Drosophila Incenp is required for cytokinesis and asymmetric cell division during development of the nervous system

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Summary

The chromosomal passenger protein complex has emerged as a key player in mitosis, with important roles in chromatin modifications, kinetochore-microtubule interactions, chromosome bi-orientation and stability of the bipolar spindle, mitotic checkpoint function, assembly of the central spindle and cytokinesis. The inner centromere protein (Incenp; a subunit of this complex) is thought to regulate the Aurora B kinase and target it to its substrates. To explore the roles of the passenger complex in a developing multicellular organism, we have performed a genetic screen looking for new alleles and interactors of *Drosophila Incenp*. We have isolated a new null allele of *Incenp* that has allowed us for the first time to study the functions of the chromosomal passengers during development. Homozygous *incenp*^{EC3747} embryos show absence of phosphorylation of histone H3 in mitosis, failure of cytokinesis and polyploidy, and defects in peripheral nervous system development. These defects are consistent with depletion of Aurora B kinase activity. In addition, the segregation of the cell-fate determinant Prospero in asymmetric neuroblast division is abnormal, suggesting a role for the chromosomal passenger complex in the regulation of this process.

Key words: *Drosophila*, Chromosomal passengers, Incenp, Cytokinesis, Prospero, Asymmetric division

Introduction

During mitosis, replicated chromosomes segregate equally to the two daughter cells on a complex molecular scaffold – the mitotic spindle. Formation of the condensed mitotic chromosomes and the spindle involves reorganization of components of the cell nucleus and cytoskeleton, with changes occurring in different domains of the mitotic cell, in a coordinated sequence. These changes are triggered by the action of a number of protein kinases, including Cdk1:cyclin B, Plk1, and the Aurora kinases (Barr et al., 2004; Carmena and Earnshaw, 2003; Nigg, 2001). Aurora B kinase is a chromosomal passenger protein that associates with chromosomes during the early stages of mitosis and transfers from chromosomes to the central spindle and cell cortex at the onset of anaphase (for a review, see Carmena and Earnshaw, 2003). It is thus a strong candidate for an activity that triggers changes in different domains of the mitotic cell at different times during mitosis.

The chromosomal passenger proteins (Adams et al., 2001a; Vagnarelli, 2004) are present in a complex that includes Incenp (inner centromere protein; known as Sli15p in budding yeast) (Cooke et al., 1987; Kim et al., 1999), Aurora B kinase (Ipl1p in budding yeast) (Adams et al., 2000; Schumacher et al., 1998; Terada et al., 1998), survivin (Bir1p in budding yeast) (Carvalho et al., 2003; Skoufias et al., 2000; Uren et al., 2000) and borealin (Gassmann et al., 2004). A fifth protein, telophase disc 60 (TD-60) (Andreassen et al., 1991; Mollinari et al., 2003), appears to be functionally linked to the complex, but is not stably associated with it (Gassmann et al., 2004). Yeast homologs of borealin or TD-60 have yet to be identified. These five proteins are all mutually interdependent for their localization during mitosis (Adams et al., 2001a; Adams et al., 2001c; Gassmann et al., 2004; Mollinari et al., 2003; Wheatley et al., 2001), further supporting the notion that their functions are also interdependent. The chromosomal passengers are required for a number of key functions during mitosis, including chromatin modifications, regulation of kinetochore-microtubule interactions, chromosome bi-orientation and stability of the bipolar spindle, mitotic checkpoint function, assembly of the central spindle and cytokinesis (Andrews et al., 2003; Carmena and Earnshaw, 2003; Gassmann et al., 2004).

It is currently thought that the various components of the chromosomal passenger complex target and regulate Aurora B kinase activity (Carmena and Earnshaw, 2003). Incenp binds to Aurora B kinase through its highly conserved C-terminal IN-BOX motif (Adams et al., 2001b; Honda et al., 2003). Incenp is phosphorylated by Aurora B kinase, and this activates the kinase through a positive-feedback loop (Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001). In addition, because Incenp is a microtubule-binding protein (Wheatley et al., 2001) that also has a well-defined centromere-

targeting domain at its N-terminus (Ainsztein et al., 1998), it was suggested to have a role in targeting the kinase to particular locations within the mitotic cell.

Genetic analysis of the chromosomal passenger complex in higher eukaryotes has thus far been limited to the mouse, where knockouts of survivin and Incenp have been described (Cutts et al., 1999; Uren et al., 2000). Genes encoding survivin and Incenp are essential, and homozygous-null embryos die early, at the 32-64-cell stage. Survivin-null embryos have high levels of failed cytokinesis and polyploidy (Uren et al., 2000). *Incenp*-null embryos are characterized by the absence of discernible metaphase or anaphase stages, absence of midbodies, multinucleated cells, multipolar mitotic spindles, micronuclei, abnormal microtubule bundling and chromatin bridges (Cutts et al., 1999). However, in both cases, the early death of the embryos made it difficult to observe a sufficient number of mitotic cells to perform a thorough analysis of the phenotype.

The aims of the present study were to investigate for the first time the function of Incenp in normal cells and tissues during development in a multicellular organism. We have used genetic analysis in *Drosophila* to identify new interactors that will be used in future studies to further define the roles of the chromosomal passenger complex in mitosis and meiosis in *Drosophila*, and we have obtained a new recessive allele of *Incenp* (*incenp*^{EC3747}). This mutation is embryonic lethal, with the maternal contribution allowing development to proceed essentially normal until embryonic stage 13. Our studies reveal that Incenp is essential for phosphorylation of histone H3 on Ser10, and also for cytokinesis in developing neurons. In addition to these defects, we have observed abnormalities in the segregation of cell-fate determinants during neuroblast division which suggests a role for the passengers in the regulation of asymmetric cell division.

Results

A genetic screen for new *Incenp* alleles and interactors

To obtain new alleles or interactors of *Incenp*, we carried out a mutagenesis screen using a previously identified allele of *Incenp* (*incenp*$^{P(EP)2340}$) (Rorth, 1996). *incenp*$^{p(EP)2340}$ is a P-element insertion into the third exon of the *Drosophila Incenp* gene (Fig. 1A). The presence and location of the P-element was confirmed by plasmid rescue and sequencing of the neighbouring genomic region (Fig. 1A). *incenp*$^{P(EP)2340}$ is a homozygous lethal mutation and was described originally as a female sterile dominant (Rorth, 1996). The chromosome carrying the insertion was recombined over a wild-type chromosome to get rid of possible second-site mutations. The

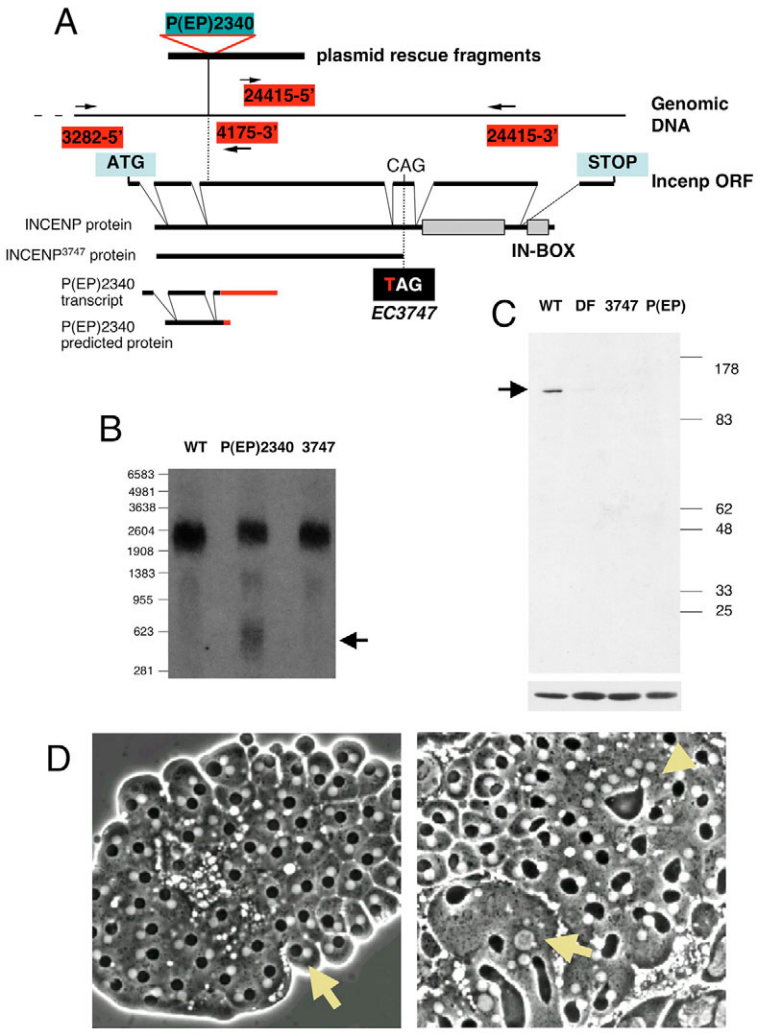

Fig. 1. *incenp*$^{P(EP)2340}$ individuals show defects in chromosome segregation and cytokinesis. (A) Diagram of the *Incenp* genomic region showing insertion site of the P-element into the third exon of the *Incenp* gene in *incenp*$^{P(EP)2340}$. Black lines on either side of the P-element represent the genomic DNA fragments cloned by plasmid rescue and sequenced. Red boxes indicate the position of primers used for the PCR characterization of imprecise excision lines. Diagram of the *Incenp* transcript below shows the position of the point mutation (C to T) in *incenp*^{EC3747}. Below, diagrams of the predicted products of both *Incenp* mutants. (B) Northern blot, showing total RNA from wild-type and both *Incenp* mutants. Arrow points to truncated transcript present in heterozygous *incenp*$^{P(EP)2340}$/+ individuals. (C) Western blot analysis of wild-type embryos (WT), mutant embryos carrying a deficiency for the *Incenp* gene (DF), *incenp*^{3747} homozygous embryos (3747) and *incenp*$^{P(EP)2340}$ homozygous embryos [3747, [P(EP)]. Arrow points to wild-type Incenp protein. Lower panel shows α-tubulin loading control. (D) Phenotype of *incenp*$^{P(EP)2340}$/+ in male meiosis. (Left) Wild-type onion-stage cyst; arrow points to individual spermatid with equal-size nucleus and mitochondrial derivative (Nebenkern). (Right) Same stage in *incenp*$^{P(EP)2340}$/+ males; notice large Nebenkerns indicative of defective cytokinesis (arrowhead), as well as variable-size nuclei indicative of problems in chromosome segregation (arrow).

P-element insertion is lethal over a chromosomal deletion uncovering the *Incenp* gene (defined by six overlapping chromosomal deficiencies of the region – see Materials and Methods for further details). *incenp*$^{P(EP)2340}$ heterozygous individuals were shown to produce a smaller transcript of around 600 bp, which is not present in the wild-type control (Fig. 1B). This is predicted to potentially produce a 91 aa N-terminal peptide, however, we could not detect any truncated peptide in protein extracts from homozygous embryos by western blot (Fig. 1C).

Male flies carrying the *incenp*$^{P(EP)2340}$ insertion show a dominant meiotic phenotype that results in low fertility. The main defects observed include misshapen meiotic spindles, tetrapolar or multipolar spindles and defects in onion stage spermatids consistent with problems both in chromosome disjunction and cytokinesis (Fig. 1D). A detailed analysis of the role of *Drosophila* Incenp in meiosis is the subject of a separate study (T. Resnick, D. L. Satinover, F.M., T. Stukenberg, W.E., T.O.-W. and M.C., unpublished data). Heterozygous individuals also show defects in larval mitosis consistent with those shown by S2 cells depleted of *Drosophila* Incenp by double-stranded RNA interference (dsRNAi) (Adams et al., 2001c). Additionally, heterozygous adults show mild external defects consistent with abnormalities during imaginal mitosis, including slightly roughened eyes, nicked wings and defects in the abdominal terguites (data not shown). The *Incenp* locus is not haplo-insufficient: individuals heterozygous for a deficiency uncovering the *Incenp* gene do not show any of the phenotypes described above (M.C., unpublished data).

The precise excision of the P-element, using an exogenous source of transposase, reverted the lethality and the dominant phenotypes, demonstrating that the insertion is responsible for both effects. Precise excision was confirmed by sequencing the genomic region of the excised chromosome. Twenty-one imprecise excisions obtained were lethal and also showed dominant meiotic phenotypes. We isolated genomic DNA from six of these lines and devised a PCR strategy to characterize them at the molecular level (Fig. 1A). All the lines analysed (Δ8, Δ9, Δ14, Δ17, Δ18 and Δ24) were internal deletions in the P-element, and are predicted to produce the same truncated peptide as the original insertion.

To date, no mutations in any of the genes coding for passenger complex proteins have been described in *Drosophila*. Therefore, to isolate new recessive alleles of *Incenp* and also possible new interactors, we performed a second-site non-complementor screen (see Halsell et al., 2000; Hays et al., 1989; White-Cooper et al., 1996) using ethyl methanesulfonate (EMS) as mutagen. We chose EMS because it is more likely to induce point mutations that can disrupt specific protein-protein interactions. The *Incenp* gene is located on the second chromosome (43A04), so to simplify the screen, we only isolated mutations in the second chromosome. We sought to isolate and analyze mutations that – when in trans to *incenp*$^{P(EP)2340}$ – were lethal, male sterile or showed an enhancement of the dominant visible phenotype.

A total of 5000 individual mutagenised chromosomes were generated and analyzed. This screen yielded 16 homozygous lethal mutations corresponding to 13 complementation groups. Most of the mutants were isolated on the basis of lethality in trans with *incenp*$^{P(EP)2340}$, except for five (EC282, EC2388, EC2394, EC2519, EC3959) that showed defects in the eyes, bristles or wings (Fig. 2A-D). Two of the mutants had a dominant female sterile phenotype (EC3322, EC4330). When analyzed carefully on a larger scale, some of the trans-heterozygote combinations turned out to be semi-lethal or viable (Table 1). The same results were obtained when we analyzed trans-heterozygote combinations with imprecise excisions of P(EP)2340 (Δ9, Δ24). However, when in trans with a precise excision of P(EP)2340 (Δ11) the EC mutations show no phenotype.

Whenever the lethal phase allowed the analysis, we studied the phenotype in male meiosis of the trans-heterozygous combinations (EMS mutant/*P(EP)2340*). Some combinations showed defects in primary spermatocytes (Fig. 2E,F) as well as in the postmeiotic onion-stage spermatids (Fig. 2H,I). This is consistent with disruption of a function required for both mitosis and meiosis. In other cases, i.e. EC666/*P(EP)2340*, the defects were found exclusively in the meiotic divisions, which raises the possibility of identifying meiotic specific functions of the chromosomal passenger proteins.

We next used deficiency and, in some cases, meiotic-recombination mapping to further characterize the new mutations. For the deficiency mapping we used a second chromosome deficiency kit (a set of overlapping deletions of chromosome 2). Four of the mutants, corresponding to two complementation groups (EC1650/EC2388 and EC2364/EC2374) are semi-lethal over a chromosomal deficiency for the *Drosophila* Aurora B kinase gene [Df(2L)1469]. In addition, two of these mutations (EC1650/EC2388) do not complement a chromosomal deficiency for the *Drosophila* borealin gene – Df(2L)2892.

These experiments showed that the chromosomes typically contained more than a single EMS hit. Further analysis of the mutants requires the separation of the different mutations by recombination. As yet, the molecular identity of only one of the new mutants has been determined (see below). A detailed description of the new mutants and their phenotypes will be the subject of a future study.

Identification of a new allele of *Incenp*

One of the newly generated mutants (EC3747) did not complement Df(2R)pk78k (42E03-43C03), a chromosomal deficiency uncovering the *Incenp* gene. EC3747 was therefore selected for further characterization as a putative new allele of *Incenp*. Genetic recombination with a multiply marked chromosome was used to minimize the presence of other spurious EMS mutations on the chromosome containing the EC3747 mutation (Materials and Methods). To further refine the mapping of EC3747 we used six strains carrying chromosomal deficiencies with breakpoints in the *Incenp* gene region (Materials and Methods). EC3747 was mapped to region 43A04, the same location as the *Incenp* gene. In addition, EC3747 failed to complement any of the imprecise excisions generated by the mobilization of the P-element. Therefore, all of the available genetic evidence suggested that EC3747 is a new allele of *Incenp*.

Molecular characterization confirmed that EC3747 was indeed a new allele of *Incenp*. To identify the mutation in EC3747, we extracted genomic DNA from single EC3747-homozygous embryos, used PCR to amplify the entire coding region of the *Incenp* gene, and then sequenced the PCR

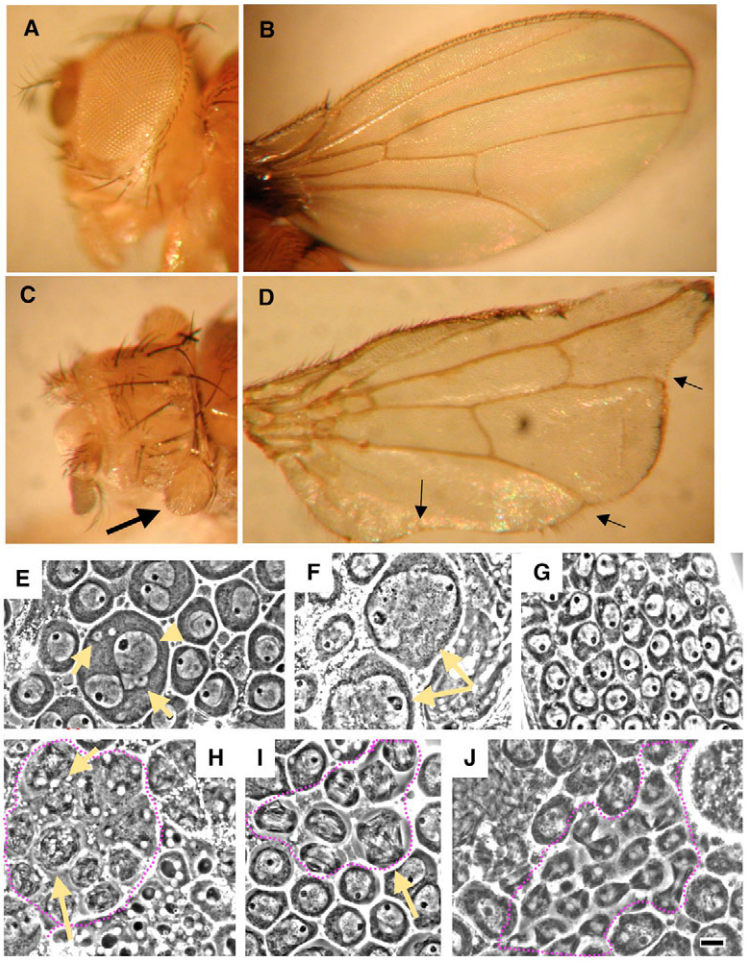

Fig. 2. Phenotypic analysis of the new genetic interactors of *incenp*$^{P(EP)2340}$. (A-B) Normal eye and wing morphology in control (*w*^{1118}) strain. (C-D) Representative examples of eye and wing phenotypes observed in trans-heterozygous combinations of EMS-induced mutations over *incenp*$^{P(EP)2340}$: (C) Reduced eyes in trans-heterozygous *EC3959/incenp*$^{P(EP)2340}$. (D) Irregular nicked wing-shape in trans-heterozygous *EC2519/incenpP*$^{(EP)2340}$ fly. (E,F) Defects in the primary spermatocytes in trans-heterozygous males as a result of abnormalities in the gonial mitoses. (E) Variation in the nuclear size in *EC2519/incenpP*$^{(EP)2340}$ is an indication of defective chromokinesis. Arrows point to micronuclei, arrowhead to polyploid nucleus. (F) The presence of giant primary spermatocytes (arrows) in *EC2394/incenpP*$^{(EP)2340}$ could be indicative of a defect in cytokinesis in gonial mitosis. (G) Wild-type primary spermatocyte cysts. (H,I) Abnormal (H) meiotic spindles and (I) tetrapolar spindles (arrows) in *EC2394/incenpP*$^{(EP)2340}$. (J) Wild-type telophase I spindles. Dotted lines in H-J delimit the meiotic cyst. Bar, 10 μm.

products directly. Genomic DNA from four single embryos was prepared as template to perform four separate PCR reactions. Comparison of the sequences revealed a point mutation in exon 4 of the *Incenp* gene. This single base change created a stop codon in position 457 of the open reading frame (Fig. 1A). The predicted molecular weight of the putative truncated gene product is 61 kDa.

Although the sequence analysis of EC3747 predicted that the product of this mutated gene would be a truncated version of *Drosophila* Incenp, when we analyzed EC3747 homozygous embryos by immunoblotting we failed to detect a product of the predicted size (Fig. 1C). The expected full-length protein with molecular weight of 110 kDa was detected in wild type, but was absent from embryos homozygous for Df(2R)pk78k, EC3747 and *incenp*$^{P(EP)2340}$. This suggests that the truncated mutant protein is unstable. The antibody used for immunoblots, Rb801, was raised against the N-terminal 348 amino acids of *Drosophila* Incenp (Adams et al., 2001c), which is wholly contained within the 457 aa polypeptide encoded by *EC3747*.

The new recessive null allele of *Incenp* shows a genetic interaction with several of the mutants generated in our screen: *incenp*^{EC3747} is semi-lethal over EC666 and EC2519, and male sterile over EC549, EC666, and EC1608. This confirms the genetic interaction between these as-yet-unidentified genes and Incenp, and excludes the notion that the interaction is specific to the dominant P-element insertion.

incenp^{EC3747} embryos show defects in late embryonic development

Analysis of the lethal phase of homozygous *incenp*^{EC3747} individuals showed that they died late in embryonic development. The rare first larval instar escapers show reduced mobility and abnormal wandering behavior (data not shown) consistent with defects in the nervous system. To investigate this further, late-stage embryos were stained for the marker Mab22C10 that stains neurons and their processes throughout the nervous system (Zipursky et al., 1984). Homozygous mutants were distinguished from heterozygous embryos using the CyOKrüppel-GFP balancer marker. Focusing on the peripheral nervous system (PNS), we observed that the heterozygous animals had a wild-type neural pattern (Fig. 3A). However, homozygous mutant embryos exhibited a range of defects, from severe cases of neuronal loss to perturbation of the neural clusters and missing subsets of neurons (Fig. 3B-D).

To determine the time in which the first defects in mitosis arose in these individuals, we fixed embryos at different stages and stained with anti-*Drosophila* Incenp and anti-tubulin antibodies. During the first 13 rapid synchronous cycles we did not observe any difference between wild-type and *incenp*^{EC3747} embryos. Since Incenp has been proposed to be required for completion of cytokinesis, we then paid special attention to the process of blastodermal cellularization. This process, which occurs in mitotic cycle 14 when membranes grow inward between the blastoderm nuclei, in some ways resembles the

Table 1. Second-site noncomplementing mutations isolated in our screen

Mutant line	Stage of lethality	Lethality over P(EP)2340
201	EL	L
282	EL	SL
497	L1	SL
549	EL	SL
666	L3	L
1608	EL	V
1650	EL	SL
2364	EL	V
2388	EL	V
2394	EL	V
2519	EL	SL
3747	EL	L
3959	EL	SL
4056	EL	SL
3322	n.d.	n.d.
4330	n.d.	n.d.

Stage of lethality column shows lethal phase of homozygous individuals. EL, embryonic lethal; L1, first instar larval stage lethal; L3, third instar larval stage lethal. Lethality over P(EP)2340 shows lethality of transheterozygous individuals. L, lethal; SL, semilethal; V, viable; n.d. not determined.

process of cytokinesis. *incenp*^{EC3747} embryos showed no defect in cellularization and Incenp could still be observed to be localized properly. This reflects perdurance of the wild-type maternal product. We could not observe significant defects in early-stage embryos, although from mitotic cycle 15, we did observe a very low percentage of cells with a reduced amount of *Drosophila* Incenp and chromosome segregation defects (data not shown).

By embryonic stage 13 (Ashburner et al., 2005; Campos-Ortega and Hartenstein, 1985) *incenp*^{EC3747} *Drosophila* Incenp was no longer detectable by immunostaining in *incenp*^{EC3747} embryos (Fig. 4B). Consistent with this, phosphorylation of histone H3 on Ser10 (a known Aurora B kinase substrate) was also no longer detected (compare Fig. 4A" with B"). This phenotype confirms that Incenp is required for Aurora B kinase to function as a histone H3 Ser10 kinase in an developing multicellular organism. At this stage, cells of the central nervous system (CNS) in *incenp*^{EC3747} embryos showed enlarged nuclei (Fig. 5B) compared with wild-type (Fig. 5A) as observed by DAPI staining for DNA. Staining with anti-γ-tubulin antibody showed that these enlarged cells contain bigger than normal centrosomes (Fig. 5D') or multiple centrosomes (Fig. 5E'). This phenotype, detectable in mutant embryos following the complete disappearance of the *Drosophila* Incenp, can be explained as the result of failure of cytokinesis in the previous division and is consistent with a lack of Aurora B kinase function (Adams et al., 2001c; Oegema et al., 2001) in the early development of the CNS.

Localization of the cell-fate determinant Prospero is abnormal in *incenp*^{EC3747} neuroblast divisions

Asymmetric cell division is key to the development of the *Drosophila* nervous system (for a review, see Bardin et al., 2004). Each dividing neuroblast produces one large daughter cell that remains a multipotent neuroblast and continues to divide, and a smaller daughter cell that becomes a ganglion mother cell that divides once more asymmetrically to produce neurons or glia cells. This cell-fate decision hinges on the segregation of Prospero, a homeodomain transcription factor that is segregated largely, if not exclusively, into the ganglion mother cell. This is accomplished by sequestering Prospero into a basal cortical crescent in the dividing neuroblast from prophase onwards (Spana and Doe, 1995).

In wild-type embryos, we observed the expected asymmetric distribution of Prospero at the basal cell surface of dividing cells (Fig. 6C). However, we were surprised to notice that, in early prophase, Prospero transiently associates with the condensing chromatin on entry into mitosis (Fig. 6A,B). The distribution of Prospero was abnormal in neuroblasts lacking detectable Incenp. Abnormalities observed in neuroblasts of *incenp*^{EC3747} embryos included defects in the shape and orientation of the basal Prospero crescent (Fig. 6D). We also observed mitotic neuroblasts with Prospero distributed all around the cell cortex, and not restricted to a basal crescent (Fig. 6D).

These results reveal that *Drosophila* Incenp and, therefore, presumably the chromosomal passenger complex, is required for the correct localization of Prospero during asymmetric cell division in the developing *Drosophila* nervous system.

Discussion

The chromosomal passenger protein Aurora B kinase , together with its auxiliary subunits Incenp, survivin and borealin/dasra (Adams et al., 2001a; Carmena and Earnshaw, 2003; Gassmann et al., 2004; Sampath et al., 2004) is a key regulator of mitotic events. This complex has been widely studied in

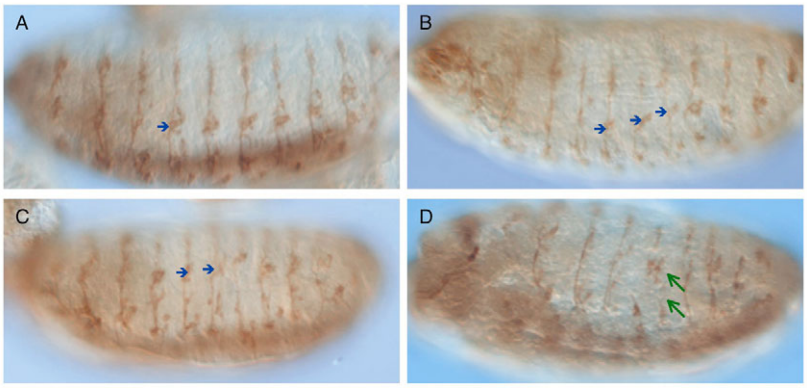

Fig. 3. Peripheral neuron system morphology *incenp*^{EC3747} embryos. (A-D) Examples of 20-hour embryos stained for the neural marker Mab22C10. Small blue arrows indicate the position of the lateral cluster of chordotonal neurons. (A) *incenp*^{EC3747}/CyO KrGFP embryo. The wild-type PNS pattern is visible. (B-D) Homozygous *incenp*^{EC3747} embryos. (B and C) Small blue arrows show examples of neural loss from the lateral cluster. (D) Green arrows show a lack of organization of neuron clusters and reduced number of neurons. In these images anterior is left, dorsal is top. The views in C and D are more ventral than those of A and B.

cultured cells, but the early lethality that is observed when its components are knocked-out by gene targeting or RNAi has thus far precluded the study of its role in development (Cutts et al., 1999; Oegema et al., 2001; Schumacher et al., 1998; Uren et al., 2000). Incenp is a key regulator responsible both for activation of the kinase and for its targeting (Adams et al., 2001b; Adams et al., 2001c; Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001).

Here, we used *Drosophila* genetics to isolate a new allele of *Incenp* (*incenp*^{EC3747}) that allowed us to investigate for the first time the role of the chromosomal passenger complex in development. The mutagenesis screen was designed to identify new genetic interactors of *Incenp*. The screen used a previously isolated allele of *Incenp* (*incenp*$^{P(EP)2340}$) that shows a dominant phenotype in mitosis and meiosis (M.C., unpublished results) (T. Resnick, D. L. Satinover, F.M., T. Stukenberg, W.E., T.O.-W. and M.C., unpublished data). The dominant phenotype associated with the *incenp*$^{P(EP)2340}$ insertion can probably be explained by the production of a N-terminal truncated peptide, predicted to be 91 aa long. Studies in higher eukaryotes have shown that, the first 43 aa of the Incenp N-terminal contain an essential domain required for centromere targeting (Mackay et al., 1998). The *incenp*$^{P(EP)2340}$ truncated peptide contains this domain, but lacks the conserved in-box required for binding to Aurora B kinase. Therefore, this peptide potentially competes with wild-type Incenp for binding to the centromere. Alternatively, the truncated polypeptide could associate with wild-type Incenp and trigger its destruction. In accordance with these hypotheses, P(EP)2340 heterozygous flies show a 4-fold decrease in the amount of centromeric Incenp compared with wild-type controls (T. Resnick, D. L. Satinover, F.M., T. Stukenberg, W.E., T.O.-W. and M.C., unpublished data).

Our screen was based on the hypothesis that in flies heterozygous for *incenp*$^{P(EP)2340}$, chromosomal passenger function might be in a precarious equilibrium that can be thrown further out of balance by mutation of a gene coding for an interacting protein. We chose EMS as a mutagen, with the aim of obtaining point mutations that specifically disrupt protein-protein interactions.

Although far from saturation, the EMS-screen yielded 16 mutants showing phenotypes characteristic of mitotic and/or meiotic defects when combined with *incenp*$^{P(EP)2340}$ (Table 1). Preliminary characterization of the trans-heterozygous combinations (mutant *incenp*$^{P(EP)2340}$) reveals a variety of distinctive phenotypes affecting different processes during cell division, including chromosome condensation, segregation and cytokinesis. Future characterization of these mutants, identification of the genes affected, their products and the nature of the interaction with Incenp should permit a functional dissection of the multiple roles of the passenger complex in a developmental context.

EC3747 is a recessive allele of *Incenp*

One of the new mutants, *EC3747*, contains a point mutation that generates a stop codon just before the coiled-coil region of *Drosophila* Incenp. The predicted molecular mass of the 456 aa protein product truncated at the C-terminal is approximately 61 kDa. Immunoblotting analysis of extracts from homozygous mutant embryos failed to detect any truncated Incenp polypeptide. This is probably due to instability of the polypeptide. Unlike *incenp*$^{P(EP)2340}$ heterozygous individuals, *EC3747* heterozygous individuals do not show any defects in mitosis or meiosis (data not shown), suggesting that *EC3747* is a recessive null allele of *Incenp*. *Incenp* is an essential gene in *Drosophila*, and individuals homozygous for *EC3747* died late during embryogenesis. As discussed below, one probable factor contributing to this embryonic lethality was abnormal development of the nervous system.

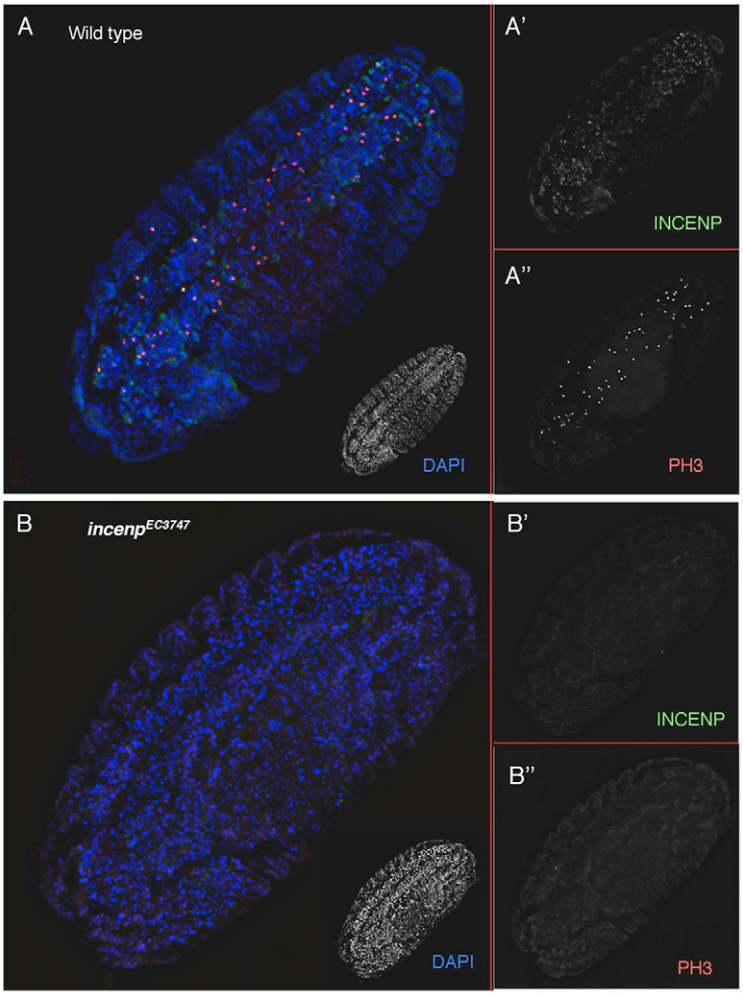

Fig. 4. *Drosophila* Incenp becomes undetectable at stage 13 of embryonic development in *incenp*^{EC3747}. Ventral view of stage-13 embryos. (A-A") Wild-type embryos, showing mitotic cells staining positive for (A') *Drosophila* Incenp and (A") phosphorylated histone H3. (B-B") Homozygous *incenp*^{EC3747} embryos at the same stage show no (B') *Drosophila* Incenp or (B") phosphorylated histone H3. Insets in A and B show the DNA staining (DAPI). Notice that the embryos have developed normally up to this stage. In the merged figures, DAPI is shown in blue, phosphorylated histone H3 in red, *Drosophila* Incenp in green.

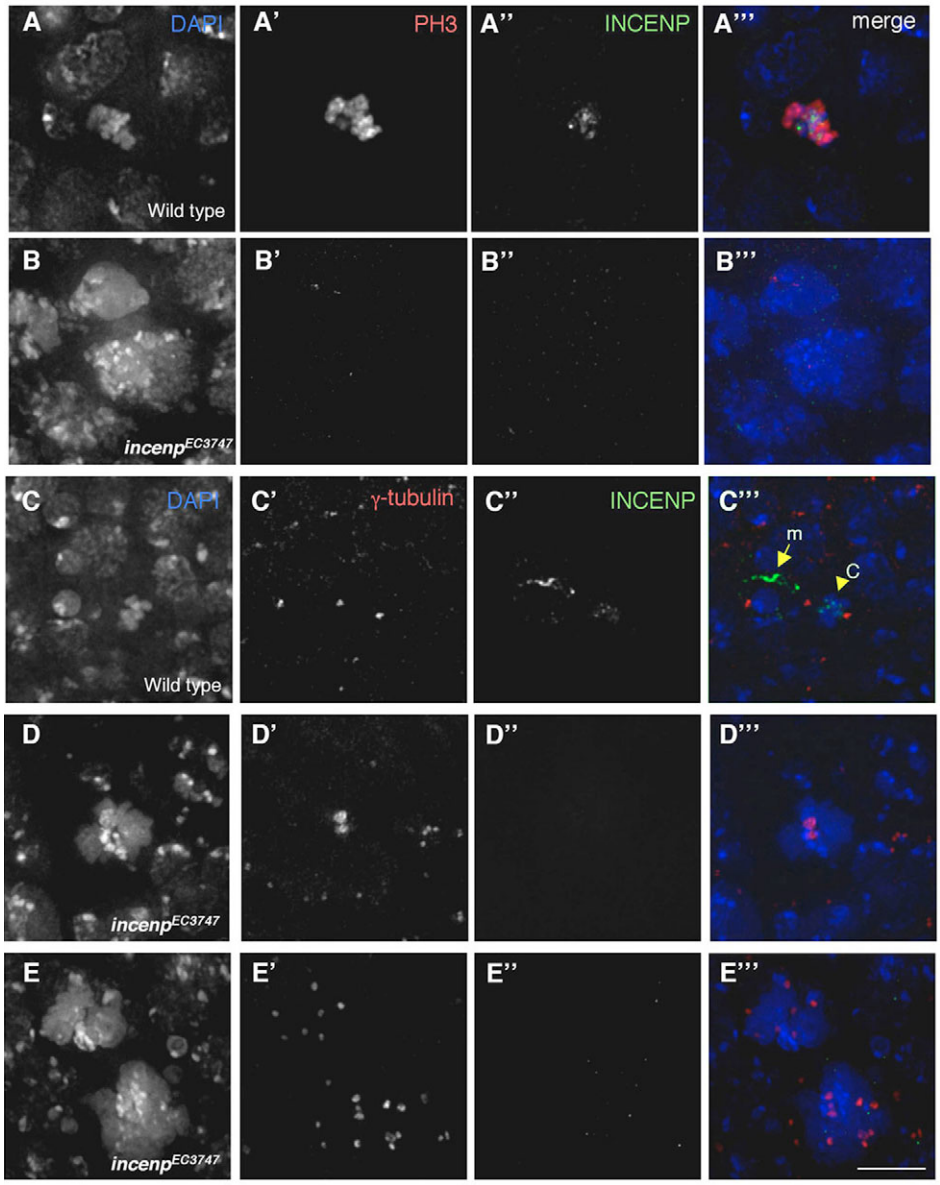

Fig. 5. *incenp*^{EC3747}enlarged CNS cells lack *Drosophila* Incenp and phosphorylated histone H3. (A-A''') Mitosis in a wild-type CNS cell showing *Drosophila* Incenp localized to the centromere at prometaphase. Histone H3 is phosphorylated in the mitotic chromosomes. (B-B''') Enlarged cells in the CNS of *Incenp*^{EC3747} embryos do not show any *Drosophila* Incenp phosphorylated histone H3 staining. In the merged figures, DAPI is shown in blue, phosphorylated histone H3 in red, *Drosophila* Incenp in green. Enlarged CNS cells in *incenp*^{EC3747} embryos are polyploid and show multiple centrosomes. (C-C''') Wild-type cell with *Drosophila* Incenp localized to centromeres at metaphase (arrowhead) and in the midzone at telophase (arrow). (D-E''') Enlarged CNS cells are polyploid and show either and abnormal accumulation of γ-tubulin in centrosomes or multiple centrosomes. The two centrosomal masses in D-D''' have not separated properly in this cell. Multiple γ-tubulin spots in E-E'''. In the merged figures, DAPI is shown in blue, γ-tubulin in red, *Drosophila* Incenp in green. Bar, 10 μm.

Incenp is required for phosphorylation of histone H3 and cytokinesis in the embryonic CNS divisions

A maternal stockpile of *Drosophila* Incenp protein allowed *incenp*^{EC3747} homozygotes to survive to late embryogenesis, therefore enabling us to study for the first time the role of this protein in development. During our phenotypic analysis of *incenp*^{EC3747}, we observed that maternal Incenp protein became generally undetectable at stage 13 of embryonic development. The only cells dividing at this stage are those of the nervous system, where we observed abnormally large mitotic cells without any phosphorylated histone H3. This is the first demonstration in a developing organism that Incenp is essential for the activity of Aurora B kinase.

Mutant neuroblasts were polyploid, and had multiple centrosomes, a phenotype that can result as a consequence of failure in chromosome segregation and cytokinesis. However, a recent study has shown that the new chromosomal passenger borealin, which is found in a complex with Aurora B kinase, Incenp and survivin, is required for maintenance of bipolar spindle in mitosis (Gassmann et al., 2004). In addition, we previously observed an elevated frequency of monopolar and multipolar spindles in S2-phase cells following dsRNAi knockdown of *Drosophila* Incenp (M.C., unpublished observations). Therefore it is possible that the centrosomal abnormalities observed in stage 13 embryos reflect a requirement for Incenp in maintenance of bipolar spindle integrity.

Incenp is required for asymmetric distribution of cell-fate determinants in the embryonic CNS

Our results reveal that Incenp is required for the asymmetric distribution of Prospero. This is likely to be a consequence of the involvement of Aurora B kinase in the regulation of asymmetric cell division rather than simply a consequence of defects in mitosis and cytokinesis. Importantly, mutations in the gene encoding pebble, a Rho GTP exchange factor that is

essential for cytokinesis (Hime and Saint, 1992; Lehner, 1995), do not disrupt the polarized localization of cell-fate determinants (Barros et al., 2003). Furthermore, the prometaphase delay resulting from the loss of Incenp-Aurora B kinase complex function, does probably not in itself alter Prospero localization because colchicine treatment does not have an effect on this process (Knoblich et al., 1995; Spana and Doe, 1995). It has been reported that cortical Prospero is highly phosphorylated and that this modification could be relevant for its localization (Srinivasan et al., 1998). This raises the possibility that Prospero is an Aurora B kinase substrate. Our observation of the partial colocalisation of Prospero with Incenp on chromatin in mitosis would agree with this.

An alternative role for the passenger complex in asymmetric cell division could be through regulation of myosin. At late prophase of neuroblast asymmetric mitosis, localization of Prospero and Numb in the basal crescent is coordinated with the orientation of the mitotic spindle and depends on two protein subcomplexes acting together with the actin cytoskeleton. Recently, it has been shown that myosin motors are involved in localization of fate determinants in different ways. The myosin VI jaguar (*Jar*) is required for localization of Miranda (Petritsch et al., 2003) and myosin II – restricted to the apical cortex by lethal giant larva (Lgl) – is necessary for apical exclusion of fate determinants (Barros et al., 2003). Myosin II regulatory light-chain has been described as a substrate of Aurora B kinase (Murata-Hori et al., 2000). Thus a potential role of the chromosomal passenger complex is to regulate myosin-motor activity essential for localization of cell-fate determinants.

The *Drosophila Incenp* mutant *incenp*^{EC3747} has allowed us to study the role of passenger proteins in development for the first time. This has revealed a previously unsuspected requirement for Incenp in the regulation of the asymmetric distribution of cell-fate determinants during CNS development in *Drosophila* embryos. Thus, the chromosomal passenger complex appears to have an essential role not only in regulating basic functions of normal mitosis, but also in regulating the more elaborate process of asymmetric cell division, which is essential for metazoan development.

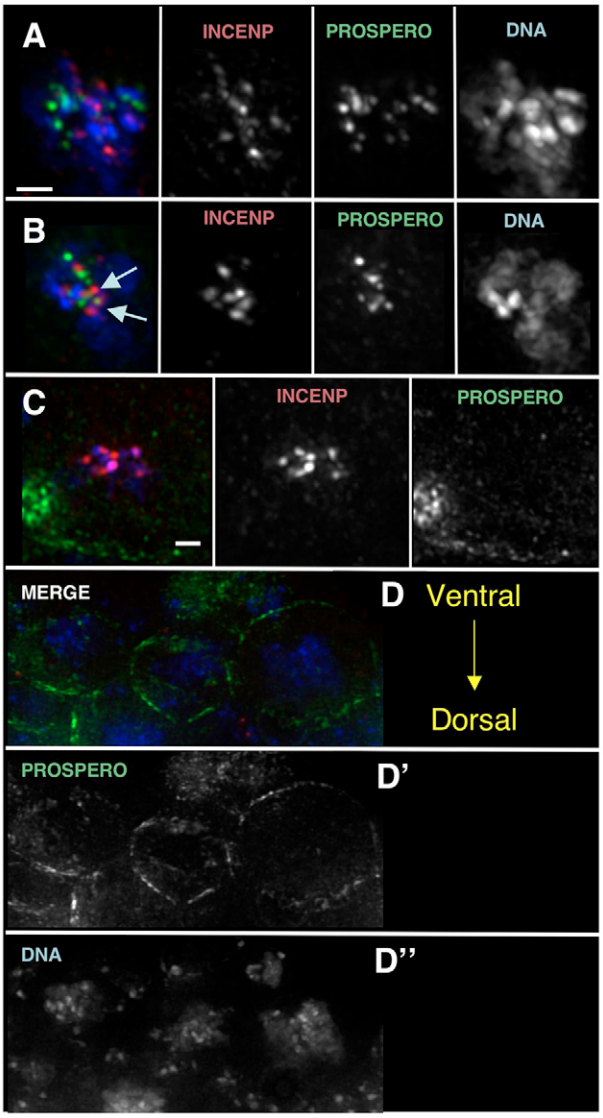

Fig. 6. Localization of the cell-fate determinant Prospero in mitosis of wild-type and *incenp*^{EC3747} embryos. (A-C) Localization of Prospero in mitosis of wild-type CNS cells. (A) In early prophase, *Drosophila* Incenp shows general association with chromatin, whereas Prospero localizes in discrete spots associated with chromatin. (B) By early prometaphase, both proteins become closer although they do not colocalise totally. (C) In metaphase, *Drosophila* Incenp remains associated with centromeres, whereas Prospero is localized in a crescent in the basal cell cortex. (D-D') In *incenp*^{EC3747} homozygous embryos, we observed enlarged polyploidy cells depleted of *Drosophila* Incenp in which Prospero localization is abnormal. All images are projections of series of deconvolved images. Bars, 5 μm.

Materials and Methods

Drosophila strains, mutagenesis and mapping methods, and embryo collection

Fly stocks were maintained at 18°C or room temperature on standard cornmeal-agar medium. The original *P(EP)2340* strain was generated in a misexpression screen (Rorth, 1996). A *w*^{1118} strain isogenic for chromosome 2 was used for the mutagenesis screen. Ethyl methanesulfonate (EMS) mutagenesis was perfomed according to standard protocols (see Grigliatti, 1998). The new mutants obtained from the screen were named EC (Edinburgh Chang) followed by the number of the mutagenised chromosome. Flies carrying mutagenised chromosomes were crossed with a 'deficiency kit' for chromosome 2 (overlapping deficiencies uncovering most of the chromosome). Multiple EMS hits were separated by meiotic recombination over a multiple-marked chromosome 2 (*al dp pr b cn cu sp*).

We refined the mapping of EC3747 using the following deficiencies: Df(2R) Drl1 (42E01-43C03), Df(2R)cn-S3 (43B01/02-43B07/09), Df(2R) pk78s (43F08-59F05/08), Df(2R) nap19 (41E02/F01-43A02), Df(2R) p32a (43A03-43F06) and Df(2R) ST1 (41B03/05-43E15/18). All the stocks used for meiotic recombination and deficiency mapping of the new mutants were obtained from the Bloomington *Drosophila* Stock Center. Embryo staging, collection and fixation was performed according to standard protocols (see Rothwell and Sullivan, 2000).

To select homozygous embryos we used the second chromosome balancer CyO-Kr-GFP. Homozygous embryos were selected by hand, using a dissecting-microscope with a fluorescence illuminator that enabled visualization of the GFP signal.

Extraction of genomic DNA

Genomic DNA from adult flies was obtained using the Berkeley *Drosophila* Genome Project protocol (http://www.fruitfly.org/p_disrupt/inverse_pcr). Genomic DNA from single embryos for sequencing was obtained as follows: embryos of the desired genotype were collected in 0.5 ml tubes (1 per tube) and homogenized in 10 μl of Gloor-Engel's extraction buffer (10 mM Tris pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μg/ml proteinase K) by using a pipette tip. The homogenate was incubated at 37°C for 30 minutes, moved to 95°C for 2 minutes, then stored at 4°C.

Northern blotting

Total RNA from w^{1118}, $incenp^{3747}$ and $incenp^{P(EP)2340}$ individuals was isolated using the RNAeasy kit (Qiagen), and 25-30 μg of RNA were loaded per lane. A 400 bp *Sal*I restriction fragment from *Incenp* cDNA, expanding over the P-element insertion site was radioactively labelled to use as probe.

Antibodies

Antibodies used for immunostaining and western blotting were as follows: rabbit polyclonal anti-*Drosophila* Incenp (Rb801) (Adams et al., 2001c); anti-phosphorylated histone H3 Ser10 (rabbit polyclonal, used 1:200; Upstate Biotechnology; mouse monoclonal, used 1:200; NEB); mouse monoclonal anti-α-tubulin B-512 (used 1:2000, Sigma); mouse monoclonal anti-γ-tubulin GTU-88 (1:50, Sigma); mouse monoclonal anti-Prospero MR1A (1:5, developed by Chris Doe, obtained from Developmental Studies Hybridoma Bank); mouse monoclonal 22C10 (1:200, gift of Andrew Jarman, University of Edinburgh, UK); rabbit polyclonal anti-GFP (1:1000, Molecular Probes). All fluorescently-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (anti-rabbit conjugated-Texas Red, anti-rabbit conjugated-FITC, anti-mouse conjugated-Texas Red, anti-rabbit conjugated-FITC, all used 1:200).

Preparation of protein extracts from homozygous embryos and western blotting

Embryos (selected as described previously) were placed in a fresh 0.5 ml microcentrifuge tube; 50 μl of 1× SDS-PAGE sample buffer were added and the embryos were homogenized for 5 seconds three times with a sonicator. After homogenization, the mixture was placed at 100°C for 5 minutes. The heated sample was spun in a table-top centrifuge at 10,000 ***g*** for 3 minutes at 4°C. The supernatant was transferrred to a fresh tube and the sample was ready for loading on an SDS-PAGE gel. Samples were run on 10% SDS-PAGE gel and corresponding blots were probed with rabbit polyclonal anti-Incenp (Rb801-1) (Adams et al., 2001c) and monoclonal anti-α-tubulin antibody B-512 (Sigma).

Immunostaining of embryos

Fixation of *Drosophila* embryos for immunofluorescence and immunohistochemistry was performed in 4% paraformaldehyde for 30 minutes. Devitellinisation and staining was done according to standard protocols (Rothwell and Sullivan, 2000). For immunohistochemistry, after the incubation with secondary antibody and subsequent washes, embryos were stained using diaminobencidine (Vectastain DAB peroxidase substrate kit, Vector Laboratories) and then mounted in Vectashield (Vector).

Microscopy

Imaging was performed using Olympus IX-70 microscope controlled by Delta Vision SoftWorx (Applied Precision, Isswqua, WA, USA). Differential interference microscopy (DIC) was used to collect images on an Olympus Provis AX70 microscope for immunohistochemical samples. Images were deconvolved, projected and saved as tiff files to be processed with Adobe Photoshop.

We like to thank Emma Rawlins and Andrew Jarman for their advice and for sharing reagents. We also acknowledge the contribution of the undergraduate students Duncan Sproul, Jayne Miller and Jane Charlesworth who participated in the initial mapping and characterization of the mutant lines resulting from the screen. The monoclonal antibody MR1A developed by Chris Q. Doe was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. Fly strains were obtained from the Bloomington *Drosophila* Stock Center. C.-J.C. was funded by a Wellcome Trust Prize Studentship. Work in W.C.E.'s laboratory is funded by The Wellcome Trust, of which W.C.E. is a Principal Research Fellow.

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