

Essential Roles of *Drosophila* Inner Centromere Protein (INCENP) and Aurora B in Histone H3 Phosphorylation, Metaphase Chromosome Alignment, Kinetochore Disjunction, and Chromosome Segregation

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Abstract. We have performed a biochemical and double-stranded RNA-mediated interference (RNAi) analysis of the role of two chromosomal passenger proteins, inner centromere protein (INCENP) and aurora B kinase, in cultured cells of *Drosophila melanogaster*. INCENP and aurora B function is tightly interlinked. The two proteins bind to each other in vitro, and DmINCENP is required for DmAurora B to localize properly in mitosis and function as a histone H3 kinase. DmAurora B is required for DmINCENP accumulation at centromeres and transfer to the spindle at anaphase. RNAi for either protein dramatically inhibited the ability of cells to achieve a normal metaphase chromosome alignment. Cells were not blocked in mitosis, however,

and entered an aberrant anaphase characterized by defects in sister kinetochore disjunction and the presence of large amounts of amorphous lagging chromatin. Anaphase A chromosome movement appeared to be normal, however cytokinesis often failed. DmINCENP and DmAurora B are not required for the correct localization of the kinesin-like protein Pavarotti (ZEN-4/CHO1/MKLP1) to the midbody at telophase. These experiments reveal that INCENP is required for aurora B kinase function and confirm that the chromosomal passengers have essential roles in mitosis.

Key words: mitosis • cytokinesis • chromosomal passengers • chromosomes • RNAi

Introduction

Successful mitosis depends on the coordination of chromosomal and cytoskeletal behavior. A group of proteins termed chromosomal passengers are prime candidates for a role in this coordination. Chromosomal passengers were originally defined by their dynamic distribution in mitosis (Earnshaw and Bernat, 1990). They are concentrated at centromeres during metaphase, transfer to the central spindle during early anaphase and to the cell cortex at the presumptive cleavage furrow shortly thereafter, and then concentrate at the midbody during cytokinesis.

To date, four chromosomal passenger proteins have been described in detail: inner centromere protein (INCENP)¹ (Mackay et al., 1993), TD-60 (Andreassen et al., 1991), aurora B kinase (Schumacher et al., 1998), and survivin (Uren et al., 2000; Skoufias et al., 2000). These proteins colocalize

throughout mitosis, and INCENP is stockpiled in a complex with aurora B in *Xenopus* eggs (Adams et al., 2000). An INCENP–aurora B complex has also been precipitated from cultured human cells (Kaitna et al., 2000). This complex appears to be functionally significant, as INCENP is required for the proper localization of aurora B on the chromosomes, central spindle, and midbody during mitosis. Thus, the hypothesis has emerged that INCENP may be a targeting subunit for aurora B kinases. It is not presently known whether this targeting is essential for aurora B function, whether aurora B has a role in targeting INCENP, or whether survivin and TD-60 are also part of this chromosomal passenger complex.

Two dominant-negative forms of vertebrate INCENP have been described. One of these, INCENP_{1–405}, interferes with prometaphase chromosome congression, sister chromatid disjunction at anaphase, and the completion of cytokinesis (Mackay et al., 1998). The other, CENP-B_{1–158}:INCENP_{45–839}, does not appear to interfere with prometaphase congression but inhibits the closing stages of cytokinesis, causing persistence of an intercellular bridge with a prominent midbody (Eckley et al., 1997). *INCENP* is an essential gene in the mouse (Cutts et al., 1999) and

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¹Abbreviations used in this paper: CB, cytoskeleton buffer; dsRNA, double-stranded RNA; GST, glutathione *S*-transferase; INCENP, inner centromere protein; KLP, kinesin-like protein; RNAi, dsRNA-mediated interference.

chicken (Vagnarelli, P., D. Hudson, and W.C. Earnshaw, unpublished material). Mouse embryos homozygous for a partial deletion of the *INCENP* gene die at the 32–64 cell stage, with multinucleate cells and abnormal microtubule bundling.

Aurora kinases were discovered in a screen of mitotic mutants in *Drosophila* (Glover et al., 1995). Budding yeast has a single aurora kinase, called Ipl1p (Chan and Botstein, 1993). Ipl1p is required for efficient chromosome segregation and appears to work, at least in part, by phosphorylating the kinetochore protein Ndc10p (Biggins et al., 1999). In addition, Ipl1p is required for the phosphorylation of histone H3 on serine¹⁰ during mitosis, a modification that is thought to be correlated with chromosome condensation (Hsu et al., 2000). Ipl1p interacts both genetically and physically with the budding yeast INCENP, Sli15p (Kim et al., 1999).

Metazoans, including *Drosophila*, have two (three in vertebrates) aurora-related kinases (Giet and Prigent, 1999; Adams et al., 2001). Aurora A kinases are centrosomal during mitosis, and mutants exhibit monopolar spindles with duplicated but not separated centrosomes (Glover et al., 1995). The role of *Drosophila* aurora B (ial) in mitosis has not previously been studied, however the aurora B kinases in mammals and *Caenorhabditis elegans* are essential for several aspects of mitotic progression, particularly cytokinesis (Schumacher et al., 1998; Terada et al., 1998). More recently, inactivation of *C. elegans* aurora B/AIR-2 by double-stranded RNA (dsRNA)-mediated interference (RNAi) has revealed that this kinase is required for histone H3 phosphorylation on serine¹⁰ and sister chromatid separation (Hsu et al., 2000). *C. elegans* aurora B is also required for normal localization and function of the ZEN-4/MKLP-1/PAV kinesin-like protein (KLP) during mitosis (Severson et al., 2000). It is aurora B that is in a complex with INCENP in *Xenopus* eggs.

Here, we report studies of chromosomal passenger function in *Drosophila* embryos and cultured cells. *Drosophila* INCENP and aurora B both behave as classical chromosomal passenger proteins, however they exhibit subtle differences in their targeting to chromosomes. Inactivation of INCENP and aurora B by RNAi in *Drosophila* cultured cells dramatically disrupted mitotic events with several significant differences from the results of recent studies in *C. elegans* (Schumacher et al., 1998; Kaitna et al., 2000). Our results demonstrate that chromosomal passenger function is interlinked and is essential for mitotic chromosome assembly, chromosome congression to the metaphase plate and segregation at anaphase, and cytokinesis.

Materials and Methods

Molecular Biology Methods and DNA Constructs

Standard molecular biology methods were followed throughout this study. INCENP and aurora B/ial *Drosophila* cDNAs were purchased from Research Genetics. INCENP_{1–755}, INCENP_{1–348}, and INCENP_{654–755} were amplified by PCR and cloned into pGEX 4T3 (Amersham Pharmacia Biotech). To produce pGEX-INCENP_{1–755}-His₆, an oligonucleotide encoding an His₆ tag flanked by NotI adapters was inserted into the NotI site of pGEX-INCENP. DmAurora B was subcloned into pET 22b (Novagen) into the NdeI site at the 5' end and the XhoI site at the 3' end. All constructs were fully sequenced. After expression in *E. coli* BL21 (DE3) *pLysS*, DmAurora B was purified by Ni²⁺-agarose chromatography.

Antibodies

Polyclonal anti-DmINCENP and anti-DmAurora B antibodies were produced in rabbits. Rabbits were immunized with the following gel-purified proteins: glutathione *S*-transferase (GST)-INCENP_{1–348} (R801), GST-INCENP_{654–755} (R803), or GST-DmAurora B_{1–58} (R963). Anti-INCENP sera were diluted 1:500 for use in immunoblotting and immunofluorescence. Anti-aurora B antibodies were affinity purified by incubating serum with Affigel 10 beads (Bio-Rad Laboratories) to which aurora B_{1–58} had been bound. After washes in 0.5 M NaCl, 10 mM phosphate (pH 7.4) antibodies were eluted in 0.2 M glycine, pH 2.0, and the eluate was neutralized with one-tenth volume 1 M Tris, pH 8.0, and dialyzed overnight into PBS. Antibodies were concentrated by ultrafiltration and stored in 50% glycerol at 20°C. For immunofluorescence, antibodies were used at 2 µg/ml.

Other antibodies used were anti- α -tubulin (YL1/2 rat monoclonal, used at 1:50; Harlan Sera Labs; or mouse mAb B512, used at 1:2,000; Sigma-Aldrich), anti-PAV-KLP (R3301, used at 1:500; Adams et al., 1998), anti-phosphorylated H3 (rabbit polyclonal, used 1:200; Upstate Biotechnology; or mouse monoclonal, used 1:100; NEB), anti-Cid (used at 1:2,000; gift of Steve Henikoff, Fred Hutchinson Cancer Research Center, Seattle, WA), anti-cyclin A and B (Rb270 and Rb271, used at 1:500; Whitfield et al., 1990; gift of W. Whitfield, University of Dundee, Dundee, Scotland), and mouse monoclonal antilamin (used at 1:50; gift of Paul Fisher, State University of New York, Stony Brook, NY). All fluorescently conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used according to the manufacturer's instructions. FITC-phalloidin was used at 200 nM.

For the blocking experiment, the affinity-purified anti-aurora B antibody was diluted 1:50 in PBS + 0.1% Triton X-100 (Bio-Rad Laboratories) with 10% FBS in the presence of GST-DmAurora B_{1–58} (0.3 mg/ml) and then incubated for 1 h at room temperature before being added directly to the cells and processed as usual.

Drosophila Embryos and Cells

Drosophila embryos derived from *w¹¹¹⁸* adults were fixed and processed for immunostaining exactly as described previously (Adams et al., 1998). Immunostaining of Dmel-2 cells for the RNAi experiments was performed as follows. Cells were grown in an incubator at 27°C in LAB-TEK Permax chamber slides (177429; GIBCO BRL) or transferred onto poly-Lys-treated slides and left to attach for 20 min at each time point. In both cases, slides were centrifuged for 10 min at 4,000 rpm before fixation. Cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (1.1 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 137 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1) for 10 min at room temperature, permeabilized in 0.2% Triton X-100 in cytoskeleton buffer, and rinsed in PBS. Blocking was performed for \leq 30 min at room temperature in PBS + 10% FBS. Antibody incubation was done in PBS + 0.1% Triton X-100 for 1 h at 37°C, followed by 4–6-min washes in PBS five times. DNA was counterstained with 0.1 µg/ml DAPI for 5 min at room temperature and rinsed in PBS. Slides were mounted in Vectashield and sealed using nail varnish. Three-dimensional data sets of selected cells were collected using a DeltaVision microscope (Applied Precision), based on an Olympus IX-70 inverted microscope with a cooled couple-charged device camera (CH350L, Photometrics). Data sets were deconvolved if required, projected onto a single plane and, exported as TIF files to be processed using Adobe® Photoshop™.

Microtubule Binding Assay

30 µg pure bovine tubulin (Cytoskeleton) was polymerized at 37°C in 20 µl BRB80 (80 mM Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP) by stepwise addition of taxol to a final concentration of 20 µM. Either BRB80, 5 µg GST-DmINCENP, or 5 µg GST, was added to preformed microtubules to a final volume of 30 µl. Reactions were incubated at 20°C for 30 min and then centrifuged at 50,000 *g* for 20 min through a 30% (wt/wt) sucrose cushion containing 1 mM GTP and 5 µM taxol. The supernatant was aspirated and retained, and the cushion washed three times with BRB80. The pellets were resuspended in protein sample buffer before analysis on 10% SDS-polyacrylamide gels.

In Vitro Assay for DmINCENP/DmAurora B Binding

Full-length GST-DmINCENP-His₆ was expressed in *E. coli* and purified by standard methods onto Ni²⁺-agarose beads (QIAGEN). After elution in 0.25 M imidazole, the protein was dialyzed into TEN buffer (10 mM Tris: HCl, pH 8.0, 100 mM NaCl, 2 mM EGTA) and bound to glutathione-

sepharose beads (Amersham Pharmacia Biotech). This procedure increased the yield of full-length INCENP over truncated INCENP. Beads were washed and incubated with TEN alone or 5 μ g DmAurora B for 30 min at 4°C. To remove nonspecifically bound protein, beads were washed five times in 20 volumes of TEN200 (TEN + 100 mM NaCl) containing 0.05% Triton X-100. As a control, DmAurora B was incubated with GST-coated sepharose beads. After washing, proteins were eluted by boiling in protein sample buffer and electrophoresed in 10% SDS polyacrylamide gels.

Measurement of Relative Levels of Phospho-H3 and Mitotic Chromosome Condensation

Three-dimensional data sets were collected with the DeltaVision microscope and were in the linear range of the CH350L camera, deconvolved, and the image intensities of four image planes bisecting the midplane of the mitotic chromosomes were averaged using the Quick Projection algorithm. We then measured the integrated intensity of a box, 5 pixels on a side, at the appropriate wavelengths for detection of phospho-H3 and DAPI, respectively, using the Data Inspector tool. This assumes that the amount of DNA within the 25-pixel box is proportional to the overall level of condensation of the chromatin within that box. Three measurements were taken per metaphase, as were three measurements of the local background. These values were entered in a Microsoft[®] Excel[™] spreadsheet, and the intensities at 617 and 457 nm were corrected by subtracting the local background. For control cells at prometaphase, metaphase, anaphase, telophase, and interphase, this method yields the following relative degrees of condensation: 2.4 ± 0.55 , 2.8 ± 1.26 , 2.7 ± 0.49 , 1.7 ± 0.49 , and 1.0 ± 0.27 , respectively. To produce the plot shown in Fig. 7 G, we averaged the three corrected values for each cell, sorted the DAPI measurements based on increasing DAPI fluorescence, and performed a linear regression on each data set. For this experiment, we obtained deconvolved three-dimensional data sets from 44 prometaphase cells, of which 34 were normal and 10 were “dumpy.” Average corrected phospho-H3 staining intensities were 2194 ± 1779 and 74 ± 123 for normal and dumpy chromosomes, respectively. These corresponded to DNA condensation values of 317 ± 109 and 474 ± 249 , indicating that dumpy chromosomes were not less condensed than normal-looking chromosomes.

RNA Interference

RNAi in *Drosophila* S2 and Dmel2 tissue culture cells was performed according to the published protocol (Clemens et al., 2000). Similar phenotypes were observed in both cell lines, only the Dmel2 data are shown here. 700-bp PCR fragments from the 5' end of the *DmINCENP* and *DmAurora B* genes were used as templates for RNA synthesis using the Megascript kit (Ambion). Experiments were performed in six-well plates or chamber slides. At each time point, cells from experiments and controls were collected and processed for immunoblotting or immunofluorescence. For immunoblotting, 10^6 cells were collected by centrifugation, resuspended in 50 μ l sample buffer, sonicated, and boiled for 3 min before loading on 10% SDS-polyacrylamide gels. Cell samples for immunostaining were processed as above.

Scoring of INCENP/Aurora-B Phenotype

Trypan blue (Sigma-Aldrich) was used to score nonviable cells. Growth curves were plotted considering only viable cells (levels of cell death were equivalent in all cultures). Microsoft[®] Excel[™] was used to plot all quantifications. Error bars represent the standard deviation, and all the values were plotted as numerical average. To determine the doubling time of the cells in the four experiments, we calculated the best fit for the growth curves (semilog scale), and the doubling time was calculated from the corresponding equations. Mitotic Index was determined as a percentage of mitotic cells in the total population. Mitotic stages were quantified in terms of percentage of mitotic cells. To determine the mitotic index in the presence of microtubule poisons, Dmel2 cells were incubated for 12 h in the presence of 1 μ g/ml of colcemid.

Results

Identification of DmINCENP

We identified a candidate DmINCENP cDNA (LD24414) by querying the Berkeley *Drosophila* Genome Project da-

tabase with the conserved COOH-terminal 90 amino acids of vertebrate INCENP. LD24414 was completely sequenced, and the cDNA sequence was mapped onto genomic P1 clone AC005425. The candidate DmINCENP gene maps to cytological region 43B1, contains six exons, and encodes a 2,441-bp cDNA with a continuous ORF of 2,265 bp (Fig. 1). Our sequence and exon/intron analysis agrees exactly with that described by Celera Genomics for the hypothetical gene CG12165. (Sequence data are available from GenBank/EMBL/DBJ under indicated accession nos.)

DmINCENP has a predicted molecular mass of 83.5 kD and a calculated isoelectric point of 9.63. Between residues 540 and 660, a coiled coil-forming region is predicted. The major region of homology with vertebrate INCENPs is in the COOH-terminal IN-BOX (Adams et al., 2000), a 40–50-amino acid domain that defines the INCENP family from yeasts to humans. The NH₂-terminal 540 amino acids are poorly conserved relative to vertebrate INCENPs, however it is this region that contains all the previously known functional domains for INCENP, such as the heterochromatin protein 1 (HP-1) and β -tubulin binding domains, the centromere targeting region and the spindle targeting domain (Mackay et al., 1993; Ainsztein et al., 1998; Wheatley et al., 2001).

DmINCENP and DmAurora B Kinase Are Chromosomal Passenger Proteins

To demonstrate whether the characteristic INCENP behavior was conserved in *Drosophila* mitosis, we raised antibodies to two nonoverlapping regions of the protein (Fig. 1). Both sera recognized a single protein of 110 kD on immunoblots of embryo extract (Fig. 2 A). This is larger than the predicted molecular mass of 83.5 kD but consistent with the behavior of INCENPs from other species, which also migrate anomalously on protein gels.

In syncytial embryos, DmINCENP associates with condensing chromatin during prophase (Fig. 2, B–E), before becoming focussed to the centromeric regions of metaphase chromosomes (Fig. 2, F–I, arrows). Upon entry into anaphase, the protein leaves the chromosomes to form a ring of spots between the segregating chromatids (Fig. 2, J–M). Each spot seems to be at the converging focus of bundles of microtubules (Fig. 2 K, arrow). As telophase progresses, the DmINCENP ring decreases in diameter until it becomes a single midbody-like structure between the central spindle microtubule bundles. A similar distribution of INCENP was also observed in cellularized embryos (Fig. 2 N) and Dmel2 cultured cells (Fig. 2, O–Q).

To localize DmAurora B in embryos and tissue culture cells, we raised antibodies against the NH₂-terminal 58 amino acids of the protein fused to GST. Although neither of the two sera we raised detected a protein on immunoblots of embryo extract, both recognized the recombinant protein expressed in bacteria (data not shown). The affinity-purified antibodies worked well for indirect immunofluorescence in both embryos and cells. This immunostaining is likely to be specific for DmAurora B for three reasons. First, affinity-purified antibodies gave a staining pattern essentially identical to the highly characteristic pattern seen with antibodies to aurora B kinases in higher

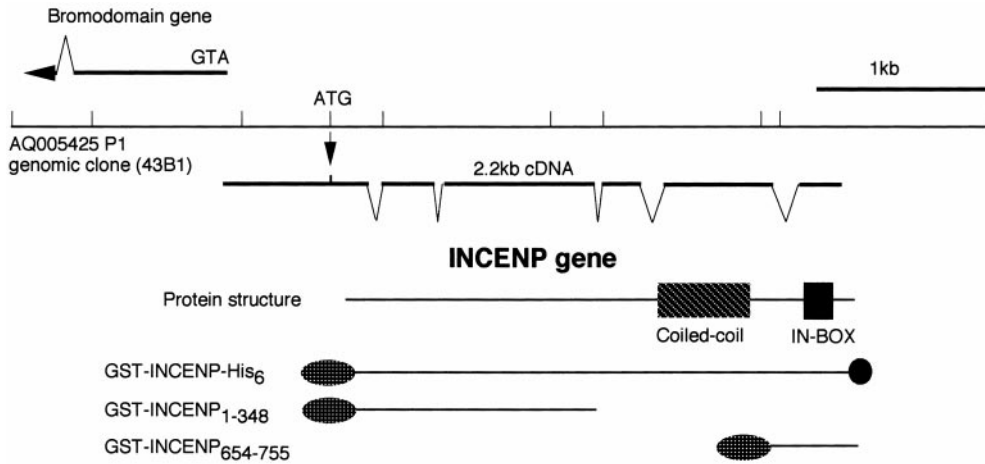


Figure 1. Map of the *DmINCENP* locus and constructs used in this study. *DmINCENP* (CG12165) is located on P1 clone, AA005425 and contains six exons. A gene encoding a bromodomain protein (CG1845) is transcribed in the opposite direction to the *DmINCENP* gene; the ORFs are separated by ~700 nucleotides. INCENP NH₂- and COOH-ter-

минаl constructs were expressed to make antigens for immunization, and full-length INCENP was expressed for microtubule binding and DmAurora B binding experiments. (Sequence data are available from GenBank/EMBL/DBJ under indicated accession nos.)

eukaryotes (Fig. 3, A–K) (Schumacher et al., 1998; Terada et al., 1998). Second, preincubation of the antibody with purified recombinant GST-aurora B_{1–58} abolished all specific staining in cultured cells (Fig. 3 L). Third, treatment of Dmel2 cells with DmAurora B dsRNA obliterated all staining in many mitotic cells after 24 h, confirming that the epitope visualized in fixed cells is the product of the *DmAurora B1al* gene (see Fig. 7 I).

The distribution of DmAurora B resembled that of DmINCENP throughout mitosis. However, interphase nuclei showed no detectable staining for DmAurora B (they did for INCENP), and as nuclei entered prophase, DmAurora B appeared first at the centromere: no staining was observed along the chromosome arms. Fig. 3 A shows a region of the embryo being traversed by a wave of mitosis. Nuclei just entering mitosis (i.e., adjacent to interphase nuclei) accumulated DmAurora B only at the centromere (Fig. 3, compare enlarged nuclei in C and D). DmAurora B remained at the centromere until the metaphase to anaphase transition, when it transferred to the central spindle and subsequently to the midbody (Fig. 3, E–H). This distribution was also observed in cellularized embryos (Fig. 3 B) and in cultured cells (Fig. 3, I–K). We conclude that DmINCENP and DmAurora B are chromosomal passenger proteins whose distribution in mitosis resembles their vertebrate counterparts.

DmINCENP Binds Directly to Microtubules and to DmAurora B

During anaphase, INCENP colocalizes with microtubules of the central spindle. Furthermore, INCENP overexpression in cultured vertebrate cells or disruption of murine INCENP leads to a dramatic remodeling of the microtubule network (Mackay et al., 1998; Cutts et al., 1999). To test whether DmINCENP binds microtubules directly, we expressed soluble full-length GST-DmINCENP in bacteria, purified it, and incubated it with taxol-stabilized microtubules prepared from purified tubulin. As a control, GST alone was incubated with polymerized tubulin. The reaction mixture was then sedimented through a sucrose cushion. GST-DmINCENP, but not GST, cosedimented

with the microtubules (Fig. 4 A). This suggests that the binding of DmINCENP to microtubules in mitosis is likely to be direct.

INCENP is stockpiled in *Xenopus* eggs in a complex with aurora B kinase (Adams et al., 2000). *Drosophila* contains two recognizable aurora-like protein kinases: the founder member of the family, Aurora (DmAurora A), which is required for centrosome separation, and the recently described DmAurora B/ial (Reich et al., 1999), whose function and localization are unknown. To determine whether DmAurora B can associate with INCENP, we incubated DmAurora B expressed in bacteria with beads laden with GST-DmINCENP. As a control, the kinase was incubated with beads carrying GST alone. Under these conditions, DmAurora B bound specifically to GST-DmINCENP but not to GST alone (Fig. 4 B).

The RNAi Method

We have used RNAi to eliminate DmINCENP and DmAurora B/ial from cultured cells. dsRNA was added to exponentially growing cultures of Dmel-2 tissue culture cells (see Materials and Methods), and at different time points samples were taken for analysis by immunoblotting and indirect immunofluorescence. Two negative controls were analyzed in parallel: cells to which no dsRNA had been added and cells to which we added dsRNA synthesized from a human intronic sequence, chosen at random to rule out unspecific effects of dsRNA on the cell cycle. The latter caused no defects and was indistinguishable from the untreated control.

Analysis of RNAi experiments is complicated by the fact that this technique causes a gradual depletion of the proteins under study, and that proteins are not necessarily lost from all cells in the population at the same rate. Furthermore, inhibition of INCENP or aurora B function causes cultures to become polyploid, and it is difficult to exclude that some of the aspects of aberrant mitosis seen in these experiments are caused by complications arising during polyploid mitosis. To minimize these complications, we examined the phenotypes described here at var-

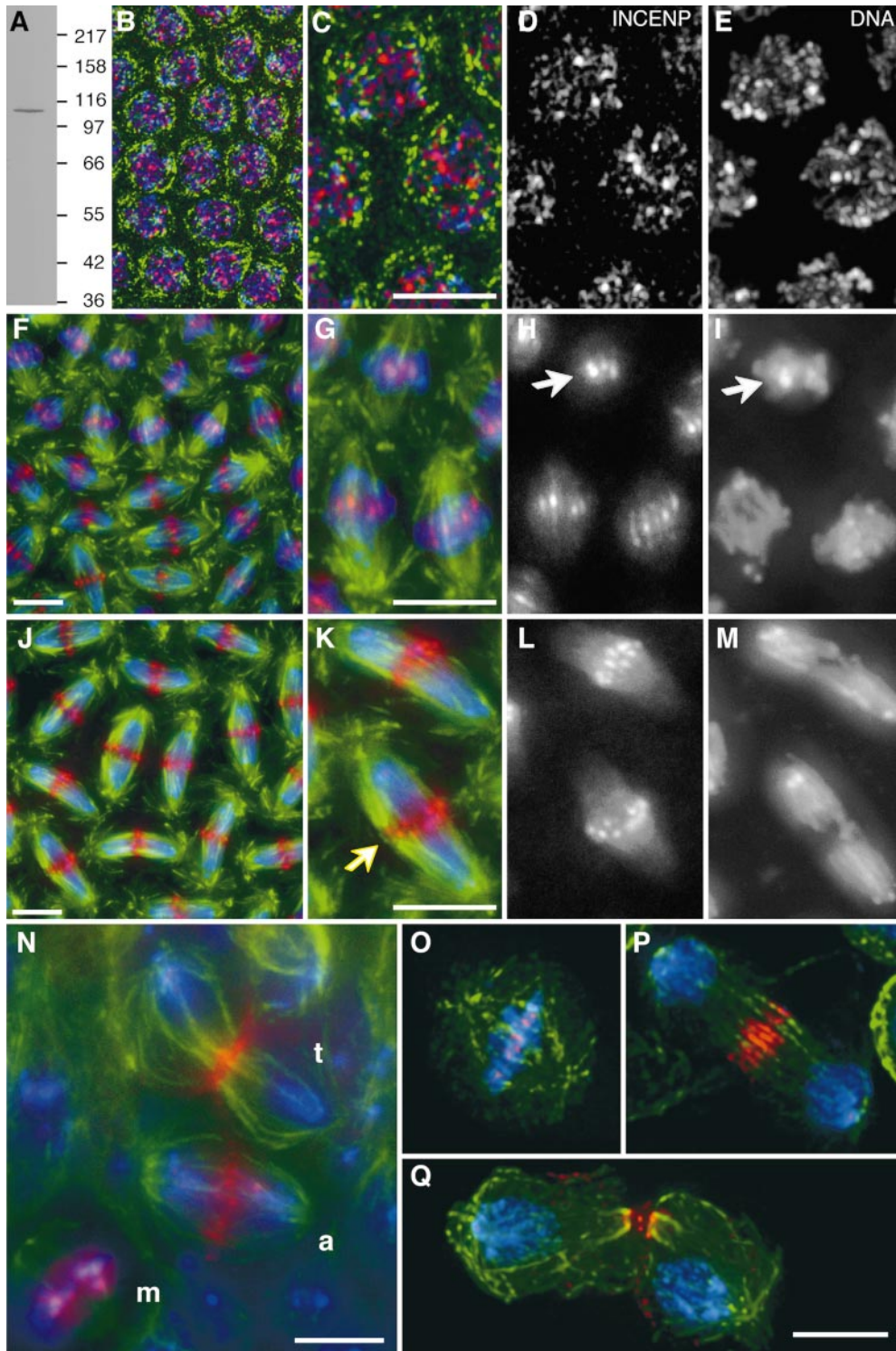


Figure 2. (A) Immunoblot of total fly embryo protein extract probed with antibody R801, raised against GST-INCENP₁₋₃₄₈. A single band of 110 kD is visible. (B–Q) Localization of DmINCENP in embryos and cultured cells. All panels are stained for INCENP with R801 (red), α -tubulin (green), and DNA (blue). (B) Syncytial prophase (note that DmINCENP is distributed along the arms of the condensing chromosomes as well as at centromeres); (F) syncytial metaphase and early anaphase; (J) syncytial anaphase. (C, G, and K) Enlargements of selected mitotic figures from B, F, and J, respectively. (D, H, and L) Corresponding INCENP staining; (E, I, and M) the corresponding DNA staining. Arrows show centromere staining in H and I, and midzone staining in K. (N) Mitotic domain of cellularized embryo showing cells at different stages of mitosis. m, metaphase; a, anaphase; t, telophase. (O–Q) Dmel2 cells at metaphase, anaphase, and telophase, respectively. Bars, 5 μ m.

ious times after the onset of RNAi treatment and could show that certain phenotypic aspects, such as defects in histone H3 phosphorylation and mitotic chromosome assembly, are observed in cells in some cases within the first cell cycle, before cultures become highly polyploid. In addition, where possible, we limited our phenotypic conclusions to cells that were demonstrably lacking INCENP or aurora B, detectable by indirect immunofluorescence.

***Elimination of DmINCENP and DmAurora B* lial Function by RNAi**

Immunoblotting analysis of cells treated with DmINCENP dsRNA showed that the levels of DmINCENP in the culture became greatly decreased 36–48 h after the addition of dsRNA to the culture (Fig. 5 A', top). In the best experiments, $\leq 95\%$ of the protein was lost. The RNAi treatment for aurora B took effect more rapidly, the protein becoming undetectable by indirect immunofluores-

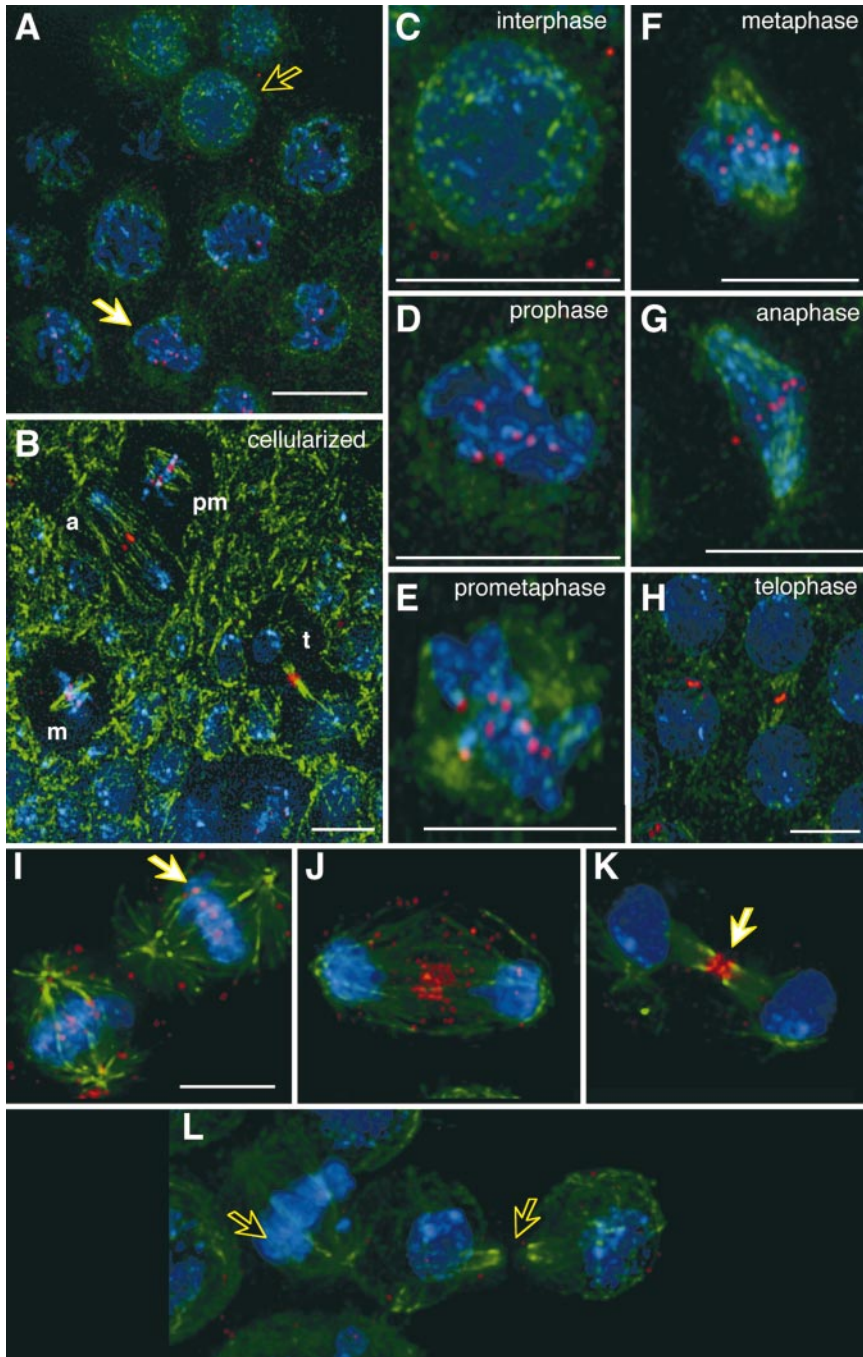


Figure 3. (A–L) Immunolocalization of DmAurora B in embryos and cultured cells. DNA (blue), DmAurora B (red), and tubulin (green) are shown. (A) Syncytial nuclei entering mitosis. Black arrow indicates a nucleus (enlarged in C) just ahead of the mitotic wave, lacking detectable aurora B staining. White arrow points to nucleus (enlarged in panel D) entering prophase, with DmAurora B accumulating at the centromere. (E–H) Localization of DmAurora B in syncytial nuclei at various mitotic stages. Note that DmAurora B is located solely at the centromeres from prophase to metaphase. DmAurora B redistributes to the spindle midzone at anaphase. (B) Mitotic domain from a cellularized cycle 14 embryo showing DmAurora B at prometaphase (pm), metaphase (m), late anaphase (a), and telophase (t). (I–K) Dmel2 staining at metaphase, anaphase, and telophase, respectively. White arrows point to centromeric (I) and midbody (K) staining. (L) Immunostaining of Aurora B is prevented by coincubation with recombinant Aurora B protein (Black arrows show absence of staining). Bars, 5 μ m.

cence in most mitotic cells by 24 h (Fig. 5 A", and see Fig. 7 H). In both cases, the loss of protein was transient, with levels beginning to recover at later times.

The phenotypes observed after INCENP and aurora B RNAi were complex, revealing defects at multiple stages of the mitotic cycle. To follow the appearance of the various phenotypes after the onset of RNAi, cultures were harvested at 24, 36, 48, and 72 h after exposure to dsRNA and assessed for the following parameters: cell number, frequency of dead (Trypan blue-positive) cells, frequency of overtly polyploid cells, mitotic index, percentage of mitotic cells negative for INCENP or DmAurora B by indirect immunofluorescence, and for the cells in mitosis, the distribution of the various mitotic phases.

RNAi treatment caused an increase in the cell doubling time from 21 h in Dmel2 cells (21.6 h in cells after exposure to control dsRNA) to 36.1 and 27.5 h in cultures after exposure to dsRNA to DmAurora B and DmINCENP, respectively (Fig. 5 B). This was accompanied by an increase in polyploid cells starting at 24 h in the DmAurora B experiment (Fig. 5 C, 36 h for DmINCENP). These correspond to the first times when significant numbers of mitotic cells were observed to be lacking DmAurora B and DmINCENP, respectively.

Strikingly, RNAi of INCENP abolished the ability of cells to achieve a metaphase chromosome alignment (Fig. 5, compare D with E). A similar phenotype was observed in the aurora B RNAi (Fig. 5 F). Instead, the population of

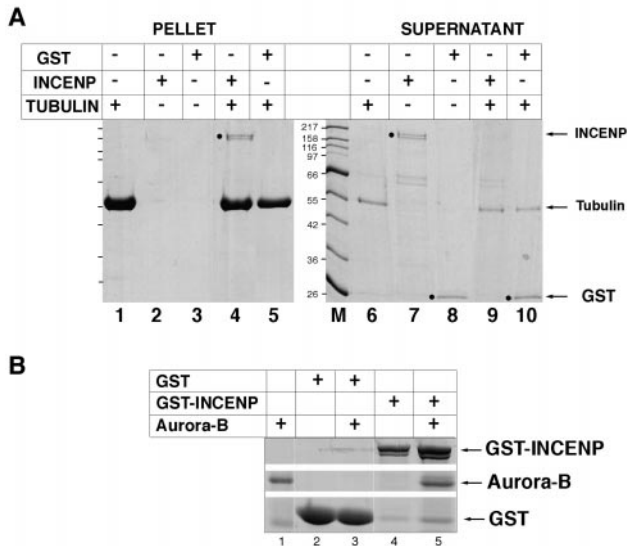


Figure 4. (A) DmINCENP binds directly to microtubules. (lane M) Molecular weight markers. (lanes 1–5) Coomassie blue–stained gel of microtubule pellet after sedimentation through a sucrose cushion. 64% of the added INCENP (lane 4) but no GST (lane 5) cosediments with microtubules. INCENP does not sediment in the absence of microtubules but remains in the supernatant (lanes 2 and 7). (lanes 6–10) Coomassie blue–stained gel of the supernatant fraction. 36% of the INCENP, but all the GST, remained unbound to microtubules (lanes 9 and 10). (B) DmINCENP binds DmAurora B in vitro. Coomassie blue–stained gel showing DmAurora B input (lane 1, 5% loaded). (lanes 2–3) GST-bound beads incubated with buffer (lane 2) or DmAurora B (lane 3, 50% loaded). No DmAurora B associates with GST beads. (lanes 4–5) GST-INCENP–bound beads incubated with buffer (lane 4) or DmAurora B (lane 5, 50% loaded). 19% of the added DmAurora B associates with GST-INCENP.

mitotic cells came to be dominated by cells with a prometaphase-like chromosome arrangement. Importantly, this increase in the percentage of prometaphase cells did not reflect an arrest in mitosis, as the mitotic index of the culture remained constant at the control level of ~5% throughout the entire experiment (Fig. 5, D–F). As discussed below, we believe that many cells in these cultures must exit mitosis directly from prometaphase without achieving a metaphase chromosome alignment.

DmINCENP and DmAurora B Are Mutually Dependent for Their Correct Localization in the Cell Cycle

INCENP is required for the correct localization of aurora B kinases in human cells and *C. elegans* early embryos (Adams et al., 2000; Kaitna et al., 2000). However, it was not known whether aurora B kinases have a role in INCENP localization.

Elimination of DmINCENP by RNAi completely abolished DmAurora B localization throughout mitosis: the protein was not detected on chromosomes, central spindle microtubules, or midbodies (Fig. 6, E and F). In contrast, DmAurora B RNAi did not block DmINCENP association with the chromosome arms during prometaphase but impaired its ability to concentrate at centromeres (Fig. 6 B) and eliminated the transfer to the midbody (Fig. 6 C).

Thus, aurora B function is not required for the initial stages of INCENP targeting to chromosomes during prophase, but it is necessary for INCENP behavior later in mitosis.

DmINCENP and DmAurora B Function Is Required for Histone H3 Phosphorylation, Mitotic Chromosome Structure, and Metaphase Chromosome Alignment

In untreated and control cultures, mitotic chromosomes invariably showed high levels of histone H3 phosphorylation on serine¹⁰, detected with a specific antibody (Fig. 7, B–D). Inhibition of DmAurora B or DmINCENP function led to both a decrease in the levels of detectable histone H3 phosphorylation (Fig. 7, A and E) and an increase in the incidence of malformed chromosomes starting as early as 24 h after exposure to dsRNA (Fig. 7 F). The phospho-H3 staining varied from cell to cell, but by 24 h after DmAurora B RNAi, the level of H3 phosphorylation was significantly reduced in 79% of the aurora-null prometaphase cells (74% of the INCENP-null cells at 36 h after DmINCENP RNAi; Fig. 7, A and E). This result suggests that, as in *C. elegans* (Hsu et al., 2000), DmAurora B is at least partially responsible for the histone H3 kinase activity in *Drosophila* cells.

Importantly, the level of histone H3-serine¹⁰ phosphorylation showed only a weak correlation with the overall degree of chromatin condensation. Fig. 7 G shows a plot in which quantitative measurements of phospho-H3 labeling and DAPI staining were compared for a series of prometaphase cells, showing a wide range of phospho-H3 labeling (see Materials and Methods). Although levels of histone H3 phosphorylation on serine¹⁰ do tend to increase with increasing chromatin condensation, it is evident that there is a huge scatter in the data from cell to cell. In other studies, we observed chromosomes completely lacking detectable aurora B kinase, which showed an apparently normal level of condensation (Fig. 7, compare H with I).

An aberrant dumpy prometaphase chromosome morphology (Fig. 7 F) was seen in 46% of INCENP-negative and 60% of aurora B–negative cells after RNAi. These dumpy chromosomes had a 28-fold lower level of phospho-H3 staining, as detected with specific antibody, than did the chromosomes with a normal morphology (see Materials and Methods). Dumpy chromosomes had an amorphous shape, and defined sister chromatids were not seen. In many cases, the dumpy chromosomes appeared to correspond to an abnormal prometaphase arrangement, characterized by a disassembled nuclear lamina (data not shown) and persistent high levels of cyclin B protein (Fig. 7, compare J with K). Although they initially appeared less condensed than normal mitotic chromosomes (Figs. 2 O, 3, I and L, and 7, B–D), in fact, their level of condensation is normal (Fig. 7 G, includes measurements from both normal and dumpy chromosomes; see also Materials and Methods). Instead, it appears that other aspects of chromosome higher order structure and behavior are aberrant. This may be due to defects in condensin binding (Giet and Glover, 2001).

Dumpy chromosomes have kinetochores, as defined by the presence of double dots of CENP-A/Cid staining. Cid is the *Drosophila* CENP-A orthologue (Henikoff et al.,

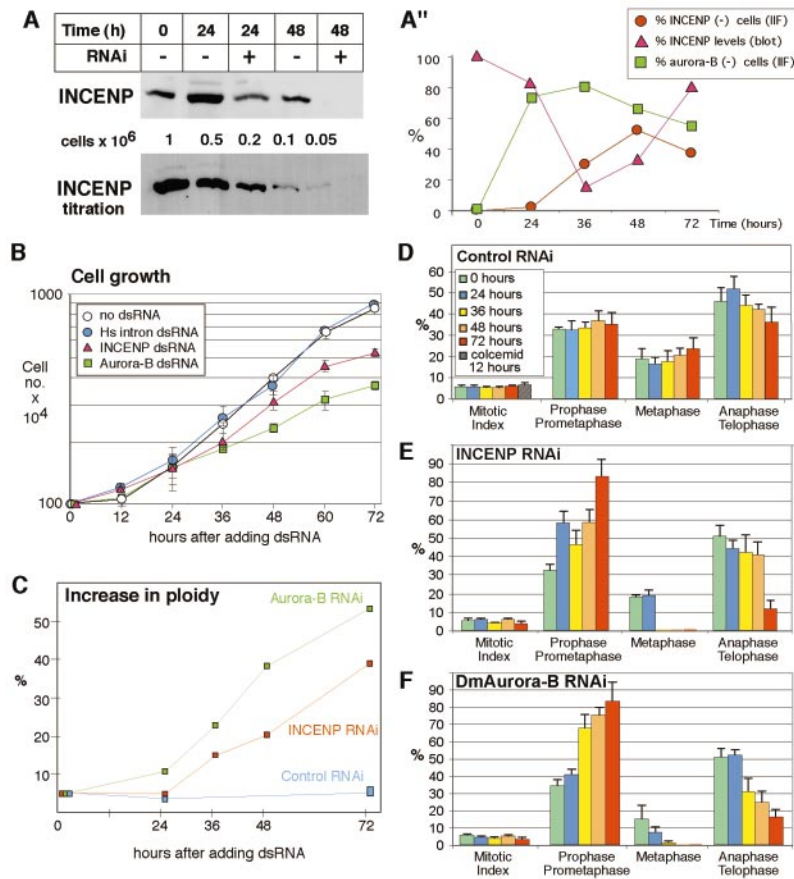


Figure 5. Summary of effects of RNAi on cell growth and mitosis. (A' and A'') Efficacy of RNAi was assessed by immunostaining (INCENP and aurora B RNAi) and by immunoblotting (INCENP RNAi only). Graph shows percentage of cells lacking detectable INCENP or aurora B staining ($n = 75$). Partially depleted cells were not scored as nulls. INCENP immunoblots were quantitated in NIH Image and normalized with respect to a tubulin-loading control, and levels were expressed as a percentage of $t = 0$ levels. (B) Growth curve of control and dsRNA-treated cells. (C) Time course of percentage of polyploid (multinucleate and abnormally large) cells. (D–F) Histograms showing the percentage of control, INCENP null, or aurora B null mitotic cells at different mitotic stages. For INCENP at $t = 0, 24$; and aurora B at $t = 0, 24$; and aurora B at $t = 0, 24$; the whole mitotic population was scored due to the scarcity of null cells. For each time point, $n = 50$.

2000), and provides a marker for the kinetochore inner plate (Warburton et al., 1997).

Role of *DmINCENP* and *DmAurora B* in Anaphase and Telophase

As expected, given the lack of normal metaphase cells, we saw few if any normal anaphase cells that were negative for INCENP or aurora B. Instead, the anaphase/telophase cells had a range of abnormalities, including anaphase-like spindles with chromosomes distributed along their length (Fig. 8, A and B), cells in various states of attempted cytokinesis with large amounts of amorphous lagging chromatin draped out behind the segregating chromatin (Fig. 8, C and D), and bizarre cells in which banana-shaped nuclei were surrounded by a mitotic-like bipolar microtubule array (Fig. 8, E–G). Fig. 8 E shows two adjacent mitotic cells in the INCENP RNAi, one of which is still expressing INCENP and is at normal metaphase, and the other of which is INCENP negative and is forming an elongate banana-shaped nucleus.

In cells with chromosomes distributed along the spindle or with banana-shaped nuclei, centromeres were seen to cluster either near opposite poles (Fig. 8, A–D) or at the opposing pointed ends of the elongate nuclei (Fig. 8, F and G). This strongly suggests that kinetochores had attached to microtubules and that anaphase A movement of chromosomes had occurred.

In cells that appeared to be in telophase, we often noticed one or more pairs of centromeres that appeared to be

stalled midway between the spindle poles (Fig. 8, C and D; and data not shown). This organization is what would be predicted if these centromeres had successfully become bioriented but were then unable to disjoin at the onset of anaphase chromosome movement. This was never seen in normal anaphases where the centromeres were typically grouped in a tight cluster at the leading edge of the segregating chromatids (Fig. 7, C and D). Consistent with difficulties in disjunction of sister kinetochores, we also saw numerous paired kinetochore spots near the spindle poles, as though nondisjoined chromatid pairs had moved together to a single pole (Fig. 8, A'', B'', and F'', double arrows).

With increasing time after RNAi treatment, we observed a dramatic increase in the number of polyploid cells in both the INCENP and aurora B RNAi so that by 72 h most of the cell population had become highly polyploid (Fig. 5 C). The simplest explanation for the origin of the many binucleate cells that we observed (Fig. 9, C and F) is that chromosome segregation and nuclear reassembly occurred, but that cytokinesis was then defective. We also observed cells with one giant nucleus (Fig. 9 G). These are likely to have arisen as a consequence of repeated failures in chromatid segregation. In addition to the chromosomal defects, we also observed spindle abnormalities in INCENP and aurora B RNAi.

Together, these observations suggest that *DmINCENP* and *DmAurora B* might be essential for a variety of anaphase/telophase events, including sister chromatid and kinetochore disjunction, chromosome structure during anaphase, and mitotic spindle architecture.

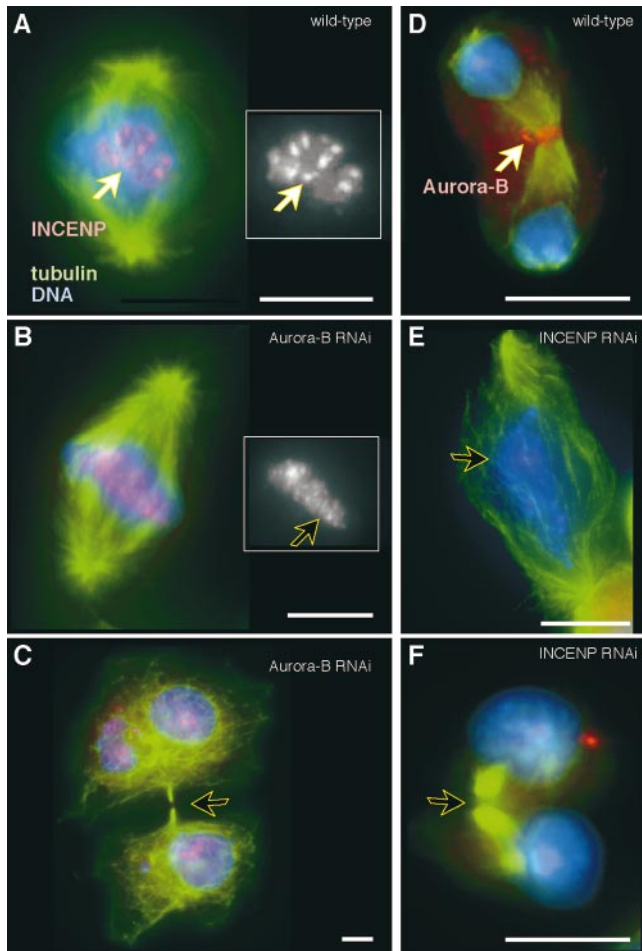


Figure 6. INCENP and DmAurora B are mutually dependent for their correct localization. (A) Control metaphase showing INCENP (red) in discrete centromeric spots. (B) Rare metaphase from DmAurora B dsRNA-treated cells. INCENP is more diffusely distributed on the chromosome arms than in controls. Boxes show INCENP staining alone. (C) After DmAurora B RNAi, INCENP does not transfer to the spindle midzone or midbody (arrowhead) at telophase. (D) Control telophase with DmAurora B (red) at the midbody. After INCENP RNAi, DmAurora B is completely delocalized from the chromosomes (E) or from the midbody at telophase (F). Throughout, microtubules are green; DNA, blue). Bars, 5 μ m.

DmINCENP Is Not Essential for the Initiation of Cytokinesis

It was possible to observe cells lacking detectable DmINCENP in which constriction of the cleavage furrow had advanced considerably and a midbody had formed (Fig. 9 B). These cells showed an accumulation of actin at the cleavage furrow similar to that in untreated cells (Fig. 9, D and E), although more actin was dispersed throughout the remainder of the cell than normal. In binucleate cells, there was no longer a focus of actin staining between the nuclei, indicating that the contractile ring had disassembled (Fig. 9 F). In contrast, binucleate cells consistently showed an abnormally high density of tubulin between the two nuclei (Fig. 9 C). This is likely to be a remnant of the central spindle.

Pavarotti, an Essential Spindle Midzone KLP Localizes Normally to the Midbody Despite Disruption of the Chromosomal Passenger Complex

In aurora B/AIR-2 ts mutants of *C. elegans*, the kinesin-related protein ZEN-4 fails to localize properly, and a spindle midzone fails to form (Severson et al., 2000). As a result, cytokinesis begins, but the furrow regresses, and binucleate cells are produced. A similar phenotype is seen with the ZEN-4 ts mutant. In *Drosophila*, however, the ZEN-4 homologue PAV-KLP appears to act at an earlier stage, as *pavarotti* mutants do not form a stable contractile ring and fail to initiate cleavage (Adams et al., 1998).

In untreated cells, PAV-KLP was invariably associated with the central spindle throughout cytokinesis (Fig. 10 A). In DmINCENP depleted cells, PAV-KLP staining was present at the midbody of 94% of cells undergoing cytokinesis ($n = 95$), however the staining was occasionally weaker than in untreated cells. To monitor the effect of the DmAurora B RNAi on PAV-KLP localization, dsRNA-treated cells from the same well were split and stained for DmAurora B and PAV-KLP on the same slide. In 90% of telophases, PAV-KLP was detected at the midbody, whereas DmAurora B staining was absent from 80% of telophases (Fig. 10, C and E). PAV-KLP was occasionally present in binucleate cells, where the cleavage furrow had regressed (Fig. 10 D).

We conclude that PAV-KLP localization is relatively unchanged after the loss of DmINCENP or DmAurora B, at least in cells that form recognizable midbody structures.

Discussion

We have characterized the mitotic distribution and function of *Drosophila* homologues of the chromosomal passengers INCENP and aurora B in early embryos and cultured cells. These studies reveal that INCENP and aurora B have essential roles in mitotic chromosome assembly, chromosome alignment at the metaphase plate, chromatid segregation at anaphase, and the completion of cytokinesis.

DmINCENP and DmAurora B Are Chromosomal Passenger Proteins That Are Interdependent for Their Targeting during Mitosis

INCENP targeting in mitosis depends on motifs at the NH₂ terminus of the protein (Mackay et al., 1993; Ainsztein et al., 1998). Thus, despite its highly divergent NH₂-terminal amino acid sequence, DmINCENP acts like a classical chromosomal passenger protein, appearing first along the chromosome arms during prophase and then subsequently accumulating at centromeres and transferring to the central spindle and midbody. This is true both during the early syncytial mitoses in *Drosophila* and in cultured Dmel2 cells. This is the first localization of INCENP in normal cells during development: previous studies have all used a variety of transformed cell lines. DmAurora B is also a chromosomal passenger but shows a subtle difference from DmINCENP in its behavior early in embryonic mitoses: DmAurora B is not chromosome associated during early prophase and, when it is first detected during late prophase/early prometaphase, it is already concentrated at centromeres.

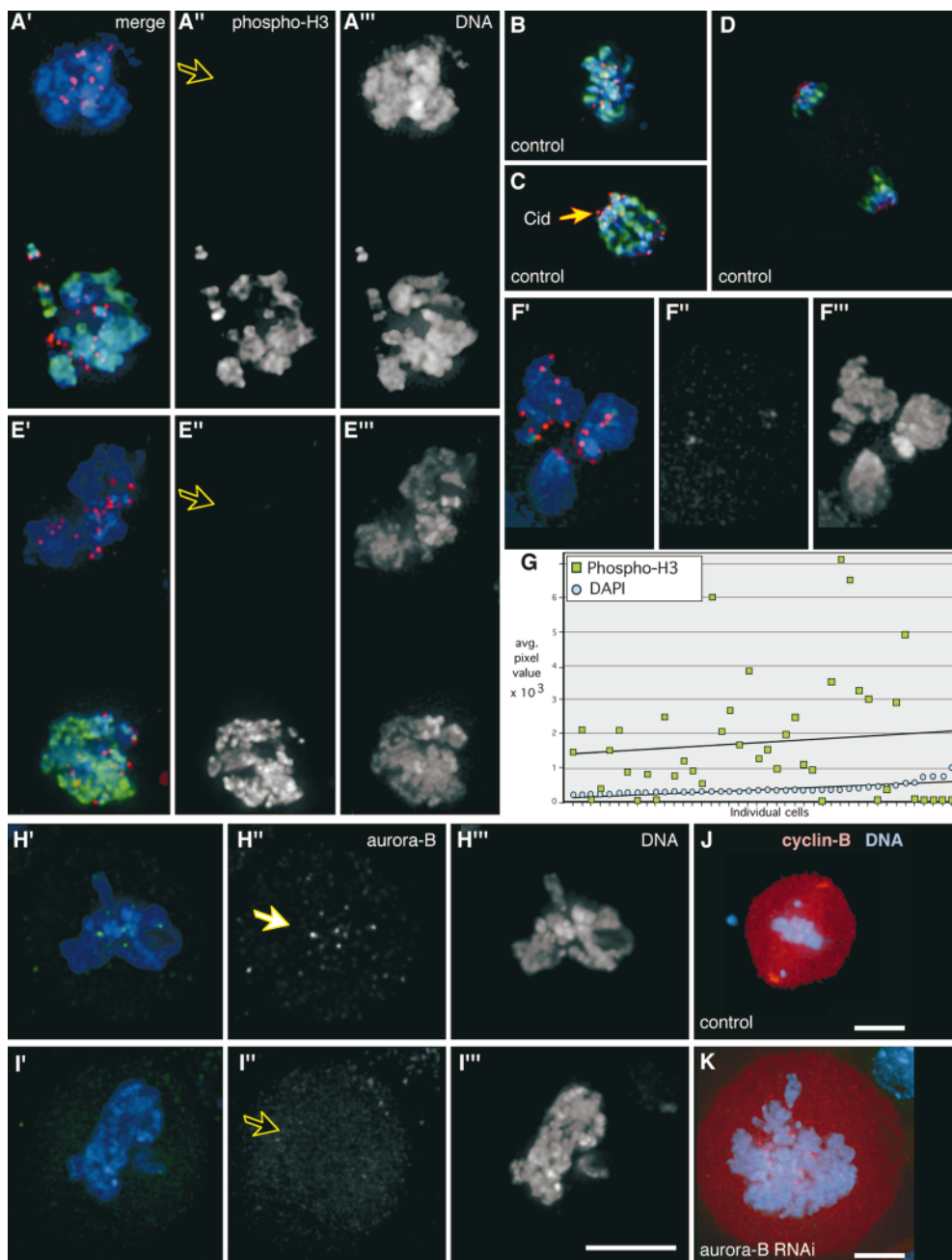


Figure 7. INCENP and DmAurora B are required for histone H3 phosphorylation, but not binding of kinetochore protein CENP-A/Cid. (A–F) Phospho-H3 (green) and CENP-A/Cid (red). (A) Condensed chromosomes lacking detectable phospho-H3 (two cells from the same image, INCENP RNAi, 36 h). (B–D) Normal mitotic chromosomes labeled for CENP-A/Cid and phospho-H3: metaphase (B), early anaphase (C), late anaphase (D). (E) Condensed chromosomes lacking detectable phospho-H3 (two cells from the same image, aurora B RNAi, 36 h). (F) Dumpy chromosomes with decreased phospho-H3 (aurora B RNAi, 36 h). (G) There is only a weak correlation between levels of detectable phospho-H3 (green boxes) and local chromatin condensation (blue circles). Note the extreme scatter in the levels of phospho-H3. Ordinate: average pixel intensity. Abscissa: each paired blue circle and green box represent the average for a different cell of three background-corrected measurements of the pixel intensity at 457 and 617 nm, respectively. 457-nm measurements were sorted in ascending order based on pixel intensity. (H and I) Similar levels of chromatin condensation in mitotic cells expressing (H) or lacking (I) Dm Aurora-B (green, both

from aurora B RNAi, 36 h). (J and K) High levels of cyclin B protein (red) in prometaphase cells: control RNAi (J) and aurora B RNAi (K). ', indicates merge; ", indicates phospho-H3 or aurora B; '", indicates DNA (DAPI). Bars, 5 μ m.

Here, we have shown for the first time that DmAurora B is required for some, but not all, aspects of INCENP localization in mitosis. In the absence of DmAurora B, INCENP localizes normally to chromosomes during prophase; however, it is subsequently unable to concentrate at centromeres and transfer to the central spindle or midbody. As predicted from previous studies, INCENP is essential for aurora B targeting: after INCENP RNAi, DmAurora B does not localize to chromosomes, midzone microtubules, or midbodies. Thus, the chromosomal passenger proteins are interdependent on one another for proper targeting during mitosis.

This interdependence, plus the fact that the two proteins are stockpiled in an 11S complex in *Xenopus* eggs, suggests that they could function in vivo in a protein complex.

DmINCENP binds microtubules in vitro and may be responsible for targeting aurora B to the central spindle, as the kinase appears to lack microtubule binding activity of its own (data not shown). However, the differences in centromere targeting in *Drosophila* early embryos suggest that the two proteins may not function in an obligate complex, at least during prophase.

DmINCENP Is Required for DmAurora B to Function Efficiently as a Histone H3 Kinase and for Mitotic Chromosome Assembly

S. cerevisiae aurora/Ipl1p and *C. elegans* aurora B/AIR-2 are required for H3-serine¹⁰ phosphorylation in mitosis

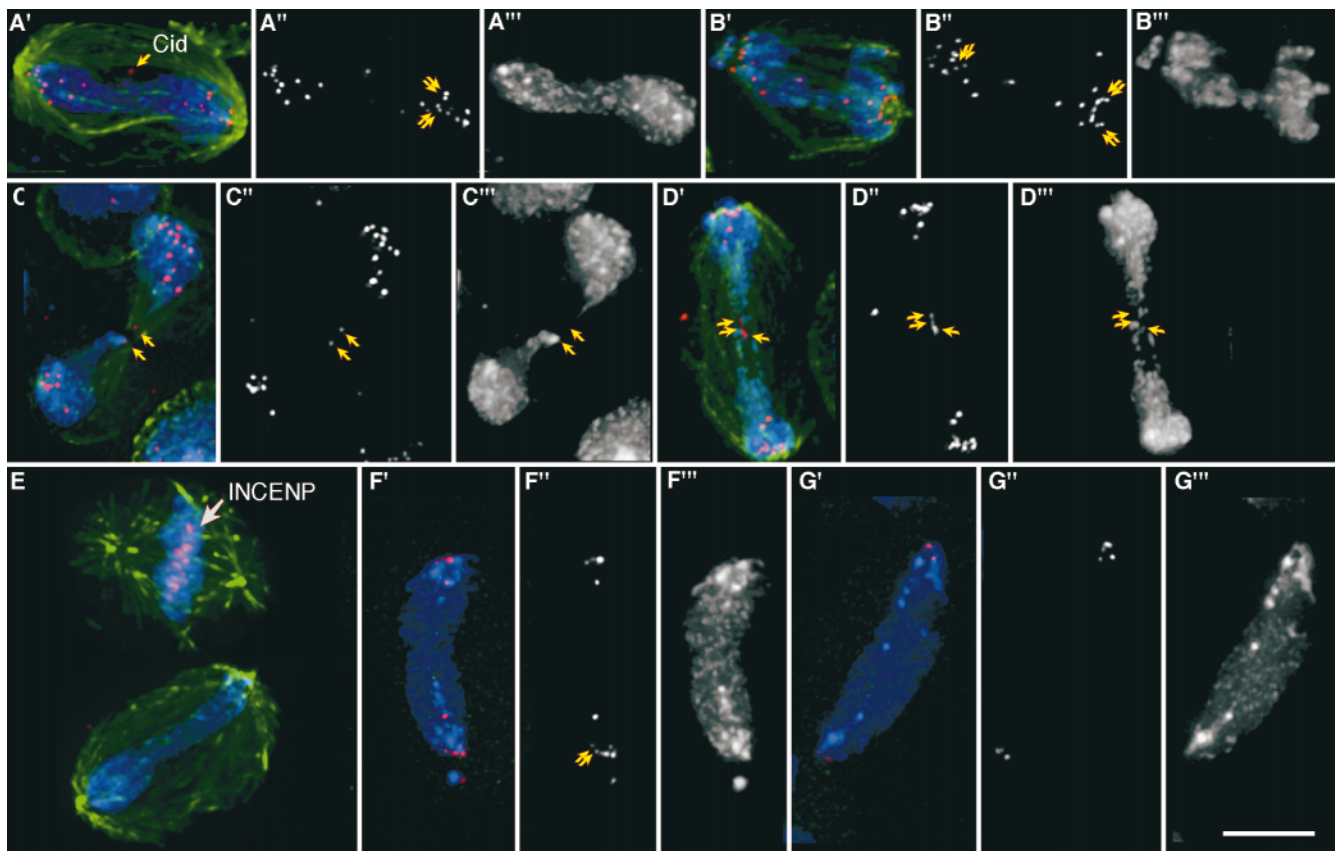


Figure 8. Aberrant centromere disjunction and lagging chromatin in the INCENP and aurora B RNAi. (A and B) Aberrant anaphases with paired CENP-A/Cid spots (red) at poles and lagging chromatin (INCENP RNAi, 36 h). (C and D) Aberrant anaphases with Cid spots at the midbody/midzone (INCENP RNAi, 36 h). (E) Metaphase and telophase/G1 cell, the latter showing an elongate nucleus with a bipolar microtubule array (INCENP RNAi, 36 h). The metaphase cell is still expressing INCENP, which is not detected in the aberrant telophase/G1 cell. (F and G) Banana-shaped nuclei similar to those shown in E, showing well-separated clusters of Cid spots joined by a continuum of chromatin (aurora B RNAi, 36 h). ', indicates merge (green, α -tubulin); '', indicates CENP-A/Cid with double arrows showing paired Cid spots; ''', indicates DNA (DAPI). Bars, 5 μ m.

(Hsu et al., 2000). Here, we have shown that not only is INCENP essential for the proper targeting of aurora B in mitotic cells, but this targeting is required for normal levels of histone H3 phosphorylation on serine¹⁰. This is the first evidence that INCENP is an essential cofactor required for aurora B kinase function in vivo.

The availability of mitotic cells containing chromosomes with a range of levels of H3 phosphorylated on serine¹⁰ enabled us to assess the widely held hypothesis that H3 phosphorylation is correlated with the degree of chromatin condensation. When phospho-H3 levels and the degree of chromatin compaction were compared by quantitative fluorescence microscopy, only a weak correlation between the two values was observed. Instead, interference with INCENP and aurora B function appears to correlate much more strongly with difficulties in assembling mitotic chromosomes of normal morphology. Mitotic chromosomes deficient in phospho-H3 had a characteristic dumpy morphology, with no evidence of resolved sister chromatids. This resembles the defects seen in *Drosophila* mutants in the SMC4 subunit of condensin (Steffensen et al., 2001) and when a *ts* mutant in *C. elegans* aurora B/AIR-2 entered mitosis at nonpermissive temperature (Severson et al., 2000). Phosphorylation of histone H3 or another chromosomal substrate by aurora B might be required for the

binding of condensins (Giet and Glover, 2001) or other chromosomal proteins that give mitotic chromosomes their characteristic morphology.

INCENP and Aurora B Are Required for Mitotic Chromosomes to Achieve a Stable Metaphase Alignment

At later times, after addition of dsRNA, we observed a dramatic increase in the percentage of mitotic cells in prometaphase coupled with a corresponding decrease in the number of metaphase cells. This was particularly dramatic in the INCENP RNAi, where we failed to observe any INCENP-negative cells in metaphase. Surprisingly, this did not lead to an increase in the mitotic index of the cultures. Therefore, in the absence of INCENP and/or aurora B function, *Drosophila* Dmel2 cells must exit mitosis from prometaphase. Elimination of INCENP and aurora B function does not trigger a mitotic checkpoint in Dmel2 cells. However, as these cells do not arrest in mitosis in colcemid (Fig. 5 D), they appear to lack a robust metaphase checkpoint anyway.

What is the ultimate fate of these prometaphase cells? We have excluded the possibility that they are removed by cell death. Although the number of dying cells in the culture showed a transient increase at 12–24 h in all cultures,

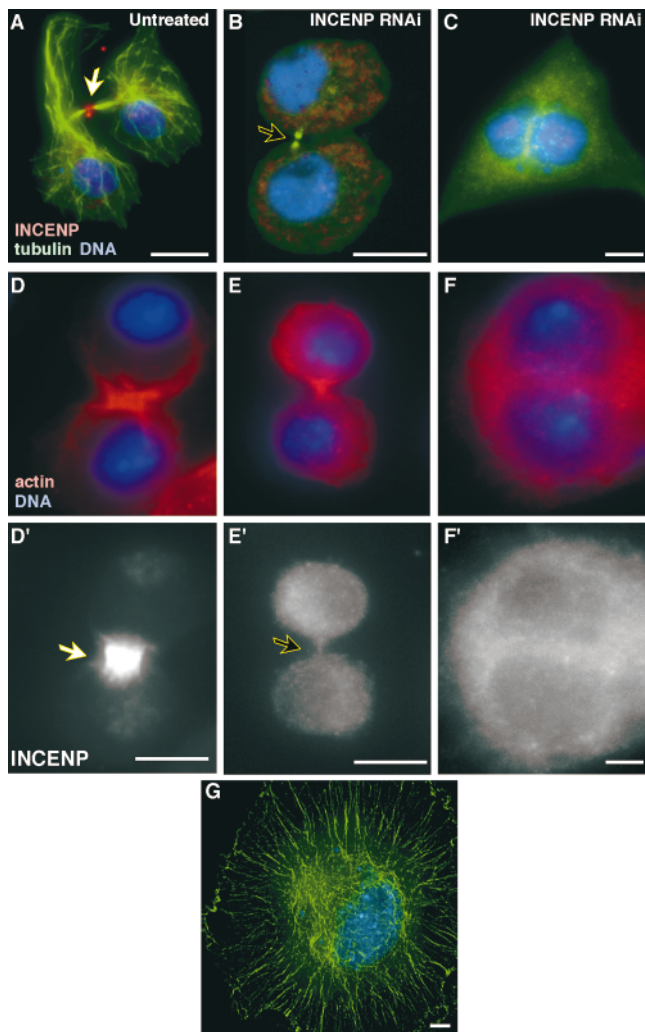


Figure 9. Removal of DmINCENP causes cytokinesis defects. (A, D, and D') Telophase figures from untreated cells. All other panels show cells after INCENP RNAi treatment. (A–C) Merged images with INCENP (red), tubulin (green), and DNA (blue). After INCENP RNAi, INCENP (red) is absent from the midbody at telophase in B (arrowhead) compared with untreated cell in A. (C) Example of microtubule structure remaining between two nuclei after a failed cleavage. (D–F) Merged images with actin (red) and DNA (blue). Actin accumulation at the cleavage furrow in late telophase is unaffected by removal of INCENP (compare D with E). However, actin is not present between nuclei in binucleate cells (F). (D'–F') The corresponding INCENP images. INCENP is efficiently removed by the RNAi procedure. (G) Grossly polyloid cell 72 h after INCENP RNAi. Radial arrays of microtubules extend to the cell cortex in these greatly enlarged cells. Bars, 5 μ m.

including controls (presumably due to some aspect of the serum-shock protocol used to induce dsRNA uptake), it then declined and remained constant in all cultures at \sim 5% throughout the remainder of the experiment (data not shown).

An alternative explanation for the lack of an increase in mitotic index would be if the cells transit directly from prometaphase into anaphase or telophase, as is the case for budding yeast cells mutant in the essential kinetochore protein Ndc10p (Goh and Kilmartin, 1993). Consistent

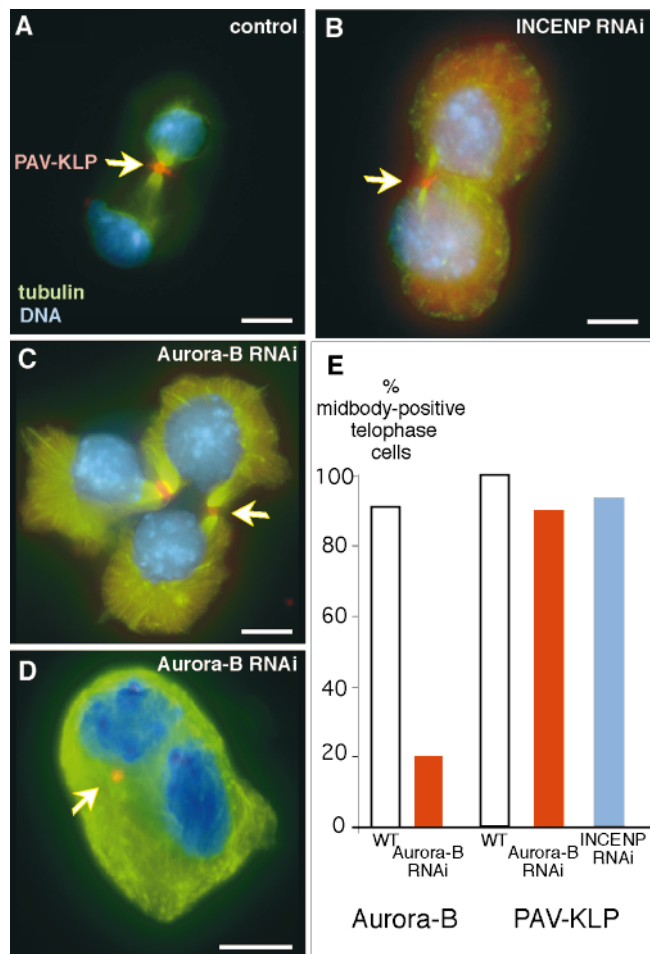


Figure 10. Pavarotti-KLP distribution is normal after DmINCENP or DmAurora B RNAi. (A) Wild-type cell showing PAV-KLP (red) at the midbody, tubulin (green), and DNA (blue). (B) PAV-KLP (arrow) is present at the midbody after INCENP RNAi. (C and D) Aberrant telophases after DmAurora B RNAi. (C) A highly abnormal polyloid cell with two midbodies, both positive for PAV-KLP (arrowhead). (D) A binucleate cell, after a failed cytokinesis, in which a spot PAV-KLP is visible at an internalized midbody remnant (arrowhead). (E) Histogram showing the percentage of telophase cells with positive midbody staining for DmAurora B or PAV-KLP in control or dsRNA-treated cells. Although DmAurora B RNAi removes DmAurora B staining from 80% of cells (left), PAV-KLP staining is removed from only 10% of telophase cells compared with control cells (right, red, $n = 30$). In the INCENP RNAi, PAV-KLP is detected at the midbody in 94% of telophase cells (blue bar, $n = 95$). Bars, 5 μ m.

with this, we saw a variety of striking abnormalities in cells either undergoing anaphase, or early in the next cell cycle (see below). Although we could observe anaphase/telophase cells with kinetochores at opposite poles of the chromatin mass, the kinetochores were often not clustered as tightly as normal (compare Fig. 7 D with Fig. 8 D). This may reflect the initiation of anaphase movement without prior alignment of the chromosomes at a metaphase plate.

Why does abrogation of INCENP and/or aurora B function prevent cells from attaining a stable metaphase chromosome alignment? One obvious possibility is that kinetochore function is impaired, much as when anticentromere

antibodies from patients with scleroderma spectrum disease are microinjected into human cultured cells (Bernat et al., 1990) or when cells express the Herpes virus protein ICP0, which causes the destruction of kinetochore proteins CENP-A and CENP-C (Everett et al., 1999). In budding yeast, the aurora kinase Ipl1p phosphorylates the essential kinetochore component Ndc10p (Biggins et al., 1999). It is therefore possible that, in metazoans, one or more kinetochore components must be phosphorylated by aurora B in order for kinetochores to function in mitosis. An obvious candidate for this is CENP-A/Cid. CENP-A retains a site homologous to serine¹⁰, which is serine⁵ in Cid (Henikoff et al., 2000). It will be important to determine whether CENP-A/Cid is phosphorylated in an aurora B kinase-dependent manner.

Arguing against this model is our observation that kinetochores assemble correctly, at least as far as CENP-A/Cid binding is concerned, and move to the spindle poles at anaphase/telophase. This implies that the ability of kinetochores to bind microtubules and to undergo anaphase A movement are preserved after abrogation of INCENP and aurora B function. However, other aspects of kinetochore function, namely the ability to form bipolar spindle attachments and disjoin at anaphase (see below), appear to be defective. How RNAi of INCENP or aurora B leads to defects in chromosome biorientation is unknown, but this is unlikely to be a result of interference with binding of the condensin subunit barren, as *barren* mutants successfully complete metaphase chromosome alignment (Bhat et al., 1996). Furthermore, we cannot exclude the possibility that some of the abnormal aspects of chromosome behavior reflect an impairment of microtubule and/or spindle function. The detailed behavior of the mitotic spindle after RNAi of INCENP and aurora B requires further analysis.

Normal INCENP and/or Aurora B Function May Be Required for Sister Kinetochore Disjunction and Chromosome Stability in Anaphase

Anaphase/telophase cells after RNAi for INCENP or aurora B exhibited three highly unusual chromosomal phenotypes. First, they often had one or more pairs of sister kinetochores located in the central spindle or flanking the midbody. Second, the foci of CENP-A/Cid staining at or near the spindle poles were often present as pairs, suggesting that sister kinetochores remained paired despite having undergone anaphase A-like poleward movement. Third, separated masses of chromatin were often connected by a mass of lagging chromatin. We refer to this as chromatin and not chromosomes because the material was amorphous, and little or no evidence of a condensed mitotic chromosome morphology could be observed.

The first two phenotypes can be explained if centromeres fail to disjoin at anaphase onset. Under these circumstances, centromeres of bioriented chromosomes would tend to accumulate near the spindle equator—later, near the midbody—and be stretched apart by the spindle forces. Monooriented kinetochores would move as pairs to one or the other spindle pole. If this occurred in cells that had attained metaphase, then the bulk of kinetochores would remain as pairs in the spindle midzone. However, as described above, abrogation of INCENP and/or aurora B

function prevents cells from reaching metaphase and would therefore be expected to lead to the observed phenotype, with most centromeres at poles and only a few remaining in the midzone. Defects in sister kinetochore disjunction could arise if INCENP and/or aurora B is involved in regulation of the cohesin complex at centromeres; experiments are under way to determine whether cohesin components are substrates for aurora B.

The presence of massive amounts of lagging chromatin is highly characteristic of anaphase/telophase after loss of INCENP and/or aurora B function. This lagging chromatin is distinct from that seen when massive problems with sister chromatid disjunction are caused by loss of topoisomerase II function. Loss of topo II function in *S. pombe* resulted in anaphase cells with a small subset of the chromatin, including centromeres at the poles and the bulk of the tangled chromatin trapped at the spindle midzone (Funabiki et al., 1993). In vertebrates, inhibition of topo II function with drugs causes a similar phenotype (Gorbisky, 1994). In contrast, RNAi for INCENP or aurora B did not appear to prevent the bulk of the chromatin from moving towards the spindle poles. However, this material trailed behind it a smear of decondensed chromatin that in extreme cases appeared to form a continuum across the dividing cell.

This lagging chromatin might arise from difficulties in sister chromatid disjunction, but we believe it more likely that it represents a failure of the chromosomes to move as integral units under the physical stress of anaphase movement. If the dumpy chromosomes observed in prometaphase cells lack an organized infrastructure then when centromeres begin to move polewards, the chromatin of the arms might simply unravel and be left behind as a smear of amorphous chromatin. This would be consistent with the observation that interference with aurora B function in *Drosophila* cells interferes with the binding of the condensin subunit *barren* to mitotic chromosomes (Giet and Glover, 2001). Indeed, *barren* mutants exhibit dramatic chromatin bridges during syncytial mitosis (Bhat et al., 1996), however such a dramatic defect was not seen in mutants affecting the condensin subunit SMC4 in *Drosophila* (Steffensen et al., 2001). It is possible that action of INCENP/aurora B on other chromosomal components in addition to condensin subunits contributes to a loss of chromosomal integrity during anaphase.

Cells with Reduced Levels of DmINCENP Can Assemble a Contractile Ring with Targeted pavarotti KLP but Are Defective in the Completion of Cytokinesis

After RNAi for DmINCENP or DmAurora B, the dramatic increase of binucleate and polyploid cells in the cultures suggests strongly that a large percentage of attempts at cytokinesis ultimately fail. However, we did observe cells lacking detectable INCENP and aurora B in the final stages of cytokinesis with a contractile ring and well-developed midbody structure (Fig. 9, B and E). This suggests that normal levels of INCENP cannot be essential for formation of a central spindle and contractile ring. Because of the nature of the RNAi method, however, we cannot exclude that at least a portion of these cells initiated mitosis with low levels of INCENP, and that this enabled them to assemble the central spindle and contractile ring.

These observations appear to exclude an essential role for the chromosomal passenger proteins in the earliest stages of cytokinesis. This is consistent with the phenotypes induced by two dominant-negative INCENP mutants in cultured cells, both of which caused a failure in the completion of cytokinesis (Eckley et al., 1997; Mackay et al., 1998). Similar late cytokinesis phenotypes were observed when aurora B (Schumacher et al., 1998; Severson et al., 2000), survivin/BIR-1 (Speliotes et al., 2000), and INCENP (Kaitna et al., 2000) were inactivated in *C. elegans*. In *C. elegans*, interference with INCENP or aurora B function blocks stable localization of the ZEN-4 kinesin-related protein in the central spindle, and it was proposed that regulation of the function of kinesin family members may be a major role of aurora B kinases (Severson et al., 2000). Because *ZEN-4* ts mutants cause a failure in the completion of cytokinesis (Severson et al., 2000), the cytokinesis defect in aurora B depleted cells was proposed to result from central spindle instability caused by this lack of *ZEN-4* targeting (Kaitna et al., 2000).

The situation in *Drosophila* cells is apparently different. The *Drosophila* ZEN-4 orthologue, Pavarotti KLP (PAV-KLP) is essential for cytokinesis but functions earlier in the process. PAV-KLP mutants do not form central spindles, do not assemble a stable contractile ring, and do not initiate furrowing (Adams et al., 1998). Furthermore, in contrast to another report (Giet and Glover, 2001), we consistently detected the Pavarotti kinesin (PAV-KLP/ZEN-4/CHO1) in midbody structures of Dmel2 cells lacking detectable INCENP or aurora B protein. This suggests that the cytokinesis defects seen after inhibition of INCENP or aurora B function cannot be entirely due to interference with the targeting of this highly conserved KLP, and that the chromosomal passengers act on other essential targets in order to promote the completion of cytokinesis. The identification of these targets, as well as those required for events earlier in mitosis, is an important goal of future research.

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References

- Adams, R.R., A.A. Tavares, A. Salzberg, H.J. Bellen, and D.M. Glover. 1998. *pavarotti* encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* 12:1483–1494.
- Adams, R.R., S.P. Wheatley, A.M. Gouldsworthy, S.E. Kandels-Lewis, M. Carmena, C. Smythe, D.L. Gerloff, and W.C. Earnshaw. 2000. INCENP binds the aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow. *Curr. Biol.* 10:1075–1078.
- Adams, R.R., M. Carmena, and W.C. Earnshaw. 2001. Chromosomal passengers and the (Aurora) ABCs of mitosis. *Trends Cell Biol.* 11:49–54.
- Ainsztein, A.M., S.E. Kandels-Lewis, A.M. Mackay, and W.C. Earnshaw. 1998. INCENP centromere and spindle targeting: identification of essential con-

- served motifs and involvement of heterochromatin protein HP1. *J. Cell Biol.* 143:1763–1774.
- Andreassen, P.R., D.K. Palmer, M.H. Wener, and R.L. Margolis. 1991. Telophase disk: a new mammalian mitotic organelle that bisects telophase cells with a possible function in cytokinesis. *J. Cell Sci.* 99:523–534.
- Bernat, R.L., G.G. Borisy, N.F. Rothfield, and W.C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement in mitosis. *J. Cell Biol.* 111:1519–1533.
- Bhat, M.A., A.V. Philp, D.M. Glover, and H.J. Bellen. 1996. Chromatid segregation at anaphase requires the barren product, a novel chromosome-associated protein that interacts with Topoisomerase II. *Cell.* 87:1103–1114.
- Biggins, S., F.F. Severin, N. Bhalla, I. Sassoon, A.A. Hyman, and A.W. Murray. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev.* 13:532–544.
- Chan, C.S., and D. Botstein. 1993. Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics.* 135:677–691.
- Clemens, J.C., C.A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B.A. Hemmings, and J.E. Dixon. 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA.* 97:6499–6503.
- Cutts, S.M., K.J. Fowler, B.T. Kile, L.L. Hii, R.A. O'Dowd, D.F. Hudson, R. Saffery, P. Kalitsis, E. Earle, and K.H. Choo. 1999. Defective chromosome segregation, microtubule bundling and nuclear bridging in inner centromere protein gene (*Incenp*)-disrupted mice. *Hum. Mol. Genet.* 8:1145–1155.
- Earnshaw, W.C., and R.L. Bernat. 1990. Chromosomal passengers: towards an integrated view of mitosis. *Chromosoma.* 100:139–146.
- Eckley, D.M., A.M. Ainsztein, A.M. Mackay, I.G. Goldberg, and W.C. Earnshaw. 1997. Chromosomal proteins and cytokinesis: patterns of cleavage furrow formation and inner centromere protein positioning in mitotic heterokaryons and mid-anaphase cells. *J. Cell Biol.* 136:1169–1183.
- Everett, R.D., W.C. Earnshaw, J. Findlay, and P. Lomonte. 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:1526–1538.
- Funabiki, H., I. Hagan, S. Uzawa, and M. Yanagida. 1993. Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* 121:961–976.
- Giet, R., and C. Prigent. 1999. Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *J. Cell Sci.* 112:3591–3601.
- Giet, R., and D.M. Glover. 2001. *Drosophila* aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.* 152:669–681.
- Glover, D.M., M.H. Leibowitz, D.A. McLean, and H. Parry. 1995. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell.* 81:95–105.
- Goh, P.-Y., and J.V. Kilmartin. 1993. *NDC10*: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 121:503–512.
- Gorbsky, G.J. 1994. Cell cycle progression and chromosome segregation in mammalian cells cultured in the presence of the topoisomerase II inhibitors ICRF-187 [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane; ADR-529] and ICRF-159 (Razoxane). *Cancer Res.* 54:1042–1048.
- Henikoff, S., K. Ahmad, J.S. Platero, and B. van Steensel. 2000. Heterochromatic deposition of centromeric histone H3-like proteins. *Proc. Natl. Acad. Sci. USA.* 97:716–721.
- Hsu, J.Y., Z.W. Sun, X. Li, M. Reuben, K. Tatchell, D.K. Bishop, J.M. Grushcow, C.J. Brame, J.A. Caldwell, D.F. Hunt, et al. 2000. Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell.* 102:279–291.
- Kaitna, S., M. Mendoza, V. Jantsch-Plunger, and M. Glotzer. 2000. Incenp and an aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis. *Curr. Biol.* 10:1172–1181.
- Kim, J.H., J.S. Kang, and C.S. Chan. 1999. Sli15 associates with the ipl1 protein kinase to promote proper chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 145:1381–1394.
- Mackay, A.M., D.M. Eckley, C. Chue, and W.C. Earnshaw. 1993. Molecular analysis of the INCENPs (inner centromere proteins): separate domains are required for association with microtubules during interphase and with the central spindle during anaphase. *J. Cell Biol.* 123:373–385.
- Mackay, A.M., A.M. Ainsztein, D.M. Eckley, and W.C. Earnshaw. 1998. A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis. *J. Cell Biol.* 140:991–1002.
- Reich, A., A. Yanai, S. Mesilaty-Gross, A. Chen-Moses, R. Wides, and B. Motro. 1999. Cloning, mapping, and expression of ial, a novel *Drosophila* member of the Ipl1/aurora mitotic control kinase family. *DNA Cell Biol.* 18:593–603.
- Schumacher, J.M., A. Golden, and P.J. Donovan. 1998. AIR-2: an Aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos. *J. Cell Biol.* 143:1635–1646.
- Severson, A.F., D.R. Hamill, J.C. Carter, J. Schumacher, and B. Bowerman. 2000. The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. *Curr. Biol.* 10:1162–1171.

- Skoufias, D.A., C. Mollinari, F.B. Lacroix, and R.L. Margolis. 2000. Human survivin is a kinetochore-associated passenger protein. *J. Cell Biol.* 151: 1575–1582.
- Speliotes, E.K., A. Uren, D. Vaux, and H.R. Horvitz. 2000. The survivin-like *C. elegans* BIR-1 protein acts with the aurora-like kinase AIR-2 to affect chromosomes and the spindle midzone. *Mol. Cell.* 6:211–223.
- Steffensen, S., P.A. Coelho, N. Cobbe, S. Vass, M. Costa, B. Hassan, S.N. Prokopenko, H. Hugo Bellen, M.M.S. Heck, and C.E. Sunkel. 2001. A role for *Drosophila* SMC4 in the resolution of sister chromatids in mitosis. *Curr. Biol.* 11:295–307.
- Terada, Y., M. Tatsuka, F. Suzuki, Y. Yasuda, S. Fujita, and M. Otsu. 1998. AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:667–676.
- Uren, A.G., L. Wong, M. Pakusch, K.J. Fowler, F.J. Burrows, D.L. Vaux, and K.H.A. Choo. 2000. Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr. Biol.* 10:1319–1328.
- Warburton, P.E., C. Cooke, S. Bourassa, O. Vafa, B.A. Sullivan, G. Stetten, G. Gimelli, D. Warburton, C. Tyler-Smith, K.F. Sullivan, et al. 1997. Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Curr. Biol.* 7:901–904.
- Wheatley, S.P., S.E. Kandels-Lewis, R.R. Adams, A.M. Ainsztein, and W.C. Earnshaw. 2001. INCENP binds directly to tubulin and requires dynamic microtubules to target to the cleavage furrow. *Exp. Cell Res.* 262:122–127.
- Whitfield, W.G., C. Gonzalez, G. Maldonado-Codina, and D.M. Glover. 1990. The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2563–2572.