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Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody

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The chromosomal passenger complex (CPC) coordinates chromosomal and cytoskeletal events of mitosis. The enzymatic core of this complex (Aurora-B) is guided through the mitotic cell by its companion chromosomal passenger proteins, inner centromere protein (INCENP), Survivin and Borealin/Dasra-B, thereby allowing it to act at the right place at the right time. Here, we addressed the individual contributions of INCENP, Survivin and Borealin to the proper functioning of this complex. We show that INCENP has an important role in stabilizing the complex, and that Borealin acts to promote binding of Survivin to INCENP. Importantly, when Survivin is directly fused to INCENP, this hybrid can restore CPC function at the centromeres and midbody, even in the absence of Borealin and the centromere-targeting domain of INCENP. Thus, Survivin is an important mediator of centromere and midbody docking of Aurora-B during mitosis.
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INTRODUCTION

Proper mitosis requires accurate nuclear division, followed by cytoplasmic partitioning into two daughter cells during cytokinesis. Nuclear division is monitored by the spindle assembly checkpoint (SAC), which is an intrinsic cell-cycle checkpoint that prevents the onset of anaphase until all sister chromatids have attained proper attachment to the mitotic spindle (Nicklas, 1997). To satisfy this checkpoint, kinetochores of paired sister chromatids have to attach in a bipolar manner, creating tension (Tanaka, 2002). Aurora-B kinase is essential to resolve naturally occurring monopolar or syntelic microtubule–kinetochore attachments (Tanaka *et al*, 2002; Lampson *et al*, 2004), and to communicate a lack of bipolarity (that is, tension) to the core SAC (Biggins & Murray, 2001; Lens & Medema, 2003). Additionally, in several organisms, interference with Aurora-B leads to a failure of cytokinesis (Kaitna *et al*, 2000; Severson *et al*, 2000; Ditchfield *et al*, 2003; Hauf *et al*, 2003).

Aurora-B acts together with Survivin, inner centromere protein (INCENP) and Borealin/Dasra-B (hereafter referred to as Borealin). These proteins physically interact and show a typical chromosomal passenger localization pattern during mitosis—at the inner centromere in (pro)metaphase, on the central spindle during anaphase and at the midbody during cytokinesis (Vagnarelli & Earnshaw, 2004). Interference with Survivin, INCENP or Borealin function disrupts Aurora-B localization and leads to similar mitotic defects, as observed after interference with Aurora-B function (Vagnarelli & Earnshaw, 2004, and references therein). Aurora-B is activated at the G2–M transition and remains active until mitotic exit (Yasui *et al*, 2004). Thus, it is likely that the specific functions of Aurora-B are mainly determined by its dynamic localization pattern. Until now, INCENP has been proposed to be the targeting module of the complex (Ainsztein *et al*, 1998), but specific functions for Survivin and Borealin in the chromosome passenger complex (CPC) have yet to be determined.

RESULTS AND DISCUSSION

An RNAi-complementation to study CPC function

We developed an INCENP RNA interference (RNAi) complementation system to study the role of INCENP in the CPC. Endogenous INCENP was knocked down by vector-driven short interfering RNA (siRNA; Fig 1A; supplementary Fig 1A–C online) and cells were reconstituted with an INCENP complementary DNA harbouring two silent mutations in the siRNA-targeting region, rendering it insensitive to the siRNA (Lens *et al*, 2003). INCENP-depleted cells showed the typical 'chromosome passenger deficiency' phenotype: defects in chromosome alignment and segregation, and a failure to undergo cytokinesis (supplementary Fig 1A–C online; Honda *et al*, 2003). Additionally, Aurora-B and BubR1 were mislocalized, and the INCENP-depleted cells failed to maintain an active SAC in response to Taxol (supplementary Fig 1B,D,E online). All these mitotic defects could be reverted by expression of a full-length INCENP containing two silent mutations (VSV–INCENP-sil; Figs 1C–E,2F; supplementary Fig 2B,C online; for INCENP constructs used, see Fig 4A), confirming the specificity of the siRNA and the functionality of the complementation approach.

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CPC formation is crucial for protein stability

In line with earlier observations (Honda *et al*, 2003), total protein levels of Aurora-B and Survivin were significantly reduced after INCENP depletion (Fig 1A). This reduction was interpreted as an indirect consequence of a failed cell division followed by a cell-cycle arrest in a tetraploid G1 state (Honda *et al*, 2003). However, we found that INCENP-depleted cells already failed to accumulate these proteins during passage through G2 (Fig 1B). Moreover, mitotic cells lacking INCENP also showed reduced Aurora-B and Survivin levels (Fig 1B,H, lane 2), which indicates that the effect of INCENP depletion on protein stability is more direct.

By introducing different INCENP truncation mutants into INCENP-depleted cells, we could show that a mutant lacking the amino-terminal 'centromere-targeting' domain (VSV–$INCENP_{47–920}$-sil; Fig 4A) restored Aurora-B levels, but failed to stabilize Survivin (Fig 1G, lane 4). In line with previous observations (Ainsztein *et al*, 1998), this mutant failed to concentrate at centromeres during prometaphase and to transfer to the midbody following the onset of anaphase (supplementary Fig 2B,C online). Consequently, this mutant was unable to restore a SAC-mediated arrest in response to Taxol and to overcome the cytokinesis defect in INCENP-depleted cells (Fig 1D,E). Because of the presence of the carboxy-terminal Aurora-B interaction domain (IN-box; Adams *et al*, 2000), $INCENP_{47–920}$-sil was able to bind to Aurora-B. In contrast, this truncation mutant failed to interact with Survivin (Fig 1F, lane 3). Conversely, a construct expressing only the first N-terminal 47 amino acids of INCENP ($INCENP_{1–47}$; Fig 4A) and thus harbouring the centromere-localization domain was able to bind to Survivin (Fig 1F, lane 4), but failed to stabilize it (Fig 1G, lane 5). Thus, this domain is both necessary and sufficient for interaction with Survivin, but the presence of Aurora-B in the complex may be needed for stabilization of Survivin. Interestingly, Survivin is an *in vivo* substrate of Aurora-B (Wheatley *et al*, 2004), but whether this phosphorylation affects protein stability is unknown. In contrast, the presence of Survivin in the complex was not essential to stabilize Aurora-B, because we found that a complex of Aurora-B and a truncated INCENP, although lacking Survivin, could exist (Fig 1F,G). However, whereas Aurora-B protein levels could be restored by expression of $INCENP_{47–920}$-sil, phosphorylation of serine-10 Histone-H3 in mitotic INCENP-depleted cells remained impaired (Fig 1H, lane 4). Thus, stabilization of Aurora-B by $INCENP_{47–920}$-sil is not sufficient to mediate Histone-H3 phosphorylation. This suggests that mitotic phosphorylation of Histone-H3 on serine-10 by Aurora-B requires either the direct presence of Survivin in the complex or centromeric localization of the kinase.

Survivin mediates mitotic localization of the CPC

Although INCENP contains a highly conserved 'centromere-targeting' domain in its N-terminus (Ainsztein *et al*, 1998), the mechanism by which this domain mediates centromeric localization remains elusive. As INCENP interacts with Survivin by means of this N-terminal domain (Fig 1F), it is possible that Survivin mediates mitotic localization of the CPC. To test this, a chimeric protein that is refractory to both INCENP and Survivin RNAi (Fig 2A; data not shown) was generated, in which the centromere-targeting domain in INCENP was replaced by Survivin (Survivin-$INCENP_{47–920}$-sil; Fig 4A). This fusion localized normally during mitosis, in both an INCENP-depleted and a combined INCENP/Survivin-depleted background (Fig 2D,G, arrows). This chimaera was also able to localize Aurora-B during mitosis (Fig 2E,F), restore serine-10 Histone-H3 phosphorylation (supplementary Fig 2D online) and restore CPC function, as expression of the chimaera in INCENP-, Survivin- or combined INCENP/Survivin-depleted cells rescued SAC function and the proper execution of cytokinesis (Fig 2B,C).

To obtain further evidence that Survivin can serve as a determining factor in localizing the CPC, we analysed the location of $INCENP_{1–47}$ in the absence or presence of endogenous Survivin. $INCENP_{1–47}$–GFP (green fluorescent protein) localized to centromeres during mitosis in control cells (Fig 2H, upper panel, see arrows), but failed to concentrate at centromeres in the absence of endogenous Survivin and instead localized diffusely in the cytoplasm during mitosis (Fig 2G, lower panel, see arrows), indicating that the centromere-targeting domain in INCENP requires at least the presence of Survivin to localize to centromeres. Taken together, these results show that Survivin is an important mediator of mitotic localization of the CPC.

Survivin can target the CPC in the absence of Borealin

Recently, it was suggested that Borealin has a role in determining centromeric localization of the CPC (Gassmann *et al*, 2004). As our results suggest that targeting of the Survivin/INCENP/Aurora-B complex is mediated by Survivin, we explored whether this depended on the presence of Borealin. We depleted Borealin using siRNA (Fig 3A–C) and introduced the Survivin–INCENP chimaera into these cells. Interestingly, expression of this chimaera in Borealin-depleted cells significantly restored CPC

Fig 1 | Protein–protein interactions within and stability of the chromosomal passenger complex. U2OS cells were transfected with 10 μg mock or inner centromere protein (INCENP) short interfering RNA (siRNA) vector combined with pBABE-puro. (**A**) Puromycin was added to enrich for transfected cells, and lysates for western blotting were prepared. (**B**) Transfected cells were released from a thymidine block and, at indicated time points, lysates were prepared for western blotting. N indicates nocodazole treatment for 24 h to enrich for mitotic cells. (**C**) 5 μg of the indicated VSV-tagged plasmids was transfected in combination with the siRNA plasmids and pBABE-puro. After puromycin selection, lysates were analysed by western blotting. (**D**) Similar to (**C**), after release from the thymidine block, cells were treated with Taxol for 18 h and collected for fluorescence-activated cell sorting (FACS) analysis. Mitotic percentages were determined by staining the cells with the MPM-2 antibody that specifically recognizes mitotic phospho-epitopes. (**E**) Similar to (**C**), 60 h after transfection, cells were collected for FACS analysis. (**F**) Human embryonic kidney (HEK) 293 cells were transfected with 5 μg of the indicated green fluorescent protein (GFP)-tagged constructs. After 48 h, cells were collected , immunoprecipitations were carried out with anti-GFP and samples were analysed by western blotting. The presence of GFP protein (arrowhead) is due to the presence of an additional transcription site in the plasmids used. (**G**) Similar to (**C**). Note that $INCENP_{1–47}$ contains both an amino-terminal VSV tag and a carboxy-terminal GFP tag. The asterisk denotes an aspecific band recognized by the anti-VSV monoclonal antibody. (**H**) Similar to (**C**). At 24 h after puromycin selection, nocodazole was added to the cells for 18 h. Mitotic cells were obtained by shake-off and lysates were analysed by western blotting. ►

function, as judged by restoration of mitotic arrest in response to Taxol and a reduction in the percentage of polyploid cells that were observed after depletion of Borealin (Fig 3D,E). Expression of neither Survivin nor INCENP individually was sufficient to restore CPC function in Borealin-depleted cells (Fig 3E; data not shown). In line with these functional data, the chimaera was found to localize to centromeres and the midbody in Borealin-depleted cells (Fig 3F) and to restore Aurora-B localization (supplementary

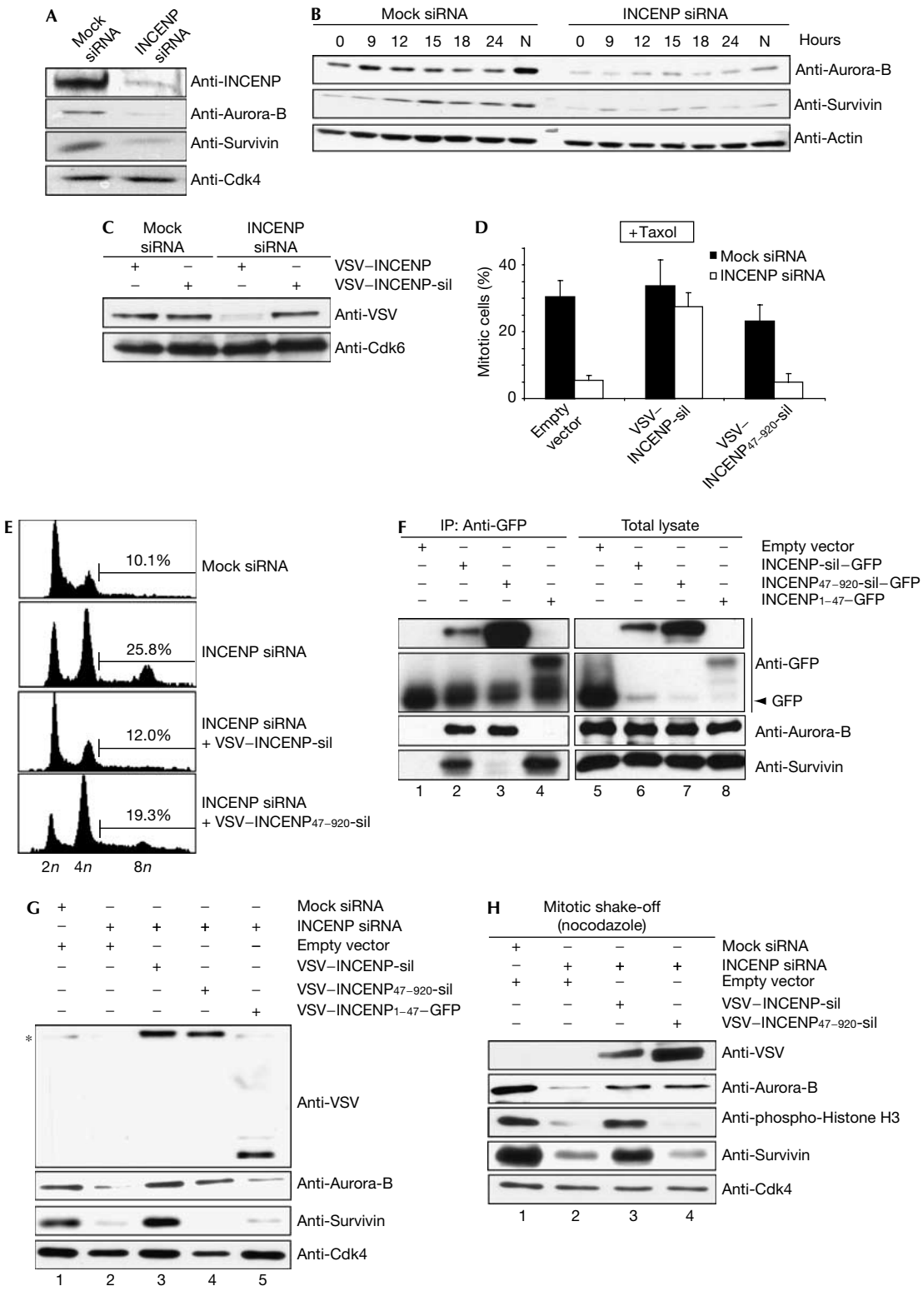


Fig 2 | Survivin is a determining factor for mitotic localization of the chromosome passenger complex. (**A**) U2OS cells were transfected with the indicated plasmids. Puromycin was added to enrich for transfected cells. Lysates were analysed by western blotting. (**B,C**) Cells were transfected with the indicated short interfering RNA (siRNA) plasmids combined with the indicated VSV-tagged constructs. (**B**) After release from a thymidine block, cells were treated with Taxol and collected for fluorescence-activated cell sorting (FACS) analysis 18 h later. Mitotic percentages were determined by MPM-2 staining. (**C**) Cells were collected for FACS analysis 60 h after transfection. (**D,E**) Cells grown on coverslips were transfected with mock or inner centromere protein (INCENP)/Survivin siRNA plasmids, VSV–Survivin–$INCENP_{47–920}$-sil and H2B–GFP (green fluorescent protein). Immunostainings were carried out with the indicated antibodies. DNA (H2B–GFP) is depicted in blue. (**F**) Cells grown on coverslips were transfected with the indicated plasmids and H2B–GFP. Cells were stained with anti-Aurora-B, and the percentage of H2B–GFP-positive prometaphase cells in which Aurora-B localized on the centromeres was scored. The number of cells counted per condition is indicated. (**G**) Time-lapse images of a representative cell transfected with INCENP siRNA plasmid, Survivin–$INCENP_{47–920}$-sil–GFP and H2B–diHcRed. Differential interference contrast (DIC), GFP and diHcRed signals are shown. (**H**) Time-lapse imaging of cells transfected with mock (upper panel) or Survivin siRNA plasmid (lower panel), $INCENP_{1–47}$-GFP and H2B–diHcRed. Scale bars, 5 μm.

Fig 3 online). However, it should be noted that the association of the Survivin–INCENP chimaera to centromeres and the central spindle was affected in Borealin-depleted cells. Instead of concentrating at centromeres and central spindle, a significant portion of the chimaera localized to chromatin throughout mitosis (Fig 3F, lower panel; data not shown). Taken together, these data show that the Survivin–INCENP chimaera is sufficient to restore a functional CPC in Borealin-depleted cells, and that Borealin might facilitate dissociation of the CPC from chromatin or enhance its concentration at centromeres and the central spindle.

Borealin facilitates Survivin–INCENP interaction

As Borealin is essential for localization of INCENP and Survivin (Gassmann *et al*, 2004), but not for a chimaera in which these two proteins are covalently linked, we reasoned that Borealin might be important for the Survivin–INCENP interaction. To further investigate the potential role of Borealin in regulating the Survivin–INCENP interaction, immunoprecipitations were carried out with GFP–INCENP from Borealin-depleted cells expressing either exogenous Survivin or Borealin. In control cells, both endogenous and exogenous Survivin could interact with INCENP (Fig 3H, lanes 1–3). In contrast, in the absence of endogenous Borealin, neither endogenous nor exogenous Survivin was able to interact with INCENP (Fig 3H, lanes 4,5). As depletion of Borealin also reduced Survivin levels, this probably explains why no endogenous Survivin was found to interact with INCENP (Fig 3H, lanes 4,10; Gassmann *et al*, 2004; Sampath *et al*, 2004). Indeed, after expression of exogenous, RNAi-resistant Borealin, endogenous Survivin levels and Survivin interaction with INCENP were restored (Fig 3E, lanes 6,12). Importantly, although exogenous Survivin levels were not affected by Borealin depletion, Survivin failed to interact with INCENP, indicating that, *in vivo*, an efficient interaction of Survivin and INCENP is dependent on the presence of Borealin (Fig 3E, lanes 5,11). The observation that Borealin can interact with INCENP that lacks the Survivin interaction domain ($INCENP_{47–920}$; Fig 3G, lanes 6,8; supplementary Fig 3 online), in combination with previous *in vitro* data that have shown the direct interaction of Survivin and INCENP (Wheatley *et al*, 2001; Romano *et al*, 2003), rules out a direct bridging function for Borealin between Survivin and the centromere-targeting domain of INCENP. Hence, similar to CSC-1, its distant homologue in *Caenorhabditis elegans* (Romano *et al*, 2003), human Borealin has a role in facilitating Survivin–INCENP interaction. By generating the Survivin–INCENP chimaera, we have circumvented the necessity for Borealin in stabilizing Survivin–INCENP interaction, which probably explains why a functional CPC can be directed to the right mitotic structures in Borealin-depleted cells.

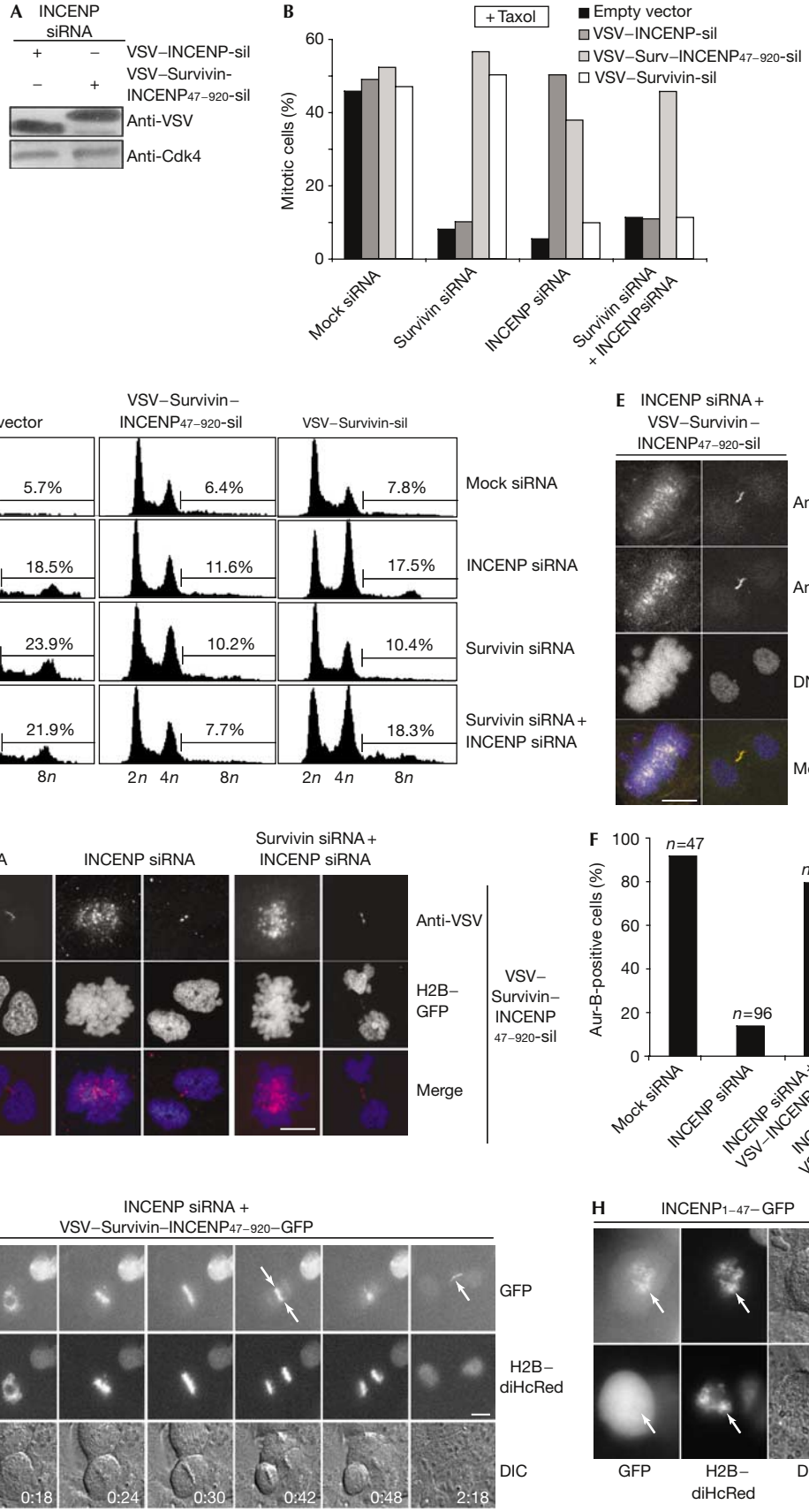
Conclusion

Given the essential functions of Aurora-B during mitosis, explaining the mechanisms behind its dynamic localization is of great importance. The observation that depletion of one CPC component by siRNA results in the destabilization of the entire complex instigates the need for more complex experiments to unravel the functions of single CPC components. Using RNAi-complementation, we have provided evidence that an important function of Survivin is to serve as an interface between the centromere/central spindle and the CPC, whereas Borealin facilitates the interaction of Survivin and INCENP (Fig 4B). Importantly, the targeting function of Survivin was revealed only when Survivin was fused to INCENP lacking its centromere-targeting domain. This suggests that although Survivin can mediate localization of the CPC to its proper mitotic structures, the other CPC components are probably needed to obtain a stable association (possibly by affecting the on/off rate of the complex). Identifying the Survivin interaction partners at the centromere and midbody will be an important next step in understanding the regulation of the CPC.

METHODS

Antibodies and reagents. The following antibodies were used: rabbit anti-INCENP and rabbit anti-Aurora-B (Abcam, Cambridge, UK), rabbit anti-Survivin (R&D, Minneapolis, MN, USA), rabbit anti-Borealin (gift from Dr H. Funabiki), mouse anti-Aurora-B (Transduction, Lexington, KE, USA), mouse anti-VSV and mouse anti-α-Tubulin (Sigma, St Louis, MO, USA), rabbit anti-Cdk6 and rabbit anti-Cdk4 (Santa Cruz, Santa Cruz, CA, USA), sheep anti-BubR1 (gift from Dr S. Taylor), rabbit anti-GFP (gift from Dr J. Neefjes), rabbit anti-phospho-Ser10-histone-H3 and mouse anti-MPM-2 (Upstate, Charlotsville, VA, USA), CREST antiserum (Cortex Biochem, San Leandro, CA, USA), peroxidase-conjugated goat anti-rabbit and peroxidase-conjugated goat anti-mouse (Dako, Glostrup, Denmark) and donkey anti-mouse/Cy5 (Jackson, West Grove, PA, USA), goat anti-rabbit/Alexa-568, goat anti-rabbit/Alexa-633, goat anti-mouse/Alexa-568 and donkey anti-sheep/Alexa-568 (Molecular Probes, Eugene, OR, USA). Reagents were from Sigma unless stated otherwise.

Plasmids. RNAi vectors were generated as described (Lens *et al*, 2003), and were based on 5′-TGACACGGAGATTGCCAAC-3′ (INCENP) and 5′-CAGCTGACCTGGATATCAC-3′ (Borealin). Human INCENP and Borealin were PCR amplified from a T-cell


A
INCENP siRNA
+
–
–
+
VSV–INCENP-sil
VSV–Survivin-INCENP47–920-sil
Anti-VSV
Anti-Cdk4
B
+Taxol
Empty vector
VSV–INCENP-sil
VSV–Surv–INCENP47–920-sil
VSV–Survivin-sil
Mitotic cells (%)
60
40
20
0
Mock siRNA
Survivin siRNA
INCENP siRNA
Survivin siRNA + INCENPsiRNA
C
Empty vector
VSV–Survivin–INCENP47–920-sil
VSV–Survivin-sil
5.7%
6.4%
7.8%
18.5%
11.6%
17.5%
23.9%
10.2%
10.4%
21.9%
7.7%
18.3%
Mock siRNA
INCENP siRNA
Survivin siRNA
Survivin siRNA + INCENP siRNA
2n 4n 8n
E
INCENP siRNA + VSV–Survivin–INCENP47–920-sil
Anti-VSV
Anti-Aurora-B
DNA
Merge
D
Mock siRNA
INCENP siRNA
Survivin siRNA + INCENP siRNA
Anti-VSV
H2B–GFP
Merge
VSV–Survivin–INCENP47–920-sil
F
Aur-B-positive cells (%)
100
80
60
40
20
0
n=47
n=96
n=87
n=62
Mock siRNA
INCENP siRNA
INCENP siRNA + VSV–INCENP-sil
INCENP siRNA + VSV–Surv–INCENP47–920-sil
G
INCENP siRNA + VSV–Survivin–INCENP47–920–GFP
GFP
H2B–diHcRed
DIC
0:00
0:18
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INCENP1–47–GFP
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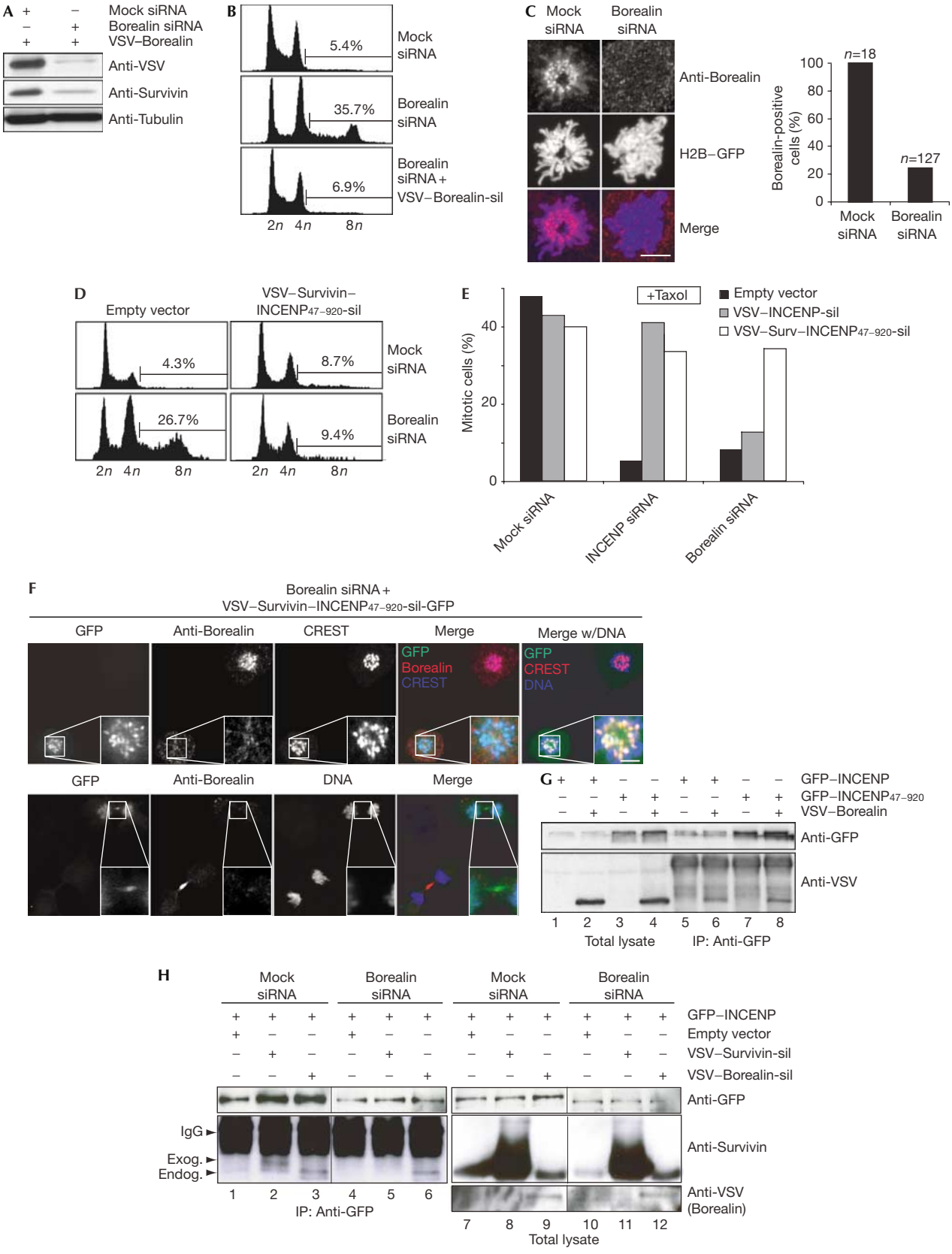

◀ **Fig 3** | Survivin can target a functional chromosome passenger complex in the absence of Borealin, but interaction with inner centromere protein (INCENP) depends on Borealin. (**A**) U2OS cells were transfected with the indicated plasmids, puromycin was added to enrich for transfected cells and lysates were analysed by western blotting. (**B–E**) Cells were transfected with the indicated plasmids. (**C**) Cells grown on coverslips were transfected with mock or Borealin short interfering RNA (siRNA) plasmid and H2B–GFP (green fluorescent protein). Immunostainings were carried out with anti-Borealin, and the percentage of H2B–GFP-positive prometaphase cells in which Borealin localized on centromeres was scored. The number of cells counted per condition is indicated. (**B**,**D**) Cells were grown asynchronously for 60 h and collected for fluorescence-activated cell sorting (FACS) analysis. (**E**) Taxol was added after thymidine release, and cells were collected for FACS analysis 18 h later. Mitotic percentages were determined by MPM-2 staining. (**F**) Cells grown on coverslips were transfected with Borealin siRNA plasmid and VSV–Survivin–INCENP$_{47–920}$-sil–GFP. Immunostainings were carried out with the indicated antibodies, and DNA was stained with 4,6-diamidino-2-phenylindole. Different colours used for the two merge pictures are indicated. (**G**) Human embryonic kidney 293 cells were transfected with 5 μg of the indicated constructs. After 48 h, cells were collected, immunoprecipitations were carried out with anti-GFP and samples were analysed by western blotting. (**H**) U2OS cells were transfected with the indicated plasmids. At 18 h after transfection, puromycin was added and 36 h later, cells were collected . Immunoprecipitations were carried out with anti-GFP and western blots were probed with the indicated antibodies. Scale bars, 5 μm.

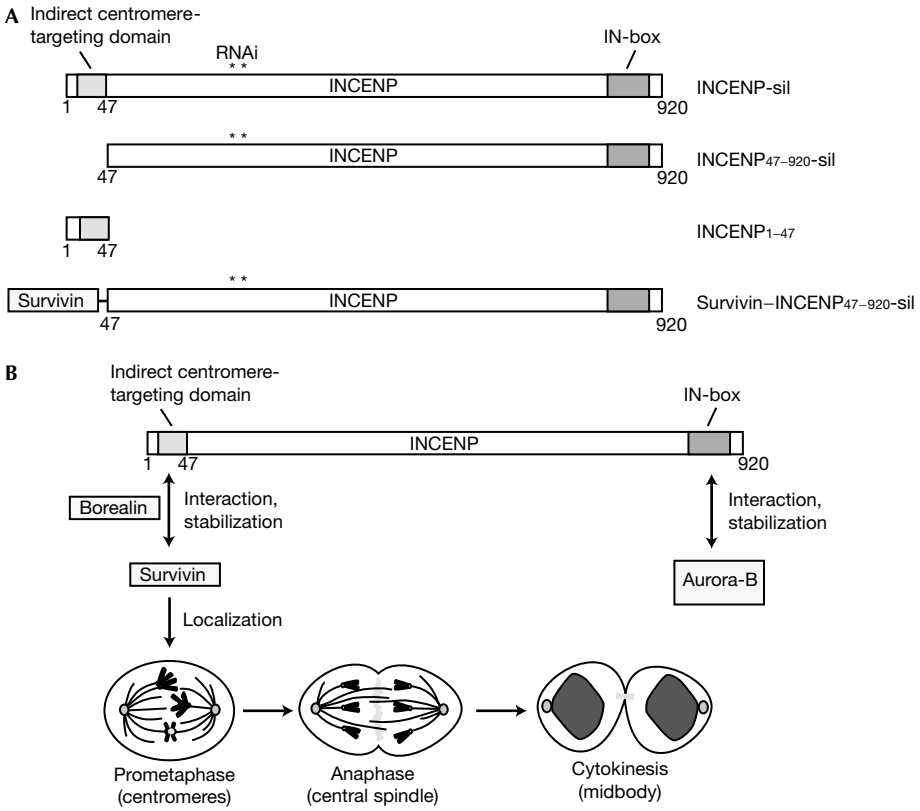

Fig 4 | Overview of inner centromere protein constructs and model. (**A**) Schematic representation of inner centromere protein (INCENP) constructs. Survivin and Aurora-B binding domains are depicted. Introduced RNA interference (RNAi) silent mutations are shown. (**B**) Model showing the proposed upstream targeting role of Survivin in the chromosome passenger complex.

cDNA library using a 5′ primer containing a VSV tag and cloned into pcDNA3.1 (INCENP) or into pCR3 containing an N-terminal VSV tag (Borealin). RNAi-resistant INCENP and Borealin constructs were generated using the QuickChange® Mutagenesis kit (Stratagene, La Jolla, CA, USA). For INCENP$_{47–920}$, INCENP was digested with *Eco*RI/*Xho*I. This fragment was cloned into pCR3 containing an N-terminal VSV tag. For INCENP$_{1–47}$, INCENP was digested with *Hin*dIII/*Eco*RI, and the product was cloned into pcDNA3.1. For Survivin–INCENP$_{47–920}$, Survivin was ligated to the N terminus of INCENP$_{47–920}$ after *Eco*RI digestion. For the C-terminal GFP constructs, inserts were cloned into various peGFP-N-vectors (Clontech, Mountain View, CA, USA). Note that the INCENP–GFP constructs also contain an N-terminal VSV tag. Histone H2B–GFP, Histone H2B–diHcRed (gifts from Dr J. Ellenberg), Survivin siRNA, VSV–Survivin, pBABE-puro and Spectrin–GFP have been described previously (Lens *et al*, 2003).

Cell culture and transfection. Human osteosarcoma U2OS and human embryonic kidney 293 cells were cultured and synchronized, as described (Lens *et al*, 2003). Cells were transfected using the standard calcium phosphate protocol.

Flow cytometry and time-lapse microscopy. The percentage of mitotic cells (MPM-2 positivity) and cell cycle distribution of spectrin–GFP-transfected cells were determined by flow cytometry, as described (Smits *et al*, 2000). H2B–GFP-expressing cells were followed by time-lapse microscopy, as described (Lens *et al*, 2003).

Immunoblotting, immunoprecipitation and immunofluorescence. Immunoblotting was carried out as described (Smits *et al*, 2000). For immunoprecipitation, cells were lysed in E1A lysis buffer with protease inhibitors (Complete®, Roche, Indianapolis, IN, USA) for 30 min at 4 °C (Smits *et al*, 2000). GFP-tagged proteins were immunoprecipitated with 3 μg anti-GFP precoupled to protein G–Sepharose (Amersham Biosciences, Pittsburg, CA, USA). Immunofluorescence was carried out as described (Lens *et al*, 2003). DNA was stained with 4,6-diamidino-2-phenylindole.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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