Kif2a depletion generates chromosome segregation and pole coalescence defects in animal caps and inhibits gastrulation of the Xenopus embryo

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ABSTRACT Kif2a is a member of the kinesin-13 microtubule depolymerases, which tightly regulate microtubule dynamics for many cellular processes. We characterized Kif2a depletion in Xenopus animal caps and embryos. Kif2a depletion generates defects in blastopore closure. These defects are rescued by removing the animal cap, suggesting that Kif2a-depleted animal caps are not compliant enough to allow gastrulation movements. Gastrulation defects are not rescued by a Kif2a mutated in an Aurora kinase phosphorylation site, suggesting that the phenotypes are caused by problems in mitosis. During animal cap mitoses, Kif2a localizes to the spindle poles and centromeres. Depletion of Kif2a generated multipolar spindles in stage 12 embryos. Kif2a-depleted animal caps have anaphase lagging chromosomes in stage 9 and 10 embryos and subsequent cytokinesis failure. Later divisions have greater than two centrosomes, generating extra spindle poles. Kif2a-depleted embryos are also defective at coalescing extra spindle poles into a bipolar spindle. The gastrulation and mitotic phenotypes can be rescued by either human Kif2a or Kif2b, which suggests that the two homologues redundantly regulate mitosis in mammals. These studies demonstrate that defects in mitosis can inhibit large-scale developmental movements in vertebrate tissues.

INTRODUCTION Microtubule dynamics must be tightly regulated to allow chromosome segregation (Ems-McClung and Walczak, 2010). The kinesin-13 family of microtubule depolymerases comprises major regulators of catastrophe events at both plus and minus microtubule ends (Sanhaji et al., 2011). Most vertebrates have two kinesin-13s, known as Kif2a and mitotic centromere-associated kinesin/Kif2c (MCAK). Mammals have a third, known as Kif2b (Manning et al., 2007). These proteins localize to distinct structures of the spindle, controlling microtubule dynamics in time and space (Manning et al., 2007). Depletion of Kif2a generates profound mitotic defects in human cancer cells, yet kif2a\textsuperscript{−/−} mice are born, albeit with brain abnormalities, and a human retardation syndrome maps to the Kif2a locus (Homma et al., 2003; Jaillard et al., 2011). There is growing evidence that kinesin-13s play important roles in both transformation and resistance to spindle poison chemotherapeutics, especially the derivatives of paclitaxel (Sanhaji et al., 2011).

The kinesin-13 microtubule depolymerase, Kif2a, is a critical regulator of the mitotic spindle in tissue culture cells, where the depletion of Kif2a produces monopolar spindles (Ganem and Compton, 2004). Kif2a is required for microtubule flux in both human cells and Xenopus extracts, where it is proposed to depolymerize the minus ends of microtubules at the pole, maintaining spindle length (Gaetz and Kapoor, 2004; Ganem et al., 2005). Kif2a is localized to spindle poles in HeLa cells but to both poles and centromeres/kinetochores of Xenopus cells and extracts (Ganem and Compton, 2004;...
Role of Kif2a in Xenopus early embryos

We characterized the phenotype of Kif2a knockdown as well as the role of p70 phosphorylation in Xenopus embryos. We depleted Kif2a by injecting specific MOs into one-cell Xenopus laevis embryos. Maternally loaded Kif2a was reduced at late blastula stages (stage 9) in MO-injected embryos, and nearly complete depletion of maternal Kif2a protein occurred by the time embryos undergo gastrulation at stage 10 (Figure 2A). The initial stages of gastrulation appeared normal in Kif2a-depleted embryos, as dorsal blastopore lips surrounded yolk plugs to generate stage 10.5 embryos with a normal external appearance. However, blastopore closure is significantly defective in Kif2a-depleted embryos, and large, round yolk plugs remain apparent at control stage 12 (Figure 2, B and B’, Supplemental Figure S1A, and Supplemental Movies S1, A and B). We imaged gastrulation events of Kif2a-depleted embryos by time-lapse imaging. The movies show circumblastoporal constriction in the morphants, but it often results in exogastrulation—the expansion and extrusion of the yolk plug rather than its internalization—which suggests lack of space for the endoderm to move into the blastocoel rather than failure of convergence (and extension) of the marginal zone. Time-lapse movies of the animal cap confirmed that loss of Kif2a interferes with epiboly (Figure 2C and Supplemental Movie S2, A and B). By tracking and measuring the movement of nuclei of cells in the animal pole during gastrulation stages, we find that control embryos exhibit normal epiboly, moving vegetally from stage 9 to stage 14. Kif morphant embryos display delayed epiboly and less vegetal movement. This phenotype is recapitulated in embryos expressing the T70A mutant but rescued by injecting RNA encoding the full-length Kif2a or Kif2b or the T70E mutant (Figure 2C’).

To learn how the animal cap and vegetal endodermal defects are related, we performed targeted injections of the Kif2a MO at the 32-cell stage of development. We injected the Kif2a MO into the A-tier blastomeres to specifically target the animal cells (Animal Cap Targeted MO, Supplemental Figure S1B, Supplemental Movie S5C) or into the B1/2 and C1/2 blastomeres to target the dorsal marginal zone cells (Dorsal MZ Targeted MO, Supplemental Figure S1B, Supplemental Movie S5D). Targeting the animal cells recapitulated the whole embryo phenotype, whereas embryos gastrulated normally if the MO was targeted to the dorsal marginal zone cells (Keller et al., 2004; Ohi et al., 2004; Lan et al., 2004; Rosasco-Nitcher et al., 2008; Knowlton et al., 2009; Ems-McClung and Walczak, 2010). Phosphorylation of a conserved serine in the neck of the kinesin domain by Aurora kinases inhibits depolymerase activity of both Kif2a and MCAK in vitro (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). Kif2a inhibited by Aurora B phosphorylation can be reactivated either by dephosphorylation or interaction with the protein ICIS (Knowlton et al., 2009). Aurora phosphorylation of the N-terminal centromere-targeting domain of MCAK regulates localization of Kif2a to spindles in a complex manner in Xenopus extracts (Zhang et al., 2007). The importance of the related site on Kif2a at T-70 has not been determined.

We investigated the role of Kif2a in spindle formation and chromosome segregation using embryonic animal caps and morpholino (MO)-mediated Kif2a depletions to explore a number of critical unanswered questions. Surprisingly, we find that Kif2a is required for the epiboly (spreading) of animal cap, and failure of epiboly has the mechanical effect of retarding or blocking blastopore closure and endoderm internalization on the other (vegetal) side of the embryo. In animal caps, Kif2a localizes to the spindle poles (centrosomes) and centromeres during mitosis and is required for spindle integrity and proper chromosome segregation, as suggested by tissue culture systems. In addition, we demonstrate the critical role of Aurora kinase phosphorylation of T70 in localizing Kif2a to both spindle poles and centromeres. We show that Kif2a is required for chromosome segregation in Xenopus tissues and that Kif2a depletion can be rescued by either human Kif2a or Kif2b. We also identify new requirements for Kif2a in both cytokinesis and pole coalescence.

RESULTS

Aurora phosphorylation of Kif2a on T70 in cells and frog egg extracts

The localization of MCAK, a kinesin-13 protein related to Kif2a, is regulated by Aurora phosphorylation on the N-terminus (Zhang et al., 2007). We noted that a similar Aurora B consensus site could be seen on the N-terminus of Kif2a (Figure 1A). To characterize this phosphorylation event, we generated a phosphospecific antibody to the 15 amino acids around T70 of Kif2a, and the antibody specifically recognized a single band that corresponds to the molecular weight of Kif2a in Xenopus extracts. This band was found only in extracts treated to activate Aurora kinases (mature oocyte extract [MOE] in Figure 1B; Lan et al., 2004). The antibody also recognized recombinant Kif2a after phosphorylation with either Aurora A or Aurora B kinase (Figure 1C). The antibody recognized two foci in the correct location for centrosomes/spindle poles in Xenopus S3 tissue culture cells and poles and centromeres in Xenopus egg extract (Figure 1D). Note that we previously showed that Kif2a is localized to centromeres in Xenopus S3 cells (Knowlton et al., 2009), suggesting that centromere phosphorylation of Kif2a by Aurora B can be regulated in a cell-type manner. In extracts the signals were lost after treatment with the Aurora kinase inhibitor Hesperadin, demonstrating that the signal is dependent on Aurora kinases (Figure 1E).

Depletion of Kif2a inhibits the completion of gastrulation in Xenopus laevis embryos

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To test this hypothesis, we injected Kif2a MO into embryos at two-cell stage and then removed the animal caps and imaged the gastrulation movements. The animal cap in the Xenopus blastula and gastrula is an epithelial tissue above the blastocoel that can be easily isolated by dissection. The removal of the animal cap from Kif2a morphant embryos largely rescues blastopore closure (Figure 3 and Supplemental Movie S3). This suggests that the blastopore closure defects are due to failure of the spreading movements of epiboly of the animal cap epithelium, probably by interfering with the radial intercalation that drives it (Keller, 1980), and this, in turn, can mechanically interfere with the vegetal internalization movements during gastrulation (Keller and Jansa, 1992, Petridou et al., 2012; also see Discussion).
Although we posit that loss of Kif2a prevents both animal cap cell division and epiboly, we do not think that the gastrulation phenotype seen after depletion of Kif2a is caused solely by a lack of cell proliferation. Blocking DNA replication by immersion of embryos into hydroxyurea and aphidicolin starting at stage 10 does not disrupt blastopore lip closure (Figure 2B and Supplemental Figure S1A; Cooke, 1973). We confirmed that proliferation was inhibited in drug-treated embryos, as visualized by larger cells (Supplemental Figure S2). These embryos gastrulated normally, indicating that a loss of cellular proliferation cannot fully account for the Kif2a-depletion phenotype; instead, disruption of radial cell polarity and intercalation is likely (see Discussion).

Xenopus Kif2a has 87% identical amino acids as human Kif2a and 53% identical amino acids as human Kif2b. Because there is no Kif2b homologue in nonmammalian vertebrates, we asked whether the phenotypes could be rescued by coinjecting RNA encoding human Kif2a or Kif2b with the MO. Both Kif2a- and Kif2b-rescued embryos continued development to tadpole stages (unpublished data). This argues strongly that Kif2a and Kif2b are redundant for their early embryonic roles. In contrast, injection of similar amounts of human Kif2A(T70A) RNA did not rescue blastopore closure or embryonic lethality (Figure 2B'). These data suggest that embryonic expression of Kif2a is required to complete gastrulation and that Aurora phosphorylation on T70 by Aurora B is critical for its function.

Kif2a is localized to mitotic centromeres and mitotic spindle poles in Xenopus animal cap epithelium

The fact that point mutants of Kif2a in a mitotic kinase site failed to rescue the gastrulation phenotypes argues strongly that the phenotypes are caused by defects in mitosis. We therefore examined whether there were defects in the animal cap mitoses that drove the lack of compliance of the tissue. Mitotic events can be imaged by dissecting animal cap tissues for fixation and immunofluorescence. All of the stages of mitosis are represented in a single field of view, and visualizing these spindles is comparable to tissue culture cells in fixed-cell imaging (Figure 4B and Supplemental Figure S3; Kieserman et al., 2008; Woolner et al., 2008).

We localized Kif2a from gastrula-stage embryos by performing immunofluorescence of animal caps, using a Kif2a antibody directed to the C-terminal region of human Kif2a (Figure 4A and Supplemental Figure S4; Knowlton et al., 2009). The caps
These staining patterns were all lost after morpholino depletion, demonstrating specificity of the antibodies (Figure 4B). The localization of Kif2a to centromeres appears cell type specific. Kif2a is found mostly at spindle poles in HeLa cells (Ganem and Compton, 2004; Knowlton et al., 2009). However Kif2a is also found at centromeres in Xenopus extracts, Xenopus S3 cells, and animal caps (Figures 1 and 4 and Supplemental Figure S4; Knowlton et al., 2009).

We stained animal caps with antibodies specific to Aurora A and Aurora B to determine their localization (Supplemental Figure S4).
FIGURE 3: Removal of animal cap tissue rescues Kif2a MO gastrulation phenotypes. Embryos were injected with water (Control) or Kif2a morpholino (Kif2aMO) at two-cell stage. At stage 8, animal caps of each treatment were removed microsurgically, and the blastopore closure was monitored through low-light time-lapse microscopy (Control-Capless, Kif2aMO-Capless). One embryo was left intact for staging purposes (Control). Failure of blastopore closure in the morphant phenotypes were rescued by removal of the cap, although closure was slightly delayed compared with the control.
Kif2a regulates mitotic spindle length, and depletion generates multipolar spindles in animal cap cells

We injected MOs into single-celled embryos to determine the cellular phenotypes of Kif2a depletion. We processed animal caps from stage 9–11 embryos for immunofluorescence to visualize Kif2a, α-tubulin, and DNA (Figure 4, B–D). The Kif2a-depleted embryos exhibited longer metaphase and anaphase spindle lengths at each of the stages (Supplemental Figure S3B). The predominant phenotype seen in Kif2a depletion in cultured cells—monopolar spindles—was a minor phenotype in stage 10 animal caps. By stage 10.5, Kif2a-depleted embryos exhibited a multipolar cellular phenotype, which became even more prevalent at stage 11, when a large percentage of the mitotic figures were multipolar (Figure 4, B and C and Supplemental Figure S3, A and B). The number of mitotic cells was also largely increased in stage 11 embryos, suggesting that the multipolar spindles caused a mitotic arrest (Figure 4D). Coinjection of human Kif2a RNA with the Kif2a MO complemented bipolar spindles and the increased spindle lengths (Figure 5). We conclude that Kif2a is required to maintain proper spindle length and prevent multipolar spindles in Xenopus animal caps. We rescued Kif2a depletion with mRNA expressing Kif2a(T70A) to determine whether Kif2a phosphorylation by Aurora kinases is required for proper spindle formation in animal caps. Neither increased spindle length nor multipolar spindles were rescued by RNA coding for Kif2a(T70A), whereas the wild-type protein fully rescued all phenotypes (Figure 5). We localized the Kif2a(T70A) protein after coinjection of RNA with the MO. Kif2a was no longer specifically found at centromeres or poles. We conclude that the Aurora
**FIGURE 5:** Kif2a morphant cellular phenotypes are rescued by Kif2a but not Kif2a(T70A). (A) Confocal micrographs of control, Kif2a-MO, and WT rescue morphants (Rescue Kif2a WT) stained for α-tubulin (green) and DNA (blue). In the control and MO-rescue animal caps, spindles were bipolar, whereas within the Kif2a-MO group, multipolar spindles were abundant. (B) Confocal images of control and Kif2a morphants rescued with a phospho-null Kif RNA (Kif2a T70A) show that Kif2a is mislocalized in the absence of T70 phosphorylation. Animal caps were stained for α-tubulin (green), Kif2a (red), and DNA (blue). (C) Confocal measurements of spindle lengths were calibrated as a pole-to-pole length (note spindle lines). The stage 10.5 Kif2a morphant cells had significantly greater metaphase and anaphase spindle lengths compared with the control and rescue animal cap cells.
phosphorylation of Kif2a is required to localize Kif2a to centromeres in X. laevis animal caps. Moreover, this phosphorylation is critical for X. laevis development and normal mitosis in X. laevis animal caps.

**Kif2a morphant phenotypes are caused by lagging chromosomes and failure of cytokinesis**

Multipolar spindles often occur because of cytokinesis failure, which generates both polyploidy and multiple centrosomes in the subsequent mitosis (Meraldi et al., 2002; Kwon et al., 2008). To determine whether Kif2a was required for cytokinesis, we injected RNA encoding green fluorescent protein (GFP)–histone H2b and red fluorescent protein (RFP)–GAP43, which acts as a plasma membrane marker, into Kif2a-depleted embryos, cut animal caps at stage 9, and imaged the layer of cells that make up the blastocoel roof by time-lapse imaging (Figure 6 and Supplemental Movie S4). In control cells, one sees faithful division of mitotic chromosomes, followed by cytokinesis between the segregated chromosomes. In >50% of the anaphase events of Kif2a-depleted embryos, we could identify chromosomes that failed to segregate and remained between the segregating masses (Figure 6, A and B). In all cases, there was a robust cytokinetic furrow that entrapped the lagging chromosomes in about half the divisions. In about half of these cells, the furrow eventually regressed, reforming a

**FIGURE 6:** Kif2a morphant phenotypes caused by lagging chromosomes and failure of cytokinesis. (A) Embryos were injected at two-cell stage with GAP43:RFP:RNA and H2B:GFP RNA (Control) and coinjected with Kif2a morpholino (Kif2aMO), MO plus human 2a RNA (Rescue 2a), or MO plus human 2b RNA (Rescue 2b). The animal caps were microdissected at stage 9 and placed in an imaging chamber for confocal microscopy. A time-lapse movie was made on a Zeiss 780 Confocal Microscope with the 25× objective and a framing rate of 30 s. Still frames of these movies are shown from the indicated time postfertilization. The morphant embryos display lagging chromosomes and failure of cytokinesis (indicated by white or yellow arrows). Scale bar, 20 μm. (B) From time-lapse movies taken from stages 9–12 (similar to those described earlier), quantification of divisions with lagging chromosomes was made for control embryos, Kif2a morphants at stage 9.5, Kif2a morphants at stage 10.5, and morphants rescued with either human Kif2a or Kif2b. Bars represent the percentage of divisions imaged with lagging chromosomes. n = 20 for each category of embryo. Control embryos displayed no divisions with lagging chromosomes. Error bars, SEM. (C) Percentage of divisions imaged with failed cytokinesis. n = 20 for the categories of embryos in B. Error bars, SEM.
one centrosome for each half-spindle (Figure 7). In contrast, Kif2a-depleted embryos had a large number of cells with more than three centrosomes. Usually, each pole had its own Cep57 foci in the multipolar spindles generated by Kif2a depletion. Thus it is likely that the multipolar spindles that arise after Kif2a depletion are caused by multiple centrosomes.

A second marker of failed cytokinesis is increased ploidy. We measured the ploidy of cells depleted of Kif2a by counting the paired kinetochores, since there is a single kinetochore per sister chromatid in mitosis. Kinetochore numbers were quantified for 50 mitotic figures from 10 separate animal caps in both the control and MO-injected embryos, using antibodies to Ndc80 as a marker. Control caps had 36 kinetochore pairs, as expected for *X. laevis*, which has 36 chromosomes. Almost all mitotic cells after Kif2a depletion had >36 chromosomes, with the number usually increasing in steps of $2N$ (Figure 7B). The multipolar spindles generally had double the wild-type ploidy, suggesting that cytokinesis failure preceded multipolarity. Thus depletion of Kif2a generated cells with extra centrosomes and increased ploidy.

To corroborate our findings that Kif2a-depleted embryos fail cytokinesis, we quantified the number of centrosomes and the ploidy in Kif2a-depleted cells. Kif2a-depleted embryos were fixed at stages 10 and 10.5, and mitotic spindles in animal caps were assessed for centrosome numbers using antibodies to centriomatrix protein Cep57 (Andersen et al., 2003; Emanuele and Stukenberg, 2007). Kif2a MO–injected multipolar cells had significantly more centrosomes than control cells (Figure 7). Control cells usually had one centrosome for each half-spindle (Figure 7). In contrast, Kif2a-depleted embryos had a large number of cells with more than three centrosomes. Usually, each pole had its own Cep57 foci in the multipolar spindles generated by Kif2a depletion. Thus it is likely that the multipolar spindles that arise after Kif2a depletion are caused by multiple centrosomes.

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**FIGURE 7:** Multipolar spindles have multiple centrosomes. Multipolarity within Kif 2a morphants results in an abnormal distribution of chromosomes to daughter cells. (A) Confocal images of animal caps stained with Cep57 (red), α-tubulin (green), and DNA (blue). Cep 57 is localized to the centrosome area within bipolar (control) and multipolar (MO) spindles. Note that the multipolar spindles generated by Kif2a depletion have additional centrosomes in each pole, and occasionally a pole has two centrosomes (white arrow tip). (B) The percentages of cells with two or fewer, three or four, or more than four centrosomes were quantified for both control and Kif2a morphants. A minimum number of 100 mitotic figures were counted from >10 different caps within each (control and MO-injected) group. **$p < 0.01$.** (C) Confocal images of control, stage 10.5, and stage 11 morphant animal cap cells. Ndc80 immunostaining is red, α-tubulin staining is green, and DNA staining is blue. (D) Scatter plot of Ndc80 paired foci. Ndc80 paired foci increase in number in Kif2a multipolar morphants, indicating an increase in cellular chromosomes. Fifty percent of the Kif 2a morphant cells ($n = 50$) had Ndc80 foci number in excess of the average number of control cells (36 pairs) and exhibited a diploid ($2N$) increase in number.
Therefore we conclude that Kif2a is required for proper chromosome segregation and cytokinesis within Xenopus embryos.

**Kif2a has a role in pole coalescence**

Normally, during stage 11, there are few mitotic figures, but Kif2a-depleted embryos had a high percentage of multipolar spindles. This suggests that Kif2a depletion causes multipolar spindles that cannot be resolved, generating a prolonged mitotic arrest at stage 11. Human cells with extra centrosomes first generate multipolar spindles early in mitosis and then have mechanisms to coalesce the extra poles to form a bipolar spindle (Kwon et al., 2008). Pole coalescence is required to fulfill the spindle checkpoint and segregate chromosomes (Kwon et al., 2008). We hypothesized that Kif2a-depleted cells were deficient in their ability to coalesce poles. Alternatively, it was possible that cells by stage 11 have failed several rounds of cytokinesis, generating multipolar structures that the pole-coalescing machinery cannot resolve. To distinguish between these two models, we generated multipolar spindles in stage 9 embryos (before cells normally fail cytokinesis) and measured the ability of Kif2a-depleted cells to coalesce poles in the first mitosis after cytokinesis failure. Stage 8/9 MO-injected and control embryos were treated with cytochalasin B (80 μM) for 1 h, and then the drug was removed by extensive washes into 0.3x MMR (see Materials and Methods) for 45 min. Animal caps were dissected at stage 10 and immunostained with antibodies against Cep57 and α-tubulin. As expected, disrupting F-actin blocks cytokinesis in embryos and leads to the precocious appearance of the multipolar spindles within both the control and MO-injected caps (Figure 8, A and B). However, multipolar spindles were more abundant within the combined cytochalasin B and MO–injected caps compared with those only treated with cytochalasin B (Figure 8B). We counted the centrosomes per pole to measure coalescence. In control cells treated with actin inhibitors, we could measure poles with more than three centrosomes, demonstrating that Xenopus embryos can coalesce poles. In contrast, we did not detect any poles with more than three centrosomes in Kif2a-depleted embryos combined with the brief cytochalasin treatment. This correlates with the increased number of multipolar spindles in Kif2a-depleted embryos. We conclude that Kif2a is required for efficient pole coalescence.

**FIGURE 8:** Kif2a-depleted cells poorly coalesce centrosomes. (A) Embryos were treated with 80 μM cytochalasin B at stages 8/9 and washed out of the drug after 1 h. At stage 10 embryos were fixed and imaged. Caps were immunostained for α-tubulin (green) and Cep57 (red) and stained for DNA (blue). Arrows (cytochalasin group) indicate centrosomes and multicentrosomes within cells. (B) Inhibition of cytokinesis generates multipolar spindles within stage 10 embryonic caps at a stage before their normal appearance in untreated MO-injected caps. Both cytochalasin-treated groups had multipolar spindles, but they were more abundant within the MO-injected group treated with DMSO instead of cytochalasin (control), depleted of Kif2a and DMSO (MO), control MO treated with cytochalasin B (CytoC control), or Kif2a-depleted embryos treated with cytochalasin (CytoC-MO). (C) Measuring pole coalescence after cytochalasin treatment by quantifying centrosomes at poles. Top, whole-cell micrographs; bottom, confocal micrographs, enlarged half-spindles of the same micrographs. Arrows indicate centrosomes within the spindles. (D) Quantification of C. For each group, 25 spindles from 10 caps were assessed.
note that poles with two centrosomes can be measured after Kif2a depletion, so Kif2a is not absolutely required for the process.

**DISCUSSION**

We characterized the loss-of-function phenotypes of the kinesin-13 microtubule depolymerase Kif2a in Xenopus embryos. Microtubule depolymerases are a major regulator of microtubule dynamics and are used for many cellular functions (Ems-McClung and Walczak, 2010). However, their requirements for tissue dynamics are poorly understood. Our work confirmed a number of in vitro results, including that Kif2a is a major regulator of spindle size and orientation and chromosome segregation (Wilbur and Heald, 2013). We identified new roles for Kif2a in cytokinesis and spindle pole coalescence, as well as in gastrulation movements. We showed that the early embryonic functions of Xenopus Kif2a can be rescued by both human Kif2a and Kif2b, suggesting that they contain overlapping functions for early embryogenesis. Finally, we demonstrated that Aurora kinase phosphorylation on T70 is required to localize Kif2a to both poles and centromeres.

**Kif2a-deficient mitoses result in failure of epiboly**

Our studies demonstrate that Kif2a is required to complete gastrulation of Xenopus embryos. We show that whole-embryo Kif2a depletion results in failure of epiboly of the animal region and failure of marginal zone/endoendal internalization and blastopore closure at the opposite, vegetal end of the embryo. Surprisingly, depletion of Kif2a in the animal cap results in the whole-embryo phenotype, whereas depletion in the marginal zone—the region that normally drives the vegetal internalization and blastopore closure—has no effect. This suggests a requirement for Kif2a in animal cap epiboly that is not found in the marginal zone and also suggests that the absence of epiboly in Kif2a morphants, both in whole-embryo and animal cap depletions, mechanically interferes with vegetal gastrulation movement. We confirmed this hypothesis by removing the offending animal cap region of whole-embryo Kif2a morphants, which rescues the vegetal internalization movements. The blastocoel roof cells of intact embryos divide to form three layers—a superficial epithelium, and two deep mesenchymal layers at stage 9. The superficial cells divide and flatten, whereas the two layers of deep cells intercalate radially (along the radii of the spherical embryo, i.e., normal to the surface of the embryo) to form a single, thinner layer of greater surface area (Keller, 1978; Keller, 1980). This spreading allows the marginal zone to move vegetally over the vegetal endoderm as it generates circumblastoporal convergence forces to internalize the yolk plug and close the blastopore. Keller and Jansa (1992) reported that the animal cap is not necessary for blastopore closure. This suggests that animal cap spreading is a rate-limiting step in blastopore closure.

Mechanical effects of failed epiboly on vegetal gastrulation movements are to be expected. Epiboly of the animal cap and embryo (decrease in area) of the vegetal region with involution of the marginal zone contribute to blastopore closure and convergence and extension (reviewed in Keller and Shook, 2004). Removal of all or part of the animal cap in normal embryos results in a consistently earlier onset and faster rates of several aspects of the internalization movements, including bottle cell formation, involution, and blastopore closure (Keller and Jansa, 1992). Epiboly does not push the marginal zone vegetally but occurs at a somewhat slower rate than the internalization movements, acting as a “brake” and supplying tissue to the internalization movements, which are slightly slower than is required, thereby preventing folding or buckling of the tissue, and smoothly integrating the spreading and internalization tissue movements. Radial intercalation drives epiboly, reducing the thickness of the animal cap, allowing for more cells on the surface to spread vegetally. The data presented here show that Kif2a depletion results in cytokinesis defects. Radial intercalation involves planarly polarized divisions and directed polarized neighbor exchange to establish and maintain fewer layers of cells of greater area (Keller, 1980; Marsden and DeSimone, 2001; Petridou et al., 2012; Woolner and Papalopulu, 2012). Both behaviors are sensitive to defects in cytokinesis. The multipolar, multicentrosomal cells with persistent connections due to failed cytokinesis and lagging chromosomes in Kif2a-depleted embryos appear incompatible with the polarized cell behavior and neighbor exchange required during radial intercalation and oriented division, and the result is a thick, multilayered animal cap of small area. Because the mutants of Kif2a at a mitotic phosphorylation (T70) site fail to rescue both chromosome segregation and gastrulation defects, we argue that the mitotic defects cause the gastrulation phenotypes. Here we show that lack of Kif2a results in failed epiboly, but we do not believe that the mechanism of this failure is primarily an effect on cell division because blocking cell division with hydroxyurea and aphidicolin to produce fewer, larger cells does not block gastrulation.

Studies from Kif2a+/− mice suggest that Kif2a plays a role in directional movement of growth cones during neuron development (Harris and Hartenstein, 1991; Homma et al., 2003). However, Kif2a+/− mice gastrulate well. Our ability to rescue the gastrulation phenotypes using RNA encoding either human Kif2a or Kif2b argues strongly that these homologues share redundant functions for the mitotic roles, which we confirmed directly. We suggest that the mouse phenotypes are caused by nonoverlapping roles of the homologues. We did not follow brain development or neuronal growth cones of our embryos rescued with either human Kif2a or Kif2b to determine whether they both retained brain development functions. However, such an approach could be used to determine whether frogs could be used as a disease model for retardation.

**Kif2a has multiple roles in animal cap mitoses**

The first observable cellular phenotype of Kif2a injection was increased spindle length in stage 10 embryos. Average metaphase spindle lengths increased ~1.4 times at both stages 10.5 and 11. Recently Aurora kinase regulation of Kif2a was shown to regulate the length of midzone microtubules (Uehara et al., 2013). Kif2a also controls spindle size during Xenopus development (Wilbur and Heald, 2013). It was argued that Kif2a was inhibited by increases in RanGTP activity in Xenopus eggs to generate larger spindles. Our finding that spindles are larger after loss of Kif2a in embryos is consistent with these observations. The fact that we see larger spindles in embryos depleted of Kif2a thus supports an emerging theme that mitotic spindle size is largely controlled by regulating microtubule dynamics through microtubule depolymerases and severing proteins (Levy and Heald, 2012; Whitehead et al., 2013; Wilbur and Heald, 2013).

The most obvious phenotype of Kif2a depletion in embryos is multipolar spindles, which first appear at stage 10.5 and increase at stage 11. Two possible “mechanisms” can promote multipolar spindle generation. One such method is centrosome fragmentation, by which spindles initially assemble with two poles but then split to form supernumerary poles by fragmentation of these original poles (Ehrhardt and Sluder, 2005). Second, cells with extra centrosomes generate a pole from each centrosome, and cells then have mechanisms to coalesce these poles to form bipolar spindles with multiple centrosomes in each pole (Meraldi et al., 2002; Kwon et al., 2008). To distinguish these models, we followed mitotic events by live-cell imaging. Kif2a-depleted cells often had anaphase-lagging chromosomes that became entrapped by the cytokinetic furrow. Although sometimes the cells were able to
resolve these defects, they resulted in eventual regression of the cytokineti
carus −50% of the time.

Kif2a had not been implicated in cytokinesis before, but this role
may not have been observed because tissue culture cells arrest with
monopolar spindles before cytokinesis can occur. Of interest, the
few cells that escape the arrest after depletion of Kif2b fail in cytoki-
nesis (Manning et al., 2007).

Cells in culture have robust methods to coalesce poles to trans-
form the multipolar spindles generated by extra centrosomes to
bipolar spindles (Kwon et al., 2008). Cell proliferation decreases sig-
ificantly at stage 11, and thus control embryos have only 7% of
cells in mitosis. However, Kif2a-depleted embryos had >25% of cells
in mitosis, and most of those had multipolar spindles. This sug-
gested that either Kif2a had an additional role in centrosome clus-
tering or clustering is deficient in embryos compared with tissue
culture systems. To distinguish these models, we generated multi-
spolar spindles in control and Kif2a at stage 9 before Kif2a depletion
caused multipolarity or cytokinesis failure at stage 10. After cytocha-
lasin treatment, we could detect multipolar spindles and extra cen-
trosomes in both control and Kif2a-depleted embryos, demonstrat-
ing that cytokinesis failure had occurred. However, the number of
multipolar spindles was higher in the Kif2a-depleted embryos, and
no pole structures contained more than two centrosomes, indicat-
ing a role for Kif2a in pole coalescence.

Regulation of kinesin-13 localization by Aurora kinases
Kinesin-13s are tightly regulated by multiple mechanisms, includ-
ing Ran-regulated importin binding, interaction with the ICIS protein
and EB1, and phosphorylation by polo and Aurora mitotic kinases.
The N-terminus of kinesin-13 plays a primary role in targeting these
kinases to subcellular structures (Zhang et al., 2007). Phosphoryla-
tion by Aurora kinase on the N-terminus of MCAK has been shown
to have a complex role in targeting MCAK to chromatin and cen-
tromes. Phosphorylation promotes binding to chromosome arms,
whereas dephosphorylation promotes centromere targeting (Zhang
et al., 2007). In this study, we characterized a related site on Kif2a.
The importance of this phosphorylation event was demonstrated by
the fact that injection of Kif2aT70A was unable to rescue any of the
observed phenotypes. Moreover, at least one of the functions of the
phosphorylation became apparent by the fact that Kif2aT70A was not
properly localized to either centromeres or spindle poles. It is
likely that Aurora A kinase phosphorylates T70 to localize Kif2a to
spindle poles, whereas Aurora B localizes to Kif2a to centromeres,
whereas Aurora A kinase phosphorylates T70 to localize Kif2a to
spindle poles, whereas Aurora B localizes to Kif2a to centromeres,
and this corresponds to the predominant pool of both kinases. We
previously showed that Aurora kinases can regulate microtubule de-
polymerase activity in both MCAK and Kif2a and localization of
MCAK. Thus Aurora regulation of both activity and localization ap-
pears to be a common regulatory theme of the kinesin-13 family.

Xenopus animal caps as a system in which to study
mitotic events
The study of mitosis has matured to a point at which the critical in
vivo experiments are knockdown and replacements with mutants.
Although Xenopus extracts are outstanding for biochemistry and
generating assays that isolate complex reactions (such as self-orga-
nization of microtubules into a bipolar spindle structure), their
robustness in generation of whole spindles can limit one’s ability to
detect subtle phenotypes. We feel that the Xenopus embryo is de-
veloping into an outstanding complement to tissue culture studies,
as one can visualize subtle phenotypes in vertebrate tissues in non-
transformed cells that would develop in a normal vertebrate animal
(Kieserman et al., 2008; Kieserman and Wallingford, 2009; Woolner
et al., 2008). The two-layer thick animal cap can be easily dissected
from above the blastocoel of a Xenopus early embryo. This explant
will live in a simple salt solution for >2 wk, using endogenous yolk as
an energy source, and, in the absence of external factors, will de-
velop into skin. Animal caps provide high-resolution imaging of cell
division events in normal euploid cells. Moreover, they are in a tissue
context with normal cell–cell interactions. Another advantage of ani-
caps is that the cells are rapidly dividing, with a 90- to 180-min
neuplet cycle time (Howe et al., 1995). Animal caps will normally de-
velop into skin, but they are pluripotent, and years of embryological
experiments have determined methods to convert animal caps into
a large number of tissue types. A long-term goal of this system is to
exploit this pluripotency to dissect cell cycle events in different tis-
ue types. Finally, animal caps allow us to use the set of Xenopus
antibodies that we have assembled to move seamlessly between
biochemistry and antibody depletions in Xenopus extracts and in
vivo knockdown and rescue experiments in animal caps.

Kif2a as a cancer target
Kinesin-13s are druggable and emerging as exciting potential can-
ter targets. Both MCAK and Kif2a have been shown to contribute to
taxol resistance, presumably because increasing the depolymerase
activity can restore more normal dynamics to taxol-treated cells
(Hedrick et al., 2008; Rizk et al., 2009). Therefore depleting kine-
sin-13 activity may increase the efficacy of taxanes. Our finding that
Kif2a has roles in pole coalescence provides another mechanism by
which Kif2a inhibitors could specifically kill cancer cells. Multiple
centrosomes are often found in the cells in human tumors, making
drugs that block pole coalescence an exciting new avenue for can-
ter therapy (Kwon et al., 2008, Godinho et al., 2009).

MATERIALS AND METHODS
Chemicals are from Sigma-Aldrich (St. Louis, MO) unless specified.

Extract and embryo preparation
Oocytes, eggs, and embryos were obtained from X. laevis females,
which were injected with 800 U of human chorionic gonadotropin
dorso to the dorsal lymph sac 18 h before use. Eggs were laid into 0.1×
MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgCl2, and 0.5 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, and
fertilized by adding macerated testes. At 30 min after fertilization,
embryos were dejellied in 2% cysteine (in 0.1× MMR, pH 8.0) and
rinsed several times with 0.1× MMR. Embryos were maintained in
0.1× MMR at 14.8°C until microinjection. IE and ME were gener-
ad as described (Lan et al., 2004). MOE is a high-speed superna-
tant of an egg extract that preserves the mitotic state. Briefly, de-
jelled eggs were lysed in an equal volume of ice-cold extraction buffer,
EB (80 mM b-glycerol phosphate, 20 mM EGTA, 10 mM MgCl2) + 1 μM microcystin, 1 mM ATP, and 10 mM DTT. Lysate was
clarified at 256,000 × g in a T-70 rotor, and the clear fraction
between the lipid and membranes was used.

Antisense morpholino, RNA preparation, and embryo
microinjection
Antisense morpholino oligonucleotides (MO; Gene Tools) were
generated based on Xenopus Kif2a genes [CTTCTGCTCCCTCTCC-
CCTGTGGCTT]. A dilution to 4 ng/nl at 10-ml injection gave Kif 2a
depletion and was used in all experiments. The 40 ng was injected
into single- or two-celled embryos. For MO + RNA rescue experi-
ments, 2–4 ng of in vitro–transcribed RNA (mMessage mMachine
Kit; Ambion) encoding hKif2a RNA was coinjected with the Kif2a-
MO mixture.
Embryos were injected at the one- and two-cell stage(s). On most occasions, controls were microinjected with MilliQ water (the morpholino diluent), but their survival was no worse than that of noninjected controls; therefore sometimes controls were not injected.

Immunofluorescence
The Kif2α, Ndc80, and Cep57 antibodies were previously described (McCleland et al., 2003; Emanuele and Stupkenberg, 2007; Knowlton et al., 2009). At stage 9, control and MO-injected embryos were assayed for viability. At stages 10, 10.5, and 11, the epithelial animal cap of these embryos were dissected and fixed overnight at 4°C in MEMFA (100 mM MOPS, 2 mM EGTA, 1 mM magnesium sulfate, 4% formaldehyde) or Dent's solution (80% methanol/20% DMSO) and rotated on a nutator. They were postfixed in methanol overnight at −20°C. After postfixing, the caps were hydrated, bleached, washed several times in Tris-buffered saline (TBS; pH 7.4) with 1% SDS detergent (TBS and Tween 20 [TBST]). Next they were blocked in 10% fetal serum/5% dimethyl sulfoxide in TBS. The caps were then incubated in the first antibody (DM1α, Kif2α; 1:500 dilution) for 48 h at 4°C. After incubation in the primary antibodies, epithelial caps were washed several times in TBST, incubated in blocking solution, and then immersed overnight at 4°C with secondary (anti-mouse and anti-rabbit) antibodies. Again after many rinses in TBST, the caps were subjected to a DNA stain, dehydrated, and then cleared in Murray’s solution. The caps were mounted on slides and observed and photographed under a confocal microscope. Immunofluorescence in cytostatic factor–arrested egg extracts and S3 cells were performed as previously described (Lan et al., 2004; Knowlton et al., 2009).

Embryo lysates and immunoblotting
Embryo lysates were prepared from control and Kif2α MO–injected embryos. Embryos were collected at stages 9, 10, and 10.5 for both groups. Staged embryos were homogenized in a lysis buffer (50 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 0.5% Triton-X, 0.5% NP-40, and protease inhibitors). They were first rinsed in lysis buffer, and then 150 μl of buffer was added to 15–20 embryos and homogenized over ice using a 100-ml pipette tip. The middle layer was carefully drawn off, avoiding the top membrane layer. This lysate was stored at −80°C until used for immunoblotting. Samples were separated by 10% SDS–PAGE, transferred to nitrocellulose, and analyzed by Western blot according to standard protocols using anti-α-tubulin (1:2500 dilution) as a standard.

Cytochalasin B treatment
Single-celled embryos were injected with Kif2α MO (4 ng/ml), and controls were not injected. For comparison purposes, Kif2α MO–injected and uninjected control embryos were not incubated in cytochalasin B. Embryos were allowed to develop to stage 8/9 and then incubated for 1 h at room temperature in 80 μM cytochalasin B in 0.3x MMR. They were washed in 0.3x MMR for 45 min, and then (stage 10) animal caps were immediately dissected, fixed, and stained for Cep57 and α-tubulin. Control animals were used to ensure that fixation occurred at stage 10.

Quantification and statistical analysis
To quantify spindle phenotypes in animal caps, we tabulated the number of bipolar metaphase spindles, multipolar spindles, and cell counts using 40× confocal micrographs of α-tubulin (DM1α antibodies) staining; >10 animal caps were analyzed for each sample. To test for a statistically significant difference between samples, we performed unpaired Student’s t tests.

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