The chromosomal passenger complex during mitotic progression: Identification of subunit-specific functions

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

Vorgelegt von

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München 2008

Dissertation eingereicht am 7.10.2008

Datum der mündlichen Prüfung: 16.01.2009
Erstgutachter: Prof. Dr. Erich A. Nigg
Zweitgutachter: Prof. Dr. Harry MacWilliams

Ehrenwörtliche Erklärung und Erklärung über frühere Promotionsversuche

Hiermit erkläre ich, dass ich, Ulf Klein, die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente wurden von mir selbst durchgeführt, soweit nicht explizit auf Dritte verwiesen wird. Ich habe weder anderweitig versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch habe ich diese Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

München, den 05.10.2008

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ACKNOWLEDGMENTS

I would like to thank Erich Nigg for his friendly and supportive mentorship, the freedom he gave me to perform these experiments and his personal advice.

I am thankful to Prof. Harry MacWilliams for reviewing this thesis.

Thanks to Francis Barr, Ulrike Grueneberg, Marsha Rosner, Elena Conti and Aarockia Jeyaprakash, Stefan Muller and Nadja Huebner for productive collaborations. I am happy that Nadja Huebner is following up on my project.

I am grateful to Ruediger Neef and Herman Sillje for their support and many helpful discussions and I like to thank Robert Kopajtich for many reagents and introduction into yeast-two hybrid screening.

Thanks to Mikael LeClech, Rainer Malik, Stefan Huemmer and Andreas Schmidt and all past and present members of the Cell Biology department for creating a stimulating and friendly atmosphere.
Especially I would like to thank Christoph Baumann for his help during the early stages of my PhD, a great time in- and outside the lab and his valuable friendship.

I am grateful to the “centrosome lab” for discussions, reagents and support.

I would like to stress that the excellent organization of the lab is in great parts achieved by the work of Alison Dalfovo, Elena Nigg, Lidia Pinto, Marianne Siebert/Durda Pavic and Klaus Weber.
I am also grateful to Albert Ries and Rainer Malik for help with computer related problems.

I like to thank my parents Maria and Sigurd Klein for their continous support.

I am indebted to the Boehringer Ingelheim Fonds and the Degussa Stiftung for generous support.

SUMMARY

Cell division involves coordinated chromosomal and cytoskeletal rearrangements to ensure the faithful segregation of genetic material into the daughter cells. The chromosomal passenger complex (CPC) consisting of the Aurora B kinase, INCENP, Survivin, and Borealin has emerged as a central player at several steps in this process (Ruchaud *et al.*, 2007). The complex is involved in chromosome condensation, kinetochore-microtubule interaction, the spindle assembly checkpoint (SAC) and cytokinesis. CPC localization parallels its functions. In early mitosis the complex binds to the inner centromere, translocates to the central spindle at the onset of anaphase and accumulates at the midbody during cytokinesis. The study of CPC targeting is complicated by the fact that all the subunits of the complex are interdependent for localization and/or protein stability.

To overcome this situation and define individual functions of the chromosomal passenger proteins we established a siRNA based complementation assay. We identified a ternary subcomplex of the CPC comprising Survivin, Borealin, and the N-terminal 58 amino acids of INCENP that is essential and sufficient for centromere binding independent of the enzymatic core of the complex, the Aurora B kinase. Our data also suggest that, within this module, Borealin might target the complex to the centromere by directly binding to DNA.

We further show that the spindle checkpoint protein PICH requires the CPC but remarkably not Aurora B kinase activity to localize to the centromere/kinetochore. Moreover, Mad2, the ultimate effector of the SAC that depends on the CPC under normal conditions, is present at kinetochores in CPC depleted cells treated with nocodazole and this may explain the mitotic arrest seen under these conditions.

The collaborating laboratory of Elena Conti recently solved the 1.4Å resolution crystal structure of most of the ternary subcomplex mentioned above, revealing that Borealin and INCENP associate with the helical domain of Survivin to form a tight three-helical bundle that creates a single structural unit. Evaluation of structure-based mutants showed that the intertwined interactions between the core CPC components lead to functional interdependence essential for CPC localization to the centromere, the central spindle and the midbody. Moreover, the composite molecular surface of the complex presents conserved residues essential for central spindle and midbody localization which we found to determine CPC function during cytokinesis.

In a yeast two-hybrid screen for Borealin interactors we identified components of the SUMO system. Subsequently, we showed that Borealin is a bona fide SUMO target and

preferentially modified by SUMO2/3. We demonstrate that Borealin sumoylation is high in early mitosis but progressively lost when cells enter anaphase. The SUMO ligase RanBP2 is essential for Borealin sumoylation and overexpression of its catalytic domain leads to chromosome missegregation during anaphase. Furthermore, 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 assign a regulatory function of RanBP2 and SENP3 in the mitotic SUMO pathway.

INTRODUCTION

The cell cycle and cell division

Self-reproduction is one of the most fundamental characteristic of cells and hence of all living organisms. The principle “omnis cellula e cellula” (each cell stems from another cell), propagated by the German pathologist Rudolf Virchow in 1858, forms the basis of the theory that new cells are formed via division of pre-existing ones. This occurs by an orderly sequence of events, called the cell cycle, in which a cell first duplicates its contents and then divides into two. The newly formed daughter cells can themselves grow and divide, generating a new cell population. In unicellular life forms such as bacteria and yeasts, each cell division produces a complete new organism. In multicellular species, multiple and complex sequences of cell divisions are required to produce a functioning organism and cell division is an essential mechanism to replace cells that die. Cell growth and DNA replication take place throughout most of the cell cycle in bacteria. The single, circular DNA molecules are then distributed to daughter cells. In eukaryotes, this process is temporally divided into four sequential phases (Figure 1). The genetic material consists of a number of discrete DNA molecules, the chromosomes, which are replicated during S (synthesis) phase and become condensed and segregated equally to the daughter cells during M (mitosis) phase. A key concept of the eukaryotic cell cycle is that S phase must always follow M phase and that M phase must not start until S phase has been completed. Thus, the integrity of the genome is maintained. Between S and M phase two gap phases called G1 and G2 exist. During G1 phase the cell is responsive to both positive and negative growth signals, and during G2 the cell prepares for entry into mitosis. G0 refers to a state in which a cell remains metabolically active, but no longer proliferates unless appropriate extracelluar signals are received (quiescent state).

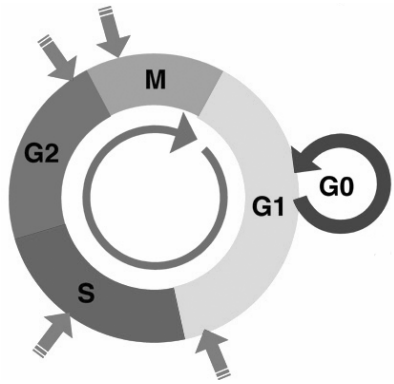


Figure 1. The eukaryotic cell cycle.

The different phases are denoted by capital letters (see text). Arrows indicate cell cycle checkpoints. G1, S and G2 phase are collectively called interphase; the time between two mitoses.

Movement through each phase of the cell cycle and transition from one phase to the next is controlled at distinct cell cycle checkpoints (Hartwell and Weinert, 1989) (Figure 1). At the restriction point in G1 phase a cell either becomes committed to enter the cell cycle or to become quiescent and enter G0. While damaged (or unreplicated) DNA is sensed late during G1, as well as in S and G2 phase, the proper attachment of chromosomes to the spindle apparatus is monitored during M phase to ensure correct segregation of the chromosomes (in the form of sister chromatids) to the daughter cells. Defects in cell cycle surveillance mechanisms contribute to chromosome instability and aneuploidy, a hallmark of cancer cells (Bharadwaj and Yu, 2004; Kastan and Bartek, 2004).

Already in 1882 Walther Flemming was the first cytologist to describe chromosome behaviour during the cell cycle in his book "Zellsubstanz, Kern und Kerntheilung" (Figure 2) and the expressions he coined (e.g. chromatin, equatorial plate, aster) are still used today. More than 100 years later, in 2001, the discovery of the key molecular determinants coordinating the cell cycle by Tim Hunt, Paul Nurse and Lee Hartwell was rewarded by the Nobelprize in Medicine. The researchers described the cell cycle dependent rise and fall in the level of proteins they therefore called cyclins and demonstrated a link between these oscillations and the discrete cell cycle stages. Cyclins were found to bind to and activate cyclin-dependent kinases (Cdk´s) that subsequently coordinate various cell cycle events via phosphorylation of target proteins.

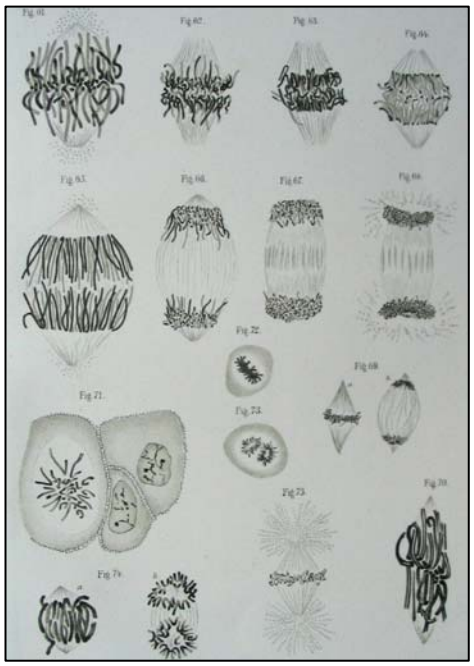


Figure 2. Illustration of chromatin behaviour during the metaphase to anaphase transition in M phase. Chromatin, microtubules and spindle poles are depicted. Adapted from the book „Zellsubstanz, Kern und Kerntheilung", F.C.W Vogel Verlag, Leipzig 1882.

The events of mitosis

Mitosis (nuclear division) is the time when the sister chromatids (the daughter DNA molecules produced in S phase) are segregated equally to the two daughter cells. This is achieved through a highly organized series of events which can be attributed to discrete phases of mitosis.

During prophase the interphase chromatin condenses and the centrosomes, which have been duplicated during S phase, start to move to opposite poles. There, they start nucleating microtubules (MTs) to build the mitotic spindle. At prometaphase, the nuclear envelope breaks down allowing the MTs to capture specialized structures called the kinetochores (KTs) on both sister chromatids. Chromosomes, which have attached to MTs emanating from opposite spindle poles are moved to the equator of the cell in a process called congression. Metaphase is the state when all chromosomes have aligned on an equatorial plate (or metaphase plate) in the center of the cell. The MT arrays now form the typical bipolar spindle with their minus-ends proximal to the poles and their plus-ends attached to the KTs. Once all chromosomes are aligned, cohesion between sister chromatids is lost at the onset of anaphase and subsequently sister chromatids are pulled apart to opposite poles. Mitosis ends with telophase, during which the nuclear envelope reforms around the decondensing sister chromatids.

Mitosis is followed by cytokinesis (cell division). During cytokinesis, the antiparallel MT arrays at the cell equator, the central spindle, is compressed into a compact midbody, the remnant of the midzone. Contraction of a ring-like structure containing actin and myosin II, which has been formed during late anaphase beneath the plasma membrane at the site of the central spindle, leads to furrow ingression. Finally, cell abscission takes place resulting in the formation of two new daughter cells.

The vertebrate centromere/kinetochore region

Each sister chromatid comprises a specialized chromatin region located at the primary constriction that consists of a series of α-satellite DNA repeats called the centromere. Specialized centromeric components are the histone H3 variants Cenp-A, H3K4 and H3K9 (histone H3 methylated on Lysine 4 or 9, respectively) and the heterochromatin-protein-1 that associates with H3K9 (Cleveland *et al.*, 2003; Sullivan and Karpen, 2004) as well as further *cen*tromeric *p*roteins (Cenps) (Foltz *et al.*, 2006). The centromere provides a structural and functional seed for the assembly of proteins building the KT. Although the exact boundaries between centromere and kinetochore are rather loosely defined, at least three different layers can be distinguished by electron microscopy (Brinkley and Stubblefield, 1966; McEwen *et*

al., 2007). They are referred to as the centromere, the inner kinetochore and the outer kinetochore (Figure 3).

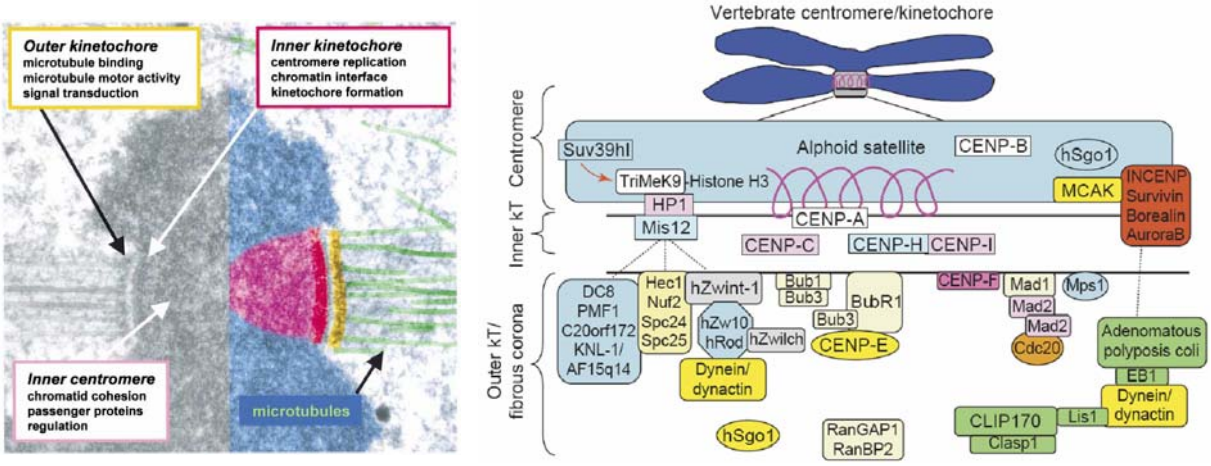


Figure 3. The vertebrate centromere/kinetochore region.

Left: Trilaminar organization of the centromere/kinetochore region as seen by electron microscopy. Adapted from Cleveland et al., Cell 2003. Right: Schematic representation of the spatial distribution of centromere/ kinetochore proteins. Proteins present within a complex are surrounded by one box. Physical interaction between proteins/protein complexes is indicated by shapes that contact each other. Adapted from Chan et al., TRENDS in Cell Biology 2005.

Besides proteins that localize constituently to the centromere (see above) a number of proteins associate with this layer only once mitosis has started. Among them are the chromosomal passenger proteins, the phosphatase PP2A, small ubiquitin-related modifiers (SUMO proteins), proteins involved in sister chromatid cohesion, the microtubule depolymerase MCAK and the ATPase PICH (Maiato *et al.*, 2004; Cheeseman and Desai, 2008; Dasso, 2008). Interestingly, when centromeres become stretched apart by pulling MTs during the early stages of mitosis, a portion of centromere components eventually extend outward towards the KT (Gorbsky, 2004). Proteins of the centromere crucially regulate the KT-MT interactions. The inner kinetochore is immediately adjacent to the centromere, also comprises centromeric chromatin, hMis12 and Cenps. In some cases (e.g. the Hec/Ndc80 complex) it can be considered as a basis for proteins that extend towards the outer kinetochore. The outer kinetochore is a proteinaceous layer only formed after nuclear envelope breakdown. To date more than 80 proteins have been identified to localize to this layer in human cells. The outer kinetochore harbours about 20-30 end-on attachment sites for the MT plus-ends and is essential for MT binding. It contains structural components that are involved in KT-MT attachment like the Hec/Ndc80 complex or the SUMO E3 ligase RanBP2

(Joseph *et al.*, 2004; DeLuca *et al.*, 2005), protein kinases like Plk1 and Mps1 (Arnaud *et al.*, 1998; Abrieu *et al.*, 2001), MT-dependent motor proteins like CENP-E (Yen *et al.*, 1991) and components of the spindle assembly checkpoint including Mad2 and BubR1 (Musacchio and Hardwick, 2002).

Using immunodepletion or protein knock-down by siRNA, hierarchic sequences of centromere/kinetochore assembly could be deduced. One major pathways is instructed by Cenp-A (Regnier *et al.*, 2005) directing the Hec/Ndc80 complex, Cenps and BubRI to the KT. Another pathway depends on the Bub1 kinase that recruits CENP-E and CENP-F, Sgo1 and additionally also checkpoint proteins (e.g. Mad2 and BubRI) to the KT (Boyarchuk *et al.*, 2007). The CPC has also been implicated in KT assembly (Vigneron *et al.*, 2004; Liu *et al.*, 2006). It emerges that a sophisticated crosstalk between the different branches exists and that centromere/kinetochore assembly occurs in a strictly linear fashion.

The trilaminar structure of the centromere/kinetochore region mediates the MT dependent chromosome movements during congression in prometaphase. Furthermore, KT associated checkpoint proteins respond to erroneous KT-MT attachment via a mechanism called the spindle assembly checkpoint (SAC), thus delaying the metaphase to anaphase transition until all chromosomes have properly aligned on the metaphase plate.

The spindle assembly checkpoint (SAC)

In 1991, two independent screens identified various genes, mutation of which bypassed the ability of yeast to induce a mitotic arrest in response to spindle poisons (Hoyt *et al.*, 1991; Li and Murray, 1991). The genes identified in these screens which include the *MAD* (mitotic-arrest deficient) and the *BUB* (budding uninhibited by benzimidazole) genes were later found to be conserved in all eukaryotes. They are involved in a surveillance mechanism termed the spindle assembly checkpoint (SAC) that is active in prometaphase to prevent the precocious separation of sister chromatids.

The SAC monitors the attachment of KTs to MTs and its function is intimately linked to the KT. A single unattached KT can block the onset of anaphase but laser ablation of it relieves the arrest (Rieder *et al.*, 1995). While checkpoint proteins become concentrated on unattached KTs creating a “wait anaphase signal”, they are absent from properly attached KTs (Chen *et al.*, 1996; Taylor and McKeon, 1997). Indeed, the SAC should only be silenced once sister chromatids are attached in a bipolar (amphitelic) fashion to the MTs (each sister KT occupied by MTs from the opposite pole); an arrangement that ensures the equal segregation during anaphase (Figure 4). Monotelic attachment is characterized by the attachment of only one KT to MTs and represents a normal condition in prometaphase. In addition, however,

MTs emanating from only one pole might become attached to both sister KTs (syntelic attachment) or a single KT might be captured by MTs nucleating from opposite poles (merotelic attachment). The SAC is activated by syntelic attachments as KT-MT attachments are destabilized at low KT tension but stabilized by high tension between amphitelic sister KTs (Nicklas *et al.*, 2001). In contrast, merotelic attachments produce sufficient occupancy and tension and are thus not sensed by the SAC. In both cases, however, destabilization of attachments is achieved by the action of Aurora B kinase, the enzymatic core of the CPC probably via regulation of the microtubule depolymerase MCAK. (Biggins and Murray, 2001; Tanaka *et al.*, 2002; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Andrews *et al.*, 2004). If uncorrected, improper KT-MT attachments can produce lagging chromatids and chromosome missegregation in anaphase (Figure 4).

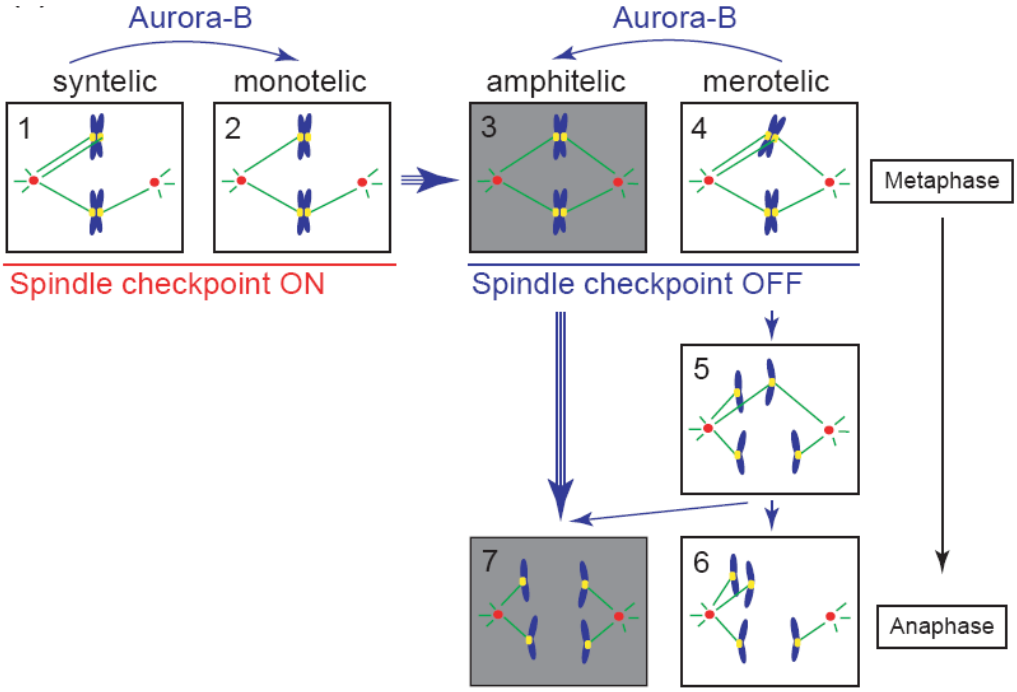


Figure 4. Illustration of correct and incorrect KT-MT attachments.

Amphitelic attachment (3) satisfies the SAC and anaphase can commence (7). During syntelic- and monotelic attachment (1 and 2) the SAC remains "on" and sister separation cannot proceed. However, the SAC does not sense merotelic attachment (4). The correction of improper attachments to establish amphitelic orientation depends on the Aurora B kinase. Uncorrected attachments will result in chromosome missegregation during anaphase (5 and 6). Adapted from Giet et al., TRENDS in Cell Biology 2005.

To which extent a complete lack of MT attachment or missing tension between KTs contribute to checkpoint activation in metazoan cells remains to be clarified (Pinsky and Biggins, 2005). The ability to sense tension once bipolar attachment is achieved seems to

have a significant role in turning off SAC activity by inhibiting the association of SAC proteins with KTs.

One major downstream effector of the SAC is Mad2, a checkpoint protein able to adopt two different conformations. SAC silencing requires the transition from an inactive so-called open conformation (O-Mad2) to an active confirmation called closed Mad2 (C-Mad2) (Musacchio and Salmon, 2007). The transition is probably mediated via a Mad1-Mad2 interaction/intermediary complex formation at the KT. C-Mad2 is a potent inhibitor of Cdc20, a KT bound protein that acts as an activator for the APC/C, a ubiquitin ligase (Peters, 2006; Yu, 2007). Once the SAC is silenced, binding of C-Mad2 and Cdc20 in complex with BubRI and Bub3, collectively called the mitotic checkpoint complex (MCC), to the APC/C results in its activation. Active APC/C induces anaphase onset by degrading two key substrates: securin and cyclin B (Pines, 2006). Securin is an inhibitor of separase, a protease that cleaves the cohesin complex which holds sister chromatids together, allowing for their separation. Destruction of cyclin B inactivates Cdk1, the master kinase regulating early mitotic events. How MCC proteins cooperate to inhibit the APC/C remains to be fully understood.

The chromosomal passenger complex (CPC): A concerted effort

The CPC is a central regulator of mitotic progression. Functions that require chromosomal passenger activity include chromatin modification, assembly of the centromere/kinetochore region, correction of KT attachment errors, aspects of the spindle assembly checkpoint, assembly of a stable bipolar spindle and the completion of cytokinesis (Vagnarelli and Earnshaw, 2004; Vader *et al.*, 2006; Ruchaud *et al.*, 2007) (Figure 5). Chromosomal passenger genes are frequently expressed at high levels in a variety of tumors, and the level of abnormal overexpression correlates with increased genetic instability and clinical outcome. Aurora B inhibition by the small molecule inhibitor VX-680 has lead to tumor regression in rodent xenograft models (Harrington *et al.*, 2004). Moreover, preliminary clinical data from phase I trials using Aurora B kinase inhibitors have largely been consistent with cytostatic effects and with disease stabilization as a response achieved in solid tumors (Gautschi *et al.*, 2008).

The CPC is conserved from yeast to man and in humans comprises the inner centromere protein (INCENP), the BIR domain containing protein Survivin, Borealin and the Aurora B kinase. Expression of the chromosomal passenger proteins is cell cycle regulated and peaks in M phase. As implicated by its name, the complex shows a dynamic localization, being centromere bound from prophase to metaphase, associated with the central spindle during anaphase and finally accumulating at the midbody during telophase/cytokinesis

(Figure 5). Strikingly, members of the CPC are interdependent for localization. Thus, knock-down of any subunit of the CPC leads to loss of the other CPC proteins from the centromere, the central spindle and the midbody.

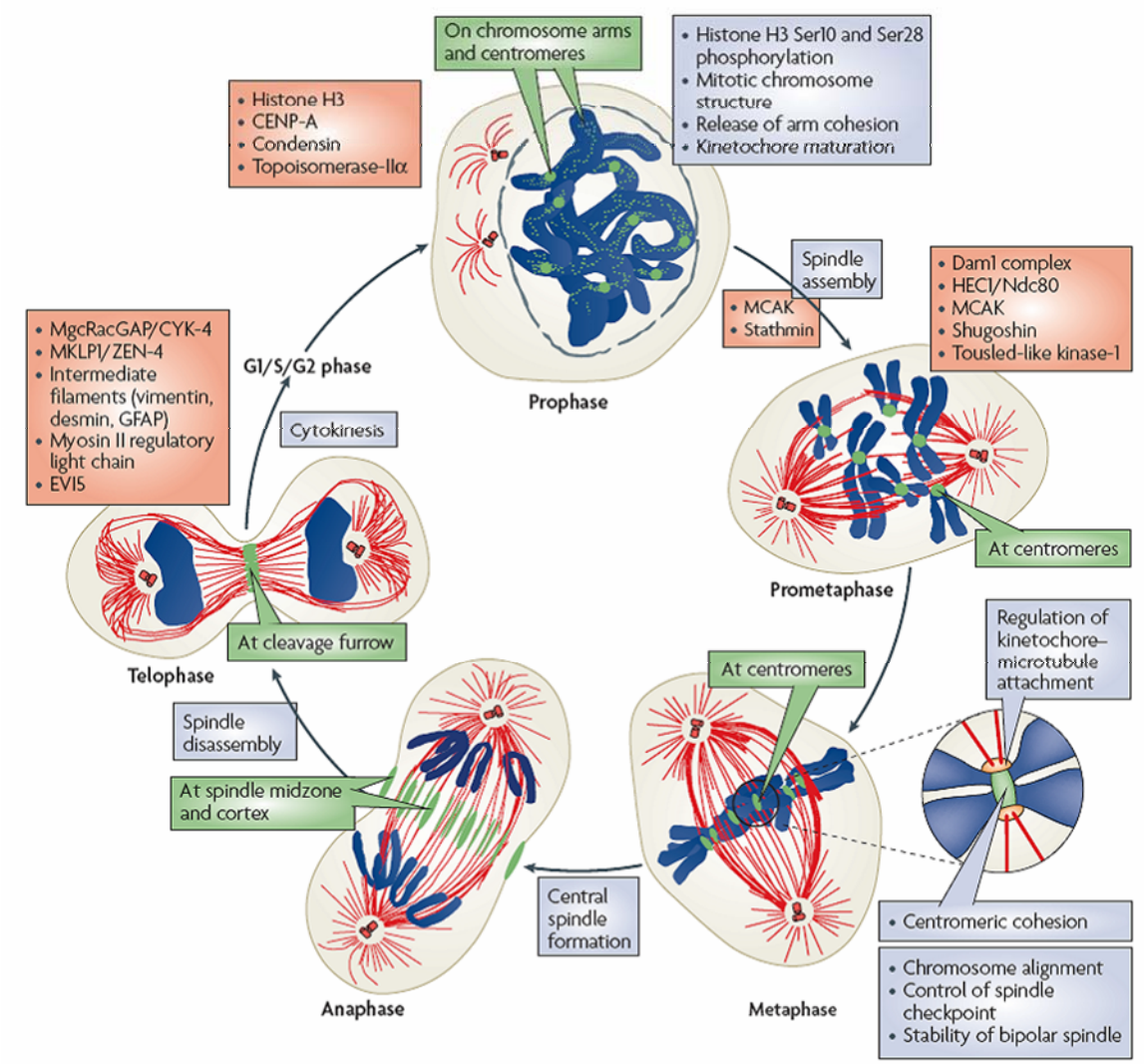


Figure 5. CPC localization and function during mitosis.

The CPC is depicted in green and Aurora B phosphorylation targets in red boxes. The different functions of the CPC during mitotic progression are given in grey boxes. In prophase the CPC is involved in the release of chromosome arm cohesion and phosphorylates histone H3, a widely used mitotic marker. The CPC is required to build a functional kinetochore/centromere region, establish proper KT-MT attachments and regulate the SAC. Upon the onset of anaphase the CPC translocates to the spindle midzone where it is essential to recruit MT associated proteins to build the central spindle. In telophase the CPC concentrates at the cleavage furrow and subsequently at the midbody where it regulates cytokinesis. Adapted from Ruchaud et al., Nat.Rev. Mol.Cell.Biol. 2007.

INCENP: INCENP was the first member of the CPC to be identified in a screen for novel components of the chromosome scaffold (Cooke *et al.*, 1987). It is the largest protein of the CPC (919 amino acids in length) and contains a 260 amino acid long coiled-coil region between its N- and C-terminus. Membership in the family is defined by the presence of a short conserved stretch of ~60–80 amino acids near the C terminus of the protein known as the IN-box (Adams *et al.*, 2000). The IN-box mediates binding to Aurora B and is subsequently phosphorylated on a conserved TSS motif by the kinase. This leads to Aurora B autophosphorylation which activates the kinase in a positive feedback-loop (Bishop and Schumacher, 2002; Honda *et al.*, 2003; Yasui *et al.*, 2004). INCENP has been shown to bind microtubules *in vitro* (Kang *et al.*, 2001; Wheatley *et al.*, 2001).

Aurora B: Aurora B is a serine/threonine kinase that belongs to the Aurora family of kinases, in mammals further comprising Aurora A and C (Meraldi *et al.*, 2004; Giet *et al.*, 2005). While Aurora A localizes to the spindle poles and regulates centrosome maturation and spindle assembly (Meraldi *et al.*, 2004), Aurora C has recently been characterized as a bona fide chromosomal passenger protein that can compensate for the loss of Aurora B (Sasai *et al.*, 2004). Aurora B kinase phosphorylates INCENP, Survivin and Borealin within the CPC. Furthermore, Aurora B has been demonstrated to phosphorylate proteins at the centromere/kinetochore region, such as CENP-A and MCAK (Zeitlin *et al.*, 2001; Andrews *et al.*, 2004; Ohi *et al.*, 2004) and at the central spindle and midbody, e.g., the kinesin Mklp1, the GTPase activating protein MgcRacGap, and the intermediate filament vimentin (Goto *et al.*, 2003; Minoshima *et al.*, 2003; Neef *et al.*, 2006). Most known phosphorylation sites of Aurora B match the consensus motif $(R/K)_{1-3}X(S/T)$ and PP1 has emerged as the major phosphatase opposing Aurora B function (Francisco *et al.*, 1994; Emanuele *et al.*, 2008). Yet, the molecular details of how phosphorylation by Aurora B influences its different substrates, is only emerging. FRAP analysis revealed that the centromeric association of Aurora B is dynamic and does not depend on microtubules or on its kinase activity (Murata-Hori and Wang, 2002). Aurora B activity is also not essential for localization of the CPC to the central spindle or the midzone. However, specific inhibition of Aurora B by small molecules results in defects, identical to the knock-down of any CPC subunit (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003).

Survivin: Survivin was originally described as an antiapoptotic protein that is overexpressed in a variety of human cancers (Ambrosini *et al.*, 1997). Survivin is a highly conserved member of the inhibitor of apoptosis protein (IAP) family as defined by the presence of a single baculoviral IAP repeat (BIR) Zn^{2+}-finger motif. Unlike other IAPs, Survivin lacks a C-terminal RING finger motif. X-ray crystallography of Survivin in solution shows the protein

to be a butterfly-shaped dimer with long alpha helices protruding laterally from the paired BIR motifs (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000). It has been reported that Survivin can bind to caspases 3 and 7, thereby causing a block in the apoptotic process (Tamm *et al.*, 1998). Survivin can also bind to the mitochondrial caspase activator Smac/DIABLO, apparently displacing XIAP, and freeing the XIAP to act as a caspase inhibitor (Song *et al.*, 2003; Song *et al.*, 2004). Besides its putative role in the regulation of apoptosis, the use of Survivin specific antibodies identified the protein to be a member of the CPC (Uren *et al.*, 2000; Wheatley *et al.*, 2001). Survivin binds MTs *in vitro* (Li *et al.*, 1998) and overexpression or depletion of the protein affects MT stability (Rosa *et al.*, 2006). Furthermore, the dynamic posttranslational modification of Survivin by ubiquitin is involved in centromere targeting of the CPC (Vong *et al.*, 2005). Survivin therefore appears to be multifunctional protein with essential roles in the regulation of both mitosis and apoptosis (Altieri, 2006).

Borealin: Borealin was discovered in a proteomic screen for mitotic chromosome scaffolds associated proteins (Gassmann *et al.*, 2004) and, simultaneously, in a screen for proteins capable of binding to chromosomes in *Xenopus* extracts where it was termed Dasra B (Sampath *et al.*, 2004). Borealin is conserved in vertebrates but also found in *Drosophila*. The *C. elegans* protein CSC-1 (Romano *et al.*, 2003) seems to be distantly related. However, no Borealin orthologue seems to be expressed in yeasts. Several species, including *Xenopus* and chicken (but apparently not human), express two Borealin isoforms, the second being termed Borealin 2/Dasra A. In *Xenopus*, Dasra A is important for the assembly of mitotic spindles that occurs in the absence of centrosomes (Sampath *et al.*, 2004). The protein, like Survivin and INCENP, is readily phosphorylated by Aurora B *in vitro* (Gassmann *et al.*, 2004). Recently, Mps1 mediated phosphorylation of Borealin was shown to influence the activity of Aurora B (Jelluma *et al.*, 2008).

In *in vitro* binding- and yeast-two hybrid assays no particular order of CPC assembly could be deduced. Intriguingly, the existence of different subcomplexes of the holo-CPC has been proposed (Gassmann *et al.*, 2004) (see Figure 6). However, the mutual interdependency between members of CPC has hampered to address this hypothesis. siRNA mediated knock-down of any subunit of the CPC leads to a delay in prometaphase, lagging chromatids in anaphase and subsequently results in a cytokinesis defect. Strikingly, CPC depleted cells show a SAC dependent arrest when cells are treated with nocodazole (no KT-MT attachment) but override a taxol induced mitotic arrest (KT-MT attachment but no establishment of tension). Thus, it has been proposed that the CPC might sense tension at the centromere/kinetochore region by an unknown mechanism (Cimini, 2007; Musacchio and

Salmon, 2007). Once the SAC has been satisfied and anaphase commences, the CPC translocates from the inner centromere to the central spindle. This relocation requires the kinesin Mklp2 (Gruneberg *et al.*, 2004) and dephosphorylation of Cdk1 phosphorylation-sites on INCENP (Murata-Hori *et al.*, 2002; Pereira and Schiebel, 2003). At the central spindle, phosphorylation of Mklp1 by Aurora B was shown to be crucial to allow proper cytokinesis (Guse *et al.*, 2005; Neef *et al.*, 2006). Additionally, Ipl1 (the yeast Aurora B homologue) was shown to negatively regulate cytokinesis by delaying cell abscission until all sister chromatids have moved out of the division plane (Norden *et al.*, 2006).

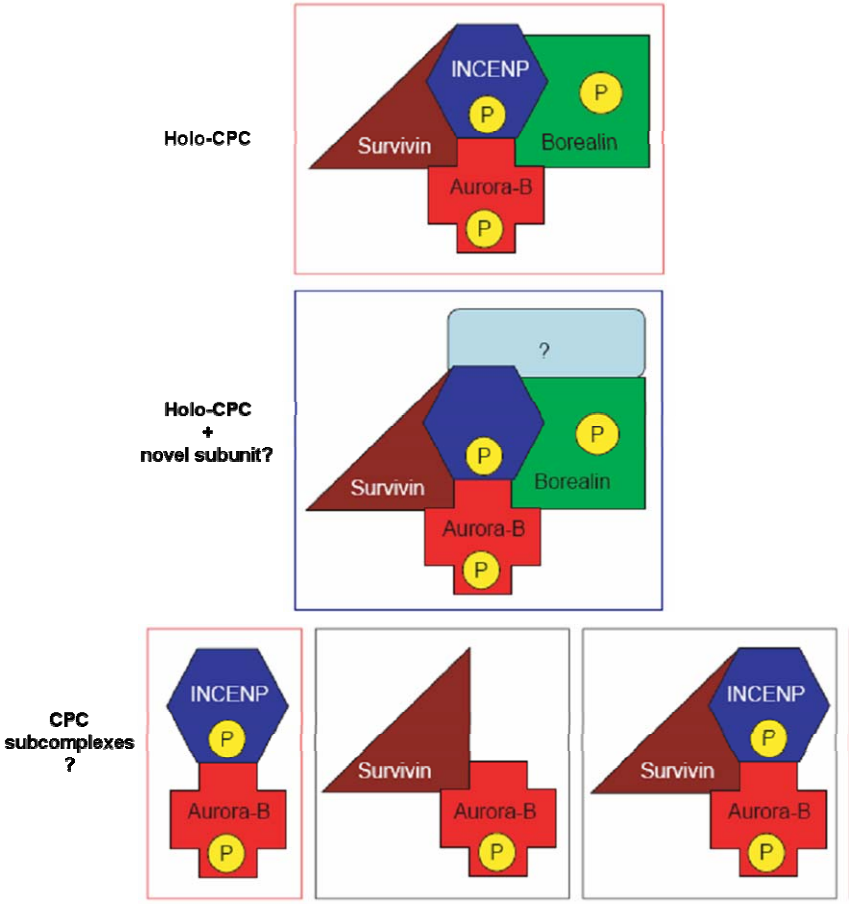


Figure 6. Architecture of the CPC (out of date 2005).

Aurora B is supposed to form different complexes with chromosomal passenger proteins. The holo-CPC comprises INCENP, Survivin, Borealin and Aurora B (first row). This complex might contain other, yet unidentified components (second row). The existence of CPC subcomplexes (bottom row) has been proposed recently e.g. a dimeric INCENP-Aurora B complex was detected using sequential immunprecipitations (Gassmann *et al.*, 2004). Adapted and modified from Giet et al., TRENDS in Cell Biology 2005.

PICH, a centromere bound helicase required for the SAC

Another kinase central to mitotic progression is Plk1. This highly conserved kinase associates with the KT, the central spindle/midbody and the centrosome and it regulates multiple key mitotic events including mitotic entry, centrosome and spindle function, chromosome cohesion and cytokinesis (Barr *et al.*, 2004). KT localization of the protein was reported to depend on the phosphorylation status of INCENP (Goto *et al.*, 2006) and antibody-mediated inhibition of Plk1 was shown to result in a prometaphase arrest (Lane and Nigg, 1996). Plk1 contains a phospho-binding motif, called the Polo-box domain (PBD) which recruits it to proteins that are phosphorylated at a specific consensus site (Elia *et al.*, 2003). PICH (Plk1 interacting checkpoint helicase) was identified in a screen for PBD binding proteins (Baumann *et al.*, 2007), a protein that belongs to the SNF2 family of helicases involved in chromatin organization (Flaus *et al.*, 2006). The protein was shown to be a substrate of Plk1 and cells depleted of Plk1 show spreading of PICH to chromosome arms. PICH, in turn, seems to be required for Plk1 to localize to chromosome arms (Santamaria *et al.*, 2007). Interestingly, a recent study reported a role for PICH in chromosome arm cohesion (Leng *et al.*, 2008). PICH depleted cells fail to arrest in mitosis when treated with drugs that disrupt KT-MT attachments and this has been speculated to be due to the inability of these cells to recruit the checkpoint protein Mad2 to the KT (Baumann *et al.*, 2007). In prometaphase cells, PICH is present at the centromere as well as the outer kinetochore. In metaphase cells, PICH localizes to numerous short threads that link sister KTs of the aligned chromosomes. These threads are progressively stretched and resolved as cells enter anaphase and shown to contain centromeric DNA (Wang *et al.*, 2008). Interestingly, PICH threads are enriched in prometaphase when sister KTs are no longer held together by cohesion (due to Sgo1 depletion) but undetectable under conditions of cohesion loss when taxol (reduced tension between sister KTs) was additionally added, indicating that PICH somehow responds to tension. Thus, if a tension sensor exists and is utilized by the SAC, PICH and/or the CPC (as outlined above) are appealing candidates.

The SUMO system: A multifunctional pathway and novel regulator of mitosis

Traditionally, mitotic progression has been considered to be primarily regulated by two posttranslational modifications, protein phosphorylation and ubiquitin mediated protein degradation. Recently however, the SUMOylation pathway has emerged as a novel regulator of mitosis (Watts, 2007; Dasso, 2008). The SUMO system is involved in the regulation of

several other cellular key processes, including transcriptional control, DNA repair and recombination and nucleo-cytoplasmic shuttling (Muller *et al.*, 2004; Hay, 2005). The unifying theme of SUMO function in all pathways appears to be the SUMO-dependent regulation of specific protein-protein interactions (Geiss-Friedlander and Melchior, 2007).

In humans, at least three SUMO proteins (SUMO1, 2 and 3) are expressed. SUMO2 and SUMO3 are highly related proteins sharing an identity of 97% (therefore collectively referred to as SUMO2/3), while SUMO1 shares only 43% identity with SUMO2/3. During mitosis, SUMO1 appears to localize to the spindle poles in prometaphase/metaphase and to the central spindle during anaphase/telophase. In contrast, SUMO2/3 binds to the chromatin region from prophase until telophase (Ayaydin and Dasso, 2004; Zhang *et al.*, 2008) and co-localizes with inner centromere markers (Azuma *et al.*, 2005). The covalent attachment of SUMO to 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 PIAS family members, hPc2 or RanBP2 (Geiss-Friedlander and Melchior, 2007) (Figure 7). In most cases the acceptor lysine is embedded in a characteristic KXE/D consensus motif. SUMO chains can be formed through linkage of additional SUMO moieties to previously conjugated SUMO proteins. Importantly, SUMO modification is a highly dynamic and reversible process in which SUMO-specific proteases (SENPs) are responsible for catalyzing desumoylation (Hay, 2007) (Figure 7). 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. SENPs are also responsible for the initial activation of SUMO proteins by catalyzing their C-terminal processing. This cleavage exposes a C-terminal glycine-glycine motif that mediates SUMO conjugation via the creation of an isopeptide bond to a lysine residue of a target protein. In addition to the covalent modification of proteins by SUMO, the non-covalent association of SUMO with target proteins has been described (Schmidt and Muller, 2003; Geiss-Friedlander and Melchior, 2007). This interaction seems to be mediated by a SUMO interacting motif (SIM) within the target protein.

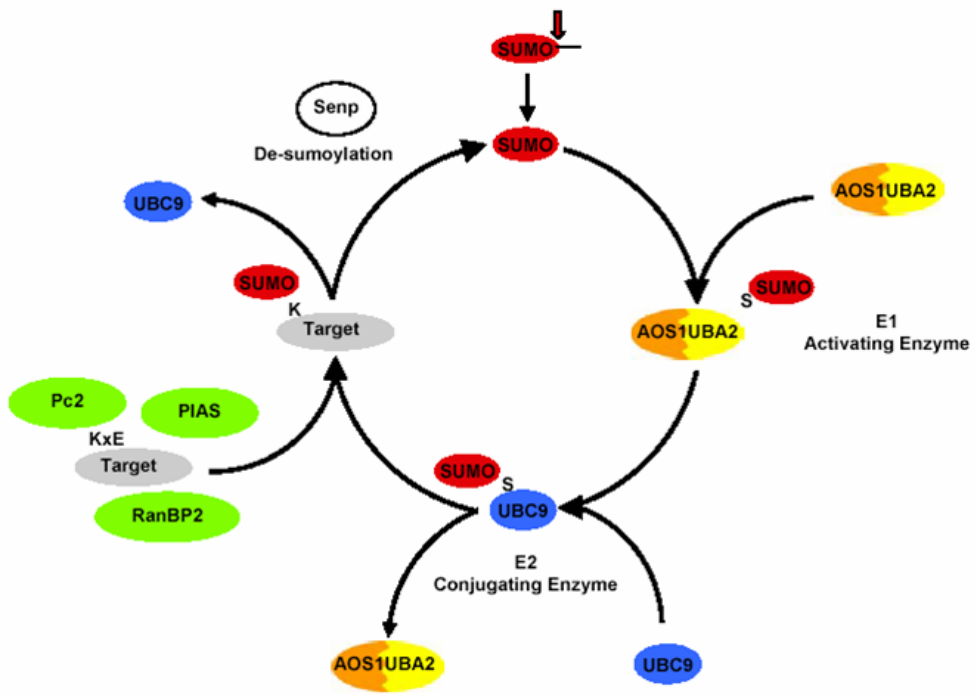


Figure 7. Illustration of the SUMO pathway.

Sumoylation is a highly dynamic process with E1 (yellow), E2 (blue) and E3 enzymes (green) regulating SUMO conjugation and SENPs (white) regulating deconjugation. Adapted and modified from Schmidt and Muller, Cell.Mol.Life.Sci 2003

Data from genetic studies in yeast indicate that a balanced equilibrium of SUMO conjugation and deconjugation is critical for mitotic events, including mitotic entry (Seufert *et al.*, 1995), chromosome cohesion (Bachant *et al.*, 2002) and proper chromosome segregation during anaphase (Tanaka *et al.*, 1999). Recent work has also revealed the importance of sumoylation for mitotic progression in vertebrates. In *Xenopus* egg extracts the SUMO E3 ligase PIASy is essential for SUMO2/3 modification of topoisomerase II, an enzyme required for resolution of catenated DNA strands which in turn is essential for sister chromatid separation. Sumoylation of topoisomerase II recruits the protein to chromatin and depletion of PIASy leads to chromosome missegregation during anaphase (Azuma *et al.*, 2005). In mouse embryonic fibroblasts, RanBP2 has been shown to act as the SUMO E3 ligase catalyzing topoisomerase II sumoylation (Dawlaty *et al.*, 2008). In the same study, adult mice with reduced RanBP2 expression showed an increase in skin tumor development. RanBP2 is found in a complex with RanGAP1 and binds to the KT region (Joseph *et al.*, 2002; Pichler *et al.*, 2002). Observations indicate that the RanGAP1/RanBP2 complex has an important mitotic role, particularly in establishing MT-KT attachments (Salina *et al.*, 2003; Joseph *et al.*, 2004) but it has not been demonstrated that the capacity of RanBP2 to promote such attachments

requires its activity as a SUMO ligase. Studies involving an unmodifiable form of RanGAP1 indicate that its sumoylation is required not only for its interaction with RanBP2 (Mahajan *et al.*, 1997) but also for the interaction of the RanBP2/RanGAP1 complex with KTs and the mitotic spindle (Joseph *et al.*, 2002). Additionally, the SUMO-specific protease SENP5 has been shown to be required for cytokinesis (Di Bacco *et al.*, 2006). However, in higher eukaryotes, only few mitotic targets of SUMO and regulatory components of the SUMO pathway are known.

AIM OF THIS WORK

The chromosomal passenger complex (CPC) consisting of the Aurora B kinase, INCENP, Survivin and Borealin plays a key role during mitotic progression. It is asscociated with the centromere from prophase until metaphase and binds to the central spindle in anaphase before accumulating at the midbody during telophase/cytokinesis.

We aimed to identify specific interactions between the different subunits to shed light on the molecular architecture of the CPC and elucidate the contribution of the individual members for targeting and functioning of the complex. Having identified a ternary subcomplex of the CPC essential and sufficient for centromere recruitment we screened for components of the centromere/kinetochore region that might be regulated by it.

Recently, it has been hypothesized that various CPCs of different composition might exist (Vagnarelli and Earnshaw, 2004). Data on the structure of the core CPC (generated by the collaborating laboratory of Elena Conti) thus prompted us to engineer different subcomplexes and investigate their functions.

Borealin is the most recently identified member of the CPC (Gassmann *et al.*, 2004; Sampath *et al.*, 2004). Hence, little is known about its role within the complex. We therefore wished to identify new interactors of the Borealin protein and analyse them in detail.

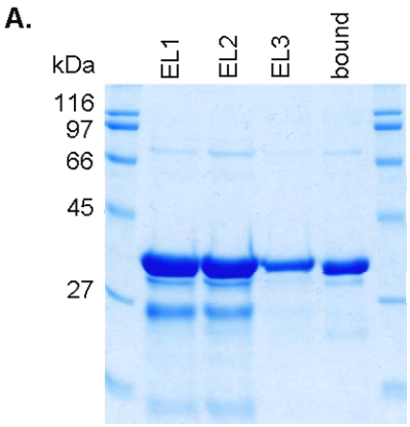
RESULTS

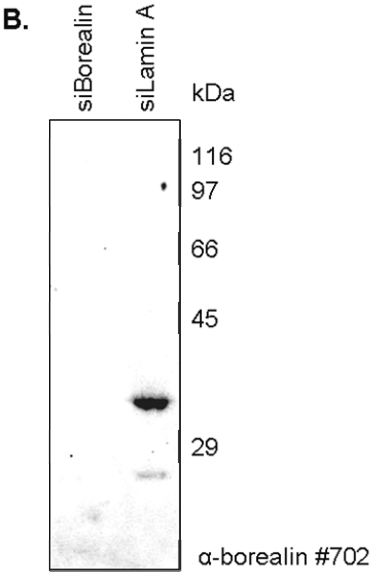
I. Centromere targeting of the CPC

Aurora B kinase function at the centromere is essential for proper chromosome segregation at the metaphase to anaphase transition via resolution of improper KT-MT attachments and creation of stable bipolar attachments (Giet *et al.*, 2005; Musacchio and Salmon, 2007). Therefore, the first goal of this work was to gain more insight into how Aurora B targets to the centromere besides the known interdependence between the members of the CPC in localization.

Production of a polyclonal Borealin antibody

At the time this study was initiated, Borealin had only been identified as a novel member of the CPC (Gassmann *et al.*, 2004) and no commercial anti-Borealin antibody was available. Thus, we set out to produce a polyclonal Borealin specific antibody. Two rabbits (#702 and #725) were immunized with full-length His-tagged Borealin produced in *E.coli* (Figure 8A). Both antibodies gave identical results. By Western blotting a band of 34kDa was detected (Figure 8B) and immunofluorescence showed the characteristic CPC staining pattern (Figure 8C). Importantly, no Western blot or immunofluorescence signal was observed when cells were treated with siRNA oligonucleotides targeting the Borealin transcript, demonstrating the specificity of the antibody.





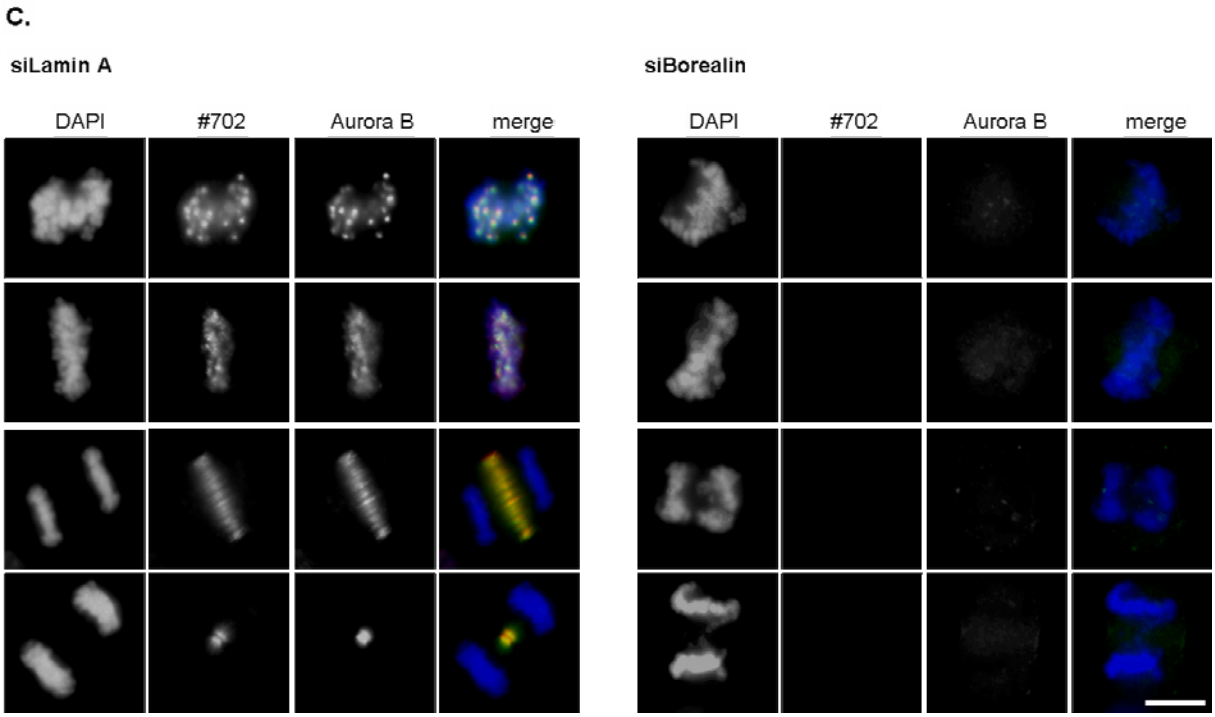


Figure 8. Production and evaluation of a polyclonal Borealin specific antibody.

(A) His-tagged Borealin full-length was purified using standard conditions and eluate fractions (EL1-3) were recovered. Bound = remaining His-Borealin on Ni-NTA beads after elution. The recombinant protein was used for immunization.

(B) HeLa cells were treated with indicated siRNA duplexes for 48hrs and cell lysates were probed with the produced anti-Borealin antibody (final bleed #702).

(C) Experiment as in (B) but cells were fixed for analysis by immunofluorescence using anti-Borealin (final bleed #702) and anti-Aurora B antibodies. The different mitotic stages are monitored. Note that in Borealin knock-down Aurora B cannot localize to the centromere, the central spindle or the midbody. Scale bar = 10µM. Throughout this work DAPI was used to visualize DNA.

The CPC targets to the centromere independent of other kinetochore/centromere poteins

In order to identify components of the kinetochore/centromere region that are required for the CPC to localize to the centromere, candidate proteins were depleted by siRNA duplexes and CPC localization was monitored by immunofluorescence. Interestingly, none of the tested candidates did affect localization of the CPC to the centromere (Figure 9A and B).

A.

siRNA	centromeric CPC
Aurora B	-
Hec1	+
Astrin	+
Cenp-E	+
Cenp-F	+
Cenp-A	+
Cenp-B	+
hMis12	+
Bub1	+
BubRI	+
Plk	+
PICH	+
Mad2	+
Sgo1	+
Sgo2	+
MCAK	+
RanBP2	+
Mps1	+
topoII	+
Ska1	+
HURP	+
PP2A	+

B.

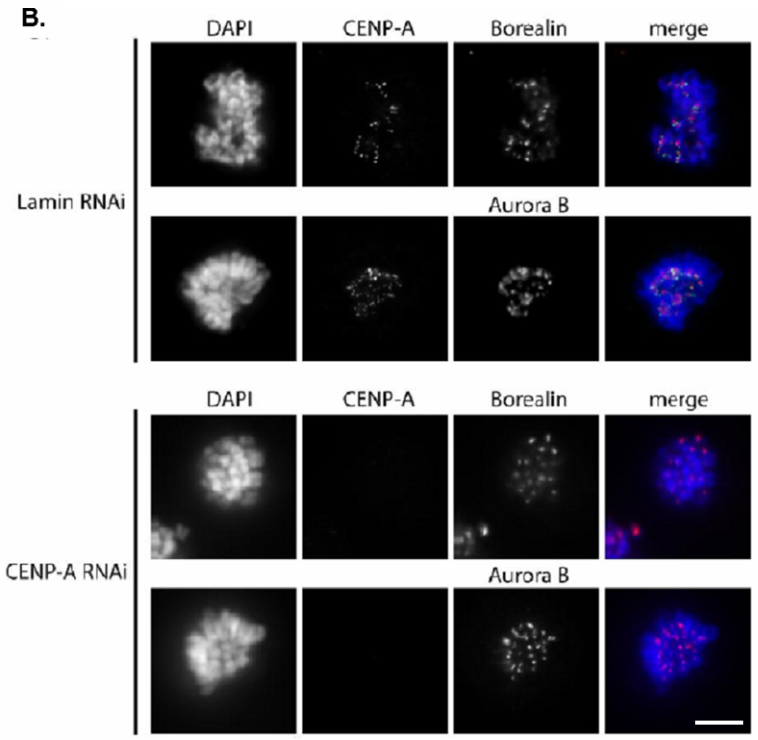


Figure 9. Centromere localization of the CPC in cells depleted of various centromere/kinetochore associated proteins.

(A and B) Indicated proteins were depleted by siRNA treatment and CPC localization was monitored by either anti-rabbit Borealin or anti-mouse Aurora B antibodies. Efficient depletion was verified by co-staining with the respective antibody. In all cases were depletion could not be monitored directly (due to the lack of respective antibodies) previously published siRNA duplexes have been used. – indicates absence of the CPC, + indicates presence of the complex at the centromere. In PP2A depletion the CPC is present at the centromere but spreads over chromosome arms.

(B) Representative result. Depletion of Cenp-A is shown. Scale bar = 10µm.

The N-terminal 58 amino acids of human INCENP are sufficient for centromere targeting

As we were unable to identify any component of the kinetochore/centromere region that acts upstream of the CPC at the centromere we next analysed the intrinsic requirements of the complex for centromere localization. Analysis of how the CPC targets to the centromere has so far concentrated on INCENP, the binding partner of Aurora B. For chicken INCENP, it has been demonstrated that amino acids 1-68 are sufficient to direct the protein to centromeres (Ainsztein *et al.*, 1998). In budding yeast, the removal of Cdk1 phosphorylation sites from INCENP seems to be important for the transfer of INCENP from the centromere to the central

spindle (Pereira and Schiebel, 2003), raising the possibility that Cdk1 phosphorylation might play a role in the centromere targeting of INCENP. Sequence analysis of the first 68 amino acids of human INCENP indicated the presence of a conserved Cdk1-consensus site at position T59 and an *in vitro* kinase assay showed that T59 is the only Cdk phosphorylation site within INCENP$^{1-68}$ (Figure 10). Similarly, we identified S72 of INCENP as a *bona fide* Aurora B phoshorylation site *in vitro* (data not shown).

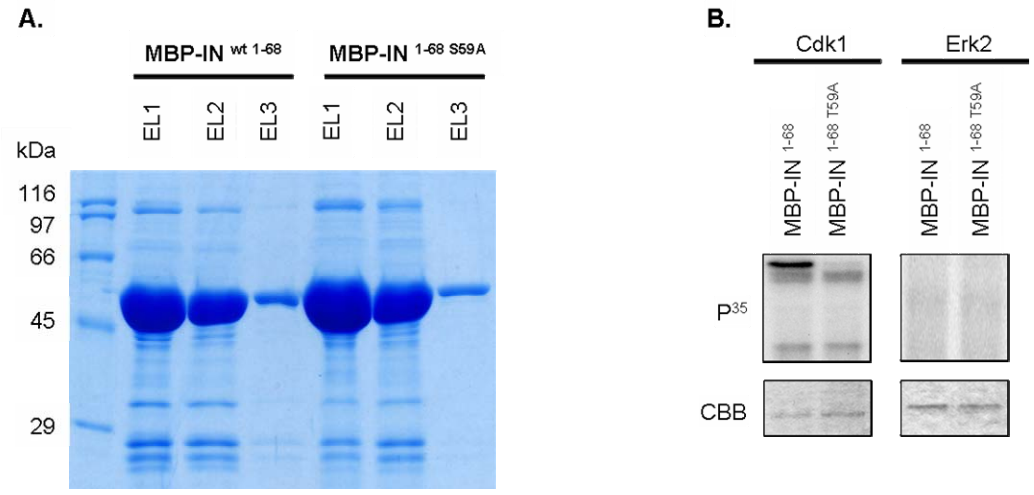


Figure 10. T59 of INCENP is a Cdk1 phosphorylation site.

(A) MBP tagged N-terminal fragments of INCENP were purified from E.coli and eluate fractions (EL1-3) were recovered.

(B) Indicated recombinant proteins were tested in kinase assays using Cdk1 (or Erk2 as control). CBB = Coomassie Brilliant Blue loading control.

To investigate the targeting of human INCENP to the centromere and the potential role of Cdk1 in this process, constructs were prepared comprising residues 1-58 (lacking the potential Cdk1-site) and residues 59-919, tagged with an N-terminal GFP-tag. INCENP$^{1-58}$ localized to the centromere from prophase to metaphase in HeLa cells (Figure 11A). Conversely, the construct lacking residues 1-58 (INCENP$^{59-919}$) was dispersed in the cytoplasm and failed to target to the centromere (Figure 11B). Neither INCENP$^{1-58}$ nor INCENP$^{59-919}$ transferred to the central spindle or the midbody, and both were cytoplasmic in anaphase and telophase cells or associated with the chromatin. These data indicate that the N-terminal 58 amino acids of the INCENP protein must carry information that determines centromere targeting and that Cdk phosphorylation of INCENP is not required for this process. However, T59 of INCENP might be involved in central spindle transfer of the CPC, similar to what has been shown in yeast (Pereira and Schiebel, 2003).

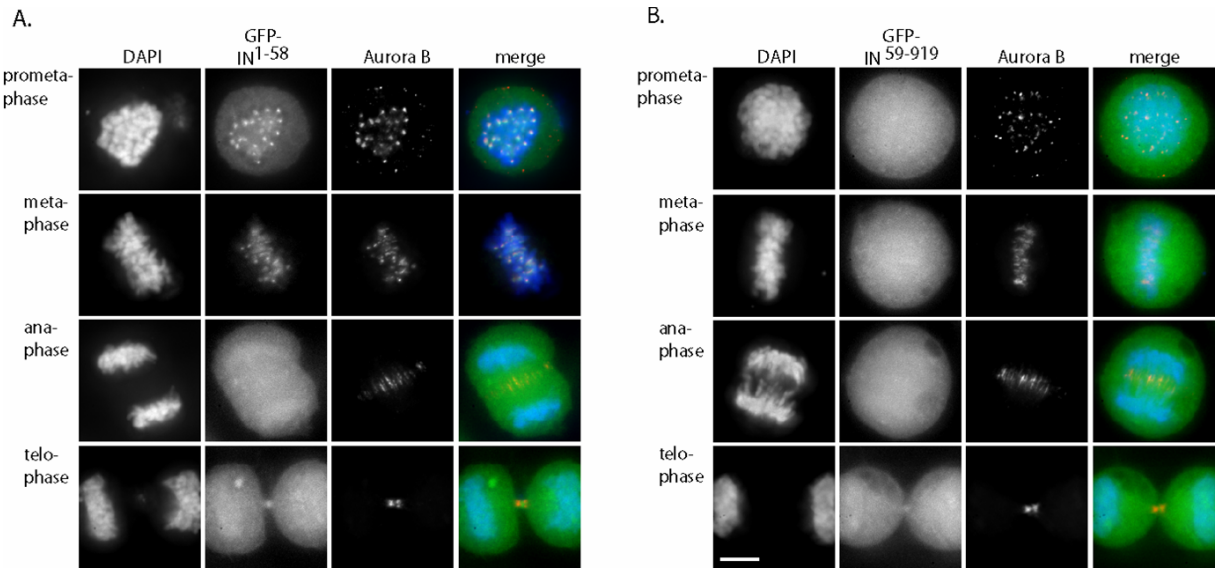


Figure 11. The N terminus of human INCENP (GFP-INCENP$^{1-58}$) targets to the centromere.

HeLa cells were transfected with INCENP$^{1-58}$ or INCENP$^{59-919}$ N-terminally fused to GFP.

(A) GFP-INCENP$^{1-58}$ targets to the centromere in prometaphase and metaphase (independently of its expression level) but remains cytoplasmic in anaphase and telophase.

(B) A GFP-INCENP construct lacking the first 58 amino acids (GFP-INCENP$^{59-919}$) does not localize to any defined cellular structure during mitosis. Bar, 10µm.

Borealin and Survivin bind to the N-terminus of INCENP *in vitro*

Despite the well established binding of Aurora B to the C-terminus of INCENP via the IN-box motif (Bishop and Schumacher, 2002; Honda *et al.*, 2003; Yasui *et al.*, 2004; Sessa *et al.*, 2005) the molecular architecture of the CPC is largely unknown. To determine whether any of the other chromosomal passenger proteins bind to the N-terminus of INCENP and may contribute to the centromere targeting a combination of directed yeast two-hybrid analyses and *in vitro* binding assays with recombinant proteins was used. In two-hybrid assays, Aurora B interacted with full-length INCENP (IN$^{1-919}$) and the C-terminus of INCENP (IN$^{790-919}$) containing the IN-box domain (Figure 12A, I, top and two bottom rows) but not with the coiled-coil domain of INCENP (IN$^{531-789}$) or its N-terminus (IN$^{1-530}$) (Figure 12A, I, rows 2 and 3). (Note that for the two-hybrid assays, an N-terminal construct of INCENP comprising residues 1-530 had to be used because shorter N-terminal fragments were self-activating.) An interaction between full-length INCENP and Borealin was found (Figure 12A, II, top row) in line with a previous report (Gassmann et al., 2004). This interaction was mapped to the N-terminus of INCENP (INCENP$^{1-530}$; Figure 12A, II, second row from top). Interestingly, depletion of the first 58 amino acids of INCENP abolished this interaction (Figure 12A, II, bottom). No interaction between INCENP and Survivin was observed in the two-hybrid assay

(Figure 12A, III). Borealin, in contrast, associated with Survivin (Figure 12A, V). Neither Survivin nor Borealin showed an interaction with Aurora B using this approach (Figure 12A, IV).

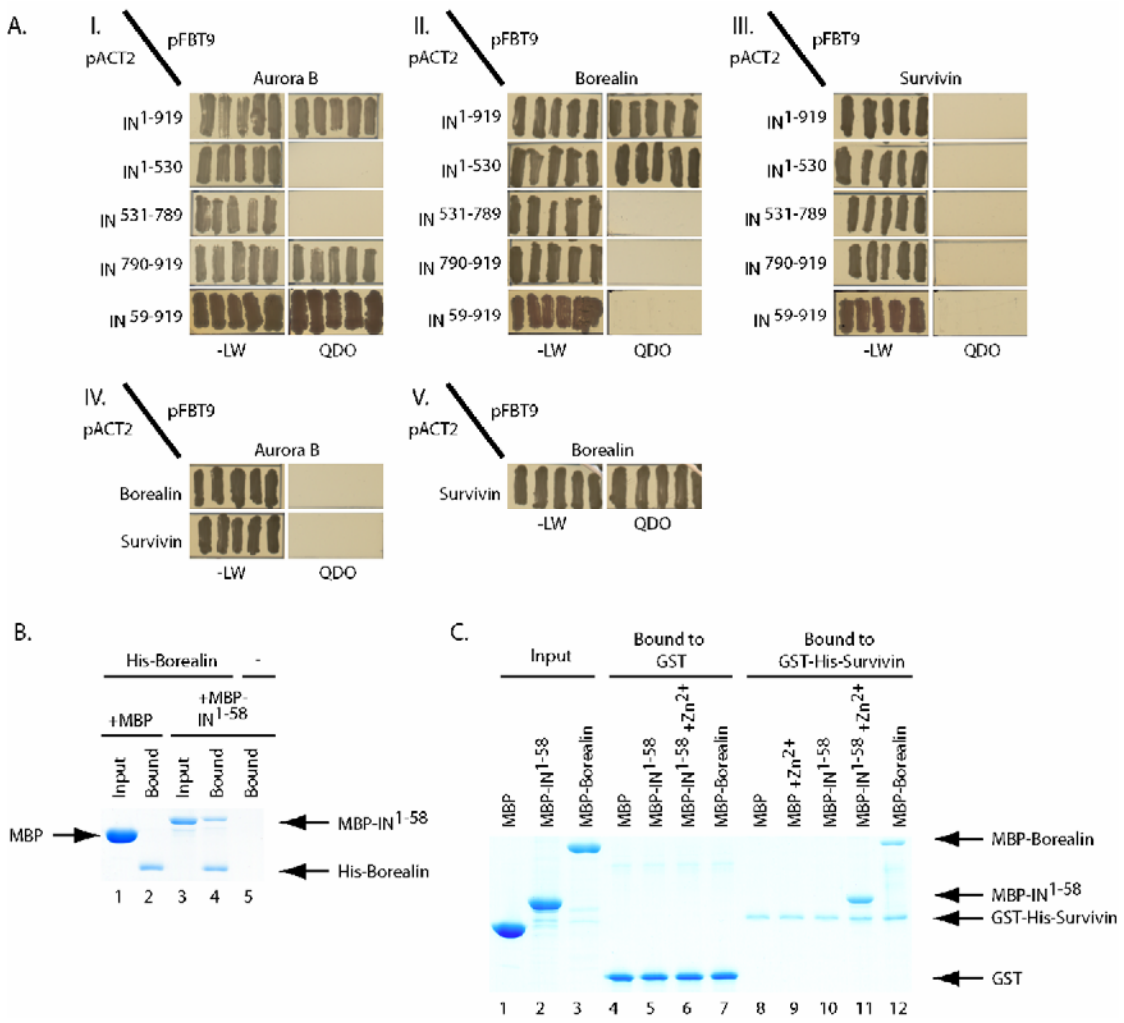


Figure 12. INCENP$^{1-58}$, Borealin, and Survivin form a complex.

(A) pFBT9-Aurora B (I and IV), pFBT9-Borealin (II and V), and pFBT9-Survivin (III) were tested against different fragments of INCENP (I–III) and full-length Borealin or Survivin (IV and V) in pACT2 by two-hybrid analysis. INCENP fragments were created based on a coiled-coil prediction tool (Berger *et al.*, 1995). INCENP$^{1-58}$ could not be used in this assay because it was self-activating. -LW indicates plates lacking leucine and tryptophane, whereas QDO indicates plates lacking leucine, tryptophane, histidine and adenine.

(B) MBP (lanes 1 and 2) or MBP INCENP$^{1-58}$ (lanes 3–5) was incubated with His- Borealin immobilized on Ni-NTA agarose (lanes 2 and 4) or Ni-NTA agarose alone (lane 5). Input and protein bound to the beads were analyzed by SDS-PAGE.

(C) MBP (lanes 1, 4, 8, and 9), MBP-INCENP$^{1-58}$ (lanes 2, 5, 6, 10, and 11), and MBP-Borealin (lanes 3, 7, and 12) were incubated with GST (lanes 4–7) or GST-His-Survivin immobilized on glutathione-Sepharose (lanes 8–12). Zn^{2+} (20mM) was added to the reactions where indicated (lanes 6, 9, and 11).

To corroborate the yeast two-hybrid observations and explore whether Borealin and Survivin could bind directly to the N-terminal 58 amino acids of INCENP, *in vitro* binding assays were used. In agreement with the two-hybrid data, an interaction between His-tagged Borealin (His-Borealin) and MBP-tagged INCENP$^{1-58}$ (MBP-IN$^{1-58}$) was observed (Figure 12B, lane 4). Furthermore, both MBP-Borealin and MBP-INCENP$^{1-58}$ bound to immobilized GST-His-Survivin (Figure 12C, lanes 11 and 12). The interaction between MBP-INCENP$^{1-58}$ and GST-His-Survivin was only observed in the presence of excess Zn^{2+} (Figure 12C, compare lanes 10 and 11), whereas the interaction between MBP-Borealin and GST-His-Survivin was not dependent on this (lane 12). As the yeast two hybrid data did not reveal an interaction between Survivin and INCENP, we presume that the yeast nucleus does not allow for the specific folding of Survivin required for this binding (see yeast two-hybrid data in Figure 12A, column III). We conclude that while Aurora B associates with the C terminus of INCENP via the IN-box, Survivin and Borealin interact with the N-terminal 58 amino acids of INCENP and bind to each other directly.

Borealin and Survivin can form higher order structures *in vivo*

The finding that both Borealin and Survivin interact with the first 58 amino acids of INCENP as well as with each other suggests the existence of a heterotrimeric complex assembled on the N-terminus of INCENP. Because oligomerization of Survivin as well as Borealin had been observed *in vitro* (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000; Gassmann *et al.*, 2004), we next asked whether this can also occur *in vivo*. To this end, HeLa cells were co-transfected with Flag- and Myc-tagged constructs of either Survivin, Borealin, or INCENP. The cells were then arrested with nocodazole to enrich for transfected mitotic cells. Coimmunoprecipitation of Flag- and Myc-tagged constructs was assessed by Western blotting. Oligomerization was observed for Borealin and Survivin but not INCENP (Figure 13, compare lanes 3 and 4 in I–III). Based on these experiments, we propose that Survivin and Borealin are prone to form higher order structures *in vivo*. However, the stochiometry of the formed complexes (e.g. with regard to INCENP binding) and/or their function is not assessed in this assay.

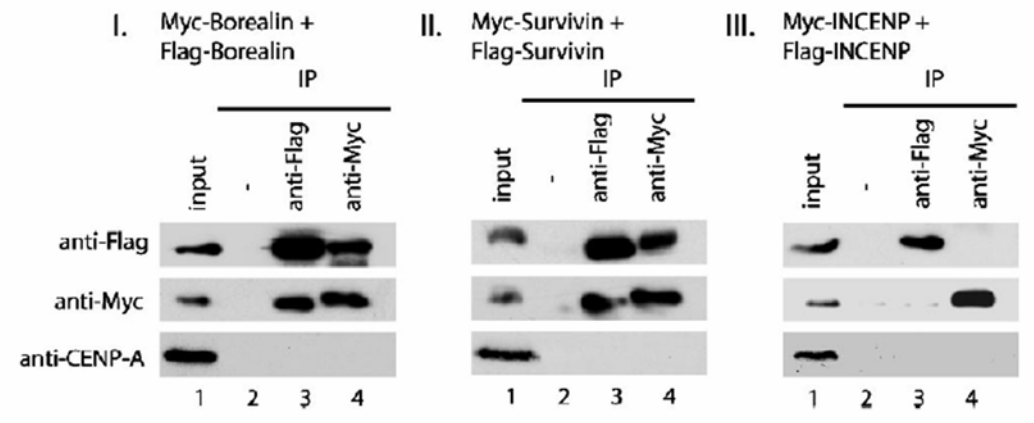


Figure 13. Borealin and Survivin can form oligomeres *in vivo*.

Myc- or Flag-tagged CPC constructs were co-overexpressed in HeLa cells arrested by nocodazole treatment followed by Myc and Flag pull-downs and Western blotting with anti-Flag or anti-Myc antibodies.

The N-terminus of INCENP forms a complex with Survivin and Borealin *in vivo*

To verify that the N-terminal domain of INCENP can also interact with Survivin and Borealin *in vivo*, different GFP-tagged INCENP constructs were transfected into HeLa cells and immunoprecipitations were performed on mitotic lysates using anti-GFP antibodies. Full-length GFP-INCENP coprecipitated Aurora B, Borealin, and Survivin (Figure 14, lane 1). INCENP fragments containing the C-terminal IN-box motif of INCENP pulled down Aurora B but not Survivin or Borealin (Figure 14, lanes 5 and 7). In contrast, N-terminal fragments of INCENP containing residues 1-58 precipitated Borealin and Survivin but not Aurora B (lanes 3 and 4) whereas the coiled-coil domain of INCENP lacking both the N- and the C-terminus did not associate with any of the other CPC components (lane 6). None of the constructs coprecipitated the centromeric protein CENP-A (lanes 1–7, second row from bottom) or the kinetochore protein Hec1 (lanes 1–7, bottom row). Together, these results support data on the interaction between the C-terminus of INCENP containing the IN-box motif and Aurora B and show that a ternary complex between Borealin, Survivin, and the first 58 amino acids of INCENP (IN^{1-58}) exists *in vivo*.

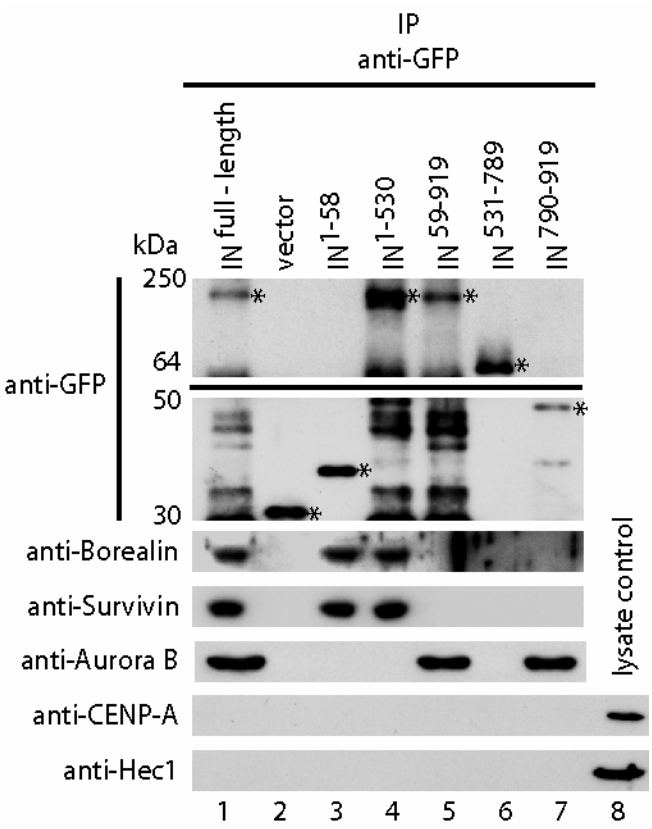


Figure 14. INCENP$^{1-58}$, Borealin, and Survivin form a ternary complex *in vivo.*

Different fragments of INCENP fused to GFP were transfected into HeLa cells and precipitated from mitotic lysates with sheep anti-GFP antibodies. Coprecipitating passenger proteins or the control centromere/kinetochore proteins CENP-A and Hec1 were visualized by Western blotting. Asterisks indicate GFP constructs. The anti-GFP blot was cut into two halves to remove the strong signal of the immunoglobulin heavy chain.

Ectopic GFP-INCENP full-length can complement for the loss of endogenous INCENP in a siRNA based complementation assay

To examine the physiological relevance of these findings for the targeting of the CPC to the centromere *in vivo*, a siRNA based rescue assay was established. INCENP or Aurora B were effectively knocked-down by a 36hrs treatment with siRNA oligonucleotides targeting the 3´-UTR of the corresponding transcripts. In line with previous results (Honda *et al.*, 2003; Gassmann *et al.*, 2004), the knock-down of Aurora B or INCENP resulted in the loss of all other CPC components from the centromere, the central spindle and the midbody (Figure 15A). Furthermore, Western blotting showed that knock-down of INCENP resulted in the simultaneous loss of Aurora B and *vice versa* and strong reduction of Survivin levels, whereas the protein level of Borealin was less affected (Figure 15B, lanes 2 and 3). Depletion of Borealin did not significantly affect Aurora B or INCENP levels but reduced the level of Survivin and the knock-down of Survivin reduced the level of all members of the CPC (Figure 15B, lanes 4 and 5). To express GFP-tagged INCENP constructs in the absence of the endogenous protein, cells were transfected

simultaneously with INCENP siRNA oligonucleotides and the respective rescue constructs (see Materials and Methods for precise protocol). The effectiveness of INCENP knock-down in these experiments was assessed by staining with antibodies against Aurora B and was found to be close to 100% (Figure 15C). The transfection efficiency of the rescue constructs was typically around 10-20%. To validate the assay, control rescue experiments using the combination of INCENP siRNA treatment and transfection with full-length GFP-tagged INCENP were performed. In cells treated with siRNA duplexes targeting endogenous INCENP, the transfected GFP-INCENP full-length construct efficiently localized to the centromere and restored Borealin, Survivin, and Aurora B staining (Figure 16). In addition, phospho-S7-CENP-A staining, a marker for Aurora B kinase activity (Zeitlin *et al.*, 2001), was lost completely in INCENP depleted cells but restored upon expression of GFP-INCENP full-length.

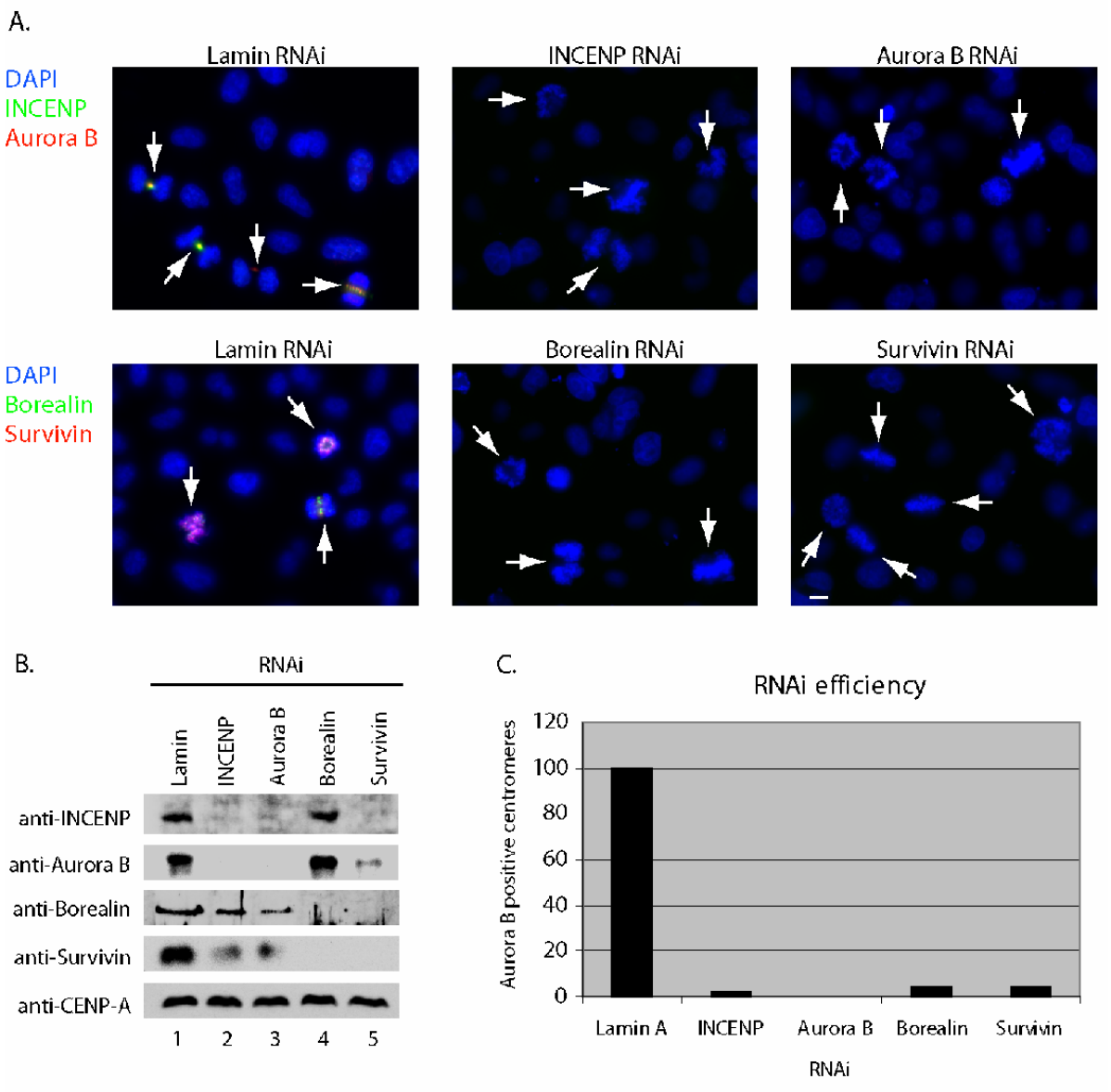


Figure 15. Evaluation of 3´-UTR siRNA duplexes used to knock-down passenger protein expression.
(A) HeLa cells were treated with siRNA oligonucleotides targeting the 3´-UTR of INCENP, Aurora B, Borealin, or Survivin transcripts, fixed, and then costained with antibodies against INCENP and Aurora B (INCENP- and Aurora B RNAi, top) or Borealin and Survivin (Borealin- and Survivin RNAi, bottom). Mitotic cells are indicated by arrows. Bar, 10 µm.
(B) Cell extracts from cells depleted for the different passenger proteins by treatment with corresponding siRNA oligonucleotides for 36hrs were analyzed by Western blotting. The blots were reprobed with antibodies against CENP-A to demonstrate equal loading (bottom row).
(C) HeLa cells treated with the indicated 3´-UTR siRNA oligonucleotides targeting chromosomal passenger proteins for 36hrs were stained with antibodies against Aurora B. One hundred cells were assessed in each case for the presence of Aurora B centromere staining.

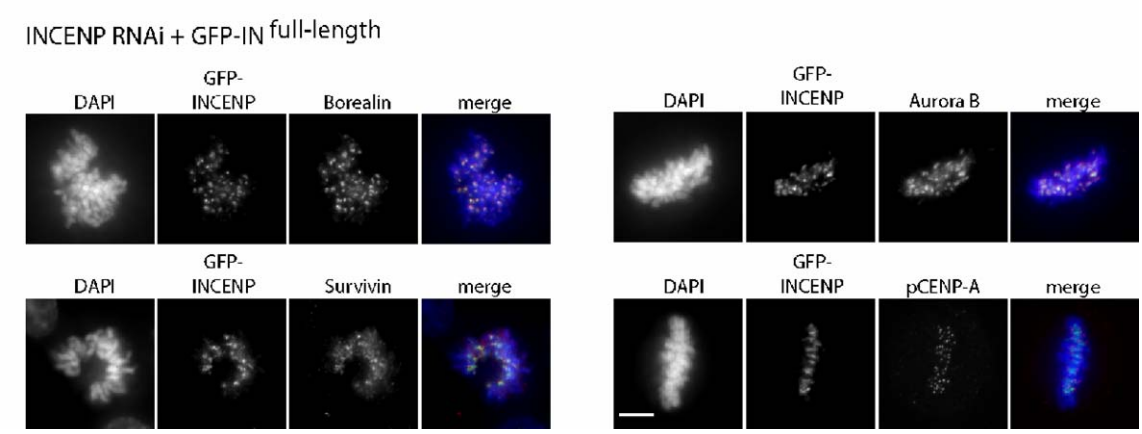


Figure 16. Rescue of INCENP depletion by ectopic expression of GFP-INCENP$^{full\text{-}length}$.
HeLa cells were treated with 3´-UTR siRNA oligonucleotides targeting INCENP and simultaneously transfected with GFP-INCENP$^{full\text{-}length}$. Transfected cells were analyzed for the presence of the other chromosomal passenger proteins or phospho-S7-CENP-A staining as a readout for Aurora B activity. For this experiment and all following CPC rescue assays usually between 8 and 25 cells could be analysed per experiment. Images shown are representatives of three independent experiments. Bar, 10 µm.

We note that phospho-S7-CENP-A staining was used as a read-out because it was found to be more sensitive to siRNA-mediated knockdown of chromosomal passenger proteins than staining with anti-phospho-S10-histone H3 antibody (Figure 17).

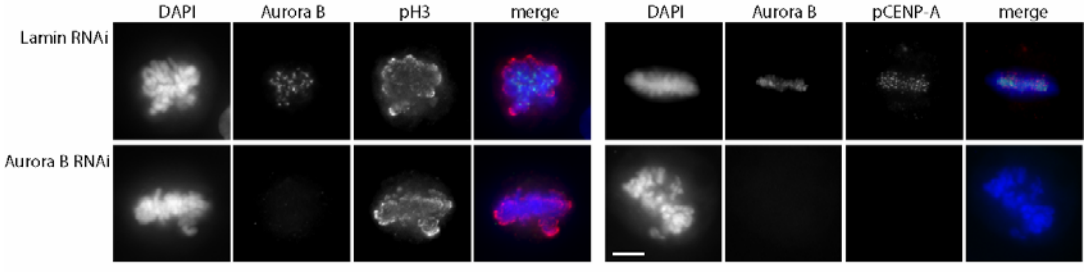


Figure 17. phospho-S7-Cenp-A is a reliable read-out for Aurora B kinase activity.

HeLa cells were treated with indicated siRNA duplexes and immunofluorescence was performed with Aurora B, phospho-S10-histone H3 and phospho-S7-CENP-A antibodies. As phospho-S7-Cenp-A but not phospho-S10-histone H3 is absent in Aurora B siRNA in immuofluorescence we used phospho-S7-CENP-A throughout the course of this work as a read-out for Aurora B activity. Bar, 10 µm.

Ectopic GFP-INCENP$^{1-58}$ can target Survivin and Borealin but not Aurora B to the centromere in the absence of endogenous INCENP

Having established the siRNA based rescue assay, this approach was used to assess the ability of GFP-tagged INCENP$^{1-58}$ to target to the centromere in cells depleted of endogenous INCENP. GFP-INCENP$^{1-58}$ efficiently localized to the centromere in this scenario. The transfected cells were also positive for Survivin and Borealin but, consistent with the coprecipitation data (Figure 14), neither for Aurora B nor phospho-S7-CENP-A (Figure 18A).

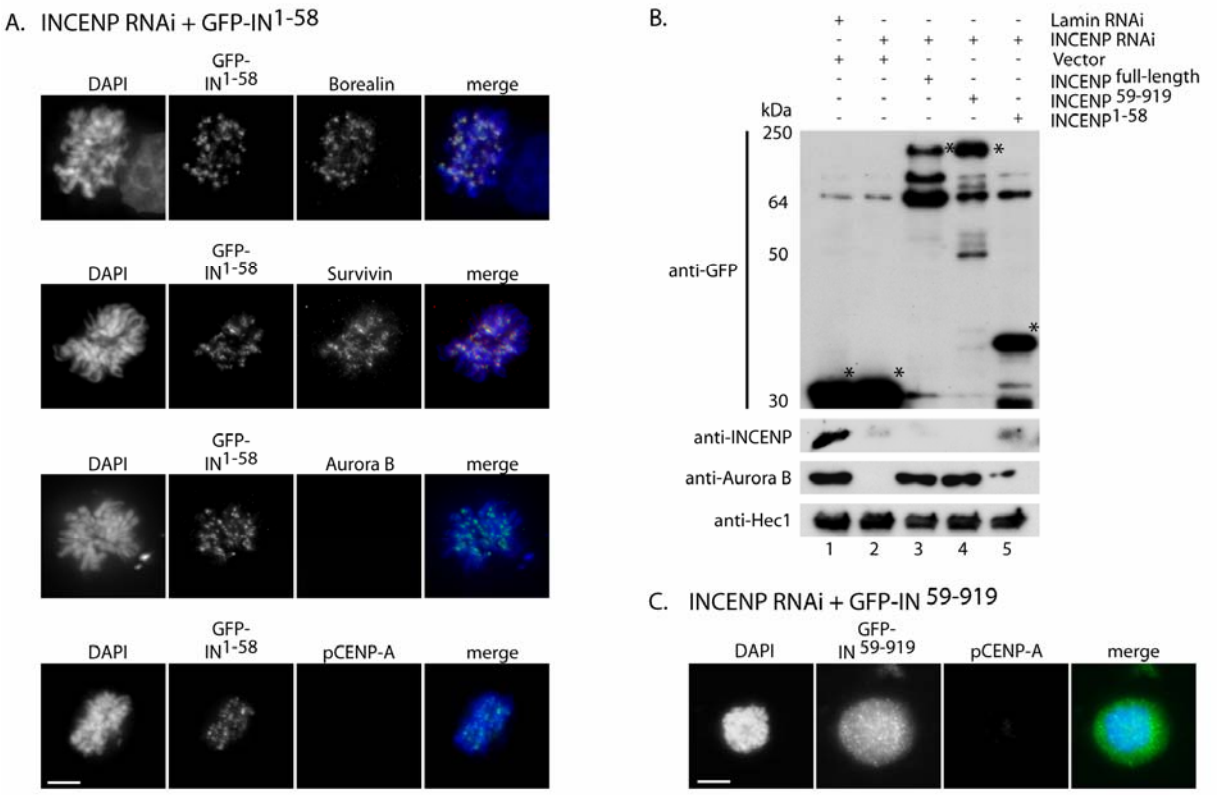


Figure 18. GFP-INCENP$^{1-58}$ targets to the centromere in the absence of endogenous INCENP and restores Survivin and Borealin but not Aurora B staining.

(A) HeLa cells were treated with 3´-UTR siRNA oligonucleotides targeting INCENP and simultaneously transfected with GFP-INCENP$^{1-58}$. Transfected cells were analyzed for the presence of the other chromosomal passenger proteins or phospho-S7-CENP-A staining as a readout for Aurora B activity.

(B) HeLa cells were transfected with the indicated constructs in pcDNA4/TO-EGFP encoding puromycin resistance and treated with the indicated siRNA oligonucleotides for 36hrs. Twenty-four hours before harvesting, the cells were treated with 2µg/ml puromycin. Asterisks indicate GFP constructs.

(C) INCENP siRNA-treated HeLa cells transfected with INCENP$^{59-919}$ were treated as in (A) and costained for phospho-S7-CENP-A. Bar, 10µm.

Biochemical analysis of cells treated as described above but enriched for transfected cells by puromycin selection showed that in contrast to GFP-INCENP full- length and GFP-INCENP$^{59-919}$ (both containing the Aurora B binding IN-box motif), GFP-INCENP$^{1-58}$ could not rescue the protein levels of Aurora B (Figure 18B, compare lane 5 with lanes 3 and 4) in cells lacking endogenous INCENP. Interestingly, the rescue of Aurora B protein levels in the absence of correct localization was not sufficient to restore Aurora B function at the centromere as indicated by the absence of phospho-S7-CENP-A staining in cells depleted of endogenous INCENP and complemented with GFP-INCENP$^{59-919}$ (Figure 18C). This stresses the importance of the first 58 amino acids of INCENP for correct Aurora B localization to the centromere which appears to be a pre-requisite for the phosphorylation of target proteins.

Together, these results indicate that a complex consisting of INCENP$^{1-58}$, Survivin, and Borealin is sufficient for centromere targeting, and, remarkably, it does not require the presence of the Aurora B protein. Although Western blotting showed that in INCENP siRNA-treated cells, Aurora B was reduced to undetectable levels (Figure 15B, lane 2), the possibility remained that the above-mentioned observations were due to minimal residual Aurora B in INCENP siRNA-treated cells, which might be sufficient to mediate centromere targeting. To exclude this, INCENP and Aurora B were simultaneously depleted and GFP-tagged INCENP$^{1-58}$ was expressed. The additional knock-down of Aurora B did not change the result that INCENP$^{1-58}$, Borealin, and Survivin could target to the centromere (Figure 19, top row). One protein that could potentially compensate for the loss of Aurora B function is the related Aurora C kinase. Aurora C was shown to localize like a chromosomal passenger protein, but it is expressed at lower levels than Aurora B and might be functionally redundant with Aurora B (Li *et al.*, 2004; Sasai *et al.*, 2004). However, simultaneous depletion of INCENP and Aurora C or INCENP, Aurora C, and Aurora B followed by rescue with GFP-INCENP$^{1-58}$

resulted in centromere localization of the ternary INCENP$^{1-58}$–Survivin–Borealin complex, excluding a compensatory role for Aurora C (Figure 19, middle and bottom rows).

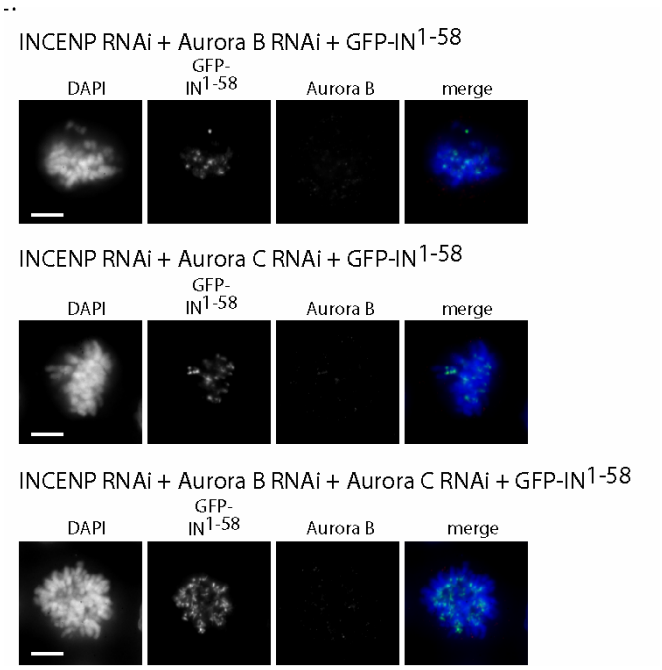


Figure 19. GFP-INCENP$^{1-58}$ targets to the centromere in the absence of Aurora B and Aurora C.
INCENP complementation assay as established above was carried out with the additional depletion of the indicated proteins. The GFP and Aurora B signals were monitored by immunofluorescence. Bar, 10µM.

Aurora B kinase activity is not required for the maintenance of the CPC at the centromere

The above-mentioned findings show that at least for the initial targeting of the CPC to the centromere Aurora B kinase activity is not required. However, prolonged highlevel expression of kinase-dead Aurora B leads to loss of Aurora B itself and the other chromosomal passengers from the centromere and spreading throughout the chromatin (Ditchfield *et al.*, 2003; Honda *et al.*, 2003). This suggests either a requirement for Aurora B kinase activity for the maintenance of the CPC at the centromere, or, alternatively, that overexpression of the kinase-dead Aurora B exerts a dominant-negative effect on the localization of the CPC. Because the prolonged direct inhibition of Aurora B kinase activity by a chemical inhibitor does not affect the association of Aurora B itself or the other chromosomal passengers with the centromere (Ditchfield *et al.*, 2003) (Figure 20), the latter explanation seems more likely.

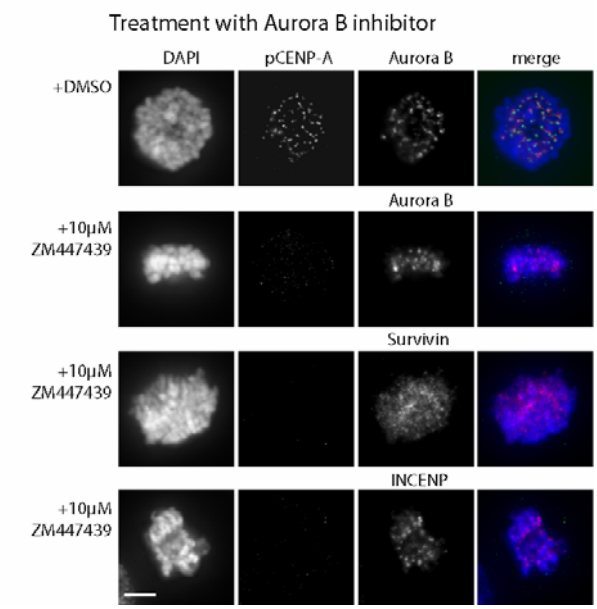


Figure 20. Inhibition of Aurora B kinase activity does not alter CPC localization to the centromere.
Hela cells were treated with the Aurora B kinase inhibitor ZM447439 (or DMSO as control) for 2hrs and immunofluorescence was performed using phospho-S7-Cenp-A and Aurora B antibodies. Bar, 10μM.

The ternary INCENP$^{1-58}$-Survivin-Borealin subcomplex cannot functionally rescue INCENP depletion

Together with the results from the siRNA rescue experiments, these data demonstrate that Aurora B kinase activity is neither required for the initial recruitment of the chromosomal passenger proteins to the centromere nor for the maintenance of the proteins at the centromere but that a subcomplex of INCENP$^{1-58}$, Survivin, and Borealin is essential for centromere targeting. We next tested if the ternary complex can functionally compensate for the loss of centromere bound Aurora B by monitoring BubR1, a spindle checkpoint protein that was shown to be absent from the kinetochore in cells treated with Aurora B inhibitors. BubR1 was present in INCENP depleted cells complemented with GFP-INCENP full-length but absent when complemented with GFP-INCENP$^{1-58}$ (Figure 21A). Furthermore, in cells complemented with GFP-INCENP$^{1-58}$ chromosome alignment on the metaphase plate was disturbed (Figure 21B). These data are consistent with the documented requirement for Aurora B kinase function in the recruitment of checkpoint proteins and chromosome congression (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003).

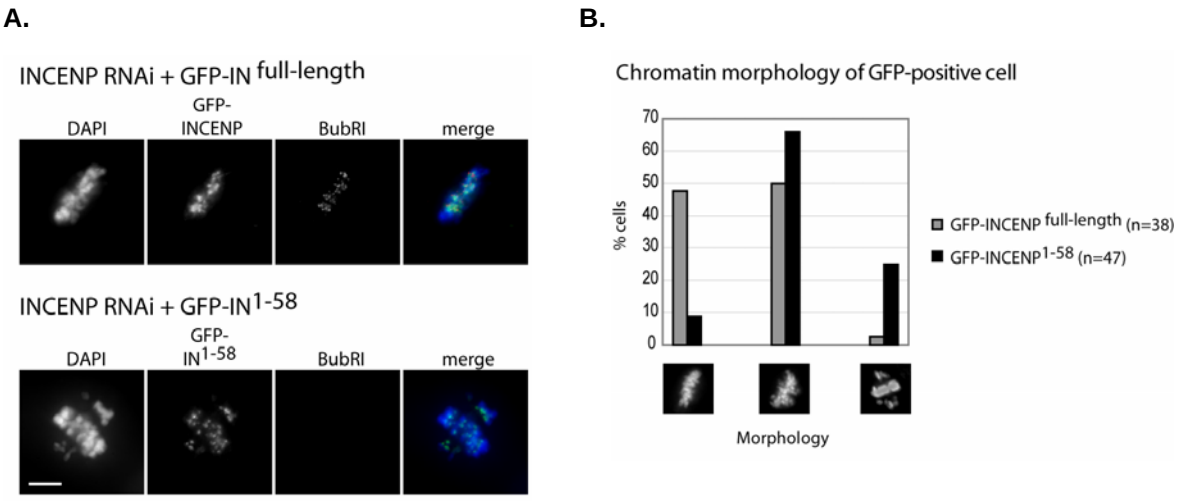


Figure 21. INCENP$^{1-58}$ is not functional at the centromere in BubR1 recruitment and proper chromosome alignment.

(A) INCENP complementation assay as described above monitoring BubR1 kinetochore recruitment.

(B) Experiment as in (A) monitoring chromatin alignment onto the metaphase plate.

Borealin binds to double-stranded DNA *in vitro*

Because no centromere/kinetochore component so far analyzed was found to influence CPC recruitment to the centromere the possibility that one or more of the CPC subunits might be able to bind to DNA directly was considered. To test this hypothesis, purified histone H3, recombinant MBP-Borealin, His-Borealin, MBP-INCENP$^{1-58}$, MBP, GST-Cdc20, and His-Plk1 were incubated with native double-stranded calf-thymus DNA-cellulose. Only His-Borealin and MBP-Borealin, as well as the positive control protein histone H3, bound to the DNA (Figure 22A, lanes 7, 9, and 2, respectively). The centromere/kinetochore proteins Cdc20 and Plk1, both of which exhibit a similar basic pI as Borealin, did not bind to the DNA cellulose, arguing against a non-specific charge-based association of Borealin with the cellulose. Furthermore, increased salt concentrations reduced binding of His-Borealin to the DNA (Figure 22B). Importantly, when His-Borealin, His-Survivin, and MBP-INCENP$^{1-58}$ were mixed and incubated with DNA-cellulose, His-Borealin could recruit the two other proteins to the DNA (Figure 22C, lane 4).

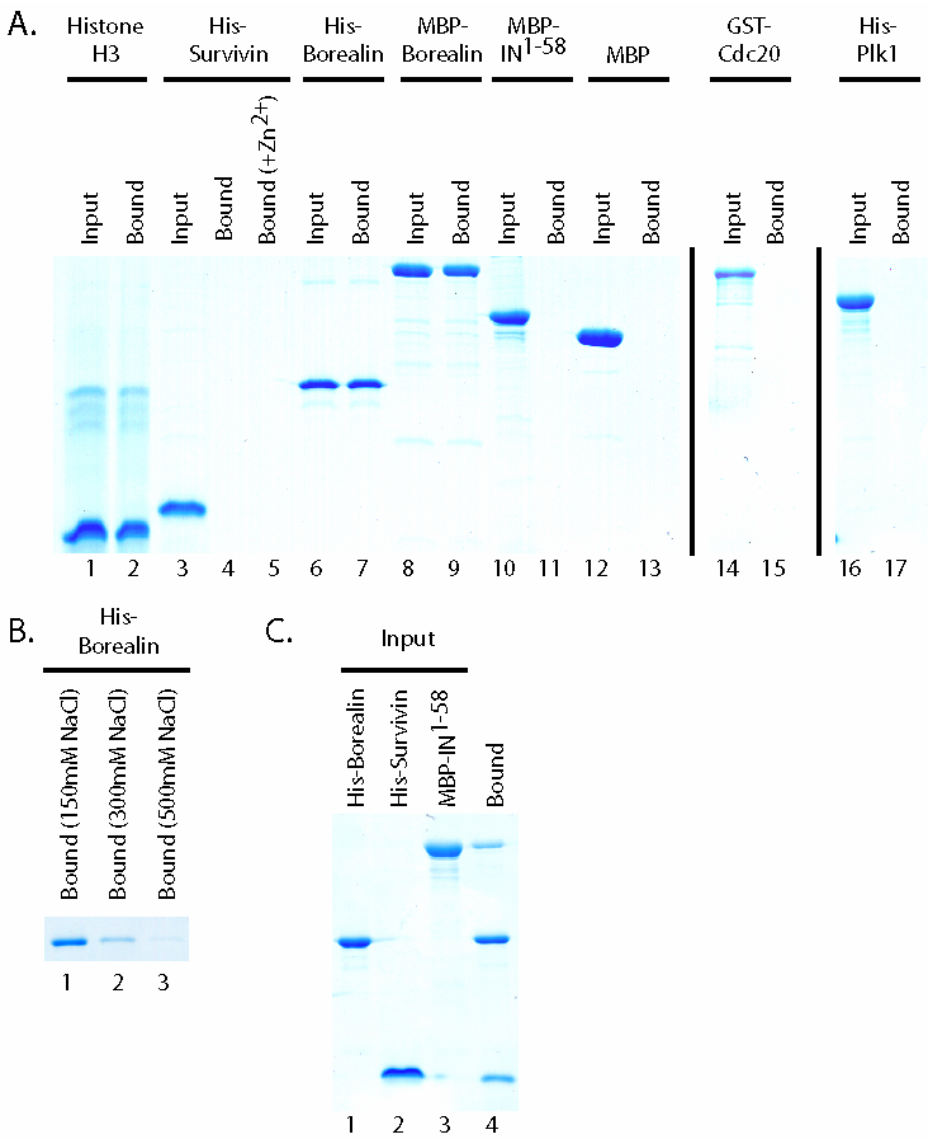


Figure 22. Borealin binds to double-stranded DNA *in vitro*.

(A) Histone H3, His-Survivin, His-Borealin, MBP-Borealin, MBP-INCENP$^{1-58}$, MBP alone, GST-Cdc20, and His-Plk1 were tested for DNA-binding activity by incubation with calf-thymus DNA-cellulose. Input (lanes 1, 3, 6, 8, 10, 12, 14, and 16) and proteins bound to the cellulose (lanes 2, 4, 5, 7, 9, 11, 13, 15, and 17) were analyzed by SDS-PAGE. Only histone H3 (lane 2), His-Borealin (lane 7), and MBP-Borealin (lane 9) bound to the DNA cellulose.

(B) His-Borealin was incubated with DNA-cellulose as in (A) in the presence of increasing NaCl concentrations.

(C) His-Borealin, His-Survivin, and MBP-INCENP$^{1-58}$ were mixed at equimolar concentrations and incubated with calf-thymus DNA-cellulose as described above. In the presence of His-Borealin, His-Survivin and MBP-INCENP$^{1-58}$ were found in the DNA-binding fraction (lane 4).

The integrity of the ternary INCENP$^{1-58}$-Survivin-Borealin subcomplex is essential for its centromere binding

The above data suggest that Borealin might be the subunit within the CPC that can directly bind to DNA and thus localize the CPC to the centromere. Hence, it was tested whether Borealin could target to the centromere *in vivo* independently of its binding partners INCENP and Survivin. Endogenous Borealin was knocked-down with siRNA duplexes targeting the 3´-UTR (see Figure 15B, lane 4, for Western blot) and replaced by transfected Myc-tagged Borealin. Myc-Borealin localized correctly to the centromere in the absence of endogenous Borealin and restored targeting of the other passenger proteins to the centromere as well as phospho-S7-CENP-A staining (Figure 23A). In contrast, centromere targeting of Myc-Borealin was abolished when INCENP (Figure 23B) or Survivin (Figure 23C) were simultaneously depleted in this assay. Thus, even though Borealin is able to bind to DNA *in vitro*, *in vivo* the presence of INCENP and Survivin is required for targeting to the centromere. Similarly, neither Myc-Survivin (Figure 23D, E and F) nor GFP-INCENP$^{1-58}$ (Figure 23G and H) were able to localize to the centromere in the absence of one of their binding partners in corresponding complementation assays. This provides evidence that the integrity of the INCENP$^{1-58}$-Survivin-Borealin subcomplex must be maintained to target these components to the centromere.

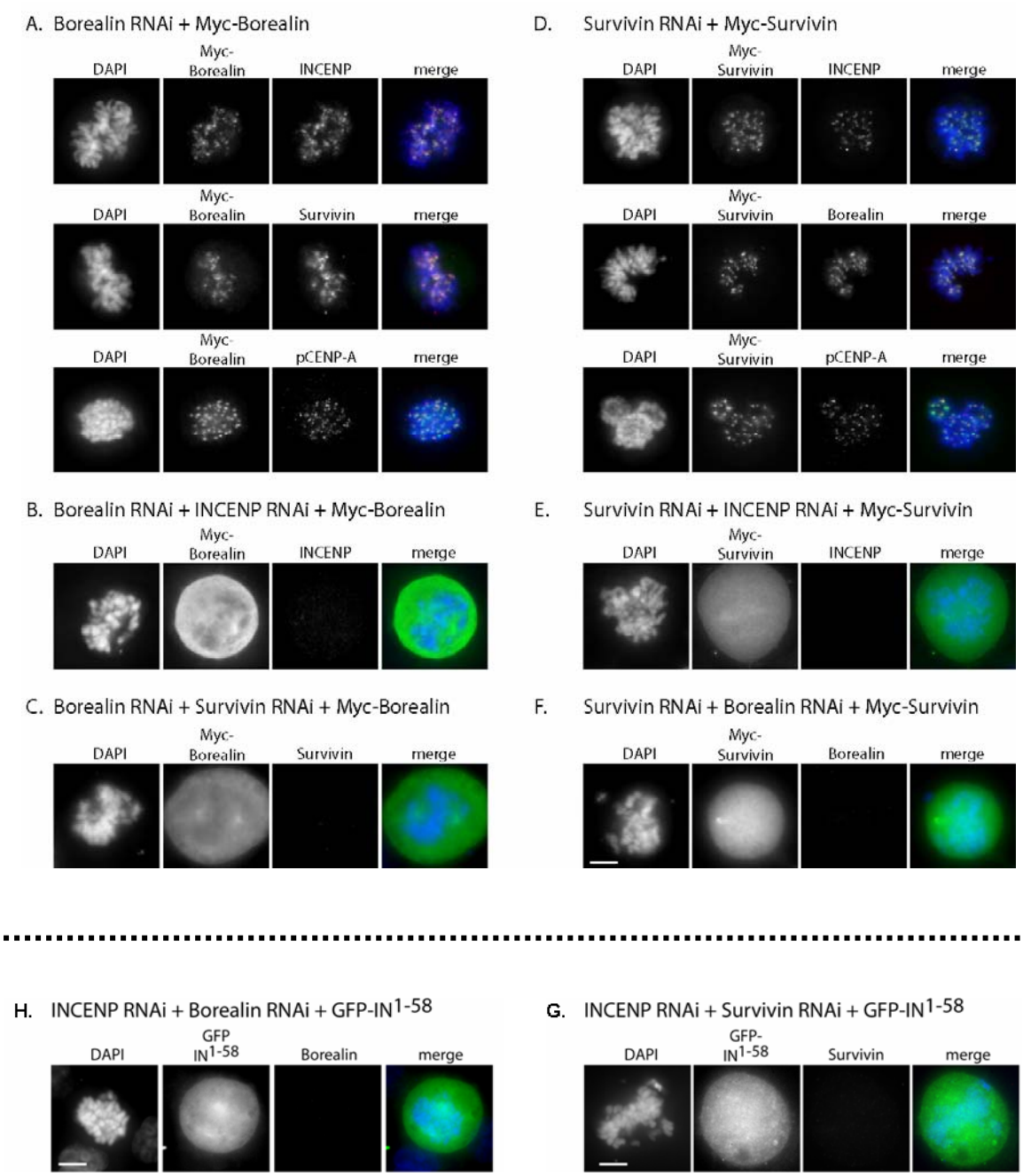


Figure 23. Centromere targeting of Borealin, Survivin and INCENP$^{1-58}$ depends on the integrity of the ternary complex.

(A-G) HeLa cells were treated with combinations of 3´-UTR siRNA oligonucleotides targeting either Borealin, Survivin or INCENP and simultaneously transfected with Myc-Borealin (A-C), Myc-Survivin (D-F) or GFP-INCENP$^{1-58}$ (H-G) as indicated. Transfected cells were analyzed for the presence of other chromosomal passenger proteins at the centromere as outlined. Bar, 10 µm. See figure 18A for controls of H and G.

Conclusion

In order to identify upstream kinetochore/centromere components that regulate CPC targeting to the centromere a focussed siRNA based screen was performed. None of the tested candidate proteins influenced the association of the complex with the centromere. This suggested that the CPC exhibits an intrinsic property that governs its centromere localization. Deletion mutants of the framework protein INCENP showed that a small N-terminal fragment of the protein (residues 1-58) is sufficient for centromere recruitment. This region of the INCENP protein was shown to bind directly to Survivin and Borealin but not to Aurora B, thus creating a heterotrimeric subcomplex within the holo-CPC.

In order to overcome the mutual interdependence of passenger proteins concerning their localization, a siRNA based complementation assay was established. This approach revealed that the ternary INCENP$^{1-58}$-Survivin-Borealin subcomplex is essential and sufficient for centromere targeting. Strikingly, Aurora B kinase, the enzymatic core of the CPC, was not required for this task. However, the ternary complex could not compensate for the lack of Aurora B kinase activity at the centromere in terms of proper chromosome alignment. We further show that the Borealin subunit can bind DNA *in vitro* and thus may directly target the CPC to centromeric DNA *in vivo*. These findings establish a functional module within the CPC that assembles on the N-terminus of INCENP and controls centromere recruitment.

II. CPC mediated recruitment of PICH to the centromere

Focused screen for components that require the presence of the CPC for kinetochore/ centromere localization

CPC functions at the centromere/kinetochore have so far been assigned exclusively to the activity of the Aurora B kinase. The role of the remaining passenger proteins was speculated to consist in governing proper localization of Aurora B during mitotic progression (Vader *et al.*, 2006). The lack of functional rescue potential of the ternary INCENP$^{1-58}$-Survivin-Borealin complex in the afore mentioned approach (Figure 21) prompted us to analyse if there is a component of the kinetochore/centromere region that relies on the presence of the CPC independently of Aurora B kinase activity. This component should fulfil two criteria: a) it should be mislocalized under conditions of CPC depletion and b) it should be localized correctly under conditions of Aurora B inhibition. Thus, we compared the localization of various kinetochore/centromere associated proteins in cells treated with siRNA duplexes targeting Aurora B to cells treated with the Aurora B inhibitor ZM447439 (Figure 24). Out of all proteins analysed only one, the DNA dependent helicase PICH, fulfilled both criteria. Strikingly, PICH has been shown to regulate SAC function and been hypothesized to sense tension at the kinetochore/centromere region (Baumann *et al.*, 2007).

Figure 24. Centromere/kinetochore localization of proteins in cells depleted of Aurora B compared to cells treated with the Aurora B inhibitor ZM447439.
Cells were analysed by immunofluorescence staining with mouse anti-Aurora B or rabbit anti-INCENP antibodies (to monitor the CPC) and antibodies against the respective candidate protein. – indicates absence of the analysed protein from the kinetochore/centromere, + indicates its presence. Only PICH (and the CPC itself) respond differently to depletion of Aurora B kinase versus its inhibition.

Protein	siAurora B	ZM447439
Aurora B	-	+
Astrin	+	+
Hec1	+	+
RanBP2	+	+
Cenp-A	+	+
BubRI	-	-
Bub1	-	-
Mps1	-	-
Cenp-E	-	-
MCAK	-	-
Mad1	-	-
Mad2	-	-
Cdc20	-	-
Plk1	-	-
PICH	-	+

This approach also showed that the KT recruitment of components directly involved in KT-MT attachments, like Hec1, Astrin and RanBP2 (Joseph *et al.*, 2004; DeLuca *et al.*, 2005; Thein *et al.*, 2007) are not regulated by the CPC while all known checkpoint proteins rely on the presence of the CPC to localize to the KT. Interestingly, Plk1 was found to depend on an active Aurora B kinase, contrasting findings that the phosphorylation status of INCENP regulates Plk1 kinetochore localization (Goto *et al.*, 2006).

To corroborate the finding that the CPC regulates PICH at the centromere/kinetochore region we made use of a dominant negative Borealin fragment (Borealin$^{1\text{-}140}$) that is unable to localize to the centromere (Gassmann *et al.*, 2004). Similar to siRNA mediated knock-down of Aurora B (or any other member of the CPC; data not shown) cells transfected with Borealin$^{1\text{-}140}$ showed mislocalization of PICH (Figure 25A). To address if the CPC might also positively regulate PICH at the centromere/kinetochore we depleted cells of the motor protein CENP-E. Under these conditions CPC levels are elevated at monooriented chromatids but PICH levels remained unaltered (Figure 25B).

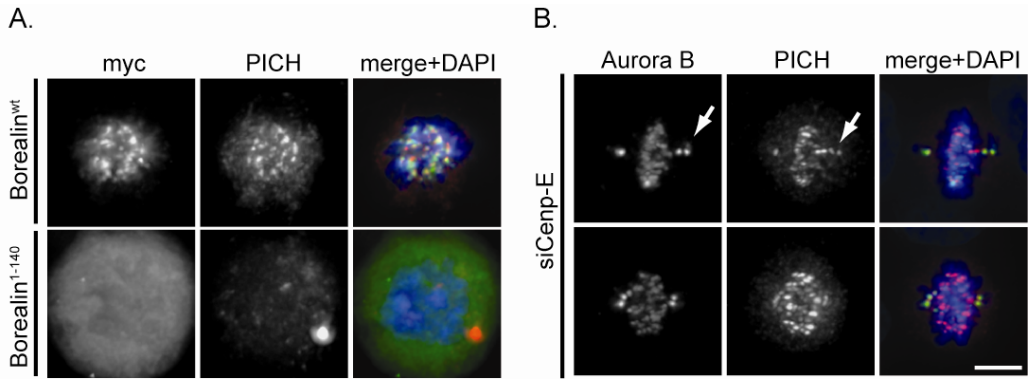


Figure 25. CPC dependent regulation of PICH.

(A) Different Myc-tagged versions of Borealin were transfected into HeLa cells and centromere/kinetochore localization of PICH was assessed.

(B) HeLa cells were treated with siRNA duplexes targeting CENP-E for 36hrs and stained for Aurora B and PICH. Arrows point to AuroraB/PICH at monooriented chromatids. Bar, 10µm.

As noted before (Baumann *et al.*, 2007) PICH is found on chromosome arms in cells depleted of Plk1. The observation that under conditions of Aurora B inhibition Plk1 (but not PICH) is absent from the kinetochore/centromere (Figure 26) indicates that cytoplasmic Plk1 is sufficient to remove PICH from chromosome arms.

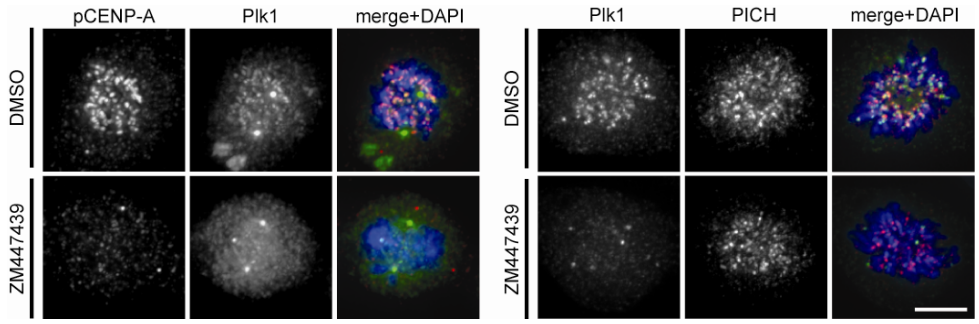
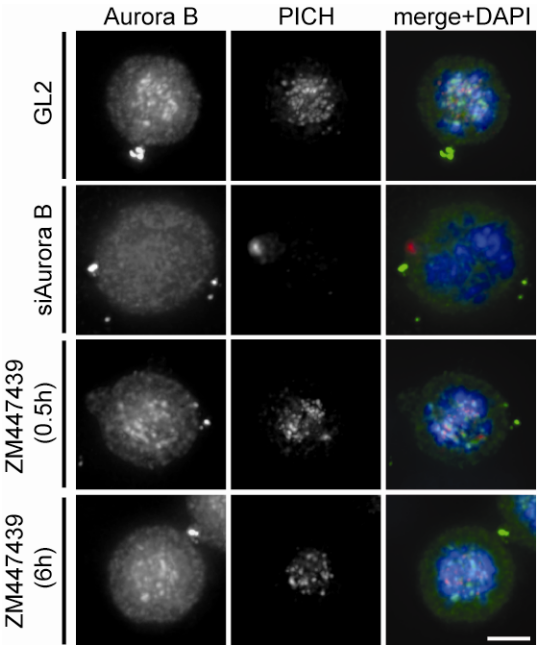


Figure 26. Aurora B kinase activity regulates Plk1 but not PICH at the kinetochore/centromere.
Hela cells were treated with ZM447429 (or DMSO as control) for 2hrs, fixed and stained with indicated antibodies. Phospho-S7-Cenp-A staining controls for Aurora B kinase activity. In ZM447439 treated cells Plk1 is absent from kinetochores but present at the centrosomes (bright dots) while PICH localization is unaffected. Bar, 10µm.

The finding that PICH centromere/kinetochore localization relies on the CPC but not Aurora B kinase activity was unique among the tested components. We therefore wished to confirm this result in another cell type. In agreement with the results obtained in HeLa cells, U2OS cells treated with siRNA duplexes against Aurora B showed mislocalization of PICH but ZM447439 treatment did also not affect PICH in U2OS cells (Figure 27).

Figure 27. Aurora B depletion but not inhibition influences PICH in U2OS cells.
U2OS cells were either treated with siRNA oligonucleotides targeting Aurora B for 36hrs or incubated with ZM447439 for different time points. Aurora B and PICH were visualized by immunofluorescence. Bar, 10µm.



INCENP$^{1-58}$ cannot rescue PICH localization to the centromere/kinetochore

Next we analysed if PICH centromere/kinetochore localization can be restored in INCENP depleted cells complemented with INCENP$^{1-58}$. Rescue assays were performed as outlined before. In cells transfected with the full-length INCENP, PICH was properly localized. But the INCENP$^{1-58}$ fragment was unable to rescue PICH centromere/kinetochore binding in transfecetd cells (Figure 28).

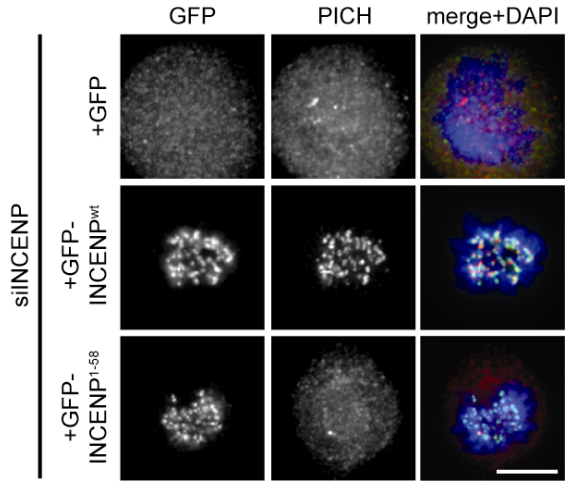


Figure 28. INCENP$^{1-58}$ cannot rescue PICH at the centromere/kinetochore.
HeLa cells were depleted of INCENP by siRNA treatment as outlined above (upper row) and transfected with either GFP (upper row), GFP-INCENP^{wt} (middle row) or GFP-INCENP$^{1-58}$ (bottom row). PICH localization was analysed by immunofluorescence. Bar, 10µm.

Mutual interplay between the CPC, PICH and Plk1 at the centromere/kinetochore

We went on to test for interdependencies between the CPC, PICH and Plk1 in terms of their centromere/kinetochore localization. HeLa cells were depleted of the respective protein by siRNA treatment and localization of the remaining components was analysed by immunofluorescence (Figure 29). Plk1 depletion did not influence centromeric localization of the CPC but resulted in PICH spreading over chromatin arms (Figure 29, top panels). Knock-down of PICH did not influence Plk1 or CPC in localization to the kinetochore/centromere (Figure 29, upper right and bottom panel).

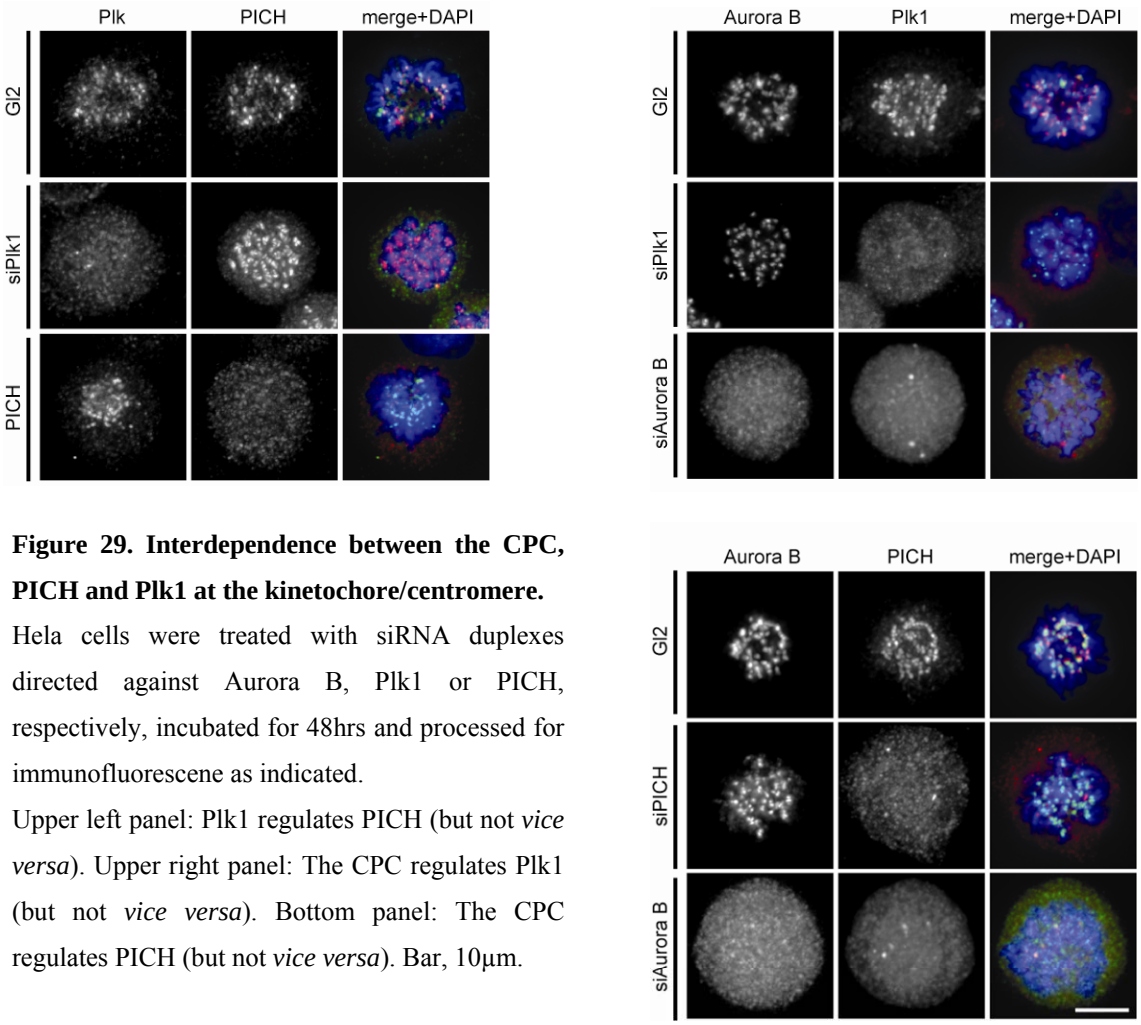


Figure 29. Interdependence between the CPC, PICH and Plk1 at the kinetochore/centromere.
Hela cells were treated with siRNA duplexes directed against Aurora B, Plk1 or PICH, respectively, incubated for 48hrs and processed for immunofluorescene as indicated.
Upper left panel: Plk1 regulates PICH (but not *vice versa*). Upper right panel: The CPC regulates Plk1 (but not *vice versa*). Bottom panel: The CPC regulates PICH (but not *vice versa*). Bar, 10µm.

Next we monitored the activity of the two kinases Aurora B and Plk1 under the above conditions. Aurora B activity was monitored by phospho-S7-Cenp-A staining and found to be unaffected by Plk1 or PICH knock-down (Figure 30A). Interestingly, staining with phospho-S676-BubR1, a Plk1 phosphorylation site (Elowe *et al.*, 2007) did not show any signal in PICH depleted cells but overall BubR1 levels were unaffected (Figure 30B). However, γ-tubulin staining, another read-out for Plk1 activity (Santamaria *et al.*, 2007) was unaffected in PICH knock-down (Figure 30C). Thus, no obvious dependencies between Aurora B, Plk1 and PICH seem to exist with regard to kinase activities of Aurora B and Plk1. The observed loss of phospho-S676-BubR1 in PICH depleted cells is likely to happen by means other than regulation of Plk1 activity.

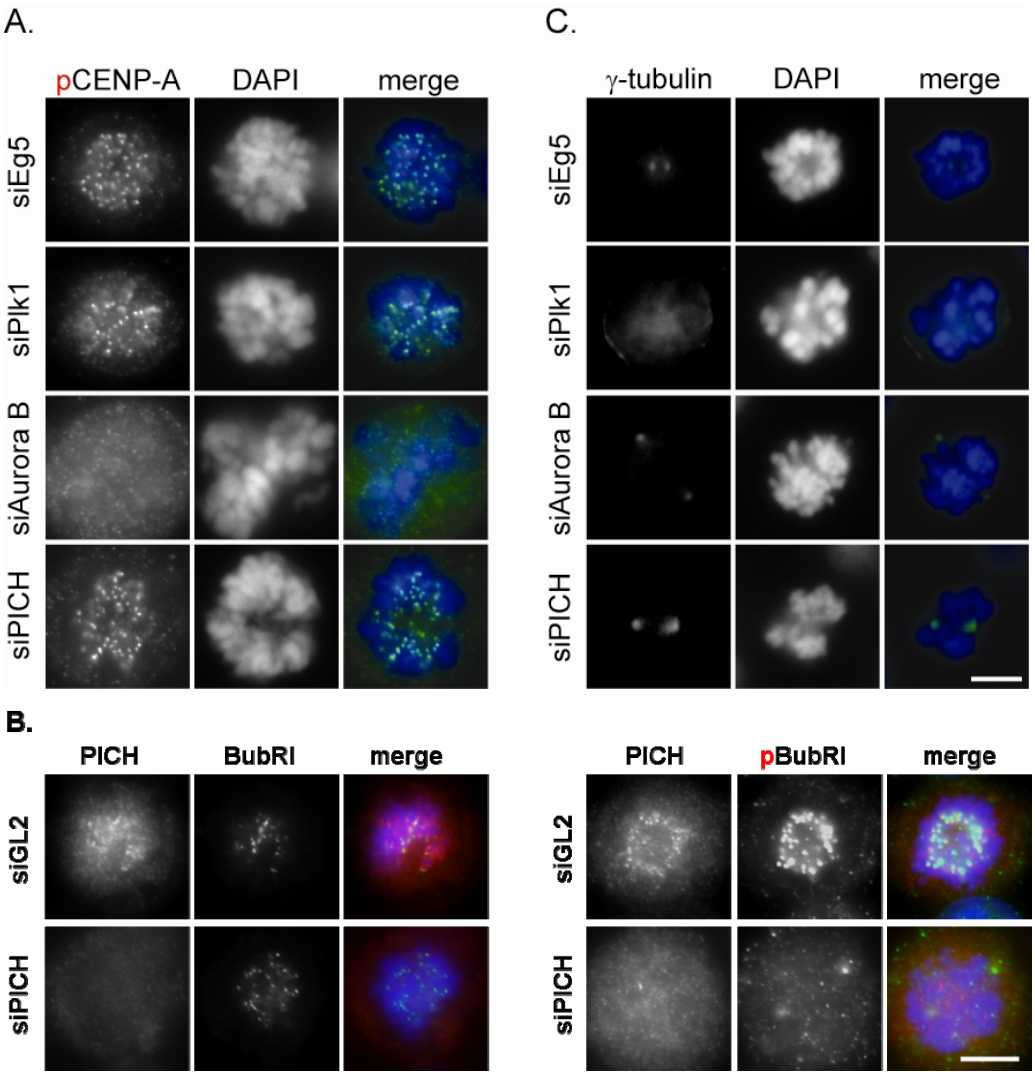


Figure 30. Aurora B and Plk1 kinase activity in cells depleted of Aurora B, Plk1 or PICH, respectively.

(A) phospho-S7-CENP-A staining was assessed in HeLa cells depleted of Plk1 (or Eg5 as control), Aurora B or PICH.

(B) phospho-S676-BubRI is absent in HeLa cells treated with PICH siRNA duplexes. BubR1 is shown as control. CPC depletion was not performed as it displaces BubR1 protein from the KT.

(C) γ-tubulin staining was assessed in HeLa cells depleted of Plk1 (or Eg5 as control), Aurora B or PICH. Bar, 10μm.

The CPC acts upstream of Plk1 in localizing PICH

In Plk1 depleted cells PICH localizes to chromosome arms (Baumann *et al.*, 2007) (Figure 31 left panel). In contrast, knock-down of Aurora B led to the absence of PICH form the centromere/kinetochore (Figure 31 left panel). Thus, we analysed the fate of PICH in Plk1/Aurora B double-depletion. In these cells PICH was absent from the centromere/ kinetochore and did not localize to the chromosome arms (Figure 31 right panel). Hence, we conclude that the CPC, in addition to regulating the centromere/kinetochore localization of PICH, is also involved in regulating the localization of PICH to chromosome arms.

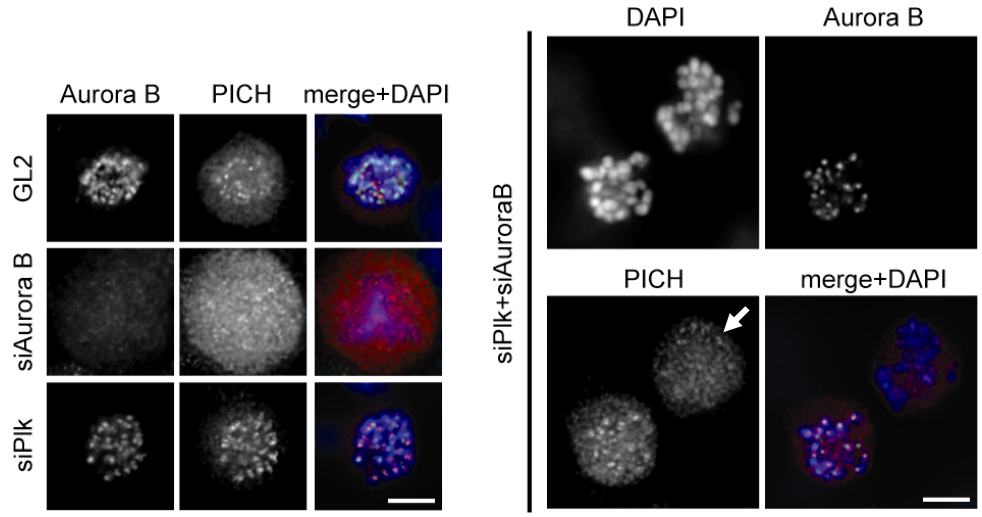


Figure 31. PICH is mislocalized in Plk1/Aurora B double-depleted cells.

HeLa cells were treated with siRNA duplexes targeting Aurora B or Plk1 (left panel) or Aurora B plus Plk1 (right panel) and incubated for 36hrs. PICH localization was analysed by immunofluorescence. Arrowhead indicates lack of PICH localization in a double depleted cell. Bar, 10µm.

The CPC might regulate SAC function via Mad2 recruitment

The function of the SAC has been speculated to depend on two ultimately intertwined mechanisms namely KT-MT attachment and the generation of tension between sister KTs. The CPC seems to constitute the tension sensing arm of the SAC. Cells depleted of the complex show a SAC dependent mitotic arrest in response to nocodazole (disrupted KT-MT attachment) but override a taxol induced mitotic arrest (reduced tension between sister KTs). The underlying determinants have not been elucidated so far. Importantly however, the checkpoint protein PICH (as outlined above) as well as the checkpoint protein Mad2 (Vigneron *et al.*, 2004) (and data not shown) are lost from the kinetochore/centromere in CPC depleted cells. Moreover, PICH knock-down also displaces Mad2 from the KT (Baumann *et al.*, 2007) (and data not shown). To gain insight into the mitotic arrest seen in CPC depleted cells arrested with nocodazole, HeLa cells were knocked-down of Aurora B and treated with

taxol or nocodazole, respectively. Treatments with either MT poison did not change the result that PICH was absent from the centromere/kinetochore under conditions of CPC depletion (Figure 32A). Strikingly, Mad2 was found to be absent from the KT in this assay when taxol was added (Figure 32B) but present when cells were grown in the presence of nocodazole (Figure 32C).

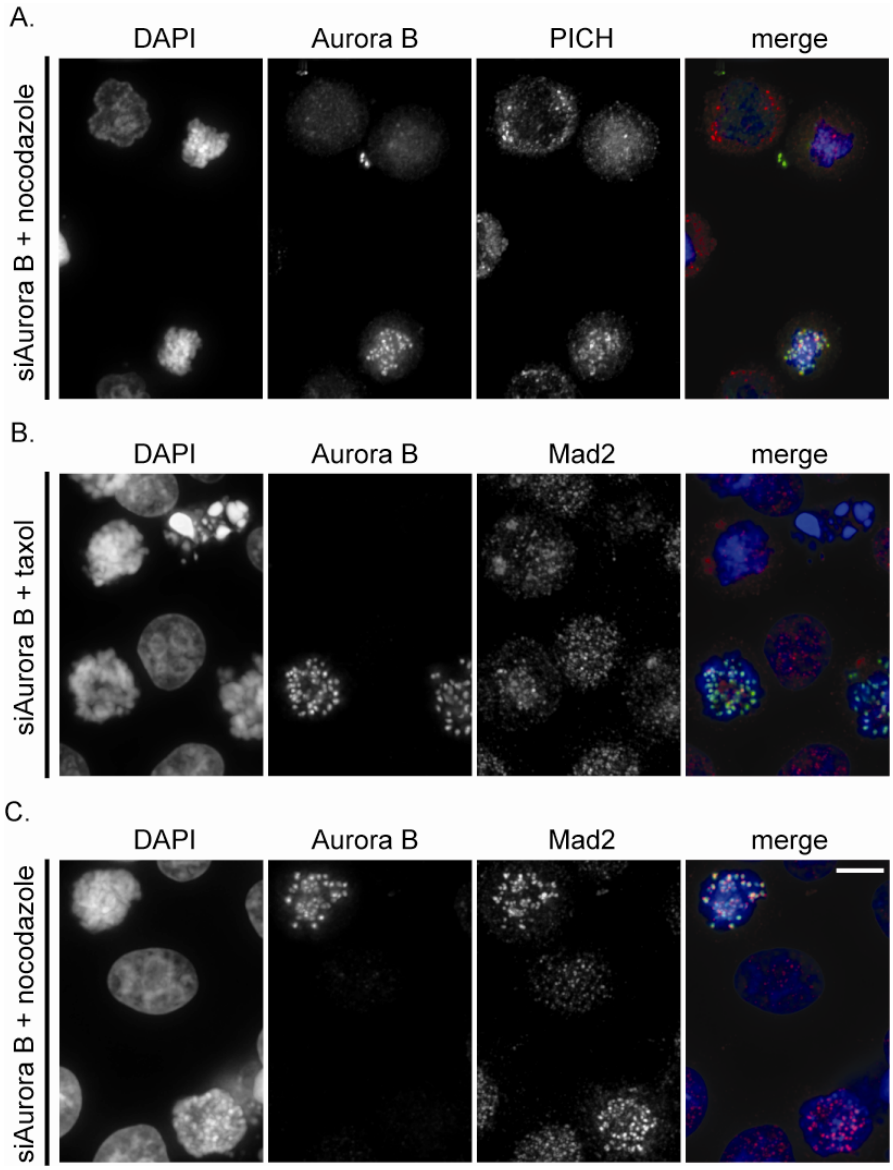


Figure 32. Mad 2 but not PICH is re-directed to KTs in Aurora B depletion under nocodazole treatment.
(A-C) Cells depleted of Aurora B were analysed in different drug treatments compared to not-depleted cells in the same image.
(A) HeLa cells were depleted of Aurora B by specific siRNA oligonucleotides for 36hrs and treated with nocodazole 12hrs before fixation. PICH localization was monitored by immunofluorescence.
(B) Experiment as in (A) but instead of nocodazole taxol was added and Mad2 was monitored.
(C) Experiment as outlined for (A), but monitoring Mad2 localization. Bar 10µm.

PICH threads form independently of the CPC

As cells start to enter anaphase Plk1 associates with the central spindle while PICH eventually forms thin threads that connect sister chromatids (Baumann *et al.*, 2007) and that contain centromeric DNA (Wang *et al.*, 2008). The exact nature of these threads and their function remain to be elucidated. We asked if Plk1 and PICH are also influenced by the CPC during anaphase. Depletion of Aurora B did not change Plk1 localization to the central spindle (Figure 33A) or PICH thread formation (Figure 33B and C). Thus, centromere/kinetochore localization of Plk1 and PICH does not appear to be a pre-requisite for central spindle binding or thread formation, respectively.

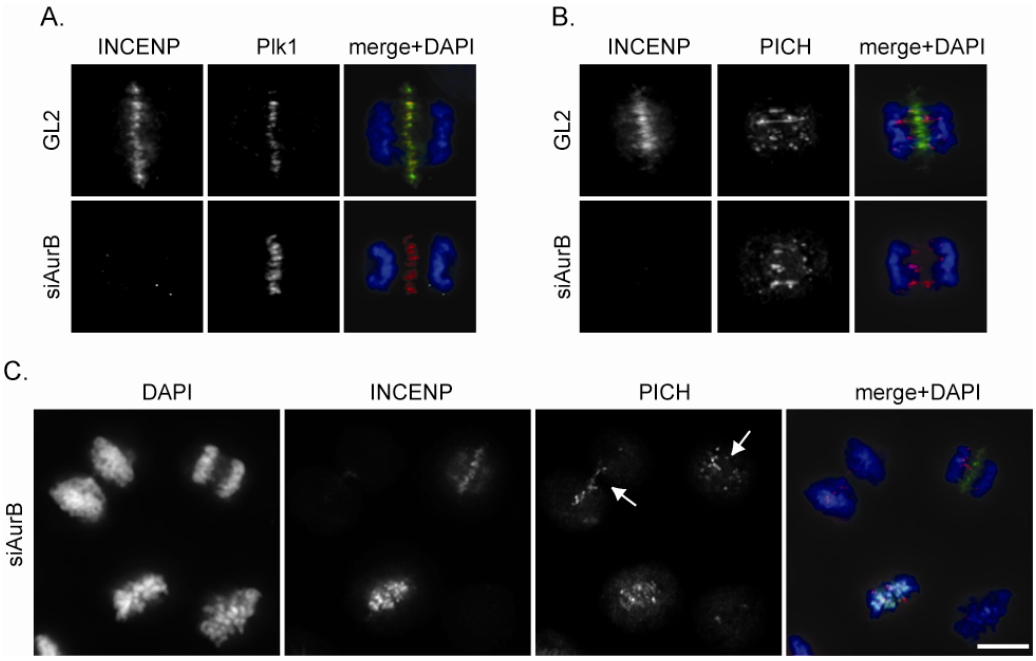


Figure 33. Plk1 and PICH localization in anaphase is independent of the CPC.

(A) HeLa cells were treated with siRNA duplexes directed against Aurora B for 48hrs and Plk1 staining was assessed in anaphase cells.

(B) Experiment as in (A), but monitoring PICH threads.

(C) Experiment as in (B). The lower region of the image shows two metaphase cells, one being depleted of the CPC (see INCENP staining). In the upper region two anaphase cells are shown, again only one being depleted of the CPC. However, in both anaphase cells PICH threads are detected (arrow heads). Bar 10µm.

Conclusion

PICH, a novel spindle checkpoint protein recently identified in our laboratory, was identified as a component that requires the presence of the CPC but, strikingly, not Aurora B kinase activity for its centromere/kinetochore localization; a behaviour that has not been reported for any other centromere/kinetochore associated protein so far. However, increased centromeric CPC levels do not elicit an increase in PICH binding to the centromere/kinetochore. Moreover, the INCENP$^{1-58}$-Survivin-Borealin subcomplex of the CPC, essential and sufficient for centromere binding, did not rescue PICH localization to the centromere/kinetochore, indicating that INCENP$^{59-919}$ and/or Aurora B must contain information that targets PICH to the centromere/kinetochore. Plk1, a PICH interaction partner and regulator of its chromosome arm recruitment, targets to the KT dependent on the Aurora B kinase activity of the CPC and was shown to act downstream of the CPC in localizing PICH.

Remarkably, PICH was also absent from the centromere/kinetochore in Aurora B depleted cells that were treated with nocodazole. In contrast, Mad2 was efficiently recruited to the KT under these conditions. Thus, Mad2 KT localization is likely to explain the mitotic arrest seen under conditions of Aurora B depletion and addition of nocodazole.

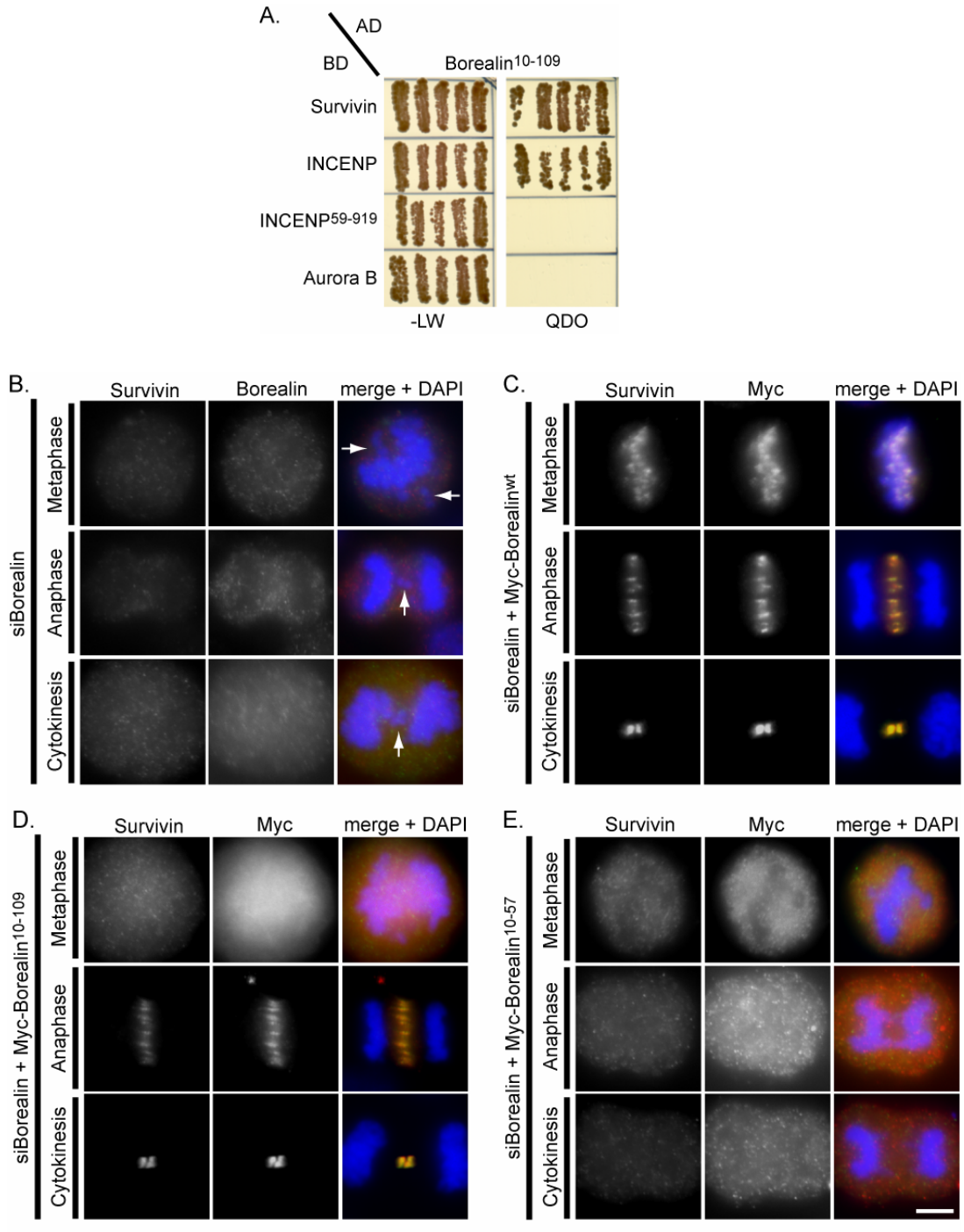
III. Combined structural and biochemical insights into CPC targeting and function

Structural studies on members of the CPC have so far been focused on Survivin, a BIR domain containing protein and on the interaction between Aurora B and INCENP. The three Survivin structures are largely consistent, showing Zn^{2+} coordination and BIR domain mediated dimerization of the protein (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000). INCENP binding to Aurora B was shown to allosterically induce kinase activation via the T-loop (Sessa *et al.*, 2005). One crucial step limiting structural studies is the production of the respective protein/proteins in high and pure amounts for crystallisation. Considering that Survivin and Borealin, as well as the N-terminus of INCENP readily could be purified from bacteria with high yields (e.g. Figure 10A), a collaboration with the laboratory of Elena Conti (EMBL, Heidelberg and MPI of Biochemistry, Munich) was established, with the aim of structure elucidation. CPC constructs and purification protocols were sent to Dr. Arockia Jeyaprakash. Subsequently, Arockia solved the solution structure of $INCENP^{1-58}$, Survivin and $Borealin^{10-109}$ at 1.4Å resolution.

Characterization of $Borealin^{10-109}$

The $INCENP^{1-58}$ fragment had been characterized by us before (see Results I) and, as mentioned above, Survivin could be crystallized as full-length protein. Thus, we proceeded to analyse $Borealin^{10-109}$. In directed yeast-two hybrid assays $Borealin^{10-109}$ interacted with Survivin (Figure 34A, upper row) but not with Aurora B (Figure 34A, bottom row) and its binding to INCENP was mediated via the first 58 amino acids of INCENP (Figure 34A, middle two rows). To assess the function of $Borealin^{10–109}$ in localizing the CPC, we used a siRNA-based complementation approach, as described before (see Result I). In this assay, Myc-tagged $Borealin^{10–109}$ was unable to restore localization of the CPC to the centromere, but it targeted the CPC to the central spindle and midbody (Figure 34D). A shorter N-terminal fragment of Borealin ($Borealin^{10–57}$) did not rescue the localization of the CPC during progression through mitosis (Figure 34E). We next tested if $Borealin^{10–109}$ could rescue the cytokinesis defect observed upon CPC depletion. While Borealin depletion resulted in 33.6% of multinucleated cells, rescue with full-length Borealin led to a decrease to 12.8%. (Figure 34F). Interestingly, cell division was largely restored in cells rescued with $Borealin^{10–109}$ but not $Borealin^{10–57}$ (12.9% multinucleation and 32.4% multinucleation, respectively). Cells expressing these fragments showed chromosome segregation defects (data not shown), as

would be expected from the loss of centromere-related functions of Aurora B. In coimmunoprecipitation experiments using the Myc-tagged Borealin constructs, Borealin$^{10–109}$ efficiently pulled down INCENP, Aurora B, and Survivin, while Borealin$^{10–57}$ did not coimmunoprecipitate any of the other CPC subunits (Figure 34G). Thus, Borealin$^{10–109}$ is functional *in vivo* to form a minimal CPC core complex that localizes to the central spindle and midbody and properly executes the role of the CPC during cytokinesis.



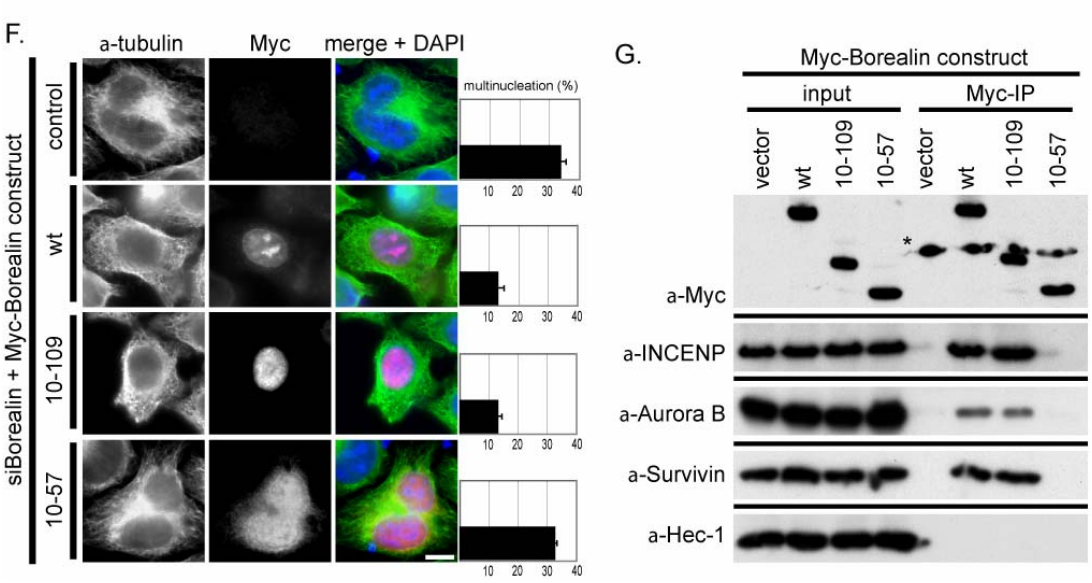


Figure 34. Borealin$^{10\text{-}109}$ is sufficient to target the CPC to the central spindle and midzone, to execute cytokinesis, and to form a complex with other CPC proteins i*n vivo*.

(A) Yeast two-hybrid assays show an interaction of Borealin$^{10–109}$ with Survivin and INCENP (via its N-terminal 58 residues) but not with Aurora B. AD indicates the pAct2 vector, BD the pFBT9 vector.

(B and C) Evaluation of a rescue assay for Borealin fragments/mutants as described (Result I). Complementation with Myc-Borealin^{wt} rescues the localization of the CPC to the central spindle and midbody and leads to proper chromosome alignment and chromosome segregation. Arrowheads indicate lagging chromatids.

(D) Myc-Borealin$^{10–109}$ fails to restore the centromere localization of the CPC during prometaphase but rescues its localization to the central spindle and midbody during anaphase and cytokinesis, respectively.

(E) Myc-Borealin$^{10–57}$ fails to localize the CPC correctly throughout mitosis. For (B)–(E) between 12 and 25 metaphase cells, between 6 and 18 anaphase cells, and between 6 and 25 cells in cytokinesis were analyzed (n = 3). The predominant localization (>80% of cells) is given in the images. Bar, 10µm.

(F) Borealin full-length and Borealin$^{10–109}$ but not Borealin$^{10–57}$ can rescue the cytokinesis defect induced by Borealin siRNA.The rescue assay was performed as in (B–E). Myc-positive interphase cells were scored for multinucleation via DAPI and α-tubulin staining. Immunofluorescence images show the prevalent phenotype for the different constructs. Quantification is illustrated next to the corresponding image (90–147 cells per experiment, n = 3; error bar represents standard deviation). Bar, 10µm.

(G) Different fragments of Myc-tagged Borealin were transfected into HeLa cells and precipitated using anti-Myc antibodies. Co-precipitating passenger proteins were visualized by western blotting. Hec-1 is shown as a negative control. Borealin$^{10–109}$ (but not Borealin$^{10–57}$) interacts with INCENP, Aurora B, and Survivin. Asterisk denotes immunoglobulines at 30 kDa.

Overall structure of the INCENP$^{1-58}$-Survivin-Borealin$^{10-109}$ complex

The INCENP$^{1–58}$-Survivin-Borealin$^{10-109}$ complex was crystallized and its structure determined by single-wavelength anomalous dispersion (SAD) by Dr. Arockia Jeyaprakash. The structure was refined to 1.4Å resolution, with an R factor of 18.5%, Rfree of 20.2%, and good stereochemistry (Figure 35).

The overall tertiary structure of Survivin in the complex is very similar to that reported previously in the absence of other CPC components (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000). Briefly, Survivin is characterized by an N-terminal globular domain (the BIR domain) and a long C-terminal helix spanning 11 helical turns (C helix) (Figure upper panels). The BIR domain (residues 15–89) consists of a three-stranded antiparallel ß-sheet surrounded by four helices. It includes a Zinc-ion that has a structural role. A short linker (residues 90–99) connects the BIR domain to the C-helix (residues 100–142).

Borealin and INCENP bind Survivin to form a three-helix bundle. The core of the helical bundle is formed by an intertwined set of hydrophobic interactions with residues contributed by all three proteins. INCENP forms a single α-helix that stacks parallel to the Survivin C-helix. The interaction between Survivin and Borealin in the CPC exhibits striking molecular mimicry of the apo-Survivin–Survivin interaction (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000). The hydrogen-bonding network engaging the backbone of Borealin parallels that of Survivin in the apo-structure. Formation of the complex involves an induced-fit rearrangement of the hydrophobic residues of Survivin, which undergo small but relevant changes in the positioning of their side chains to recognize Borealin and INCENP. Borealin is characterized by a long α-helix (αB1, residues 15–60), which stacks against the Survivin C-helix in an antiparallel fashion. In addition, Borealin contains two small α helices (αB2, residues 63–67 and αB3, residues 70–75,) arranged almost perpendicular to the bundle. This part of the molecule caps the first turn of the Survivin C-helix and wraps around the linker. A structure-based sequence alignment of the human proteins compared to *Xenopus laevis* and *Danio rerio* CPC components revealed that residues forming the core of the BIR domain and the three-helix bundle are highly conserved (all structural data provided by Dr. Arockia Jeyaprakash).

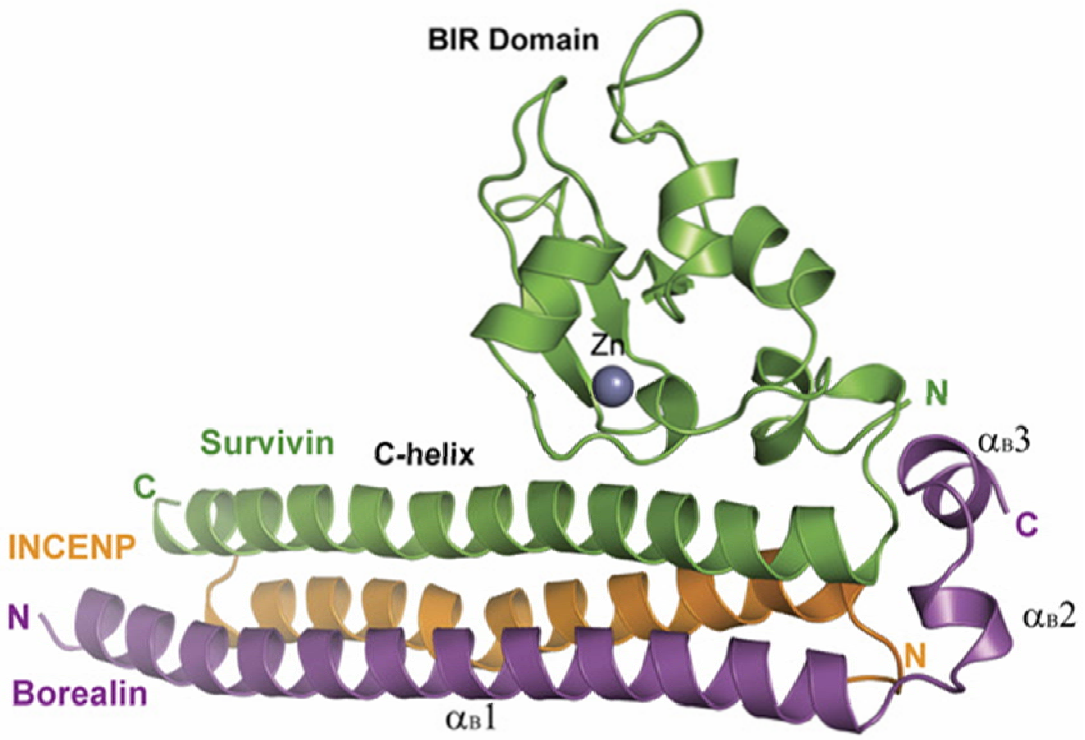
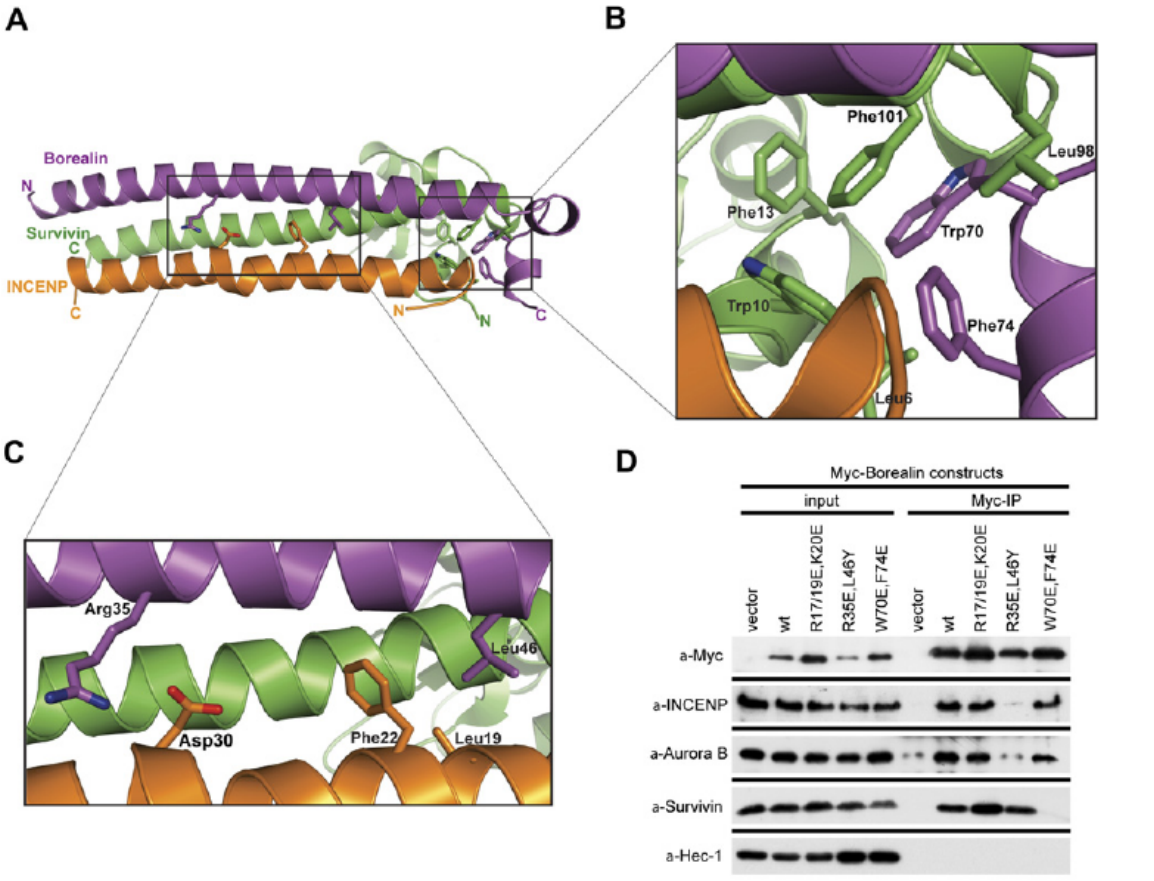


Figure 35. Overall structure of the core CPC formed by INCENP$^{1-58}$, Survivin and Borealin$^{10-109}$.
Survivin (green) has a Zinc-binding globular domain (BIR domain) connected to the long C-terminal helix (C helix). Borealin (magenta) and INCENP (orange) interact with the Survivin C-helix to form a triple helical bundle. In its CPC bound form the hydrophobic pocket of Survivin mediating homodimerization is occupied by Borealin residues. Data Dr. Arockia Jeyaprakash.

Dissociation of Survivin or INCENP from Borealin impairs CPC localization

The finding of a stable Survivin–Borealin subcomplex of the CPC *in vitro* (data by Dr. Arockia Jeyaprakash) and data on the existence of an INCENP–Aurora B subcomplex (Gassmann *et al.*, 2004) prompted us to investigate the effect of disrupting specific subsets of protein-protein interactions. First, we analyzed the structure to identify residues of Borealin whose mutation would specifically disrupt its binding to either Survivin or INCENP. In the globular part of the CPC core structure, Borealin residues Trp70^{Bor} and Phe74^{Bor} dock into the hydrophobic pocket present on the BIR domain of Survivin (Leu6^{Sur}, Trp10^{Sur}, Phe13^{Sur}, Phe101^{Sur}) and have only marginal contacts with INCENP (Figure 36A and B). On the other hand, in the helical bundle of the CPC, Borealin Arg35^{Bor} interacts electrostatically with INCENP Asp30^{INC}, and Leu46^{Bor} interacts hydrophobically with Phe22^{INC} and Leu19^{INC} (Figure 36A and C). However, neither Arg35^{Bor} nor Leu46^{Bor} contact Survivin. Therefore, in order to create a complex in which Borealin binds INCENP (and thus Aurora B) but not Survivin, we mutated residues Trp70^{Bor} and Phe74^{Bor} to negatively charged amino acids

(W70E, F74E). By mutating Arg35^{Bor} with a reverse-charge substitution and Leu46^{Bor} with a bulky side-chain substitution (R35E, L46Y) we attempted to generate a second complex in which Borealin binds Survivin but not INCENP. In line with the structural data, the full-length Borealin W70E, F74E mutant was able to coimmunoprecipitate INCENP and Aurora B but not Survivin, while the Borealin R35E, L46Y mutant coimmunoprecipitated Survivin but not INCENP or Aurora B (Figure 36D). Being able to constitute different subcomplexes of the CPC *in vivo* we utilized the siRNA rescue assay mentioned above to evaluate their functionality (see controls in Figure 34B and C). Borealin W70E, F74E as well as Borealin R35E, L46Y did not rescue the localization of the CPC to either the centromere or to the central spindle and midbody (Figure 36E and F). Thus, dissociation of Survivin from the CPC impairs the overall localization of the CPC, and a subcomplex consisting of Survivin and Borealin (lacking INCENP and Aurora B) is unable to target to any defined subcellular site. Notably, Aurora B did not coimmunoprecipitate with Borealin and Survivin in the absence of INCENP (Borealin mutant R35E, L46Y, Figure 36D), while it was coimmunoprecipitated with Borealin and INCENP in the absence of Survivin (Borealin mutant W70E, F74E, Figure 36D).



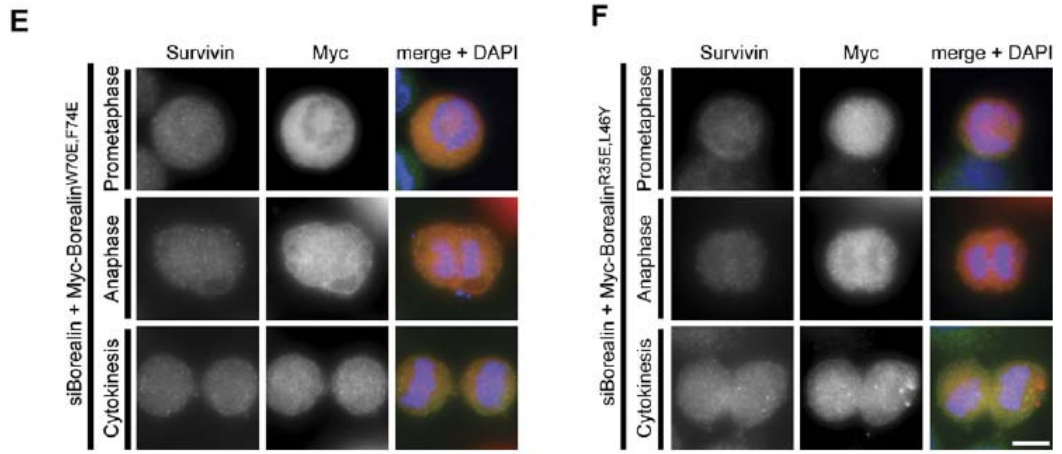


Figure 36. Subcomplexes of the CPC lacking either Survivin or INCENP/Aurora B fail to localize correctly throughout mitosis.

(A) Cartoon representation of the structure of core CPC highlighting the regions where Borealin mutations have been introduced.

(B) Close-up view of the hydrophobic interaction of the Survivin hydrophobic core with $Trp70^{Bor}$ and $Phe74^{Bor}$. These Borealin residues do not interact significantly with INCENP.

(C) Close-up view of the interaction network of Borealin residues $Arg35^{Bor}$ and $Leu46^{Bor}$ with INCENP $Asp30^{INC}$, $Phe22^{INC}$, and $Leu19^{INC}$. These Borealin residues do not interact significantly with Survivin.

(D) Transfection of different Myc-tagged Borealin mutants into HeLa cells followed by respective immuno-precipitations reveal the constitution of different subcomplexes *in vivo*. Coprecipitating passenger proteins were visualized by western blotting. Hec1 serves as a negative control. Borealin W70E, F74E mutant binds INCENP and Aurora B but not Survivin. Borealin R35E, L46Y binds Survivin but not INCENP/Aurora B. The Borealin surface mutant R17/19E, K20E (see also Figure 38) forms a holocomplex like the wild-type protein.

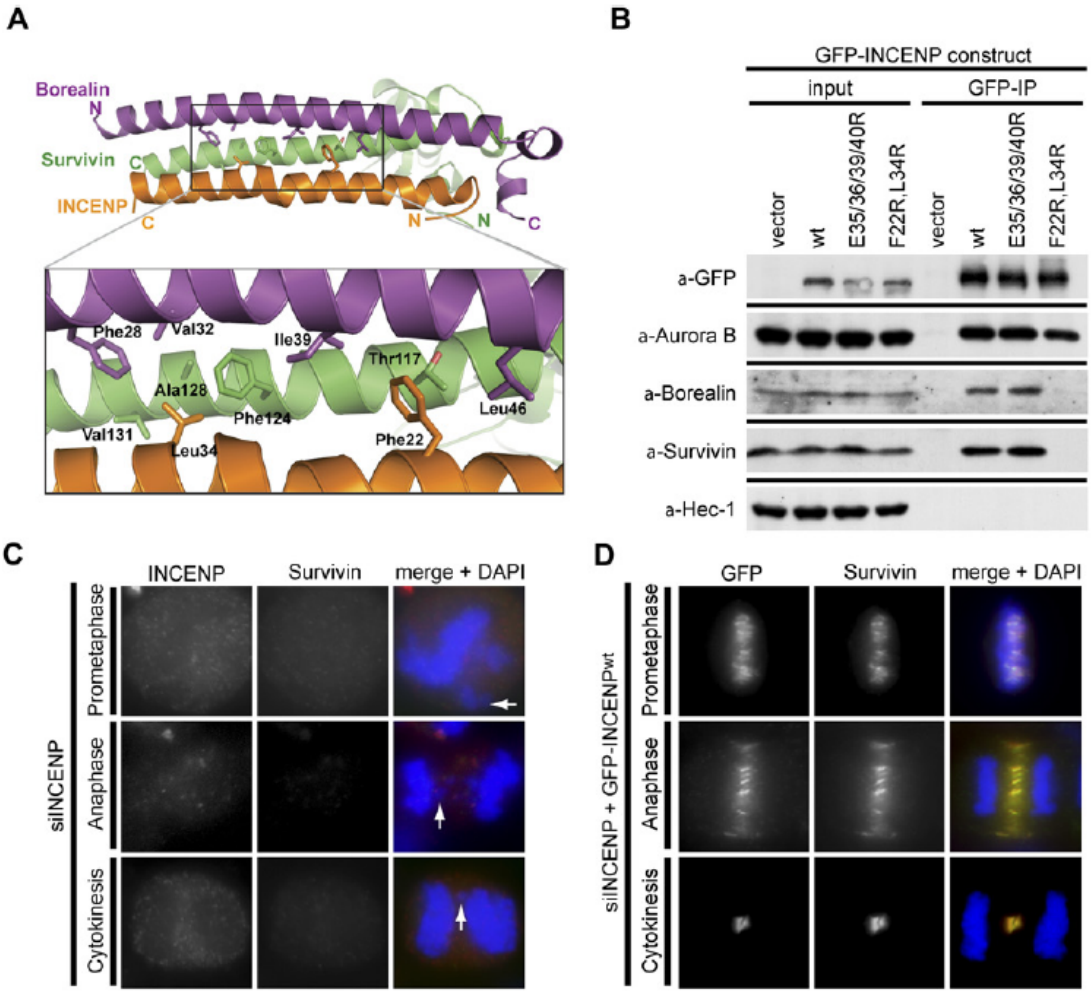
(E and F) Borealin W70E, F74E mutant (E) and Borealin R35E, L46Y (F) failed to properly localize respective CPC subcomplexes in rescue assays.

Quantification was done as in Figures 1A–D with equal numbers of cells analyzed. Bar, 10μm.

An INCENP–Aurora B subcomplex does not provide holo-CPC function *in vivo*

The recent hypothesis that multiple chromosomal passenger complexes might coexist and fulfill different functions has attracted broad attention (reviewed in (Vagnarelli and Earnshaw, 2004)). In particular, it has been shown that a subcomplex of INCENP and Aurora B (lacking Survivin and Borealin) is present in Borealin-depleted HeLa lysates, prompting the speculation that this subcomplex might accomplish mitotic functions (Gassmann *et al.*, 2004). Based on our structure of the core CPC, we set out to assemble an INCENP–Aurora B complex devoid of Survivin and Borealin. Specifically, we targeted $Phe22^{INC}$ and $Leu34^{INC}$ of INCENP, which participate in a network of interactions with Borealin ($Phe28^{Bor}$, $Ile39^{Bor}$, and

Leu46^{Bor}) and Survivin (Thr117^{Sur}, Phe124^{Sur}, and Val131^{Sur}) within the helical bundle (Figure 37A). Coimmunoprecipitation assays confirmed that the INCENP F22R, L34R mutant retained binding to Aurora B but was unable to interact with Borealin and Survivin (Figure 37B). To evaluate the functionality of this INCENP–Aurora B subcomplex, we again made use of the established siRNA rescue approach (see Results I). The INCENP F22R, L34R mutant neither targeted to the centromere nor the central spindle and midbody (Figure 37E). Notably, the INCENP–Aurora B subcomplex also failed to rescue the mitotic functions of the holo-CPC, as determined by staining with phospho-S7-specific antibodies against CENP-A (Figure 37F). Thus, an engineered INCENP–Aurora B subcomplex unable to bind Survivin and Borealin does not target to any defined structure during mitosis and is not able to restore holo-CPC function on chromatin.



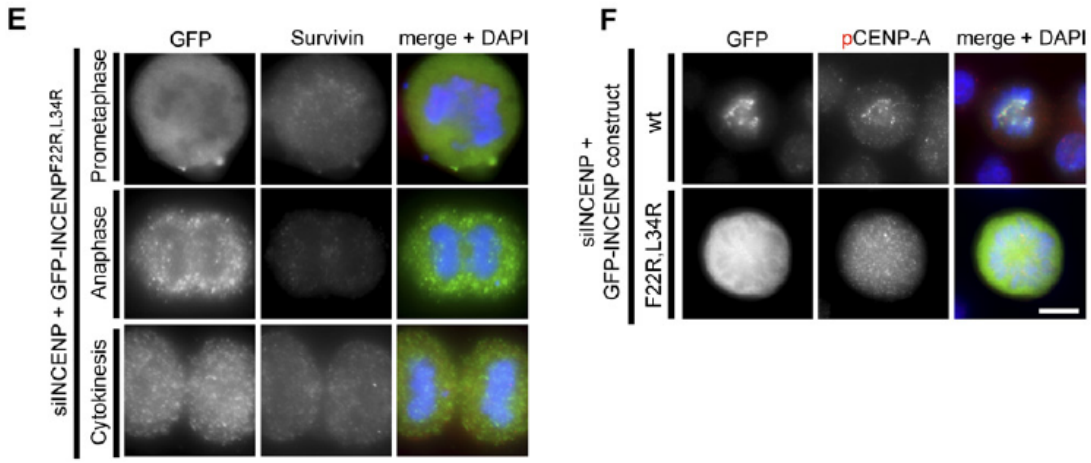


Figure 37. An engineered INCENP–Aurora B subcomplex fails to rescue CPC localization and function.

(A) Cartoon representation of the overall structure of the core CPC, highlighting the region where INCENP mutations were engineered to disrupt Survivin and Borealin binding. A close-up view shows that Phe22^{INC} and Leu34^{INC} are engaged in multiple interactions with Survivin (Thr117, Phe124) and Borealin (Phe28, Leu46).

(B) HeLa cells were transfected with GFP-tagged INCENP mutants followed by immunoprecipitations using GFP antibodies. Western blotting revealed the constitution of different subcomplexes *in vivo*. While wild-type INCENP and the INCENP E35/36/39/40R surface mutant (see also Figure 38) form a holo-CPC, INCENP F22R, L34R failed to bind Borealin and Survivin but retained binding to Aurora B.

(C-D) A rescue assay to assess the function of INCENP constructs as described (see Result I). GFP-INCENP^{wt} could restore CPC localization to the centromere, the central spindle, and the midbody and proper progression through mitosis. Arrowheads indicate lagging chromatids.

(E) INCENP F22R, L34R mutant failed to localize to any defined subcellular structure during mitosis.

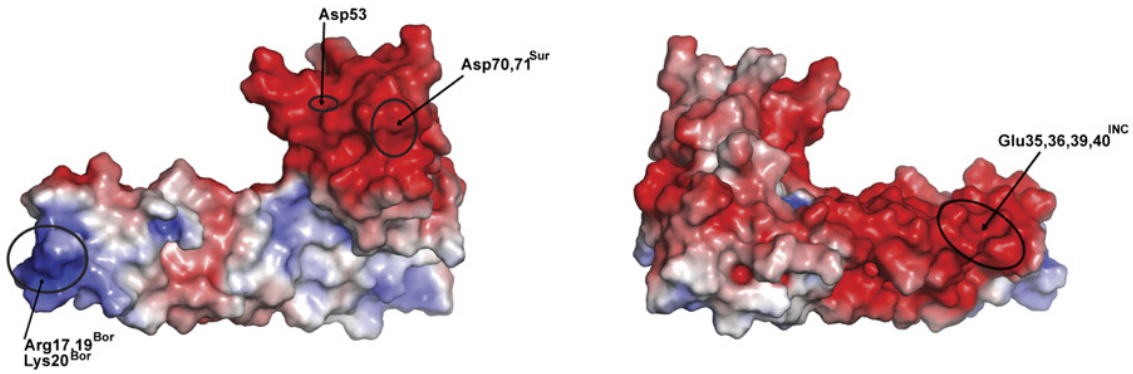
(F) INCENP F22R, L34R failed to show phospho-S7-CENP-A signal. Bar, 10 µm.

The helical bundle is essential for the central spindle and midbody localization of CPC

Earlier work has demonstrated that INCENP residues 32–44 (Xenopus numbering) are essential for centromere targeting (Ainsztein *et al.*, 1998). This observation can be rationalized in light of the structure, which suggests that deletion of residues 32–44 of INCENP might result in a considerable loss of interaction with Survivin and Borealin (Figure 35). Interestingly, however, residues Glu35^{INC}, Glu36^{INC}, Glu39^{INC}, and Glu40^{INC} are not involved in structural interactions of the CPC but form a conserved negatively charged patch on the surface (Figure 38A). We therefore tested the effect of specifically mutating this conserved set of exposed glutamic acids to positively charged residues in the context of full-length INCENP. In line with the structural analysis, the INCENP E35/36/39/40R mutant was able to assemble with the other CPC components to form the holo-complex *in vivo* (as shown

by coimmunoprecipitation in Figure 37B) and localized correctly to centromeres. However, INCENP E35/36/39/40R did not localize to the central spindle and midbody in anaphase or cytokinesis but remained on the chromatin (Figure 38B). Adjacent to the INCENP negative cluster, at the tip of the helical bundle, Borealin features several conserved positively charged surface residues (Figure 38A). Remarkably, mutation of $Arg17^{Bor}$, $Arg19^{Bor}$, and $Lys20^{Bor}$ to glutamic acids resulted in a phenotype similar to that of the INCENP E35/36/39/40R mutant. The BorealinR17/R19/K20E mutant was able to bind all other CPC members in vivo (coimmunoprecipitation in Figure 36D) but was unable to dissociate from chromatin and associate with the central spindle or the midbody (Figure 38C). This phenotype might be due to the impairment of modifications on either the CPC or chromatin that would cause persistent binding to chromosomes in anaphase or to the inability to bind a factor that might promote the dissociation from chromatin. We next ask if INCENP E35/36/39/40R and Borealin R17/R19/K20E can execute the normal CPC function during metaphase and cytokinesis. Like the wild-type proteins, both, Borealin R17/R19/K20E and INCENP E35/36/ 39/40R restored chromosome alignment on the metaphase plate and phosphorylation of CENP-A (Figure 38D and E), suggesting that Aurora B kinase activity was not affected by these mutants. However, both mutants were unable to rescue the cytokinesis defect seen upon depletion of Borealin or INCENP (Figure 38F and G). This observation suggests that the central spindle and midbody binding of the CPC is required for proper execution of cytokinesis.

A.



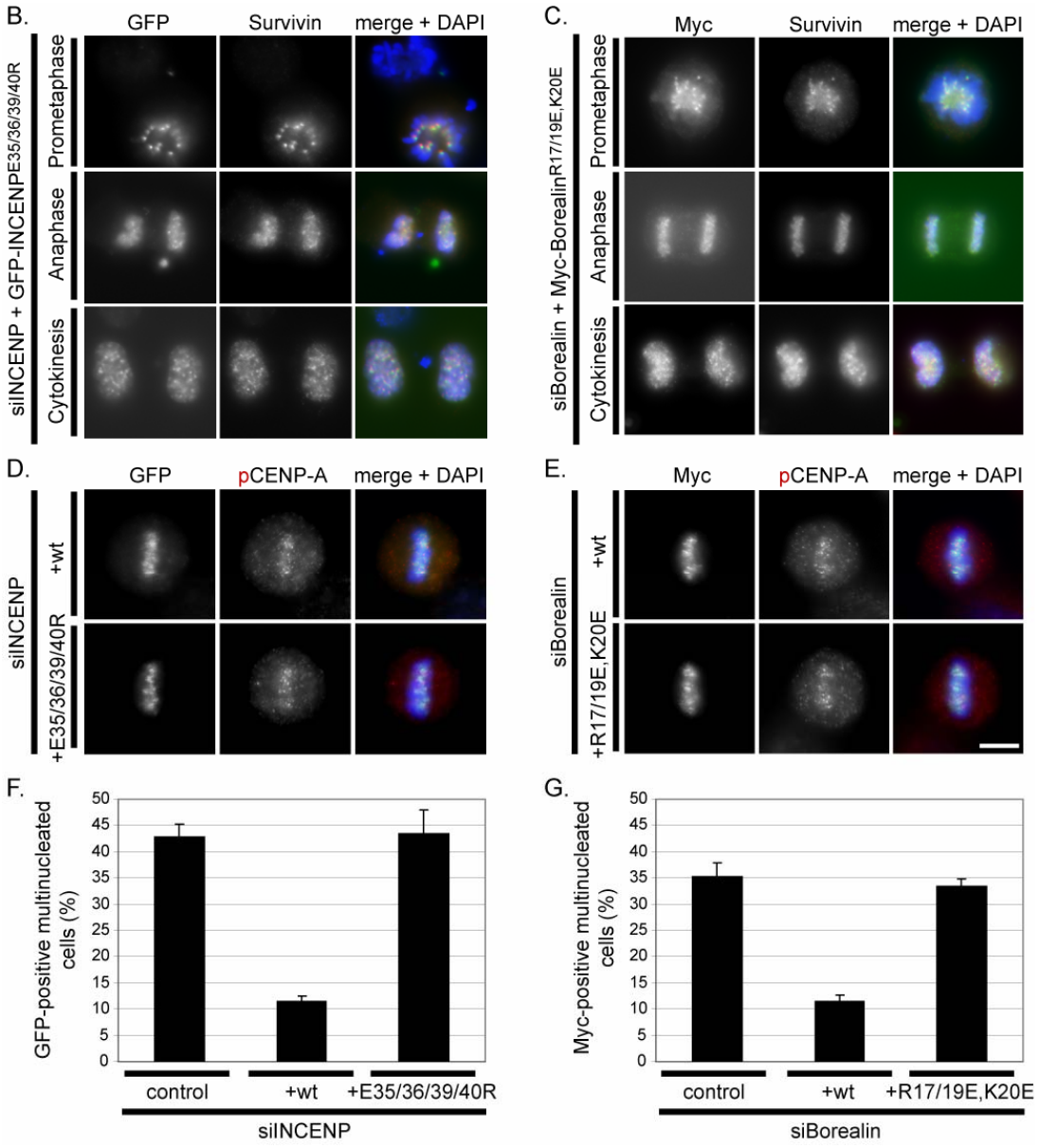


Figure 38. A conserved composite molecular surface formed by Borealin–Survivin–INCENP is crucial for central spindle and midbody localization of the CPC and proper cell division.

(A) Calculation of electrostatic surface charge reveals a highly acidic (red) and basic patch (blue) on the molecular surface of the INCENP$^{1–58}$-Survivin-Borealin$^{10-109}$ complex. The two views are related by a 180° rotation about the vertical axis. Data Dr. Arockia Jeyaprakash.

(C–H) Evaluation of surface charge mutants in respective rescue assays. (C) Charge reversal of a conserved acidic patch on INCENP affects binding of the CPC to the central spindle and midbody. (D) Charge reversal of a conserved basic patch on Borealin phenocopies the localization observed for the INCENP E35/36/39/40R mutant. Chromosome alignment onto the metaphase plate and phosphorylation of (S7)-CENP-A is unaffected by INCENP 35/36/39/40R (E) and Borealin R17/19E, K20E (F). (G) GFP-INCENP E35/36/39/40R and (H) Myc-Borealin R17/19E, K20E rescued interphase cells were analyzed for multinucleation as in Figure 34F (n = 3; error bar represents standard deviation). Both mutants do not rescue the cytokinesis defect observed upon depletion of INCENP or Borealin, respectively.

Conclusion

Both Survivin and Borealin were shown previously to be able to form dimers (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000; Gassmann *et al.*, 2004) (and see Figure 14). In the context of the core CPC, however, the proteins associate with INCENP in a 1:1:1 stochiometry.

Additionally, the structural basis for the interdependence of the chromosomal passenger proteins becomes evident in light of the crystal structure of the core CPC. A complex network of intermolecular interactions observed within the passenger proteins stabilizes the core of the CPC and holds the subunits together. Although the function and regulation of Aurora B involves three different proteins, these seem to operate as a single structural unit.

Besides the essential interaction between INCENP, Survivin and Borealin for centromere recruitment, the C-terminus of Borealin (Borealin$^{109-280}$) must carry information that determines centromere binding. This would be in line with our earlier hypothesis that Borealin might bind to DNA. Specific disruption of any single passenger protein results in the impairment of the structural unit and in the failure of CPC targeting, leading to the defects seen under conditions of CPC depletion. Intriguingly, we failed to define any function of an engineered INCENP/Aurora B subcomplex. Moreover, results obtained with a deletion fragment of Borealin (Borealin$^{10-109}$) and surface charge mutants (INCENP 35/36/39/40R and Borealin R17/19E, K20E) revealed that the CPC exerts its function in cytokinesis from the central spindle/midbody.

IV. The MAP kinase pathway and the CPC

Borealin is phosphorylated by Erk kinase

Results obtained in collaboration with the laboratory of Marsha Rosner (Ben May Department for Cancer Research, Chicago) suggested that the MAP kinase pathway is involved in CPC regulation. In particular, the Raf kinase inhibitory protein (RKIP) was shown to regulate centromeric Aurora B kinase activity but the mechanism has remained elusive (Eves *et al.*, 2006). To analyse if MAP kinase mediated phosphorylation of the CPC might be involved in this regulation we tested chromosomal passenger proteins for phosphorylation by the Erk kinase. Interestingly, Borealin, but no other passenger protein, was readily phosphorylated in an *in vitro* kinase assay (Figure 39). Erk kinase phosphorylated Borealin showed an electrophoretic mobility shift indicating a strong modification and/or structural rearrangements.

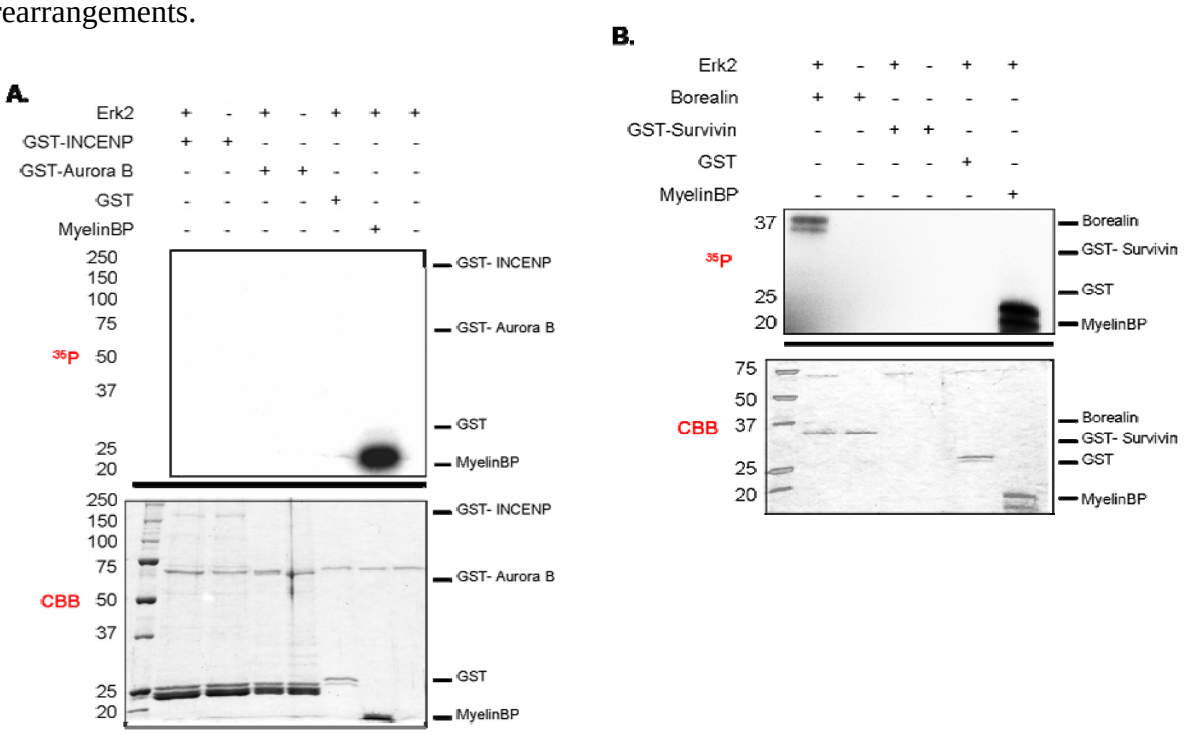


Figure 39. Erk2 *in vitro* kinase assay monitoring CPC phosphorylation.

(A and B) Recombinant chromosomal passenger proteins were tested for phosphorylation by Erk kinase. Only Borealin and the positive control myelin basic protein (MyelinBP) are phosphorylated in this assay. Phosphorylation was monitored by autoradiography (^{35}P) and loading was controlled by Coomassie Brilliant Blue staining (CBB).

Identification of Borealin T106 as an Erk kinase phosphorylation site *in vitro*

Several phosphorylation sites on the Borealin protein have been identified by our mass spectrometry group (personal communication by Roman Koerner). Based on these data we mutated corresponding residues and found that mutation of Borealin residue T106 abolished Erk kinase mediated phosphorylation (Figur 40A). As the consensus phosphorylation motives of MAP kinases and the master mitotic kinase Cdk1 are similar and residue T106 is followed by a lysine residue at position +3 (rather indicative of a Cdk1 site) we tested Cdk1 phosphorylation of Borealin wild-type compared to the T106A mutant. Indeed, Borealin was phosphorylated by Cdk1 in *in vitro* kinase assays but we did not observe an electrophoretic shift or any influence of mutating the residue T106 (Figure 40B).

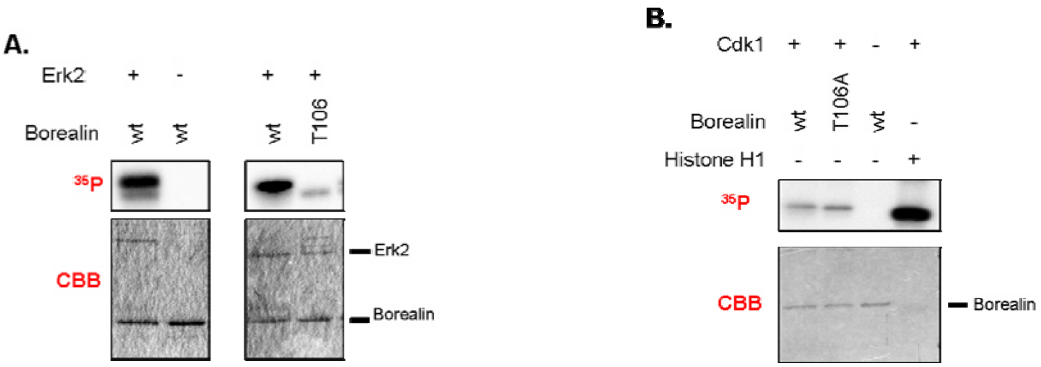


Figure 40. Borealin T106 is an Erk kinase site *in vitro*.

(A) Experiment as in Figure 24. Recombinant Borealin wild-type and the BorealinT106A mutant were tested in an *in vitro* kinase assay for phophorylation by Erk. Note almost complete absence of the ^{35}P signal in the BorealinT106A reaction.

(B) *In vitro* Cdk1 kinase assay on Borealin wild-type compared to BorealinT106A mutant. No difference in phosphorylation was observed for the two constructs.

Production of a BorealinT106 phospho-specific antibody

To address if Borealin can be assigned as an Erk kinase target *in vivo* we immunized rabbits with a peptide comprising phosphorylated T106 (H-AEAIQT(PO_3H_2)PLKS-OH) to produce a phospho-specific antibody against this site. Antibodies were purified from the final bleeds and the specificity tested by immunofluorescence. (Antibodies produced in two different rabbits #127 and #352 gave identical results). Phospho-T106-Borealin showed CPC characteristic centromere staining from prophase until metaphase but did not stain the central spindle or the midbody (Figure 41A and B). Interestingly, a few metaphase cells did not stain with phospho-T106-Borealin (Figure 41C), suggesting a dependency on the SAC status. Importantly, no signal was detected in cells treated with siRNA duplexes directed against the

Borealin transcript (Figure 42 middle row). Attesting for the phospho-specificity of the antibody, no signal was observed in cells treated with calf intestine phosphatase (Figure 42 bottom row). Next we investigated localization and function of the Borealin T106A mutant. As we did not observe any difference between this mutant and wild-type Borealin in corresponding rescue assays (data not shown) we did not proceed further to analyse this phosphosite of Borealin.

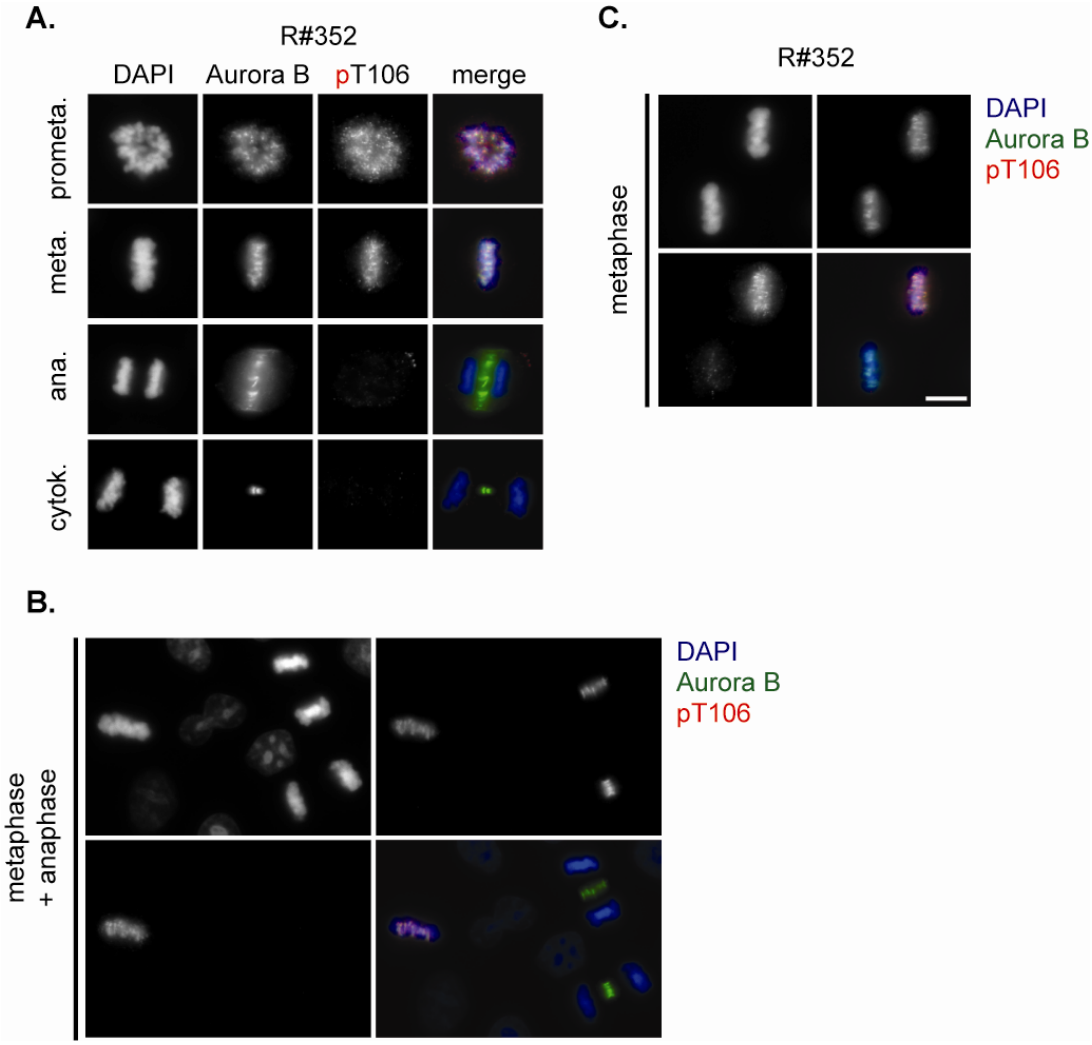


Figure 41. Phospho-T106-Borealin antibody stains the centromere but not the central spindle or midbody.

(A-C) HeLa cells were methanol fixed and stained for Aurora B and phospho-T106-Borealin. Individual mitotic stages are shown in (A). (B) shows a side-by-side comparison of metaphase to anaphase cells. (C) A few metaphase cells were negative for phospho-T106-Borealin. One speculative explanation for this observation could be that phosphorylation at T106 is SAC dependent. Bar, 10µm.

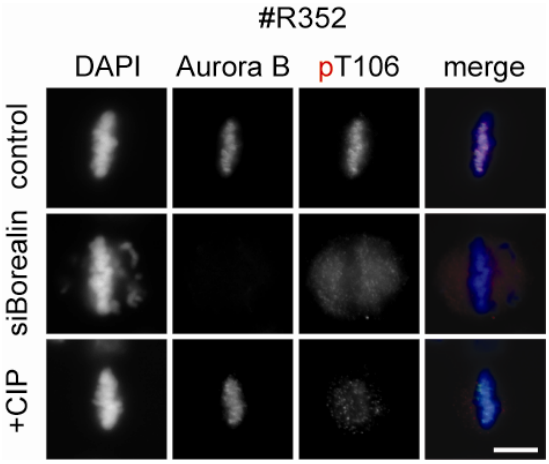


Figure 42. Phospho-T106-Borealin antibody responds to CPC depletion and phosphatase treatment. HeLa cells were treated with siRNA duplex directed against Borealin (or control) and incubated for 48hrs. Where indicated calf intestine phosphatase (CIP) was added for 15min before methanol fixation. Cells were stained with anti-Aurora B and anti-phospho-T106-Borealin antibodies. In both siRNA mediated depletion of Borealin and CIP treatment phospho-T106-Borealin signal was abolished. Bar, 10µm.

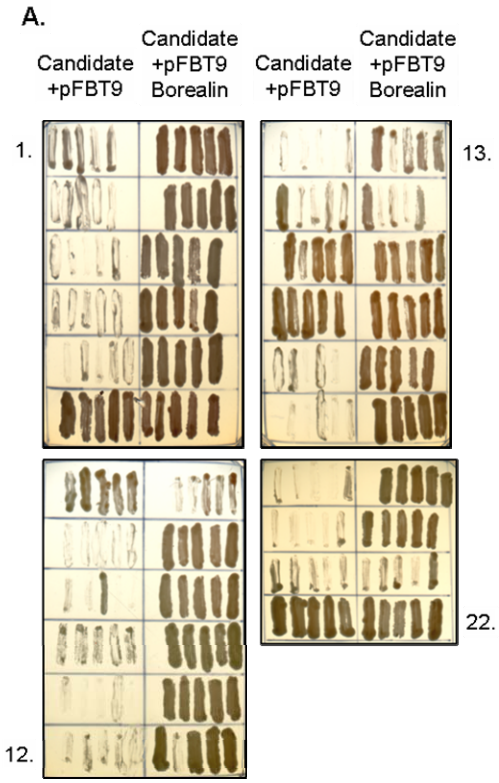
Conclusion

At this point it is not clear how the MAP kinase pathway regulates the CPC. Borealin residue T106 has been identified as an Erk kinase site *in vitro* and it remains to be seen if this can be confirmed *in vivo*. However, the observed phosphorylation was remarkably strong and led to a striking electrophoretic shift. A phosphospecific antibody directed against the Borealin T106 site stained the centromere region from prophase to metaphase, as expected for the CPC, but was undetectable at the central spindle and the midbody indicating regulation of this site during mitotic progression. However, this temporal regulation would also be consistent with Cdk1 mediated phosphorylation and would require further analysis. The absence of a detectable difference between the T106A mutant and wild-type Borealin in a rescue approach led us to abandon the project.

V. The CPC is linked to the SUMO system

A yeast-two hybrid screen identifies SUMO pathway components as interaction partners of Borealin

To obtain further insights into the role of the N-terminus of INCENP and/or the Borealin subunit of the CPC, we screened a yeast two-hybrid HeLa cDNA library with INCENP$^{1-530}$ or full-length Borealin as the bait. Among interactors of the Borealin protein we identified several components of the ubiquitin-like SUMO modification system, e.g. the E2 conjugating enzyme Ubc9 (Figure 43A and B). Among the (few) identified interactors of INCENP$^{1-530}$ only heterochromatin-protein1 looked interesting to us (data not shown). However, this interaction has been described previously (Ainsztein *et al.*, 1998) and was therefore not investigated further.



B.

nr.	Interactor	function	interaction
1	LOC441376	ND	m
2	Kif3A	kinesin	s
3	PIAS2	SUMO	s
4	ATP6V1D	lysosomal	s
5	SUMO1	SUMO	s
6	s-a.		
7	s-a.		
8	Ubc9	SUMO	s
9	C21ORF45	ND	s
10	ARHGEF11	ND	s
11	Ubc9	SUMO	s
12	Tex11	male germ	s
13	FLJ23825	ND	m
14	s-a.		
15	s-a.		
16	s-a.		
17	PIAS2	SUMO	s
18	EXOSC9	exosome	s
19	Ubc9	SUMO	s
20	PDCD7	apoptosis	s
21	PBXIP1	transcription	w
22	s-a.		

Figure 43. Borealin interactors identified by yeast-two hybrid screening.

(A) Putative Borealin interaction partners were tested for self-activation with the empty vector (candidate + pFBT9) or real interaction (candidate + pFBT9 Borealin).

(B) Interactors identified. Numbers refer to (A). The strength of the interaction is given (w = weak, m = moderate, s = strong). s-a. = self-activating. ND = not determined. Kif3A (nr. 2) was found to have mitotic functions only after this screen has been performed (Haraguchi *et al.*, 2006).

Borealin is covalently modified by SUMO

Next we analysed if Borealin interacts with SUMO pathway components in directed yeast-two hybrid assays. These assays confirmed the interaction with Ubc9 and SUMO1 and additionally revealed an interaction of Borealin with SUMO2 (Figure 44A and B). The other known members of the CPC, INCENP, Aurora B and Survivin, did not bind Ubc9 or SUMO forms (Figure 44A and data not shown). The binding of Borealin to both SUMO paralogs 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 44B lower two panels).

To test this, 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 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 42kDa was detected (Figure 44C). 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 44C) and SUMO2 (Figure 44D). The major conjugates migrated at ~57, ~70 and ~83kDa, 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 modified by SUMO (Figure 44C).

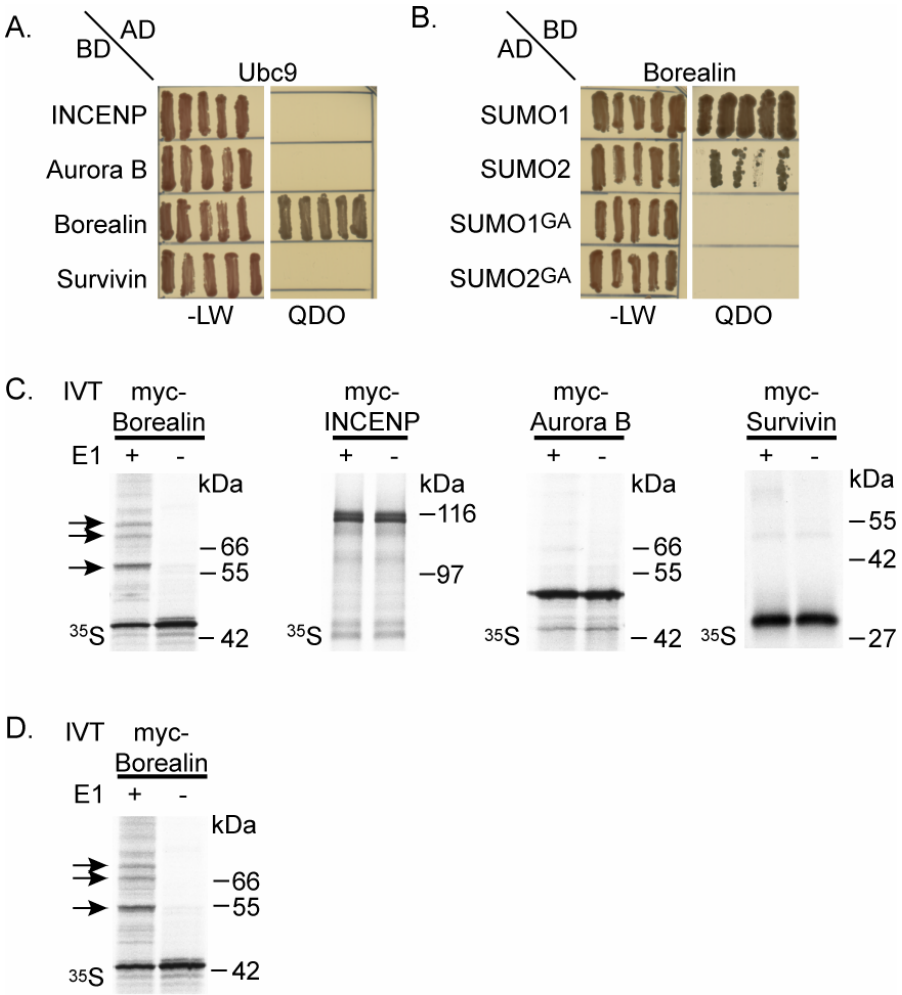


Figure 44. Borealin interacts with components of the SUMO system and is modified by SUMO *in vitro*.
(A and B) Interaction of Borealin with Ubc9 (A) and the conjugatable forms of SUMO1 and SUMO2 (B, upper two rows), but not the unconjugatable forms (B, lower two rows) in directed yeast two-hybrid assays.
(C and D) Borealin is modified by SUMO *in vitro*. ^{35}S-labelled 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.

To examine whether SUMO paralogs 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 45A). Survivin, a direct binding partner of Borealin within the CPC (Gassmann *et al.*, 2004) was neither conjugated to SUMO1 nor SUMO2 (Figure 45B). SUMO mutants (SUMO1^{K16R} and SUMO2^{K11R}) that are unable to form chains

(Hay, 2005) generated an identical pattern of SUMO-Borealin conjugates (Figure 45C) 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 were 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, reminiscent of the Borealin-SUMO conjugates described above (Figure 45D). 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 detect any of the higher molecular weight bands. To test modification of endogenous Borealin by SUMO2/3, total cellular SUMO2/3-conjugates from mitotic cell lysates were immunoprecipitated by anti-SUMO2/3 antibodies (Figure 45E). We validated the experimental setup by monitoring sumoylation of topoisomerase II (Azuma *et al.*, 2003; Azuma *et al.*, 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 64kDa 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.

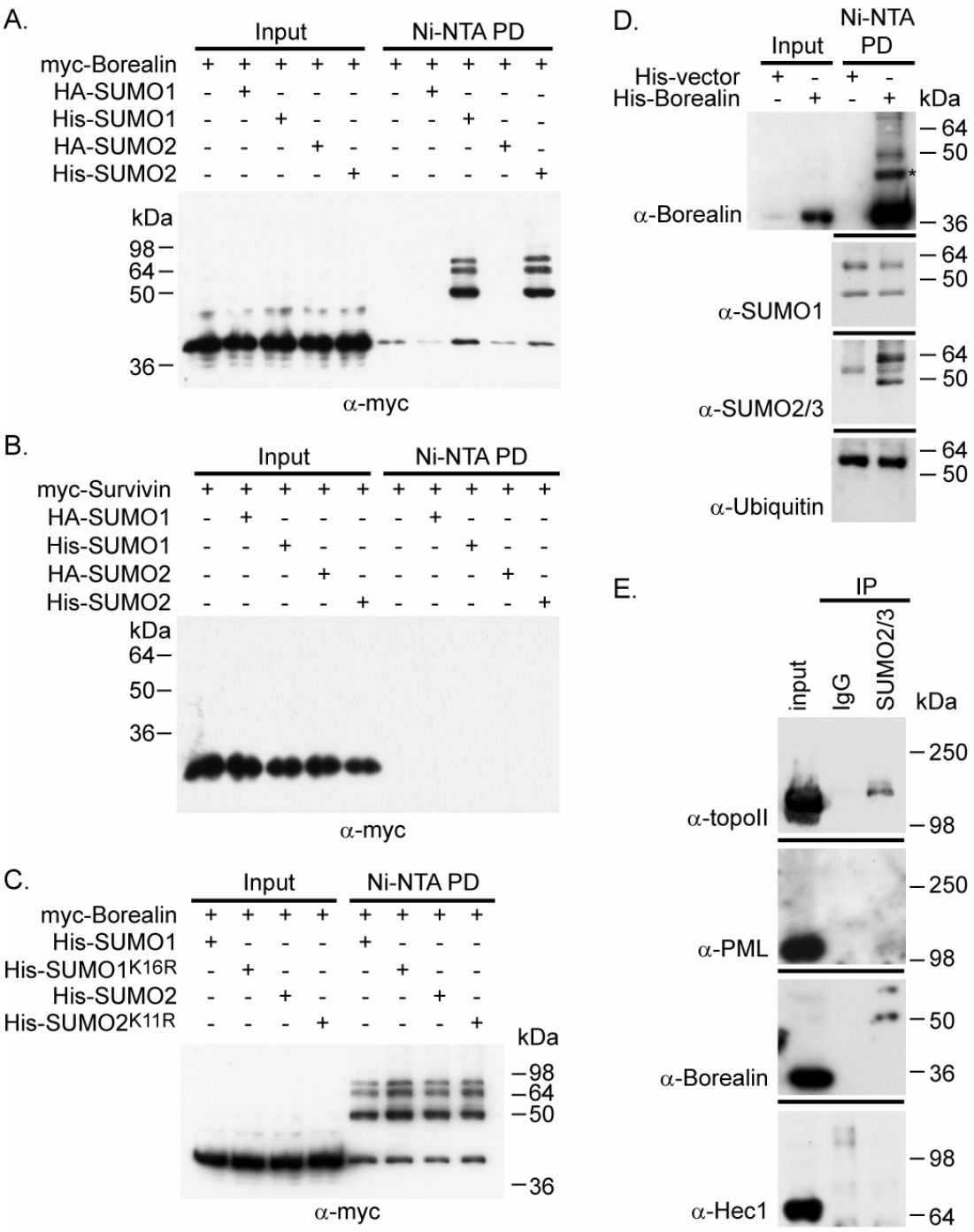


Figure 45. Borealin is modified by SUMO *in vivo* and is preferentially targeted by SUMO2/3.

(A) Myc-tagged Borealin and HA- or His-tagged versions of SUMO1 or SUMO2, respectively, were co-expressed in COS-7 cells. His-SUMO conjugates were recovered on Ni-NTA beads and subjected to Western blotting 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 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 16hr. 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 ~45kDa (asterisk) is 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.

Borealin is a target of ubiquitination

Modification of Survivin by ubiquitin has been shown to regulate the dynamic association of the CPC with the centromere and to be required for proper chromosome segregation (Vong *et al.*, 2005). Ubiquitination, like sumoylation, occurs via attachment of the moiety to a given lysine residue and thus may block sumoylation. We therefore asked if Borealin is ubiquitinated and performed the assay outlined above for His-SUMO (Figure 45A-C) using His-tagged ubiquitin. In agreement with the above mentioned study Survivin was readily ubiquitinated. However, only mono-ubiquitination was observed (Figure 46A). In the same assay Borealin showed ubiquitination (Figure 46B).

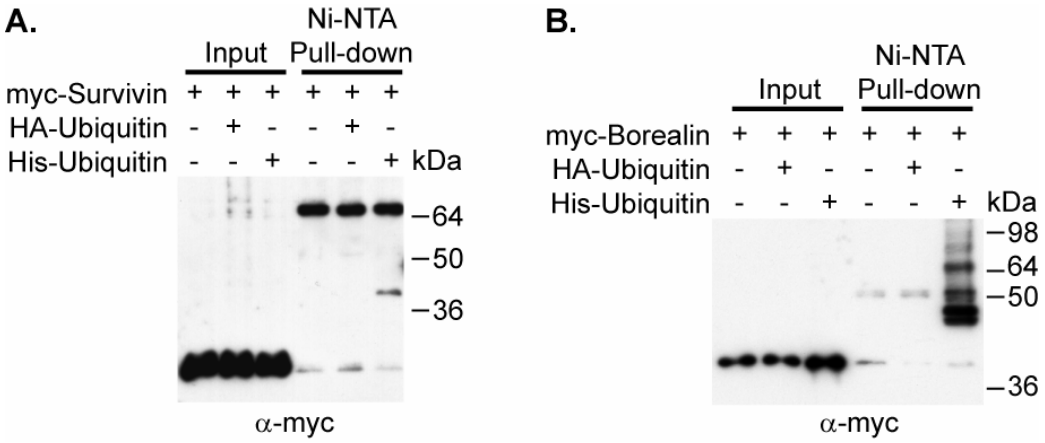


Figure 46. Borealin is ubiquitinated.

(A) Myc-tagged Survivin was tested in an ubiquitination assay as outlined above for SUMO (Figure 47). A single Survivin-ubiquitin conjugate was detected.

(B) Assay as in (A) testing for Borealin ubiquitination. Borealin is polyubiquitinated.

Sumoylation of Borealin is independent of CPC formation

The ability to dissociate individual members of the CPC from the complex based on the crystal structure described above led us to analyse if Borealin incorporation into the CPC is required for its sumoylation. We tested the Borealin mutants (described above in Figure 36D) that are unable to bind INCENP/Aurora B or Survivin in the *in vivo* sumoylation assay.

Notably, both mutants showed sumoylation like the wild-type protein (Figure 47). Hence, sumoylation of Borealin can occur independently of CPC formation.

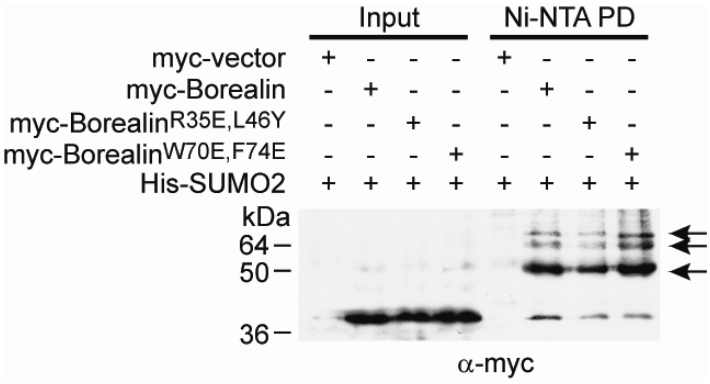


Figure 47. CPC independent sumoylation of Borealin.

Myc-tagged Borealin mutants unable to bind INCENP/Aurora B (Borealin$^{R35E, L46Y}$) or Survivin (Borealin $^{W70E, F74E}$) where tested in the *in vivo* sumoylation assay. Both mutants showed sumoylation (arrowheads).

Sumoylation of Borealin is cell cycle regulated

To address if 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 48A). In G1 arrested cells the level of Borealin-SUMO2/3 conjugates was low (Figure 48A, lane 5). Strikingly, the amount was strongly increased in metaphase-arrested cells (Figure 48A, lane 6 and 42B), before the conjugates were progressively lost as cells entered anaphase (Figure 48A, lane 7 and 8). Proteasome inhibition by MG132 used to prepare mitotic lysates did not affect Borealin sumoylation (Figure 48B). We conclude that sumoylation of Borealin is dynamically regulated during cell cycle progression with a peak in early mitosis.

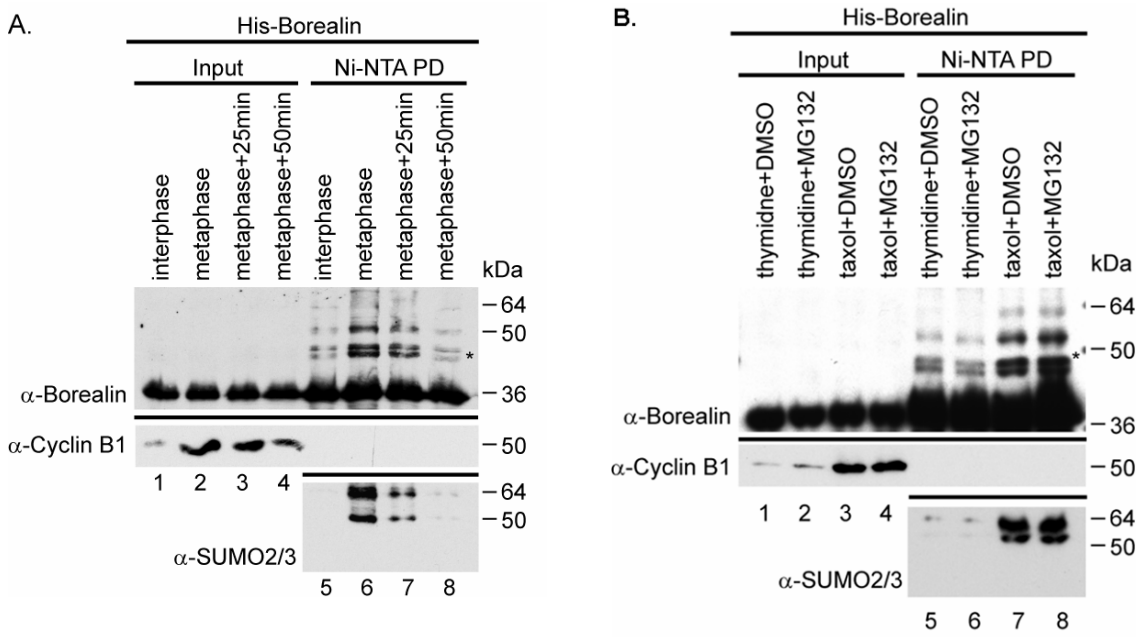


Figure 48. Sumoylation of Borealin is cell cycle regulated and independent of proteasome inhibition.
(A) HeLa cells expressing His-Borealin were arrested in G1 (lane 1), metaphase (lane 2) or allowed to enter anaphase (lane 3 and 4). His-Borealin and His-Borealin-conjugates were detected by Western blotting 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) Proteasome inhibition used for mitotic arrest does not affect sumoylation of Borealin. Experiment as in (A) using different drug combinations as indicated. DMSO or MG132 were added 2hrs before lysate preparation.

The SUMO E3 Ligase RanBP2 is essential for sumoylation of Borealin

The notion that Borealin sumoylation is regulated during the cell cycle led us to search for regulatory components that control its dynamic modification. To this end, candidate SUMO E3 ligases, notably PIAS family members, RanBP2 and hPC2, were analyzed for their ability to interact with Borealin in the yeast two-hybrid system. Binding of Borealin was observed to the α and β splice variants of PIAS2 and the catalytically fragment of RanBP2 ($RanBP2^{\Delta FG}$) (Figure 49A). To test for these interactions in mammalian cells, endogenous Borealin was immunoprecipitated from mitotic HeLa cells (Figure 49B). 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. 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 previously shown to localize to the centromere/kinetochore region and the mitotic spindle (Joseph *et al.*, 2002).

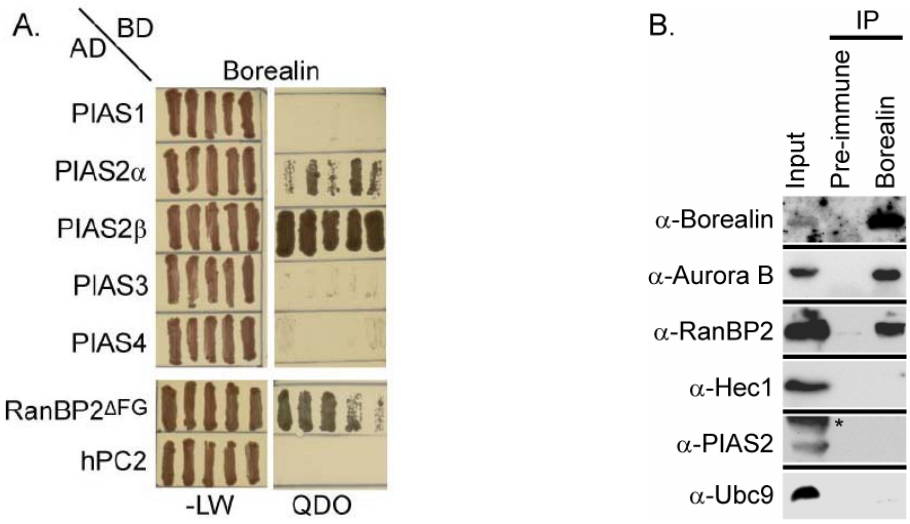


Figure 49. Borealin interacts with RanBP2.

(A) Borealin was tested for interaction with SUMO E3 ligases in directed yeast two-hybrid assays.

(B) Immunoprecipitations were performed with rabbit anti-Borealin antibodies or pre-immune 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.

To address a putative SUMO E3 ligase function of RanBP2 towards Borealin, recombinant RanBP2$^{\Delta FG}$ was added to an *in vitro* sumoylation reaction on Borealin, which was performed under limiting Ubc9 concentrations (70nM). Under these conditions basal E1-E2 mediated sumoylation of Borealin was weak (Figure 50A, upper panel, lane 2), but addition of RanBP2$^{\Delta FG}$ in a concentration range of 5nM to 50nM enhanced the formation of the three Borealin-SUMO conjugates (Figure 50A, lane 4 and 5), while it did not stimulate sumoylation of the control substrate p53 (Figure 50A, lower panel). In agreement with previous data (Pichler *et al.*, 2002), higher concentrations of RanBP2 (500nM) exert an inhibitory effect on sumoylation (Figure 50A, upper panel, 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 siRNA (Figure 50B). Sumoylation of Borealin was monitored by Ni-NTA pulldown 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 knock-down of the essential E2 enzyme Ubc9. In contrast, Borealin sumoylation was unaltered in cells depleted of the α and β forms of PIAS2. Furthermore, depletion of Eg5, which, similar to the knock-down of RanBP2 (Joseph *et al.*, 2004), leads to a prometaphase-like arrest (Blangy *et al.*, 1995), did not influence sumoylation of Borealin. Noteworthy,

depletion of RanBP2 did not affect the level of total SUMO2/3-conjugates (Figure 50C) or sumoylation of the unrelated control substrate PARP-1 (Figure 50D), indicating that RanBP2 does act on specific SUMO target proteins.

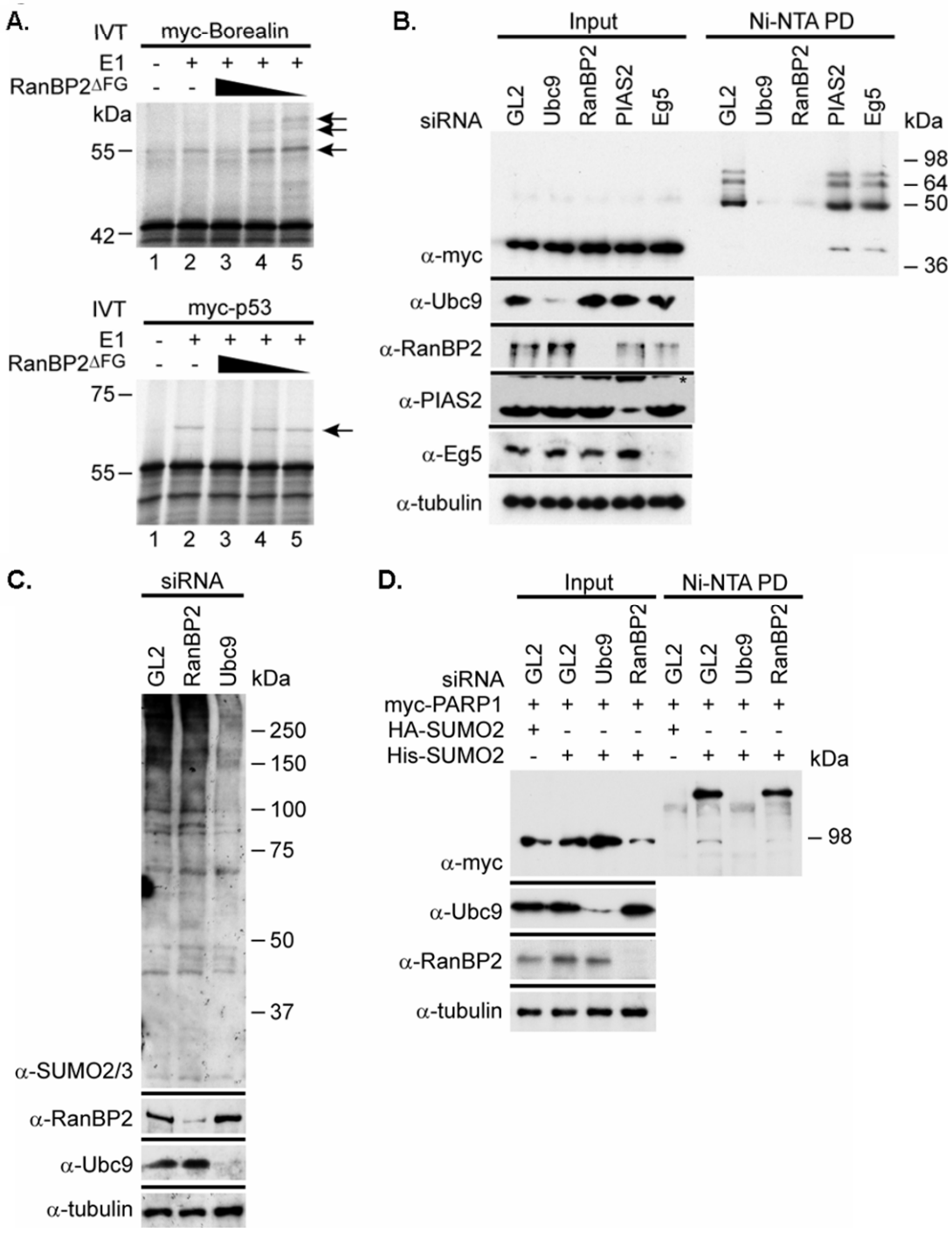


Figure 50. RanBP2 regulates sumoylation of Borealin.

(A) ^{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 500ng (lane 3), 50ng (lane 4) and 5ng (lane 5). SUMO conjugates are indicated by arrows. Data Dr. Stefan Muller.

(B) 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 crossreactive band. His-SUMO2 conjugates were recovered on Ni-NTA beads and Western blotting was performed with anti-myc antibodies.
(C) Depletion of RanBP2 does not lead to a general loss of SUMO2/3 conjugates. HeLa cells were treated with siRNA oligonucleotides directed against indicated proteins. Depletion was verified by Western blotting and SUMO2/3 conjugates were detected by anti-SUMO2/3 antibodies.
(D) PARP1 sumoylation occurs independent of RanBP2. Myc-tagged PARP1 and HA- or His-SUMO constructs were co-expressed in HeLa cells treated with siRNA oligonucleotides directed against indicated proteins. Depletion was verified by Western blotting. His-SUMO conjugates were recovered on Ni-NTA beads and Western blotting was performed with anti-myc antibodies.

To address if 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 51A). 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 51B - E). Taken 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 51F). This effect is due to posttranscriptional regulation, since the mRNA level of RanBP2 does not significantly change during cell cycle progression (Figure 51G).

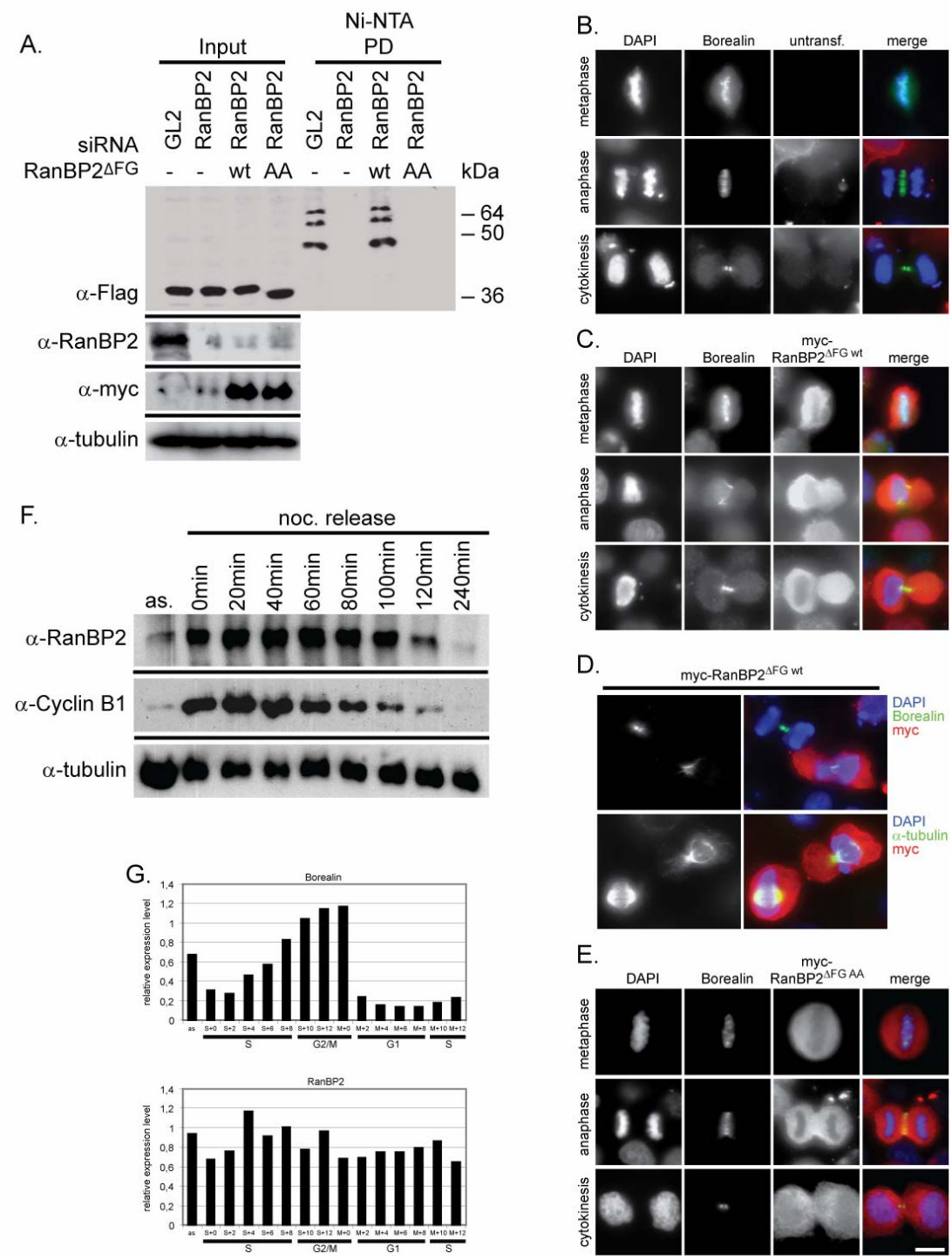


Figure 51. RanBP2$^{\Delta FG}$ restores sumoylation of Borealin in RanBP2 depleted cells.

(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 massive chromosome missegregation after anaphase onset. (D) Experiment as in (C). Lower panel shows co-staining 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 DAPI. Scale bar = 10μm.

(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. Data Markus Haindl.

(G) RanBP2 and Borealin mRNA levels upon cell cycle progression were determined in synchronized HeLa cells using quantitative RT-PCR. Data provided by Patrick Déscombes.

RanBP2 mediated Borealin sumoylation is likely to occur before CPC centromere binding

Interaction of RanBP2 and the CPC was found in taxol arrested cells when both components bind to the centromere/kinetochore region, suggesting that sumoylation of Borealin occurs during this stage (Figure 49B). Arguing against this, however, we did not observe binding of Ubc9 to the RanBP2-CPC complex in this experiment (Figure 49B). To address if RanBP2 KT-binding is crucial for Borealin sumoylation we exploited the finding that the SUMO E3 ligase is absent from the KT when cells are treated with nocodazole (Joseph *et al.*, 2004). Indeed, we observed RanBP2 KT localization in taxol but not in nocodazole treated cells. CPC localization was unaffected in both treatments (Figure 52A). However, no difference in the sumoylation status of Borealin was detected comparing the two conditions (Figure 52B). Thus, Borealin seems to become sumoylated before the CPC targets to the centromere.

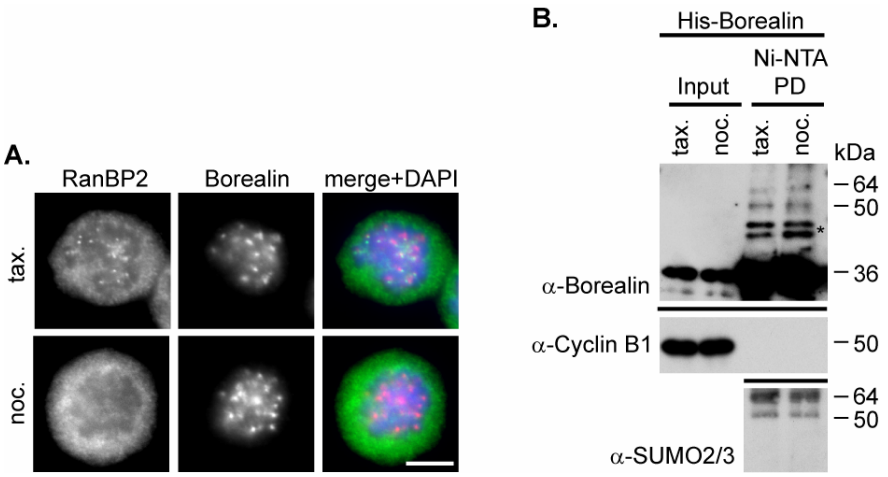


Figure 52. RanBP2 localization to the KT is not required for Borealin sumoylation.

(A) HeLa cells were treated with the indicated drugs for 16hrs and stained with anti-RanBP2 and anti-Borealin antibodies. In the presence of nocodazole RanBP2 is absent from the KT. Bar, 10μm.

(B) HeLa cells were transfected with His-Borealin and incubated with the indicated drugs for 16hrs. His-Borealin and His-Borealin-conjugates were detected by Western blotting with anti-Borealin and anti-SUMO2/3 antibodies. Bands marked by asterisk are interpreted as Borealin-SUMO2/3 degradation products. The mitotic status was assayed by monitoring Cyclin B1 levels. Tax. = taxol, noc. = nocodazole.

Attempts to map the SUMO acceptor site/s of Borealin

To asses the function of Borealin sumoylation experiments were performed in order to map the SUMO acceptor lysine/s. Spot-blot analysis of Borealin peptides and *in vitro* sumoylation of Borealin fragments spanning 3-6 lysine residues gave inconsistent results (data not shown). Surprisingly, we found that every lysine residue present in *in vitro* translated Borealin could serve as a SUMO acceptor site in our assay. (This phenomenon was even observed for lysine residues present in protein tags, e.g. the flag-tag). We therefore created two Borealin fragments cutting the protein in half and tested these in our *in vivo* sumoylation assay. We observed sumoylation for the C-terminal half of Borealin but not its N-terminus (Figure 53A). We next mutated all 12 lysine residues present in the C-terminal half (Borealin^{12KR}). Surprisingly, however, this mutant still showed normal levels of sumoylation (Figure 53B). Thus, we conclude that a) either more subtle assays are required to map the SUMO site of Borealin or b) "jumping" of the SUMO moiety on Borealin can occur.

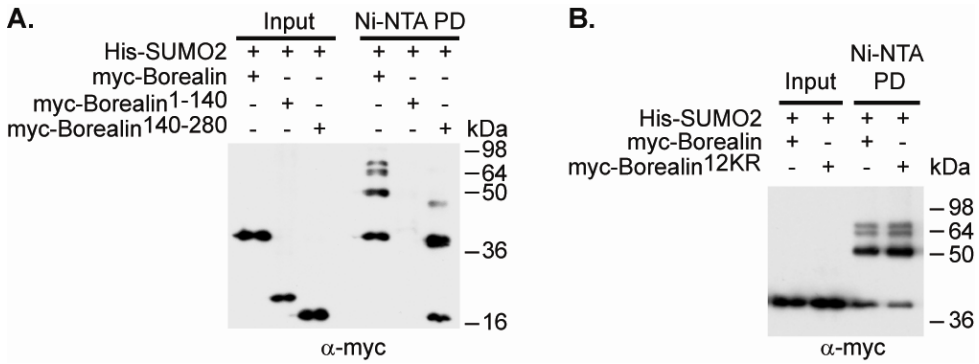


Figure 53. The SUMO acceptor site of Borealin could not be mapped.

(A) Borealin was cut in half and both constructs were tested for *in vivo* sumoylation as outlined before. Only the C-terminal half shows sumoylation.

(B) All 12 lysine residues present in the C-terminal half were mutated (Borealin^{12KR}) and sumoylation of the mutant was analysed. Inconsistently, Borealin^{12KR} was still sumoylated.

CPC formation and localization are sumoylation independent

It has been reported very recently that RanBP2-mediated SUMO modification of topoisomerse II is required for its localization to centromeres (Dawlaty *et al.*, 2008). In contrast, depletion of RanBP2 or Ubc9 did not affect the centromeric localization of Borealin (Figure 54A and data not shown), indicating that centromere targeting of the CPC occurs independently of Borealin sumoylation. As we were unable to map the exact site of SUMO modification we created a non-sumoylatable variant of Borealin, where all 25 lysine residues were mutated to arginine (Figure 55A and B) in order to rule out that the above result is due to residual amounts of RanBP2 (or Ubc9) left after corresponding siRNA treatment. 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 54B). In line with the notion that the CPC requires the Borealin subunit to target to its distinct locations (see Results I and III) HA-Borealin^{25KR} associated with all other CPC components (Figure 54C). Interestingly, this mutant fails to exhibit normal localization to the nucleolus in interphase cells depleted from endogenous Borealin (Figure 55C). However, this effect is unlikely due to the loss of SUMO modification, as the general inhibition of sumoylation did not affect the nucleolar localization of wild-type Borealin (Figure 55D). 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 (Figure 56A and B).

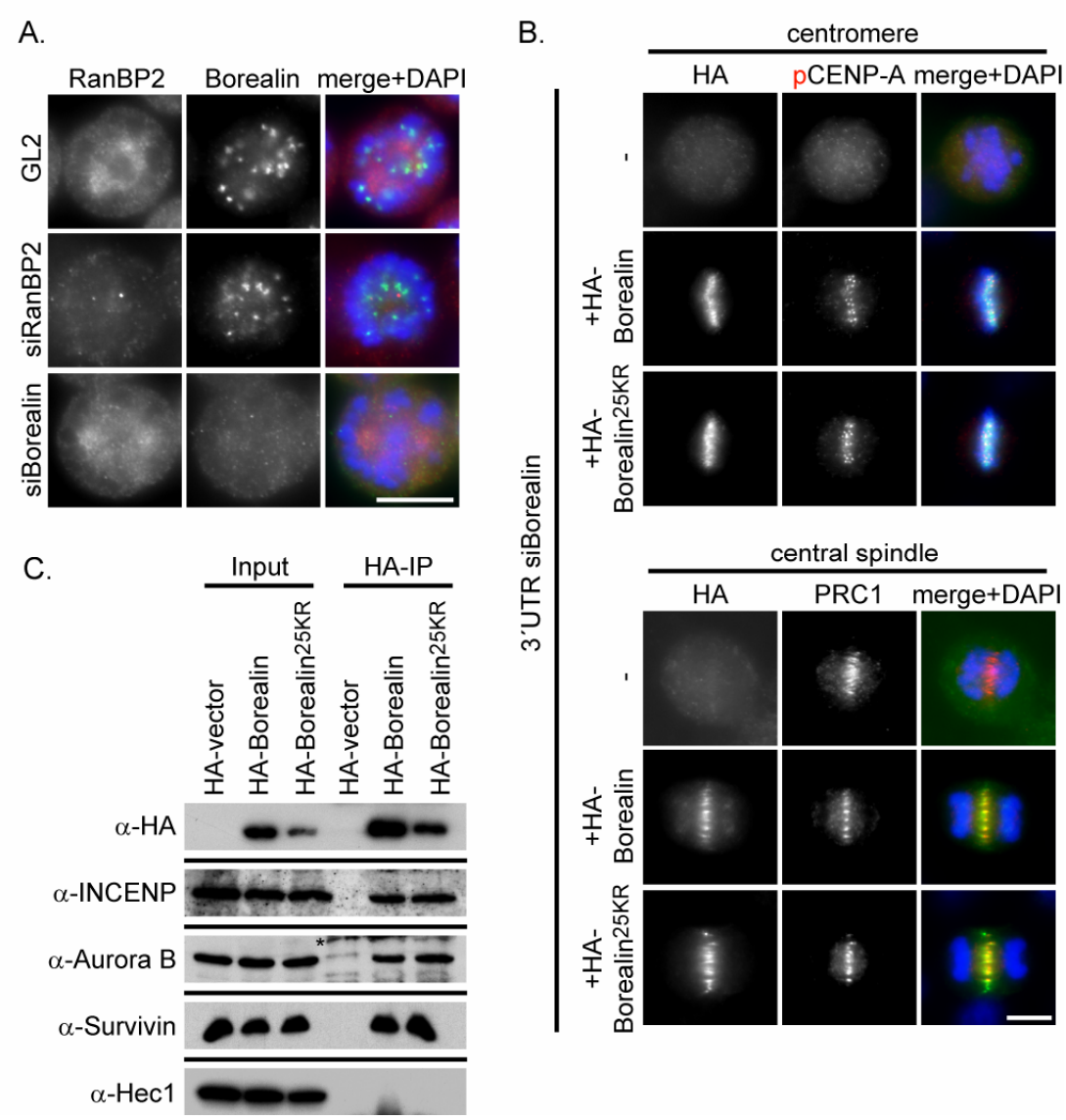


Figure 54. CPC assembly and localization to the centromere and central spindle are independent of Borealin sumoylation.

(A) RanBP2 does not influence CPC localization to the kinetochore/centromere region and *vice versa*. HeLa cells were treated with indicated siRNA duplexes for 48hrs and stained for RanBP2 and Borealin. Bar = 10µm.

(B) HeLa cells were treated with siRNA duplexes specific for the 3´UTR of Borealin and simultaneously transfected with HA-Borealin or HA-Borealin25KR. Immunofluorescence was performed with antibodies directed against HA, phospho-S7-CENP-A and PRC1. Scale bar = 10µm.

(C) HeLa cells were transfected with the indicated constructs, arrested in S phase by thymidine treatment and released for 10hr 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 immunoglobuline heavy chain.

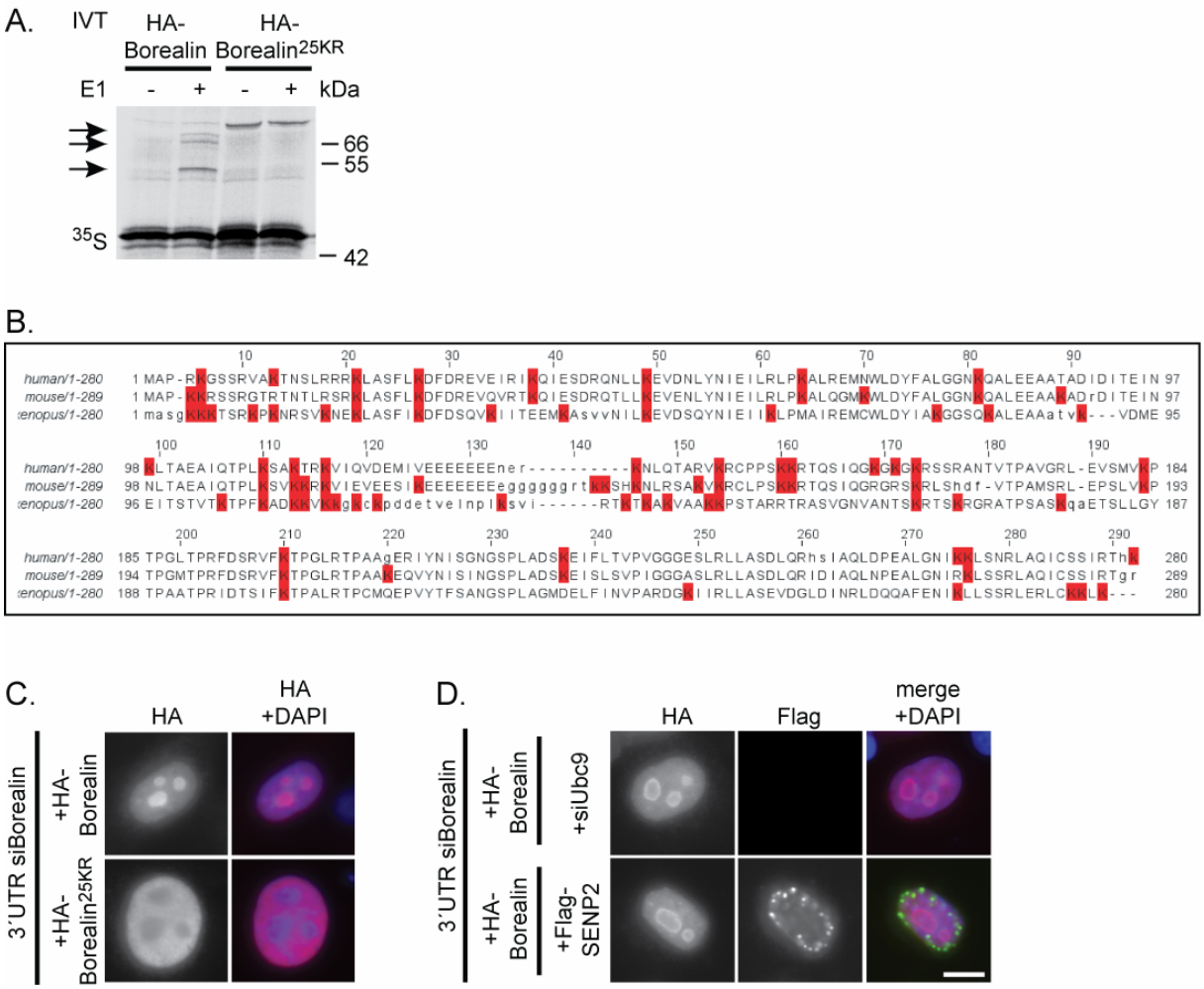


Figure 55. Construction of a non-sumoylatable mutant of Borealin.

(A) *In vitro* sumoylation of Borealin and a Borealin mutant in which all lysine residues have been mutated to arginine (Borealin^{25KR}) was performed as outlined in Figure 35. SUMO-Borealin conjugates are indicated by arrows.

(B) Illustration of lysine residues within human, mouse and Xenopus Borealin. Of note, none of the lysine residues confers to the proposed minimal SUMO consensus motif KxE/D.

(C) HeLa cells were treated with siRNA duplexes specific for the 3´UTR of Borealin and simultaneously transfected with HA-Borealin or HA-Borealin^{25KR}. Immunofluorescence was performed on interphase cells with antibodies directed against HA.

(D) Experiment as in (C) showing interphase cells expressing HA-Borealin that have been depleted of Ubc9 (upper panel) or co-transfected with Flag-tagged SENP2. Scale bar = 10µm.

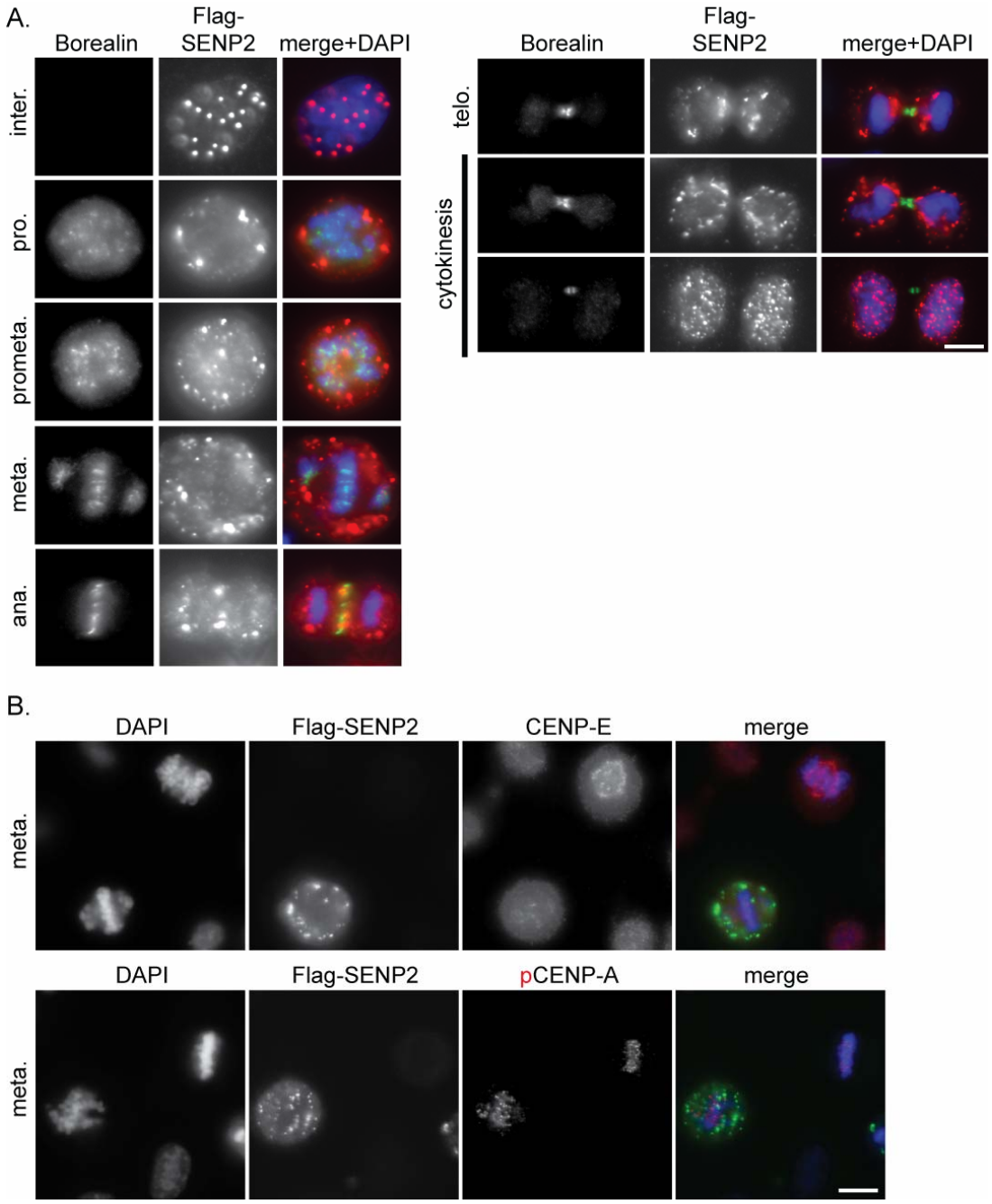


Figure 56. Localization and function of the CPC is unaffected in cells overexpressing SENP2.

(A) HeLa cells were transfected with Flag-SENP2 and incubated for 48hrs. Immunostaining was performed with anti-Flag and anti-Borealin antibodies.

(B) Experiment as in (A) staining for Flag-SENP2 and CENP-E (upper panel) or Flag-SENP2 and phospho-S7-CENP-A (lower panel). As previously reported, SENP2 overexpression results in a loss of CENP-E from kinetochores. However, phospho-S7-CENP-A staining was unaffected. Scale bar = 10µm.

SENP3 catalyzes desumoylation of Borealin

We next asked whether one of the known SUMO specific isopeptidases might be involved in the desumoylation of 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 (Figure 57A). This finding was corroborated by coimmunopreciptation experiments in mammalian cells using Flag-tagged SENP3 and myc-tagged constructs of Borealin. Full-length Borealin as well as an N-terminal fragment of Borealin (Borealin$^{1-140}$) coprecipitated SENP3, while no interaction was detected between SENP3 and the C-terminal fragment of Borealin (Borealin$^{141-280}$) (Figure 57B).

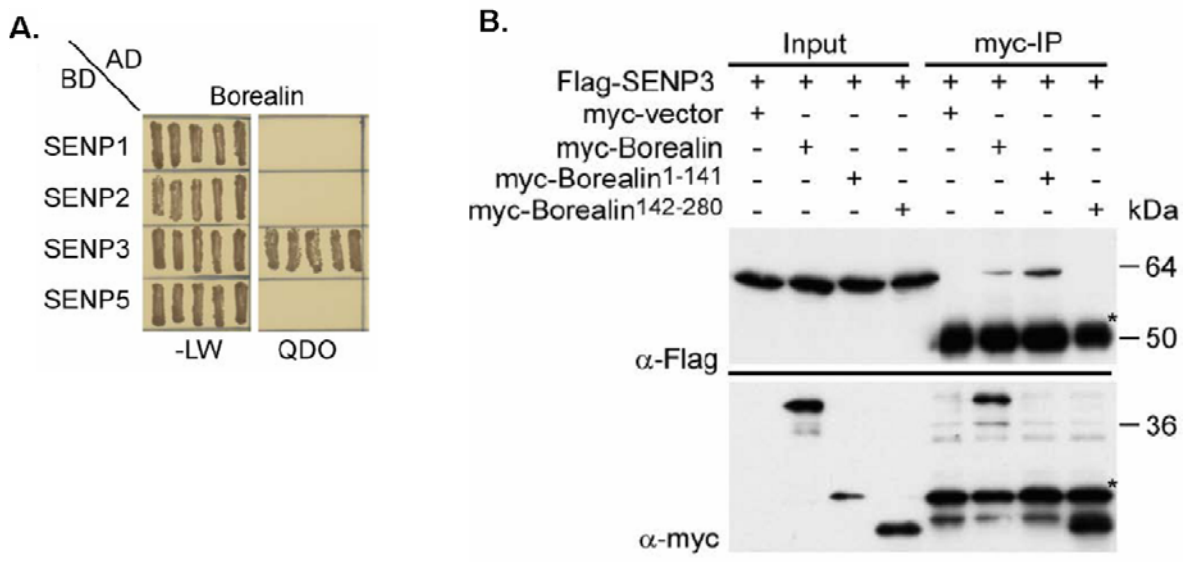


Figure 57. The SUMO protease SENP3 interacts with Borealin.

(A) Borealin was tested for interaction with SUMO proteases in directed yeast two-hybrid assays as described in Figure 35.

(B) 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.

Consistent with this finding and previously reported data on their subcellular distribution, Flag-SENP3 and HA-Borealin exhibit colocalization in the nucleolus in interphase cells (Figure 58A). In early mitosis after nucleolar disassembly SENP3 is found evenly distributed in the cytosol thus showing partial overlap with Borealin (Figure 58B). In late mitosis it accumulates at the reforming nuclear envelope and re-enters the nucleolus during cytokinesis (Figure 58C). Noteworthy, the overexpression of Flag-SENP3 did not affect the normal localization of endogenous Borealin in distinct mitotic stages.

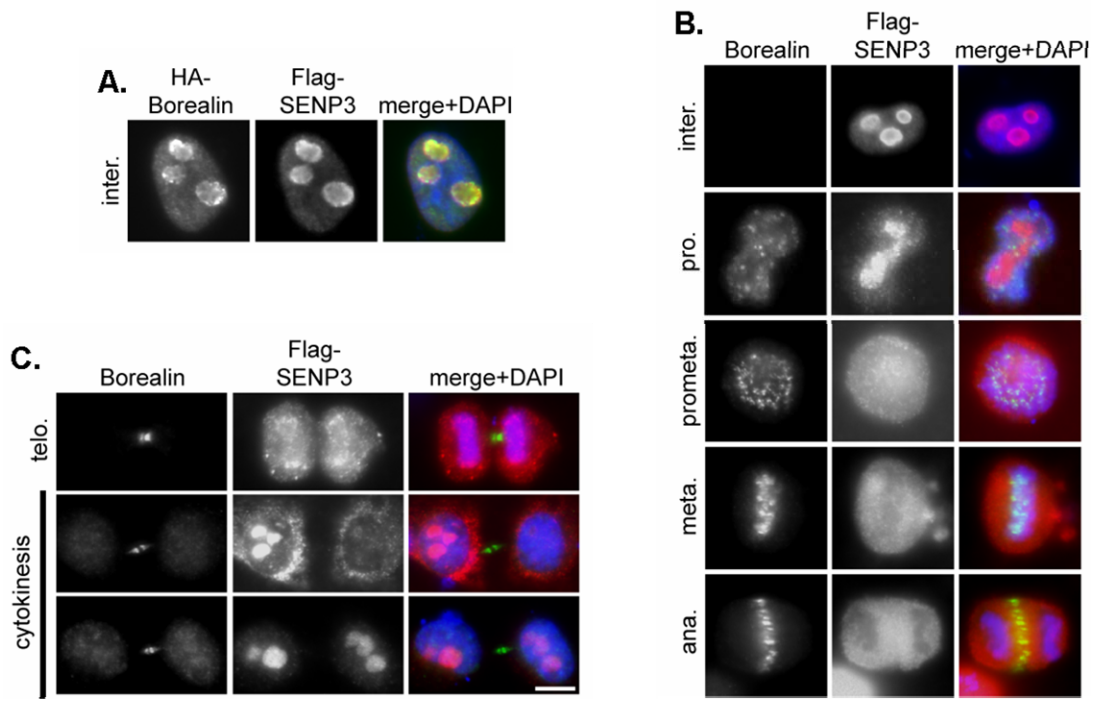


Figure 58. SENP3 and Borealin show overlapping localization.

(A) 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.

(B) Localization of SENP3 during mitosis. HeLa cells were transfected with Flag-tagged SENP3 and incubated for 48hrs. 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 re-enters the nucleolus during cytokinesis. Scale bar = 10μm.

To directly analyze whether SENP3 can catalyze desumoylation of Borealin, an *in vitro* demodification assay was performed. Borealin, pre-modified by either SUMO1 or SUMO2, was incubated with wild-type or catalytically inactive mutant forms of either SENP3 or SENP5, the closest homolog of SENP3 in humans. While Borealin-SUMO1 conjugates were not influenced by the addition of either SENP (Figure 59A, lane 3-6), the amount of Borealin-SUMO2 conjugates was greatly reduced in reactions supplemented with the wild-type SENP3 protein, but unaffected by the inactive protein (Figure 59A, compare lane 8 and 9). In comparison, SENP5 only marginally reduced the amount of Borealin-SUMO2 conjugates (Figure 59A, lane 10). 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 59B, compare lane 11 and 12), whereas the level of Borealin-SUMO1 species was not affected (Figure 59A, lane 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 downregulation of the protein was verified by immunoblotting with an anti-SENP3 antibody (Figure 59C´). 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 59C). Taken together, these data strongly indicate that SENP3 exerts protease activity on Borealin and specifically reverts the modification of Borealin by SUMO2/3.

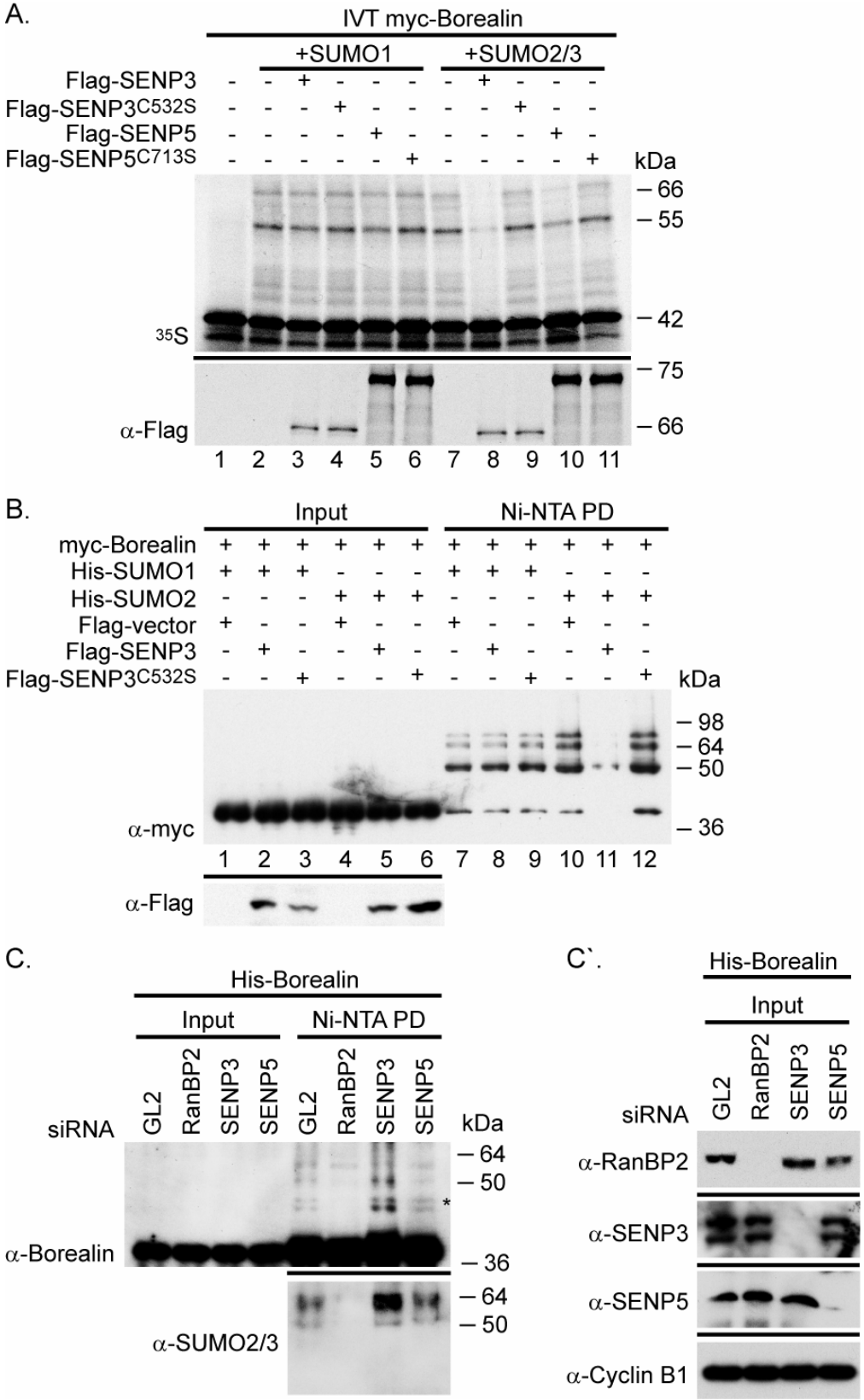

A.
IVT myc-Borealin
+SUMO1
+SUMO2/3
Flag-SENP3
Flag-SENP3C532S
Flag-SENP5
Flag-SENP5C713S
kDa
66
55
42
75
66
35S
α-Flag
1 2 3 4 5 6 7 8 9 10 11
B.
Input
Ni-NTA PD
myc-Borealin
His-SUMO1
His-SUMO2
Flag-vector
Flag-SENP3
Flag-SENP3C532S
kDa
98
64
50
36
α-myc
1 2 3 4 5 6 7 8 9 10 11 12
α-Flag
C.
His-Borealin
Input
Ni-NTA PD
siRNA
GL2
RanBP2
SENP3
SENP5
kDa
64
50
36
α-Borealin
α-SUMO2/3
C`.
His-Borealin
Input
siRNA
GL2
RanBP2
SENP3
SENP5
α-RanBP2
α-SENP3
α-SENP5
α-Cyclin B1

Figure 59. SENP3 catalyzes desumoylation of Borealin.

(A) Flag-tagged versions of wild-type SENP3 ($SENP3^{wt}$, lane 3 and 8) and SENP5 ($SENP5^{wt}$, lane 5 and 10) or the catalytically inactive mutants ($SENP3^{C532S}$, lane 4 and 9, and $SENP5^{C713S}$, lane 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 $SENP3^{C532S}$. Data Markus Haindl.

(B) Myc-tagged Borealin and His-SUMO constructs were coexpressed with Flag-tagged $SENP3^{wt}$ (lane 2, 5, 8 and 11) or $SENP3^{C532S}$ (lane 3, 6, 9 and 12) in HeLa cells. His-SUMO conjugates were recovered on Ni-NTA beads (lane 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 10hr 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 ~45kDa (asterisk in C) is interpreted as a Borealin-SUMO2/3 degradation product. Note that knock-down of SENP3, but not SENP5, enhances Borealin sumoylation compared to control depleted cells. In contrast, RanBP2 knock-down results in a loss of Borealin modification.

Conclusion

In a yeast two-hybrid screen for interaction partners of Borealin we identified components of the SUMO system. Subsequently, we identified Borealin as a mitotic substrate of sumoylation as well as ubiquitination. Borealin is preferentially modified by SUMO2/3 during early mitosis and found to become demodified at the onset of anaphase. Sumoylation of Borealin was shown to be independent of CPC formation and seems to occur before centromere targeting.

Intriguingly, the SUMO E3 ligase RanBP2 interacts with the CPC, stimulates SUMO modification of Borealin *in vitro* and is required for its modification *in vivo*. The overexpression of a catalytically active RanBP2 fragment results in massive chromosome missegregation during anaphase, but the critical target responsible for this phenotype is not definitively identified. The SUMO acceptor site/s of Borealin could not be mapped but we ruled out that sumoylation affects CPC assembly or localization during mitosis. Moreover, we found the SUMO protease SENP3 to interact with Borealin and to mediate deconjugation of SUMO2/3 from Borealin. Our data thus show RanBP2 and SENP3 to dynamically regulate sumoylation of Borealin during mitotic progression.

DISCUSSION

A novel module on INCENP required for centromere targeting of the CPC

It has been well established that INCENP binds the Aurora B kinase via its C-terminal IN-box domain. This module of the CPC is required for full kinase activation via a positive feed-back loop (Bishop and Schumacher, 2002; Honda *et al.*, 2003; Yasui *et al.*, 2004; Sessa *et al.*, 2005). Our data on the INCENP fragment comprising residues 1-58 extends the concept of a modular structure of the INCENP protein. A second module assembles on the N-terminus of INCENP and is required for centromere targeting of the CPC. (We note, that we did not observe any interaction between Survivin and Aurora B, neither in yeast two hybrid assays nor in *in vivo* co-immunoprecipitation, contrasting a previous study (Wheatley *et al.*, 2001) which reported *in vitro* binding of the proteins even under conditions of 3M NaCl). We envision a module at the N-terminal end of INCENP rigidly bound to centromeric DNA (probably mediated via Borealin) and a flexible active kinase module at the C-terminal end of the INCENP protein that regulates centromere/kinetochore assembly and MT-KT interaction (Figure 60), possibly via Hec1 phosphorylation (DeLuca *et al.*, 2006). The two modules are connected by the long coiled-coil region of INCENP. Supporting evidence for this model has been reported in yeast (Sandall *et al.*, 2006).

CPC function at the centromere is mediated by the kinase activity of Aurora B. The regulation of Aurora B kinase involves its partners within the complex. The concept of auxiliary subunits for kinases is not unusual in the cell cycle field and is exemplified best by the cyclin-dependent kinases with their associated cyclin subunits. So far, Aurora B has appeared to be unusual in requiring association with three additional subunits. It is noteworthy, however, that these three subunits appear to act as one functional element (Figure 35-37).

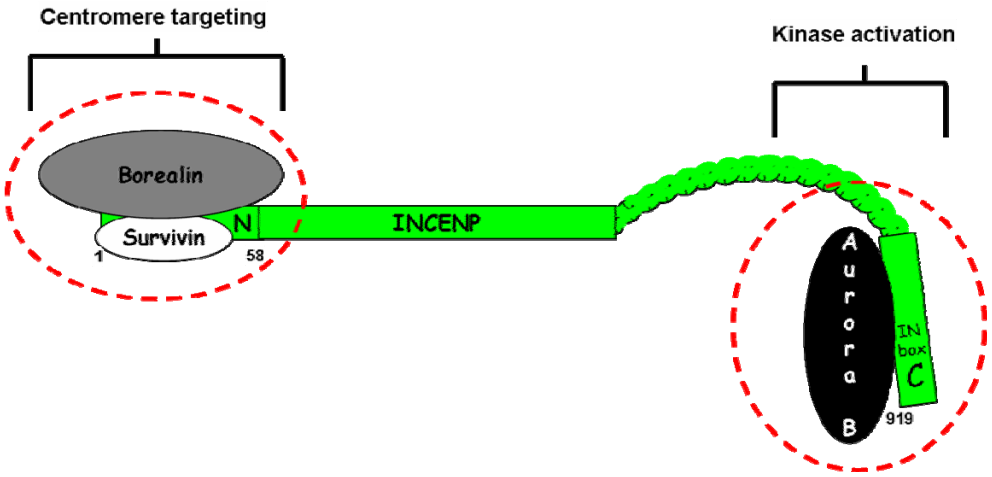


Figure 60. Model of CPC architecture.

Our data suggest that Survivin and Borealin directly bind to each other and to the first 58 amino acids of INCENP. Aurora B, in contrast, associates with the C-terminal IN-box of INCENP to become fully active, as documented previously. We propose that Borealin targets the CPC to the centromere by binding to DNA directly but can only do so when present within a functional subcomplex. Aurora B kinase itself is not involved.

Targeting the CPC to the centromere via Borealin-mediated DNA binding

The chromosomal passenger proteins bind to the inner centromere during prometaphase and metaphase, but the precise mechanism(s) by which the CPC proteins localize to this region have not been elucidated previously. Importantly, no kinetochore/centromere protein has yet been found whose knock-down results in the loss of the CPC from the centromere. Our present data raise the intriguing possibility that CPC targeting is ultimately determined by a DNA binding activity associated with Borealin but that *in vivo* this activity is only displayed in the context of a functional ternary complex of Borealin with INCENP$^{1\text{-}58}$ and Survivin (Figure 23). This assumption predicts that fragments and/or deletion mutants of INCENP$^{1\text{-}58}$, Borealin, or Survivin that do not interact with their binding partners of the ternary complex cannot target to the centromere. In agreement with this, it has been reported that Borealin fragments that do not bind INCENP as well as an N-terminal fragment of Survivin lacking the Borealin-binding domain were unable to localize to the centromere (Gassmann *et al.*, 2004; Lens *et al.*, 2006a). Furthermore, attempts of restoring the CPC by supplementing Aurora B depleted *Xenopus* egg extract with INCENP, Aurora B, and Survivin failed (Vigneron *et al.*, 2004). This result is likely due to the absence of Borealin in the corresponding experiment and indicates that localization and stability of the CPC requires the Borealin protein. In agreement with our data on Borealin DNA binding, a subsequent study (Gao *et al.*, 2008) showed DNA binding of Australin (the Drosophila Borealin homolog). In yeast no homolog of Borealin was identified. We suggest that the large

yeast Survivin protein (Bir1p ~100kDa) combines functions of the smaller Survivin (~16kDa) and Borealin (~34kDa) proteins present in higher eukaryotes. It has been proposed that in addition to the holo-CPC, Aurora B and INCENP can form a separate, independent complex (Gassmann *et al.*, 2004). Data obtained with the ternary INCENP$^{1-58}$-Survivin-Borealin complex suggested that such a complex should not be able to target to the centromere but rather be cytoplasmic and this has been confirmed through the analysis of our structure-based mutants (Figure 37). The result is consistent with the observation that GFP-Aurora B exhibits a dynamic behaviour at the centromere, exchanging rapidly with a cytoplasmic pool (Murata-Hori *et al.*, 2002). Besides the CPC composition other factors such as specific modification states of (peri)centromeric histones (Sullivan and Karpen, 2004) and chromatin structure may also contribute to the centromere localization of the complex. Recruitment of the CPC to the centromere might therefore include a larger number of regulatory steps than previously assumed. Cooperation of these mechanisms may be necessary to ensure that the CPC targets specifically to the centromeres rather than the entire chromosome. Interestingly, we have observed spreading of the CPC over the entire chromosome in cells depleted of PP2A phosphatase or Haspin kinase (data not shown).

Aurora B independent recruitment of the CPC to the centromere

How can the finding of Aurora B-independent targeting of a CPC subcomplex be reconciled with the fact that siRNA-mediated knock-down of Aurora B leads to loss of the other CPC components from the centromere? We suggest that the requirement for Aurora B is indirect and results from the instability of full-length INCENP in the absence of Aurora B. That GFP-INCENP$^{1-58}$ is stable in the absence of Aurora B suggests that the region that confers instability to INCENP must lie within the central coiled-coil domain or the C terminus of the protein, the region that interacts with the Aurora B kinase (Bishop and Schumacher, 2002; Honda *et al.*, 2003; Yasui *et al.*, 2004; Sessa *et al.*, 2005). Furthermore, the rescue results with GFP-INCENP$^{1-58}$ show that expression of the first 58 amino acids of INCENP rescues the protein levels of Survivin and Borealin in the transfected cells (Figure 18A). Similarly, we found Aurora B levels to be rescued by an INCENP construct that comprises the IN-box (Figure 18B). These findings argue that the interaction with INCENP stabilizes its binding partners of the CPC. Interestingly, in *C. elegans*, in contrast to mammalian cells, localization of CSC-1 (a remote Borealin homologue), BIR-1 (*C. elegans* Survivin), and ICP-1 (*C. elegans* INCENP) is not dependent on Aurora B (AIR-2), whereas, conversely, AIR-2 depends on all three for localization (Speliotes *et al.*, 2000; Romano *et al.*, 2003). This

difference might be explained by the different sizes of *C. elegans* and mammalian ICP-1/INCENP. *C. elegans* ICP-1 (~70 kDa) is much smaller than mammalian INCENP (~120 kDa) and its stability might therefore be independent of AIR-2/Aurora B.

So far, attempts to target the CPC for cancer therapy have focused on inhibiting Aurora B kinase activity (Gautschi *et al.*, 2008). In light of our results, alternative antineoplastic reagents could aim at interfering with binding of CPC components to each other. In particular, peptides competing with respective protein binding sites within the ternary subcomplex might allow to interfere with CPC targeting thus ultimately leading to cell death.

The CPC and the tension sensing arm of the SAC

The primary signal that activates the SAC has remained a matter of debate (Pinsky and Biggins, 2005), but experiments indicate that the checkpoint recognizes the lack of MT attachment to the KT and can sense the absence of tension generated on the KT by pulling MTs. The interdependence between tension and MT attachment makes it difficult to determine whether these signals are separable. Nevertheless, cells depleted of the CPC show a mitotic arrest only in response to lacking MT attachment (by nocodazole treatment) but not when tension between KTs is abolished (by taxol treatment). The CPC is therefore considered to represent the tension sensing arm of the SAC (Tanaka *et al.*, 2002; Musacchio and Salmon, 2007; Ruchaud *et al.*, 2007).

We have shown that PICH, a recently described checkpoint protein, is regulated by the CPC. In particular, PICH is absent from the centromere/kinetochore in CPC depleted cells but is not influenced by Aurora B kinase inhibition. This difference was unique among the tested candidates and to our knowledge the requirement for the CPC in localizing centromere/ kinetochore components has so far always been assigned to the enzymatic activity of Aurora B (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Pouwels *et al.*, 2007; Emanuele *et al.*, 2008). As PICH localization could not be rescued with the ternary INCENP$^{1-58}$-Survivin-Borealin subcomplex, the region of the CPC required for PICH centromere/kinetochore binding must be present within INCENP$^{59-919}$ or the Aurora B protein. The established rescue assay should allow for the identification of the corresponding region of the CPC. We also tested if PICH localization depends on the the sumoylation status of Borealin and found that the lysine-less Borealin mutant (Borealin^{25KR}) could rescue the centromere/kinetochore localization of PICH (data not shown).

Interestingly, the CPC is also involved in regulating PICH localization to the chromosome arms (Figure 31). The CPC, PICH and Plk1 have all been demonstrated to regulate chromosome arm cohesion. While Plk1 phosphorylates the SA2 subunit of the cohesin complex, thereby releasing it from the chromatin (Sumara *et al.*, 2002; Gimenez-Abian *et al.*, 2004; Hauf *et al.*, 2005), PICH is essential to localize Plk1 to the chromosome arms (Santamaria *et al.*, 2007) and cells depleted of PICH show closed chromosome arms (Leng *et al.*, 2008). The requirement for the CPC in the resolution of arm cohesion has been reported previously (Losada *et al.*, 2002; Gimenez-Abian *et al.*, 2004) but the molecular basis has remained elusive. We envision that the CPC is required for PICH to localize to chromosome arms. This in turn then recruits Plk1 to release cohesin via phosphorylation of SA2.

The SAC is regulated by PICH supposedly via the KT recruitment of Mad2. Furthermore, PICH has been hypothesized to sense tension due to its unique localization between sister KTs (Baumann *et al.*, 2007). As the CPC is required for centromere/kinetochore targeting of PICH we tested if the SAC override seen in CPC depleted cells treated with taxol but not nocodazole might be assigned to PICH centromere/kinetochore localization. However, we observed absence of the protein from the centromere/kinetochore in both conditions. Remarkably, the checkpoint protein Mad2 that similarly depends on the CPC was re-directed to the KT in Aurora B depleted cells that have been treated with nocodazole. The difference in Mad2 KT binding provides an explanation for the different response of CPC depleted cells to the two MT poisons. These results also indicated that Mad2 KT localization is independent of PICH centromere/kinetochore recruitment. We did not observe any influence of CPC depletion on thread formation of PICH. Similar to what was shown for the microtubule depolymerising kinesin MCAK (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004), we speculate that upon stretching of the inner centromere region (due to bipolar attachment and pulling MTs), negative regulation of PICH by the CPC is lost (and PICH association with forming threads can occur when anaphase commences).

More than one chromosomal passenger complex?

A recent study has claimed the existence of at least two chromosomal passenger complexes in mitotic HeLa cells (Gassmann *et al.*, 2004). Immunoprecipitation of endogenous Borealin pulled down essentially all of the Survivin, about half of the Aurora B, and about two thirds of the INCENP. Re-precipitation of the unbound fraction with an

antibody to Aurora B brought down the remainder of the kinase plus most of the soluble INCENP, but no further detectable Borealin or Survivin. The interpretation was that a holo-complex of INCENP, Aurora B, Borealin and Survivin and a subcomplex of Aurora B plus INCENP exist. We have found that IN-box mediated binding of INCENP to Aurora B stabilizes the kinase (Figure 18B). An engineered INCENP-Aurora B complex did not phophorylate CENP-A or localize to any defined structure during mitosis. Evaluation of other engineered subcomplexes did show identical results. Thus, to us it seems that during mitosis only the holo-CPC can fulfil functions required for mitotic progression but targeting of the complex to the centromere occurs independently of Aurora B. The above mentioned data on sequential immunoprecipitation of chromosomal passenger proteins could easily be explained by different binding affinities between the proteins. The fact that in the absence of Borealin and Survivin the Aurora B kinase and INCENP are competent in binding to each other has previously been shown (Honda *et al.*, 2003; Sessa *et al.*, 2005) (see also yeast two-hybrid data in Figure 12A).

Data on the oligomerization status of chromosomal passenger proteins have been controversial. Borealin can bind to itself in yeast-two hybrid assay (data not shown), *in vitro* and *in vivo* (Figure 13). Within the CPC, however, Borealin is monomeric (Figure 35). Recombinant Survivin forms dimers in solution (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000) and can form higher order structures *in vivo*. Via molecular mimicry its dimeric interface is occupied by Borealin within the CPC resulting in a 1:1:1 stochiometry of Borealin, Survivin and INCENP (Figure 35). Intriguingly, *in vitro* a homodimeric complex comprising two molecules of Survivin and two molecules of Borealin can be reconstituted (data by Dr. Arockia Jeyaprakash). At this point it is not clear if this molecule is a classical Survivin dimer with two associated Borealin molecules or if its architecture is different. Is a Survivin-Borealin homodimer relevant *in vivo* and/or is there a relevant role for a Survivin dimer? A possible answer might be found outside mitosis. In interphase cells Survivin is found in the cytoplasm while INCENP and Borealin are nuclear. In particular, Borealin resides in the nucleolus. This scenario would probably allow for Survivin dimerization and a number of studies have suggested that Survivin´s antiapoptotic function is indeed exerted from the cytoplasm. Therefore a possible explanation for some of the controversy surrounding Survivin function during mitosis versus its role in regulating apoptosis (Altieri, 2006; Lens *et al.*, 2006b) might lie within its dimerization status. In this context it is interesting to note that the sumoylation of Borealin does not seem to require its interaction with the CPC (Figure 47). Hence, SUMO modified Borealin might function

independently of the CPC (e.g. outside mitosis). However, the tight control of its modification at the metaphase to anaphase transition (Figure 48) rather points to a CPC dependent role. Determination of the oligomerization status of sumoylated Borealin might reveal further aspects of the modification. In light of a recent report on the activation of Aurora B by the chromosomal enrichment of CPC (Kelly *et al.*, 2007), it is possible that in the presence of appropriate interacting proteins at the centromeres and central spindle, the CPC components might assemble into a larger complex of oligomeric nature to regulate mitotic progression. In contrast to their closely related localization during mitosis, the nucleocytoplasmic localization of the chromosomal passenger proteins before NEB is largely unrelated (Rodriguez *et al.*, 2006) and indicates that the functional unit in interphase cells might not be the holo-CPC.

Central spindle and midbody binding of the CPC: spindle transfer?

One of the most striking observations regarding the CPC is its dynamic localization. However, only few factors are known that regulate the passage of the CPC from the centromere to the central spindle and midbody. In yeast, the dephosphorylation of Cdk1 sites on INCENP seems to be a pre-requisite for spindle transfer (Pereira and Schiebel, 2003) and this might be a regulatory mechanism in human cells, too (Murata-Hori *et al.*, 2002). Additionally, cells depleted of the kinesin motor protein Mklp2 do not show transfer of the CPC from the centromere to the central spindle (Gruneberg *et al.*, 2004). Although the term “spindle transfer” is widely used when referring to CPC localization a real transfer from one location to the other has not been proven yet. In contrast, our data on the Borealin fragment comprising residues 10-109 shows that spindle and midbody binding can occur independently of prior centromere targeting (Figure 34). Interestingly, this mutant showed proper cytokinesis (during the first round of division) arguing that the function of the CPC during cytokinesis is excecuted from the central spindle and midbody. In agreement with this, a study in Drosophila secondary spermatocytes mutants that lack chromosomes revealed Aurora B localization to the spindle and midbody during anaphase and cytokinesis, respectively (Bucciarelli *et al.*, 2003). Thus, centromere and central spindle/midbody bound CPC may represent two independent pools of the complex that are differently regulated.

Like centromere recruitment, spindle and midbody binding of the CPC is independent of Aurora B kinase activity (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). But Aurora B kinase activity is needed to build a functional mitotic spindle (Murata-Hori *et al.*, 2002; Guse *et al.*, 2005). The ternary INCENP$^{1\text{-}58}$-Survivin-Borealin subcomplex as well as all other engineered subcomplexes that missed one of the core components INCENP, Survivin or Borealin were

unable to target to the central spindle or midbody. In contrast, Aurora B was dispensable for centromere targeting. This raises the question if Aurora B kinase is only a passive passenger carried by the core CPC components? To rigorously rule out a role for Aurora B in localizing the complex to the spindle, an INCENP mutant defective in Aurora B binding should be monitored in anaphase cells. Based on the INCENP-Aurora B structure (Sessa *et al.*, 2005) the mutant INCENP$^{F825A, F837A}$ might make this experiment feasible.

The overall charge of the molecular surface of the CPC is important for central spindle and midbody binding. A charge-reversal of two conserved clusters on different proteins of the CPC (INCENP and Borealin) in different directions (negative cluster mutated to positive and vice versa) led to chromatin binding of the CPC in anaphase (Figure 38). How does the surface charge of the CPC influence its localization? First, corresponding mutations might have affected Cdk1 mediated phosphorylation of the complex required for its "transfer". Of note, T59, which we have identified as a Cdk1 site on INCENP (Figure 10), is in close proximity to the mutated INCENP residues. Second, it is tempting to speculate that the mutated CPC versions are no longer recognized as appropriate cargo-proteins for their putative kinesin Mklp2 at the central spindle. Strikingly, all scenarios mentioned above where defects in spindle binding of the CPC in anaphase are observed exhibit one common feature; the CPC does not mislocalize to the cytoplasm but is associated with the chromatin. Is this due to the inability to leave the chromatin (trap hypothesis) or does this localization reflect the default localization when spindle binding is perturbed (acceptor hypothesis)? One possible way to address this point is to introduce corresponding charge-reversal mutations in the context of the Borealin$^{10-109}$ mutant that does localize to the spindle but not to the centromere. A recruitment to the centromere would clearly be in favour of the trap hypothesis.

MAP kinase mediated regulation of the CPC

Inhibition of MAP kinase has been shown to override a SAC dependent arrest in *Xenopus* extract and cultured human cells (Takenaka *et al.*, 1997; Takenaka *et al.*, 1998). Additionally, it has been shown in HeLa cells that activated Erk kinase targets to the KT (Shapiro *et al.*, 1998). A collaboration with the laboratory of Marsha Rosner revealed that cells depleted of Raf-kinase inhibitory protein (RKIP) showed decreased Aurora B kinase activity (Eves *et al.*, 2006). Herein, we further show that within the CPC, Borealin is a target of Erk kinase mediated phophorylation *in vitro*. The identified Erk kinase site, residue T106 of Borealin, shows phosphorylation from prophase until metaphase consistent with Erk kinase being active at the centromere/kinetochore. Of note, Mps1 mediated phosphorylation of

Borealin was recently shown to influence Aurora B kinase activity (Jelluma *et al.*, 2008) and Mps1 is a substrate of MAP kinase (Zhao and Chen, 2006; Borysova *et al.*, 2008). Thus, the observed influence of the MAP kinase pathway on Aurora B might be indirectly mediated by Mps1. We could not link phosphorylation of T106 of Borealin to a change in Aurora B kinase activity and inhibitor treatment did not unequivocally show that T106 is a site of Erk kinase phosphorylation. However, the phospho-T106-Borealin antibody might proof useful as a mitotic marker once the corresponding kinase has been identified.

Sumoylation of Borealin

The data reported herein define a mitotic pathway of SUMO2/3 conjugation/ deconjugation on the CPC component Borealin (Figure 61). Importantly, this pathway is controlled by the E3 SUMO ligase RanBP2 and the SUMO-specific protease SENP3 (Figure 61). Modification of Borealin occurs at up to three lysine residues, but we were unable to assign distinct residues for attachment of SUMO. Notably, none of the 25 lysine residues of Borealin is embedded in a KxE/D motif, which serves as a preferential SUMO attachment site in the majority of SUMO substrates (Geiss-Friedlander and Melchior, 2007). Individual and combinatorial mutations of highly conserved lysine residues did not abolish the modification in the assays we used. A possible explanation for this phenomenon might be “jumping” of the SUMO moiety, a mechanism that has been described for ubiquitin. Interestingly, recent work in yeast described sumoylation of Bir1p, the yeast relative of Survivin (Montpetit *et al.*, 2006). We show that Survivin is not a major target of SUMO modification in human cells. Remarkably, however, yeast lacks a Borealin homolog and we have proposed above that the functions of human Survivin and Borealin are combined in the single yeast Bir1p protein. Therefore, these data support the idea that sumoylation of the CPC is an evolutionarily conserved mechanism. However, the functional impact of SUMO on CPC activity in both lower and higher eukaryotes remains to be elucidated. The defect in nucleolar targeting of the lysine-less Borealin mutant might be linked to other lysine-based modifications. We also show that Borealin is a target of ubiquitination (Figure 46). An exciting new aspect in the SUMO/ubiquitin field is the emerging interplay of both modification systems on a given target protein. Of note, recent work has shown that a balanced non-degradative 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, for both modification systems targeting

distinct components of a multiprotein complex. An interesting new concept that awaits to be confirmed for other complexes.

Interestingly, as demonstrated for CENP-E, dynamic association of centromeric proteins to these structures also involves non-covalent interactions between a SUMO-modified acceptor protein and a specific binding partner, which harbours 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, we have shown that the CPC constitutes one of the most upstream components of centromere/kinetochore assembly (Figure 24). Continuing our collaboration with the laboratory of Elena Conti we are currently trying to crystallize Borealin in complex with SUMO2/3 (Dr. Arockia Jeyaprakash).

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) and see (Figure 56).

The SUMO pathway components RanBP2 and SENP3 during mitosis

We have identified Borealin as a physiological target for RanBP2-mediated sumoylation. RanBP2 is found at the cytoplasmic site of the nuclear pore in interphase cells, but redistributes to the mitotic spindle and the KT upon entry of cells into mitosis (Joseph *et al.*, 2002; Joseph *et al.*, 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*. However, displacing RanBP2 from the KT by means of nocodazole treatment did not abolish Borealin sumoylation (Figure 52). Additionally, Borealin mutants defective in Survivin or INCENP

binding, respectively, show SUMO modification (Figure 47) even though they do not target to the centromere (Figure 36D). Moreover, Borealin as well as topoisomerase II bind to the inner centromere. RanBP2-mediated sumoylation of both proteins might therefore occur before centromere binding. In contrast to what was observed for topoisomerase II, sumoylation of Borealin does not seem to be required for centromere targeting of the CPC. Strikingly, a drastic defect in chromosome segregation is seen upon ectopic overexpression of the catalytically fragment of RanBP2 in anaphase cells while no defects were observed with the catalytically inactive fragment (Figure 51B-E). The misregulation should therefore be based on misregulated (upregulated) sumoylation of RanBP2 targets at this stage. So far, Borealin and topoisomerases II are the only substrates identified *in vivo* and remarkably, the depletion phenotype of both proteins is indeed characterized by chromosome missegregation (among other defects).

The dynamics of sumoylation are controlled by SUMO proteases 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, while Ulp2, which appears 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; Bachant *et al.*, 2002). In mammalian cells, depletion of SENP5, the closest homolog of SENP3 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 towards Borealin-SUMO2/3 conjugates *in vitro*. By contrast, we identified SENP3 as a specific interaction partner of Borealin and show 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 towards 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. Interestingly, SENP3 was recently identified in a siRNA based screen as a potential component of the SAC (Stegmeier *et al.*, 2007). However, 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 independently of CPC recruitment to the centromere. Moreover, a proteomic study identified SENP3 at the mitotic spindle (Sauer *et al.*, 2005), indicating that the protease might act to desumoylate spindle associated components. Notably, Borealin becomes desumoylated at the time the CPC transfers to the central spindle in anaphase. 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. It is also interesting to speculate that RanBP2 and SENP3 form a (mitotic?) couple that dynamically regulates the sumoylation of other target proteins than Borealin.

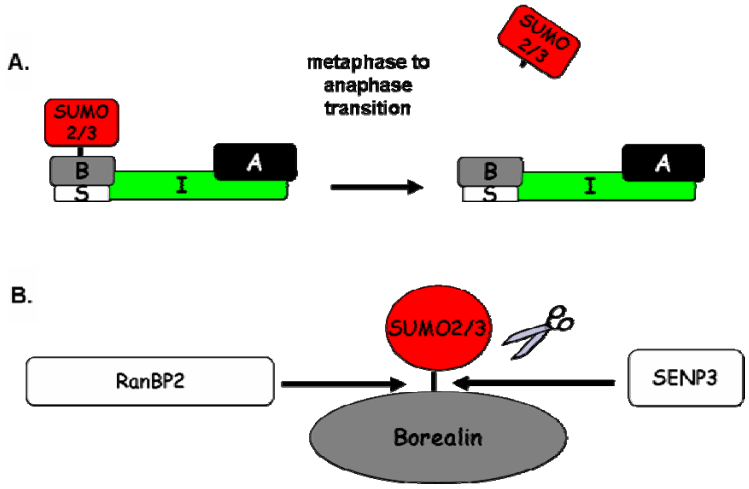


Figure 61. Model of Borealin sumoylation.

(A) Sumoylation of Borealin is prominent in early mitosis when the CPC is bound to the centromere but lost as cells enter anaphase and the complex associates with the central spindle.

(B) Sumoylation of Borealin is regulated by the SUMO E3 ligase RanBP2 and the SUMO protease SENP3. The modification may occur in the context of the CPC or be independent of complex formation (as depicted here).

MATERIALS AND METHODS

Cloning procedures

All cloning procedures were performed according to standard techniques as described in Molecular Cloning, A Laboratory Manual, 2nd edition, Sambrook, J., Fritsch, E.F., Maniatis, T., Cold Spring Harbor Laboratory Press, 1989 and Current Protocols in Molecular Biology, Wiley, 1999. Restriction enzyme reactions were carried out as specified by the suppliers (NEB, Ipswich, MA) and ligation reactions were done using Rapid Ligation Kit (Roche Diagnostics Mannheim, Germany). Extraction of DNA from agarose gels and preparation of plasmid DNA were performed using standard kits purchased from Qiagen according to the manufacturer's instructions. For PCR reactions, Pfu DNA polymerase was used as recommended by the manufacturer (Stratagene, La Jolla, CA) and reactions were carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA). Site-directed mutagenesis was carried out using the QuickChange Mutagenis Kit (Stratagene, La Jolla, CA). All PCR products obtained were analysed by sequencing (Medigenomix, Martinsried, Germany). See Appendix for table of plasmids that have been constructed during the course of this work.

Expression and purification of recombinant proteins

For gene fusions with the hexa-histidine tag (His-tag), Survivin and Borealin cDNAs were cloned into pQE vectors (QIAGEN, Hilden, Germany). For fusion to the maltose binding protein (MBP), Borealin and INCENP$^{1-58}$ cDNA were cloned into the pMAL vector (New England Biolabs, Beverly, MA). Glutathione *S*-transferase (GST) was expressed from the pGEX vector (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and GST-His-Survivin was a gift of Dr. Francis Barr. His-Plk1 purified from insect cells was a gift of Christoph Baumann. MBP-Borealin was expressed in *E. coli* BL21 cells by induction with 1mM IPTG for 4h at 30°C. All other recombinant proteins were expressed by induction with 0.1mM IPTG at 18°C overnight. His-, GST-, and MBP-fusion proteins were purified according to standard protocols. Purified proteins were dialyzed over night at 4°C in PBS and subsequently frozen at -80°C in 10% glycerol.

In vitro kinase assay

To obtain active Aurora B kinase, ten 15cm plates of Sf9 insect cells were co-infected with 1mL of the P3 baculoviruses of His-tagged Aurora B present in pHI100-His and GST-tagged INCENP present in pHI100-GST. After incubation for 60h, cells were harvested spun

down at 500rpm for 5min and washed once with cold PBS. The pellet was resuspended in 10mL lysis buffer (50mM Na-phosphate pH7.8, 150mM NaCl, 1%IGEPAL, 0.1mM ATP, 5mM ß-mercaptoetahnol, 1mM NaF, 20mM ß-gycerolphosphate, protease inhibitor cocktail tablets) pipetted twice through a needle (0.40 x 20mm) and incubated on ice for 30min. Lysates were then spun down using an ultracentrifuge (27000rpm for 35min in a SW40 rotor). The supernatant was added to a 25mL plastic-column (Qiagen) and 300µL pure GST beads were added. The column was incubated on a rotating wheel at 4°C for 2h. Subsequently, the column was allowed to empty by gravity flow and washed three times with lysis buffer. 50µL of elution buffer (lysis buffer + 20mM glutathione) was added to obtain eluate fractions.

For in *vitro kinase* assays, the active Aurora B-INCENP complex or Cdk1/cyclin B1 (Upstate, Charlottesville, VA) and respective substrates at ~100ng were incubated in BRB80 buffer (80mM Pipes, pH 6.8, 1mM MgCl2, 1mM EGTA). For Erk2 kinase (Upstate, Charlottesville, VA) reactions Erk2 kinase buffer was used (20mM Hepes pH7.4, 10mM $MgCl_2$, 1mM DTT, 0.2mM Na_3VO_4, 5mM para-nitrophenylphosphate). CaM kinase reactions were carried out using a CaM kinase kit (NEB) according to manufacturer´s instruction. Kinase reactions were carried out at 30°C for 60min in these buffers supplemented with 10µM ATP and 2µCi [γ-32P] ATP (Amersham Corp.). Reactions were stopped by the addition of SDS sample buffer and heating at 95°C for 5min. Protein samples were separated by SDS–PAGE followed by Coomassie Blue staining. The gels were dried on filter paper and ^{32}P incorporation was visualized by autoradiography.

Antibody production

In order to produce Borealin specific antibodies, the His-tagged protein was expressed in *E. coli* from pQE81L vectors (Qiagen) and purified under denaturing conditions over a Ni_2+-NTA column (Qiagen). Following further purification on a preparative 12% SDS-PAGE gel, 250µg of Borealin was injected several times into New Zealand white rabbits (Charles River Laboratories, Romans, France). The obtained sera were checked for Borealin reactivity by Western blotting and immunofluorescence.

Phosphospecific antibodies directed against a peptide of Borealin pT106 (H-AEAIQT(PO_3H_2)PLKS-OH) were raised in New Zealand white rabbits (Charles River Laboratories, Romans, France) and then isolated from a protein-A purified IgG fraction of the serum over the same peptide immobilized on Sulfolink resin according to the manufacturer's instructions (Pierce Biotechnology, PERBIO Science GmbH, Bonn, Germany).

Cell culture and synchronisation

HeLa S3, U2OS, 293T, and COS-7 cells were grown at 37°C under 5% CO2 in DMEM (Invitrogen), supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml and 100µg/ml, respectively). Sf9 insect cells were grown at 30°C in TC-100 medium supplemented with penicillin-streptomycin (100 IU/ml and 100µg/ml, respectively).

Cell cycle arrest of HeLaS3 cells in S-phase was induced by thymidine treatment (2mM) for 16hr. Mitotic cell cycle arrest in prometaphase was induced by nocodazole (0.5µg/mL) or taxol (10µM) treatment for 16hr. Mitotic cells were collected by mitotic shake off. Aurora B kinase activity was efficiently blocked by using ZM447439 inhibitor (Tocris) at 10µM. The proteasome inhibitor MG132 (Calbiochem) was used at 20µM.

Transient transfection and siRNA

Plasmid transfections were performed using FuGENE6® transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. SiRNA duplexes were transfected using Oligofectamine (Invitrogen, Carlsbad, CA) as described previously (Elbashir *et al.*, 2001). Sequences of siRNA duplexes used in this study (Dharmacon RNA Technologies, Lafayette, CO and Qiagen, Hilden, Germany) are listed in Appendix.

siRNA rescue assays

For rescue experiments, siRNA and plasmid transfection were performed in parallel. Cells were incubated for the indicated times and subsequently fixed for immunostaining. For rescue assays on chromosomal passenger proteins 30 mitotic, nontransfected cells on each coverslip were analyzed for the presence of chromosomal passenger proteins by staining with the appropriate antibodies. Slides were discarded if more than two of these 30 cells showed chromosomal passenger staining (siRNA efficiency higher than 94%). On the remaining coverslips, transfected mitotic cells were analyzed for staining of indicated proteins. Each rescue experiment was done in triplicate. To biochemically analyze the potential of GFP-INCENP constructs to rescue Aurora B levels in a background free of endogenous INCENP, HeLa S3 cells were treated with siRNA oligonucleotides and transfected with corresponding constructs as described above. INCENP wild-type and deletion constructs were cloned into a modified version of pcDNA4/TO (Invitrogen) encoding the enhanced green fluorescent protein (EGFP)-tag and puromycin resistance (a gift from Dr. Francis Barr). Puromycin was added at 2µg/ml to enrich for transfected cells 24hrs before lysate preparation.

In vitro binding assay

5µg of purified His-Borealin, GST-His-Survivin, or 10µg of purified GST was bound to Ni-NTA agarose (QIAGEN) or glutathione-Sepharose beads (GE Healthcare), respectively, in binding buffer (20mM Tris-Cl, pH 8.0, 150mM NaCl, 1mM DTT, and 0.1% (vol/vol) Triton X-100) for 2h at 4°C. After incubation, the beads were washed two times in binding buffer. Binding partners were added at 10µg/ml (MBP-INCENP1-58 and MBP-Borealin) or 20µg/ml (MBP), respectively, in binding buffer. Zn^{2+} was added to a final concentration of 20mM where indicated. Samples were incubated on a rotating wheel at 4°C for 2h. The beads were washed three times with binding buffer, boiled in 2xSDS sample buffer, and analyzed by SDS-PAGE. For DNA-binding assays, 5µg of purified protein (Histone H3, His-Survivin, His-Borealin, MBP-Borealin, MBP-$INCENP^{1-58}$, GST-Cdc20, and His-Plk1) or 10µg of purified protein (MBP) was added into binding buffer (50mM Tris-Cl, pH 8.0, 4mM $MgCl_2$, 1mM DTT, 150mM NaCl, and 0.1% (vol/vol) Triton X-100) and bound to calf-thymus double-stranded DNAcellulose (Sigma-Aldrich) for 2h at 4°C. Zn^{2+} was added at 20mM where indicated. DNA-cellulose was washed three times with binding buffer, boiled in 2xSDS sample buffer, and analyzed by SDS-PAGE.

Immunofluorescence microscopy

Cells were grown on coverslips and either fixed with 3% paraformaldehyde (immunostaining for kinetochore associated proteins) for 10min at RT followed by a 5min permeabilisation with 0.5 % Triton-X100 at 4°C or fixed in –20°C methanol (immunostaining for centrosomal and phospho-specific antibodies). Primary antibodies used in this study are listed in the Appendix and were detected with Alexa-Fluor-488-(green) and Alexa-Fluor-555-(red) conjugated antibodies. DNA was stained with 2µg/ml DAPI. Cover slips were mounted in phenylenediamine in 90% glycerol.

Immunofluorescence microscopy was performed using a Zeiss Axioplan II microscope (Carl Zeiss, Jena, Germany) equipped with an Apochromat 63x oil immersion objective, and images were acquired using a Micromax charge coupled device (CCD) camera (model CCD-1300-Y; Princeton Instruments, Trenton, NJ) and MetaView software (Visitron Systems, Puchheim, Germany). Alternatively, a Deltavision microscope on a Nikon Eclipse TE200 base (Applied Precision, Issaquah, WA) equipped with an Apo 60x/1.4 oil immersion objective and a CoolSnap HQ camera (Photometrics) was used for collecting 0.2µm distanced optical sections in the *z*-axis. Images at single focal planes were processed with a deconvolution algorithm (Nikon_60x_140_12601.otf). Settings were conservative, with noise

filtering set to medium and 3 deconvolution cycles. Images were projected into one picture using the Softworx software (Applied Precision). Exposure times and settings for image processing (deconvolution) were constant for all samples to be compared within any given experiment. Images were cropped in Adobe Photoshop CS and then sized and placed in figures using Adobe Illustrator CS (Adobe Systems, Mountain View, CA).

Coimmunoprecipitation

For coimmunoprecipitation of endogenous or overexpressed chromosomal passenger proteins HeLa S3 cells in 15cm dishes were synchronized by thymidine release (unless otherwise stated) followed by mitotic shake-off to enrich for mitotic cells. Cell pellets were washed in PBS twice and subsequently lysed in 500µl of lysis buffer (50mM Tris, pH 7.4, 400mM NaCl, 40mM ß-glycerol phosphate, 10mM NaF, 0.5% (vol/vol) IGEPAL, 0.1% deoxycholate, 30µg/ml RNase, 80U/ml micrococcal nuclease (Sigma-Aldrich), 2mM Prefabloc, protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany), 100µM ATP, 100µM $MgCl_2$, 100nM okadaic acid, and 0.3mM Na-vanadate) for 30min at 4°C on ice. Antibodies and corresponding IgG´s as control were either coupled to Affi-Prep Protein A Support beads (Bio-Rad Laboratories) if antibodies were raised in rabbits, or to Protein G beads (Pierce Biotechnology, Rockford, IL) if antibodies were of mouse origin.

GFP-tagged proteins were precipitated from the cleared lysate with sheep anti-GFP antibodies, Flag-tagged proteins with anti-Flag (M2) antibodies, Myc-tagged proteins with anti-Myc (9E10) antibodies and HA-tagged proteins with anti-HA (12CA5) antibodies. Respective beads, antibodies and the cleared lysate were incubated on a rotating wheel for 2h at 4°C. Immune complexes were spun down, washed 3 times in lysis buffer and subsequently boiled in SDS sample buffer. 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 above using anti-Myc antibodies (9E10).

***In vitro* sumoylation**

Proteins were generated by *in vitro* transcription/translation in the presence of ^{35}S-labeled methionine using the TNT Quick Coupled T7 kit (Promega). Sumoylation was carried out using the Sumoylation control kit (LAE biotech). In experiments using recombinant RanBP2$^{\Delta FG}$ (Biomol) the amount of Aos1/Uba2 and Ubc9 was reduced to 100nM and 70nM, respectively. For *in vitro* demodification assays Flag-tagged proteases, generated by *in vitro*

transcription/translation, were added to an *in vitro* modification reaction after incubation and samples were incubated for an additional 90min at 30°C.

In vivo sumoylation

Following transfection of respective constructs, HeLa S3 or COS-7 cells were incubated for 48h and Ni-NTA precipitations were done as described (Muller et al., 2000). In experiments, where protein knock-down was performed siRNA duplexes were simultaneously transfected. Depletion of proteins was verified by Western blotting using input fractions.
To demonstrate endogenous sumoylation HeLa S3 cells were arrested by a 16h taxol treatment before harvesting. Lysates were prepared in buffer A (50mM Tris/HCl pH 7.5, 150mM NaCl, 1% NP-40, 0,5% sodium deoxycholate, 0.1% SDS, 1mM DTT, 10mM $CaCl_2$, 200u/mL micrococcal nuclease (Fermentas), 10mM NEM (Pierce) and protease inhibitor cocktail tablets) and subjected to immunoprecipitation with 10µg SUMO2/3 antibody or control mouse IgG.

Cell cycle dependent sumoylation

HeLa S3 cells were transfected with His-tagged Borealin and incubated for 30h. Thymidine or taxol were added at a concentration of 2mM and 1µM, respectively and cells were incubated for an additional 16h. Taxol arrested cells were released into 10µM MG132 for one hour to obtain metaphase cells. To harvest anaphase/telophase cells, corresponding cell populations were released from this block for 25 and 50min, respectively. Cell lysates and Ni-NTA pull-downs were performed as described above.

Yeast-two hybrid analysis

Yeast two-hybrid screens for Borealin and $INCENP^{1-530}$ were performed according to the yeast protocol handbook (Clontech). For the directed interaction screening, yeast colonies were selected by pACT2 (Leu^-) and pFBT9 (Trp^-) selection markers for plasmid uptake on plates lacking these amino acids (–LW). Specific interaction between the GAL4 activation domain (AD) and the GAL4 binding domain (BD) and subsequent expression of markers (His^-/Ade^-) was monitored by streaking yeasts on QDO selective plates (Quadruple Drop Out: Leu^-/Trp^-/His^-/Ade^-). Construct were tested in the same assay for self activation first by cotransfection with an empty vector.

In vitro coupled transcription translation

The respective proteins were produced by *in vitro* coupled transcription translation (IVT) in the presence of ^{35}S-methionine using the TNT T7 Quick Coupled Transcription/ Translation System (Promega, Madison, WI, USA) by incubation at 37°C for 90min.

APPENDIX

I. List of plasmids

Name	Gene	Insert	Tag	Vector	Expression
UK 1	INCENP	wt	GFP	peGFPC2	mammalian expression
UK 2	INCENP	wt	myc	pcDNA3.1 3x myc A	mammalian expression
UK 3	INCENP	wt	flag	pcDNA3.1 flag A	mammalian expression
UK 4	INCENP	wt	GAL-BD	pFBT9	Y2H
UK 5	INCENP	wt	GAL-AD	pACT2	Y2H
UK 6	INCENP	wt		pCR II topo	cloning vector
UK 7	INCENP	INCENP (1-58)	GFP	peGFP C2	mammalian expression
UK 8	INCENP	INCENP (1-68)	GFP	peGFP C2	mammalian expression
UK 9	INCENP	INCENP (1-77)	GFP	peGFP C2	mammalian expression
UK 10	INCENP	INCENP (1-68 T59D)	GFP	peGFP C2	mammalian expression
UK 11	INCENP	INCENP (1-68 T59A)	GFP	peGFP C2	mammalian expression
UK 12	INCENP	INCENP (1-77 S72A)	GFP	peGFP C2	mammalian expression
UK 13	INCENP	INCENP (1-77 S72D)	GFP	peGFP C2	mammalian expression
UK 14	INCENP	INCENP (1-530)	GFP	peGFP C2	mammalian expression
UK 15	INCENP	INCENP (531-789)	GFP	peGFP C2	mammalian expression
UK 16	INCENP	INCENP (790-919)	GFP	peGFP C2	mammalian expression
UK 17	INCENP	INCENP (1-790)	GFP	peGFP C2	mammalian expression
UK 18	INCENP	INCENP (59-919)	GFP	peGFP C2	mammalian expression
UK 19	INCENP	INCENP (69-919)	GFP	peGFP C2	mammalian expression
UK 20	INCENP	INCENP (T59A T412A)	GFP	peGFP C2	mammalian expression
UK 21	INCENP	INCENP (T59D T412D)	GFP	peGFP C2	mammalian expression
UK 22	INCENP	INCENP (T219A,S275A)	GFP	peGFP C2	mammalian expression
UK 23	INCENP	INCENP (S832A,T833A)	GFP	peGFP C2	mammalian expression
UK 24	INCENP	INCENP (T219A,S275A,Y822A, S828A,S832A,T833A)	GFP	peGFP C2	mammalian expression
UK 25	INCENP	INCENP (1-58)		pCR II topo	cloning vector
UK 26	INCENP	INCENP (1-68)		pCR II topo	cloning vector
UK 27	INCENP	INCENP (1-77)		pCR II topo	cloning vector
UK 28	INCENP	INCENP (1-68 T59D)		pCR II topo	cloning vector
UK 29	INCENP	INCENP (1-68 T59A)		pCR II topo	cloning vector
UK 30	INCENP	INCENP (1-77 S72A)		pCR II topo	cloning vector
UK 31	INCENP	INCENP (1-77 S72D)		pCR II topo	cloning vector

UK 32	INCENP	INCENP (1-530)		pCR II topo	cloning vector
UK 33	INCENP	INCENP (531-789)		pCR II topo	cloning vector
UK 34	INCENP	INCENP (790-919)		pCR II topo	cloning vector
UK 35	INCENP	INCENP (1-790)		pCR II topo	cloning vector
UK 36	INCENP	INCENP (59-919)		pCR II topo	cloning vector
UK 37	INCENP	INCENP (69-919)		pCR II topo	cloning vector
UK 38	INCENP	INCENP (T59A T412A)		pCR II topo	cloning vector
UK 39	INCENP	INCENP (T59D T412D)		pCR II topo	cloning vector
UK 40	INCENP	INCENP (T219A,S275A)		pCR II topo	cloning vector
UK 41	INCENP	INCENP (S832A)		pCR II topo	cloning vector
UK 42	INCENP	INCENP (T833A)		pCR II topo	cloning vector
UK 43	INCENP	INCENP (S832A,T833A)		pCR II topo	cloning vector
UK 44	INCENP	INCENP (T219A,S275A,Y822A, S828A,S832A,T833A)		pCR II topo	cloning vector
UK 45	INCENP	INCENP (1-530)	myc	pcDNA3.1 3x myc A	mammalian expression
UK 46	INCENP	INCENP (531-789)	myc	pcDNA3.1 3x myc A	mammalian expression
UK 47	INCENP	INCENP (790-919)	myc	pcDNA3.1 3x myc A	mammalian expression
UK 48	INCENP	INCENP (1-77)	myc	pcDNA3.1 3x myc A	mammalian expression
UK 49	INCENP	INCENP (1-68)	myc	pcDNA3.1 3x myc A	mammalian expression
UK 50	INCENP	INCENP (1-530)	BD	pFBT9	Y2H
UK 51	INCENP	INCENP (531-789)	BD	pFBT9	Y2H
UK 52	INCENP	INCENP (790-919)	BD	pFBT9	Y2H
UK 53	INCENP	INCENP (1-530 T59A)	BD	pFBT9	Y2H
UK 54	INCENP	INCENP (1-530 T412A)	BD	pFBT9	Y2H
UK 55	INCENP	INCENP (1-530 T59A,T412A)	BD	pGBT9	Y2H
UK 56	INCENP	INCENP (1-530)	AD	pACT2	Y2H
UK 57	INCENP	INCENP (531-789)	AD	pACT2	Y2H
UK 58	INCENP	INCENP (790-919)	AD	pACT2	Y2H
UK 59	INCENP	wt	MBP	pMAL	bacterial expression
UK 60	INCENP	INCENP (1-58)	MBP	pMAL	bacterial expression
UK 61	INCENP	INCENP (1-68)	MBP	pMAL	bacterial expression
UK 62	INCENP	INCENP (1-77)	MBP	pMAL	bacterial expression
UK 63	INCENP	INCENP (1-68 T59A)	MBP	pMAL	bacterial expression
UK 64	INCENP	INCENP (1-77 S72A)	MBP	pMAL	bacterial expression
UK 65	INCENP	INCENP (790-919)	MBP-HIS	pMALHis	bacterial expression
UK 66	Aurora B	wt		pCRII topo	cloning vector

UK 67	Aurora B	Aurora B (Y12D)		pCRII topo	cloning vector
UK 68	Aurora B	Aurora B (Y12F)		pCRII topo	cloning vector
UK 69	Aurora B	wt	flag	pcDNA3.1 flag A	mammalian expression
UK 70	Aurora B	wt	myc	pcDNA3.1 3x myc A	mammalian expression
UK 71	Aurora B	Aurora B (Y12D)	GFP	peGFPC2	mammalian expression
UK 72	Aurora B	Aurora B (Y12F)	GFP	peGFPC2	mammalian expression
UK 73	Aurora B	wt	GFP	peGFPC2	mammalian expression
UK 74	Aurora B	wt	MBP-HIS	pMALHis	bacterial expression
UK 75	Aurora B	wt		pAct2	Y2H
UK 76	Aurora B	wt		pFBT9	Y2H
UK 77	Survivin	wt	flag	pcDNA3.1 flag A	mammalian expression
UK 78	Survivin	wt	myc	pcDNA3.1 3x myc A	mammalian expression
UK 79	Survivin	wt	HIS	pQE 32L	bacterial expression
UK 80	Survivin	wt		pACT2	Y2H
UK 81	Survivin	wt		pFBT9	Y2H
UK 82	Aurora C	wt	flag	pcDNA3.1 flag	mammalian expression
UK 83	Aurora C	wt	GFP	peGFP	mammalian expression
UK 84	Borealin	wt	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 85	Borealin	Borealin (S165A)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 86	Borealin	Borealin (S219A)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 87	Borealin	Borealin (S165A, S219A)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 88	Borealin	Borealin (S165, S219, T106, T204 AAAA)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 89	Borealin	Borealin (T106A)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 90	Borealin	Borealin (T204A)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 91	Borealin	Borealin (T106, T204 AA)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 92	Borealin	Borealin (T106E)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 93	Borealin	Borealin (T204E)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 94	Borealin	Borealin (T106E, T204E)	myc	pcDNA3.1 3xmyc C	mammalian expression
UK 95	Borealin	wt		pFBT9	Y2H
UK 96	Borealin	wt		pGAD	Y2H
UK 97	Borealin	wt	HIS	pAcHIS-Tev	insect expression
UK 98	Borealin	wt	HIS	pQE81L	bacterial expression
UK 99	Borealin	wt	GST	pGEX	bacterial expression

UK 100	Borealin	wt	MBP	pMAL	bacterial expression
UK 101	Borealin	Borealin (S165A)	HIS	pQE81L	bacterial expression
UK 102	Borealin	Borealin (S219A)	HIS	pQE81L	bacterial expression
UK 103	Borealin	Borealin (S165A, S219A)	HIS	pQE81L	bacterial expression
UK 104	Borealin	Borealin (S165, S219, T106, T204 AAAA)	HIS	pQE81L	bacterial expression
UK 105	Borealin	Borealin (T106A)	HIS	pQE81L	bacterial expression
UK 106	Borealin	Borealin (T204A)	HIS	pQE81L	bacterial expression
UK 107	Borealin	Borealin (T106, T204 AA)	HIS	pQE81L	bacterial expression
UK 108	Borealin	wt	flag	pcDNA3.1 flag C	mammalian expression
UK 109	INCENP	INCENP1-58	GFP	pcDNA4/TO eGFP puro	mammalian expression
UK 110	INCENP	INCENP full-lenght	GFP	pcDNA4/TO eGFP puro	mammalian expression
UK 111	INCENP	INCENP 59-919	GFP	pcDNA4/TO eGFP puro	mammalian expression
UK 112	Borealin	Borealin 1-55	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 113	Borealin	Borealin 30-105	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 114	Borealin	Borealin 55-140	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 115	Borealin	Borealin 105-155	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 116	Borealin	Borealin 140-170	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 117	Borealin	Borealin 155-230	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 118	Borealin	Borealin 170-280	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 119	Borealin	Borealin 1-55	GAL-BD	pFBT9	Y2H
UK 120	Borealin	Borealin 30-105	GAL-BD	pFBT9	Y2H
UK 121	Borealin	Borealin 55-140	GAL-BD	pFBT9	Y2H
UK 122	Borealin	Borealin 105-155	GAL-BD	pFBT9	Y2H
UK 123	Borealin	Borealin 140-170	GAL-BD	pFBT9	Y2H
UK 124	Borealin	Borealin 155-230	GAL-BD	pFBT9	Y2H
UK 125	Borealin	Borealin 170-280	GAL-BD	pFBT9	Y2H
UK 126	Sumo1	full-lenght	GAL-BD	pFBT9	Y2H
UK 127	Sumo2	full-lenght	GAL-BD	pFBT9	Y2H
UK 128	Sumo1	full-lenght	GAL_AD	pAct2	Y2H

UK 129	Sumo2	full-lenght	GAL_AD	pGAD C1	Y2H
UK 130	PIAS-y	full-lenght	GAL-BD	pFBT9	Y2H
UK 131	PIAS3	full-lenght	GAL-AD	pGAD C1	Y2H
UK 132	Borealin	Borealin 1-105	GAL_BD	pFBT9	Y2H
UK 133	Borealin	Borealin 105-170	GAL_BD	pFBT9	Y2H
UK 134	Borealin	Borealin 1-140	GAL_BD	pFBT9	Y2H
UK 135	Borealin	Borealin 140-280	GAL_BD	pFBT9	Y2H
UK 136	Borealin	Borealin 1-105	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 137	Borealin	Borealin 105-170	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 138	Borealin	Borealin 1-140	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 139	Borealin	Borealin 140-280	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 140	PIASx-alpha	full-lenght	GAL-BD	pFBT9	Y2H
UK 141	Borealin	Borealin 10-109	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 142	Borealin	Borealin 110-280	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 143	Borealin	Borealin 10-57	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 144	INCENP	INCENP F22R, L34R	GFP	peGFPC2	mammalian expression
UK 145	INCENP	INCENP E35,36,39,40R	GFP	peGFPC2	mammalian expression
UK 146	Borealin	Borealin R35E, L46Y	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 147	Borealin	Borealin W70E, F74E	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 148	Borealin	Borealin R17,19E, K20E	myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 149	Borealin	full-lenght	myc-HIS	pcDNA4/TO/myc-HIS	mammalian expression
UK 150	Borealin	(K20,48,109,112,162,183,198,225,263,264R)	myc-HIS	pcDNA4/TO/myc-HIS	mammalian expression)
UK 151	Borealin	full-lenght	Cherry	pcDNA3.1 Cherry-C	mammalian expression
UK 152	Borealin	full-lenght 25KR (all lysines >arginine)	Cherry	pcDNA3.1 Cherry-C	mammalian expression
UK 153	Borealin	full-lenght	His	pcDNA3.1-His-C	mammalian expression
UK 154	Borealin	full-lenght (lysine in spacer eliminated)	His	pcDNA3.1-His-C	mammalian expression
UK 155	Borealin	full-lenght 25 KR (all lysines >arginine)	His	pcDNA3.1 His-C	mammalian expression
UK 157	Borealin	full-lenght	HA	pcDNA3.1 HA-C	mammalian expression
UK 158	Borealin	full-lenght 25 KR (all lysines >arginine)	HA	pcDNA3.1 HA-C	mammalian expression
UK 159	RanBP2	RanBP2deltaFG	GAL-AD	pGAD C1	Y2H

UK 160	RanBP2	RanBP2deltaFG	GAL-BD	pFBT9	Y2H
UK 161	RanBP2	RanBP2deltaFG	GFP	peGFPC2	mammalian expression
UK 162	RanBP2	RanBP2deltaFG	myc	pcDNA3.1-3xmyc-C	mammalian expression
UK 163	RanBP2	RanBP2deltaFG	Myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 164	AuroraB	Aurora B K106R	GFP	peGFPC1	mammalian expression;((kinase dead)
UK 165	Pc2	Pc2 full-length	AD	pGAD	Y2H
UK 166	Pc2	Pc2 full-length	Myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 167	SUMO2	SUMO2	flag	pCI	mammalian expression
UK 168	SENP3	SENP3 full-length	flag	pCI	mammalian expression
UK 169	SENP3	SENP3 full-length C532S	flag	pCI	mammalian expression;(protease dead)
UK 170	SENP5	SENP5 full-length	flag	pCI	mammalian expression
UK 171	Borealin-SUMO2 fusion	Borealin-SUMO2 fusion	flag	pCI	mammalian expression
UK 172	SUMO1-Borealin fusion	SUMO1-Borealin fusion	Myc	pcDNA3.1 3xmyc-C	mammalian expression
UK 173	AuroraB	Aurora B delta 1-66	flag	pcDNA3.1 flag-A	mammalian expression
UK 174	AuroraB	Aurora B delta 1-66	GFP	peGFPC2	mammalian expression
UK 175	SUMO1	SUMO1-GA	BD	pGBD	Y2H
UK 176	SUMO2	SUMO2-GA	BD	pGBD	Y2H
UK 177	Centrin-1	Centrin-1 full-length	BD	pFBT9	Y2H
UK 178	Centrin-2	Centrin-2 full-length	BD	pFBT9	Y2H
UK 179	Centrin-3	Centrin-3 full-length	BD	pFBT9	Y2H
UK 180	Centrin-1	Centrin-1 full-length	AD	pAct2	Y2H
UK 181	Centrin-2	Centrin-2 full-length	AD	pAct2	Y2H
UK 182	Centrin-3	Centrin-3 full-length	AD	pAct2	Y2H
UK 183	Centrin-2	Centrin-2 full-length	Cflag	pcDNA3.1-Cflag	mammalian expression
UK 184	Centrin-2	Centrin-2 R16,K17,R18	GFP	peGFPC2	mammalian expression
UK 185	Centrin-2	Centrin-2 full-length	GFP	peGFPC2	mammalian expression
UK 186	Centrin-2	Centrin-2 K22,30,65,66,103	GFP	peGFPC2	mammalian expression
UK 187	Centrin-1	Centrin-1 full-length	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 188	Centrin-2	Centrin-2 full-length	Myc	pcDNA3.1 3xmyc-A	mammalian expression

UK 189	Centrin-3	Centrin-3 full-length	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 190	Centrin-2	Centrin-2 K22R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 191	Centrin-2	Centrin-2 K65,66R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 192	Centrin-2	Centrin-2 K103R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 193	Centrin-2	Centrin-2 K22,30,65,66,103	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 194	Centrin-2	Centrin-2 K65,66,103R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 195	Centrin-2	Centrin-2 K22,30,65,66R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 196	Centrin-2	Centrin-2 K30,65,66R	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 197	Centrin-2	Centrin-2 R16,K17,R18	Myc	pcDNA3.1 3xmyc-A	mammalian expression
UK 198	Centrin-2	Centrin-2 full-length	Myc	pcDNA3.1 His-A	mammalian expression
UK 199	XPC	XPC full-length	flag	pReceiver-M11	mammalian expression
UK 200	Centrin-2	Centrin-2 full-length	flag	pcDNA3.1flag	mammalian expression
UK 201	Centrin-2	Centrin-2 R16,K17,R18	flag	pcDNA3.1flag	mammalian expression
UK 202	Centrin-2	Centrin2		pcDNA3	mammalian expression
UK 203	Centrin-2	Centrin-2 (5KR)		pcDNA3	mammalian expression
UK 204	Centrin-2	Centrin-2 (5KR)	GFP	peGFPN3	mammalian expression
UK 205	Centrin-2	Centrin-2	GFP	peGFPN3	mammalian expression

II. List of siRNA oligonucleotide sequences

mRNA target	Sequence
INCENP	5´-GGCTTGGCCAGGTGTATATdTdT-3´ (Klein et al., 2006)
Aurora B	5´-GGAAAGAAGGGATCCCTAAdTdT-3´ (Klein et al., 2006)
Aurora-C	5´-GCTGAATCATTTCATACCAdTdT-3´ (Klein et al., 2006)
Survivin	Qiagen, cat. no. SI02652958 (Klein et al., 2006)
Borealin	5´-AGGTAGAGCTGTCTGTTCAdTdT-3´ (Klein et al., 2006)
hMis12	5´-GGACATTTTGATAACCTTTdTdT-3´ (Goshima et al., 2003)
Cenp-A	5´-CTCGTGGTGTGGACTTCAAdTdT-3´
Haspin	5´-GGCATCTGATGCTGAAAAGdTdT-3´ (Dai et al., 2006)
Plk1	5´-CGAGCTGCTTAATGACGAGdTdT-3´ (Baumann et al., 2007)
Eg5	5´-CTAGATGGCTTTCTCAGTAdTdT-3´ (Baumann et al., 2007)
Cenp-E	5´-ACTGGAGAGCAGTAAGAGTdTdT-3´
RKIP	5´-GGUGGCGUCCUUCCGUAAAdTdT-3´ (Eves et al., 2006)
MCAK	5´-GGTATCTGCTGGCTCTAAAdTdT-3´
TD-60	5´-AAGAGATGAAAGTGAGACTdTdT-3´ (Mollinari et al., 2003)
XPC	5´-TCAGCAGATGGTCCAGCAAdTdT-3´
Centrin-2	5´-GCACATGTAACTAGATTTAdTdT-3´
Ubc9_1	5´-GGGATTGGTTTGGCAAGAAdTdT-3´
Ubc9_SM	5´-GCAGAGGCCTACACGATTTdTdT-3´ (Klein et al., 2009)
PIAS2	5´-AAGATACTAAGCCCACATTdTdT-3´ (Yang et al., 2004)
SUMO1	Santa Cruz cat. no. sc-29498A
SUMO1	5´-GGTGAATATATTAAACTCAdTdT-3´
SUMO2/3	5´-GTCAATGAGGCAGATCAGAdTdT-3´
RanBP2	5´-GGACAGTGGGATTGTAGTGdTdT-3´ (Joseph et al., 2004)
hPc2_1	5´-CGTGGGAACCGGAGGAGAAdTdT-3´
hPc2_2	5´-GTTTGTACGTGGTGTTATTdTdT-3´
Senp3(Q3)	5´-GGCAGAGGCGGTAAAGAAAdTdT-3´ Qiagen
Senp3 (9)	5´-CTGGCCCTGTCTCAGCCATdTdT-3´ (Stegmeier et al., 2007)
Senp5_2	5´-GAACATCGTTCTAATACCAdTdT-3´ (Klein et al., 2009)
Senp5_3	5´-AGAAAGCTCTTCAAATCCAdTdT-3´
PDCD7	5´-GCAGCCGCTGATGGCGTACdTdT-3´ (Will et al., 2002)
GL2	(Elbashir et al., 2001)
Lamin-A	(Elbashir et al., 2001)
Kif3A	Qiagen, cat. no. SI02655415
Kif3A_2	Qiagen, cat. no. SI03019765
Kif3B	Qiagen, cat. no. SI02655303
Kif3B_2	Qiagen, cat. no. SI03019772

III. List of antibodies

Antigen	Species	Dilution IF	Dilution_WB	Company
Aurora B	M	1:500	1:250	BD Bioscience
Aurora C	R	-	1:200	Zymed
BubRI	M	1:500	1:500	Chemicon
pCenp-A (Ser7)	R	1:500	-	upstate
Cenp-A	M	1:1000	1:1000	MoBiTec
Survivin	R	1:1000	1:1000	abcam
Survivin	M	1:1000	1:500	Santa Cruz
Flag (M2)	M	1:2000	1:1000	SIGMA
MBP	M	-	1:250	NEB
pHistoneH3 (Ser10)	R	1:3000	1:2000	upstate
PIAS2	M	-	1:250	SIGMA
Ubc9	M	-	1:1000	BD Transduction Laboratories
SUMO1 (21C7)	M	1:2000	1:500	Zymed
His	M	-	1:500	Amersham Bioscience
Ubc9	G	-	1:1000	Santa Cruz
Hec1	M	1:1000	1:1000	GeneTex
Senp5	R	-	1:500	Gift from Ed Yeh
Senp3	R	-	1:500	Gift from Stephan Muller
Borealin	R	1:2000	1:2000	Homemade
pBorealin (Thr106)	R	1:500		Homemade
SUMO2/3	M	1:1000	1:500	ABGENT
RanBP2	G	1:500	1:500	Gift from Frauke Melchior
RanGAP1	G	1:500	1:500	Gift from Frauke Melchior
RanGAP1	M	1:500	1:500	Zymed
GFP	M		undiluted	Homemade
Myc (9E10)	M	undiluted	undiluted	Homemade
Topoisomerase IIα	M	1:2000	1:2000	Stressgen
HA (12CA5)	M	undiluted	undiluted	Homemade
HA (16B12)	M	1:2000	1:1000	COVANCE
PRC1	R	1:1000	-	Santa Cruz
pVimentin (Ser72)	R	1:500	-	EPITOMICS
Centrin-2	R	1:1000	1:1000	Homemade
γ-tubulin	M	1:1000	1:1000	SIGMA
α-tubulin (FITC labeled)	M	1:1000	-	Santa Cruz
XPC	M	1:500	1:500	GeneTex
XPC	R	1:1000	1:1000	Santa Cruz
CPDs	M	1:3000		MBL
6-4PPs	M	1:400		MBL
Plk1	M	undiluted	undiluted	Homemade
MCAK	R	1:1000	1:1000	Gift from Thomas Mayer
BubI	M	1:1000	1:1000	Homemade
Astrin	R	1:2000	1:1000	Homemade
PICH	Rat	1:1000	-	Homemade
Cenp-B	M	undiluted	-	ATCC
pBubRI (S676)	R	1:2000		Homemade
Mad2	R	1:500	1:500	Bethyl laboratories
Eg5	R		1:500	Homemade
Cenp-E	G	1:500		Santa Cruz
CyclinB1	M		1:1000	upstate
CREST	H	1:5000		Immunovision

IV. Abbreviations

All units are abbreviated according to the International Unit System.

aa: amino acid
AD: activation domain
BD: binding domain
Cdk: cyclin-dependent kinase
BSA: bovine serum albumin
CPC: chromosomal passenger complex
C-terminus; carboxy-terminus
DAPI: 4´,6-diamidino-2-phenylindole
DTT: dithiothreitol
E. coli: Escherichia coli
ECL: enhanced chemiluminescence
EDTA: ethylene-dinitrilo-tetraacetic acid
EGTA: ethylene-gycol-tetraacetic acid
FCS: Fetal calf serum
GFP: green fluorescent protein
GST: glutathione S-transferase IF: immunofluorescence
h:hour
IgG: Immunoglobulin G
INCENP: inner centromere protein
IP: immunoprecipitation
IPTG: isopropyl-beta-D-thiogalactopyranoside
kDa: kilo Dalton
KT: kinetochore
Mad: mitotic arrest deficient
MAP: mitogen activated protein
MBP: maltose binding protein
min: minute(s)
MT: microtubule
N-terminus: amino-terminus
PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PICH: Plk1-interacting checkpoint helicase

PIPES: 1,4-Piperazinediethanesulfonic acid

Plk: Polo-like kinase

PMSF: phenylmethylsulfonyl fluoride

RT: room temperature

SAC: spindle assembly checkpoint

SDS-PAGE: Sodium dodecylsulfate polyacrylamid gelelectrophoresis

siRNA: small interference ribonucleic acid

T106: threonine at position 106

wt: wildtype

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PUBLICATIONS

Parts of this work have been published (see below).
In collaboration with Nadja Huebner (PhD student in the laboratory of Prof. Nigg) a manuscript on the regulation of PICH by the CPC is in progress. A study on the regulation of Centrin-2 by sumoylation and its functional impact (not mentioned herein) has just been finished by Prof. Erich Nigg and myself. A manuscript is in preparation.

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10/2003 – 03/2004	**Institute of Molecular Pathology (Vienna, Austria)** Master project (Group of Prof. Nasmyth) Master thesis: "Purification of the cohesin holocomplex from *Saccharomyces cerevisiae*" Grade: A

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04/2002 – 08/2002	**The Institute of Cancer Research (London, UK)** Cancer Research UK Centre for Cancer Therapeutics (Prof.Workman) "Prostate Cancer and the androgen receptor"

10/2001 – 03/2002 **Max-Planck Institute of Biochemistry (Munich, Germany)**
Department of Molecular Structural Biology (Prof. Baumeister)
"Characterization of the AAA+ ATPases PAN (*M. janaschii*) and the membrane protease LON (*T.acidophilum*)"

10/1999 – 10/2001 **University of Hannover (Hannover, Germany)**
Diploma Program in Biology
Overall grade "Pre-diploma": A

MILITARY SERVICE

09/1998 – 09/1999 Transport soldier

PRIMARY & SECONDARY EDUCATION

08/1989 – 06/1998 Grammar school (Gymnasium Marianum Meppen)
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08/1985 – 07/1989 Elementary school (Marienschule Meppen)

AWARDS & FELLOWSHIPS

- 2008 Junior Research Award (Max-Planck Institute of Biochemistry)
  > Prize money
- 2005-2007 Boehringer Ingelheim Phd fellowship
  > PhD salary. Seminars and courses and regular meetings of scholarship-holders.
- 2002-2004 Degussa Stiftung Stipend
  > Financial support and regular meetings of scholarship holders
- 2002/2003 International Max-Planck Research School Stipend
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- 2002 Leonardo da Vinci Stipend by German Academic Exchange Service (DAAD) EU Placement Program
  > Financial support.
- 2001 Best pre-diploma in biology at the University of Hannover
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