP2 in metaphase progression

Zhang et al. reported that overexpression of SENP2 can lead to prometaphase arrest, possibly due to its regulatory activity towards the targeting of factors to kinetochore (Zhang et al., 2008). When probing for a functional difference between SENP1 and SENP2, we discovered that siRNA depletion of SENP2 leads to accumulation of metaphase arrested cells (Figure 3.12). In contrast, depletion of SENP1 or Nup153 does not block metaphase progression, suggesting a specific role of SENP2 in metaphase progression. It is unclear whether SENP2 depletion can lead to hyperSUMOylation of BubR1 and Nuf2 whose SUMOylation have been proposed to be important for the recruitment of the microtubule motor protein CENPE to the kinetochore

(Zhang et al., 2008). In addition, these analyses were performed with only one set of siRNA oligo against individual target; the observation needs to be further verified with an independent siRNA oligo.

3.4 Mutual dependency of Nup153, SENP1, and SENP2 for stability

3.4.1 Downregulation of Nup153 destabilizes SENP1

Previous studies in yeast have indicated that mutations within the Nup60/Mlp1/Mlp2 complex or the Nup84 complex destabilize Ulp1 and mislocalize Ulp1 into perinuclear aggregates (Zhao et al., 2004; Palancade et al., 2007). With our findings that SENP1 and SENP2 can interact with Nup153 at the NPC, it was thus of interest to determine whether disruption of Nup153 perturbs the stability and/or localization of SENP1 and SENP2. To this end, Nup153 was RNAi depleted, followed with analysis by indirect immunofluorescence analysis using antibody against SENP1 (antibodies to endogenous SENP2 suitable for immunofluorescence are not currently available). As shown in Figure 3.13A, severe depletion of Nup153 leads to deformation of the nuclear envelope as previously reported (Mackay et al., 2009). However, SENP1 is still detected at the nuclear envelope of interphase cells. Since residual levels of Nup153 are still present at the nuclear envelope after the siRNA treatment, it is difficult to rule out that this population of Nup153 is sufficient to maintain the localization of SENP1 at the pore. However, what was clear is that the overall staining intensity of SENP1 is drastically decreased upon the depletion of Nup153 (Figure 3.13A), indicative of SENP1 destabilization. To verify this possibility, western analysis of SENP1 was performed. As shown in Figure 3.13B, SENP1 antibody detects two bands migrating at slightly different mobilities. Both bands are depleted upon treatment with two independent siRNA oligos

of SENP1, indicating that they reflect isoforms of SENP1 or SENP1 molecules with different post-translational modification. Interestingly, RNAi depletion of Nup153 resulted in significant downregulation of SENP1 but not SENP2 (Figure 3.13C), suggesting a role of the nuclear basket protein Nup153 in stabilizing SENP1. Since we have also detected interaction between SENP1 and the NPC cytoplasmic fibril component RanBP2, we examined whether depletion of RanBP2 can similarly destabilize SENP1. RNAi depletion of RanBP2 did not downregulate SENP1 (Figure 3.13C), indicative of a specific role for Nup153 in stabilizing SENP1. Previous studies have shown that severe downregulation of Nup153 can also destabilize and mislocalize a number of nuclear basket nups, including Nup50 and TPR (Hase and Cordes, 2003). To determine whether the destabilization of SENP1 upon Nup153 depletion can be attributed to alterations of Nup50 or TPR, we performed RNAi depletion against these nups. As shown in Figure 3.13D, depletion of TPR leads to only minor downregulation of SENP1 and depletion of Nup50 has no effect on SENP1 stability, highlighting Nup153 as the predominant player at the nuclear basket in stabilizing SENP1.

3.4.2 SENP1 and SENP2 contribute to Nup153 stability

As described in Chapter 2, codepletion of SENP1 and SENP2 results in a similar, albeit less severe, NPC biogenesis defect compared to Nup153 depletion. This leads to the possibility that SENP1, SENP2, and Nup153 work in a common pathway in NPC biogenesis. Since SENP1 and SENP2 can regulate the SUMOylation of Nup153, it is possible that codepletion of SENP1/SENP2 leads to a SUMO-mediated downregulation of Nup153, which in turn, contributes to defects in NPC biogenesis. To test this possibility, levels of Nup153 were examined upon the depletion of SENP1 and SENP2.

As shown in Figure 3.14A, B, using two independent oligo sets, single depletion of SENP1 or SENP2 did not affect the stability of Nup153; however, codepletion of SENP1 and SENP2 resulted in a partial but reproducible decrease in Nup153 level. While it is unclear whether SENP1 and SENP2 mediate NPC biogenesis through regulating the stability of Nup153, these observations collectively suggest an interesting mutual dependency of Nup153, SENP1, and SENP2 for stability.

3.4.3 Post-mitotic assembly defect leads to mislocalization of SENP1

Mackay et al. have previously reported that mild downregulation of Nup153 can lead to defect in post-mitotic nuclear basket assembly, with mislocalization of numerous nuclear basket nups in the cytoplasm of cells undergoing cytokinesis. To determine whether SENP1 NPC localization is affected by a post-mitotic defect of nuclear basket assembly, cells that were mildly depleted of Nup153 were subjected to indirect immunofluorescence using antibodies against SENP1 and tubulin, which marks the midbody structure of cells undergoing cytokinesis. As shown in Figure 3.15A, mild depletion of Nup153 did not mislocalize SENP1 in interphase cells; however, cytoplasmic mislocalization of SENP1 was observed in cells undergoing cytokinesis as indicated by the tubulin midbody structure. Since depletion of Nup153 is known to mislocalize multiple nuclear basket components, including Nup50 and TPR, in midbody-staged cells, our observations suggest that functional nuclear basket assembly is required for the post-mitotic recruitment of SENP1. Consistent with this interpretation, dominant interference of post-mitotic nuclear basket assembly by overexpression of Nup153 C-terminal domain (Mackay et al., 2010) similarly leads to cytoplasmic delocalization of SENP1 in cells undergoing cytokinesis (Figure 3.15B). In contrast, overexpression of

Nup153's N-terminal domain or zinc finger domain did not lead to delocalization of SENP1 (Figure 3.15B). We failed to detect delocalization of SENP1 upon severe depletion of Nup153 in interphase cells (see Figure 3.13A), but did observe significant downregulation of SENP1 levels (see Figure 3.13B, C, D). Thus, it is possible that SENP1 was in fact delocalized into nucleoplasm/cytoplasm in the absence of Nup153 but rapidly degraded. Collectively, these findings suggest that binding determinant(s) at the nuclear basket, such as Nup153, play an important role in the localization and stability of SENP1.

3.4.4 Cdk1/Cdk2 inhibition delocalizes components of cytoplasmic fibril and downregulates SENP1 and SENP2

Interphase NPC assembly has been recently reported to be regulated by Cdk1/Cdk2 (Maeshima et al., 2010). Inhibition of Cdk1/Cdk2 with roscovitine led to colocalization of multiple nucleoporins, including Nup62, Nup133, and Nup107 in cytoplasmic foci. Interestingly, the transmembrane Nup Pom121 and the nuclear basket component Nup153 were not delocalized upon Cdk1/Cdk2 inhibition. While this study did not address whether Cdk1/Cdk2 inhibition can also delocalize components of cytoplasmic fibrils of the NPC (RanGAP1, Ubc9, RanBP2), the reported observations share striking similarity with our findings that Nup62 and Nup133, but not Pom121 and Nup153, show accumulation of cytoplasmic foci upon codepletion of SENP1 and SENP2. These findings suggest that Cdk1/Cdk2 and SENP1/SENP2 may share a common regulatory pathway in interphase NPC biogenesis. As shown in Figure 3.16, we found that inhibition of Cdk1/Cdk2 for 16 hr by roscovitine significantly downregulates SENP2 levels while SENP1 exhibited only subtle downregulation. Roscovitine treatment has also

been reported to decrease expression of numerous nucleoporins including Nup153 (Maeshima et al., 2010). Thus, the defective interphase NPC biogenesis induced by Roscovitine treatment may be due to the downregulation of SENP1/SENP2 or Nup153 or both, as depletion of these proteins leads to similar pattern of defective NPC biogenesis.

3.5 Conclusions

In this study, the NPC-associated SUMO proteases and the nucleoporin are found to be target of SUMOylation. The presence of SUMO proteases at the vicinity of the NPC is likely to confer functional regulation on NPCs. However, we failed to correlate the SUMO regulation of Nup153 to its interaction with the nuclear basket component TPR. In addition, SUMOylation does not modulate Nup153 localization at the NPC. Nonetheless, we have found a function of Nup153 SUMOylation in modulating interaction with SENP1/SENP2, opening a gateway for its role in the SENP1/SENP2-mediated regulation of NPC biogenesis. Interestingly, the SENP1 and SENP2 are themselves targets of SUMOylation, leading to the possibility that self-deSUMOylation or trans-deSUMOylation among SENP1 and SENP2 may regulate their SUMOylation status. While it is unclear what the main regulatory purpose of SENP SUMOylation is, we have found that SUMOylated SENP1 displays compromised binding to Nup153 and nonSUMOylatable SENP2 displays defective nuclear localization, opening new doors in understanding the regulatory mechanism of the SENPs. We have also uncovered that Nup153 is a binding platform for both the SUMO conjugation machinery Ubc9 and the deconjugation machinery SENP1/SENP2. These findings lead to an interesting possibility that Nup153 may coordinate the dynamic SUMO conjugation and deconjugation of interacting proteins at the nuclear basket, including the SENPs and Ubc9 whom are all

targets of SUMOylation. In support of this possibility, we have found that depletion of the nuclear basket components Nup153 or Nup50 lead to aberrant accumulation of specific SUMO1 substrate. With the finding the Nup153 and SENP1/SENP2 could be downstream of the Cdk1/Cdk2 activity, the nuclear basket could be a busy hub where various enzymatic activities converge to regulate the myriad of activities of NPCs.

3.6 References

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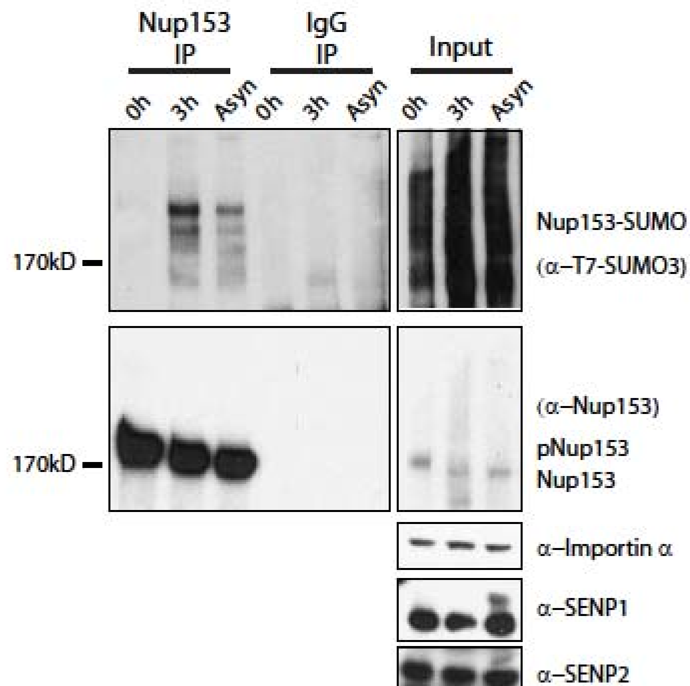

Figure 3.1 Nup153 SUMOylation is abrogated upon metaphase arrest. Hela cells that stably express T7-SUMO3 were treated with nocodazole overnight. Cells arrested at metaphase were collected and released into fresh media for 0hr or 3hr. Asynchronous cells (Asyn), as well as metaphase arrested cells (0h) and cells 3hr post nocodazole release (3h) were lysed for Nup153 immunoprecipitation followed by western analysis using antibodies against T7, Nup153, Importinß, SENP1 and SENP2. pNup153: phosphorylated Nup153.

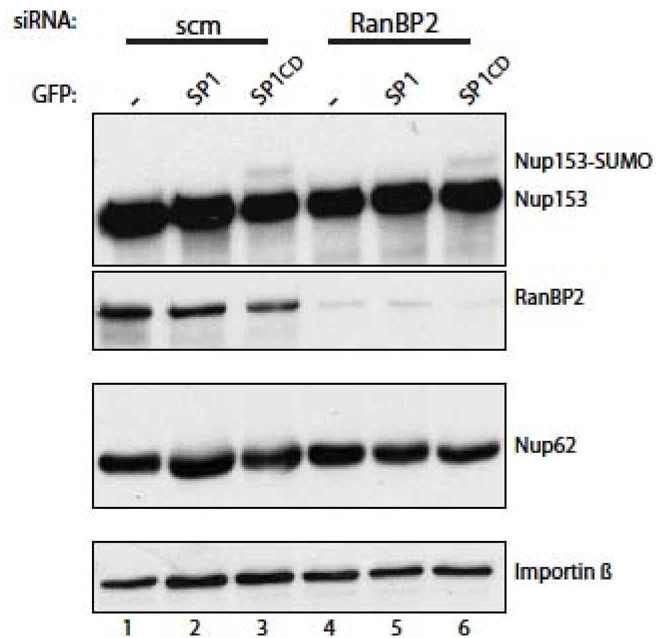

Figure 3.2 The SUMO E3 ligase RanBP2 does not mediate the SUMOylation of Nup153. Hela cells were transfected with RanBP2 siRNA for 24hr followed by the transfection of plasmid constructs of GFP (-), GFP-SENP1 (SP1), or GFP-SENP1 catalytic dead (SP1 CD) for another 24hr. Cells were harvested and subjected to western analysis using antibodies against Nup153, Nup358, Nup62, and Importin ß.

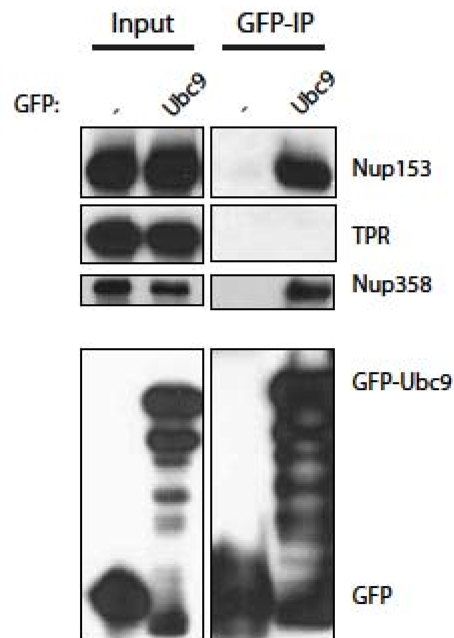

Figure 3.3 Ubc9 interacts with Nup153. Hela cells were transfected with plasmid constructs encoding GFP or GFP-Ubc9. After 24hr, cells were harvested and GFP fusion proteins were affinity purified followed by western analysis using antibodies against Nup153, TPR, RanBP2, and GFP.

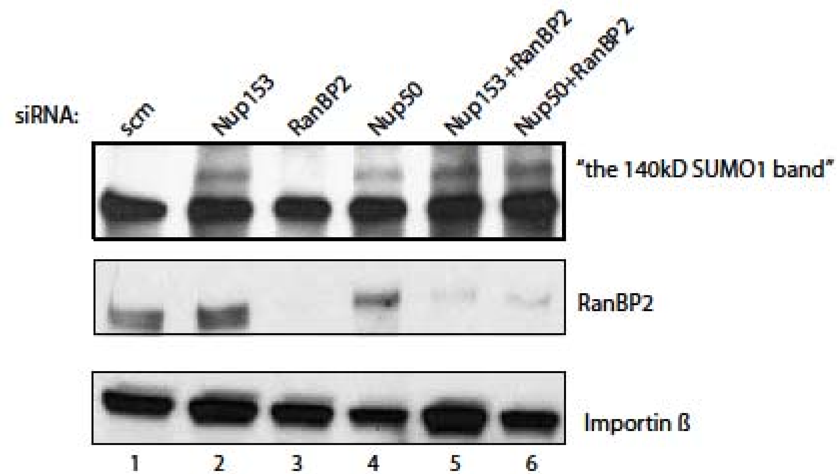

Figure 3.4 Depletion of Nup153 or Nup50 perturbs SUMO1 modification. siRNA against Nup153, RanBP2, or Nup50 were transfected into Hela cells for 48hr. Cells were harvested for western analysis using antibodies against Nup153, RanBP2, and Importin ß.

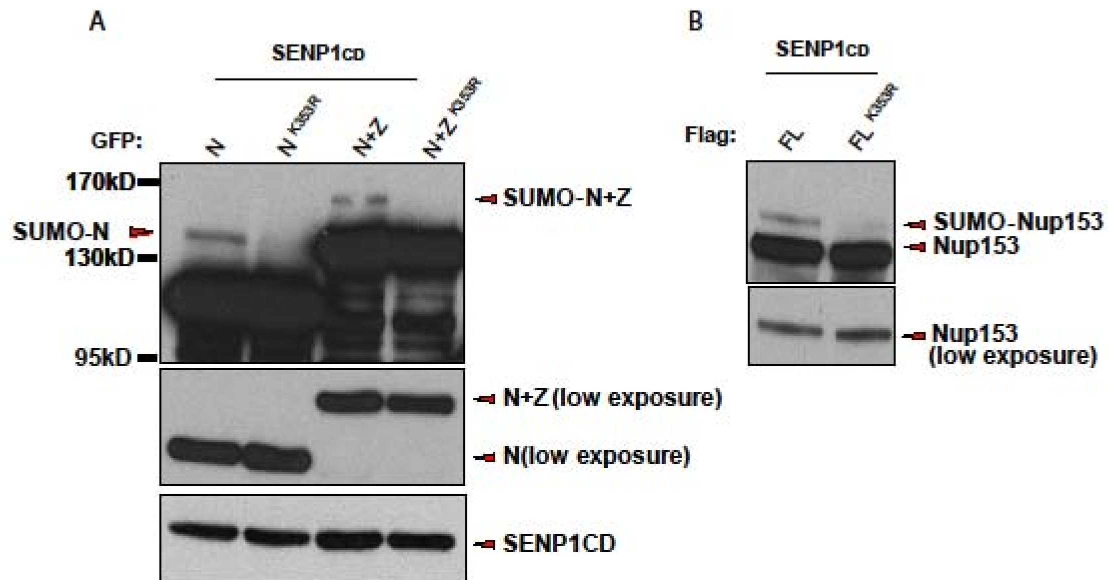

Figure 3.5 Lysine 353 of Nup153 is the SUMOylation site. (A, B) Flag contructs of Nup153 N-terminal domain (N), N-terminal domain with zinc finger domain (N+Z), or full-length Nup153 (FL), together with their K353R counterparts, were cotransfected with construct encoding GFP- catalytic dead SENP1 (SENP1CD) into Hela for 24hr. Cells were harvested for western analysis using antibodies against Flag and GFP.

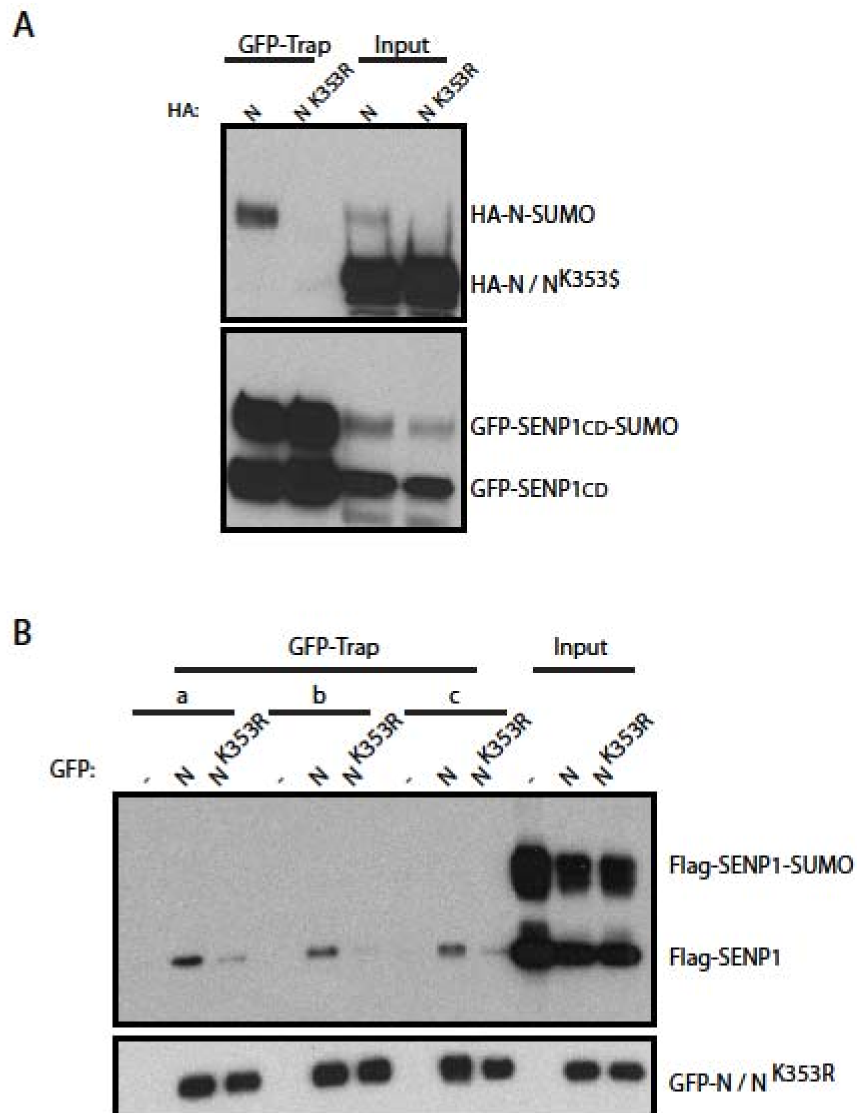

Figure 3.6 SUMOylation of lysine 353 modulates Nup153 interaction with SENP1. (A) HA-tagged fusion proteins of the Nup153 N-terminal domain (HA-N) or its K353R counterpart (HA-N K353R) were cotransfected with a construct encoding GFP- catalytic dead SENP1 (SENP1CD) into Hela cells. After 24hr, GFP proteins were recovered from cell lysates followed by western analysis using antibodies against Flag and GFP. (B) Flag-tagged fusion protein of catalytic dead SENP1 was coexpressed with GFP-tagged fusion proteins of Nup153 N-terminal domain (GFP-N) or its K353R counterpart (GFP-N K353R). After 24hr, GFP proteins were recovered from cell lysates and washed with washing buffer containing 1% Tx100 (a), or with addition of 0.1% Deoxycholate (b), or 10% glycerol (c), and subjected to western analysis using antibodies against Flag and GFP.

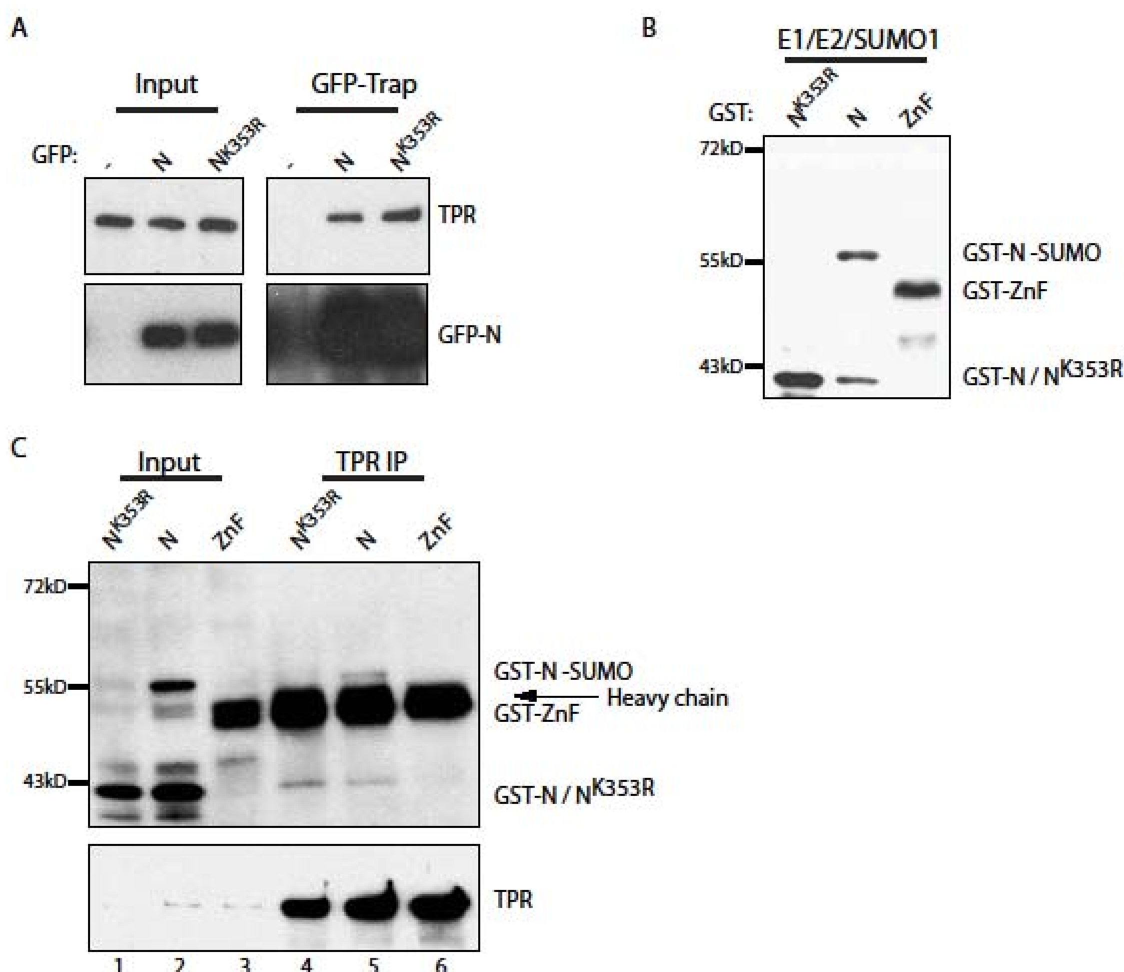

Figure 3.7 Nup153 SUMOylation does not modulate binding with TPR. (A) GFP (-) or GFP fusion proteins of N-terminal domain of Nup153 (N), or its K353R counterpart (N K353R) were expressed in Hela cells. After 24hr, GFP proteins were recovered from cell lysates followed by western analysis using antibodies against TPR and GFP. (B) Constructs encoding GST-tagged fusion protein of a TPR-binding fragment (aa 254-400) of N-terminal domain (N) or its K353R counterpart (NK353R) or the zinc finger domain (ZnF) were cotransformed with a polycistronic construct that encode the SUMO activation/conjugation enzymes (E1/E2) and SUMO1 into E. coli for protein expression. Affinity-purified GST proteins were subjected to western analysis using GST specific antibody. (C) Affinity purified GST fusion protein of ZnF, N, or NK353R were mixed with Hela cell lysates. TPR were then immunoprecipitated from lysates and subjected to western analysis using antibodies against GST or TPR.

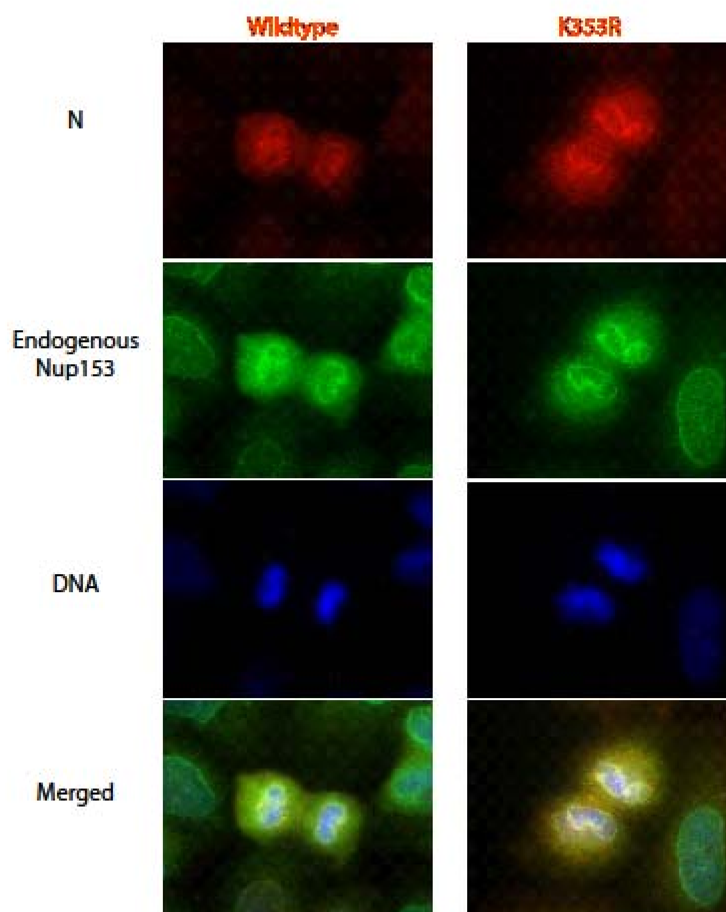

Figure 3.8 SUMOylation of Nup153 is not required for its targeting to post-mitotic NPC assembly site. Flag-tagged fusion proteins of Nup153 N-terminal domain (N, panel at left) or its K353R counterpart (N K353R, panel at right) were expressed in Hela cells for 24hr. Cells were examined by indirect immunofluorescence analysis using antibodies against Flag (red) and Nup153 (green). DNA was detected with Hoechst stain (blue).

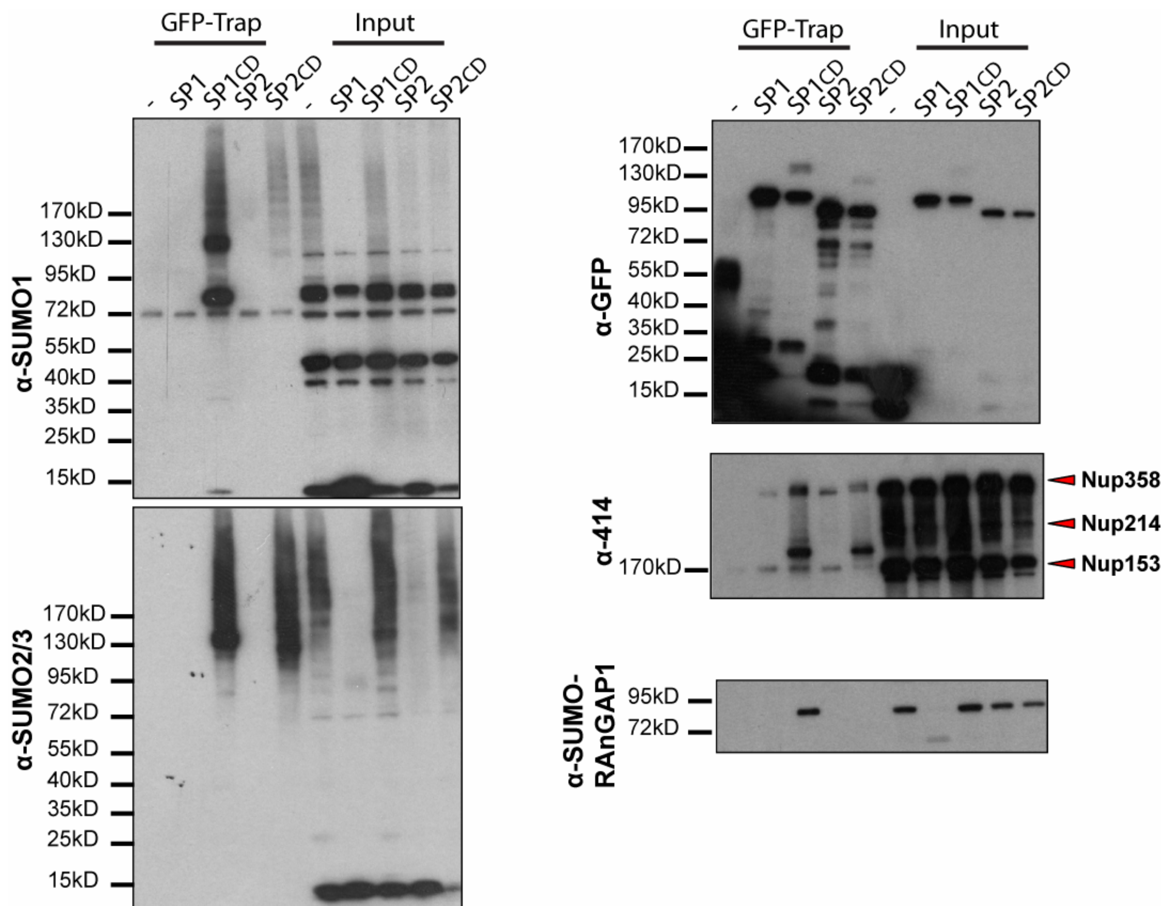

Figure 3.9 SENP1 and SENP2 have targeting preference for SUMO paralogs. GFP (-) or GFP fusion proteins of SENP1 (SP1), SENP2 (SP2) and their respective catalytic dead counterparts (CD) were expressed in Hela cells. After 24hr, GFP proteins were recovered followed by western analysis using antibodies against SUMO1, SUMO2/3, GFP, RanGAP1, and the 414 antibody (recognizes Nup358, Nup214, Nup153).

Figure 3.10 SENP1 and SENP2 are targets of SUMO modification. (A) GFP fusion proteins of SENP1 (SP1), SENP2 (SP2), or SENP3 (SP3), as well as their catalytic dead counterparts (CD) were overexpressed in Hela cells. After 24hr, cell lysates were then subjected to western analysis using antibody against GFP. (Part of this data was presented in Chapter 2 Figure 2.12B) (B) GFP fusion proteins of catalytic dead SENP1 or SENP2 were coexpressed with T7-tagged fusion proteins of SUMO1 or SUMO2. After 24hr, GFP proteins were recovered from cell lysates and subjected to western analysis with antibodies against T7 or GFP.

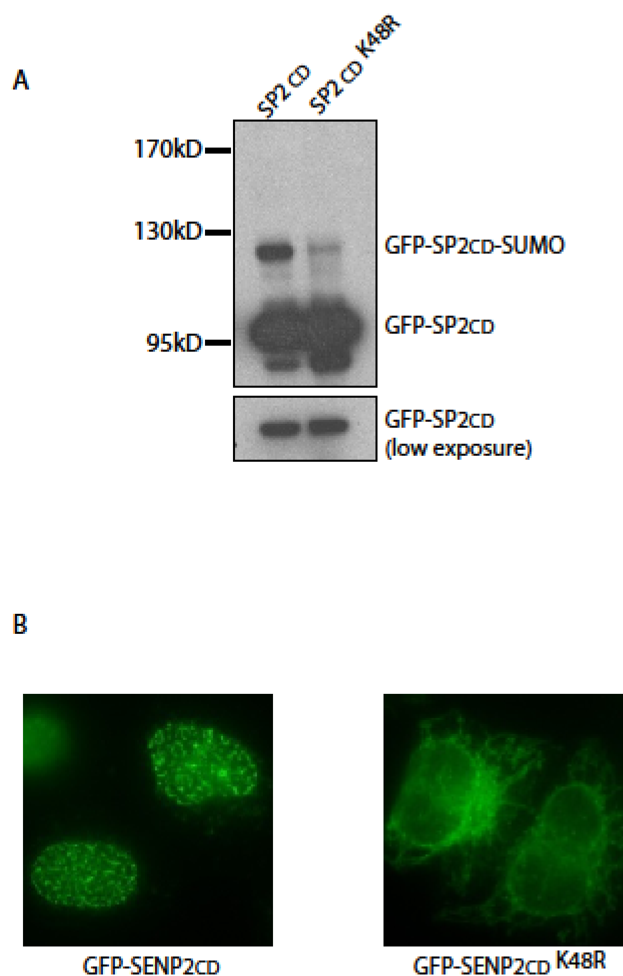

Figure 3.11 Characterization of SENP2 SUMOylation. (A) GFP fusion protein of catalytic dead SENP2 (SP2CD) or its K48R counterpart was overexpressed in Hela cells. After 24hr, cell lysates were then subjected to western analysis using antibody against GFP. Levels loaded were adjusted to have equal amount of unmodified SENP2 protein. (B) GFP fusion protein of catalytic dead SENP2 (SP2CD) or its K48R counterpart was overexpressed in Hela cells. After 24hr, cells were subjected to immunofluorescence analysis using GFP-specific antibody. The GFP-SENP2CD decorates the nuclear rim as well as nucleoplasm, its K48R counterpart, however, shows nuclear exclusion.

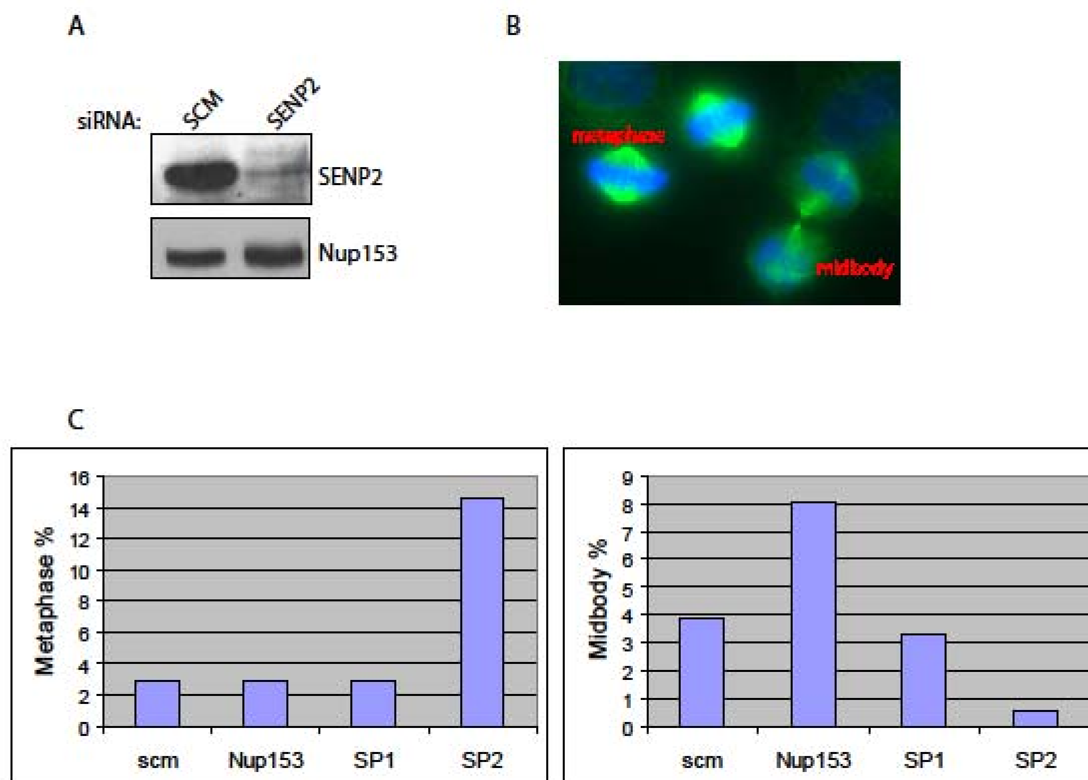

Figure 3.12 Characterization of SENP2 SUMOylation. (A) Hela cells were treated with siRNA against SENP2. After 48hr, cell lysates were collected and subjected western analysis using antibodies against SENP2 and Nup153. (B, C) Hela cells were treated with siRNA against Nup153, SENP1 (SP1)or SENP2 (SP2). After 48hr, cells were subjected to indirect immunofluorescence analysis using tubulin-specific antibody (green) and DNA (blue) (B). Cells in midbody or metephase stage were quantified (C), with an average of two independent sets of experiments graphed.

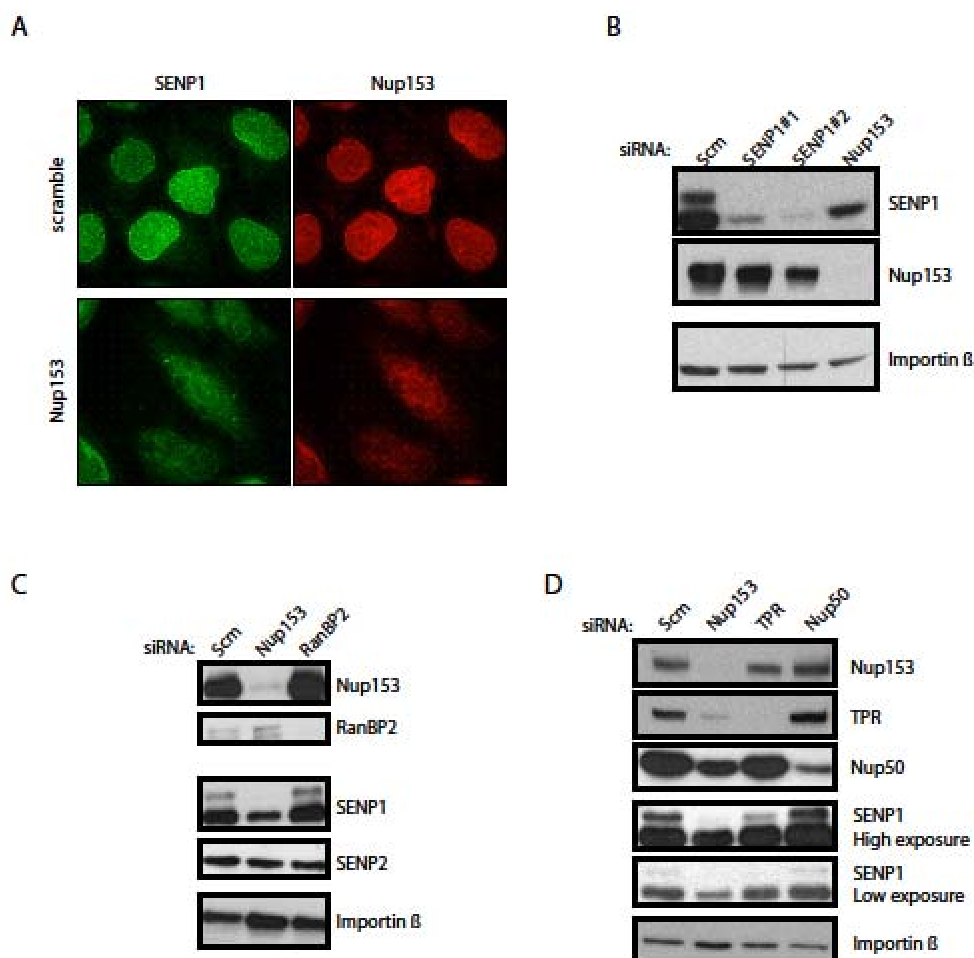

Figure 3.13 Downregulation of Nup153 destabilizes SENP1. (A) Hela cells were treated with siRNA against Nup153 (or its scramble oligo) for 48hr, and were subjected to immunofluorescence analysis with antibodies against SENP1 (green) and Nup153 (red). (B, C, D) Hela cells were treated with two independent sets of siRNA against SENP1 (B), or siRNA against Nup153, Nup358, TPR, and Nup50 (C, B). After 48hr, cell lysates were harvested and subjected to western analysis using antibodies against indicated proteins.

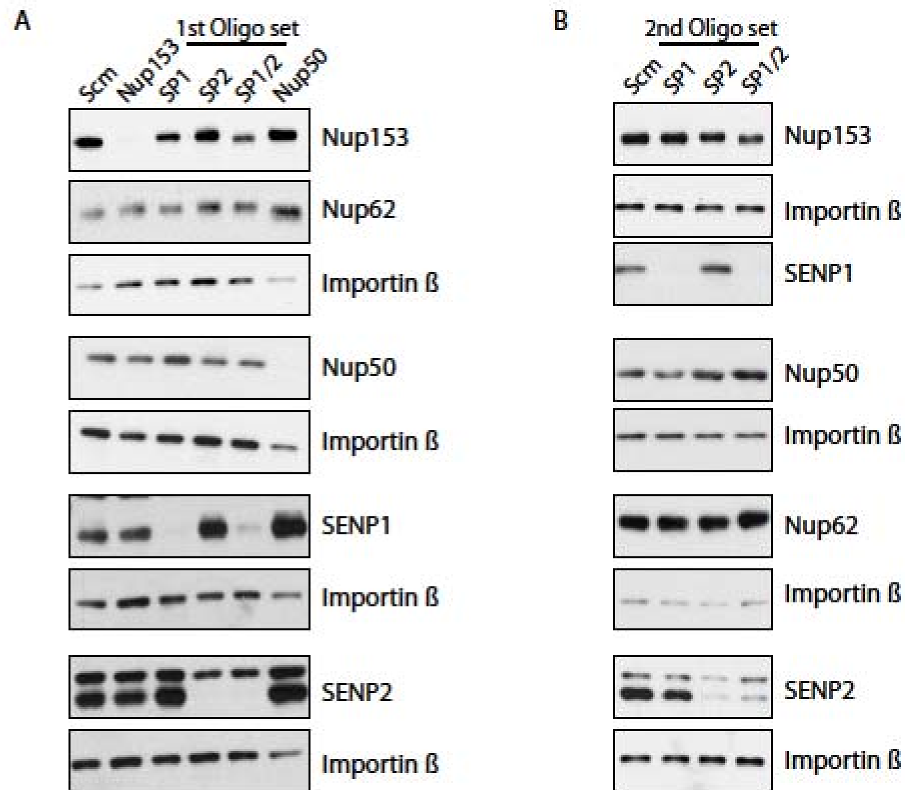

Figure 3.14 SENP1 and SENP2 regulate the stability of Nup153.
(A, B) Hela cells were treated with two independent sets of siRNA oligos against SENP1 and SENP2, as well as Nup153 and Nup50, for 48hr. Cell lysates were harvested and subjected to western analysis with antibodies against indicated proteins.

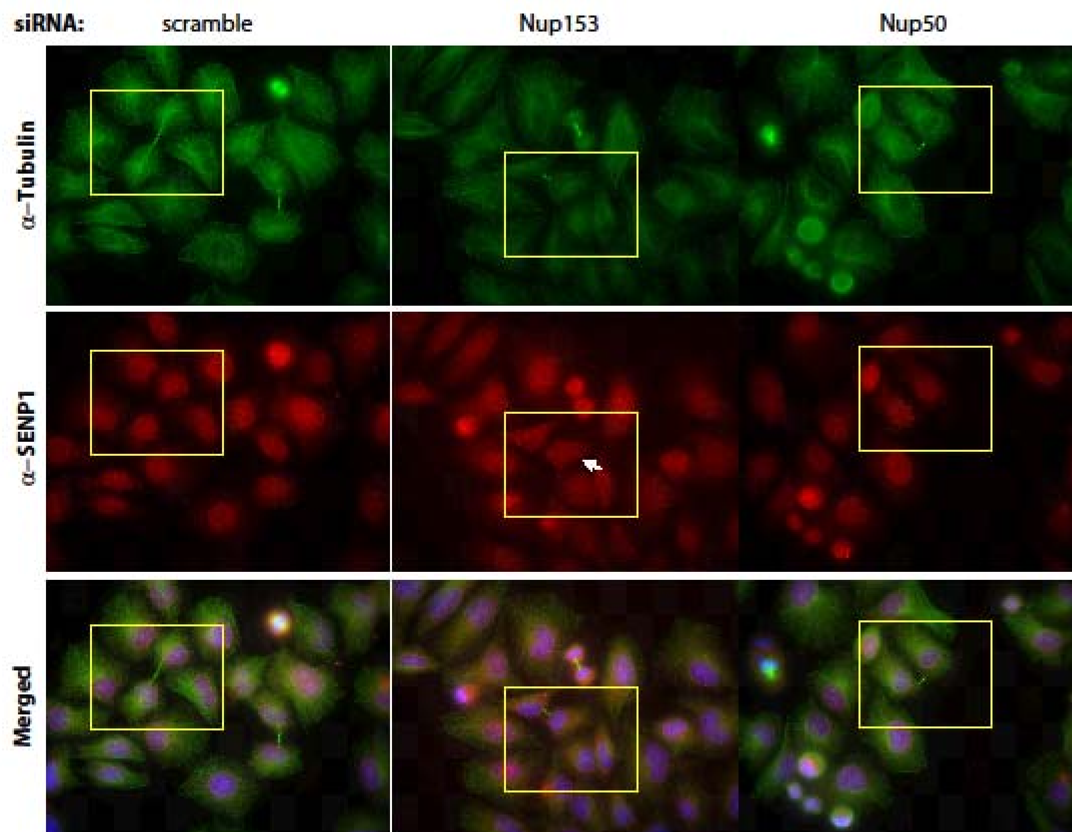

Figure 3.15A Post-mitotic nuclear basket assembly defect delocalizes SENP1. Hela cells were treated with siRNA against Nup153 or Nup50 (with control scramble). After 48hr, cells were subjected to indirect immunofluorescence using antibodies against tubulin (green) and SENP1 (red), with DNA stained (blue). Midbody-staged cells are highlighed by yellow boxes. Delocalized SENP1 is indicated by a small white arrow.

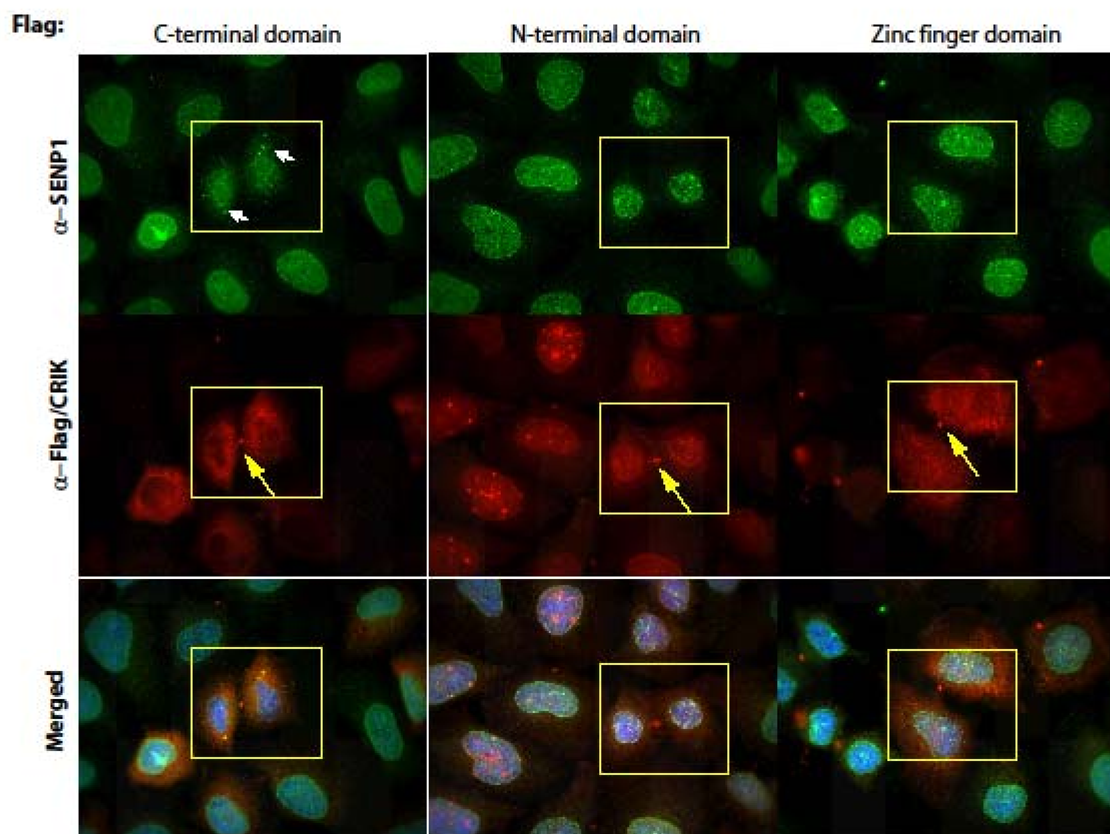

Figure 3.15B Dominant interference of post-mitotic nuclear basket assembly disrupts SENP1 localization. Flag-tagged fusion proteins of Nup153 C- or N-terminal domain, as well as the zinc finger domain were overexpressed in Hela cells. After 24hr, cells were subjected to indirect immunofluorescence analysis using antibodies against SENP1 (green), Flag (red) and CRIK (red). [CRIK is a midbody marker stained as a red dot in-between cells undergoing cytokinesis indicated by yellow arrows; both the C-terminal domain and zinc finger domain have cytoplasmic localization while the N-terminal has nuclear rim and nucleoplasmic localization]. The DNA were stained in blue. Delocalized SENP1 is indicated by a small white arrow.

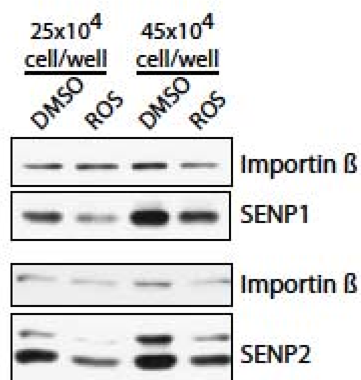

Figure 3.16 Inhibition of Cdk1/Cdk2 downregulates SENP1 and SENP2. Hela cells seeded at two density 25x104 or 45x104 cell/well were treated with the Cdk1/Cdk2 inhibitor roscovitine (ROS). After 16hr, cell lysates were subjected to western analysis using antibodies against SENP1, SENP2, and Importin b.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 A role for SENP1 and SENP2 NPC biogenesis

Previous studies from yeast and from mammalian tissue culture system have found numerous nucleoporins as well as nonnuclear pore components to contribute to interphase NPC biogenesis (Doucet and Hetzer, 2010), but little is known about the spatiotemporal control of this process. In this study, SENP1 and SENP2 are identified to have a regulatory role in NPC biogenesis, with the exciting implication that deSUMOylation activity at the nuclear envelope may regulate recruitment of nucleoporins to the NPC assembly site. A key question that remains to be verified is whether the catalytic activity of SENP1/SENP2 is required for this regulatory function. To determine this, siRNA resistant expression constructs of SENP1/SENP2 or their catalytic dead counterparts could be individually/dually transfected into SENP1/SENP2 depleted cells and assess for their ability to rescue the NPC biogenesis defect. Nup153's role in coordinating NPC biogenesis is proposed in this study to be mediated through its

interaction with SENP1/SENP2. Since the SUMOylation of Nup153's N-terminal domain was shown to modulate its interaction with SENP1/SENP2, the above rescue strategy could also be employed to probe for a role of Nup153 SUMOylation in NPC biogenesis, specifically determining whether the nonSUMOylatable mutant K353R of Nup153 can rescue the NPC biogenesis defect induced through Nup153 depletion.

4.2 Distinguishing between a *de novo* and a post-mitotic NPC assembly defect upon Nup153/SENP1/SENP2 depletion

New NPCs are constantly assembled from newly translated nucleoporins and incorporated into the expanding nuclear envelope during interphase (*de novo* NPC assembly). In addition, existing nucleoporins present from NPCs that are disassembled during mitosis are reassembled at telophase (post-mitotic NPC assembly) (Doucet and Hetzer, 2010). While cytoplasmic delocalization of nucleoporins upon Nup153/SENP depletion is observed predominantly in G1/S cells, these phenotypes are not excluded from cells undergoing cytokinesis. Preliminary tracking of cells undergoing cytokinesis with G1 markers like phospho-Rb or PCNA (not shown) were not conclusive to distinguish whether these phenotypes are a reflection of a *de novo* NPC assembly defect as the NE becomes closed or a defect in post-mitotic NPC assembly.

Makio et al. have previously employed a strategy to monitor *de novo* NPC synthesis through tracking nucleoporins fused to a photoactivatable fluorescent protein Dendra, which switches from green fluorescence to red upon exposure to visible blue light (Makio et al., 2009). Since depletion of Nup153/SENP leads to accumulation of Nup133 in cytoplasmic foci, a Dendra fusion protein of Nup133 would be an ideal reagent to distinguish *de novo* vs post-mitotic NPC assembly. Briefly, Hela cells stably

expressing the Dendra-Nup133 fusion protein would be subjected to live-cell imaging and cells at early stages of mitosis (prophase / metaphase) photoactivated to switch its green fluorescence to red, enabling us to track them as a mitotic disassembled nup population. The Dendra-red Nup133 would be tracked in these mitotic cells as they undergo cytokinesis untill entry to G1/S. If red cytoplasmic foci of these Dendra-red Nup133 fusion proteins appeared in cells undergoing cytokinesis (when post-mitotic NPC assembly occurs) upon the depletion of Nup153/SENP, then Nup153/SENP may have roles in mediating post-mitotic NPC assembly. However, if these Dendra-red Nup133 population were properly assembled at post-mitotic NPC assembly sites with the appearance of newly translated Dendra-green Nup133 foci in cells undergoing cytokinesis and transitioning to early G1, this would be indicative of a *de novo* NPC assembly defect.

4.3 Possible redundancy of SENP1 and SENP2 in NPC biogenesis

In this study, depletion of SENP1 alone does not lead to NPC biogenesis defect whereas depletion of SENP2 can mildly deregulate the assembly of multiple nucleoporins. However, codepletion of SENP1 and SENP2 leads to a more severe NPC assembly defect. These observations suggest that SENP2 may be the predominant SUMO protease to mediate NPC biogenesis. However, in its absence, SENP1 may partially substitute for the role of SENP2 in deSUMOylating SUMO targets for NPC assembly regulation. In Chapter 3, SENP2 is demonstrated to have a bias toward binding/targeting SUMO2/3 substrates; however, SENP1 can bind/target both SUMO1 and SUMO2/3 substrates. In addition, both SENP2 and SENP1 localize to the NPC and interact with the nuclear basket Nup153. Their overlapping SUMO paralog specificity and localization may

explain why SENP1 could partially substitute the function of SENP2. Such interpretation points toward that deSUMOylation of SUMO2/3-modified target(s) as an important regulatory event during NPC biogenesis. Since the catalytic domains of SENP1 and SENP2 are highly similar, the sequence divergence of their N-terminal domains could mediate specific recognition of regulatory targets during NPC biogenesis.

4.4 Distinct requirements for recruiting and assembling cytoplasmic fibrils and nuclear basket of the NPC

The basic architecture of NPC consists of the central nups core, the cytoplasmic fibrils, and the nuclear basket. Sequential recruitment of transmembrane and scaffolding nucleoporins to the assembly site has been illustrated by Doucet et al. that Pom121 recruitment to the NPC assembly site precedes the scaffolding Nup107 complex during *de novo* assembly (Doucet et al., 2010). It is proposed that NPC assembly at the NE begins with the formation of a prepore structure by transmembrane nups that subsequently recruit the assembly of the core scaffolding nups and peripheral nups. The nucleated core scaffolding/peripheral nups will then recruit components that form the cytoplasmic fibril and nuclear basket structures (Doucet et al., 2010; Doucet and Hetzer, 2010). Emerging evidence suggests that the central core nups independently recruit elements of the cytoplasmic fibrils and nuclear basket. A recent study from Makio et al. has demonstrated that disruption of Nup170 and Nup157 scaffolding nups in yeast leads to an assembly defect of cytoplasmic fibrils, while nuclear basket intermediates are still found to decorate INM sites that failed to fuse with ONM (Makio et al., 2009). In addition, depletion of the mammalian peripheral Nup98 specifically leads to assembly defects in components of cytoplasmic fibril but not components of the nuclear basket.

In this study, the depletion of SENP1/SENP2 is found to lead to assembly defects in the scaffolding/peripheral nups Nup133/Nup62, as well as the cytoplasmic fibril nup RanBP2. In contrast, components of the nuclear basket appear to undergo normal assembly upon SENP1/SENP2 depletion. These observations imply the dependency of cytoplasmic fibril assembly on the scaffolding/peripheral nups Nup133/Nup62. In contrast, the presence of Nup133/Nup62 may not be essential for the assembly of nuclear basket, suggesting that in the absence of SENP1/SENP2, the nuclear basket can be assembled without a complete scaffolding/peripheral core. Such a nuclear basket intermediate might be similar to the intermediate reported by Makio et al. that is associated with the INM at a site where fusion with the ONM is absent. Immunogold EM analysis of NPCs structures using antibodies against components of scaffolding/peripheral, cytoplasmic fibrils, and nuclear basket could be employed to address this hypothesis.

To determine if the assembly of the scaffolding/peripheral nups Nup133/Nup62 is required prior to recruitment of the cytoplasmic fibrils, Nup133 and Nup62 could be depleted through RNAi and the assembly pattern of cytoplasmic fibril components such as RanGAP1 and RanBP1/Nup358 examined. If assembly of the scaffolding/peripheral core precedes the recruitment of cytoplasmic fibril components, Nup133/Nup62 depletion will likely lead to accumulation of RanGAP1 and RanBP1/Nup358 in cytoplasmic foci. In addition, disruption of the scaffolding/peripheral core through Nup133/Nup62 RNAi is not expected to perturb the assembly pattern of the nuclear basket.

If these proposed experiments yield the predicted results, the observed assembly defects in scaffolding/peripheral nups and cytoplasmic fibrils upon SENP1/SENP2

depletion would suggest an upstream role of SENP1/SENP2 in mediating the assembly of scaffolding/peripheral nups, entailing the possibility that certain scaffolding/peripheral nups could be SUMO targets regulated by SENP1/SENP2. Indeed, dominant interference of SENP1 activity through overexpression of catalytic dead SENP1 resulted in Nup133 SUMOylation (not shown). This dominant interference of SENP1/SENP2 approach may be a feasible method to systematically identify which of the scaffolding/peripheral nups could be SUMO targets regulated by SENP1/SENP2. A similar approach could be applied to examine potential SENP1/SENP2 regulation on nonnucleoporin factors identified to mediate NPC biogenesis such as the ER protein Rtn4, the trafficking factors Ran, Importin α, Importin β. Interestingly, some members of the scaffolding nups Nup43, Nup107, Nup133, Nup155, Nup205, and Nup93 (Vertegaal et al., 2006; Golebiowski et al., 2009; Blomster et al., 2009), as well as the trafficking factors Importin α, Importin β, and Ran (Nie et al., 2009; Rosas-Acosta et al., 2005; Matafora et al., 2009), have been previously identified in proteomic studies as potential SUMO targets (see Table 1.1 in Chapter 1).

It is unclear how dynamic SUMOylation of nucleoporins could regulate the assembly process of NPC biogenesis. It is possible that the SUMOylation of Nup153, Nup133 or other nups can act as a switch to impose temporal regulation of their interactions with other nups/factors during NPC assembly. Such regulation may be important to prevent premature incorporation of newly translated nups to the NPC assembly sites. In the absence of SENP1/SENP2, the SUMOylation of the scaffolding Nup133 may block its interaction with other components of the scaffolding/peripheral core, therefore interfering with the assembly of this structure and, in turn, delocalization

of NPC components in the cytoplasm. Nup133 has been recently characterized to mediate NPC assembly through its curvature sensing domain (Doucet et al., 2010). It is proposed that the transmembrane nup Pom121 first establishes the NPC assembly site by inducing the fusion of INM/ONM. The induced membrane curvature is then thought to be detected by the sensing domain of Nup133, which subsequently targets itself and the binding components of the scaffolding core to the NPC assembly site (Doucet et al., 2010). SUMOylation may be involved in regulating the membrane curvature sensing mechanism of Nup133.

Before any of these possibilities can be tested, the SUMOylation site(s) of Nup133 needs to be mapped. This can be done by first mutating individual lysines from the four predicted SUMO consensus sites. If SUMOylation occurs at a nonconsensus site, one could map the SUMO modification to individual domain(s) of Nup133 and narrow in for the candidate lysine. To determine whether Nup133 SUMOylation interferes with its interaction with other nucleoporins, GST fusion proteins of SUMOylated Nup133 can be purified using the bacterial SUMOylation system (Chapter 2) and test for interaction with candidate scaffolding binding partners of Nup133 such as Nup107, Nup43, Nup37, Sec13, and Seh1 (Loïodice et al., 2004; Belgareh et al., 2001). To determine whether Nup133 SUMOylation could modulate its membrane curvature sensing, one could monitor the *de novo* NPC biogenesis of nonSUMOylatable mutant of Nup133 through immunofluorescence analysis in Hela cells. If mutating its SUMOylation site does not perturb its incorporation into nuclear envelope, this would indicate that SUMOylation of Nup133 may not be required for NPC biogenesis. In contrast, SUMOylation may interfere with its curvature sensing function. Since SUMOylation of Nup133 is not a

robust event and it is technically challenging to introduce SUMOylated Nup133 recombinant fusion protein into tissue culture cells to examine its NPC incorporation, this hypothesis will have to be tested in an *in vitro* NPC assembly assay using *Xenopus* egg extract similarly employed by D'Angelo et al. In this assay, GFP-fusion protein of SUMOylated Nup133 would be purified using the bacterial SUMOylation strategy as described in Chapter 2. In the egg extract system, *de novo* NPC assembly can be studied in isolation by forming nuclei in the presence of the calcium chelator BAPTA which blocks the assembly of NPC. As BAPTA is diluted with untreated egg extract, NPC assembly/incorporation will be initiated (Macaulay and Forbes, 1996). If this is done in the presence of fluorescent SUMOylated Nup133 fusion protein, the incorporation of SUMOylated Nup133 into *de novo* NPC can be monitored following BAPTA removal. If Nup133 SUMOylation blocks its curvature sensing function, the SUMOylated GFP-Nup133 recombinant protein would be defective in incorporation at the nuclear rim in this assay.

Similar to Nup133, the SUMOylation of the nuclear basket component Nup153 may interfere with its interaction with components of the scaffolding/peripheral core, thereby blocking the recruitment of these components to the NPC assembly site. An unexpected role of Nup153 in mediating the recruitment of scaffolding/peripheral core has been identified in this study in that Nup153 depletion blocks proper incorporation of Nup133 and Nup62 into NPCs. In this study, the function of Nup153 SUMOylation has been examined in the context of its interaction with another nuclear basket component, TPR. However, how Nup153 SUMOylation modulates its interaction with components of the scaffolding/peripheral core nups has not been tested. A similar strategy of purifying

SUMOylated Nup153 as described in Chapter 3 could be employed to more globally determine how SUMOylation of Nup153 impacts its network of interactions.

4.5 The nuclear basket may regulate assembly of core scaffolding/peripheral nups

Since the core scaffolding/peripheral nups are known to be critical for NPC biogenesis, the assembly defect of cytoplasmic fibril components upon the disruption of core scaffolding/peripheral nups may reflect a lack of core "seeding" sites for the assembly of cytoplasmic fibril components. While a postulated role of SENP1/SENP2 in regulating the scaffolding Nup133 or Nup205 awaits further verification, it is still unclear how the regulatory activities of SENP1/SENP2 are directed to the assembling scaffolding nups. Since nuclear basket intermediates can be assembled in the absence of full scaffolding/peripheral assemblies, it is possible that nuclear basket may serve as a platform to present SENP1/SENP2 activities to the assembling scaffolding nups. This possibility is supported by the finding that the nuclear basket component, Nup153 can interact with SENP1/SENP2, which suggests that a partnership of Nup153-SENP1/SENP2 at the nuclear basket may impose regulation on the assembling scaffolding/peripheral core components. This model predicts that disruption of the Nup153-SENP1/SENP2 partnership would similarly lead to a NPC biogenesis defect. Indeed, depletion of Nup153 alone can lead to profound assembly defect of the scaffolding Nup133, the core peripheral Nup62, and components of the cytoplasmic fibril. Nup153 has been previously found to associate with Nup160, a component of the Nup107 complex with Nup133 as its constituent. In addition, Nup153 is also known to interact with numerous core transmembrane/scaffolding/peripheral nups including

Pom121, Nup160, Nup155, and Nup98 that reside in close proximity to Nup133. These interaction networks may allow Nup153 to present the regulatory activities of SENP1/SENP2 to Nup133 during NPC biogenesis.

4.6 The N- and C-terminal domains of Nup153 may confer domain-specific targeting of SENP1/SENP2 substrates

In this study, Nup153 is found to interact with SENP1/SENP2 through both its N- and C-terminal domains. Nup153 C-terminal domain is likely to have an importin-mediated interaction with SENP1/SENP2; however, the SUMOylation of lysine 353 at Nup153 N-terminal domain provides a reinforcing recruitment signal for SENP1/SENP2. Such bimodal interactions of SENP1/SENP2 with Nup153 may confer unique regulation of SENP1/SENP2 activity. One possible model is that SENP1/SENP2 binding to different Nup153 domains allows the targeting SENP1/SENP2 activities towards specific subset of proteins that differentially bind to different domains of Nup153. Both N- and C-terminal domains of Nup153 are binding platforms for a variety of macromolecules. Regulated targeting of SENP1/SENP2 to these binding domains may allow regulated deSUMOylation activities towards proteins that bind to these Nup153 domains. Mapping of Nup153's domains required for NPC biogenesis may lead to new insights into how the different SENP-binding platforms of Nup153 regulate specific deSUMOylation targets. Mackay et al. have previously employed a panel of Nup153 truncation mutants targeting its N- and C-terminal domains to map functional domains of Nup153 in mediating its role in mitotic progression (Mackay et al., 2009). A similar domain mapping strategy would be useful in identifying the minimal functional domain of Nup153 required to coordinate

NPC biogenesis to fully decipher the involvement of SENP binding to individual domains.

4.7 Ending remarks

This study has made new discovery about the function of NPC-associated SUMO proteases, with an exciting new model of NPC biogenesis involving the deSUMOylation activity of SENP1/SENP2. This model predicts that SUMO modification of nucleoporins, especially the central scaffolding/peripheral nups, could be a key event in imposing spatiotemporal regulation on nucleoporin assembly. In addition, components of the nuclear basket may establish an regulatory platform at NPC assembly site to coordinate the activity of the SENPs. Elucidating these possibilities may lead to more new insights of NPC biogenesis.

4.8 References

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