

# INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow

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**Cytoskeletal rearrangements during mitosis must be co-ordinated with chromosome movements. The ‘chromosomal passenger’ proteins [1], which include the inner centromere protein (INCENP [2]), the Aurora-related serine-threonine protein kinase AIRK2 [3,4] and the unidentified human autoantigen TD-60 [5], have been suggested to integrate mitotic events. These proteins are chromosomal until metaphase but subsequently transfer to the midzone microtubule array and the equatorial cortex during anaphase. Disruption of INCENP function affects both chromosome segregation and completion of cytokinesis [6,7], whereas interference with AIRK2 function primarily affects cytokinesis [3,8]. Here, we report that INCENP is stockpiled in *Xenopus* eggs in a complex with *Xenopus* AIRK2 (XAIRK2), and that INCENP and AIRK2 kinase bind one another *in vitro*. This association was found to be evolutionarily conserved. Sli15p, the binding partner of yeast Aurora kinase Ipl1p, can be recognized as an INCENP family member because of the presence of a conserved carboxy-terminal sequence region, which we term the IN box. This interaction between INCENP and Aurora kinase was found to be biologically relevant. INCENP and AIRK2 colocalized exactly in human cells, and INCENP was required to target AIRK2 correctly to centromeres and the central spindle.**

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## Results and discussion

As INCENP is a highly insoluble chromosomal protein [2], we were interested to find it stockpiled in *Xenopus* eggs in a soluble form: affinity purified polyclonal antibody against

a peptide from the carboxyl terminus of *Xenopus* INCENP (XINCENP) recognized a doublet of 125 and 120 kDa on immunoblots of soluble *Xenopus* egg extract proteins (Figure 1a). Immunostaining with this antibody in cultured *Xenopus* cells gave a classical INCENP distribution: the protein concentrated at centromeres during metaphase, before transferring to the central spindle and equatorial cortex in anaphase (Figure 1b).

Sucrose density gradient centrifugation of egg extract proteins revealed that XINCENP exists in two complexes, a soluble 11S form and a larger (>40S) form (Figure 1c). The larger complex shifted to 11S if extracts were sedimented through gradients containing 1% triton X-100. In clarified egg extract, only the 11S form was present. The INCENP peak shifted to 5S when the extracts were sedimented through gradients containing 0.5 M NaCl. Thus, XINCENP is stockpiled in eggs in a macromolecular complex that is disrupted by moderate ionic strengths.

To characterize the 11S complex, we used purified anti-XINCENP antibodies to immunoprecipitate the protein from soluble egg extract. Purified immunoglobulin G (IgG) from preimmune serum of the same rabbit was used for mock immunoprecipitations. When immunoprecipitated proteins were analyzed by SDS-PAGE, three bands of molecular weight 125, 120 and 41 kDa were specifically enriched in the anti-XINCENP immunoprecipitates (Figure 1d). These polypeptides were excised from gels and peptide sequences obtained by mass spectroscopy. As expected, p125 and p120 were identified as XINCENP. About 2–5 µg of XINCENP was immunoprecipitated from the 250 mg extract protein, representing >99% of the XINCENP originally present. XINCENP was extremely rare, accounting for only 0.001–0.003% of total extract protein.

Several peptide sequences comprising a total of 28 residues were obtained from p41. These sequences revealed that p41 was similar to the Aurora/Ipl1-related protein kinases (AIRKs), a family of serine-threonine kinases required for progression through mitosis and cytokinesis [9,10]. We cloned the cDNA encoding p41 by PCR. On the basis of sequence homology with other AIRKs, we propose that p41 is a *Xenopus* homologue of the chromosomal passenger AIM-1/AIRK2 (Figure 2). XAIRK2 is a basic protein of 361 amino acids and predicted molecular weight 41.8 kDa. The protein might be targeted for ubiquitin-mediated proteolysis at the end of mitosis as it contains both an RxxL motif

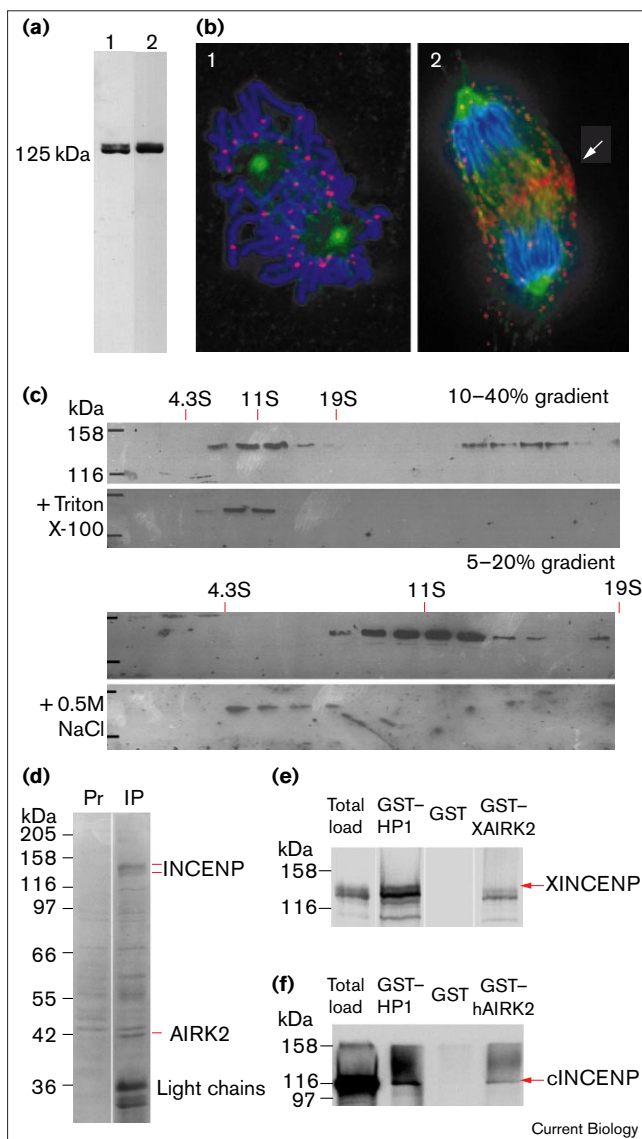


Figure 1

INCENP is associated with AIRK2 kinase. (a) Antibody R644 recognized a doublet of 125 and 120 kDa on western blots of total soluble egg extract (lane 1), and immunoprecipitated a protein doublet that was recognized by another anti-XINCENP antibody (Rb1186). (b) *Xenopus* A6 cells in prometaphase (panel 1) or anaphase (panel 2) were stained for XINCENP (red), tubulin (green), and with 4,6-diamidino-2-phenylindole (DAPI) to show DNA (blue). The arrow in panel 2 indicates accumulation of INCENP at the presumptive cleavage furrow. (c) INCENP is stockpiled in a salt-sensitive 11S complex in *Xenopus* egg extracts. Crude extract contained an additional > 40S detergent-sensitive complex (upper panels). Molecular weight markers at 158 and 116 kDa are the same for all four gels. (d) Large-scale immunoprecipitation of XINCENP revealed an association with XAIRK2. Pr, control immunoprecipitation with preimmune serum; IP, immunoprecipitation with affinity-purified R644 serum. Three bands specific for the anti-XINCENP immunoprecipitation were present at 41, 125 and 130 kDa. (e) *In vitro* translated XINCENP binds GST-XAIRK2 *in vitro*. GST-XAIRK2 was expressed in *Escherichia coli* and purified on glutathione-agarose; <sup>35</sup>S-methionine-labeled XINCENP translated *in vitro* was incubated with the glutathione beads, and material that remained bound after washing was subjected to SDS-PAGE and autoradiography. INCENP bound GST-AIRK2 and a fusion between GST and the heterochromatin protein 1 (GST-HP1) but not GST alone. (f) Chicken INCENP (cINCENP) binds human AIRK2 *in vitro*. Binding conditions were as in (e).

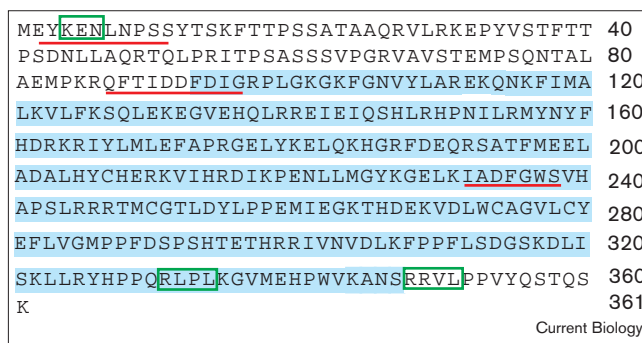
Previous searches of the yeast sequence database had failed to identify an INCENP homologue and we had assumed that INCENP might be specific for organisms with an open mitosis. Subsequent database searches using the carboxyl terminus of INCENP revealed the existence of INCENP homologues in flies and worms, based on a short stretch of homology (64–84 amino acids) that we term the IN box (Figure 3). Using PSI-BLAST [13], we found that fission yeast also has an INCENP homologue. Use of the *Schizosaccharomyces pombe* sequence to rescreen the *Saccharomyces cerevisiae* database revealed a related protein, Sli15p, with the conserved IN box sequence, albeit in slightly truncated form. Importantly, SLI15 was originally identified in a genetic screen for genes that interact with the budding yeast Aurora kinase IPL1 [14]. Both *Ipl1* and *Sli15* mutants show identical chromosome segregation defects, and the two proteins form a stable complex *in vitro* and *in vivo* [14]. It has been proposed that Sli15p might be a positive regulator of Ipl1p function. Ipl1p can phosphorylate the kinetochore component Ndc10p [15] and its function is opposed by protein phosphatase 1 [16]. Although the vertebrate homologue of Ndc10p has yet to be identified, it will be very interesting to look for interactions between INCENP/AIRK2 and kinetochore components. Interestingly, neither *Sli15* nor *Ipl1* mutants in budding yeast exhibit obvious primary cytokinesis defects. It is possible that these proteins may have acquired additional specificities concomitant with the evolution of open mitosis and the AIRK2 branch of the Aurora kinases.

The interaction between vertebrate INCENP and AIRK2 suggests that the two proteins might act in a common

at the carboxyl terminus and a possible KEN box at the amino terminus [11,12].

To confirm the interaction between INCENP and AIRK2, we performed *in vitro* binding assays using *Xenopus* or human AIRK2 fused to glutathione-S-transferase (GST), and *in vitro* translated, <sup>35</sup>S-methionine-labelled *Xenopus* or chicken INCENP. In these experiments, INCENP bound specifically to GST-AIRK2 and not to GST or the beads alone. Binding was observed both for the homologous pairing of *Xenopus* INCENP and AIRK2 (Figure 1e) and for the heterologous pairing of chicken INCENP and human AIRK2 (Figure 1f). The latter result is consistent with the observation that chicken INCENP undergoes a normal pattern of cell-cycle-related movements in mammalian cells. This interaction between INCENP and Aurora was found to be conserved through evolution: budding yeast INCENP and Aurora kinase showed both genetic and physical interactions (see below).

Figure 2

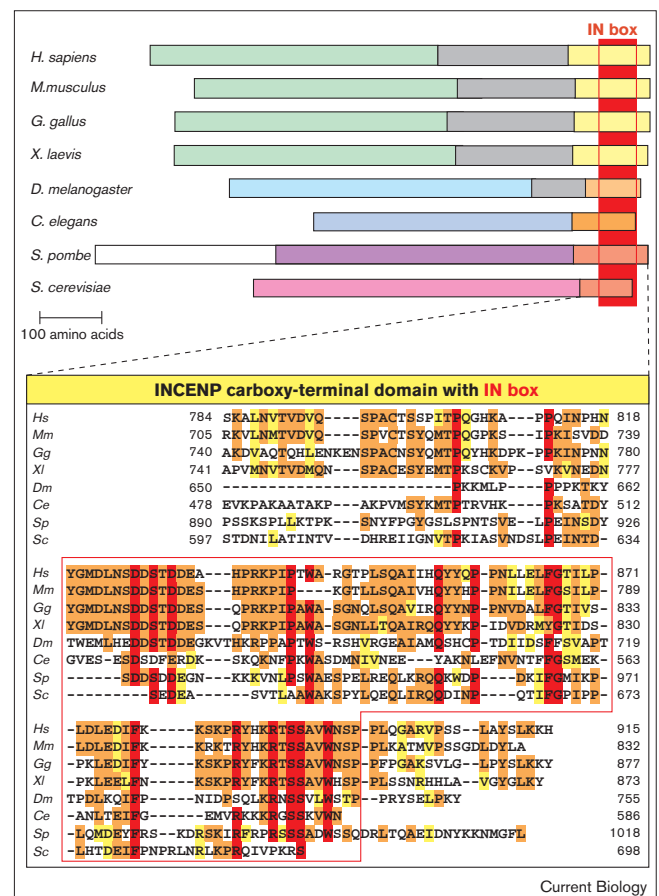


Amino acid sequence of XAIRK2 (accession number AF292096). Red underlines, peptide sequences derived by mass spectrometry; green boxes, potential degradation motifs; blue shading, predicted kinase domain. See Supplementary material for alignment with other aurora-like kinases.

process during mitosis. For example, INCENP could either be a targeting or regulatory subunit of the AIRK2 kinase. In support of this hypothesis, dominant mutations and loss of function mutations in AIRK2 in both vertebrate cultured cells and *C. elegans* result in defects in late cytokinesis similar to those seen with two dominant INCENP mutations [3,6,8,17]. It is also possible that these proteins may function in earlier stages of mitosis and that INCENP is a substrate for the kinase. A recent study has found independently that *C. elegans* INCENP and AIRK2 form a complex and that both are required for chromosome segregation and cytokinesis (M. Glotzer, personal communication).

To investigate the hypothesis that INCENP may target AIRK2 to the central spindle and cortex, we expressed in HeLa cells a dominant-negative INCENP (INCENP<sub>1-405</sub>), which targets to centromeres, but cannot transfer to the central spindle. This truncated protein causes endogenous INCENP to become diffusely localized throughout the cell, and causes chromosome congression and segregation defects, as well as failure of cytokinesis [6]. Cells transiently transfected with INCENP<sub>1-405</sub> were subsequently fixed and stained with antibodies specific for endogenous INCENP and AIRK2/AIM-1. The INCENP antibody used (Ra-2 [18]) was raised against the carboxy-terminal 22 kDa of INCENP, and therefore does not bind to the exogenous INCENP<sub>1-405</sub>. In non-transfected cells, both INCENP and AIRK2/AIM-1 were co-localized throughout mitosis in a classical chromosome passenger pattern, consistent with their being complexed with one another (Figure 4a–c). In cells exhibiting mitotic abnormalities, however, both the endogenous INCENP and AIRK2/AIM-1 were completely delocalized: staining was weak and diffusely spread throughout the cell (Figure 4d,e). In contrast, 0% (*n* = 30) of cells transfected with empty vector and

Figure 3



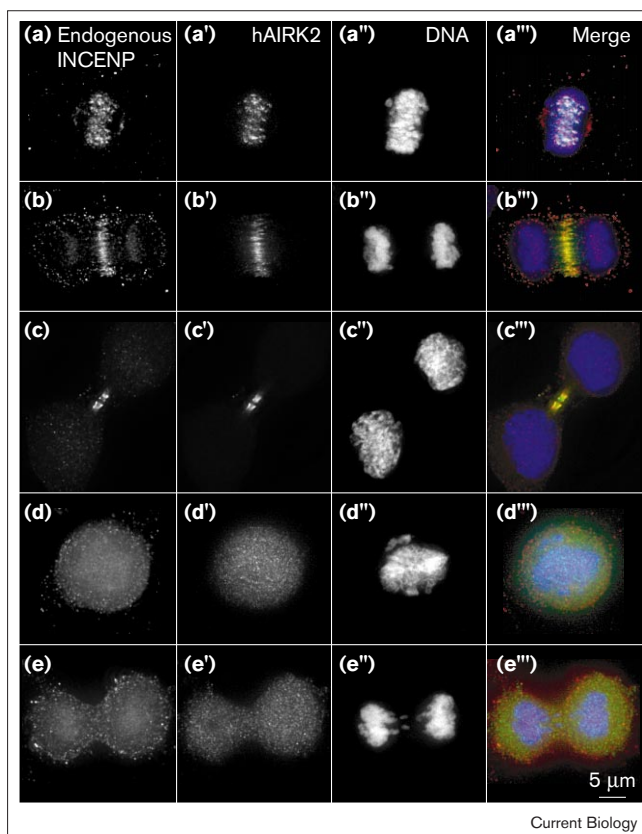
Putative lengths and domain boundaries of INCENP in different species. Identical colors indicate closely homologous domains; grey regions are predicted to adopt coiled-coil conformation with > 40% probability by the program MultiCoil [21]. The highly conserved, characteristic IN box is highlighted in the multiple sequence alignment of the carboxy-terminal domains (CLUSTALX with manual adjustments). Similar residues (yellow), highly conserved (orange) and identical positions (red) are indicated.

GFP–H2B [19] showed a diffuse AIRK2 localisation in mitotic cells (see Supplementary material).

Thus, when INCENP localisation is perturbed, the localisation of AIRK2 is similarly perturbed. This supports the hypothesis that INCENP is a targeting or regulatory subunit for AIRK2 kinase, although we cannot exclude the possibility that INCENP might be a downstream effector of AIRK2 kinase. It will be very interesting in future studies to determine whether other chromosomal passenger proteins that show an identical distribution, including TD-60 and survivin (S.P.W., unpublished data), are also involved in the activity of the INCENP–AIRK2 complex. Our results demonstrate that correct AIRK2 localisation is dependent on normal INCENP function in human cells. AIRK2 is upregulated in certain colorectal tumours, and elevated levels of AIRK2 induce multinuclearity, presumably because



Figure 4



An INCENP dominant-negative mutant disrupts AIRK2 localisation in HeLa cells. **(a–c)** INCENP and AIRK2/AIM-1 colocalize in HeLa cells undergoing a normal mitosis at (a) prometaphase, (b) late anaphase and (c) telophase. **(d,e)** Disruption of endogenous INCENP localisation is accompanied by disruption of AIRK2/AIM-1 localisation at prometaphase and late anaphase. Note the lagging chromosomes in (e). Cells were stained with the following reagents: (a–e) anti-INCENP antibody Ra-2 [18]; (a'–e') anti-AIM-1/AIRK2 antibody (Transduction laboratories); (a''–e'') DNA stained with DAPI. The merged image is shown in (a'''–e''').

of failures in cytokinesis [20]. This could be explained by the inability of INCENP to sequester the extra AIRK2, leading to its delocalisation and aberrant function.

#### Supplementary material

Supplementary material including a full description of the materials and methods, a sequence comparison of AIRK2 kinases and further data in support of Figure 4 is available at <http://current-biology.com/supmat/supmatin.htm>.

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