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Molecular Biology of the Cell
Vol. 20, 410–418, January 1, 2009

RanBP2 and SENP3 Function in a Mitotic SUMO2/3 Conjugation-Deconjugation Cycle on Borealin

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Submitted May 22, 2008; Revised October 8, 2008; Accepted October 14, 2008
Monitoring Editor: Yixian Zheng

The ubiquitin-like SUMO system controls cellular key functions, and several lines of evidence point to a critical role of SUMO for mitotic progression. However, in mammalian cells mitotic substrates of sumoylation and the regulatory components involved are not well defined. Here, we identify Borealin, a component of the chromosomal passenger complex (CPC), as a mitotic target of SUMO. The CPC, which additionally comprises INCENP, Survivin, and Aurora B, regulates key mitotic events, including chromosome congression, the spindle assembly checkpoint, and cytokinesis. We show that Borealin is preferentially modified by SUMO2/3 and demonstrate that the modification is dynamically regulated during mitotic progression, peaking in early mitosis. Intriguingly, the SUMO ligase RanBP2 interacts with the CPC, stimulates SUMO modification of Borealin in vitro, and is required for its modification in vivo. Moreover, the SUMO isopeptidase SENP3 is a specific interaction partner of Borealin and catalyzes the removal of SUMO2/3 from Borealin. These data thus delineate a mitotic SUMO2/3 conjugation–deconjugation cycle of Borealin and further assign a regulatory function of RanBP2 and SENP3 in the mitotic SUMO pathway.

INTRODUCTION

The ubiquitin-like SUMO system is involved in the regulation of several cellular key processes, including transcriptional control, DNA repair, and recombination (Gill, 2004; Muller *et al.*, 2004; Hay, 2005). The unifying theme of SUMO function in these pathways seems to be the SUMO-dependent regulation of specific protein–protein interactions (Geiss-Friedlander and Melchior, 2007). In humans, at least three SUMO forms (SUMO1, 2, and 3) are expressed. SUMO2 and SUMO3 are highly related proteins sharing an identity of 97% (we therefore refer to them as SUMO2/3), whereas SUMO1 shares 43% identity with SUMO2/3. The covalent attachment of SUMO to a lysine residue within a target protein is catalyzed by an enzymatic cascade involving the E1-activating enzyme (Aos1/Uba2), the E2-conjugating enzyme Ubc9, and, at least in some cases, additional E3 ligases, such as protein inhibitor of activated signal transducers and activators of transcription (PIAS) family members, human Polycomb protein 2 (hPc2), and Ran binding protein 2 (RanBP2) (Pichler *et al.*, 2002; Kagey *et al.*, 2003; Schmidt and Muller, 2003). Although PIAS-mediated sumoylation has been observed on many substrates, only recently a first in vivo substrate for RanBP2-dependent sumoylation has been identified (Dawlaty *et al.*, 2008).

Importantly, SUMO modification is a highly dynamic and reversible process. The abundance of a given SUMO-conjugate is dynamically regulated by a balance between SUMO modification mediated by the E1, E2, and E3 enzymes and demodification, which is governed by SUMO-specific isopeptidases of the ubiquitin-like protease (Ulp/SENP) family (Hay, 2007; Mukhopadhyay and Dasso, 2007). In humans, six members of this family, termed SENP1-3 and SENP5-7, have been identified so far. A characteristic feature of distinct SENPs is their distribution to specific subcellular regions, indicating that their activity is spatially regulated.

Data from genetic studies in yeast indicate that a balanced equilibrium of SUMO conjugation and deconjugation is critical for mitotic events, including mitotic entry and proper chromosome segregation during anaphase (Watts, 2007; Dasso, 2008). Recent work has also revealed the importance of sumoylation for mitotic progression in mammalian cells and has delineated a critical role of SUMO in kinetochore/centromere function (Di Bacco *et al.*, 2006; Dawlaty *et al.*, 2008; Zhang *et al.*, 2008). However, in higher eukaryotes, only few mitotic targets of SUMO and regulatory components of the SUMO pathway are known.

Progression through mitosis is tightly coordinated by mitotic kinases. The kinase Aurora B is important for key mitotic events, including chromosome congression, the spindle assembly checkpoint, and cytokinesis (Meraldi *et al.*, 2004; Giet *et al.*, 2005; Ruchaud *et al.*, 2007). Aurora B is part of a multiprotein complex, termed the chromosomal passenger complex (CPC), which additionally comprises INCENP, Survivin, and Borealin (also known as DasraB; Sampath *et al.*, 2004). The CPC binds to the centromere in prometaphase and metaphase, relocates to the central spindle at the onset of anaphase, and accumulates at the midbody during telophase and cytokinesis. Accordingly, phosphorylation targets of Aurora B have been identified at each of these sites (Zeitlin *et al.*, 2001; Andrews *et al.*, 2004; Ohi *et al.*, 2004; Neef *et al.*, 2006). The components of the CPC are physically and functionally interdependent in that depletion of any one subunit by RNA interference abolishes correct localization of the others and inhibits proper CPC function (Honda *et al.*, 2003; Gassmann *et al.*, 2004; Jeyaprakash *et al.*, 2007).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–05–0511) on October 22, 2008.

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Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2008/10/22/E08-05-0511.DC1

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Recent work has underscored the importance of post-translational modification for proper activity and accurate spatiotemporal control of the CPC. For example, phosphorylation of Borealin by Mps1 is crucial for Aurora B activity and chromosome alignment (Jelluma *et al*., 2008). Moreover, the ubiquitin system regulates the dynamic association of passenger proteins with the centromere and chromatin (Vong *et al*., 2005; Ramadan *et al*., 2007).

Here, we report that Borealin is dynamically modified by SUMO2/3 during mitosis. We identify RanBP2 as an essential regulator of the conjugation process and SENP3 as the SUMO protease-catalyzing deconjugation.

MATERIALS AND METHODS

Cell Culture, Transfection and Western Blotting

HeLaS3 or COS-7 cells were grown under standard conditions. Plasmid transfections were performed using FuGENE 6 reagent (Roche Diagnostics, Mannheim, Germany). The following antibodies were used for Western blotting: anti-SUMO1 (clone 21C7; Zymed Laboratories, South San Francisco, CA), anti-SUMO2/3 (clone 1E7, MBL, Woburn, MA), anti-ubiquitin (Epitomics, Burlingame, CA), anti-PIAS2 (clone 116, Sigma Chemie, Deisenhofen, Germany), anti-RanBP2 (gift of Frauke Melchior, University of Göttingen, Göttingen, Germany), anti-Ubc9 (clone 50; BD Biosciences, San Jose, CA), anti-SENP5 (gift of Ed Yeh, MD Anderson Cancer Center, Houston, TX), anti-cyclin B1 (BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-Eg5 (Blangy *et al*., 1995), anti-PML (rabbit polyclonal; Millipore Bioscience Research Reagents, Temecula, CA), anti-topoisomerase II (clone 1C5; Assay Designs, Ann Arbor, MI), anti-hemagglutinin (HA) (clone 16B12; Covance Research Products, Princeton, NJ), goat anti-mouse immunoglobulin G (IgG) (Invitrogen, Carlsbad, CA). Anti-SENP3 antibodies have been described previously (Haindl *et al*., 2008). All other antibodies have been described previously (Klein *et al*., 2006).

Cloning and Mutagenesis

The cDNAs for chromosomal passenger proteins have been described previously (Klein *et al*., 2006) and were cloned into pcDNA3.1 (Invitrogen) encoding a 3xmyc tag. For His-tagging and HA-tagging, Borealin was cloned into pcDNA3.1 encoding a 6xHis-tag or a HA-tag, respectively. Myc-PARP1 and myc-RanBP2$^{\Delta FG}$ were cloned into pCI (Invitrogen). SUMO constructs have been cloned into pSG5 (Stratagene, La Jolla, CA) for His-tagging or pcDNA3.1 for HA-tagging. SENPs were cloned into pCI (Invitrogen) for FLAG-tagging. Site-directed mutagenesis was carried out using the QuickChange mutagenesis kit (Stratagene).

RNA Interference (RNAi) Sequences

mRNAs were targeted as follows: Ubc9, 5′-GCAGAGGCCUACACGAUUU dTdT-3′; and Eg5, 5′-CUAGAUGCCUUUCUCAGUAdTdT-3′. Small interfering RNA (siRNA) oligonucleotides for RanBP2, PIAS2 (targeting the α and β isoforms), Borealin, SENP3, and SENP5 have been described previously (Joseph *et al*., 2004; Yang and Sharrocks, 2005; Klein *et al*., 2006, Haindl *et al*., 2008).

Yeast Two-Hybrid Assay

cDNAs encoding the respective prey or bait proteins were cloned in frame with the GAL activation domain of pACT2 or pGAD vectors or the GAL binding domain of pFBT9 or pGBD vectors and tested for self-activation. Directed yeast two-hybrid assays were performed as described previously (Klein *et al*., 2006).

In Vitro Sumoylation and Desumoylation

Proteins were generated by in vitro transcription/translation in the presence of ^{35}S-labeled methionine using the TNT Quick Coupled T7 kit (Promega, Madison, WI). Sumoylation was carried out using the Sumoylation control kit (LAE Biotech, Rockville, MD). In experiments using recombinant RanBP2$^{\Delta FG}$ (BIOMOL Research Laboratories) the amount of Aos1/Uba2 and Ubc9 was reduced to 100 and 70 nM, respectively. In vitro demodification assays were done as described previously (Haindl *et al*., 2008). In vitro processing activity of SENP3 and SENP5 was assayed on N-terminal FLAG-tagged SUMO2 full-length, C-terminal fused to the HA-tag (FLAG-SUMO2-HA).

In Vivo Sumoylation

After transfection of respective constructs, HeLaS3 or COS-7 cells were incubated for 48 h and nickel-nitrilotriacetic acid (Ni-NTA) precipitations were done as described previously (Muller *et al*., 2000). Where indicated, siRNA duplexes were transfected simultaneously.

Cell Cycle-dependent Sumoylation

HeLaS3 cells were transfected with His-tagged Borealin and incubated for 30 h. Thymidine or taxol was added at a concentration of 2 mM and 1 μM, respectively, and cells were incubated for an additional 16 h. Taxol-arrested cells were released into 10 μM MG132 (Calbiochem, San Diego, CA) for 1 h to obtain metaphase cells. To harvest anaphase/telophase cells, corresponding cell populations were released from this block for 25 and 50 min, respectively. Cell lysates and Ni-NTA pull-downs were performed as described above. For the experiment in Supplemental Figure 1, cells where arrested by thymidine or taxol as outlined above, and MG132 was added at 10 μM for 2 h before lysate preparation.

Coimmunoprecipitation

To immunoprecipitate endogenous Borealin, HeLaS3 cells were harvested by mitotic shake-off after 16-h taxol treatment (1 μM). To test for a Borealin-SENP3 interaction, COS-7 cells were simultaneously transfected with myc-tagged Borealin and FLAG-tagged SENP3 constructs and immunoprecipitations were done as described previously (Klein *et al*., 2006). To demonstrate endogenous sumoylation HeLaS3 cells were arrested by a 16 h taxol treatment before harvesting. Lysates were prepared in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 10 mM $CaCl_2$, 200 U/ml micrococcal nuclease [MBI Fermentas, Hanover, MD], 10 mM N-ethyl maleimide [Pierce Chemical, Rockford, IL], and protease inhibitors [Complete; Roche Diagnostics, Basel, Switzerland]) and subjected to immunoprecipitation with 10 μg of SUMO2/3 antibody or control mouse IgG.

Immunofluorescence Microscopy

Immunofluorescence was performed as described previously (Baumann *et al*., 2007) with the following primary antibodies: PRC1 (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG-M2 (Sigma Chemie), RanBP2 (a kind gift of Frauke Melchior), HA (clone 16B12; Covance Research Products), and CENP-E (Santa Cruz Biotechnology). All other antibodies have been described previously (Klein *et al*., 2006). Secondary antibodies were Cy2/Cy3-conjugated donkey antibodies (Dianova, Hamburg, Germany).

Gene Expression Analysis of RanBP2 and Phosphorylation of SENP3

Analysis of mRNA levels by quantitative reverse transcription-polymerase chain (RT-PCR) reaction were done as described previously (Graser *et al*., 2007). For normalization, expression of β-glucuronidase was measured. To monitor RanBP2 protein level, lysates were prepared from asynchronous growing HeLaS3 cells or HeLaS3 cells released from a double thymidine block into nocodazole (0-min time point). To determine protein levels at later stages of mitosis, cells were released from the nocodazole block for different time points. To study phosphorylation of SENP3, lysates were prepared from asynchronous growing HeLaS3 cells or HeLaS3 cells arrested by nocodazole or thymidine. For dephosphorylation the lysates were treated with 500 U of λ phosphatase (New England Biolabs, Ispwich, MA) or for control reactions λ phosphatase together with phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate).

RESULTS

The CPC Subunit Borealin Is Covalently Modified by SUMO

To obtain insight into the role of the Borealin subunit of the CPC, we screened a yeast two-hybrid HeLa cDNA library with full-length Borealin as the bait and identified two components of the ubiquitin-like SUMO modification system, the E2-conjugating enzyme Ubc9, and the modifier SUMO1 as interaction partners of Borealin. Directed yeast two-hybrid assays confirmed these interactions and additionally revealed an interaction of Borealin with SUMO2 (Figure 1, A and B). The other known members of the CPC—INCENP, Aurora B, and Survivin—did not bind Ubc9 or SUMO forms (Figure 1A; data not shown). The binding of Borealin to both SUMO paralogues was dependent on the integrity of their C-terminal double-glycine motif, which is essential for conjugation, suggesting that SUMO is covalently attached to Borealin (Figure 1B, bottom two panels).

To test this hypothesis, we used a reconstituted in vitro SUMO modification system. ^{35}S-labeled myc-tagged Borealin, generated by in vitro transcription/translation, was incubated with recombinant components of the sumoylation

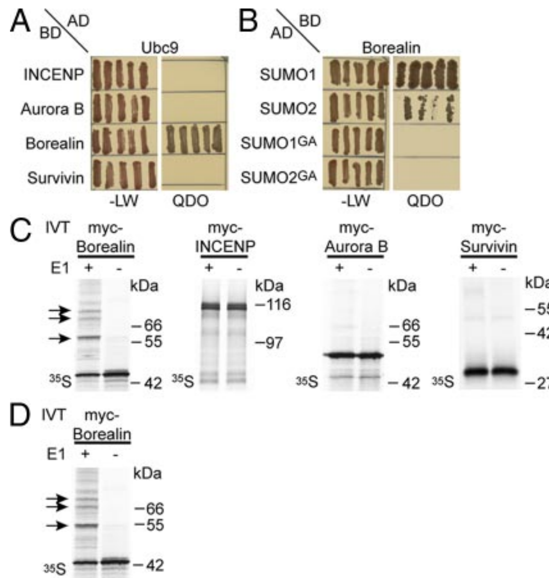

Figure 1. Borealin interacts with components of the SUMO system and is modified by SUMO in vitro. (A and B) Interaction of Borealin with Ubc9 (A) and the conjugatable forms of SUMO1 and SUMO2 (B, top two rows), but not the unconjugatable forms ($SUMO^{GA}$) (B, bottom two rows) in directed yeast two-hybrid assays. −LW indicates plates lacking leucine and tryptophane, whereas QDO indicates plates lacking leucine, tryptophane, histidine, and adenine. (C and D) Borealin is modified by SUMO in vitro. ^{35}S-Labeled CPC subunits, generated by in vitro transcription/translation, were incubated with recombinant E1, E2, and either SUMO1 (C) or SUMO2 (D) in the presence of ATP. In control reactions, the E1 enzyme was omitted. SUMO–Borealin conjugates are indicated by arrows.

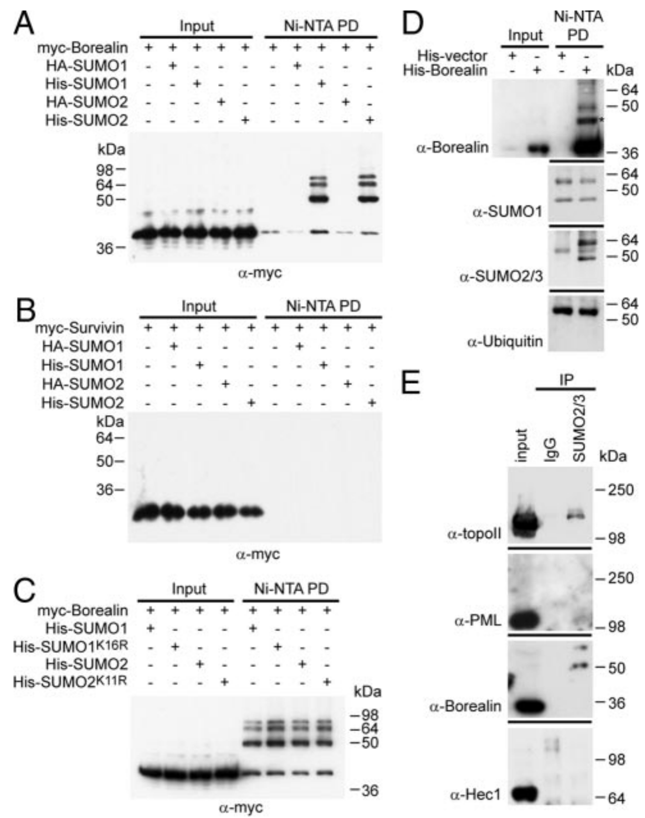

Figure 2. Borealin is modified by SUMO in vivo and is preferentially targeted by SUMO2/3. (A) Myc-tagged Borealin and HA- or His-tagged versions of SUMO1 or SUMO2, respectively, were coexpressed in COS-7 cells. His-SUMO conjugates were recovered on Ni-NTA beads and subjected to Western blotting by using anti-myc antibody. (B) Myc-Survivin was tested for sumoylation as described in A. (C) Myc-tagged Borealin and either wild-type ($SUMO1^{wt}$, $SUMO2^{wt}$) or mutant versions of His-tagged SUMO forms ($SUMO1^{K16R}$, $SUMO2^{K11R}$) were coexpressed in COS-7 cells and analyzed as described in A. (D) Borealin is modified by endogenous SUMO2/3, but not SUMO1. HeLa cells expressing His-tagged Borealin were arrested in prometaphase by taxol treatment for 16 h. His-Borealin was recovered on Ni-NTA beads, and immunoblotting was performed with anti-Borealin, anti-SUMO1, anti-SUMO2/3, or anti-ubiquitin antibodies. The Borealin-reactive bands at ~45 kDa (asterisk) are interpreted as a Borealin–SUMO2/3 degradation product. (E) Endogenous Borealin is conjugated to SUMO2/3. Immunoprecipitations were performed with anti-SUMO2/3 or control IgGs from taxol-arrested HeLa cells and probed by Western blotting with the indicated antibodies.

machinery, i.e., the E1 activating enzyme Aos1/Uba2, the E2 conjugating enzyme Ubc9, and either SUMO1 or SUMO2 in the presence of ATP. In the control reaction, which lacked the E1 enzyme, a single major Borealin band, migrating at the predicted size of 42 kDa was detected (Figure 1C). In contrast, addition of the E1 enzyme to the reaction resulted in the formation of at least three distinct higher-molecular-weight Borealin conjugates both with SUMO1 (Figure 1C) and SUMO2 (Figure 1D). The major conjugates migrated at ~50, ~64, and ~75 kDa, being consistent with the attachment of up to three SUMO moieties to Borealin. In line with the finding that only Borealin but no other chromosomal passenger protein interacted with SUMO pathway components in the yeast two-hybrid assay, neither Survivin, nor Aurora B and INCENP were significantly modified by SUMO (Figure 1C).

To examine whether SUMO paralogues can also modify Borealin in vivo, a myc-tagged construct of Borealin was coexpressed with either HA or His-tagged constructs of SUMO1 or SUMO2. His-SUMO-conjugates were affinity purified under denaturing conditions on Ni-NTA beads. Consistent with the result obtained in the in vitro sumoylation assay, Western blotting with an anti-myc antibody detected three SUMO-Borealin species in His-SUMO1 and His-SUMO2 pull-downs (Figure 2A). Survivin, a direct binding partner of Borealin within the CPC (Gassmann *et al*., 2004; Klein *et al*., 2006), was neither conjugated to SUMO1 nor SUMO2 under these conditions (Figure 2B). SUMO mutants ($SUMO1^{K16R}$ and $SUMO2^{K11R}$), where potential residues for chain formation were mutated (Hay, 2005), generated an identical pattern of SUMO–Borealin conjugates (Figure 2C), arguing for sumoylation of distinct lysine residues within Borealin. To determine whether Borealin is preferentially modified by either SUMO1 or SUMO2/3, when these modifiers are expressed at their endogenous levels, a His-tagged Borealin construct or an empty His-vector control was expressed in HeLa cells and proteins were purified on Ni-NTA beads under denaturing conditions from mitotic cell lysates. Western blotting with an anti-Borealin antibody allowed for the detection of higher-molecular-weight species (Figure 2D), reminiscent of the Borealin–SUMO conjugates described above. When blotted with SUMO2/3-specific antibodies, at least two bands were identified as SUMO2/3 conjugates. By contrast, antibodies directed against SUMO1 or ubiquitin did not specifically detect these bands. To test modification of endogenous Borealin by SUMO2/3, total cellular SUMO2/3-conjugates from mitotic cell lysates cells were immunoprecipitated by anti-SUMO2/3 antibodies

(Figure 2E). We validated the experimental setup by monitoring sumoylation of topoisomerase II (Azuma *et al*., 2003, 2005; Zhang *et al*., 2008), a SUMO2/3 substrate that is specifically conjugated in early mitosis, and PML, a SUMO2/3 substrate that is unmodified at this stage (Everett *et al*., 1999). Consistently, we detected topoisomerase II–SUMO2/3 conjugates, but no PML–SUMO2/3 forms in the mitotic SUMO2/3 precipitates. Importantly, anti-Borealin–reactive forms migrating at 50 and 64 kDa were specifically enriched in the anti-SUMO2/3 pull-down fraction. The amount of Borealin–SUMO2/3 conjugates was comparable to the amount of topoisomerase II–SUMO2/3 species. In summary, these data define the CPC subunit Borealin as a mitotic substrate for sumoylation and indicate that it is preferentially modified by SUMO2/3.

The SUMO E3 Ligase RanBP2 Is Essential for Sumoylation of Borealin

To address whether sumoylation of Borealin is cell cycle regulated, HeLa cells expressing His-tagged Borealin were harvested at different stages of the cell cycle and Borealin-SUMO2/3 conjugates were purified on Ni-NTA beads (Figure 3A and Supplemental Figure 1). In G1-arrested cells, the level of Borealin-SUMO2/3 conjugates was low (Figure 3A, lane 5). Strikingly, the amount was strongly increased in metaphase-arrested cells (Figure 3A, lane 6 and Supplemental Figure 1), before the conjugates were progressively lost as cells entered anaphase (Figure 3A, lane 7 and 8). Thus, we conclude that sumoylation of Borealin is dynamically regulated during cell cycle progression with a peak in early mitosis.

This notion led us to search for regulatory components that control the dynamic modification of Borealin. To this end, candidate SUMO E3 ligases, notably, PIAS family members RanBP2 and hPC2, were analyzed for their ability to interact with Borealin in a yeast two-hybrid system. Binding of Borealin was observed to the α and β splice variants of PIAS2 and the catalytically active fragment of RanBP2 (RanBP2$^{\Delta FG}$) (Pichler *et al*., 2002) (Supplemental Figure 2A). To test for these interactions in mammalian cells, endogenous Borealin was immunoprecipitated from mitotic HeLa cells (Figure 3B). Importantly, RanBP2, but not PIAS2 isoforms, were specifically detected in anti-Borealin precipitates. Attesting to the specificity of this interaction, coimmunoprecipitation was also observed for Aurora B, but not for Hec1, another component of the centromere/kinetochore region. This indicates that RanBP2 can bind to the CPC and thus represents a potential SUMO E3 ligase for Borealin. In line with this assumption, RanBP2 was shown previously to localize to the centromere/kinetochore region and the mitotic spindle (Yokoyama *et al*., 1995; Joseph *et al*., 2002, 2004).

To address a putative SUMO E3 ligase function of RanBP2 toward Borealin, recombinant RanBP2$^{\Delta FG}$ was added to an in vitro sumoylation reaction on Borealin, which was performed under limiting Ubc9 concentrations (70 nM). Under these conditions, basal E1-E2–mediated sumoylation of Borealin was weak (Figure 3C, top, lane 2), but addition of RanBP2$^{\Delta FG}$ in a concentration range of 5 to 50 nM enhanced the formation of the three Borealin–SUMO conjugates (Figure 3C, lanes 4 and 5), whereas it did not stimulate sumoylation of the control substrate p53 (Figure 3C, bottom). In agreement with previous data (Pichler *et al*., 2002), higher concentrations of RanBP2 (500 nM) exert an inhibitory effect on sumoylation (Figure 3C, top, lane 3), probably reflecting a competitive automodification of RanBP2.

To further analyze the role of RanBP2 in the conjugation of SUMO to Borealin in vivo, cells expressing myc-tagged Borealin and His-SUMO2 were depleted of RanBP2 and control proteins by small interfering RNA (siRNA) (Figure 3D). Sumoylation of Borealin was monitored by Ni-NTA pull-down of His-SUMO2 conjugates followed by anti-myc immunoblotting. Remarkably, depletion of RanBP2 resulted in an almost complete loss of sumoylation and reduced the level of Borealin–SUMO conjugates to almost the same extent as knockdown of the essential E2 enzyme Ubc9. In contrast, Borealin sumoylation was unaltered in cells depleted of the α and β forms of PIAS2. Noteworthy, depletion of RanBP2 did not affect the level of total SUMO2/3-conju-

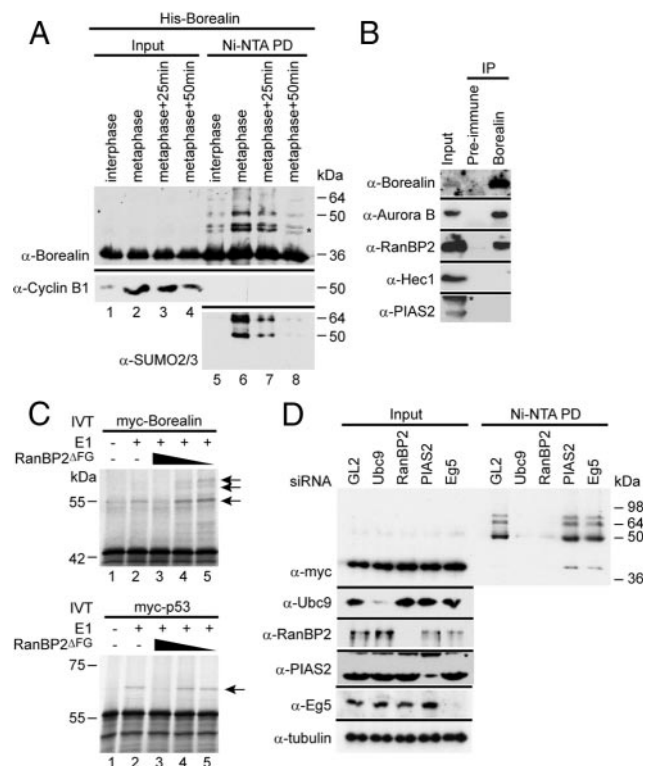

Figure 3. Sumoylation of Borealin is cell cycle regulated and requires RanBP2. (A) Sumoylation of Borealin is cell cycle regulated. HeLa cells expressing His-Borealin were arrested in G1 (lane 1), metaphase (lane 2), or allowed to enter anaphase (lanes 3 and 4). His-Borealin and His-Borealin-conjugates were detected by Western blotting with anti-Borealin and anti-SUMO2/3 antibodies (lane 5–8). Bands marked by asterisk are interpreted as Borealin–SUMO2/3 degradation products. The mitotic status was assayed by monitoring Cyclin B1 levels. (B) RanBP2 binds to the CPC. Immunoprecipitations were performed with rabbit anti-Borealin antibodies or preimmune serum from taxol-arrested HeLa cells and probed by Western blotting with the indicated antibodies. The anti-PIAS2 antibody is directed against the α and β isoforms. Asterisk denotes an anti-PIAS2 cross-reactive band. (C) RanBP2 stimulates sumoylation of Borealin in vitro. ^{35}S-labeled Borealin or p53, generated by in vitro transcription/translation, were subjected to an in vitro sumoylation assay under limiting E2 concentrations. RanBP2$^{\Delta FG}$ was added at a concentration of 500 ng (lane 3), 50 ng (lane 4), and 5 ng (lane 5). SUMO conjugates are indicated by arrows. (D) RanBP2 is required for sumoylation of Borealin in vivo. Myc-tagged Borealin and His-SUMO2 were coexpressed in HeLa cells treated with siRNA oligonucleotides directed against indicated proteins. Depletion was verified by Western blotting. The siRNA directed against PIAS2 targets the α and β isoform (Yang *et al*., 2005). The asterisk in the anti-PIAS2 Western blot denotes a cross-reactive band. His–SUMO2 conjugates were recovered on Ni-NTA beads, and Western blotting was performed with anti-myc antibodies.

gates (Supplemental Figure 3A) or sumoylation of the unrelated control substrate poly(ADP-ribose) polymerase (PARP)1 (Gocke *et al*., 2005) (Supplemental Figure 3B), indicating that RanBP2 does act on specific SUMO target proteins. Furthermore, depletion of Eg5, which, similar to the knockdown of RanBP2 (Joseph *et al*., 2004), leads to a prometaphase-like arrest (Blangy *et al*., 1995), did not influence sumoylation of Borealin.

To address whether the observed loss of Borealin sumoylation can be assigned to the enzymatic activity of RanBP2, HeLa cells were depleted of endogenous RanBP2 and complemented with either wild-type or catalytically inactive RanBP2$^{\Delta FG}$ (Dawlaty *et al*., 2008). Importantly, the wild-type catalytically fragment of RanBP2 (RanBP2$^{\Delta FG\ wt}$), but not the catalytically inactive variant (RanBP2$^{\Delta FG\ AA}$) restored SUMO modification of Borealin (Figure 4A). Moreover, when overexpressed, wild-type RanBP2$^{\Delta FG}$, but not the inactive mutant, lead to a dramatic defect in chromosome segregation after anaphase onset (Figure 4, B–E).

Together, these data show that RanBP2 is essential for SUMO modification of Borealin and suggest that unbalanced RanBP2-mediated SUMO conjugation prevents the equal distribution of genetic material to daughter cells. The critical role of RanBP2 in mitosis is further supported by the observation that its protein level is strongly elevated upon onset of mitosis (Figure 4F). This effect is due to posttranscriptional regulation, because the mRNA level of RanBP2 does not significantly change during cell cycle progression (Figure 4G).

It has been reported very recently that RanBP2-mediated SUMO modification of topoisomerase II is required for its localization to centromeres (Dawlaty *et al*., 2008). Notably, depletion of RanBP2 or Ubc9 did not affect the centromeric localization of Borealin (Figure 5A; data not shown), indicating that centromere targeting of the CPC occurs independently of Borealin sumoylation. To rule out that this result is due to residual amounts of RanBP2 (or Ubc9) left after corresponding siRNA treatment, we created a nonsumoylatable variant of Borealin. Because we were unable to assign one or several specific lysine residues of Borealin as attachment sites for SUMO, we had to mutate all its 25 lysine residues to arginine to abolish sumoylation (Supplemental Figure 4, A–D). During mitosis, HA-Borealin^{25KR} exhibits wild-type localization to the centromere, rescued the phosphorylation of the Aurora B substrate CENP-A and bound to the central spindle in anaphase cells (Figure 5B). In line with the notion that the CPC requires the Borealin subunit to target to its distinct locations (Klein *et al*., 2006, Jeyaprakash *et al*., 2007) HA-Borealin^{25KR} associated with all other CPC components (Figure 5C). Interestingly, this mutant fails to exhibit normal localization to the nucleolus in interphase cells depleted from endogenous Borealin (Supplemental Figure 4E). However, this effect is unlikely due to the loss of SUMO modification, because the general inhibition of sumoylation did not affect the nucleolar localization of wild-type Borealin (Supplemental Figure 4F).

These data suggest that complex formation of the CPC and localization of the complex to the centromere and central spindle during mitosis occur independently of sumoylation. Consistent with this idea, general abrogation of SUMO2/3 conjugation by expression of SENP2 (Zhang *et al*., 2008) did not affect localization of Borealin and CPC activity (Supplemental Figure 5, A and B).

SENP3 Catalyzes Desumoylation of Borealin

We next asked whether one of the known SUMO-specific isopeptidases might be involved in the desumoylation of

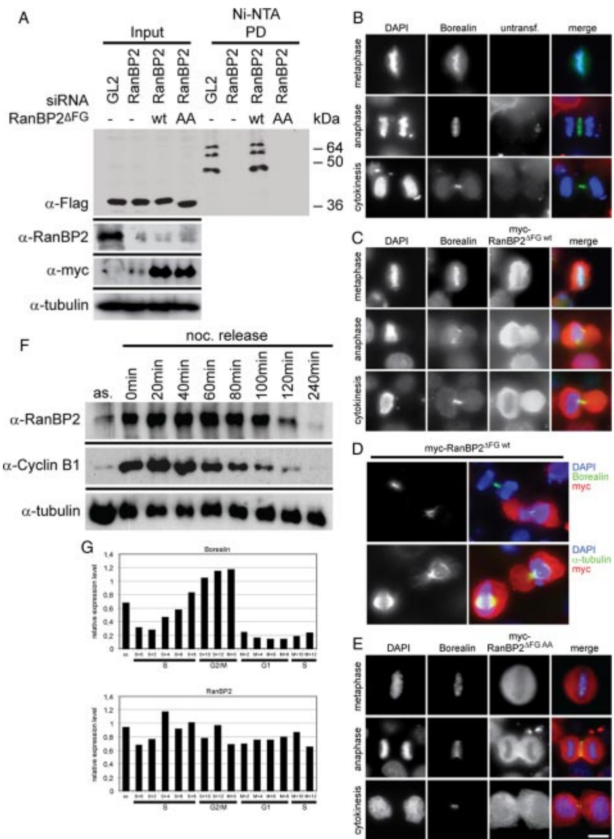

Figure 4. RanBP2$^{\Delta FG}$ restores sumoylation of Borealin in RanBP2 depleted cells. (A) FLAG-tagged Borealin and His-SUMO2 were coexpressed in HeLa cells treated with siRNA oligonucleotides directed against RanBP2 or GL2 as control. In parallel, cells were transfected with either empty myc-vector (−), myc-tagged wild-type RanBP2$^{\Delta FG}$ (wt) or catalytically inactive RanBP2$^{\Delta FG}$ (AA) bearing the mutations L2651A and L2653A. Depletion of RanBP2 and expression of myc-tagged RanBP2$^{\Delta FG}$ constructs was verified by Western blotting. His-SUMO2 conjugates were recovered on Ni-NTA beads, and Western blotting was performed with anti-FLAG antibodies to detect Borealin-SUMO conjugates. (B–E) Overexpression of RanBP2$^{\Delta FG}$ causes chromosome missegregation during mitosis. (B) Untransfected mitotic control cells stained with Borealin antibodies. (C) HeLa cells were transfected with myc-tagged RanBP2$^{\Delta FG}$ and stained with myc- and Borealin antibodies. Note the massive chromosome missegregation after anaphase onset. (D) Experiment as in C. Bottom, costaining of myc- and tubulin antibodies. (E) Experiment as in C, but the catalytically inactive RanBP2$^{\Delta FG}$ construct (RanBP2$^{\Delta FG\ AA}$) was transfected. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm. (F) RanBP2 protein level at distinct cell cycle stages. HeLa cells were released from a double thymidine block into nocodazole, and lysates were prepared at different time points after nocodazole release and detected by anti-RanBP2 antibodies. The mitotic status was assayed by monitoring Cyclin B1 levels. Tubulin protein level serves as a loading control. as. = asynchronous growing cells. (G) RanBP2 and Borealin mRNA levels upon cell cycle progression were determined in synchronized HeLa cells by using quantitative RT-PCR.

Borealin. Thus, we first searched for a physical interaction of Borealin with members of the human SENP family (SENP1, SENP2, SENP3, or SENP5) in the yeast two-hybrid system. Among the tested candidates, only SENP3 showed binding to Borealin in this experimental system (Supplemental Figure 2B). This finding was corroborated by coimmunoprecipitation experiments in mammalian cells using FLAG-tagged SENP3 and myc-tagged constructs of Borealin. Full-length

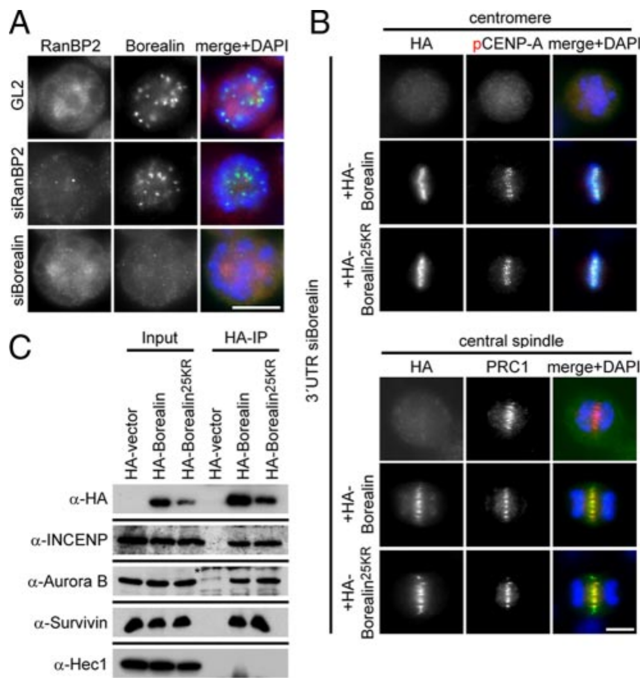

Figure 5. CPC assembly and localization to the centromere and central spindle are independent of Borealin sumoylation. (A) RanBP2 does not influence CPC localization to the kinetochore/centromere region and vice versa. HeLa cells were treated with indicated siRNA duplexes for 48 h and stained for RanBP2 and Borealin. DNA was stained with DAPI. Bar, 10 μm. (B) HeLa cells were treated with siRNA duplexes specific for the 3′ untranslated region of Borealin and simultaneously transfected with HA-Borealin or HA-Borealin^{25KR}. Immunofluorescence was performed with antibodies directed against HA, phospho(S7)-CENP-A, and PRC1. DNA was stained with DAPI. Bar, 10 μm. (C) HeLa cells were transfected with the indicated constructs, arrested in S phase by thymidine treatment, and released for 10 h to enter mitosis. Mitotic lysates were prepared and immunoprecipitations were performed with anti-HA antibodies and probed by Western blotting with the indicated antibodies. Asterisk denotes immunoglobulin heavy chain.

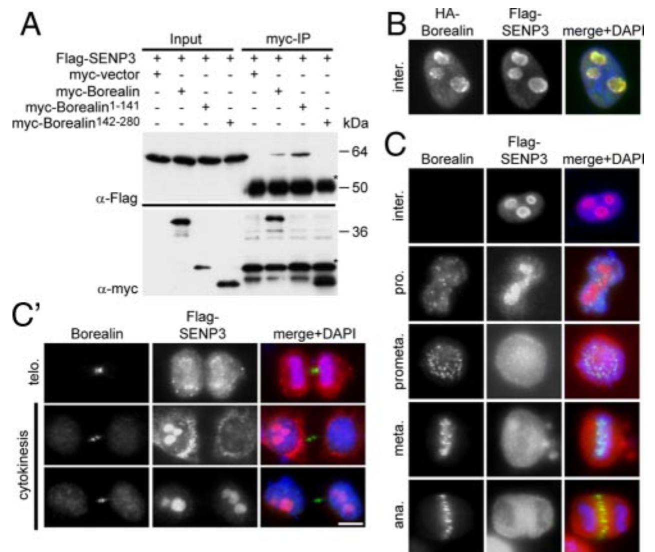

Figure 6. SENP3 colocalizes and interacts with Borealin. (A) FLAG-tagged SENP3 and myc-tagged constructs of Borealin were coexpressed in HeLa cells. Immunoprecipitations were performed using anti-myc antibodies. The two fragments of Borealin show a different electrophoretic mobility due to different isoelectric points. Asterisks denote immunoglobulins. (B) SENP3 and Borealin colocalize in interphase nucleoli. HeLa cells were transfected with FLAG-SENP3 and HA-Borealin. Localization was determined by immunostaining with anti-HA and anti-FLAG antibodies. (C) Localization of SENP3 during mitosis. HeLa cells were transfected with FLAG-tagged SENP3 and incubated for 48 h. Immunostaining was performed with anti-FLAG and anti-Borealin antibodies. SENP3 localizes to the cytoplasm during mitosis and showed partial overlap with Borealin from prophase until anaphase. (C′) During telophase SENP3 accumulates at the reforming nuclear envelope and reenters the nucleolus during cytokinesis. DNA was stained with DAPI. Bar, 10 μm.

Borealin as well as an N-terminal fragment of Borealin (Borealin$^{1\text{-}140}$) coprecipitated SENP3, whereas no interaction was detected between SENP3 and the C-terminal fragment of Borealin (Borealin$^{141\text{-}280}$) (Figure 6A). Consistent with this finding and previously reported data on their subcellular distribution (Nishida *et al*., 2000; Gassmann *et al*., 2004), FLAG-SENP3 and HA-Borealin exhibit colocalization in the nucleolus in interphase cells (Figure 6B). In early mitosis, after nucleolar disassembly SENP3 is found evenly distributed in the cytosol, thus showing partial overlap with Borealin (Figure 6C). In late mitosis, it accumulates at the reforming nuclear envelope and reenters the nucleolus during cytokinesis (Figure 6C′). Noteworthy, the overexpression of FLAG-SENP3 did not affect the normal localization of endogenous Borealin in distinct mitotic stages. Importantly, SENP3 is heavily phosphorylated in cells that were arrested in early mitosis by treatment with nocodazole (Supplemental Figure 6).

To directly analyze whether SENP3 can catalyze desumoylation of Borealin, an in vitro demodification assay was performed. Borealin, premodified by either SUMO1 or SUMO2, was incubated with wild-type or catalytically inactive mutant forms of either SENP3 or SENP5, the closest homologue of SENP3 in humans. Although Borealin-SUMO1 conjugates were not influenced by the addition of either SENP (Figure 7A, lanes 3–6), the amount of Borealin–SUMO2 conjugates was greatly reduced in reactions supplemented with the wild-type SENP3 protein, but it was unaffected by the inactive protein (Figure 7A, compare lanes 8 and 9). In comparison, SENP5 only marginally reduced the amount of Borealin–SUMO2 conjugates (Figure 6A, lane 10). Noteworthy, however, SENP5 used in these assays is enzymatically active as demonstrated by its ability to catalyze C-terminal processing of the SUMO2 precursor (Supplemental Figure 7).

Next, we assessed SENP3-catalyzed demodification of Borealin in vivo. Confirming and extending the in vitro results, expression of wild-type SENP3, but not the inactive mutant, induced an almost complete loss of Borealin–SUMO2 conjugates (Figure 7B, compare lane 11 and 12), whereas the level of Borealin–SUMO1 species was not affected (Figure 7A, lanes 8 and 9). To further study the involvement of endogenous SENP3 in desumoylation of Borealin, His-Borealin was expressed in HeLa cells and SENP3 was depleted from cells by siRNA duplexes. Efficient down-regulation of the protein was verified by immunoblotting with an anti-SENP3 antibody (Figure 7C′). Importantly, upon depletion of SENP3 the amount of Borealin–SUMO2/3 conjugates was significantly increased. Noteworthy, depletion of SENP5 did not affect Borealin-SUMO2/3 conjugates, whereas depletion of RanBP2 reduced the amount of sumoylated Borealin (Figure 7C).

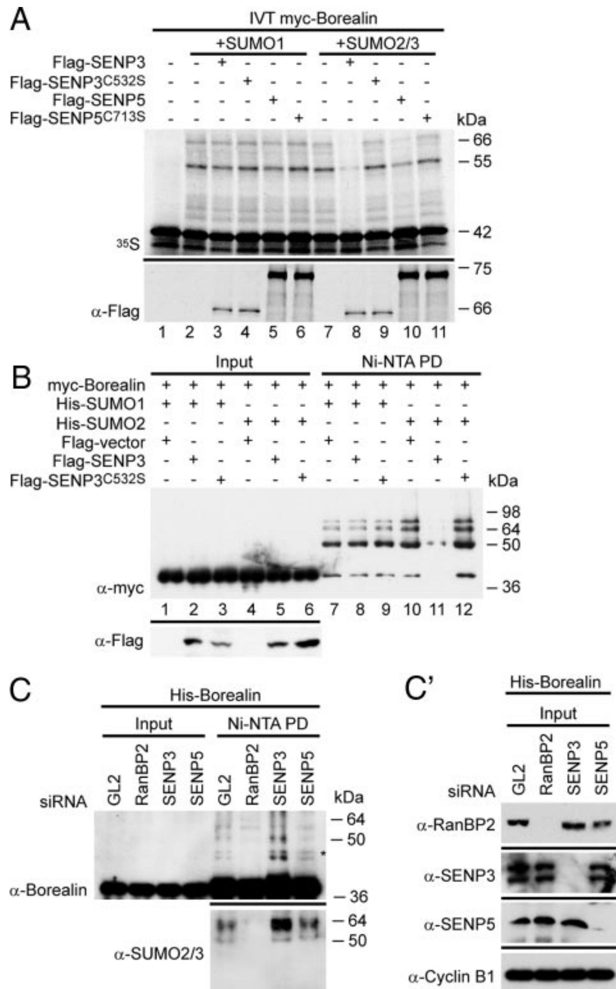

Figure 7. SENP3 catalyzes desumoylation of Borealin. (A) FLAG-tagged versions of wild-type SENP3 ($SENP3^{wt}$, lanes 3 and 8) and SENP5 ($SENP5^{wt}$, lanes 5 and 10), or the catalytically inactive mutants ($SENP3^{C532S}$, lanes 4 and 9, and $SENP5^{C713S}$, lanes 6 and 11), generated by in vitro translation/transcription, were added to in vitro sumoylated Borealin. The anti-FLAG Western blot serves as a loading control for the proteases. Note deconjugation of SUMO2/3, but not SUMO1, of Borealin when incubated with $SENP3^{wt}$ but not $SENP3^{C532S}$. (B) Myc-tagged Borealin and His-SUMO constructs were coexpressed with FLAG-tagged $SENP3^{wt}$ (lanes 2, 5, 8, and 11) or $SENP3^{C532S}$ (lanes 3, 6, 9, and 12) in HeLa cells. His-SUMO conjugates were recovered on Ni-NTA beads (lanes 7–12), and Western blotting was performed with anti-myc antibodies. Expression of SENP3 constructs was verified by anti-FLAG Western blotting. (C and C′) SENP3 depletion leads to accumulation of SUMO2/3 modified Borealin. HeLa cells were transfected with His-tagged Borealin and indicated siRNA duplexes, arrested in S phase by thymidine treatment, and released for 10 h to enter mitosis. Mitotic lysates were prepared and Ni-NTA precipitation was performed as described above. Immunoblotting was performed with indicated antibodies to demonstrate depletion of corresponding proteins (C′) and monitor the sumoylation status of Borealin (C). The Borealin reactive bands at ~45 kDa (asterisk in C) is interpreted as a Borealin–SUMO2/3 degradation product. Note that knockdown of SENP3, but not SENP5, enhances Borealin sumoylation compared with control-depleted cells. In contrast, RanBP2 knockdown results in a loss of Borealin modification.

Together, these data strongly indicate that SENP3 exerts protease activity on Borealin and specifically reverts the modification of Borealin by SUMO2/3.

DISCUSSION

The data reported here define a mitotic pathway of SUMO2/3 conjugation–deconjugation on the CPC component Borealin. Importantly, this pathway is controlled by the E3 SUMO ligase RanBP2 and the SUMO-specific isopeptidase SENP3. Modification of Borealin occurs at up to three lysine residues, but by individual or combinatorial mutations of a series of lysine residues we were unable to assign distinct residues for attachment of SUMO. This scenario may be explained by the fact that none of the 25 lysine residues of Borealin is embedded in a KxE/D motif, which serves as preferential SUMO attachment sites in the majority of SUMO substrates (also see Supplemental Figure 5D). Due to the difficulties in obtaining a SUMO-deficient version of Borealin by mutating distinct lysine residues, we had to generate a lysine-less version of Borealin to abolish sumoylation. Notably, however, this mutant does show sumoylation-independent defects, such as impaired nucleolar localization in interphase (Supplemental Figure 4E). This complicates its further use in complementation experiments because it would be difficult to directly link functional differences between $Borealin^{25KR}$ and wild-type Borealin to a loss of sumoylation rather than the loss of other modifications at these residues or structural alterations.

Interestingly, recent work in yeast described sumoylation of Bir1, the yeast relative of Survivin (Montpetit *et al*., 2006). We show that Survivin is not a major target of SUMO modification in human cells. Notably, however, yeast lacks a Borealin homologue, and it has been proposed that the functions of human Survivin and Borealin are combined in the single yeast Bir1 protein (Vader *et al*., 2006). Therefore, these data support the idea that sumoylation of the CPC is an evolutionary conserved mechanism. However, the functional impact of SUMO on CPC activity in both lower and higher eukaryotes remains to be elucidated.

An exciting new aspect in the SUMO/ubiquitin field is the emerging interplay of both modification systems on a given target protein. Interestingly, recent work has shown that a balanced nondegradative ubiquitination–deubiquitination cycle of Survivin is required for the chromosome segregation function of the CPC at the centromere (Vong *et al*., 2005). Modification of Borealin and Survivin by SUMO and ubiquitin, respectively, thus provides an example, where both modification systems target distinct components of a multiprotein complex.

Borealin–SUMO2/3 conjugates are most prominent in early mitosis when the CPC is associated with the centromere, suggesting that the SUMO-modified fraction of Borealin is associated with these structures. This would be in line with observations from *Xenopus* egg extracts and human cells, which show that during prometaphase/metaphase SUMO2/3 is found at centromeres and chromatin, whereas SUMO1 localizes to the mitotic spindle and the spindle midzone (Azuma *et al*., 2003; Ayaydin and Dasso, 2004; Zhang *et al*., 2008). The loss of Borealin sumoylation observed during later mitotic phases (around anaphase onset) when the CPC translocates to the central spindle is in agreement with SUMO2/3 being chromatin associated also during these stages (Zhang *et al*., 2008). The importance of SUMO function at the kinetochore/centromere is underscored by the recent finding that overexpression of SENP2 in HeLa cells leads to a loss of SUMO2/3 from these structures and induces a prometaphase-like arrest due to a failure in kinetochore targeting of the microtubule motor CENP-E (Zhang *et al*., 2008; Supplemental Figure 6B).

We could assign the centromere/kinetochore protein Borealin as a physiological target for RanBP2-mediated sumoylation. RanBP2 is found at the nuclear pore in interphase cells, but it redistributes to the mitotic spindle and the kinetochore/centromere upon entry of cells into mitosis (Yokoyama *et al*., 1995; Joseph *et al*., 2002, 2004). Similarly to what was shown for topoisomerase II (Dawlaty *et al*., 2008), we found that RanBP2 associates with the CPC in mitosis, stimulates SUMO modification of Borealin in vitro, and is essential for Borealin sumoylation in vivo. Interestingly, we observe a drastic defect in chromosome segregation upon ectopic overexpression of the catalytically fragment of RanBP2. However, in contrast to what was observed for topoisomerase II, sumoylation of Borealin does not seem to be required for centromere targeting of the CPC. In line with this observation, CPC components are still present at centromeres in cells overexpressing SENP2 (Zhang *et al*., 2008; Supplemental Figure 6A). Interestingly, as demonstrated for CENP-E, dynamic association of centromeric proteins to these structures also involves noncovalent interactions between a SUMO-modified acceptor protein and a specific binding partner, which harbors a SUMO interaction motif (SIM) (Zhang *et al*., 2008). Thus, one may envision that the attachment of the SUMO moiety to Borealin may provide a centromeric docking site for a yet to be identified SIM-containing binding partner. Indeed, it was shown that the CPC constitutes one of the most upstream components of centromere/kinetochore assembly (Vigneron *et al*., 2004; Liu *et al*., 2006; Emanuele *et al*., 2008).

The dynamics of sumoylation are controlled by SUMO isopeptidases and the importance of SUMO deconjugation is illustrated by genetic data from yeast and mice showing that like conjugation, deconjugation is needed for viability. In yeast, Ulp1 has an essential role in the G2/M phase of the cell cycle, whereas Ulp2, which seems to be particularly important for the depolymerization of SUMO chains, is involved in the control of chromosome cohesion at centromeric regions (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000 Bachant *et al*., 2002). In mammalian cells depletion of SENP5, the closest homologue of SENP3 in humans causes a cytokinesis defect, indicating that SENP5 functions in mitosis (Di Bacco *et al*., 2006). Noteworthy, however, depletion of SENP5 did not affect Borealin–SUMO2/3 conjugates and did not interfere with CPC localization or function (our unpublished observation), indicating that the defect in cytokinesis is not related to an altered CPC function. Accordingly, SENP5 did not bind to Borealin and exhibited only a low catalytic activity toward Borealin–SUMO2/3 conjugates in vitro. By contrast, we identified SENP3 as a specific interaction partner of Borealin and showed that SENP3 catalyzes the removal of SUMO2/3 from Borealin both in vitro and in vivo. Thus, SENP3 features an activity that catalyzes desumoylation of Borealin in mitosis. The preferential activity of SENP3 toward SUMO2/3 conjugates confirms previous reports (Nishida *et al*., 2000; Gong and Yeh, 2006; Haindl *et al*., 2008) and strengthens the concept of a functional divergence of distinct SUMO forms in mitosis. In interphase cells, SENP3 is found in the nucleolus, where it functions as an essential factor of ribosome biogenesis (Haindl *et al*., 2008), but little is known about SENP3 function during mitosis. SENP3 was recently identified in an siRNA screen as a potential component of the spindle assembly checkpoint (SAC) (Stegmeier *et al*., 2007), but depletion of SENP3 did not interfere with CPC targeting to the centromere or activity of the CPC at these sites (our unpublished observation), suggesting that SENP3 affects SAC function downstream of CPC recruitment to the centromere. Interestingly, SENP3 is phosphorylated in mitosis, suggesting that its activity and/or localization are controlled by mitotic kinases. Recent proteomic studies indeed found SENP3, and in particular a phosphorylated form of SENP3, at the mitotic spindle (Sauer *et al*., 2005; Nousiainen *et al*., 2006). This indicates that at least a subfraction of the protease acts on spindle associated components, which is consistent with the loss of Borealin sumoylation in anaphase, when the CPC is transferred to the mitotic spindle.

In summary our data indicate that in addition to its role in ribosome biogenesis SENP3 exhibits an important mitotic function. Because cell cycle progression and ribosome biogenesis are tightly interconnected (Dez and Tollervey, 2004), SENP3 may represent a critical factor that coordinates both processes. Identification of novel SENP3 targets may thus shed more light on the question of how the SUMO system regulates key mitotic processes.

ACKNOWLEDGMENTS

We thank Andreas Ledl, Per Stehmeier, Frauke Melchior, and Ed Yeh for reagents; Jochen Rech for experimental help; and P. Descombes for the quantitative PCR data. We are grateful to Christoph Baumann, Rainer Malik, Sabine Elowe, Stefan Hümmer, Alexander Buchberger, Olaf Stemmann, and Stefan Jentsch for discussions and comments on the manuscript. We acknowledge financial support from the "Max-Planck Gesellschaft" and the "Fonds der chemischen Industrie." U.R.K is supported by the Boehringer Ingelheim Fonds. This work was supported by the DFG Priority Programme SPP 1365, "The Regulatory and Functional Network of Ubiquitin Family Proteins. S.M. is indebted to Stefan Jentsch for generous and continuous support.

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