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The Survivin–Crm1 interaction is essential for chromosomal passenger complex localization and function

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The chromosomal passenger complex (CPC) of Aurora-B, Borealin, INCENP (inner centromere protein) and Survivin coordinates essential chromosomal and cytoskeletal events during mitosis. Here, we show that the nuclear export receptor Crm1 is crucially involved in tethering the CPC to the centromere by interacting with a leucine-rich nuclear export signal (NES), evolutionarily conserved in all mammalian Survivin proteins. We show that inhibition of the Survivin–Crm1 interaction by treatment with leptomycin B or by RNA-interference-mediated Crm1 depletion prevents centromeric targeting of Survivin. The genetic inactivation of the Survivin–Crm1 interaction by mutation of the NES affects the correct localization and function of Survivin and the CPC during mitosis. By contrast, CPC assembly does not seem to require the Survivin–Crm1 interaction. Our report shows the functional significance of the Survivin–Crm1 interface and provides a novel link between the mitotic effector Crm1 and the CPC.

Keywords: cell cycle; leptomycin B; nucleocytoplasmic transport; *trans*-dominance
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INTRODUCTION

Regulated development and cellular homeostasis relies on proper mitosis. It has emerged that the chromosomal passenger proteins Aurora-B, Borealin, inner centromere protein (INCENP) and Survivin form a conserved complex and are key regulators of mitotic events (Vagnarelli & Earnshaw, 2004). These proteins show a typical chromosomal passenger localization pattern during mitosis—at the centromere in prometaphase, on the central spindle during anaphase and at the midbody during cytokinesis (Vagnarelli & Earnshaw, 2004). Interference with each of the subunits of the chromosomal passenger complex (CPC) results in severe mitotic defects and apoptosis (Vagnarelli & Earnshaw, 2004).

Although the low molecular weight would theoretically allow Survivin to access intracellular compartments by passive diffusion, regulated subcellular localization has been suggested for CPC proteins and for other cell-cycle regulators (Rodriguez *et al*, 2002, 2006; Xu & Massague, 2004). Active nucleocytoplasmic transport takes place through the nuclear pore complex and is regulated by specific signals binding to transport receptors (Weis, 2003). The best-characterized nuclear export signals (NESs) are leucine rich, interact with the export receptor chromosome region maintenance 1 (Crm1) and depend on the small GTPase Ran, which controls the Crm1–substrate interaction (Weis, 2003). The guanine-nucleotide-exchange factor RCC1 (regulator of chromosome condensation) facilitates Ran binding to Crm1, whereas RanBP1, the main regulator of Ran, promotes Crm1 dissociation from Ran.

Besides the CPC, cellular components that regulate nucleocytoplasmic transport in interphase cells are crucially involved in controlling mitotic processes. These are accomplished by the Ran–GTPase system in conjunction with specific receptors of the importin-β family (Dasso, 2005). Recently, Crm1 has been identified as an essential mitotic effector in mammalian cells (Arnaoutov *et al*, 2005; Wang *et al*, 2005).

At present, the molecular mechanisms and requirements for centromere targeting and function of the chromosomal passengers, as well as for the Ran–GTPase system, are under intense investigation (Earnshaw, 2005). Here, we provide evidence that an evolutionarily conserved NES in Survivin is crucial for the biological activity of Survivin and show that inactivation of the Survivin–Crm1 interaction affects the correct localization and function of CPC proteins.

RESULTS AND DISCUSSION

Leptomycin B affects CPC subunit localization

To provide a molecular link between the CPC and Crm1, we first examined whether the centromeric localization of CPC proteins was impaired on application of the Crm1-specific inhibitor

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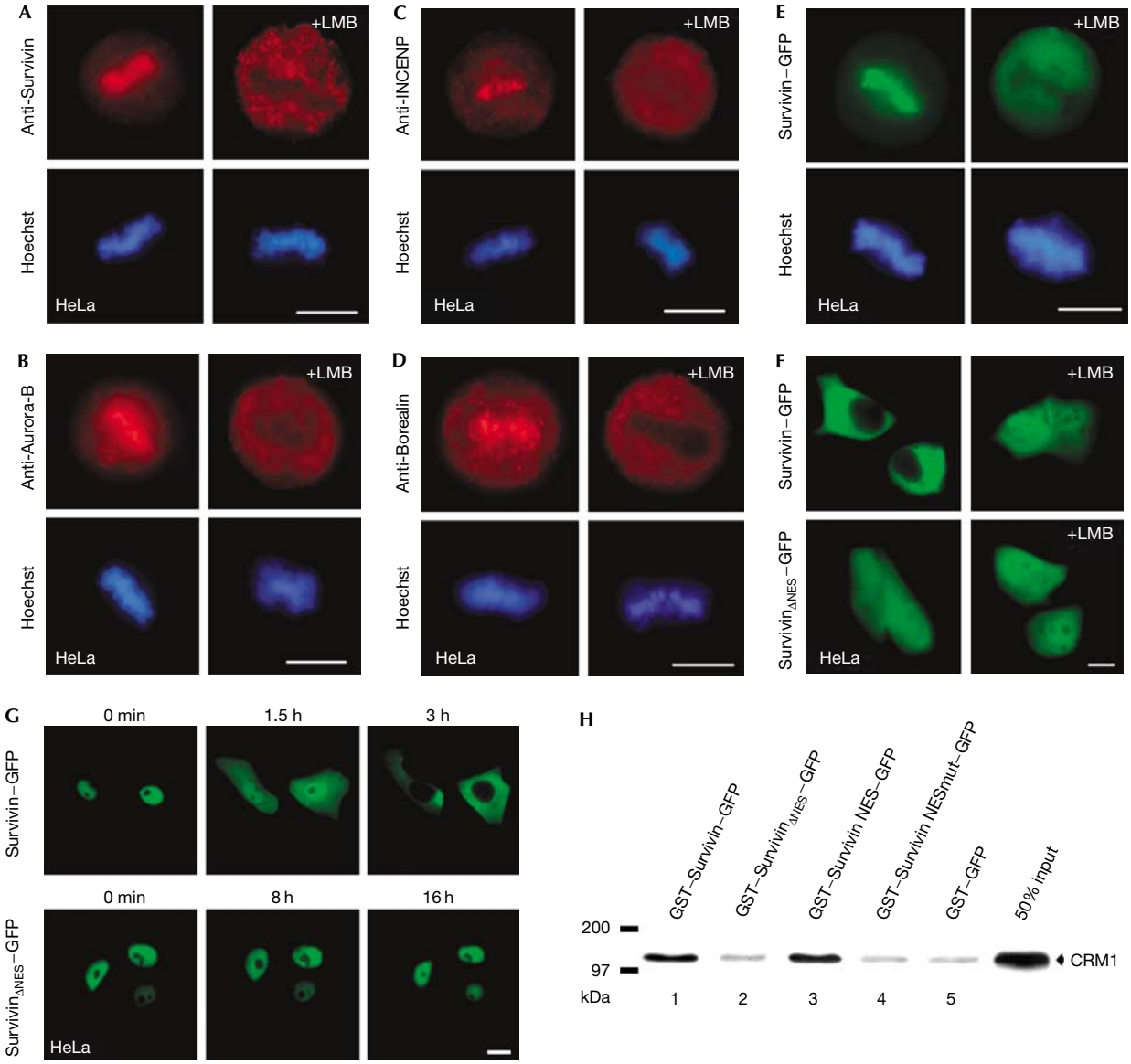

Fig 1 | Survivin contains a leptomycin-B-sensitive nuclear export signal interacting with Crm1. (**A–E**) The centromeric localization of (**A**) Survivin, (**B**) Aurora-B, (**C**) INCENP, (**D**) Borealin and (**E**) Survivin–GFP is affected by treatment with leptomycin B (LMB) for 6 h. Endogenous chromosomal passenger complex (CPC) proteins were detected by immunostaining (red). DNA was stained with Hoechst. (**F**) Survivin–GFP localizes to the cytoplasm and accumulates in the nucleus on LMB treatment, whereas Survivin$_{\Delta NES}$–GFP is equally distributed between the nucleus and the cytoplasm and does not respond to LMB. (**G**) Nuclear-injected GST–Survivin–GFP is efficiently exported, in contrast with export-deficient GST–Survivin$_{\Delta NES}$–GFP. GFP fusions were detected by fluorescence microscopy. Scale bars, 10 μm. (**H**) Survivin binds to Crm1 *in vitro*. ^{35}S-labelled Crm1 protein was incubated with 4 μg of the indicated GST–GFP fusion proteins prebound to glutathione Sepharose beads in the presence of Ran–GTP. Binding of Crm1 to the NES-containing substrates is abolished by mutating the NES. GST–GFP served to control for non-specific binding. GFP, green fluorescent protein; GST, glutathione *S*-transferase; ΔNES, mutant nuclear export signal; NES, nuclear export signal.

leptomycin B (LMB). We observed that Survivin, Aurora-B, INCENP and Borealin no longer efficiently localized to the centromere on LMB treatment (Fig 1A–D). Similar results were found for Borealin–green fluorescent protein (GFP), and it was found that LMB also affected the centromeric and midbody localization of Survivin–GFP (Fig 1E; supplementary Fig S1A–D online).

Survivin contains a Crm1 dependent NES

The localization of ectopically expressed Aurora-B–GFP, INCENP or Borealin–GFP was not affected by LMB in interphase cells (not shown), whereas Survivin–GFP accumulated in the nucleus on LMB treatment, as reported previously (Rodriguez *et al*, 2002, 2006; Fig 1F). As LMB impairs numerous cellular pathways, the effects of LMB need to be verified genetically and biochemically.

Deletion mutagenesis (data not shown), inspection of the NES consensus sequence and microinjection experiments identified the Survivin amino acids 89–98 (^{89}VKKQFEELTL^{98}) as the NES (SurvNES). Nuclear-injected full-length glutathione *S*-transferase (GST)–Survivin–GFP or GST–SurvNES–GFP were quantitatively exported (Fig 1G; supplementary Fig S1E online). By contrast, GST–Survivin$_{\Delta NES}$–GFP and GST–SurvNESmut–GFP, in which two essential leucine residues of the NES were mutated (^{89}VKKQFEELTL^{98} → ^{89}VKKQFEEATA^{98}; mutated residues are underlined), were not exported (Fig 1G; supplementary Fig S1E online). In contrast with other experimental systems used to identify NESs (Rodriguez *et al*, 2002, 2006), microinjection of GST–GFP fusions allows the quantification of transport independent of drug treatment or passive diffusion (Heger *et al*, 2001). Inactivation of the NES in the context of the full-length protein (Survivin$_{\Delta NES}$–GFP) resulted in a protein that no longer localizes to the cytoplasm but is equally distributed between the nucleus and the cytoplasm, and does not respond to LMB (Fig 1F). These results unequivocally define the evolutionarily conserved NES in all mammalian Survivin proteins (supplementary Fig S1F online). The direct NES-mediated Survivin–Crm1 interaction was verified by *in vitro* interaction studies (Fig 1H). Recombinant GST–Survivin–GFP bound to Crm1 in the presence of Ran–GTP, in contrast with inactive GST–Survivin$_{\Delta NES}$–GFP or GST–GFP alone. No efficient binding was detectable without Ran–GTP or in the presence of LMB (not shown).

Survivin–Crm1 interaction is needed to localize CPC

We observed that in contrast with Survivin–GFP, Survivin$_{\Delta NES}$–GFP failed to localize correctly during mitosis (Fig 2A; supplementary Fig S2A online) and did not localize with Crm1 at the centromere (Fig 2B). The presence of centromeres in cells expressing Survivin$_{\Delta NES}$–GFP was verified by staining with a CREST antiserum (Fig 2C), confirming that impairment of centromeric localization is not due to loss of the centromere structure. A similar localization of NES-deficient Survivin was observed in cell lines on RNA interference (RNAi)-mediated ablation of endogenous Survivin (supplementary Fig S1G online).

Similar to LMB, RNAi-mediated depletion of Crm1 also impaired targeting of Survivin–GFP or Aurora-B to the centromere (Fig 2D; supplementary Fig S1H online; data not shown). Further evidence that Crm1 and Survivin are physically associated early in mitosis is illustrated in Fig 2E. Crm1 could be recovered in a complex with endogenous Survivin–Aurora-B from mitotic cells, whereas complex formation was abolished on pretreatment with LMB. Equally, the Crm1–Survivin–GFP complex could be precipitated from Survivin–GFP-expressing cells but not from Survivin$_{\Delta NES}$–GFP- or GFP-expressing cells (not shown).

To understand whether CPC assembly or CPC targeting to the centromere requires Crm1, we examined the localization of CPC proteins during the transition from late G2 to prophase in the absence and presence of LMB. At late G2, the localization of Survivin–GFP gradually changed from a predominantly cytoplasmic to a cytoplasmic–nuclear distribution, with a subsequent accumulation at the centromeres in early prophase (Fig 2F; supplementary Fig S2E online). Aurora-B–GFP and Borealin–GFP accumulated at the centromeres with similar kinetics (supplementary Fig S2C–E online). A similar change in localization, except for the centromeric accumulation, was observed for a RevNES–GFP fusion (Rev, for regulator of expression of virion proteins; supplementary Fig S2B online), indicating that the export competence of the cell is gradually lost during breakdown of the nuclear envelope and no active nuclear import of Survivin seems to be involved. By contrast, 30 min pretreatment of late G2 cells with LMB interfered with the centromeric localization of Survivin–GFP (Fig 2G) and Aurora-B–GFP (not shown), whereas the already assembled CPCs at the centromeres were not affected (Fig 2H). By contrast, Ran binding protein 2 (RanBP2) was lost from the centromeres under these conditions (data not shown). As expected, Survivin$_{\Delta NES}$–GFP was not detectable at the centromeres during G2/M transition (supplementary Fig S2A,E online).

Also, Crm1 could not be recovered in a complex with Survivin–GFP from metaphase-enriched cells (supplementary Fig S2F online). Thus, Crm1 seems to be transiently involved in the transport of the CPC to the centromere, rather than in CPC anchoring, consistent with the results from the study of Rodriguez *et al* (2006). As such, the CPC behaves differently compared with factors such as RanBP2 (Arnaoutov *et al*, 2005; Rodriguez *et al*, 2006). This is further supported by showing that Crm1 and Survivin only partly colocalize at the centromere (supplementary Fig S1J online). Other reports indicate that Survivin localizes to the inner centromere, whereas Crm1 has been detected at the outer centromere (Arnaoutov *et al*, 2005) or even at the centrosomes (Wang *et al*, 2005), underscoring the dynamic nature of Crm1 during cell-cycle regulation. However, the molecular details of the localization of Crm1 are not yet understood. The NES–Crm1 interaction in nucleophosmin seems to be regulated by phosphorylation (Wang *et al*, 2005), whereas no phosphorylation of the NES sequence was reported for Survivin (Nousiainen *et al*, 2006). Also, Survivin mutants, in which the Thr 97 residue in the NES was changed to either alanine (mimicking non-phosphorylation) or aspartic acid (mimicking phosphorylation), still localized to the centromeres (not shown).

NES-deficient Survivin binds to CPC components

We further controlled that the effects of NES-deficient Survivin on CPC targeting and activity are mediated by preventing the Survivin–Crm1 interaction rather than by affecting the ability of Survivin to dimerize or to interact with CPC proteins. First, we immunoprecipitated CPCs from mitotic cells expressing Survivin–GFP, Survivin$_{\Delta NES}$–GFP or GFP. CPCs containing Survivin–GFP or Survivin$_{\Delta NES}$–GFP were recovered with similar efficiencies, whereas the non-CPC kinetochore protein centromere protein A (CENP-A) was not precipitated (Fig 3A). Similarly, immunoprecipitation of the crucial Survivin binding partner Borealin (Gassmann *et al*, 2004) recovered both Survivin–GFP and Survivin$_{\Delta NES}$–GFP (supplementary Fig S2G online). By precipitating CPCs containing Survivin and also Survivin$_{\Delta NES}$ with similar efficiencies from G2-enriched transfectants (Fig 3B), we could show that the inhibitory effects of Survivin$_{\Delta NES}$ are not caused by impaired CPC assembly late at the G2/M transition. G2 enrichment was controlled by staining for targeting protein for Xklp2 (TPX2) and Cyclin B1 expression, and no CPC proteins could be precipitated using an antibody against actin, which served as the control (not shown).

Second, *in vitro* interaction assays were used to verify Survivin dimerization and binding of immobilized GST–Survivin–GFP or

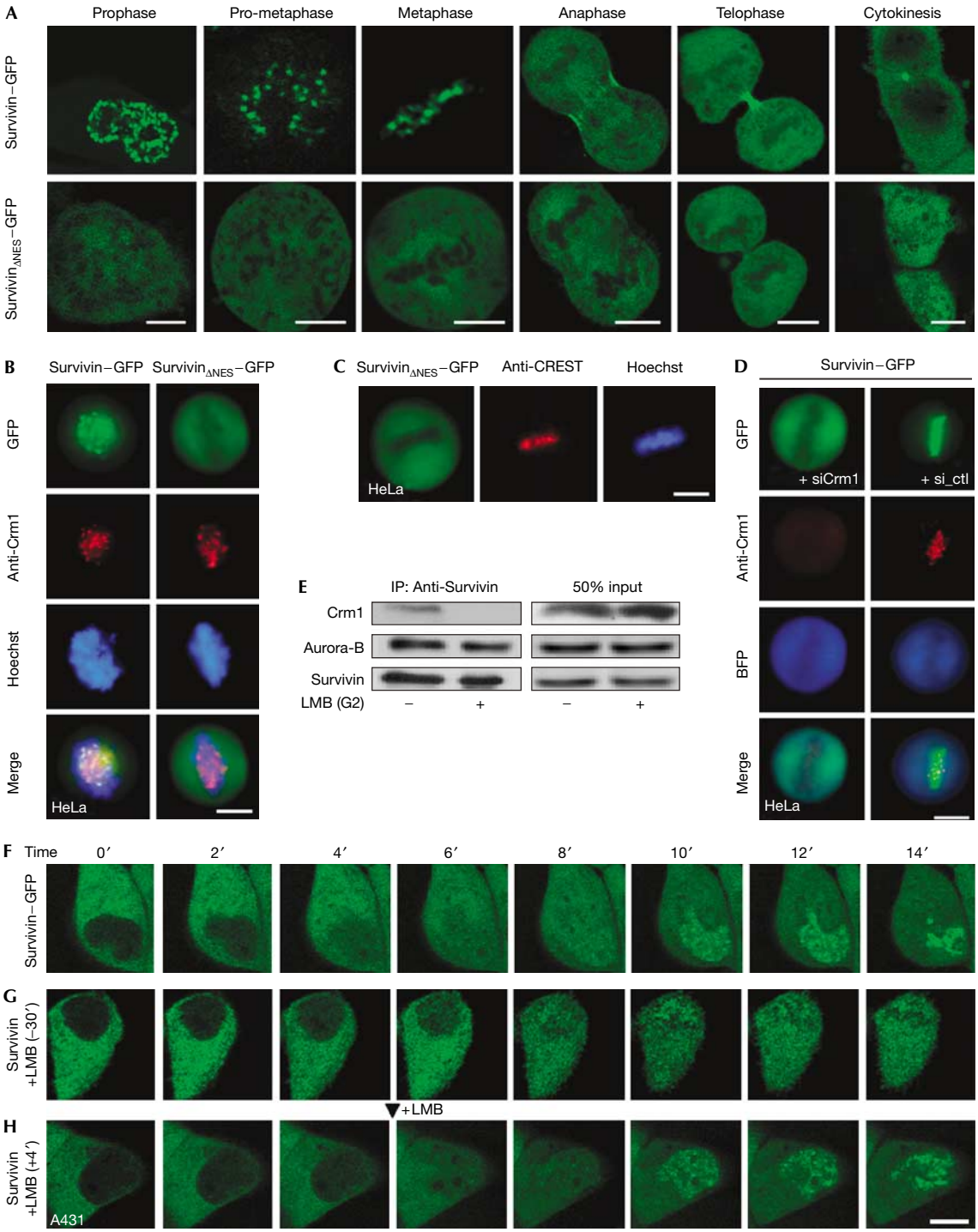

Fig 2 | The Survivin–Crm1 interaction is required to tether Survivin to the centromere. (**A**) The localization of Survivin–GFP and Survivin$_{\Delta NES}$–GFP during mitosis was followed in live A431 cells. (**B**) Survivin–GFP but not Survivin$_{\Delta NES}$–GFP localizes with Crm1 at the centromere. (**C**) Staining of centromeres in cells expressing Survivin$_{\Delta NES}$–GFP using a CREST antiserum. (**D**) RNA-interference-mediated ablation of Crm1 impairs targeting of Survivin–GFP to the centromere. Cells were transfected with a Crm1 or a control (ctl) short interfering RNA together with a BFP expression plasmid as the transfection control. Crm1 was detected by immunostaining (red). (**E**) Survivin–Crm1 complex formation is abolished by leptomycin B (LMB). Immunoprecipitation of mitotic HeLa cell extracts was performed using Survivin antibody. Crm1, Survivin and Aurora-B were detected by immunoblot. (**F–H**) Time-lapse imaging of Survivin–GFP during G2/M transition. (**G**) Pretreatment of late G2 cells with LMB interfered with the (**F**) centromeric localization of Survivin–GFP, whereas (**H**) assembled chromosomal passenger complexes at the centromeres were not affected. The arrowhead indicates addition of LMB. Scale bars, 10 μm. GFP, green fluorescent protein; ΔNES, mutant nuclear export signal.

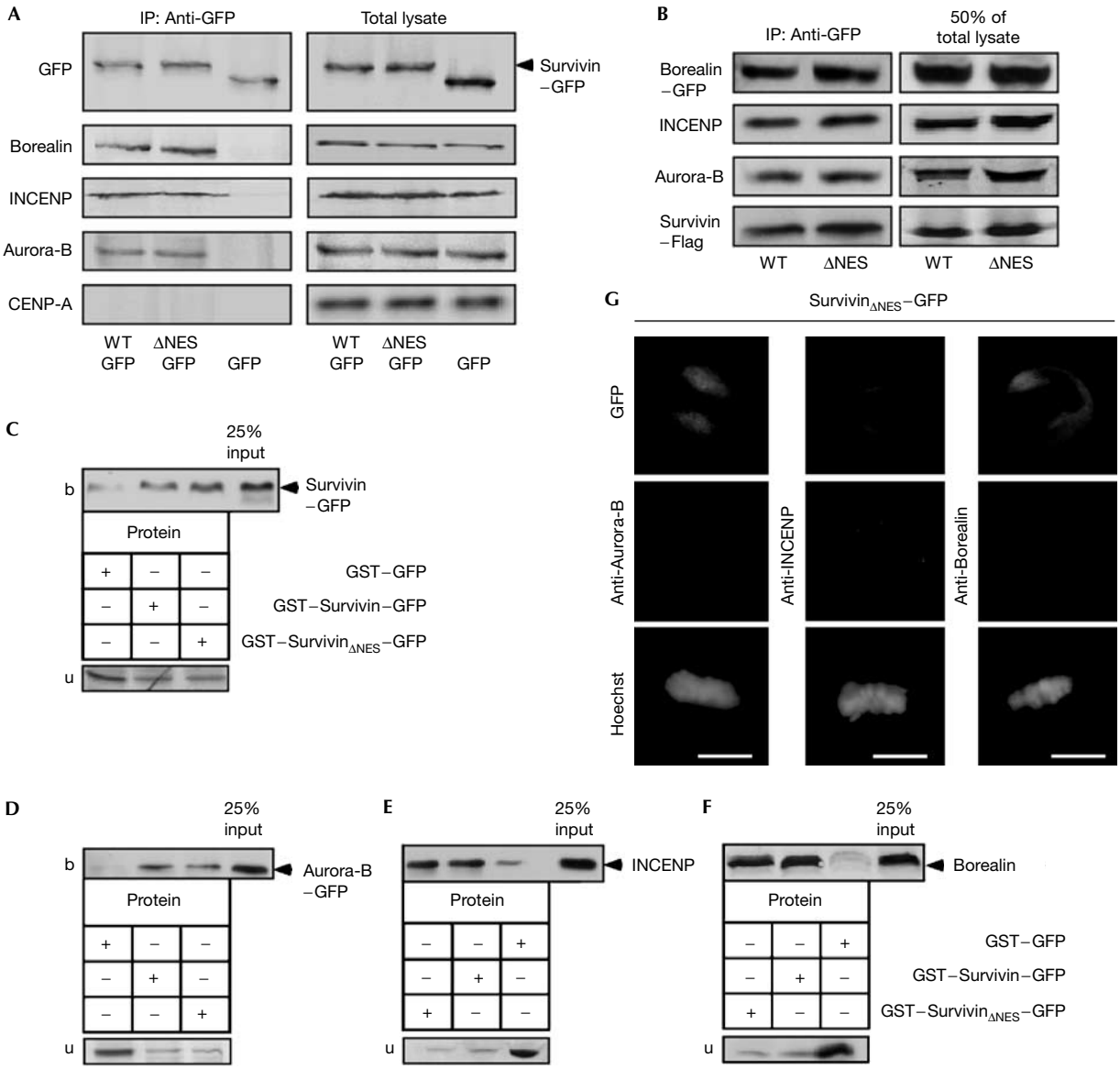

Fig 3 | Survivin and Survivin$_{\Delta NES}$ interact with subunits of the chromosomal passenger complex *in vivo* and *in vitro*. (**A**) Immunoprecipitations (IPs) of mitotic HeLa cell extracts were carried out with green fluorescent protein (GFP) antibody, and immunoblots were probed with the indicated antibodies. (**B**) IPs of chromosomal passenger complexes (CPCs) from G2 cells expressing the indicated proteins. CPCs were recovered using immobilized GFP antibody, and precipitated proteins were identified by immunoblot. (**C–F**) Survivin and Survivin$_{\Delta NES}$ bind to CPC proteins *in vitro*. *In vitro*-translated, labelled (**C**) Survivin–GFP, (**D**) Aurora-B–GFP, (**E**) INCENP or (**F**) Borealin was incubated with 4 μg of recombinant GST–Survivin–GFP or GST–Survivin$_{\Delta NES}$–GFP prebound to glutathione Sepharose beads. GST–GFP controlled non-specific binding. Bound (b) and unbound (u) fractions were separated by SDS–polyacrylamide gel electrophoresis, and the proteins were visualized by phosphorimaging. Input: Total amount of *in vitro*-translated protein added to the immobilized GST–GFP proteins. (**G**) Expression of Survivin$_{\Delta NES}$–GFP interferes with the centromeric localization of CPC subunits. In Survivin$_{\Delta NES}$–GFP-expressing HeLa transfectants, Aurora-B, INCENP and Borealin were detected by immunostaining (red). DNA was stained with Hoechst. Scale bars, 10 μm. GST, glutathione *S*-transferase; LMB, leptomycin B; ΔNES, mutant nuclear export signal; WT, wild type.

GST–Survivin$_{\Delta NES}$–GFP to the respective *in vitro*-translated CPC proteins (Fig 3C–F).

In addition, on overexpression of Survivin$_{\Delta NES}$–GFP, Aurora-B, which colocalized with Survivin–GFP at the centromeres (supplementary Fig S1A online), was not detectable at the centromeres but was found colocalized with Survivin$_{\Delta NES}$–GFP (Fig 3G). Similar results were observed for INCENP and Borealin (Fig 3G). Expression of GFP alone showed no effect on the localization of chromosomal passenger (CP) proteins (not shown). Thus, the genetic inactivation of the NES does not seem to affect significantly the ability of Survivin to dimerize or to interact with other CPC subunits. However, we cannot

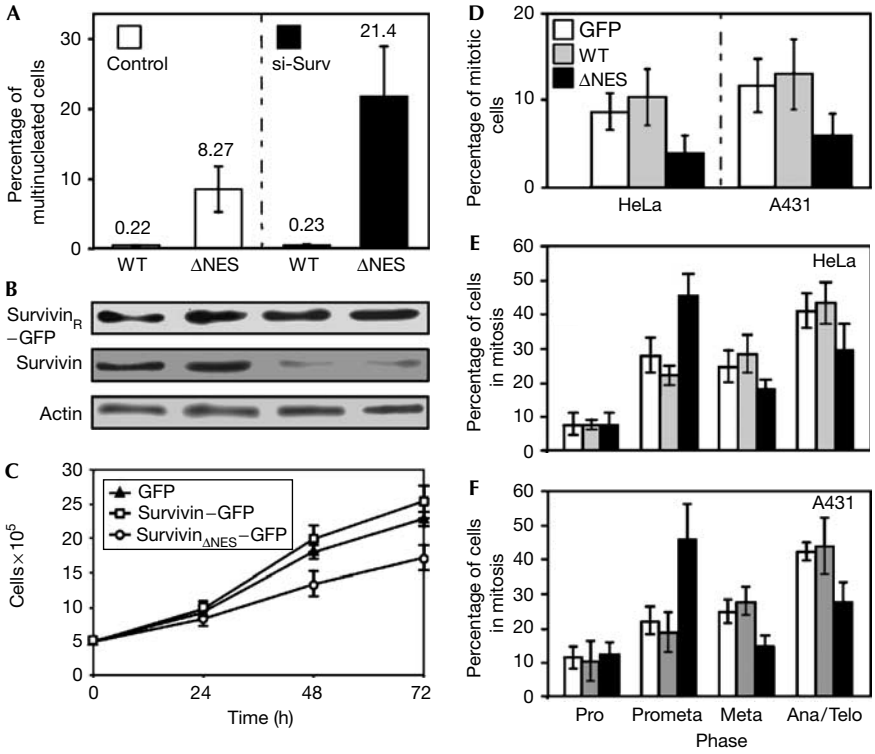

Fig 4 | The Survivin–Crm1 interaction is important for correct mitotic progression. (**A**) HeLa cells stably expressing short interfering RNA (siRNA)-resistant Survivin–GFP fusions (Survivin$_{R\text{-}WT}$–GFP or Survivin$_{R\text{-}\Delta NES}$–GFP) were transfected with Survivin siRNA (si-Surv) or a control siRNA together with an RFP expression plasmid. The number of cells with two or more nuclei was examined in 200 GFP- and RFP-double-positive cells, and the percentages of multinucleated cells were determined (mean ± s.d., $n = 3$). (**B**) Depletion of endogenous Survivin and similar expression levels were controlled by immunoblot using Survivin antibody. Actin was used as the loading control. (**C**) Proliferation of A431 cell lines expressing the indicated GFP fusions (mean ± s.d., $n = 2$). Survivin$_{\Delta NES}$–GFP-expressing cells show reduced proliferation and (**D**) less mitotic cells. Mitotic stages were analysed in (**E**) HeLa or (**F**) A431 cell lines expressing the indicated GFP proteins (mean ± s.d., $n = 3$). At least 80 GFP-positive cells were evaluated for each group. Survivin$_{\Delta NES}$–GFP expression increased the number of cells in pro-metaphase and decreased the number of metaphase and anaphase/telophase cells. GFP, green fluorescent protein; ΔNES, mutant nuclear export signal; WT, wild type.

exclude that the mutations might affect other unknown functions of Survivin.

Survivin–Crm1 interaction is required for CPC function

Various reports show several mitotic defects on interference with Survivin by RNAi or by the expression of *trans*-dominant Survivin mutants, resulting in multinucleate cells (Vagnarelli & Earnshaw, 2004). Short interfering RNA (siRNA)-resistant Survivin–GFP efficiently counteracted the formation of multinucleate cells on RNAi-mediated ablation of endogenous Survivin, whereas siRNA-resistant Survivin$_{\Delta NES}$–GFP was unable to rescue full mitosis (Fig 4A,B). The dose-dependent increase in multinucleated cells (supplementary Fig S2H,J online), the inhibition of cell proliferation (Fig 4C) and the defects in cell-cycle progression (Fig 4D–F) induced by Survivin$_{\Delta NES}$–GFP characterize NES-deficient Survivin as a *trans*-dominant protein, and underscore the biological relevance of the Survivin–Crm1 axis.

CONCLUSION

This study provides evidence for a conserved molecular link between the tethering of the CPC to the centromere and the Crm1–Ran–GTP axis (supplementary Fig S3 online). Borealin forms a complex with Survivin, which can bind to Aurora-B kinase and is incorporated into the CP-holocomplex by interacting with INCENP (Klein *et al*, 2006). The NES in Survivin mediates the recruitment of Crm1–Ran–GTP, which seems to be involved in guiding the CPC to the centromeres in early prophase by an unknown mechanism. This process might be catalysed by the activity of the guanine nucleotide-exchange factor RCC1 or of TD60 (Vagnarelli & Earnshaw, 2004). Hydrolysis of Ran–GTP, by factors such as RanBPs/Ran–GAP1 (GTPase activating protein 1), might facilitate the release of Crm1. On reassembly of the nuclear envelope, Crm1 functioning as an export receptor might further regulate Survivin function in post-mitotic cells.

METHODS

Plasmids. Eukaryotic and bacterial expression constructs for GFP- or FLAG-tagged and untagged versions of Survivin wild type (WT) and mutants were constructed by PCR and by cloning into the vectors pc3 (Invitrogen, Karlsruhe, Germany), pc3–GFP/BFP (blue fluorescent protein) or pGEX–GFP, as described previously (Knauer *et al*, 2005a; for details, see supplementary information

online). Plasmid pc3AuroraB and pc3AuroraB_GFP were constructed by PCR. Plasmids pc3Crm1–HA, pc3-RevNES–GFP and pDsRed have been described previously (Knauer *et al*, 2005b). Plasmids encoding INCENP (pBSK_hINCENP; Honda *et al*, 2003), Aurora-B kinase (pGEXAurora-B; Wheatley *et al*, 2004) and Borealin–GFP (pEGFPN1-Borealin; Gassmann *et al*, 2004) were reported.

Antibodies and reagents. The following antibodies were used: anti-Survivin, anti-CENP-A, anti-Cyclin B1, anti-TPX2 (Novus Biologicals, Littleton, CO, USA); anti-Aurora-B, anti-β-actin, anti-γ-tubulin, anti-Flag (Sigma-Aldrich, Munich, Germany); anti-Crm1 (Santa Cruz Biotechnology, Heidelberg, Germany); anti-GFP (BD Biosciences, San Jose, CA, USA); and CREST antiserum (Europe Bioproducts Ltd, Cambridge, UK). Antibodies to INCENP and Borealin were from W. Earnshaw. Antibodies to RanBP2 were from F. Melchior. Appropriate Cy-3-conjugated secondary antibodies were used (Dianova, Hamburg, Germany). Reagents were from Sigma, unless stated otherwise.

Cells, transfection and microinjection. Cell lines were maintained and prepared for microinjection or transfected, as described previously (Knauer *et al*, 2005b). Microinjection and purification of recombinant GST–GFP fusions were performed as described by Knauer *et al* (2005b). Cells were treated with 10 nM LMB.

Flow cytometry analysis and sorting. Cytometry of GFP-expressing cells was performed as described by Stauber *et al* (1998).

Immunofluorescence and imaging of cells. Immunofluorescence, observation of cells, quantification, image analysis and presentation were performed as described previously (Stauber *et al*, 1998; Knauer *et al*, 2005b). For Crm1 staining, cells were extracted with 0.005% digitonin before fixation. DNA was visualized with Hoechst 33258 or TO-PRO®-3 iodide (Invitrogen).

Immunoprecipitation and immunoblotting. Immunoprecipitation was carried out as described previously (Knauer *et al*, 2005a). For lysate preparation of mitotically enriched cells, HeLa cells were synchronized at G1/S by treatment for 24 h with 2.5 mM thymidine. Cells were then released for 12 h into medium containing 50 ng/ml nocodazole in the absence or presence of 5 nM LMB. Mitotic cells were collected by shake-off and lysed in immunoprecipitation buffer. For lysate preparation of G2-enriched cells, A431 cells were synchronized with 2.5 mM thymidine and released for 10 h before lysis. Survivin, Borealin or GFP fusion proteins were immunoprecipitated with 3 μg of Survivin, Borealin or GFP antibodies precoupled to protein G-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoprecipitated products were detected by immunoblot (Knauer *et al*, 2005a).

***In vitro* protein binding assay.** Coupled transcription and translation was performed using plasmids pc3Crm1–HA, pc3Survivin–GFP, pBSK_hINCENP, pc3Borealin and pc3AuroraB_GFP, as described previously (Knauer *et al*, 2005a). Crm1 pull-down assays with recombinant GST–GFP substrates bound to glutathione Sepharose beads and recombinant RanQ69L were carried out as described earlier (Knauer *et al*, 2005a). For details, see supplementary information online.

RNA interference. RNAi was performed using double-stranded siRNA (Eurogenetec, Searing, Belgium). The targeted regions are as follows: Crm1, 5′-TGTGGTGAATTGCTTATAC-3′; Survivin, 5′-CTGGACAGAGAAAGAGCCA-3′ (residues mutated in siRNA-resistant Survivin are underlined). Cells were treated in parallel with a non-specific control siRNA duplex: 5′-GGT GTGCTGTTTGGAGGTC-3′. siRNA duplexes (each 50 nM) were transfected together with 0.2 μg of red fluorescent protein or BFP expression plasmids, and cells were analysed after 48 h.

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

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