Two distinct sites in Nup153 mediate interaction with the SUMO proteases SENP1 and SENP2

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Abbreviations: NPC, nuclear pore complex; GFP, green fluorescent protein; SENP1, SENP2 or SP1, SP2, sentrin/SUMO specific protease 1 and 2; CD, catalytically dead

Numerous enzymes of the mammalian SUMO modification pathway, including two members of the SUMO protease family, SENP2 and SENP1, localize to the nuclear periphery. The SUMO proteases play roles both in processing SUMO during the biogenesis of this peptide moiety and also in reversing SUMO modification on specific targets to control the activities conferred by this post-translational modification. Although interaction with the C-terminal domain of the nucleoporin Nup153 is thought to contribute to SENP2 localization at the nuclear pore complex, little is known about the binding partners of SENP1 at the nuclear periphery. We have found that Nup153 binds to both SENP1 and SENP2 and does so by interacting with the unique N-terminal domain of Nup153 as well as a specific region within the C-terminal FG-rich region. We have further found that Nup153 is a substrate for sumoylation, with this modification kept in check by these two SUMO proteases. Specifically, either RNAi depletion of SENP1/SENP2 or expression of dominantly interfering mutants of these proteins results in increased sumoylation of endogenous Nup153. While SENP1 and SENP2 share many characteristics, we show here that SENP1 levels are influenced by the presence of Nup153, whereas SENP2 is not sensitive to changes in Nup153 abundance.

Introduction

Post-translational modification by the small ubiquitin-like peptide moiety SUMO is critical to many processes, from DNA damage response to mitotic execution. Sumoylation also plays a role in maintaining the RanGTP gradient that underlies nuclear-cytoplasmic trafficking, as well as regulating the nuclear response to a range of signaling pathways, including Wnt-signaling, androgen signaling and hypoxia. Three paralogues of SUMO are widely used. SUMO1 shares only ~50% identity with SUMO2, but SUMO2 and SUMO3 are so similar (96% identical) that they are not considered to have distinct properties and are often referred to as SUMO2/3. While there is considerable overlap in the targets of modification for these three paralogues, SUMO2/3 is distinguished by its ability to form polymer chains and by its modulation in response to cellular stress.

A key aspect of SUMO-dependent regulation is the dynamic nature of this modification and thus the proteases responsible for reversing sumoylation play important roles. There are six mammalian SUMO-specific proteases or SENPs (sentrin/SUMO proteases), note that SENP4 has specificity for distinct peptide modifier, NEDD8, which can be broadly classified into two groups based on evolutionary relationships: Ulp1-like (SENP1, SENP2, SENP3 and SENP5) and Ulp2-like (SENP6 and SENP7). Notably, DeS1-1, which belongs to a distinct family of proteases, was recently reported to have desumoylating activity; further expanding the possible players in this node of regulation. Substrate preference and sub-cellular localization are thought to contribute to the selectivity of SUMO protease activity, but the full picture of how their activity is controlled remains to be elucidated. SENP1 and SENP2 are a particularly related sub-group, but are not redundant, as evidenced by the distinct phenotypes that result from their genetic knockout in mice, as well as the differential requirements for these proteases in specific functions. A better understanding of what features are shared or unique to these two proteases will provide insight into their roles.

One hallmark characteristic shared by SENP1 and SENP2 is their ability to target to the nuclear rim. Although this is not their exclusive localization, such subcellular localization is likely to be an important aspect of their function. First, localization to the nuclear rim, and the nuclear pore more specifically, is an evolutionarily conserved feature of SUMO proteases and second, interactions with nuclear pore proteins have been found to control the activity of the associated SENPs. In mammalian cells, the targeting of SENP2 has been the subject of more analysis and separable interactions with both Nup153 and the Nup107 complex have been characterized. A specialized interaction with transport receptors has also been found as a

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conserved bridge between SENP2 or yeast Ulp1 and nuclear pore proteins.24,27,28 Here, our aim was to push forward the understanding of the interface between pore targeted SENPs and the nuclear pore protein Nup153. In particular, we have delved further into the determinants within Nup153 that are important for interaction with SENP2 and we have probed SENP1 in parallel to better understand what characteristics are shared with or distinct from those of SENP2. Interestingly, these studies have also revealed that Nup153 undergoes a cycle of sumoylation, which is both modulated by the SENPs and contributes to their recruitment to Nup153. A discrete site at the C-terminal tail of Nup153 was found to provide a second site of interaction between Nup153 and SENP1 or SENP2. While SENP1 and SENP2 share similarity in their interface with Nup153, distinctions between these SENPs were also brought to light in this analysis. One difference lies in their sensitivity to reduction in Nup153 levels, with SENP1 levels selectively decreasing under these conditions.

**Results**

Nup153 interacts with SENP1 and SENP2 and its SUMO status is regulated by these SUMO proteases. To look at the interface between Nup153 and SENPs, we started by comparing the binding ability of Nup153 to SENP1 and SENP2. SENP1 has been observed at the nuclear rim both when ectopically expressed and when endogenous protein is detected (Fig. S1A). To date, however, molecular partnerships between SENP1 and proteins that reside at the nuclear envelope have not been characterized. GFP along with GFP fusions of both SENP1 and SENP2 were expressed in HeLa cells and an affinity matrix was then used to trap these GFP proteins along with associated proteins from cell lysates.6 Nup153 was recovered with both GFP-SENP1 and GFP-SENP2, but not with GFP alone (Fig. 1A, lanes 6, 7 and 9). Other nucleoporins tracked in parallel, Nup62 and Nup50, were not recovered with these SENPs, underscoring the specificity of the interaction.

As the catalytic activity of SENP1 has been reported to impact its localization and presumably might alter protein partnerships involved in its localization, catalytically dead (CD) mutants of SENP1 and SENP2 were analyzed in parallel samples. Unexpectedly, expression of both SENP1CD and SENP2CD resulted in the presence of a slower migrating form of Nup153 (Fig. 1A, lanes 3 and 5, arrow). Moreover, this slower migrating Nup153-reactive species was enriched with ectopically expressed SENP1 in these samples. This result suggests that a modification of Nup153 revealed by SENP1CD and SENP2CD increases its affinity for these SENPs (Fig. 1A, lanes 8 and 10). Expression of mutant SUMO protease did not broaden the migration of proteins that are at or in contact with the nucleopore, as we did not detect additional species of Nup30, Nup62, Nup98, lamin A, lamin B2 or Importin β (Fig. 1A; Fig. S2). It is notable, however, that the mutant SENPs themselves displayed a prominent slower migrating form (Fig. 1A, lanes 3 and 5, double-arrow). This has been reported previously for SENP1 and found to correspond to SUMO-modification (see below).

Expression of catalytically dead SENP is predicted to interfere with desumoylation of proteins normally targeted by that SUMO protease. To determine if the slower migrating form of Nup153 is due to SUMO modification, SUMO1, SUMO2 and SUMO3 were expressed as T7-tagged peptides in conjunction with catalytically dead SENP2. Nup153 was then immunoprecipitated from cell lysates. Upon probing the samples for the presence of the T7 tag, responding in size to the slower migrating form of Nup153 was detected primarily in samples expressing either T7-SUMO2 or T7-SUMO3 and absent from the control samples (Fig. S3, lanes 3 and 4, vs. lanes 1 and 5–8). Thus the slower migrating form of Nup153 is indeed sumoylated.

If expression of SENP1CD and SENP2CD interfere with ongoing desumoylation of Nup153, then dephosphorylation of endogenous proteases would be expected to result in the appearance of SUMO-modified Nup153. To test this, siRNAs were used to deplete either SENP1 or SENP2 or the two processes together in cells expressing T7-SUMO3. The efficacy and specificity of this depletion is tracked in the lower parts of Figure 1B, lanes 1–4. Nup153 was then recovered from these cell lysates by immunoprecipitation (Fig. 1B, lanes 5–8, middle part). Depletion of either protease alone resulted in an increase in a T7-reactive species not seen in control samples that corresponds in size to the slower migrating form of Nup153 (Fig. 1B, lane 5–7, upper part). Simultaneous depletion of both proteases resulted in robust detection of this sumoylated species (Fig. 1B and lane 8). This series of experiments reveals that Nup153 is normally targeted for a cycle of sumoylation and desumoylation and that both SENP1 and SENP2 are involved in the desumoylation arm of this pathway.

The specificity of the role for SENP1 and SENP2 in maintaining desumoylation of Nup153 is further highlighted by the finding that similar ectopic expression of a catalytically dead mutant of SENP3 does not result in a modified form of Nup153 (Fig. 1C, lane 6, upper part), despite its ability to interfere more generally with desumoylation (Fig. 1C, lane 6, lower part). To delineate the region of Nup153 that is subject to this sumoylation cycle, we divided Nup153 into its three main domains—a unique N-terminal region (N), a tandem zinc finger motif (Z) and an FG-rich C-terminal domain—and expressed each as a Flag-tagged protein in conjunction with SENP1, in wild-type and catalytically dead form. The N-terminal domain of Nup153 displayed a slower migrating form when coexpressed with SENP1CD (Fig. 1D, lane 1), this was the case for SENP2CD as well (see Fig. 4). This slower migrating form of the Nup153 N-terminal domain is not present with expression of wildtype SENP1 or SENP2. We did not observe modified species of either the zinc finger or C-terminal domain, although expression of SENP1CD in these same samples was sufficient to reveal modification of endogenous Nup153 (Fig. 1D, lanes 3 and 5). Thus, the site of cyclical sumoylation of Nup153 resides within its unique N-terminal region.
Catalytically inactive SENP1 and SENP2 maintain specific attributes, although both have enhanced interaction with sumoylated Nup153. As mentioned above, SENP1cD has been previously shown to become SUMO1-modified. To determine if this modification also explains the shifted species that we observe for SENP2cD, we expressed SENP1cD and SENP2cD as GFP fusion proteins in cells expressing either T7-SUMO1 or T7-SUMO2. Material isolated on a GFP affinity matrix was then probed for T7 reactivity and the resulting pattern clearly indicated that SENP1cD is modified by both SUMO1 and SUMO2 (Fig. 2A, lanes 8 and 9). SENP2cD is targeted robustly by SUMO2 (Fig. 2A, lane 12). Lack of SUMO1-modified SENP2cD is not due to secondary problems with the sample as a slower migrating form of SENP2cD is evident when the same samples are probed for the GFP moiety (Fig. 2A, lane 22–24), but may be limited by levels of exogenous SUMO1. Overall, these results underscore
that SUMO modification of both SENP1 and SENP2, but not SENP3 (Fig. 1C), is observed when the catalytic activity of these proteases is inactivated.

We further investigated phenotypes that result from overexpression of wild-type or mutant SENP1 and SENP2 by tracking RanGAP, the major SUMO1-modified protein in the cell. Expression of exogenous SENP1, but not SENP2, resulted in desumoylation of RanGAP (Fig. 2B, lane 2 vs. 4, upper left part). Similarly, SUMO-RanGAP associated with SENP1\textsubscript{CD}, but not SENP2\textsubscript{CD} (Fig. 2B, lane 8 vs. 10, upper left part). In general, SUMO1 modified targets associate more robustly with SENP1\textsubscript{CD} than with SENP2\textsubscript{CD} (Fig. 2B, lane 18 vs. 20, upper part; note that the two prominent bands are likely sumoylated RanGAP and GFP-SENP1CD itself). These interactions correspond to substrate specificities, as SENP1 can broadly target the SUMO paralogues, whereas SENP2 is more specific for SUMO2/3. The recovery of modified Nup153 with both SENP1\textsubscript{CD} and SENP2\textsubscript{CD} (Figs. 1B and 2B, lanes 8 and 10, middle part) is consistent with the observation that Nup153 is modified by SUMO2/3 (Fig. S3), which is targeted by both proteases when they are enzymatically active.
Notably, Nup358/RanBP2 is also recovered with SENP1<sub>CD</sub> and SENP2<sub>CD</sub> (Fig. 2B), indicating that these proteases may normally turnover a transient modification of this nucleoporin as well, perhaps in this case related to its SUMO E3 activity.

Nup153 contains two independent regions that interact with both SENP1 and SENP2. Nup153 has been previously reported to interact via its FG-rich C-terminal domain with SENP2<sup>20,26</sup>. This interaction was mapped to the N-terminal, pore targeting region of SENP2<sup>21,22</sup> which shares only limited similarity to the non-catalytic region of SENP1<sup>23</sup>. We therefore took an unbiased approach to characterizing the Nup153-SENP1 association and first tested the three main domains of Nup153 for recovery with GFP-SENP1. Both the N-terminal region of Nup153 and the C-terminal domain co-isolated with SENP1 (Fig. 3A, lanes 9 and 10), whereas the zinc finger domain of Nup153 did not (Fig. 3B, lane 6). While the interaction between SENP2 and the C-terminal domain was observed as expected, the N-terminal domain of Nup153 was also recovered with GFP-SENP2 (Fig. 3A, lanes 11 and 12), albeit a less robust recovery of Nup153-N than Nup153-C with both SENP1 and SENP2.

To gain further insight into these two regions of interaction, the N- and C-terminal domains of Nup153 were co-expressed with GFP fusions of wild-type and catalytically dead SENP1 and SENP2. As expected from our previous domain analysis (Fig. 1), when desumoylation is blocked by expression of catalytically inactive SENP1 or SENP2, a population of the N-terminal region there are distinctions: the proximal end of the C-terminal domain contains FG linkers that are rich in charged residues facilitating 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 charged residues when compared with the FG linkers at the distal end (Fig. 1A). We therefore looked at the interactions between SENP1/2 and the C-terminal region of Nup153 in more detail. In the case of SENP2, interaction with the C-terminal region of Nup153 has been shown to be mediated by Importin α/β<sup>23</sup> similar to requirements for the tethering of Ulp1p to the nuclear pore in yeast.<sup>24,25</sup> While sequence determinants within the SUMO proteases critical for recognition by these transport factors are characterized,<sup>26,27</sup> specific requirements for the interaction on the nucleoporins have not been delineated. The most prominent feature of the C-terminal domain of Nup153 is its FG rich nature, a property shared by several nucleoporins that in general 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 charged residues when compared with the FG linkers at the distal end (Fig. 1A). In addition, a specific binding site for Importin α has been mapped to the terminal residues (Fig. 1A, underlined).<sup>28</sup> To test the contribution of these features, we engineered four additional constructs. These encompassed the proximal (875–1,262) and distal (1,263–1,475) regions of the Nup153 C-terminal domain, as well as deletion of the Importin α binding site in the context of the full C-terminal domain (Δ, 875–1,457) and the distal fragment (distal<sup>Δ</sup>, 1,263–1,457). This panel of GFP fusion proteins was expressed in HeLa cells and then recovered and analyzed for association of endogenous SENP1 and SENP2. As shown in Figure 6B, the C-terminal Nup153 domain and its proximal and distal ends all interact to some degree with SENP1 and SENP2; however, the interaction of SENP1/SENP2 with the distal region

**Figure 3.** Nup153 has a bimodal interaction with SENP1<sub>CD</sub> and SENP2<sub>CD</sub>. Flag-tagged fusion proteins of Nup153 N- and C-terminal domain (A) or zinc finger domain (B) were coexpressed with GFP<sup>+</sup> (1) or GFP fusion proteins of SENP1<sup>Δ</sup> (SP1), catalytically dead SENP1<sup>ΔK353R</sup> (SP1<sup>Δ</sup>) or SENP2<sup>Δ</sup> (SP2) in HeLa cells as indicated. After 24 h, GFP proteins and associated factors were recovered from cell lysates followed by western analysis using antibodies against Flag and GFP. Asterisk indicates truncated GFP-fusion products, as described in Figure 1 legend. Arrow indicates sumoylated GFP-SENP1<sub>ΔK353R</sub>.
is consistently more robust than the interaction with the proximal region (Fig. 6, lanes 10 and 12). 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 (Fig. 6B, lanes 9 and 11). The distinctions seen in binding to Nup153-C-terminal constructs were not explained by differences in localization (all are primarily cytoplasmic) nor are there signs of aggregation that would indicate differences in accessibility (Fig. S4).

These observations collectively suggest that one facet of the interaction between Nup153 and SENP1/SENP2 is an interaction that takes place at a discrete site at the C-terminal tail of Nup153.

Link between Nup153 and SENP1 level distinguishes SENP1 and SENP2. While this biochemical analysis demonstrates similarities in the requirements for partner-ship between Nup153 and both SENP1 and SENP2, we noticed that Nup153 depletion elicits a distinct phenotype with respect to these proteases. Namely, when Nup153 levels are robustly depleted by siRNA, SENP1 but not SENP2 levels are concomitantly reduced (Fig. 7, lane 2). This effect was also observed at the level of immunofluorescence detection (Fig. S1) and was further substantiated by quantitative analysis of additional independent experiments (Fig. 7B), which showed a 42% reduction in SENP1 levels, whereas...
SENPS2 levels were not significantly different following Nup153 depletion. As the link to Nup153 levels may be confounded by additional effects at the nuclear pore basket, we also tracked and individually depleted two other nucleoporins that localize to this substructure of the nuclear pore, including Tpr, which is characterized as an integral and functional unit of the pore basket. Depletion of Nup153 to this degree resulted in some reduction of Tpr levels, as has been previously observed, and Nup50, whereas abundance of the transport factors Importin α and Importin β were unaffected (Fig. 7A, lane 2). To determine whether the reduced levels of SENP1 under these conditions are attributed to reduction of other basket components, Tpr and Nup50 were individually targeted for depletion. In these cases, we did not see a concomitant reduction of Nup153, although such an effect has been observed under some circumstances. The reduced levels of Tpr or Nup50 did not alter SENP1 levels, underscoring that SENP1 levels are not dependent on the general nuclear pore basket structure, but rather are sensitive specifically to the presence of Nup153. Moreover, SENP2 differs in this dependence. Thus, although these two SUMO proteases share many similarities, both their paralogue specificity and differential sensitivity to Nup153 levels underscore biological specialization.

Discussion

This study has revealed several new features of the association between the nuclear pore protein Nup153 and SUMO proteases. First, we have found that this interaction takes place with SENP1 as well as the previously characterized association with SENP2. We also established that, in both cases, there is a dual interaction platform on Nup153. The C-terminal domain of Nup153 had been previously identified as a binding site for SENP2. Here, we defined a specific region (residues 1,458–1,475) to be especially critical for this interaction and further found this site to associate similarly with SENP1. Under the experimental conditions here, we discovered an additional interaction with the N-terminal domain of Nup153. This interface may have escaped previous detection as SUMO modification of the N-terminal domain contributes to recognition by SENP1 and SENP2, making its detection more readily revealed when SUMO protease activity is inhibited. The C-terminal domain of Nup153, in contrast, is not targeted for sumoylation and its interaction with SENP1 and SENP2 is independent of their protease activity.

The ~600 amino acid Nup153 C-terminal domain is characterized overall by FG sequence motifs, which are important as interaction sites for karyopherins (transport receptors). Although the sequence most critical for SENP1 and SENP2 association does not contain FG repeats, this short tail of Nup153 (17 amino acids) nonetheless associates with the karyopherins Importin α and Importin β (data not shown). Roles for transport receptors as specialized bridges to the NPC, rather than solely transporting cargo through the NPC, has been demonstrated for SENP2, as well as the yeast SENP1/SENP2 homolog, Ulp1. Our results confirm this paradigm, and moreover point to a non-canonical (FG-independent) site as the tethering point of this bridge for SENP1/2. A similar sequence at the tail of yeast Nup1p—a protein that like Nup153 bears FG motifs and is localized to the nuclear pore basket—brings up the interesting possibility that this is an evolutionarily conserved aspect of the interface between SUMO proteases/karyopherins and the NPC.

The two regions of Nup153 that are sites of interaction with SENP1 and SENP2 are separated by over a thousand amino
acids (sumoylation at amino acid 353 vs. the distal tail of Nup153 starting at amino acid ~1,460; Fig. 5A). Although the structure of Nup153, with the exception of its zinc finger motifs, is not known, an important feature of this protein that has been characterized is its inherent flexibility, notably of the C-terminal, FG-rich domain. This suggests that these two interfaces may be brought into close proximity. For example, docking of SENP1 at the C-terminal tail of Nup153 may facilitate intramolecular recognition of the N-terminal region of Nup153.

Our results have also revealed that Nup153 is dynamically sumoylated, as knockdown of SENP1 and SENP2 result in sumoylated Nup153. Several other proteins in the vicinity of the nuclear pore, including two previously reported to be sumoylated, Lamin A\(^\text{\textregistered}\) and Importin \(\beta\), were not noticeably modified under these conditions. Although this does not rule out changes in the sumoylation status of these proteins, it does underscore a level of specificity. The cycle of sumoylation on Nup153 may contribute to localization of SENP1 and SENP2 to the vicinity of the nuclear pore, but also raises new questions about how sumoylation of Nup153 affects its other interactions and roles. The basis of SENP1 and SENP2 interaction with sumoylated Nup153 is not yet understood. The catalytic domains of SENP1 and SENP2 are known to bind SUMO with high affinity. In addition, the noncatalytic N-terminal domains of SENP1 and SENP2 each harbor a consensus SUMO interaction motif (SIM) expected to interact with SUMO moieties. This raises the possibility that there are two modes of interaction at the Nup153 N-terminal region: one in which a SIM within SENP1/2 is engaged and one facilitated by SUMO recognition and its consequent cleavage by the catalytic domain. These could be alternate or sequential steps in binding. The influence of the SUMO status of the SENP itself on the SENP-Nup153 association (Fig. 5) also suggests a regulatory mechanism that warrants further investigation. Sumoylated Nup153 was reported in a systematic analysis of sumoylation changes in response to heat shock. Interestingly, levels of SUMO-modified Nup153 decreased significantly following heat shock. Our results here suggest that modulation of the interaction between Nup153 and SENP1/2 could provide an explanation for this shift in the balance of sumoylation-desumoylation.

It has been reported that forced cytoplasmic localization of SENP2 results in its ubiquitin-mediated degradation.\(^{44}\) Ulp1 depends on multiple nucleoporin binding partners for its NPC localization and genetic disruption of several nucleoporins also leads to destabilization of Ulp1.\(^{45}\) Interactions with additional nucleoporins have recently been reported for SENP2 and shown to contribute to NPC targeting. SENP1 may similarly depend on more than one nucleoporin for NPC targeting, but the influence of Nup153 on SENP1 levels indicates that Nup153 may be of particular importance for maintenance of SENP1. It will be of interest to determine if this cross-talk is at the level of protein stability or an earlier event such as SENP1 transcription. This influence of Nup153 on SENP1 levels prompts us to speculate that Nup153 is not purely a substrate for this enzyme, but also plays an active role in its targeting and biology. Finally, the differential response of SENP1 and SENP2 to changes in Nup153 levels underscores that, although these enzymes share many properties, including the bimodal interface with Nup153 described here, their targets, regulation and overall roles are not identical.

**Experimental Procedures**

**Plasmid constructs and antibodies.** SENP1 (in pEGFP-C2), SENP2 (in pEGFP-N2), SENP3 (in pEGFP-C3) and Nup153 N-terminal domain in pEGFP-N2 (Clontech) and p3xHA (made by replacing the eGFP coding sequence within pEGFP-N2 with three repeats of HA tag coding sequence) were subjected to site-directed QuickChange mutagenesis (Stratagene) to generate catalytically dead mutants of SENP1 (C602S), SENP2 (C549S), SENP3 (C532S), and the SUMO mutant Nup153 N-terminal domain (K353R). Other Flag and GFP fusion constructs of SENP1 and the Nup153 N-(1–657), zinc finger (658–879) and C-(880–1,475)-terminal domains were cloned in p3xFLAG-CMV (Sigma) and pEGFP-N2 plasmids. Nup153

Figure 7. SENP1 levels are sensitive to the abundance of Nup153. (A) HeLa cells were treated with siRNA against Nup153, a corresponding scrambled oligo (Scm) or siRNAs that target Tpr, Nup50, SENP1 or SENP2 for 48 hr. Cell lysates were then subjected to western analysis, here loaded following normalization with an internal control, using antibodies against indicated proteins. The arrows indicate specific bands. (B) Similarly treated HeLa cells were used to assess levels of SENP1 and SENP2 quantitatively. Equal protein levels were run on gels for quantitative detection, numbers were adjusted normalized to an internal standard (actin), and then graphed relative to the control (Scm), with an average and standard deviation shown. SENP1 (n = 4) was significantly reduced (p < 0.0001) only when Nup153 was depleted; SENP2 levels (n = 3) did not correlate with changes in the nucleoporin expression.
C-terminal domain truncation mutants: C (875–1,457), C-distal (1,263–1,457), C-distal (1,263–1,457) and C-proximal (875–1,262) were all constructed in pGFP-N2 plasmid. Full-length SUMO1 and SUMO3 with an N-terminal T7 tag were cloned into pCXN3-MycHisB plasmid (Invitrogen). Antibodies against SENP1, SENP2, SENP3, NUP153, SUMO2/3 and Nup50 were generated in the Ullman and the Dasso laboratories. The hybridoma 12CA5 was used for anti-HA antibody. Nup53 antibody (SA1) is a gift from Brian Burke.8 Other antibodies were commercially available (TMR (Bethyl HIC-00099), mAb414 (Covance MMS-120P), T7 (Novagen 69522), GFP (Clonetech JL-8 633820), Importin β (BD Biosciences 610485) and Flag (Sigma M2 F1804).

Plasmid transfection and generation of stable Hela cell lines expressing T7-tagged SUMO1/3. All plasmid transfection were performed using Lipofectamine LTX (Invitrogen) according to manufacturer instruction. For generation of stable lines, T7-tagged SUMO1 and SUMO3 expressing constructs were transfected into Hela cells. Twenty four hr post transfection, cells were then selected with medium containing 600 μg/mL Geneticin (Invitrogen) for 1 week. Geneticin resistant clones were isolated and screened for uniform expression of T7-tagged SUMO1/SUMO3 using indirect immunofluorescence and western analysis. Stable transgenic clones were then maintained in media containing 200 μg/mL Geneticin.

siRNA depletion of SENP1, SENP2, Nup53 and Nup50 siRNA oligos against control16,17,18,19,20 were transfected into Hela cells (in 10 mM final concentration) for 48 h using Lipofectamine RNAi MAX (Invitrogen) as previously described in Mackay et al.21 Scrambled siRNA oligo used in Figure 7 is a scrambled sequence based on the Nup53 siRNA oligo.18

GFP affinity trap and co-recovery analysis. Hela cells were seeded with lysis buffer (1x PBS, 1% NE-40, 40 mM β-Glucosidase, 10 μM Sodium Orthovanadate, 2x COMPLETE protease inhibitor, 40 mM N-Ethylmaleimide). Cell lysates were later incubated with protein-G beads, with Nup53 antibody (SA1) pre-immobilized, for 4 h at 4°C. Beads were subsequently washed 3 times with the above lysis buffer (without N-Ethylmaleimide). Proteins were eluted with SDS loading buffer and subjected to western analysis.

Quantification of SENP1 and SENP2 levels upon depletion of nucleoporins. Hela cells were treated with siRNA oligo against Scn, Nup53, TPR, Nup50, SENP1 and SENP2 as described above. The harvested cell lysates were subjected to western analysis using the Odyssey infrared imaging system (Li-Cor) according to the manufacturer’s instruction. SENP1 and SENP2 levels were normalized against actin levels after background adjustment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: www.landesbioscience.com/journals/nucleus/article/20902

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