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Cell cycle-independent furrowing triggered by phosphomimetic mutations of the INCENP STD motif requires Plk1

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ABSTRACT
Timely and precise control of Aurora B kinase, the chromosomal passenger complex (CPC) catalytic subunit, is essential for accurate chromosome segregation and cytokinesis. Post-translational modifications of CPC subunits are directly involved in controlling Aurora B activity. Here, we identified a highly conserved acidic STD-rich motif of INCENP that is phosphorylated during mitosis in vivo and by Plk1 in vitro and is involved in controlling Aurora B activity. By using an INCENP conditional-knockout cell line, we show that impairing the phosphorylation status of this region disrupts chromosome congression and induces cytokinesis failure. In contrast, mimicking constitutive phosphorylation not only rescues cytokinesis but also induces ectopic furrows and contractile ring formation in a Plk1- and ROCK1-dependent manner independent of cytokinesis but also induces ectopic furrows and contractile ring formation in a Plk1- and ROCK1-dependent manner independent.

INTRODUCTION
Mitosis and cytokinesis are coordinated by essential kinases whose expression and activity are deregulated in many cancers (Li and Li, 2006; Malumbres and Barbacid, 2007). Cyclin-dependent kinase-1 (CDK1), Aurora A and Polo-like kinase 1 (Plk1) cooperate to regulate mitotic entry (Lindqvist et al., 2009). Subsequently, Plk1 and Aurora B direct bipolar spindle formation, spindle stability, chromosome congression and bi-orientation (Sunkel and Glover, 1988; Sampath et al., 2004; Sumara et al., 2004; Lénárt et al., 2007; Petronczki et al., 2008). At anaphase onset, as CDK1 activity drops, both Aurora B and Plk1 control cytokinesis (Ruchaud et al., 2007; Petronczki et al., 2008; Carmena et al., 2009; Hümmer and Mayer, 2009; Carmena et al., 2012; van der Horst and Lens, 2014).

Cytokinesis is the process by which cells divide to yield two daughter cells (Pollard and O’Shaughnessy, 2019). Cytokinesis initiates with cleavage furrow ingression mediated by the action of an acto-myosin contractile ring triggered by local membrane activation of the small GTPase RhoA (D’Avino et al., 2005; Piekny et al., 2005; D’Avino and Glover, 2009). RhoA drives contractile ring assembly through the activation of formin actin assembly factors and ROCK1 kinase, which subsequently phosphorylates members of the myosin regulatory light chain (MLC) family (Matsumura, 2005; Otomo et al., 2005). RhoA activation requires recruitment of the active form of the RhoGEF Ect2 to the plasma membrane (Wagner and Glotzer, 2016; Basant and Glotzer, 2018) by the centralspindlin complex composed of the kinesin-like protein MKLP1 (also known as KIF23) and the RhoGAP Cyk4 (also known as MgcRacGAP and RACGAP1) (Mishima et al., 2002; Yüce et al., 2005; Zhao and Fang, 2005; Nishimura and Yonemura, 2006; Pavicic-Kaltenbrunner et al., 2007; Lekomtsev et al., 2012).

Plk1-mediated phosphorylation of Cyk4 is essential for cleavage furrow ingression and cytokinesis in part by creating a binding site for the BRCT domain of Ect2 (Burkard et al., 2007, 2009; Petronczki et al., 2007; Wolfe et al., 2009). It also prevents premature midzone formation by regulating the activity of the microtubule-bundling protein PRC1 (Hu et al., 2012). Ultimately, Plk1 phosphorylation of PRC1 releases centralspindlin from the central spindle allowing its recruitment at the plasma membrane (Adriaans et al., 2019).

Aurora B kinase regulates many aspects of mitosis ranging from chromosome and spindle structure to the correction of kinetochore–microtubule attachment errors, regulation of mitotic progression and completion of cytokinesis (Vagnarelli and Earnshaw, 2004; Vander et al., 2006; Ruchaud et al., 2007; Carmena et al., 2012; van der Horst and Lens, 2014; D’Avino et al., 2015). Aurora B is part of the chromosomal passenger complex (CPC) composed of INCENP (Cooke et al., 1987; Terada et al., 1998; Adams et al., 2000), survivin (also known as BIRC5) and Borealin (also known as Dasra B and CDCA8) (Honda et al., 2003; Gassmann et al., 2004; Sampath et al., 2004; Klein et al., 2006). INCENP is required for Aurora B activation via direct binding and a phosphorylation feedback loop (Bishop and Schumacher, 2002; Sessa et al., 2005; Ruchaud et al., 2007), and, with Survivin and Borealin, forms a localization module for the CPC (Jeyaprakash et al., 2007; Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). Knockdown by RNA interference (RNAi) of any CPC member destabilizes the others and disrupts spindle midzone transfer and cytokinesis (Adams et al., 2001; Carvalho et al., 2003; Honda et al., 2003;
Lenses et al., 2003; Gassmann et al., 2004; Vader et al., 2006). Aurora B phosphorylation of the central spindle component MKLP1 releases it from inhibition by the PAR5 protein (14-3-3 family in mammals) and allows it to oligomerize with CytK4 (Minoshima et al., 2003; Guse et al., 2005; Basant et al., 2015).

Even prior to discovery of the CPC, INCENP was suggested to have a role in the initiation of membrane furrowing, as it could be detected at the equatorial cortex in anaphase before any detectable furrowing and before myosin had begun to concentrate in a contractile ring (Earnshaw and Cooke, 1991; Eckley et al., 1997). However, it was later suggested that INCENP and the CPC may not be essential for furrow initiation during mitotic exit (Adams et al., 2001; Guse et al., 2005; Ahoen et al., 2009). All studies agree that CPC activity is required to complete cytokinesis (Schumacher et al., 1998; Tatsuka et al., 1998; Tenada et al., 1998; Adams et al., 2001; Honda et al., 2003; Gassmann et al., 2004; Vader et al., 2006; Yue et al., 2008; Xu et al., 2009), where it plays a key role in regulating the final events of abscission (Capalbo et al., 2016; Pike et al., 2016).

To better understand the role of INCENP in regulating mitosis and cytokinesis, we have performed a functional analysis of conserved serine and threonine sites on the INCENP polypeptide that are specifically phosphorylated during mitosis. Here, we describe a highly conserved negatively charged region located in the N-terminus of INCENP IN-box whose phosphorylation is essential for cytokinesis and chromosome alignment. Surprisingly, we show that mimicking constitutive phosphorylation of this domain triggers constitutive Plk1-dependent cell cycle-independent contractile ring assembly and ectopic furrow formation.

RESULTS
Phosphorylation on INCENP during mitosis
Phosphoproteomics analyses have previously shown that a conserved motif near the N-terminal end of the INCENP IN-box is phosphorylated during mitosis while INCENP is on the spindle (Noussiainen et al., 2006; Dephoure et al., 2008; Malik et al., 2009; Oppermann et al., 2009). We used a recently described cell cycle fractionation procedure (Ly et al., 2017) to confirm that phosphorylation of this region is significantly increased in flow-sorted mitotic human TK6 cells that were histone H3 serine 28 phosphorylated (H3S28ph)-high, Cdc15+ and CycB+ (i.e. in early prometaphase; Fig. 1A–B; Fig. S1). Our analysis identified 22 phosphorylation sites on human (Hs)INCENP. Two sites at amino acids 420 and 424 show a particularly marked increase in phosphorylation. These two sites are not conserved in chicken INCENP, so we turned our attention to three other sites at S828, S831 and T832 (S749, S752, T753 in chicken INCENP class I) that also show significantly increased phosphorylation during mitosis. This very highly conserved region lies just within the IN-box, as originally defined (Adams et al., 2000), and is adjacent to the portion of the IN-box shown to bind Aurora B (Sessa et al., 2005) (Fig. 1C).

Phosphorylation on INCENP residues S752 and T753 regulates Aurora B activity and chromosome alignment
In order to determine the function of these phosphorylation events in mitotic regulation by the CPC, we expressed phosphodeficient and phosphomimetic forms of chicken (Gg)INCENP class I protein mutated at S749, S752 and T753 in an INCENP conditional (tet-off) knockout prepared in chicken DT40 (B-lymphoblastoid) cells (protocol in Fig. S2A) (Samejima et al., 2008; Xu et al., 2009). Here, we refer to cells with the genotype INCENP<sup>WT</sup> growing normally in culture as INCENP<sup>ON</sup>. ‘c’ is a conditional allele of INCENP in which the promoter has been hijacked so that both INCENP class I and II isoforms are expressed from the endogenous gene, but under tetracycline control (Samejima et al., 2008; Xu et al., 2009). [Chicken INCENP has two isoforms that differ by the insertion of 38 residues near the C’ end of the SAH domain. Either isoform can support life in DT40 cells (Mackay et al., 1993).] We refer to the cells as INCENP<sup>OFF</sup> when they are grown in the presence of doxycycline for a minimum of 24 h, by which time INCENP protein becomes undetectable in immunoblots (Fig. 2A; Fig. S2A, lane 2). Stable INCENP<sup>OFF</sup> clones expressing the mutant proteins were isolated. We selected clones in which, after shutoff of the conditional endogenous allele, the mutant proteins were expressed at levels similar to INCENP in wild-type DT40 cells for subsequent analysis (Fig. 2A). Addition of doxycycline allowed us to analyze the behavior of each mutant in an INCENP<sup>OFF</sup>-null background. Cells expressing the S749A mutant were normal in all assays tested, so this site is not discussed further here.

Immunofluorescence analysis of H3S10ph levels on prometaphase chromosomes from the various mutant cell lines suggested that Aurora B activity in vivo was reduced when INCENP was mutated at S752 and T753 (Fig. 2B–C; Fig. S2B). INCENP<sup>OFF</sup> cells expressing INCENP<sup>S752A,T753A</sup> (from here on abbreviated INCENP<sup>S752A</sup>) showed significantly reduced levels of H3S10ph similar to INCENP<sup>OFF</sup> cells. In contrast, INCENP<sup>OFF</sup> cells expressing the double phosphomimetic INCENP<sup>S752E,T753E</sup> (from here on abbreviated INCENP<sup>S752E</sup>) exhibited ~50% of H3S10ph levels of cells expressing exogenous INCENP<sup>WT</sup> (Fig. 2B–C).

The decreased H3S10 phosphorylation did not result from a lack of Aurora B binding by the various INCENP mutants or from incorrect localization of the CPC. GST pulldowns with baculovirus-expressed human His-tagged Aurora B and bacterially expressed wild-type and mutant human GST–INCENP peptides showed that in vitro, similar levels of Aurora B were bound to a C-terminal peptide from INCENP wild type (aa 880–918), INCENP<sup>S831AA</sup> or INCENP<sup>S831EE</sup> (Fig. 2D, lanes 1–3). This was confirmed in vivo by using DT40 INCENP<sup>OFF</sup> cells expressing triple affinity purification (TrAP)-tagged (Hudson et al., 2008) full-length chicken INCENP (wild type, the INCENP<sup>S752AA</sup> or INCENP<sup>S752EE</sup> mutants). Similar amounts of Aurora B kinase were pulled down in all cases (Fig. 2D, lanes 5–7). Thus, defects in Aurora B binding cannot explain the lower H3S10ph levels seen in cells expressing the INCENP mutations. Consistent with these observations, these INCENP mutants localized normally to centromeres during metaphase (Fig. 3A).

INCENP depletion severely disrupted chromosome alignment, with more than 70% of early mitotic cells having non-aligned chromosomes (Fig. 3A–B). All phosphodeficient and phosphomimetic single and double mutants failed to rescue chromosome alignment in the INCENP<sup>OFF</sup> background, with the exception of INCENP<sup>S749A</sup> and to a lesser extent the INCENP<sup>S752A</sup> mutant (between 35 and 64% misalignment compared to 21% rescue by exogenous INCENP<sup>WT</sup>; Fig. 3B). The phosphodeficient INCENP<sup>S752AA</sup> double mutant showed the most severe effects, closely resembling the INCENP<sup>OFF</sup> cells (64% and 74% misalignment, respectively). Thus, controlled phosphorylation of these residues is required for chromosome alignment.

This region of INCENP is also implicated in normal function of the spindle assembly checkpoint (SAC) in DT40 cells. The involvement of the CPC in the SAC in DT40 cells appears to be less prominent than it is in cell lines from other vertebrates (Yue et al., 2008; Xu et al., 2009), nonetheless loss of INCENP causes a significant decrease in mitotic index in cells exposed to low doses of...
taxol (Fig. S2C). This checkpoint defect was also seen in INCENP<sup>OFF</sup> cells expressing INCENP<sup>S752AA</sup> and INCENP<sup>T752EE</sup>. An example of an INCENP<sup>S752EE</sup>-expressing cell entering anaphase with an unaligned chromosome is shown in Fig. S2D.

Taken together, our results suggest that phosphorylation of INCENP on S752 and T753 represents a novel mechanism regulating Aurora B activity in vivo that is necessary for normal chromosome alignment and checkpoint function in early mitosis. We refer to this conserved domain of INCENP as the STD motif. This highly negatively charged motif at the N-terminal end of the IN-box is the most highly conserved region of the INCENP polypeptide.

**Phosphorylation of INCENP on both S752 and T753 is required for cytokinesis**

As we reported previously, INCENP<sup>OFF</sup> cells suffer profound defects in cytokinesis. This can be observed through an increase of multinucleated cells in fixed samples and by time-lapse live-cell imaging (Xu et al., 2009) (Fig. 4A–C; Movies 1–4). By 26 h...
doxycycline, 48% of surviving INCENP\textsuperscript{OFF} cells were bi- or multinucleated, compared with 3% for INCENP\textsuperscript{OFF} cells expressing INCENP\textsuperscript{WT} (Fig. 4B). INCENP\textsuperscript{OFF} cells expressing phosphodeficient INCENP\textsuperscript{ST752AA} showed failed cytokinesis to a degree similar to INCENP\textsuperscript{OFF} cells. Consistent with this, localization of INCENP\textsuperscript{ST752AA} appeared to be defective during mitotic exit—the protein was either diffuse throughout the cell or present in lower than normal amounts across the midzone (Fig. S3A). Nonetheless, these cells appeared to assemble spindles with midzones capable of supporting anaphase B spindle elongation, despite the reduced levels of INCENP (Fig. S4, Movies 3, 5 and 6). Comparison of multinucleation indexes seen with phosphodeficient single mutations INCENP\textsuperscript{ST752A} (7%), INCENP\textsuperscript{ST753A} (18.3%) and the double mutation INCENP\textsuperscript{ST752AA} (41%) suggested that phosphorylation on these two residues acts in a synergistic manner (Fig. 4B).

Remarkably, the INCENP\textsuperscript{ST752EE} double phosphomimetic mutant fully rescued cytokinesis in INCENP\textsuperscript{OFF} cells, with levels of multinucleation similar to wild type (4.6% and 3%, respectively; Fig. 4B). The localization of the mutant protein appeared to be normal late in mitotic exit, although we could occasionally observe an apparent delay in INCENP transfer from the chromosomes to the central spindle early in anaphase (Fig. S3A). As expected given the lower levels of Aurora B activity (H3S10ph) observed in INCENP\textsuperscript{OFF} cells expressing INCENP\textsuperscript{ST752EE}, cytokinesis in these cells was more sensitive to Aurora B inhibition with ZM447439 than in wild type. Treatment with 100 nM ZM447439 was sufficient to induce binucleation in INCENP\textsuperscript{ST752EE} cells whereas wild-type cells were largely unaffected by treatment with this inhibitor concentration (Fig. S3B,C).

Our data show that despite its correct localization to centromeres in early mitosis, INCENP\textsuperscript{ST752EE} does not enable the CPC to correct chromosome attachment errors but is sufficient to allow spindle transfer and cytokinesis. The low level of multinucleated cells in cultures of INCENP\textsuperscript{OFF} cells expressing INCENP\textsuperscript{ST752EE} also indicates that furrowing driven by this mutant can apparently proceed to completion in mitosis.
**INCENP**

**INCENPTT752EE triggers formation of cell cycle-independent ectopic furrows**

Live and fixed-cell analysis of INCENPOFF cells expressing INCENP TT752EE revealed a remarkable increase in plasma membrane blebbing, with ectopic furrows forming in both interphase and mitotic cells (Fig. 5). Substantial plasma membrane contractions were observed in mitosis as early as prophase (Fig. 5Ab) and, remarkably, even in interphase cells (Fig. 5Aa). Ectopic contractile furrows were also observed during cytokinesis (Fig. 5Ac). Although normal in many other respects (see below), these ectopic furrows lacked normal cortical localization of INCENP.

Using time-lapse video microscopy to track and evaluate membrane blebbing and ectopic furrowing events, we measured two different parameters: the time spent in contraction expressed as a percentage of the total movie time and the percentage of elongation that cells undergo from their resting state (Fig. 5C and Fig. 5A, respectively). We obtained a strong correlation between these two methods ($r=0.91$) suggesting that both parameters are accurate monitors of blebbing/furrowing events. This correlation is consistent with early reports showing that equatorial contraction is accompanied by polar relaxation resulting in cell elongation (Bray and White, 1988).

INCENPOFF cells expressing either INCENP TT752EE or INCENP TT752EE exhibited a remarkable increase in contractility (Fig. 5C,D). For INCENP TT752EE, we observed an 8-fold increase in the time spent in contraction (16.8±2.3% of the movie time, compared to 2.1±0.7% in control INCENPOFF cells expressing INCENPTT; mean±s.e.m.; Fig. 5C). We also measured a 3-fold increase in the degree of elongation (average of 45.5±4.4% compared to 16.9±2.1% in control cells; Fig. 5A). These values for INCENPOFF cells expressing INCENP TT752EE rose dramatically to 55.8±5.8% time in contraction and a 100.3±6.3% degree of elongation in the presence of colcemid (Fig. 5B; Fig. 5A). Thus, cells display enhanced contractility in the absence of a microtubule network. A similar increase in contractility had previously been reported for KE37 lymphoblastoid cells following colcemid treatment (Bornens et al., 1989).

Cytokineti furrowing is a classical cell cycle-coupled event, with Aurora B, Plk1 and other proteins required for cytokinesis typically accumulating in G2. We therefore determined whether the ectopic furrows triggered by INCENP STD motif mutants occur preferentially in G2 or also in other cell cycle phases. INCENPOFF cells expressing INCENP TT752EE were treated with BrdU in order to label S phase and G2 and M phase cells. Brief colcemid treatment avoided slippage of BrdU-positive cells out of mitosis (Fig. S5B). Expression of the mutant protein had no detectable effect on cell cycle progression (i.e. the percentage of the culture in S phase). A similar frequency of ectopic furrowing events was observed in BrdU negative (G1) and positive (S+G2+M) cells (Fig. 5E). Thus, furrowing induced by INCENP TT752EE can occur regardless of the cell cycle stage.

**INCENP TT752EE-dependent ectopic furrows partly resemble cytokinetic furrows**

In order to further characterize the ectopic furrows, we investigated the localization of several proteins required for contractile ring formation. We observed prominent F-actin rings in both interphase and mitotic cells undergoing INCENP TT752EE-induced furrowing (Fig. 5G). We also observed an enrichment of both exogenously expressed GFP-anillin and GFP–RhoA at constriction sites and at the cortex in INCENP TT752EE cells expressing INCENP TT752EE (Fig. 5F,G). This contrasts with the normal localization of anillin, which is localized within the nucleus of interphase cells (Field and Alberts, 1995).

Ectopic furrows almost always appeared in the vicinity of chromosome clusters in mitotic cells or close to the nucleus in interphase cells (Fig. 5; Fig. S5). This suggests that the contractile signal may be initiated on or near chromatin and then transmitted to the cell cortex. However, we cannot at this point determine whether furrowing is triggered by local action of low levels of membrane-associated CPC containing INCENP TT752EE, or whether the CPC activates a signaling pathway that acts at a distance.

The ectopic furrows exhibit at least two important differences from classical cytokinetic furrows. INCENP and the other members of the CPC normally localize to the equatorial cortex, even before furrowing (Earnshaw and Cooke, 1991; Eckley et al., 1997). In contrast, the INCENP mutant protein was not detectable at the ectopic furrows despite localizing normally to the midbody in dividing cells (Fig. 5Ac).
Secondly, the ectopic furrows do not seem to be associated with PRC1 (Fig. S5C). This can be explained by their lack of association with a central spindle. Together, the lack of INCENP and PRC1 at ectopic furrows suggests that they may frequently fail to complete abscission, as both PRC1 and the CPC have been reported to be important regulators of this process (Carmena et al., 2012; Hu et al., 2012). We note, however, that we have observed ‘cells’ lacking any detectible DNA in these cultures, suggesting that the ectopic furrows occasionally lead to abscission despite the absence of key proteins.

Taken together, our data indicate that mimicking constitutive phosphorylation on S752 and T753 in the INCENP STD motif triggers a potent contractile signal uncoupled from normal cell cycle controls. We therefore set out to further characterize this signaling pathway.

**PLk1 specifically phosphorylates INCENP T753 after priming on S752 in vitro**

Because phosphorylation of the INCENP STD motif appears to be required both for accurate chromosome alignment and for
Fig. 5. ST752EE mutant triggers the formation of ectopic cleavage furrows. (A) Immunostaining of INCENP (green) and α-tubulin (red) together with DNA (DAPI, blue) on INCENPOFF cells expressing INCENP ST752EE mutant protein. White arrowheads are pointing at ectopic cleavage furrow in formation where INCENP is not recruited. Cells in interphase (a), prometaphase (b) and cytokinesis (c) are shown. (B) Immunostaining of INCENP (green) and Plk1T210ph (red) together with DNA (DAPI, blue) on colcemid-treated INCENPOFF cells expressing wild-type, ST752AA and ST752EE INCENP mutant proteins. White arrowheads are pointing at ectopic membrane contractions. (C) Measurement of time in contraction from DIC movies (as a percentage of total movie time) on INCENPOFF cells expressing INCENP wild-type or ST752EE mutation in the presence or absence of colcemid (left graph); a minimum of 18 cells per condition were analyzed. (D) Quantification of furrowing events in INCENPOFF cells, treated in colcemid expressing wild-type, ST752AA, T753E and ST752EE mutant proteins. White arrowheads are pointing at ectopic membrane contractions. (E) Quantification of furrowing events in INCENPOFF cells expressing INCENP ST752EE in S-G2 and G1 phase of the cell cycle. Results in C–E are mean ± s.e.m. [n=3 (C,D) or n=2 independent experiments (E)]. **P < 0.01; ***P < 0.001; n.s., not significant, P > 0.05 (two-tailed unpaired t-test). (F) Immunostaining of α-tubulin (red) together with DNA (DAPI, blue) in GFP–anillin (GFP:anillin)-expressing INCENP ST752EE. Arrowheads highlight enrichment of GFP–anillin at the cortex. Scale bars: 10 μm. (G) Upper and middle row, staining of F-actin (red) together with DNA (DAPI, blue) on colcemid-treated INCENPOFF cells expressing INCENP ST752EE. Lower row, GFP–RhoA (GFP:RhoA) together with DNA (DAPI, blue) labeling of INCENPOFF interphase cells expressing INCENP ST752EE. Arrowheads highlight enrichment of F-actin and GFP–RhoA at constriction sites. Scale bars: 5 μm.
cytokinesis (Fig. 6A), we undertook a candidate approach in order to uncover upstream kinase(s) capable of phosphorylating this motif. S752 and T753 are located within putative Plk1 and casein kinase 2 (CK2) consensus sites (D/E-X-S/T-phi-X-D/E and S/T-X-X-D/E/pS, respectively) (Meggio et al., 1994; Nakajima et al., 2003), prompting us to investigate whether either of these kinases could phosphorylate the C-terminal domain of INCENP (aa 737–839) fused to GST. CDK1 was used as a negative kinase control and the triple non-phosphorylatable mutation of the STD motif (S749A, S752A and T753A) as a negative substrate control. We refer to these substrates as GST-IN:WT and GST-IN:AAA, respectively.

GST-IN:WT was very efficiently phosphorylated by CK2 (Fig. 6B) whereas GST-IN:AAA was not. Thus, the STD motif can be specifically phosphorylated by CK2. In contrast, neither Plk1 nor CDK1 phosphorylated GST-IN:WT to detectable levels (Fig. 6B). Looking at single mutants of the STD motif in CK2 kinase assays, we observed a stronger phosphorylation signal on the phosphomimic mutant of S752 (GST-IN:S752E) when compared to phosphorylation of the wild-type peptide (Fig. 6C). Building on this result, and since we previously identified a synergistic effect between S752 and T753 phosphorylation in rescuing cytokinesis (Fig. 4B), we next tested whether mimicking phosphorylation on either S752 or T753 could act as a priming event for Plk1 phosphorylation of the GST–C-term-INCENP peptide. Indeed, mimicking phosphorylation on S752 (GST-IN:S752E) strongly stimulated phosphorylation of the GST–C-term-INCENP peptide by Plk1 in vitro (Fig. 6D). Mimicking phosphorylation of T753

Fig. 6. Plk1 specifically phosphorylates Thr753 after priming on Ser752 in vitro.
(A) Diagram showing effects of INCENP mutations on S752 or T753 or both on chromosome alignment or cytokinesis. (B) GST–GgINCENP737-839 wild type (GST-IN:WT), GST–GgINCENP737-839 bearing a triple mutation S749A–S752A–T753A (GST-IN:AAA), casein or histone H1 were used as substrates in kinase assays for PLK1, CK2 and CDK1. A lower exposure is shown and the star indicates auto-phosphorylated CK2. The Coomassie gel staining shows protein loading. (C) GST–GgINCENP737-839 wild type or GST–GgINCENP737-839 bearing different mutations on the SDDSTDD motif were used as substrates in kinase assays for CK2. The Coomassie gel staining shows protein loading. (D) Phosphorylation of GST–GgINCENP737-839 wild type by PLK1 compared with GST–INCENP737-839 bearing different mutation on the SDDSTDD motif. Arrows in B–D highlight GST–GgINCENP737-839 polypeptide. (E) \(^{32}P\) signal quantification of two independent PLK1 kinase assay experiments such as in D, \(n=2\) independent experiments. Values are means±s.e.m. (F) Model showing the priming event on S752 allowing PLK1 phosphorylation on the T753 of GgINCENP.
The ectopic furrowing pathway triggered by expression of INCENPST752EE, and then examined the frequency of furrowing with the treatment with ROCK1 inhibitor Y27632 led to a 91% decrease in the time spent in contraction (Fig. 7A,B; Movie 11). Blebbistatin, a drug affecting myosin 2 ATPase activity (Straight et al., 2003), reduced ectopic furrowing by 80% compared to untreated cells, thus confirming the role of myosin II in ectopic furrow ingression (Fig. 7D). Indirect evidence supporting the involvement of anillin in the ectopic furrowing events observed during interphase is provided by the moderate (45%) inhibition of furrowing seen in the presence of the nuclear export inhibitor leptomycin B (Fig. 7F). Anillin is reported to normally be nuclear in interphase (Chen et al., 2015), but we observed cortical anillin in cells undergoing ectopic furrowing (Fig. 5F).

Taken together, these results suggest that phosphorylation of the INCENP STD motif creates a highly anionic patch that triggers a furrowing initiation pathway that is dependent on Plk1 activity and can be uncoupled from normal cell cycle controls. Ectopic furrowing induced by STD motif mutations shares many characteristics with the formation of normal cleavage furrows seen during mitotic exit.

**DISCUSSION**

Cytokinesis is spatially and temporally coordinated with chromosomal events to ensure accurate chromosome segregation (D’Avino et al., 2015; Pollard and O’Shaughnessy, 2019). This coordination is achieved at least partly through the actions of Plk1 and Aurora B kinase in the chromosomal passenger complex (CPC). Aurora B and Plk1 work together in cytokinesis to release scaffolding proteins from the central spindle, to allow them to oligomerize and to recruit the RhoA GEF Ect2 to the plasma membrane where it initiates the assembly of the contractile apparatus (Su et al., 2011; Kotynkova et al., 2016; Adriaans et al., 2019).

Here, we describe a highly conserved phosphorylated region of the CPC scaffolding protein INCENP that regulates Aurora B activity and, when mutated to mimic constitutive phosphorylation, initiates a dominant furrowing signal that is uncoupled from cell cycle controls. Our results suggest that controlled phosphorylation of this region is required for chromosome alignment and full SAC activity in early mitosis. They also suggest a novel switch mechanism in which sustained INCENP phosphorylation by Plk1 at anaphase onset may trigger furrowing initiation. Thus, the CPC may act earlier in triggering furrowing than has previously been appreciated.

**The STD motif regulates Aurora B activity and cytokinesis**

The IN-Box is a short stretch of homology between INCENP proteins in vertebrates, *Drosophila*, *C. elegans* and yeasts (Adams et al., 2000). The most highly conserved region of the INCENP polypeptide lies near the N-terminal end of the IN-Box and is extremely rich in serine, threonine and aspartate residues. We therefore term this region the STD motif. Crystal structures reveal that the IN-box drapes around the small lobe of Aurora B kinase like a crown (Sessa et al., 2005; Elkins et al., 2012). At the other end of this region the STD motif to promote Plk1 phosphorylation. Thus, another kinase must be capable of priming phosphorylation of the 9STD motif to promote Plk1 phosphorylation. CDK1, a key kinase implicated in early mitotic events is apparently not involved in this pathway, as its inhibitor roscovitine had no inhibitory effect on membrane contractility, CK2, is also apparently not essential for this pathway, as TBCA also had no inhibitory effect on membrane contractility (Fig. 7B; Fig. S6D). Thus, another kinase must be capable of priming phosphorylation of the STD motif to promote Plk1 phosphorylation.

As expected, the actin-depolymerizing drug cytochalasin B totally abolished furrowing events (Fig. 7A,B; Movie 11). Blebbistatin, a drug affecting myosin 2 ATPase activity (Straight et al., 2003), reduced ectopic furrowing by 80% compared to untreated cells, thus confirming the role of myosin II in ectopic furrow ingression (Fig. 7D). Indirect evidence supporting the involvement of anillin in the ectopic furrowing events observed during interphase is provided by the moderate (45%) inhibition of furrowing seen in the presence of the nuclear export inhibitor leptomycin B (Fig. 7F). Anillin is reported to normally be nuclear in interphase (Chen et al., 2015), but we observed cortical anillin in cells undergoing ectopic furrowing (Fig. 5F).

Taken together, these results suggest that phosphorylation of the INCENP STD motif creates a highly anionic patch that triggers a furrowing initiation pathway that is dependent on Plk1 activity and can be uncoupled from normal cell cycle controls. Ectopic furrowing induced by STD motif mutations shares many characteristics with the formation of normal cleavage furrows seen during mitotic exit.

**Ectopic contractile furrows induced by INCENPST752EE require Plk1 and ROCK1 activation**

The ectopic furrowing pathway triggered by expression of INCENPST752EE is strongly dependent on active ROCK1 and Plk1. We used small-molecule inhibitors to specifically inhibit Aurora B, Plk1, ROCK1, CDK1 and CK2 (ZM447439, GW843682X, Y27632, roscovitine and TBCA, respectively) as well as several downstream effectors in cells expressing INCENPST752EE, and then examined the frequency of furrowing in live cells and in fixed images.

Analysis of the live-cell imaging revealed that the ROCK1 pathway was essential for furrowing (Movies 7 and 10). Treatment with ROCK1 inhibitor Y27632 led to a 91% decrease in the time spent in contraction (Fig. 7A,B). We also observed a substantial inhibition of the maximum elongation upon Y27632 treatment (74% decrease; Fig. S6D). These observations were confirmed by measurements of fixed cells, in which the percentage of furrowing cells was decreased by 88% (Fig. 7C).

This analysis also revealed a critical role for Plk1 in the ectopic furrowing pathway induced by INCENPST752EE. Plk1 inhibition with GW843682X reduced the percentage of furrowing cells to the level observed in colcemid-treated INCENP OFF cells expressing wild-type INCENP (a 64% reduction; Fig. 7C; Movies 7 and 9). As with the treatment with ROCK1 inhibitor, treatment with the Plk1 inhibitor also caused a highly significant decrease in the time spent in contraction (68% decrease; Fig. 7B) and in the maximum elongation (44% decrease; Fig. S6C,D), as scored in the live-cell analysis.

Aurora B also plays a role in the ectopic furrowing. Although inhibition of this kinase did not have as dramatic an effect as the inhibition of ROCK1 or Plk1, addition of ZM447439 led to a 36% reduction in the percentage of furrowing cells and a 37% decrease in the time spent in contraction (Fig. 7B,C; Movies 7 and 8). This suggests that the ectopic signaling pathway must at least partly involve CPC activity (Fig. 7A–C; Fig. S6D).
Coincidentally, the shorter version of the IN-Box used in structural studies just misses the STD motif, beginning with its C-terminal aspartate (Sessa et al., 2005; Elkins et al., 2012). Examination of those crystal structures suggests that the STD motif is located in the vicinity of the catalytic cleft of Aurora B. This might explain why STD motif mutants negatively impact on H3S10 phosphorylation by

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Fig. 7. Ectopic cleavage furrows generated by ST752EE mutation are Plk1-, ROCK1- and Aurora B-dependent. (A) Frames, 100 s apart from time-lapse live-cell DIC imaging of INCENPOFF cells expressing INCENP ST752EE in interphase treated or not (-) with ZM447439 (ZM), GW843682X (GW), Y27632 (Y) or Cytochalasin B (CytB) in the presence of colcemid. Scale bar: 10 µm. (B) Measurement of time in contraction from DIC movies (as a percentage of total movie time) in INCENPOFF cells expressing ST752EE mutation in the presence of colcemid treated or not with ZM447439, GW843682X, Y27632, Roscovitine, TBCA or cytochalasin B; from 17 to 40 cells per condition were analyzed. (C) Quantification of furrowing events on fixed sample of INCENPOFF colcemid-treated cells expressing INCENP wild-type compared with INCENPOFF colcemid-treated cells expressing the ST752EE mutation treated with ZM447439, GW843682X or Y27632, n=3 independent experiments. (D) Quantification of furrowing events on fixed sample of INCENPOFF colcemid-treated cells expressing ST752EE mutation treated with Blebbistatin or Leptomycin B, n=3 independent experiments. Results are mean±s.e.m. *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001; n.s., not significant, P>0.05 (two-tailed unpaired t-test).
the kinase. This highly negative region could modulate the structure of the catalytic cleft or it could influence the binding or release of highly positive substrates such as the histone N-terminal tails. Phosphorylation of the STD motif appears to reflect a previously undescribed mechanism for regulating Aurora B activity.

Proper phosphorylation of the STD motif is essential for mitotic regulation. Mutations of S752 or T753 to an alanine have the strongest effects, failing to rescue both chromosome alignment and cytokinesis. Expression of phosphomimetic mutants of these residues gives more-complex results, apparently separating INCENP functions in chromosome alignment from those in cytokinesis. Single and double mutations of S752 or T753 to glutamate all fail at chromosome alignment but are not significantly different from wild-type INCENP at rescuing cytokinesis in

The INCENP STD motif is a highly conserved putative Plk1 consensus site. Previous phosphorylation analysis of human mitotic protein complexes found that phosphorylation of HsINCENP S828, S831 and T832 (corresponding to GgINCENP S749, S752 and T753) is sensitive to the specific Plk1 inhibitor BI4834 (Hegemann et al., 2011). In addition, S831 and T832 phosphorylation was downregulated in prometaphase cells either depleted of Plk1 or treated with the Plk1 inhibitor TAL (Santamaria et al., 2011).

Sequence alignment of the STD motif shows that in yeasts, only the serine (S752 equivalent) is phosphorylatable. The T753 equivalent is replaced by glutamate or aspartate in budding and fission yeasts, respectively. Nonetheless, we see the most severe effects on both chromosome alignment and cytokinesis in DT40 cells when INCENP is mutated on T753. Importantly, the overall negative charge of the domain is conserved in yeasts, suggesting that it is likely involved in electrostatic interactions related to a conserved function. This could include acting as a priming site for yeast Plk1 (Cdc5 and Plol). Thus, there may be an additional level of regulation in vertebrates compared to yeast.

The priming kinase that phosphorylates INCENP on S752 in vivo is unknown. However, CK2 can phosphorylate INCENP in vitro on both S752 and T753. CK2 was reported to be involved in early mitosis (St-Denis et al., 2009) and it could phosphorylate INCENP, priming it for subsequent Plk1 phosphorylation.

Alternatively, Plk1 may act as its own priming kinase for S752 and T753. Phosphorylation of both homologous residues in human INCENP is sensitive to Plk1 inhibition or depletion (Hegemann et al., 2011; Santamaria et al., 2011). Furthermore, we found that phosphorylation of S752 can prime Plk1-dependent phosphorylation on T753, and vice versa, although to a lesser extent. Together, these findings could point towards a feed-forward activation loop between the two sites upon Plk1 phosphorylation.

Whatever the mechanism of Plk1 activation, the kinase must act at multiple steps. Firstly, it phosphorylates the STD motif, and this is essential for both chromosome alignment and cytokinesis. Secondly, it must act again downstream of the phosphorylated STD motif, since furrowing induced by expression of INCENPST752EE was strongly inhibited following Plk1 inhibition with GW843682X.

Control of the ectopic furrowing pathway

Although ectopic furrows induced by INCENPST752EE share numerous features with normal contractile furrows, including the presence of RhoA, cortical anillin and an actin–myosin contractile ring, remarkably, furrowing occurs without local INCENP accumulation. Indeed, INCENP localization was known to be dispensable for the initiation of furrowing and furrow positioning (Mackay et al., 1998; Shannon et al., 2005). Current data do not rule out a mechanism in which INCENP and the CPC act at a distance to release a signal that initiates the furrowing pathway. The lack of a local concentration of factors originating from a spindle midzone, such as INCENP (Cooke et al., 1987; Earnshaw and Cooke, 1991) and PRC1 (Mollinari et al., 2002), could explain why furrows in cells expressing INCENPST752EE do not typically complete abscission.

Importantly, the furrowing signal from INCENPST752EE is apparently upstream of Ect2 recruitment and RhoA activation at the plasma membrane. Optogenetic activation of RhoA at the membrane is insensitive to Plk1 inhibitors (Wagner and Glotzer, 2016), whereas the INCENPST752EE pathway is blocked by inhibition of Plk1 and, more weakly, Aurora B. The latter finding suggests that Aurora B facilitates but is not essential for this signaling pathway. Indeed, phosphomimetic STD motif mutations
diminish Aurora B activity by ~50% in prometaphase cells. Thus, the novel dominant furrow-initiation pathway described here does not involve hyperactivation of Aurora B and indeed ectopic furrowing was not observed when Aurora B was overexpressed in a mouse model (González-Loyola et al., 2015).

In the normal mitotic furrowing pathway, Plk1 phosphorylation of Cyk4 creates a docking site that recruits Ect2 in the anaphase spindle midzone (Burkard et al., 2009). Plk1 then releases the complex from PRC1 (Adriaens et al., 2019) and Aurora B kinase allows its oligomerization and association with the cortex, where it can activate RhoA in a microtubule-independent manner (Basant et al., 2015). Indeed, furrowing during interphase is actually promoted by microtubule disassembly as shown here and in KE37 cells (Bornens et al., 1989). Furthermore, PRC1 regulation is unlikely to be involved in the interphase furrowing pathway, as PRC1 appears to be nuclear and is specifically expressed and activated in mitosis (Jiang et al., 1998; Mollinari et al., 2002). It is possible, however that in the presence of constitutive INCENP expression, Aurora B might function throughout the cell cycle in regulating Cyk4 oligomerization.

How the phosphorylated STD motif promotes furrowing requires further study. This extremely anionic patch could bind to a positively charged ligand to target the CPC to specific substrates or to modulate the kinase activity towards particular substrates. If phosphorylation of the patch regulates CPC binding to histones, this might help to explain the delay in INCENPST752E transfer to the spindle at anaphase onset. Whatever the target, the STD motif is critical for INCENP function, since the INCENPST752AA mutant behaves essentially as INCENP-null both for chromosome biorientation and cytokinesis.

**Conclusion**

INCENP is an essential cofactor for Aurora B kinase in the CPC. The analysis of the highly conserved STD motif presented here reveals that INCENP is also both a target of Plk1 and involved in a downstream Plk1-dependent pathway to promote plasma membrane furrowing. Thus, as speculated previously (Carmina and Earnshaw, 2006), INCENP appears to be a central player in coordinating, relaying and tuning essential signals sent by kinases orchestrating the different steps of mitosis.

**MATERIALS AND METHODS**

**Cell culture and model**

DT40 cells were grown as previously described (Buerstedde and Takeda, 1991). The INCENP conditional knockout cells were described and analyzed in our previous studies (Samejima et al., 2008; Xu et al., 2009). Doxycycline, at a final concentration of 1 µg/ml, was added to the culture medium for a minimum of 24 h to repress transcription of the INCENP gene. TK6-CDK1as cells were obtained and synchronized with 1NM-PP1 at 2 µM for 12 h as described previously (Gibcus et al., 2018; Samejima et al., 2008; Xu et al., 2009).

**Immunoblotting and antibodies**

Whole-cell lysates were prepared by lysing the cells in sample buffer. SDS-PAGE and immunoblotting were performed following standard procedures. Anti-α-tubulin antibody (clone B512, T5168, 1:2000) and anti-H3 phospho-Ser10 (06-570, 1:1000) were purchased from Sigma and Upstate Biotech (now Merck-Millipore), respectively, and anti-Plk1 pt210 from Abcam (ab39068, 1:500). Rabbit polyclonal (WCE1186, 1:500) and mouse monoclonal anti-INSEN (3D3, 1:500) were previously described (Cooke et al., 1987; Mackay et al., 1993). For F-actin staining, phalloidin conjugated monoclonal anti-INCENP (3D3, 1:500) were previously described (Cooke et al., 1987; Mackay et al., 1993). For F-actin staining, phalloidin conjugated monoclonal anti-INCENP (3D3, 1:500) were previously described (Cooke et al., 1987; Mackay et al., 1993).

**Kinase assays and pull-down experiments**

Recombinant baculovirus coding for His-tagged Plk1 was generated using Bac-to-Bac system (Invitrogen) and used to infect SF-9 insect cells. After 48 h, infected cells were pelleted and lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.5% NP-40, 0.25% deoxycholate, 1 mM PMSF, protease inhibitors, 20 mM β-glycerophosphate and 0.3 mM sodium...
vanadate), followed by a short sonication then centrifugation (14,000 g for 30 min). The clear lysate was incubated with Ni-NTA-agarose beads (Qiagen) in presence of 10 mM imidazole for 1 h at 4°C. Beads were washed twice with lysis buffer supplemented with 15 mM imidazole, once with 50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 15 mM imidazole, 0.1% NP-40, 1 mM PMSF, and once in 10 mM Tris-HCl pH 8.0, 15 mM imidazole. The kinase was eluted by incubation of the beads in 250 mM imidazole, 10 mM Tris-HCl, pH 7.5 and 150 mM NaCl then dialyzed against 10 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 5 mM β-glycerophosphate, 0.05 mM sodium vanadate, 0.1 mM ATP, and 1 μM of [γ-32P]ATP. After 30 min at 30°C, reactions were stopped by the addition of SDS sample buffer. Samples were separated by SDS-PAGE, gels dried and phosphoprotein incorporation determined by PhosphorImager. Signal quantification was performed after measuring 32P levels in two independent sets of experiments.

Various mutants of INCENP with an N-terminal Triple Tag (Hexa-His, S, SBP; TrAP) were constructed and stable DT40 INCENP conditional knockout cells expressing these constructs generated. After doxycyclin treatment, cells were lysed using 50 mM Tris-HCl pH 8.0, 250 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, protease inhibitors, 20 mM β-glycerophosphate, 0.3 mM sodium vanadate and 50 U benzonase, then sonicated. After centrifugation (20,000 g; 15 min, 4°C), lysates were incubated with pre-washed Streptavidin MyOne-C1 Dynabeads for 90 min (Invitrogen). After washing, proteins bound to the beads were separated by SDS-PAGE. For the in vitro binding assay, bacterially expressed GST, or wild-type or mutant GST–His6INCENP C-terminus (aa 820–918) bound to glutathione–Sepharose beads, were incubated with lysates from Sf9 infected cells producing His6–HsINCENP (HsINCENP established in this laboratory) (Ly et al., 2014). After washing, proteins bound to the beads were separated by SDS-PAGE. The for in vitro binding assay, bacterially expressed GST, or wild-type or mutant GST–His6INCENP C-terminus (aa 820–918) bound to glutathione–Sepharose beads, were incubated with lysates from Sf9 infected cells producing His6–HsAurora B (lysis buffer, 50 mM Tris-HCl pH 8.0, 250 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, protease inhibitors, 20 mM β-glycerophosphate and 0.3 mM sodium vanadate) (previously reported in Gassmann et al., 2004) for 2 h at 4°C followed by 30 min at room temperature. After washing twice with lysis buffer, once with 50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.1% NP-40, 1 mM PMSF and once in 10 mM Tris-HCl pH 8.0, proteins bound to the beads were analyzed by SDS-PAGE and immunoblotting.
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MCP200 assembly of CYK-4/MgcRacGAP and ZEN-4/MKLP1 to form the centralspindlin
18 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle.

RESEARCH ARTICLE


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