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The *Xenopus* Chromokinesin Xkid Is Essential for Metaphase Chromosome Alignment and Must Be Degraded to Allow Anaphase Chromosome Movement

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Summary

At anaphase, the linkage between sister chromatids is dissolved and the separated sisters move toward opposite poles of the spindle. We developed a method to purify metaphase and anaphase chromosomes from frog egg extracts and identified proteins that leave chromosomes at anaphase using a new form of expression screening. This approach identified Xkid, a *Xenopus* homolog of human Kid (kinesin-like DNA binding protein) as a protein that is degraded in anaphase by ubiquitin-mediated proteolysis. Immunodepleting Xkid from egg extracts prevented normal chromosome alignment on the metaphase spindle. Adding a mild excess of wild-type or nondegradable Xkid to egg extracts prevented the separated chromosomes from moving toward the poles. We propose that Xkid provides the metaphase force that pushes chromosome arms toward the equator of the spindle and that its destruction is needed for anaphase chromosome movement.

Introduction

During cell division, interactions between chromosomes and microtubules segregate the chromosomes into two sets (Murray and Hunt, 1993). Errors in this process cause birth defects and contribute to tumor progression. At the beginning of mitosis, the chromosomes condense and microtubules form a bipolar spindle with a microtubule organizing center at each end. The microtubules are polar structures whose minus ends are located near the spindle pole and are moved poleward by microtubule flux (Mitchison, 1989; Mitchison and Salmon, 1992; Desai et al., 1998). As mitosis proceeds, the kinetochores (the proteinaceous complex assembled on the centromeric DNA) attach to microtubules, the pairs of replicated sister chromatids align with their sister kinetochores attached to opposite poles of the spindle (biorientation), and migrate to the equator of the spindle, equidistant from the two poles (congression). When all the chromosomes have attached and congressed, the cell is in metaphase and the chromosomes are said to lie on the metaphase plate. During anaphase, the sister chromatids separate from each other and move toward the spindle poles. Although the mechanical details of mitosis are known, the molecular mechanisms of chromosome positioning and segregation are poorly understood.

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In vertebrate cells, at least three microtubule-dependent forces influence chromosome movement and positioning: forces generated at the kinetochore, forces that move microtubules toward the spindle poles, and the polar ejection force (Rieder et al., 1986), which pushes chromosome arms away from the spindle poles (reviewed in Rieder and Salmon, 1994; Skibbens et al., 1995). In tissue culture cells, individual kinetochores oscillate between moving toward and away from the poles to which they are attached (Skibbens et al., 1993; Khodjakov and Rieder, 1996; Waters et al., 1996). After bioriented sister chromatid pairs congress, these movements oscillate about the metaphase plate. One model postulates that tension on the linkage between a kinetochore and a spindle pole regulates the direction of chromosome movement; the greater the tension at the kinetochore the more likely the kinetochore is to move away from the pole (Salmon, 1989; Murray and Mitchison, 1994). If the intensity of the polar ejection force decreased with distance from the poles, this model could explain why bioriented chromosomes congress.

Several microtubule motors play roles in chromosome movement and positioning (Rieder and Salmon, 1998; Heald and Walczak, 1999). These include three molecules found at the kinetochore: CENP-E, a plus-end-directed, kinesin-like motor (Yen et al., 1991), cytoplasmic dynein, a minus-end-directed motor (Pfarr et al., 1990; Steur et al., 1990), and XKCM1, a kinesin-like molecule that stimulates microtubule depolymerization (Desai et al., 1999a). CENP-E is required for metaphase chromosome alignment (Schaar et al., 1997; Wood et al., 1997), whereas dynein appears to capture the first microtubules that interact with the kinetochore (Rieder and Alexander, 1990; Skibbens et al., 1993). Chromosome bound motors (chromokinesins) appear to contribute to the polar ejection force, the bipolarity of the spindle, and chromosome positioning on the spindle (reviewed in Vernos and Karsenti, 1995) . Interfering with the *Xenopus* chromokinesin, Xklp1, alters the distribution of microtubules in the spindle and induces defects in metaphase chromosome alignment (Vernos et al., 1995). In female *Drosophila* meiosis I, the chromokinesin Nod is required for proper alignment of chromosomes that have not recombined with each other (Theurkauf and Hawley, 1992; Afshar et al., 1995). Mutants in another *Drosophila* chromokinesin, Klp38B, cause chromosome missegregation in meiosis and mitosis (Molina et al., 1997; Ruden et al., 1997).

Cells trigger anaphase by dissolving the linkage between sister chromatids and segregating the separated sisters to opposite poles of the spindle. Activating the anaphase promoting complex (APC, also known as the cyclosome, reviewed in Zachariae and Nasmyth, 1999) induces the destruction of several mitotic proteins. Securin degradation allows sister chromatids to separate (reviewed in Biggins and Murray, 1998), and mitotic cyclin destruction (Murray et al., 1989) inactivates the mitosis inducing protein kinase, Cdk1 (cyclin dependent kinase 1, also known as Cdc2 or Cdc28) (reviewed in Morgan, 1997). Although sister separation requires securin destruction, it does not require microtubule dependent forces or the inactivation of Cdk1 (Rieder and Palazzo, 1992; Straight et al., 1996). Anaphase chromosome

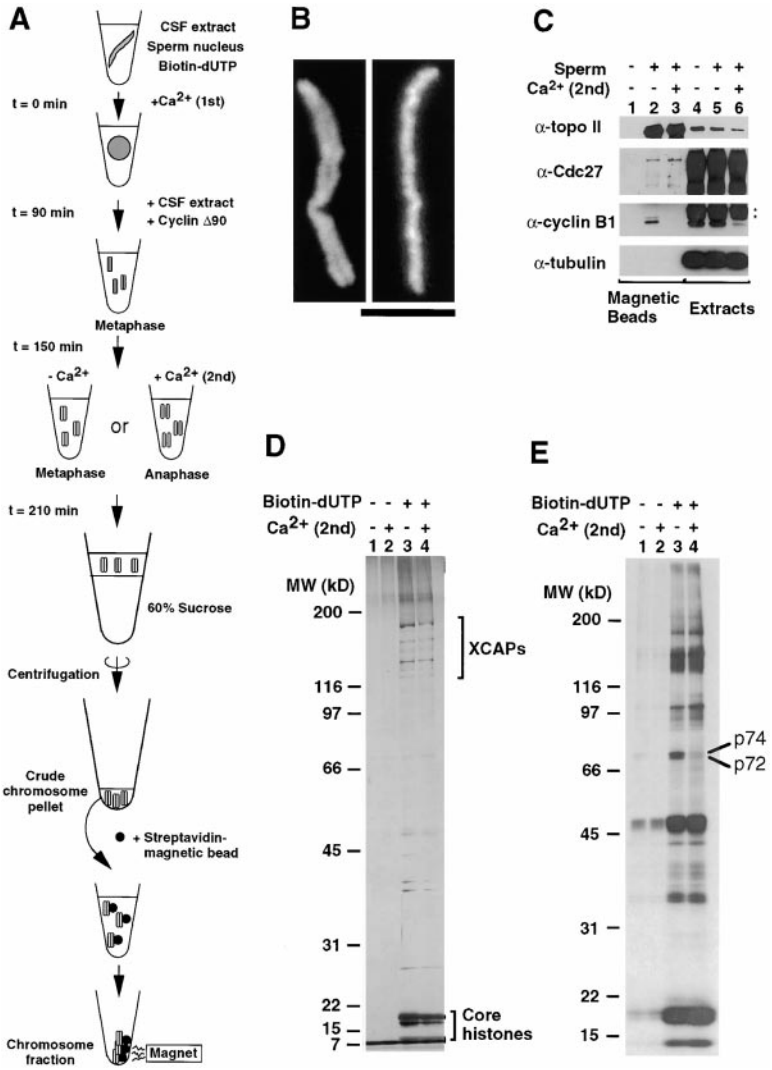


Figure 1. Purification of Metaphase and Anaphase Chromosomes from *Xenopus* Egg Extracts

(A) The protocol to purify biotinylated chromosomes using streptavidin-coated magnetic beads.

(B) Hoechst 33258 staining of chromosomes from a metaphase (left) or an anaphase (right) extract after dilution into chromosome dilution buffer. Bar, 10 μm.

(C) Total extract proteins (lanes 4–6) or chromosomal proteins copurified with streptavidin-magnetic beads (lanes 1–3) were immunoblotted with anti-topoisomerase II, anti-Cdc27, anti-cyclin B1, or anti-α-tubulin. Asterisks represent cross-reacting proteins with anti-cyclin B1 antibodies. Samples were prepared from metaphase (lanes 1, 2, 4, and 5) or anaphase (lanes 3 and 6) extracts containing 5 μM biotin-dUTP, in the presence (lanes 2, 3, 5, and 6) or absence (lanes 1 and 4) of sperm nuclei. The chromosomal proteins are the elute from magnetic beads prepared from 180 μl of extract, whereas the extract lanes contain the protein from 1.2 μl of extract.

(D) Silver staining of chromosomal proteins that copurified with streptavidin-magnetic beads from metaphase (lane 3) or anaphase (lane 4) extract containing 5 μM biotin-dUTP (lanes 3, 4). Lanes 1 and 2 are control samples from extracts that lacked biotin-dUTP. Positions of high molecular weight XCAPs (*Xenopus* chromosome-associated polypeptides, such as topoisomerase II and condensins) and core histones are indicated.

(E) Autoradiograph of chromosomal proteins from a ^{35}S-methionine labeled extract that copurified with streptavidin-magnetic beads from metaphase (lane 3) or anaphase (lane 4) extract containing 5 μM biotin-dUTP. Lanes 1 and 2 are control samples from extracts that lacked biotin-dUTP.

movement requires sister separation and microtubule dependent forces (Shamu and Murray, 1992), but can still occur if the inactivation of Cdk1 is blocked (Holloway et al., 1993; Surana et al., 1993).

We purified metaphase or anaphase chromosomes from *Xenopus* egg extracts and looked for proteins that left chromosomes at the onset of anaphase. This approach identified a single protein, Xkid, which is the *Xenopus* homolog of Kid, a human, kinesin-like, DNA binding protein (Tokai et al., 1996). Manipulating Xkid affects chromosome movement: removing the protein prevents normal chromosome alignment at metaphase, and blocking its degradation prevents poleward chromosome movement in anaphase.

Results

Isolation of Metaphase and Anaphase Chromosomes

We used frog egg extracts to look for proteins that left chromosomes at anaphase. Unfertilized frog eggs are arrested in metaphase of meiosis II by cytostatic factor (CSF [Masui and Markert, 1971]), which inhibits the APC. Fertilization triggers a rise in cytoplasmic calcium that overcomes this arrest and induces anaphase. This process can be mimicked by adding calcium to CSF-arrested extracts prepared from unfertilized *Xenopus* eggs (CSF extracts).

To compare metaphase and anaphase chromosomal proteins, we developed a novel chromosome purification method (Figure 1A). Sperm nuclei and biotin-dUTP were added to CSF extracts. The extracts were released into interphase by adding calcium, inducing the incorporation of biotin-dUTP into the replicating chromosomes. During interphase, the added calcium was sequestered in intracellular stores, making it possible to induce entry into mitosis, by adding additional CSF extract to the interphase extracts. After spindle assembly, anaphase was induced by adding calcium for the second time. In a normal cell cycle, chromosomes would decondense at the end of anaphase because the destruction of mitotic cyclins inactivates Cdk1 (Shamu and Murray, 1992). To prevent this, nondegradable cyclin (cyclin Δ90) was added to maintain Cdk1 activity and chromosome condensation (Figure 1A) (Holloway et al., 1993). Anaphase depends on two events: dissolving the linkage between sister chromatids (sister separation) and segregating the separated chromosomes to opposite poles of the

spindle. We developed a method to visualize paired sister chromatids. Diluting metaphase extracts into chromosome dilution buffer revealed pairs of sister chromatids whereas the same treatment of anaphase extracts produced separated chromatids (Figure 1B).

We used the binding of streptavidin to biotin incorporated during DNA replication to isolate the chromosomes (Figure 1A and Experimental Procedures). Western blotting confirmed that known chromosomal proteins, such as DNA topoisomerase II (Hirano and Mitchison, 1994), were enriched in the bead-bound fraction (Figure 1C). A small fraction of Cdc27 (a component of APC) (King et al., 1995) and cyclin B1 also purified with the beads. Neither α-tubulin nor proteins that cross reacted with anti-cyclin B1 antibodies (asterisks) copurified with chromosomes. Besides cyclin B1, which is known to be degraded in anaphase, none of these proteins were lost from the chromosomes at anaphase.

We compared the proteins bound to metaphase and anaphase chromosomes. The proteins detected by silver staining were indistinguishable between metaphase and anaphase chromosomes (Figure 1D). Although eggs have large stockpiles of most chromosomal proteins, those like cyclin, which are degraded in one cell cycle, must be resynthesized in the next (Murray and Kirschner, 1989). To look for such proteins, we examined the newly synthesized proteins that associate with chromosomes. When ^{35}S-methionine was added to the initial extracts, a pair of proteins (p72 and p74) copurified with metaphase chromosomes but not with anaphase chromosomes (Figure 1E). On two dimensional gels, we found that p74 and p72 comigrated in the isoelectric focusing dimension (Figure 2B) and that no protein other than p74/p72 showed reproducible difference between metaphase and anaphase (data not shown).

Identification of p74/p72 as Xkid

We used expression cloning (Lustig et al., 1997) to obtain a cDNA clone encoding p74/p72. We examined metaphase chromosomes prepared from extracts that had been supplemented with labeled proteins expressed from pools of a cDNA expression library. After screening 54 pools representing 27,000 clones, we found one pool that produced chromosome binding proteins of 74 kDa and 50 kDa (Figure 2A). The in vitro translated 74 kDa protein behaved like the endogenously labeled p74/p72 protein: calcium-induced APC activity led to a decline in the amount of the protein recovered with the chromosomes. By subdividing the p74-containing pool we identified a cDNA clone encoding the 74 kDa protein. When the mRNA from this clone was translated in reticulocyte lysate and added to extracts, a small amount of the 72 kDa protein copurified with chromosomes in addition to the 74 kDa protein (data not shown), suggesting that both proteins are derived from a single mRNA, by using alternative initiation codons or post-translational modification. Reticulocyte translated p74 comigrates on a two dimensional gel with endogenously labeled p74/p72 from egg extracts, supporting the idea that the cDNA encodes both p72 and p74 (Figure 2B). The same protein was also identified in a screen for proteins whose destruction was induced by the Cdk1-dependent activation of the APC (Zou et al., 1999; T. McGarry, H. Zou, T. Bernal, and M. Kirschner, personal communication).

The 651 amino acid open reading frame encoding p74 and p72 (Figure 2C) is 54% identical to human Kid (kinesin-like DNA binding protein) (Tokai et al., 1996). We therefore named the *Xenopus* protein Xkid (*Xenopus* Kid). A short stretch of the Xkid tail (Leu 585–Ser651) is 31% identical to *Drosophila* Nod (Zhang et al., 1990). The C-terminal domains of Kid and Nod have been shown to bind DNA in vitro (Afshar et al., 1995; Tokai et al., 1996). Both Xkid and Kid have an N-terminal motor domain followed by a predicted coiled-coil region and two copies of a putative class 1 helix-hairpin-helix DNA binding motif (HhH1). Both proteins have a Cdk1 phosphorylation consensus sequence at an equivalent position in the nonmotor domain. Xkid has five putative destruction boxes that contain the RxxL motif first identified in mitotic cyclins (Glotzer et al., 1991), but only one of these is present in Kid.

If p74/p72 is Xkid, endogenously labeled p74/p72 should be immunodepleted by antibodies against Xkid. We raised rabbit antibodies against the C-terminal 304 amino residues of Xkid. The affinity-purified antibody recognizes a protein at 74 kDa in CSF extracts whose abundance declines rapidly after adding calcium to CSF extracts, showing that the antibody is specific to Xkid and that Xkid is degraded in anaphase (Figure 2D). Using anti-Xkid antibodies coupled to magnetic beads, more than 90% of Xkid was depleted from CSF extracts that had been labeled with ^{35}S-methionine. These Xkid-depleted CSF extracts were added to cycloheximide-treated interphase extracts containing biotinylated chromosomes where 90% of Xkid had been degraded, and metaphase chromosomes were prepared from the mixed extracts (Figure 2E, lane 2). Neither p74 nor p72 copurified with metaphase chromosomes from Xkid-depleted extracts (Figure 2E, lane 4). The reduction of p74/p72 was not caused by premature anaphase in the Xkid-depleted extracts (data not shown).

Xkid Localizes to Metaphase Chromosomes in Frog Egg Extracts

We examined the localization of Xkid during mitosis. Anti-Xkid immunofluorescence was strong on metaphase chromosomes and weak on spindle poles and along spindle microtubules (Figure 3A, left). Twenty minutes after adding calcium, when sister chromatids had segregated, the level of anti-Xkid immunofluorescence associated with chromosomes fell (Figure 3A, right). To obtain more detailed images of chromosomes, we diluted extracts with chromosome dilution buffer to disperse the spindles and chromosomes. Punctate Xkid staining was seen on metaphase chromosomes but no signal was detected on anaphase chromosomes, even though these had been prepared from an extract in which the Cdk1 activity had been kept high by adding nondegradable cyclin (Figure 3B).

Xkid Is Required for Metaphase Chromosome Alignment

We investigated the role of Xkid during spindle assembly by immunodepleting the protein from extracts. Since metaphase spindles are assembled by adding a CSF extract to an interphase extract, both of these extracts must be depleted of Xkid (Figure 4A and Experimental Procedures). Forty minutes after adding CSF extracts, bipolar metaphase spindles were observed in mock-treated extracts (Figures 4B and 4C). In contrast, chromosomes were stretched and misaligned in 83% of spindles assembled in Xkid-depleted extracts. Similar

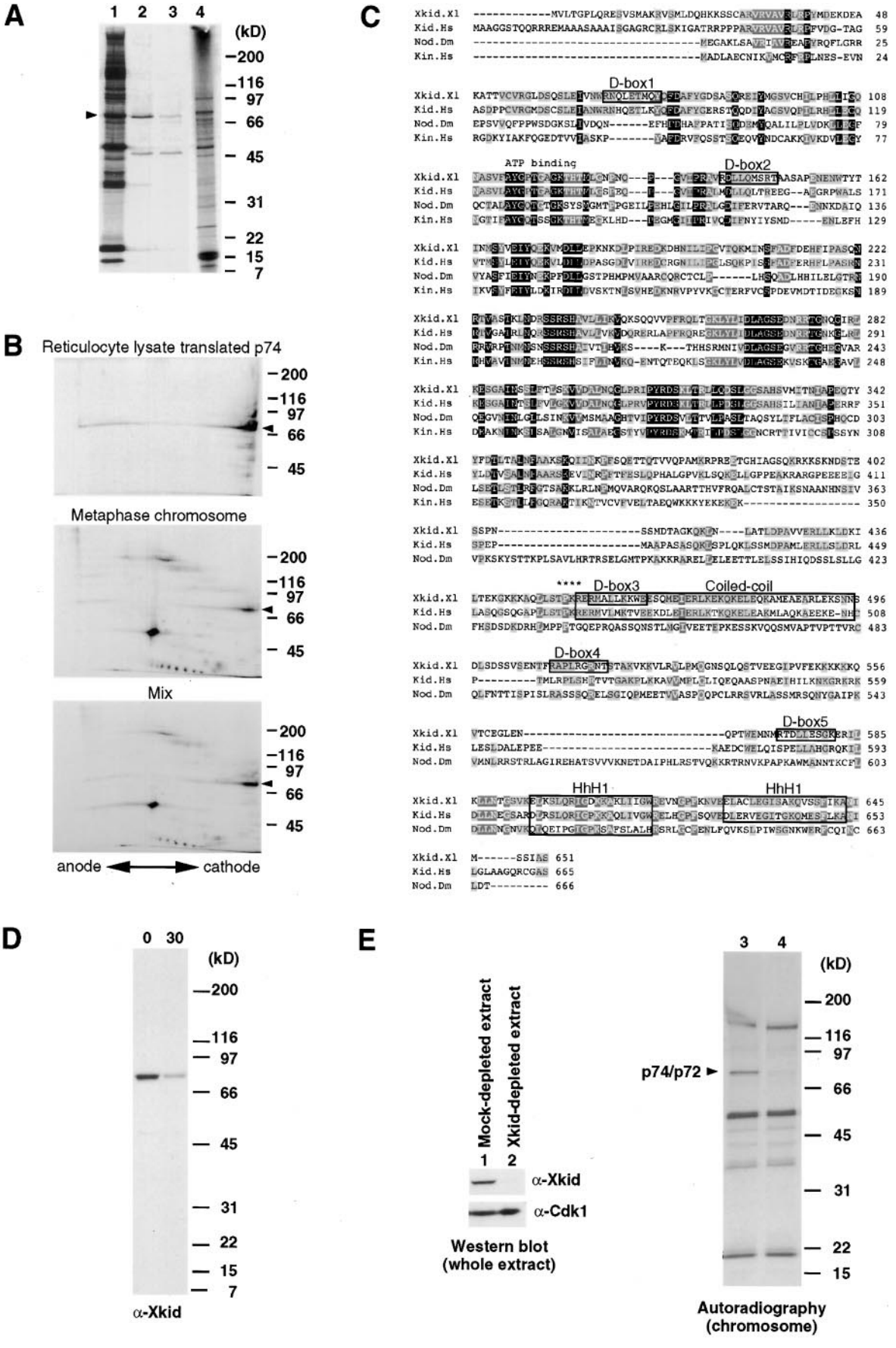


Figure 2. p74/p72 Is a *Xenopus* Homolog of Kid

(A) Cloning of a gene encoding the metaphase chromosomal protein p74/p72. Pools of a cDNA expression library were transcribed and translated in reticulocyte lysates in the presence of ^{35}S-methionine. Metaphase chromosomal proteins were obtained from egg extracts

defects were seen when affinity-purified Xkid antibodies were added to the extracts to a final concentration of 68 μg/ml (Figure 4C).

To show that this chromosome misalignment phenotype was caused solely by loss of Xkid, we asked if adding recombinant Xkid protein could restore normal chromosome alignment. We purified baculovirus-expressed Xkid tagged with 6 histidine residues at its N termini (6×His-Xkid) (Figure 4D) and added it to Xkid-depleted extracts. Adding purified 6×His-Xkid rescued the misalignment of chromosomes in spindles (Figures 4B and 4E). Whereas 40 nM 6×His-Xkid is required to fully rescue immunodepleted extracts, adding 10 nM 6×His-Xkid (similar to the concentration of endogenous Xkid) increased the fraction of properly aligned spindles to 53%.

Chromosomes' Arms Move to Poles in the Metaphase Spindle in the Absence of Xkid

To examine the consequences of Xkid depletion in more detail, we followed the formation of individual spindles by video microscopy. We collected images of rhodamine-labeled microtubules and DAPI-stained DNA at 45 s intervals after the addition of CSF extracts that induce spindle formation (Figure 5).

Figures 5A and 5B show the comparison between mock-depleted and Xkid-depleted extracts. In both extracts, chromosomes started to condense between 4 and 7 min after adding CSF extract, and microtubules nucleated from centrosomes and chromosomes around 10 min. In all the control spindles, the majority of chromosomes stayed at the metaphase plate throughout the experiment, although occasional chromosomes moved substantial distances away from the equator before returning to it (the chromosome marked "b" in Figure 5A). In one extreme case, an individual chromosome moved rapidly to one spindle pole and then returned to the equator (the chromosome marked "a", between 35 and 43 min in Figure 5A).

In Xkid-depleted extracts, the initial stages of spindle assembly resembled those in control extracts. The chromosomes condensed, bipolar spindles formed, and the chromosomes were initially located at the spindle equator (Figure 5B). By 15 min after the onset of mitosis, however, the chromosomes began to move toward the poles. As well as being abnormally positioned along the length of the spindle, many of the chromosomes in the depleted extracts were stretched along the length of the spindle as if one end was being held at the spindle equator and the other was being pulled toward the pole. For one chromosome, the end located at the center of the spindle appeared to become detached from the spindle (the chromosome marked "c" at 49 min in Figure 5B) allowing the chromosome to shrink rapidly toward the pole. For most stretched chromosomes, the end that moved toward the pole did so continuously. As chromosomes stretched, the speed of poleward movement was 1.0 ± 0.3 μm/min (n = 18) in Xkid-depleted extract.

We considered several ideas to explain how Xkid depletion alters the position and stretch of chromosomes on the spindle. The first was that Xkid is required to maintain normal chromosome structure. This is unlikely because the extent of chromosome condensation is the same in Xkid-depleted and control extracts, even when spindle formation was inhibited by nocodazole (data not shown). In addition, there was no morphological difference between chromosomes from control and Xkid-depleted extracts when they were examined in chromosome dilution buffer (data not shown). The second possibility is that the sister chromatids partially separate from each other allowing the sister kinetochores to move toward opposite poles. We used anti-CENP-E antibodies, which recognize a kinesin found at kinetochores (Desai et al., 1997), to see the kinetochores in Xkid-depleted extracts (Figure 6). Most of the stretched chromosomes (72%) had a pair of CENP-E dots at the chromosome end closest to the center of the spindle (arrows in Figure 6) whereas 24% had a pair of dots at the chromosome end closest to the pole and 4% had only a single visible dot. These results argue that there is no loss of sister chromatid linkage in metaphase, Xkid-depleted extracts. Instead, they support the idea that the positioning of the kinetochores on the spindle is relatively normal, but that the chromosome arms are moved toward the spindle poles.

Xkid Is Degraded by Ubiquitin-Mediated Proteolysis

Xkid is required for metaphase chromosome alignment, but is degraded in anaphase. The APC triggers polyubiquitination and proteolysis of cyclins and securins

containing cycloheximide, to which a reticulocyte lysate containing translation products from a pool (G3/2) that consists of 870 clones (lane 4) was added either just before the induction of mitosis (lane 2) or before the initial exit from CSF arrest induced by calcium addition (lane 3). The control lanes contain metaphase chromosomal proteins purified from egg extracts labeled with ^{35}S-methionine (lane 1) and the total translation products from pool G3/2 (lane 4). The position of p74/p72 is marked with an arrowhead.

(B) The cloned 74 kDa protein comigrates on two dimensional gels with p74/72 isolated from metaphase chromosomes. Top, chromosomal proteins prepared from a metaphase extract supplemented with the ^{35}S-methionine-labeled reticulocyte lysate programmed with the p74-encoding clone. Center, endogenously ^{35}S-methionine-labeled proteins associated with metaphase chromosomes purified from egg extracts. Lower, mixture of the upper two samples. The first dimension gel was isoelectric focusing (pH 3–10) and second dimension was SDS-(7.5%) polyacrylamide gel electrophoresis.

(C) Sequence alignment of *Xenopus laevis* Xkid, *Homo sapiens* Kid, *Drosophila melanogaster* Nod, and the motor domain *of Homo sapiens* kinesin heavy chain is shown. Positions of putative destruction boxes (D boxes) in Xkid are shown as small boxes. Cdc2 phosphorylation consensus sequences are marked with asterisks. HhH1 and coiled-coil domains predicted by simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de) are indicated with boxes.

(D) Specificity of affinity-purified anti-Xkid. Samples from CSF-arrested extracts were taken 0 and 30 min after calcium addition, run on a 7.5%–15% gradient gel, transferred to nitrocellulose, and probed with affinity-purified anti-Xkid. No band was detected with the IgG fraction of preimmune serum (data not shown).

(E) Immunodepletion of Xkid from egg extracts. CSF extracts, labeled with ^{35}S-methionine and immunodepleted with control IgG beads or anti-Xkid beads, were added to an interphase extract containing biotinylated chromosomes and cycloheximide, which had been added to prevent protein synthesis in interphase. Sixty minutes later, chromosomes from each extract were purified. A Western blot of total proteins using anti-Xkid or anti-Cdk1 (loading control) from mock-depleted extract (lane 1) or Xkid-depleted extract (lane 2), and an autoradiograph of ^{35}S-labeled chromosomal proteins from mock-depleted extract (lane 3) or Xkid-depleted extract (lane 4) is shown.

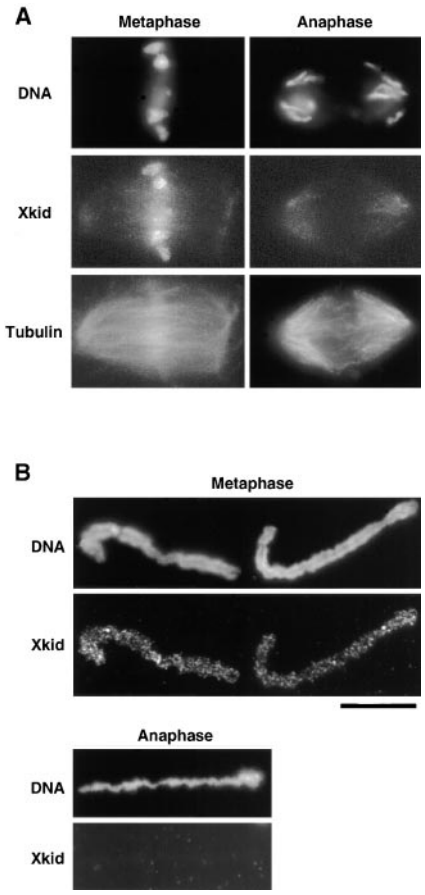


Figure 3. Xkid Is Localized to Metaphase Chromosomes in Egg Extracts

(A) Anti-Xkid immunofluorescence on spindles. Spindles assembled on replicated chromosomes were spun down onto a coverslip at 0 (metaphase) or 20 (anaphase) min after calcium was added to induce the onset of anaphase. DNA, Xkid, and microtubules were visualized with Hoechst 33258, anti-Xkid, and rhodamine-labeled tubulin. No fluorescent signal was observed with control IgG (data not shown). (B) Xkid localization on individual chromosomes. A metaphase egg extract containing replicated chromosomes and cyclin Δ90 was treated with calcium to obtain anaphase chromosomes or incubated without further additions to obtain metaphase chromosomes. Sixty minutes later, the reactions were diluted with chromosome dilution buffer and processed for indirect immunofluorescence using anti-Xkid antibodies and stained with Hoechst 33258 to visualize DNA. Bar, 10 μm.

during the transition from metaphase to anaphase, and these modified proteins are degraded by the 26S proteasome. We therefore investigated whether Xkid degradation depends on the activity of the APC and the proteasome. A proteasome inhibitor, MG115, (Rock et al., 1994) and the N-terminal region (amino residues 13–110) of cyclin B (N-Cyc), which competitively inhibits APC-mediated ubiquitination (Holloway et al., 1993; King et al., 1995), both inhibited anaphase degradation of Xkid (Figure 7A). The degradation of Xkid was not inhibited by the N-terminal region of cyclin B containing point mutations in the destruction box that render it unrecognizable by the APC (N-CycND). These data suggest that APC and proteasome activity are both required for anaphase degradation of Xkid.

When ^{35}S-methione-labeled Xkid was added to a CSF extract and anaphase was induced by adding calcium, a higher-molecular weight smear increased in intensity during Xkid degradation (Figure 7B, left panel). This smear and a ladder of labeled bands are retained by a metal affinity column when extracts are supplemented with ubiquitin tagged with 6 histidine residues (6×His-Ubiquitin) (Figure 7C, upper panel). More of the smear was recovered from extracts with added calcium. This calcium-stimulated smear was suppressed by supplementing the extracts with the N terminus of cyclin B, but not the mutant version that lacks a destruction box, suggesting that anaphase-specific ubiquitination of Xkid depends on the APC.

The C terminus of Xkid appears to play a role in its destruction at anaphase. A version of the protein that carries 12 repeats of the Myc epitope at its C terminus (Xkid-12Myc) is not degraded when calcium is added to a CSF extract (Figure 7B, right panel). This form of the protein produces much smaller amounts of ubiquitin-conjugated high molecular weight material than the wild-type protein (Figure 7C, lower panel), reinforcing the conclusion that Xkid destruction depends upon its ubiquitination.

Xkid Degradation Is Required for Chromosome Segregation during Anaphase

Is APC-dependent proteolysis of Xkid required for any anaphase events? To answer this question, we exploited the observation that Xkid-12Myc was stable in anaphase (Figure 7B). Xkid-12Myc with 6 histidine residues at its N terminus (6×His-Xkid-12Myc) was purified from insect cells (Figure 4D). This modified form of the protein is functional since adding it rescued the misalignment of chromosomes in Xkid depleted extracts, although about twice as much protein is required for rescue compared to the amount of 6×His-Xkid (Figure 4E).

We investigated whether excess Xkid or 6×His-Xkid-12Myc affects anaphase in egg extracts. Metaphase spindles were formed in mock-depleted extracts or Xkid-depleted extracts supplemented with recombinant forms of Xkid before adding calcium to induce anaphase. Bipolar spindles were classified into four categories (Figure 8A): metaphase, early anaphase, mid anaphase, and late anaphase. In mock-depleted extracts or Xkid-depleted extracts containing 20 nM 6×His-Xkid, adding calcium induces an orderly progression from metaphase through early (16 min after adding calcium) and mid anaphase (27 min) to late anaphase (36 and 46 min) (Figures 8A and 8B). Xkid is degraded after adding calcium although more slowly than cyclin B1 (Figure 8D). In contrast, adding 40 nM 6×His-Xkid or 6×His-Xkid-12Myc to Xkid-depleted extracts greatly slowed progress through anaphase (Figures 8A and 8B). This slower anaphase chromosome movement correlated with the persistence of some of the added Xkid; 6×His-Xkid persisted well after the destruction of endogenous Xkid and there was little destruction of the added 6×His-Xkid-12Myc (Figure 8D). This reduced proteolysis is not a reflection of decreased APC activity since cyclin B1 destruction was similar in all of the extracts (Figure 8D). Adding exogenous Xkid had no effect on the timing of sister chromatid separation: in all extracts, more than 93% of sister chromatids had separated by the time the control extracts were in mid anaphase (27 min after adding calcium) (Figure 8C). These results show that excess

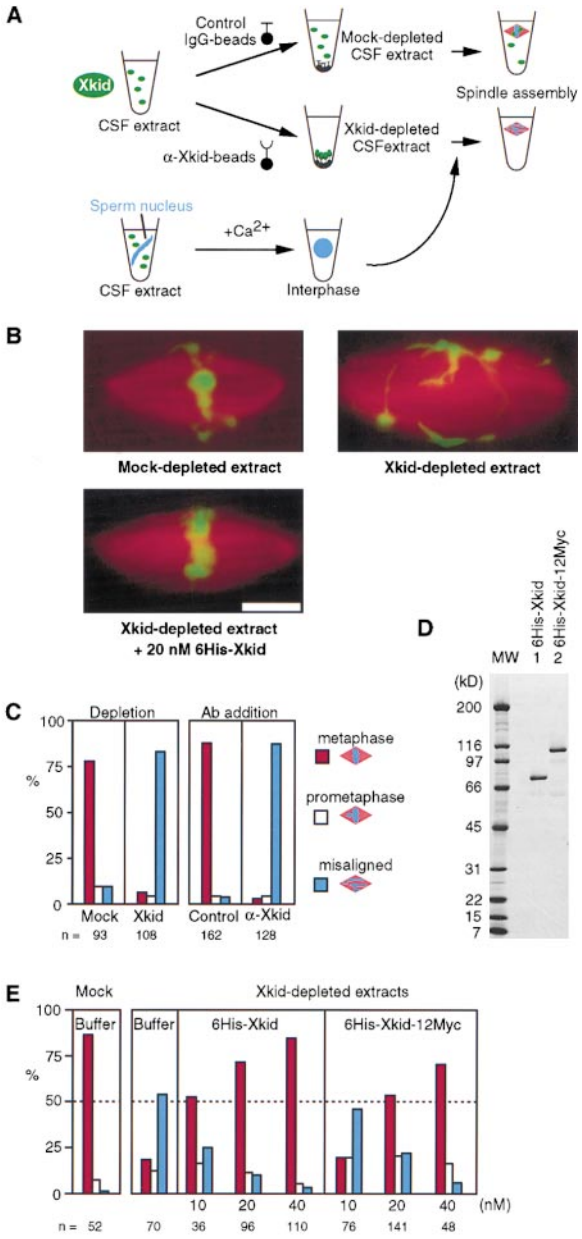


Figure 4. Xkid Depletion Induces Defects in Chromosome Alignment on the Mitotic Spindle

(A) A schematic representation of the protocol to assemble metaphase spindles in Xkid-depleted extracts.

(B) Spindles assembled in a mock-depleted extract, an Xkid-depleted extract, or an Xkid-depleted extract supplemented with 20 nM 6×His-Xkid, are shown. Chromosomes were visualized by Hoechst 33258 staining (green) and microtubules with rhodamine-labeled tubulin (red). Bar, 10 μm.

(C) Quantitation of spindle structures assembled on replicated chromosomes in mock- or Xkid-depleted extracts (left panel), and spindle structures in extracts containing 68 μg/ml control IgG or 68 μg/ml anti-Xkid (right panel) are shown. Spindles were scored between 44 and 56 min after entry into mitosis. Similar results were obtained in eight independent experiments. Spindle classification: metaphase, all chromosomes aligned at the equator of a bipolar spindle; prometaphase, all chromosomes aligned at the equator of the bipolar spindle except for one or two misaligned, unstretched chromosomes; misaligned, multiple chromosomes stretched and misaligned at the equator of the spindle. The rest of the population (always less than 10% of the total number of spindles) are multipolar spindles, probably representing the fusion of spindles assembled from two or more nearby nuclei.

(D) Coomassie blue staining of purified recombinant Xkid proteins. 0.25 μg (3.2 pmol) of 6×His-Xkid (lane 1) and 0.3 μg (3.2 pmol) of 6×His-Xkid-12Myc (lane 2) purified from insect cells are shown.

(E) Quantitation of spindle structures assembled on replicated chromosomes in mock-depleted extracts (Mock) or Xkid-depleted extracts supplemented with buffer (25 mM Na-HEPES [pH 7.2], 100 mM imidazole, 300 mM NaCl, and10% glycerol), 6×His-Xkid (10, 20, and 40 nM final), and 6×His-Xkid-12Myc (10, 20, and 40 nM final). Spindles were scored 75 min after exit from interphase as described in (B).

6×His-Xkid inhibits anaphase chromosome movement but not sister chromatid separation.

Discussion

Xkid Is Required for the Metaphase Chromosome Alignment

Perturbing Xkid disrupted the balance of forces that normally positions chromosomes on the mitotic spindle. In Xkid-depleted extracts, chromosomes did not stay at the metaphase plate and stretched toward the poles. A majority of the stretched chromosomes had their centromeres closest to the equator, suggesting that kinetochores remained attached to microtubules and attempt to maintain their position at the metaphase plate, whereas the chromosome arms move toward the spindle pole. Human Kid has a plus-end directed microtubule motor activity (J. Yajima, M. Edamatsu, and Y. Y. Toyoshima, personal communication), suggesting that Xkid is likely to be a microtubule motor. We propose that Xkid is the chromosome-bound motor that generates the polar ejection force, which plays an important role in opposing the forces that move chromosome arms toward the pole.

What moves chromosome arms toward the poles in the absence of Xkid? In *Xenopus* spindles, the rate of poleward microtubule flux (2–3 μm/min) (Sawin and Mitchison, 1991b; Desai et al., 1998) is faster than the poleward movement of stretched chromosome arms (about 1 μm/min) in Xkid-depleted extracts. We suggest that the arms attach to microtubules and are moved toward the poles by microtubule flux, and that the force required to stretch the chromosomes slows microtubule movement to the poles. Minus-end directed motors are an alternative to flux for the source of the poleward force that Xkid opposes. The known minus-end directed motors in egg extracts are cytoplasmic dynein and XCTK2 (Walczak et al., 1997; Walczak et al., 1998), whose human homolog, Cho2, is a minus-end directed motor (Kuriyama et al., 1995). Neither protein is a good candidate for a chromosome-arm motor. Cytoplasmic dynein is associated with kinetochores rather than chromosome arms in mitosis (Desai et al., 1997), and XCTK2 is found at the poles and on spindle microtubules, rather than on chromosomes (Walczak et al., 1997) .

The Role of Kinetochores and the Polar Ejection Force in Chromosome Alignment

Is the polar ejection force enough to align chromosomes? In tissue culture cells, the kinetochore is essential for chromosome alignment. Ablating the leading

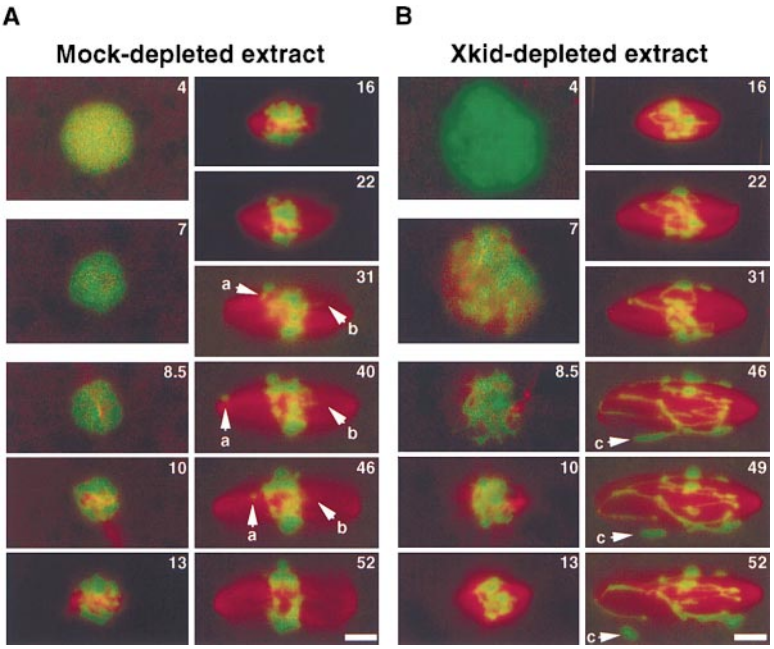


Figure 5. Chromosome Movements in Xkid-Depleted Extract during Spindle Assembly

Immediately after adding mock-depleted CSF extract or Xkid-depleted CSF extract to interphase extracts containing replicated chromosomes, spindle formation was monitored by time-lapse video microscopy. Rhodamine-labeled microtubules are shown in red; DAPI labeled chromosomes are shown in green. Time is given in minutes after adding CSF extracts. Arrowheads (a and b) indicates a chromosome that moved back and forth between the poles in the mock-depleted extract. The other arrowhead (c) indicates stretched chromosomes in the Xkid-depleted extract, which eventually detaches from the metaphase plate. Bars, 10 μm.

kinetochore of a congressing chromosomes keeps it from reaching the metaphase plate (Khodjakov and Rieder, 1996), and microinjecting anti-centromere or anti-CENP-E antibodies inhibits congression (Bernat et al., 1990; Simerly et al., 1990; Schaar et al., 1997).

In frog egg extracts, the situation is more complex. Immunodepleting CENP-E interferes with metaphase chromosome alignment, showing that perturbing one kinetochore component can disrupt congression (Wood et al., 1997). In other situations, congression occurs without biorientation of sister kinetochores. Spindles formed from unreplicated DNA appear to lack kinetochores, but have a clear metaphase plate, implying that forces that act on the acentric arms can be sufficient to align these acentric chromosomes at metaphase (Sawin and Mitchison, 1991a). During anaphase, excess Xkid can keep separated sister chromatids at the spindle equator despite forces being exerted at their kinetochores. This observation suggests that, under some circumstances, the polar ejection force can equal forces acting at the kinetochore and play a dominant role in determining chromosome position on the spindle.

Functions of Chromokinesins

We believe that chromokinesins fall into two classes, those that position chromosomes on the spindle and those involved in spindle assembly. Xkid and the *Drosophila* Nod protein play important roles in chromosomes alignment on the spindle. In *Drosophila* female meiosis, chromosome IV rarely recombines. The two nonrecombinant chromosomes lie on either side of the metaphase plate and this positioning is disrupted in *nod* mutants (Theurkauf and Hawley, 1992). Bipolar spindles assemble on sperm and DNA beads (Antonio et al., 2000 [this issue of *Cell*]) in egg extracts that lack Xkid and in *nod* mutant oocytes, suggesting that neither protein is needed for spindle assembly. We propose that Xkid and Nod generate the polar ejection force by exerting force between chromosome arms and microtubules. The polar ejection force required for chromosome alignment varies among different cell types. Recombined chromosomes are positioned normally in *nod* mutants and the misaligned chromosomes are not stretched like those in Xkid-depleted extracts (Theurkauf and Hawley, 1992), whereas in the African blood lily, *Haemanthus*, the centromeres align at the metaphase plate in the apparent absence of the polar ejection force, with chromosome arms stretched toward the poles (Khodjakov et al., 1996).

Two chromokinesin families play roles in spindle assembly: the *Xenopus* Xklp1/chicken chromokinesin proteins (Vernos et al., 1995; Wang and Adler, 1995) and the *Drosophila* Klp38B protein (Molina et al., 1997; Ruden et al., 1997). Inhibiting Xklp1 prevented the formation of normal bipolar spindles around chromosomes (Vernos et al., 1995) or DNA-coated beads (Walczak et al., 1998). The effects of anti-Xklp1 antibodies on chromosome alignment (Vernos et al., 1995) may have been a secondary effect of defects in spindle assembly and the primary function of Xklp1 seems to be sorting microtubules according to their polarity during the early stages of spindle assembly (Vernos and Karsenti, 1995; Walczak et al., 1998). Mitotic cells in the *Drosophila* Klp38B mutant, *tiovivo*, show circular mitotic figures, suggesting that Klp38B helps establish spindle bipolarity (Molina et al., 1997; Ruden et al., 1997).

Induction of Anaphase Chromosome Movement by Xkid Degradation

What induces chromosome segregation at anaphase? Sister chromatid separation is essential for anaphase chromosome movement (Shamu and Murray, 1992), but is it sufficient? Studies in tissue culture cells (Skibbens et al., 1995), budding yeast (Yamamoto et al., 1996), and fission yeast (Furuya et al., 1998) support the idea that destroying the linkage between sister chromatids allows them to move toward the spindle poles without changing the forces acting at the kinetochore or chromosome

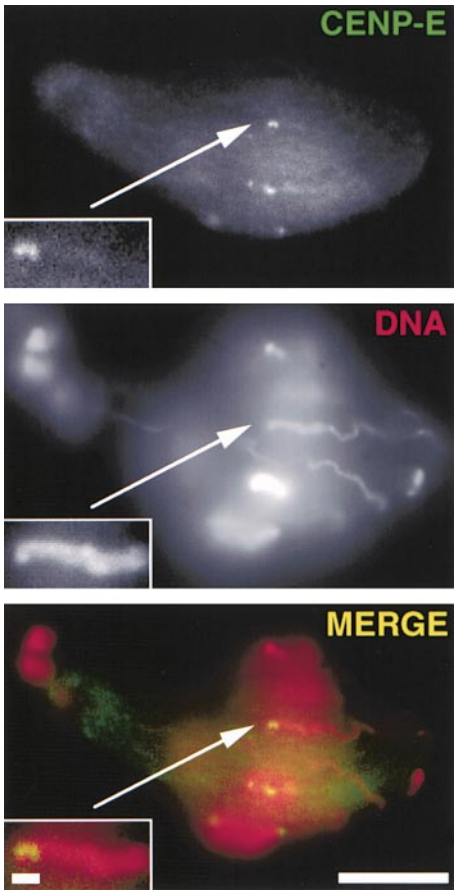


Figure 6. CENP-E Localization in an Xkid-Depleted Extract

Spindles formed in an Xkid-depleted extract were fixed on a coverslip and treated with Oregon green–labeled anti-CENP-E antibodies and Hoechst 33258 to visualize the DNA. Anti-CENP-E immunofluorescence of a single focal plane is shown in green while Hoechst 33258–labeled chromosomes are shown in red in the merge panel. White arrows indicate the position of a chromosome containing a pair of anti-CENP-E dots at the equatorial end of the chromosome whose magnified image is shown in insets. Bar, 10 μm (1 μm in the inset).

arms. In animal cells, however, the final stage of anaphase movement must reflect changes in these forces: without them, the separated chromosomes could only get to the position occupied by monooriented chromosomes during spindle assembly. Changes could include a reduction in the polar ejection force, abolishing the ability of kinetochores to move away from the pole they are attached to, or cross-linking the kinetochore to microtubules that are being moved poleward (Skibbens et al., 1993; Rieder and Salmon, 1994). The demonstration that Xkid degradation is required for anaphase chromosome movement strongly suggests that reducing the polar ejection force plays an important role in anaphase of early frog embryos.

The destruction of Xkid depends on the activity of the APC. Chromosomes' movement to the poles requires the destruction of Xkid, but not that of cyclin B (Holloway et al., 1993). Thus, activating the APC independently triggers events that interact with each other to segregate the chromosomes. These include sister separation, poleward chromosome movement, and spindle disassembly (Juang et al., 1997). Using a single biochemical step to trigger anaphase resembles the ability of Cdk1/cyclin B to assemble spindles by independently inducing nuclear envelope breakdown, chromosome condensation, and altered microtubule dynamics.

Our observations on Xkid differ from those on its closest human homolog. Human Kid is on metaphase chromosomes and anaphase centromeres in tissue culture cells (Tokai et al., 1996). In contrast, Xkid is absent from anaphase chromosomes in egg extracts. Staining *Xenopus* tissue culture cells with anti-Xkid antibodies reveals patterns similar to those seen for human Kid (H. F. and A. Straight, unpublished data; Antonio et al., 2000 (this issue of *Cell*]) and human Kid translated in reticulocyte lysates is not degraded in frog anaphase extracts (H. F., unpublished data). There are two possible explanations for the different observations in egg extracts and tissue culture cells. First, human Kid is the true homolog of Xkid, but the proteins differ in their anaphase stability. Second, human Kid is a somatic version of the Kid protein family and Xkid is a meiotic or early embryonic version. In this scenario, the destruction of Kid homologs in anaphase is controlled by regulating which version is expressed. Xkid may play a role in the arrest of unfertilized eggs in metaphase of meiosis II, which lasts for many hours. High levels of chromosome bound Xkid may help to ensure that chromosomes are stably maintained at the metaphase plate. Once the egg is fertilized, destruction of Xkid would allow the separated chromosomes to move to the poles. Xkid may also help early embryonic cells pass rapidly through mitosis despite the absence of a functional spindle checkpoint (Minshull et al., 1994). A high level of chromosome bound Xkid would increase the strength of the polar ejection force, thus keeping monooriented chromosomes near the spindle equator, where it is easy for the unattached kinetochore to capture microtubules from the other pole.

A Novel Strategy for Identifying Chromosomal Proteins

We report a method for isolating highly purified chromosomes from *Xenopus* egg extracts. On two-dimensional gels, Xkid was the only protein whose abundance or phosphorylation changed between metaphase and anaphase chromosomes (unpublished data). This may seem surprising, since proteins such as cohesin and INCENP (Earnshaw and Cooke, 1991) leave chromosomes during anaphase. Our inability to see cohesins disappearing at the onset of anaphase agrees with work showing that these proteins leave frog chromosomes at the onset of mitosis, substantially before sister chromatids separate (Losada et al., 1998). In addition, since we added nondegradable cyclin B to keep Cdk1 active during anaphase, proteins that cannot leave the chromosomes until Cdk1 is inactivated would not be detected in our experiments. The purity of the recovered chromosomes allowed us to clone Xkid by screening the translation products of large pools of clones. This is a general strategy for finding new chromosomal proteins and could be modified to identify proteins that bind to chromosomes only after specific perturbations, such as the activation of various checkpoints. The ability to deplete such proteins and replace them with mutant versions gives frog egg extracts many of the assets of genetically tractable organisms. This is especially true for studying molecules that are essential for cell viability and reproduction.

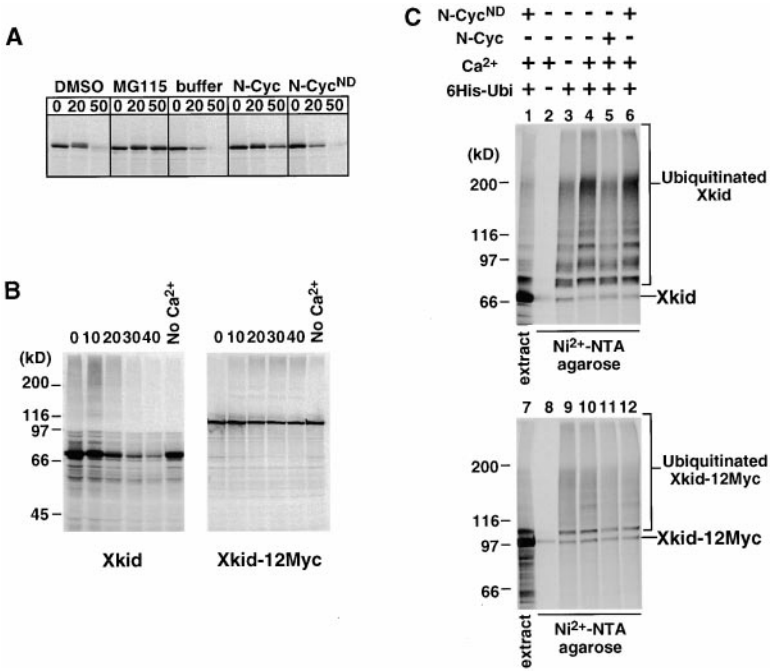


Figure 7. Xkid Is Degraded through Ubiquitin-Mediated Proteolysis

(A) Stabilization of anaphase Xkid by a proteasome inhibitor and an APC inhibitor. One-tenth volume of reticulocyte lysate containing ^{35}S-labeled Xkid was added to a CSF extract containing cyclin Δ90 and cycloheximide. Anaphase was induced by adding calcium (final 0.4 mM) to the reaction mixtures containing 1.6 mM MG115 (in DMSO), 75 μg/ml N-Cyc (the N-terminal amino acids 13–110 of cyclin B), or 75 μg/ml N-CycND (N-Cyc containing a mutated destruction box, R42A, A44R) (Holloway et al., 1993). Aliquots of samples were taken at 0, 20, or 50 min, run on an SDS-polyacrylamide gel, and analyzed by autoradiography.

(B) Adding 12 repeats of Myc epitope to the C terminus of Xkid inhibited Xkid degradation. One-tenth volume of a reticulocyte lysate containing ^{35}S-labeled Xkid or Xkid-12Myc was added to a CSF extract containing cyclin Δ90 and cycloheximide. After anaphase was induced by adding calcium, aliquots of samples were taken at the indicated times (in minutes). The metaphase control was incubated for 40 min without adding calcium.

(C) Ubiquitination of Xkid. One-tenth volume of a reticulocyte lysate containing ^{35}S-labeled Xkid (upper panel) or Xkid-12Myc (lower panel) was added to a CSF extract containing 0.5 mg/ml untagged ubiquitin (lanes 2 and 8), 0.5 mg/ml 6×His-ubiquitin (lanes 1, 3–7, and 9–12), 90 μg/ml N-Cyc (lanes 5 and 11), 90 μg/ml N-CycND (lanes 1, 6, 7, and 12)). Extracts were incubated for 10 min after adding buffer (lanes 3 and 9) or calcium to 0.4 mM (lanes 1, 2, 4–8, and 10–12). Autoradiograms of total extract proteins (lanes 1 and 7) and proteins affinity purified with Ni^{2+}-NTA-agarose (lanes 2–6 and 8–12) are shown.

Experimental Procedures

A detailed description of the Experimental Procedures is posted on the internet at http://www.cell.com/cgi/content/full/102/4/411/DC1.

Visualizing Individual Chromosomes

One microliter egg extract containing condensed chromosomes was mixed with 4 μl chromosome dilution buffer (10 mM K-HEPES, (pH 7.6), 200 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EGTA, and 250 mM Sucrose). Fifteen minutes after incubation at room temperature, 1 μl of the diluted chromosomes were mixed with 3 μl Fix (Murray, 1991) on a glass slide and covered with an 18 × 18 mm coverslip.

Purification of Biotinylated Chromosomes

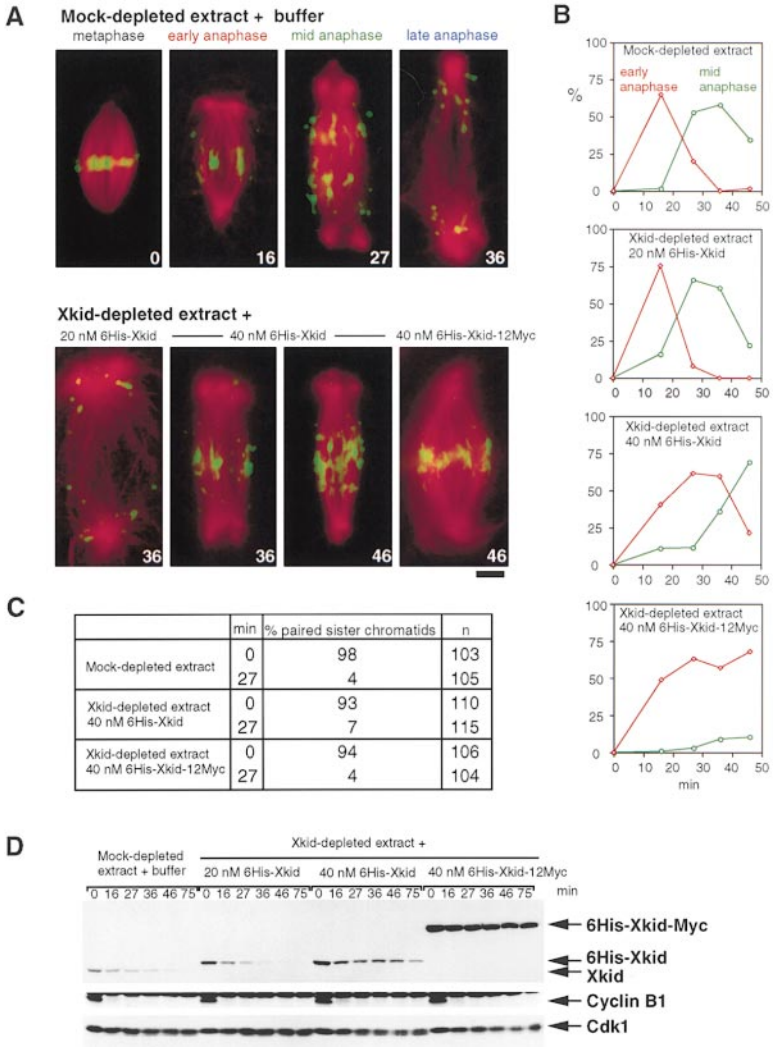
Metaphase spindles were assembled as described (Shamu and Murray, 1992). For a standard experiment, biotinylated-dUTP (final concentration 5 μM), 20 μCi Tran^{35}S-label (ICN), sperm nuclei (final concentration 1400/μl), and calcium chloride (final 0.3 mM) were added to 120 μl CSF extract to obtain an interphase extract. After 90 min of incubation at 20°C, 60 μl fresh CSF extract and cyclin B Δ90 (Glotzer et al., 1991) (final 24 μg/ml) were added to interphase extracts, and the reactions were incubated for a further 60 min. Buffer or 0.4 mM calcium chloride was added and the reactions were incubated for a further 60 min to obtain metaphase or anaphase chromosomes, respectively. Sister chromatid separation was monitored by diluting a sample of the extract into chromosome dilution buffer, after which the remainder of the extract was frozen in liquid nitrogen and stored at −80°C.

To purify chromosomes, frozen extracts (180 μl reactions) were quickly thawed and mixed with 540 μl of dilution buffer (DB; 10 mM K-HEPES [pH 7.6], 100 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermine, 250 mM Sucrose, 1 mM PMSF, and 10 μg/ml LPC (leupeptin, pepstatin, and chymostatin)). Diluted extract was layered over 0.5 ml of 60SCB (DB plus 60% (w/v) sucrose, 0.05% Triton X-100, 1 mM PMSF, and 10 μg/ml LPC) in a 1.5 ml microfuge tube and centrifuged at 8000 rpm for 60 min in a Sorvall HB-4 rotor. The crude chromosome pellet (10–15 μl) was mixed with 15 μl Dynabeads M-280 streptavidin (Dynal) resuspended in 15 μl 30SCB (DB plus 30% (w/v) sucrose, 0.05% Triton X-100, 1 mM PMSF, and 10 μg/ml LPC), and gently rotated at 4°C for 4 hr. Dynabeads were collected with a magnetic particle separator and washed six times with 30 μl of 30SCB. Chromosomal proteins were eluted with standard sample buffers and applied to 7.5%–15% gradient SDS-polyacrylamide gels or 2-D gels.

Cloning Xkid cDNA

Poly A$^+$ RNA was purified with Oligo(dT)-Cellulose Type 3 (Collaborative Biomedical Products) from total RNA which had been isolated using a commercial extraction reagent (Trizol, GibcoBRL) from *Xenopus* CSF extracts. The first strand of the cDNAs were synthesized using reverse transcriptase (SuperScript II, GibcoBRL). The 3′ end of the first strand was annealed with an oligonucleotide that contained the SP6 promoter sequence and terminated GGG, exploiting the tendency of reverse transcriptase to add several C residues to the 3′ end of cDNAs synthesized from capped mRNA molecules. The second strand was synthesized and the double-stranded cDNA was amplified using additional oligonucleotides that introduced AscI and NotI sites, following the manufacturer's instructions (Clontech). After PCR products were digested with AscI and NotI, DNA fragments were purified from a 7.5% agarose gel and ligated between NotI and AscI sites of a pBluescript KS(+)-based cloning vector. The ligation products were transformed into DH10B cells plated to LB-ampicillin plates to obtain 500–1000 colonies / plate. Each plate was replica printed and the original plates were stored at 4°C. Pools of bacterial cells were collected from the replica prints, plasmid DNA was prepared and added to a coupled trasncription-translation reticulocyte lysate (TNT, Promega). After the translation was complete, the ^{35}S-labeled lysates were added to interphase extracts containing 1400 sperm nuclei/μl, 100 μg/ml cycloheximide, 1 mM L-methionine, and 0.4 mM L-cysteine just before adding fresh CSF extracts and cyclin Δ90. After chromosomes were purified as described above, chromosomal proteins were run on an 7.5%–15% gradient polyacrylamide gel.

A single pool (G3/2), representing 870 clones, was analyzed further because a 74 kDa chromosomal protein was detected in this pool. The individual colonies that constituted this pool were placed in 29



	min	% paired sister chromatids	n
Mock-depleted extract	0	98	103
	27	4	105
Xkid-depleted extract 40 nM 6His-Xkid	0	93	110
	27	7	115
Xkid-depleted extract 40 nM 6His-Xkid-12Myc	0	94	106
	27	4	104

Figure 8. A Mild Excess of Xkid Inhibits Anaphase Movement of Separated Sister Chromatids.

Metaphase spindles were assembled on replicated chromosomes using mock-depleted CSF extract or Xkid-depleted CSF extract supplemented with 6×His-Xkid or 6×His-Xkid-12Myc. (The purity of the protein preparations is shown in Figure 4D). Times are in minutes after adding calcium. Nondegradable cyclin had been added to inhibit chromosome decondensation and reformation of cytoplasmic microtubules.

(A) Representative spindles at the indicated times. Rhodamine-labeled microtubules are shown in red and chromosomes are shown in green. Bar, 10 μm.

(B) Quantitation of spindle structures scored in mock-depleted extract (n = 55–118), Xkid-depleted extract containing 20 nM 6×His-Xkid (n = 49–121), Xkid-depleted extract containing 40 nM 6×His-Xkid (n = 34–107), and Xkid-depleted extract containing 40 nM 6×His-Xkid-12Myc (n = 66–113). Spindles in early anaphase (red lines) and mid anaphase (green lines).

(C) Adding excess Xkid has no effect on sister chromatid separation. Sister separation in mock-depleted extract containing buffer alone and Xkid-depleted extract containing 40 nM 6×His-Xkid or 40 nM 6×His-Xkid-12Myc after treatment with chromosome dilution buffer at the indicated times. At least 100 chromosomes were scored for each sample.

(D) Western blot analysis. Samples taken at the time indicated were immunoblotted with anti-Xkid, anti-cyclin B1, and anti-Cdk1 antibodies. The upper band in the anti-cyclin B1 blot is a cross-reactive species. The concentration of 6×His-Xkid-12Myc was determined by Coomassie staining as shown in Figure 4D.

subpools. After screening the subpools by the chromosome purification method, a single well was identified as the location that encoded the 74 kDa chromosomal protein. Plasmids recovered from 4 independent colonies obtained from this well have a cDNA insert of 2.6 kb and can be transcribed to produce an RNA that encodes a 74 kDa protein in reticulocyte lysates, which binds to *Xenopus* chromosomes. We named the plasmid encoding this protein pSP70-1. Sequence analysis revealed that this cDNA (GenBank accession number: AF267850) is identical, except for minor variations, with the *XKID* clone which had been identified previously (pTP70, GenBank accession number; AF267849 [Zou et al., 1999]).

Preparation of Xkid Antigen and Antibodies

GST-Xkid-C304, which contains a GST tag on the N terminus of the C-terminal 304 amino acids of Xkid was produced in *E. coli* BL21 cells from the plasmid pGXKID-3, constructed from pGEX-6P-2 (Amersham Pharmacia). The GST-fusion protein was purified as described (Kellogg et al., 1995), the C-terminal Xkid fragment was cleaved and purified from GST following the manufacturer's instructions (Amersham Pharmacia), and further purified on a 1 ml HiTrap SP column. The peak fraction containing Xkid-C304 was used to raise polyclonal antibodies in rabbits at Cocalico Biologicals, which were affinity purified. Control IgG was purified from preimmune serum as described (Harlow and Lane, 1988).

Ubiquitination Assays

^{35}S-methionine-labeled Xkid or Xkid-12Myc were produced in transcription-translation coupled reticulocyte lysate using pTP70 (a generous gift of H. Zou, T. McGarry and M. Kirschner) or pTP70MYC, respectively. 1.8 μl of reticulocyte lysate was mixed with 18 μl of CSF extract containing 100 μg/ml cycloheximide, 1 mM MG115 (Peptides International), and either 0.5 mg/ml 6×His-Ubiquitin ([Klotzbucher et al., 1996], a gift of A. Szidon) or human ubiquitin (Sigma) for a control. APC was activated by adding calcium chloride to 0.4 mM and the reaction mixture was incubated for 10 min at 20°C. A 2 μl sample was resuspended in SDS sample buffer to monitor the proteins in total extract, and the rest of the reaction was added to 1 ml of binding buffer, and 6×His-ubiquitinated proteins were isolated as described (Klotzbucher et al., 1996).

Expression and Purification of Recombinant Proteins from Insect Cells

6×His-Xkid or 6×His-Xkid-12Myc was produced in Sf-9 cells by baculovirus expression from recombinant baculoviruses using two pFastBac HTb (Gibco BRL) based plasmids. A slightly modified version of published methods (Takizawa et al., 1999) was used to purify 6×His-tagged proteins. The peak fraction of proteins eluted from the Co-coupled HiTrap chelating column (Amersham Pharmacia) was used for this study.

Immunofluorescence Microscopy

Methods described by Desai et al. (1999b) were used. Spindles spun onto coverslips were stained with 1 μg/ml affinity-purified anti-Xkid or Oregon green–conjugated anti-human CENP-E (Lombillo et al., 1995; Desai et al., 1997) (a gift of A. Desai and T. Yen) in AbDil. Anti-Xkid antibodies were visualized by FITC-conjugated goat anti-rabbit antibody (Cappel).

For visualization of Xkid on individual chromosomes, 20 μl of extract containing chromosomes was mixed with 80 μl chromosome dilution buffer, incubated for 15 min, and fixed 5 min by adding 400 μl of fixation buffer (20% glycerol, 1×MMR, 0.5% Triton X-100, and 2.7% formaldehyde). Chromosomes were sedimented onto a coverslip through a 5 ml cushion (1×MMR, 40% glycerol) at 10,000g for 30 min in a Sorvall HB-4 rotor and the coverslips were processed for immunofluorescence. DNA was visualized by 0.5 μg/ml Hoechst 33258 followed by a wash with AbDil.

Immunodepletion and Antibody Blocking

2.5 μg of affinity-purified anti-Xkid or control IgG was incubated with 25 μl of protein A-Dynabeads (Dynal) for at least 1 hr in a 0.6 ml low retention microtube (United Scientific Products). Antibody-coupled beads were washed twice with TBS, and twice with sperm dilution buffer (5 mM K-HEPES [pH 7.7]), 1 mM $MgCl_2$, 100 mM KCl, and 150 mM sucrose) containing 10 μg/ml LPC. After removing excess buffer, 50 μl of CSF crude extract containing 50 mM sucrose, 100 μg/ml cycloheximide, and 120 μg/ml rhodamine-labeled tubulin was added to the beads, mixed gently by tapping the tube with a finger, and incubated for 90–105 min on ice without any further agitation. The supernatants from multiple tubes, separated from beads with a magnetic particle separator, were collected in a 1.5 ml microtube. Supernatants were separated from residual beads by the magnetic particle separator and used as the immunodepleted CSF extract.

Interphase extracts lacking Xkid were prepared by adding calcium chloride (final 0.3 mM) to CSF extracts containing 50 mM sucrose, 100 μg/ml cycloheximide, 120 μg/ml rhodamine-labeled tubulin and 800 sperm nuclei /μl, and incubated for 100 min at 20°C. Thirty microliters of immunodepleted CSF extract, either preincubated with 0.125–1 μl recombinant proteins or with buffer (25 mM Na-HEPES [pH 7.2], 100 mM imidazole, 300 mM NaCl, and 10% glycerol) alone for 10 min on ice, was added to 10 μl of interphase extracts containing sperm nuclei. After 90 min incubation, calcium chloride (final 0.4 mM) and Cyclin Δ90 (final 16 μg/ml) were added to the extract to induce anaphase.

To immunodeplete ^{35}S-labeled proteins from egg extracts, 25 μCi of ^{35}S-methionine and cysteine (Tran^{35}S-label, ICN) was added to 50 μl of CSF extract, incubated for 30 min at 20°C, and then cycloheximide was added to 100 μg/ml. After incubating the ^{35}S-labeled CSF extract with protein A-Dynabeads coupled with anti-Xkid or control IgG for 90 min, the supernatant was separated from beads and added to 120 μl of interphase extracts containing biotinylated chromosomes, together with cyclin Δ90 (final 24 μg/ml).

For antibody blocking experiments, 0.36 μl of 8.5 mg/ml affinity-purified anti-Xkid antibody or control IgG was added to 30 μl of interphase extract containing sperm nuclei at the concentration of 200/μl. Fifteen microliters of CSF extract was added to the interphase extract and the spindle morphology was monitored between 44 and 75 min later.

To monitor spindle and chromosome morphology, 1 μl of extract was put on a glass slide, 4 μl of Fix was added, and an 18 × 18 mm coverslip was placed on the top of the drop.

Time-Lapse Microscopy

The methods of Murray et al. (1996) were used. Immediately after an immunodepleted CSF extract was added to an interphase extract containing sperm nuclei, DAPI was added to the final concentration at 0.05 μg/ml, 4 μl of mixed extract was placed under an 18 × 18 mm coverslip and sealed with valap (Desai et al., 1999b). Fluorescent images of DNA and microtubules were recorded in time lapse by epifluorescence microscopy using a Nikon Microphoto-SA microscope, with a Nikon Plan Fluor 20× lens and a Princeton Instruments cooled CCD camera (RTE/CCD-1300-V). The camera, shutters, and the filter wheel were controlled by Metamorph software (Universal Imaging). DAPI and rhodamine images were recorded into separate image stacks at 45 s intervals. The rate of 18 smooth movements of stretching chromosomes in Xkid-depleted extract was determined to obtain an average rate.

Western Blots

Immunoblots were blocked with PBS containing 4% Carnation nonfat dry milk (Nestle). Primary and secondary antibodies were diluted in blocking solution. Primary antibodies were used at the following concentrations: 3 μg/ml anti-Xkid, 2 μg/ml anti-human Cdk1 (SC-54, Santa Cruz Biotechnology), 1/2000 dilution of anti-topo II (Hirano and Mitchison, 1993) (gift of T. Hirano), 1/1000 dilution of anti-CDC27Hs sera (Tugendreich et al., 1995) (a gift of P. Hieter), and affinity purified anti-*Xenopus* cyclin B1 (a gift of D. Kellogg). Antibodies were detected using enhanced chemiluminescence (Amersham).

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GenBank Accession Number

The *Xenopus* chromokinesin cDNA sequence, Xkid, reported in this paper, has been deposited in GenBank with accession number AF267580.