The Aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis

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Background: The Aurora/Ipl1p-related kinase AIR-2 is required for mitotic chromosome segregation and cytokinesis in early *Caenorhabditis elegans* embryos. Previous studies have relied on non-conditional mutations or RNA-mediated interference (RNAi) to inactivate AIR-2. It has therefore not been possible to determine whether AIR-2 functions directly in cytokinesis or if the cleavage defect results indirectly from the failure to segregate DNA. One intriguing hypothesis is that AIR-2 acts to localize the mitotic kinesin-like protein ZEN-4 (also known as CeMKLP1), which later functions in cytokinesis.

Results: Using conditional alleles, we established that AIR-2 is required at metaphase or early anaphase for normal segregation of chromosomes, localization of ZEN-4, and cytokinesis. ZEN-4 is first required late in cytokinesis, and also functions to maintain cell separation through much of the subsequent interphase. DNA segregation defects alone were not sufficient to disrupt cytokinesis in other mutants, suggesting that AIR-2 acts specifically during cytokinesis through ZEN-4. AIR-2 and ZEN-4 shared similar genetic interactions with the formin homology (FH) protein CYK-1, suggesting that AIR-2 and ZEN-4 function in a single pathway, in parallel to a contractile ring pathway that includes CYK-1. Using *in vitro* co-immunoprecipitation experiments, we found that AIR-2 and ZEN-4 interact directly.

Conclusions: AIR-2 has two functions during mitosis: one in chromosome segregation, and a second, independent function in cytokinesis through ZEN-4. AIR-2 and ZEN-4 may act in parallel to a second pathway that includes CYK-1.

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Background

During cytokinesis, the microfilament and microtubule cytoskeletons appear to interact extensively. The genetic pathways that execute this ultimate step in cell division remain largely undefined [1–3]. Cytokinesis begins when an actomyosin contractile ring assembles at the equatorial cortex in response to an unidentified signal from the mitotic apparatus [4,5]. The contractile ring then ingresses through the cytoplasm to encircle the spindle interzone, generating the midbody. Microtubules may be required throughout furrow ingression, as the loss of microtubules during cytokinesis can result in regression of cleavage furrows [6,7]. Additionally, interactions between the contractile ring and the spindle midbody may stabilize cleavage furrows near the end of cytokinesis, and the completion of cytokinesis may require the co-ordinated disassembly of the midbody and the contractile ring.

Genetic evidence that the mitotic spindle regulates assembly of the contractile ring has come from studies of the *Drosophila* gene *pavarotti* (*pav*), which encodes a homolog of the mammalian mitotic kinesin-like protein-1 (MKLP-1) [8]. MKLP-1 can bind to and crosslink antiparallel microtubules *in vitro* [9], and both MKLP-1 and Pav protein localize to the central spindle, or interzone, where microtubules of opposite polarity interdigitate during mitosis. Mutations in *pav* affect both the mitotic spindle and the contractile ring. The central spindle in mutant embryonic cells is depleted of microtubule bundles, and actin, anillin and the septin protein Peanut fail to assemble into a contractile ring [8]. In the absence of conditional alleles, it has not been possible to address whether *pav* is also required later in cytokinesis, after the contractile ring has assembled.

Evidence that mitotic kinesin-like proteins may be required later in cytokinesis has come from studies of the *Caenorhabditis elegans* MKLP gene *zen-4* [10,11]. Inactivation of *zen-4*, either by a null mutation or by RNA-mediated interference (RNAi), results in a late cytokinesis defect: the cleavage furrows retract after extensive ingression [10,11]. In addition to the cytokinesis defect, the central spindle fails to form in *zen-4* mutant embryos. Because other spindle-dependent processes like DNA

segregation and anaphase spindle elongation occur normally, ZEN-4 may be required specifically for cytokinesis.

While it is clear that MKLP-1 family members play an essential role in cytokinesis, little is known about the mechanisms that control their localization and activity. The Aurora/Ipl1p-related kinase AIR-2 may be one important regulator, as ZEN-4 is undetectable at the mitotic spindle of *air-2* mutant embryos and cytokinesis fails after extensive furrowing [12,13]. The mammalian Aurora homolog AIM-1 is also required for cytokinesis, suggesting that this function may be widely conserved [14]. However, Aurora/Ipl1p-related kinases appear to act primarily in centrosome separation and chromosome segregation in other systems [15], and *air-2(RNAi)* embryos have defects in DNA segregation that result in the formation of a single daughter nucleus in the path of the advancing cleavage furrow. Thus, it has been suggested that the cytokinesis defects in *air-2* embryos could result indirectly from the failure to segregate DNA [13].

Here, we report the identification of conditional, loss-of-function alleles of *air-2* and *zen-4*. We found that ZEN-4 functions late in cytokinesis, as well as during the two-cell stage to stabilize cleavage furrows. In contrast, AIR-2 is required at metaphase of the first mitotic cell cycle for DNA segregation, ZEN-4 localization and cytokinesis. Because DNA segregation defects alone were not sufficient to disrupt furrow ingression in other mutants, we suggest that AIR-2 acts specifically in cytokinesis, through the localization of ZEN-4. Genetic interactions with the formin homology (FH) protein CYK-1 also support models in which AIR-2 and ZEN-4 act in a linear pathway, and we have found that AIR-2 and ZEN-4 interact directly *in vitro*. We conclude that AIR-2 performs two distinct functions during mitosis, one in DNA segregation and a second, independent function in cytokinesis.

Results

AIR-2 and ZEN-4 localize to the mitotic spindle midzone and are required for a late step in cytokinesis
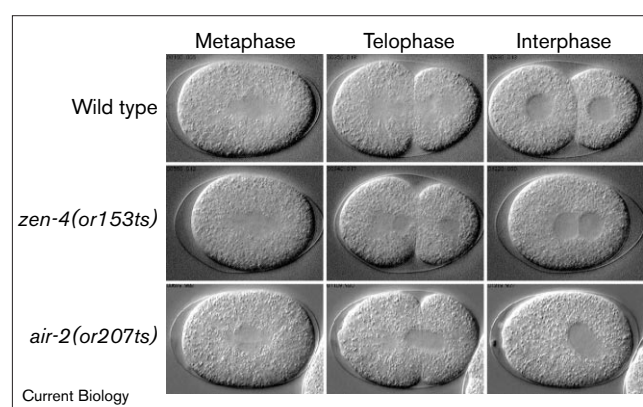
Because many maternally expressed genes are also essential zygotically, screens for non-conditional, maternal-effect mutations may fail to identify important genes that govern cell division during early embryogenesis. We therefore chose to screen for temperature-sensitive (ts) mutations that result in embryonic lethality at the restrictive temperature (25°C), but are viable at the permissive temperature (15°C) [16]. Mothers homozygous for *zen-4(or153ts)* or *air-2(or207ts)*, two of the mutations identified in this screen, produced embryos with highly penetrant defects in cytokinesis at the restrictive temperature. Hereafter, we will refer to embryos produced at the restrictive temperature as mutant embryos. Our analysis focused largely on the first mitotic cell cycle in the *C. elegans* embryo; however, AIR-2 and ZEN-4 are also required in subsequent divisions,

and for polar body extrusion during meiosis ([10–13], data not shown).

In *zen-4(or153ts)* mutant embryos, cleavage furrows ingressed extensively through the cytoplasm during the first two mitotic cell cycles but ultimately regressed to produce a multinucleate single cell (Figure 1). Although ZEN-4 localized normally to the central spindle in *zen-4(or153ts)* embryos at the permissive temperature (Figure 2a; 13/13 one-cell-stage embryos), ZEN-4 protein was undetectable at the restrictive temperature and the mitotic spindle midzone was disorganized or absent (Figure 2a; 10/10 one-cell-stage embryos). Therefore, *zen-4(or153ts)* behaves like a null allele at the restrictive temperature (see also Materials and methods).
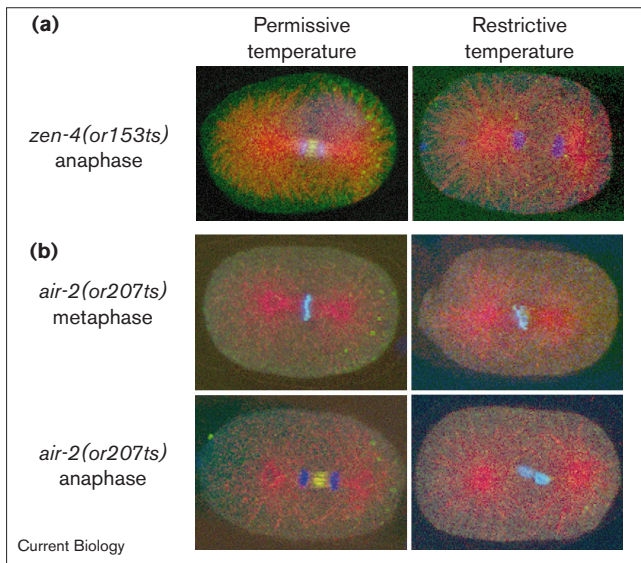
Because AIR-2 is required for ZEN-4 localization [12], we reasoned that AIR-2 and ZEN-4 may function in a pathway. In fact, *air-2(RNAi)* embryos [13] and *air-2(or207ts)* mutant embryos resembled *zen-4* mutants: cleavage furrows regressed after extensive contraction (Figure 1), and the spindle interzone was abnormal (Figure 2b). Furthermore, AIR-2 localized first to mitotic chromosomes during metaphase, then redistributed to the spindle midzone at anaphase in wild-type embryos [12], and in *air-2(or207ts)* embryos at the permissive temperature (Figure 2b; 11/11 metaphase embryos, 8/8 anaphase embryos). Thus, AIR-2 is in the right place to interact with ZEN-4. AIR-2 protein remained on anaphase chromosomes in *air-2(or207ts)* mutant embryos (Figure 2b, 6/6 embryos), suggesting that AIR-2 activity may be important for its redistribution to the mitotic spindle. In addition to the cytokinesis defects, *air-2* mutant embryos had defects in DNA segregation (Figures 1 and 2b) [12,13].

Figure 1

Cytokinesis in wild-type, *zen-4* and *air-2* mutant embryos. Extensive cleavage furrows formed during the first attempt at cytokinesis in *zen-4(or153ts)* and *air-2(or207ts)* mutant embryos. These furrows inevitably regressed. The *air-2(or207ts)* mutant embryos had additional defects in chromosome segregation that resulted in the formation of a large, centrally positioned nucleus late in mitosis.

Figure 2

	Permissive temperature	Restrictive temperature
(a) *zen-4(or153ts)* anaphase		
(b) *air-2(or207ts)* metaphase		
air-2(or207ts) anaphase		

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ZEN-4 and AIR-2 both localize at the mitotic spindle interzone. **(a)** In *zen-4(or153ts)* embryos at the permissive temperature, ZEN-4 protein (green) localized at the spindle midzone between separating anaphase chromosomes (blue). The central spindle contained dense bundles of interzone microtubules (red). At the restrictive temperature, ZEN-4 protein could not be detected in the central spindle and the interzone was depleted of microtubules. **(b)** In *air-2(or207ts)* embryos at the permissive temperature, AIR-2 protein (green) accumulated first on chromosomes, then at the midzone during anaphase. At the restrictive temperature, AIR-2 remained associated with anaphase chromosomes and the interzone failed to form.

Because *zen-4* mutants did not share this defect (Figures 1 and 2a), ZEN-4 cannot mediate all AIR-2 functions.

AIR-2 functions at metaphase or early anaphase

AIR-2 is required for chromosome segregation and ZEN-4 localization, which occur before cytokinesis. For this reason, we determined when, during the first cell cycle, AIR-2 was required for normal cytokinesis. When *air-2(or207ts)* mutant embryos were shifted to the restrictive temperature after the onset of anaphase, but before the initiation of a visible cleavage furrow, DNA segregation and cytokinesis occurred normally (Figure 3a,d; 7/7 embryos). Only when embryos were shifted to the restrictive temperature before anaphase did cytokinesis fail (Figure 3d; cytokinesis failed in 8/11 embryos shifted 2 minutes or more before anaphase). Conversely, downshift experiments showed that AIR-2 is not required during pronuclear migration, but first becomes necessary when the pronuclei meet, during prometaphase or metaphase (Figure 3f). Incomplete cytokinesis always coincided with defects in DNA segregation in these experiments (upshift, 15/15 embryos; downshift, 12/12 embryos). We conclude that AIR-2 functions at metaphase

or early anaphase, and that the cytokinesis defect may result from an earlier requirement for AIR-2 in DNA segregation or ZEN-4 localization.

ZEN-4 functions late in cytokinesis

To determine when ZEN-4 functions relative to AIR-2, we performed temperature-shift experiments using *zen-4(or153ts)* mutant embryos (Figure 3). Downshift experiments established that ZEN-4 is dispensable until cytokinesis has begun: embryos maintained at the restrictive temperature, then shifted to the permissive temperature before or shortly after the initiation of cytokinesis, divided normally (Figure 3e; 5/5 embryos). Only when the downshift occurred late in cytokinesis were defects apparent (Figure 3e; cytokinesis failed in 11/19 embryos). Thus, ZEN-4 is required after AIR-2, consistent with ZEN-4 functioning downstream of AIR-2 in a pathway.
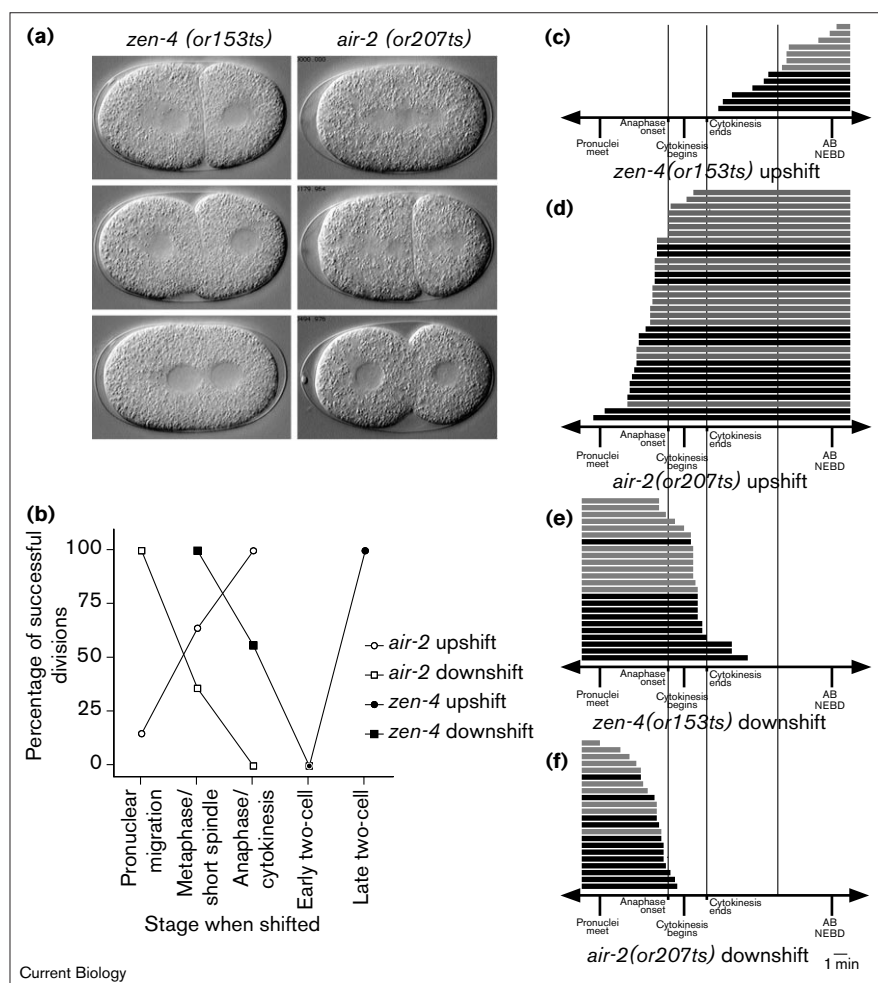
While we expected to find a requirement for ZEN-4 during cytokinesis, we were surprised to find that ZEN-4 is also required to maintain the separation of daughter cells well after the apparent completion of cytokinesis. If two-cell stage embryos were shifted to the restrictive temperature more than 4 minutes before nuclear envelope breakdown during the ensuing mitosis, the plasma membrane regressed to reform a single cell (Figure 3a,c; 6/6 embryos). Only when shifted within 4 minutes of nuclear envelope breakdown — over halfway through interphase — were cleavage furrows maintained (Figure 3c; 7/7 embryos). We conclude that ZEN-4 acts late in cytokinesis, and to maintain cell separation.

AIR-2 is required to recruit, but not maintain, ZEN-4 at the spindle interzone

ZEN-4 accumulates at the spindle midzone during metaphase and anaphase, when AIR-2 is required for cytokinesis. We therefore determined whether AIR-2 is required for ZEN-4 localization at the same time it is required for cytokinesis. When we shifted *air-2(or207ts)* mutant embryos to the restrictive temperature before or during pronuclear migration (Figure 4a; 8/8 embryos), or during metaphase (Figure 4b; 3/3 embryos), chromosome segregation was defective and ZEN-4 was not detectable in the central spindle. In contrast, when *air-2(or207ts)* embryos were shifted to the restrictive temperature in early anaphase, then fixed approximately 2–3 minutes later, after cleavage furrows had ingressed over 50% of the diameter of the embryo, ZEN-4 localization and DNA segregation both appeared normal (Figure 4b, 3/3 embryos). Therefore, AIR-2 may be required only for the initial localization of ZEN-4 at the mitotic spindle but not for its maintenance (see Discussion). Furthermore, the temporal requirement for AIR-2 in ZEN-4 localization coincides with the requirement for AIR-2 in cytokinesis, consistent with AIR-2 functioning through the localization of ZEN-4.

Figure 3

Temperature-shift analysis suggests that ZEN-4 is required during cytokinesis and to maintain cell separation at the two-cell stage, whereas AIR-2 is required during metaphase. **(a)** When *zen-4(or153ts)* mutant embryos were shifted to the restrictive temperature at the two-cell stage, cleavage furrows regressed within 1 min. In contrast, *air-2(or207ts)* mutant embryos divided successfully when shifted to the restrictive temperature before cytokinesis. **(c–f)** Each bar indicates the time in which an individual embryo was observed at the restrictive temperature. Gray bars and black bars represent embryos that completed or failed to complete cytokinesis, respectively. These experiments are summarized in the graph in **(b)**. (c) The *zen-4(or153ts)* embryos were shifted to the restrictive temperature at the two-cell stage. Cleavage furrows were stable only when embryos were shifted less than 4 min before nuclear envelope breakdown (NEBD) in AB, the anterior daughter blastomere produced by the first division. (d) The *air-2(or207ts)* embryos could divide normally when maintained at the restrictive temperature throughout cytokinesis. Only when embryos were raised to the restrictive temperature during metaphase were cleavage defects observed. (e) The *zen-4(or153ts)* embryos grown at the restrictive temperature divided normally if shifted to the permissive temperature during the early stages of furrow ingression. When shifted late in cytokinesis or at the two-cell stage, embryos failed to divide. (f) When *air-2(or207ts)* mutant embryos were shifted to the permissive temperature after metaphase, defects in cytokinesis and chromosome segregation were apparent. When embryos were maintained at the permissive temperature for most or all of metaphase, chromosome segregation and cytokinesis were normal.

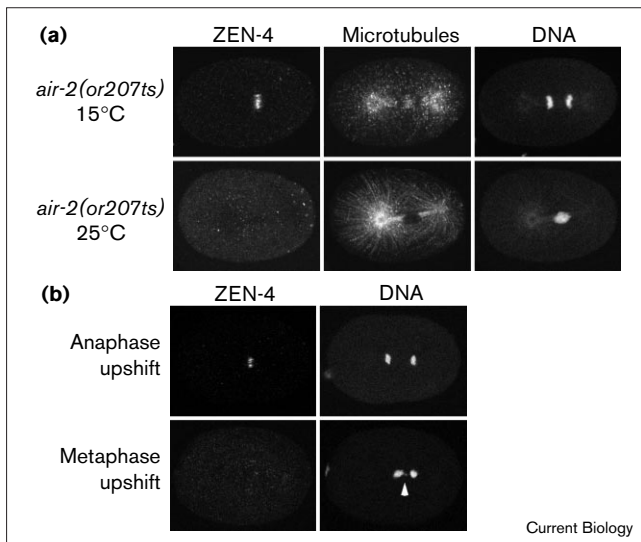## Defects in DNA segregation are not sufficient to disrupt ZEN-4 localization or cytokinesis

Although AIR-2 may act in a pathway with ZEN-4, the defects in ZEN-4 localization and in cytokinesis could also result indirectly from the failure of *air-2* mutants to segregate DNA. To address this issue, we observed cytokinesis in embryos lacking the *C. elegans* centromere protein-A (CENP-A) homolog HCP-3, which localizes to kinetochores and is required for chromosome segregation [17]. Cleavage furrows ingressed successfully through reforming nuclei in these embryos, resulting in the formation of closely opposed daughter nuclei connected by a DNA bridge (Figure 5a; 5/5 embryos). Furthermore, ZEN-4 localized normally to the central spindle in *hcp-3(RNAi)* embryos (Figure 5b; 7/7 embryos), indicating that proper DNA segregation was not required for ZEN-4 localization or the formation of interzone microtubules. To compare the segregation defects in *air-2* and *hcp-3* mutant embryos

in the absence of cytokinesis, we examined *hcp-3(RNAi); zen-4(or153ts)* double mutant embryos. A single, large nucleus reformed in these embryos (5/5 embryos), as occurred in *air-2* embryos (Figure 5a). Thus, by inactivating ZEN-4 and HCP-3 together, we were able to reproduce fully the *air-2* mutant phenotype. Cytokinesis also occurred normally in embryos mutant for topoisomerase II (data not shown), and in *hcp-1; hcp-2* double mutant embryos ([18]; M. Roth, personal communication), both of which fail to segregate chromosomes. We conclude that defects in DNA segregation are not sufficient to disrupt cytokinesis, and that AIR-2 is unusual in having fully penetrant defects in both segregation and cytokinesis.

ZEN-4 and AIR-2 may act in parallel to the FH protein CYK-1 during embryonic cytokinesis

Consistent with AIR-2 and ZEN-4 functioning in a single pathway, we detected similar genetic interactions between
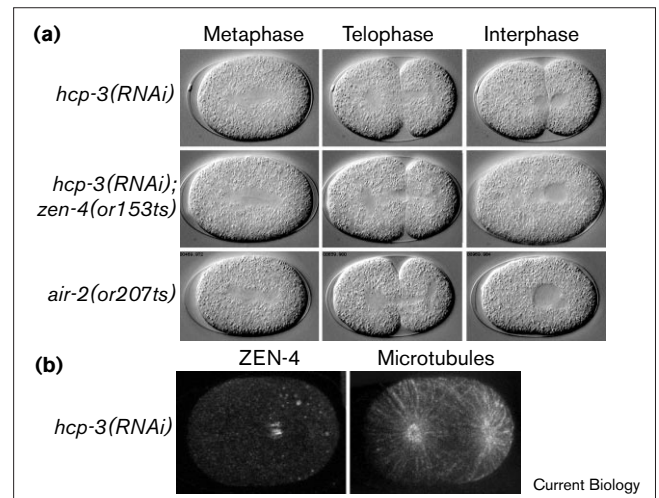
Figure 4

AIR-2 is required for the initial localization of ZEN-4, but not for its maintenance. **(a)** ZEN-4 protein localized at the interzone of the mitotic spindle in *air-2(or207ts)* embryos dividing at the permissive temperature (15°C). At the restrictive temperature (25°C), ZEN-4 protein was not detectable. Although dense bundles of kinetochore microtubules formed in mutant embryos, the interzone was depleted of microtubules. **(b)** Shifting *air-2(or207ts)* embryos to the restrictive temperature during anaphase did not eliminate ZEN-4 localization. However, ZEN-4 protein failed to localize to the central spindle in *air-2(or207ts)* embryos upshifted less than 2 min earlier, in metaphase. The arrowhead points to a DNA bridge resulting from the defect in DNA segregation.

Figure 5

Defects in DNA segregation are not sufficient to disrupt ZEN-4 localization and cytokinesis. **(a)** In *hcp-3(RNAi)* embryos, cleavage furrows ingressed through the reforming central nucleus, resulting in the formation of two closely opposed daughter nuclei. In *hcp-3(RNAi); zen-4(or153ts)* double mutant embryos, cytokinesis failed and a single central nucleus reformed. The segregation and cytokinesis defects in these embryos resembled those in *air-2(or207ts)* embryos. **(b)** ZEN-4 protein localized normally to the spindle interzone in *hcp-3(RNAi)* embryos.

AIR-2 and ZEN-4 and the FH protein CYK-1. CYK-1 is required for cleavage furrow ingression early in cytokinesis during the first and second cell cycles (A.F.S., David L. Baillie, B.B., unpublished data). However, mothers homozygous for weak loss-of-function alleles of *cyk-1* produce embryos that make extensive cleavage furrows during the second attempt at cytokinesis, resembling *air-2* and *zen-4* mutants at this division (Figure 6a, Table 1) [10–14,19]. Like both single mutants, *air-2; zen-4* double mutant embryos made deep furrows during cytokinesis (Figure 6b, Table 1), consistent with AIR-2 and ZEN-4 functioning in a single pathway. In contrast, cleavage furrows failed to ingress in 22 out of 22 *air-2; cyk-1* and 22 out of 24 *cyk-1; zen-4* double mutant embryos, forming at most shallow dimples (Figure 6b, Table 1), suggesting that CYK-1 acts in a second pathway. Further consistent with parallel functions, CYK-1 and ZEN-4 localization occur independently (Figure 6c). We therefore conclude that AIR-2 and ZEN-4 act in a single, linear pathway, in parallel to the FH protein CYK-1.

Interestingly, *cyk-1; zen-4* double mutant embryos had defects in pronuclear migration as well as in cytokinesis (Figure 6d). Following fertilization, the oocyte pronucleus migrates to meet the sperm pronucleus, which is anchored in the posterior of most wild-type, *cyk-1* and *zen-4* embryos. In contrast, pronuclei often met in the center of *cyk-1; zen-4* double mutant embryos. In *Saccharomyces cerevisiae*, the FH protein Bni1p and the kinesin Kip3p are required to position the spindle pole body during budding [20,21]. Perhaps CYK-1 and ZEN-4 function similarly to posteriorly position the sperm pronucleus and its associated centrosomes during the time of pronuclear migration.
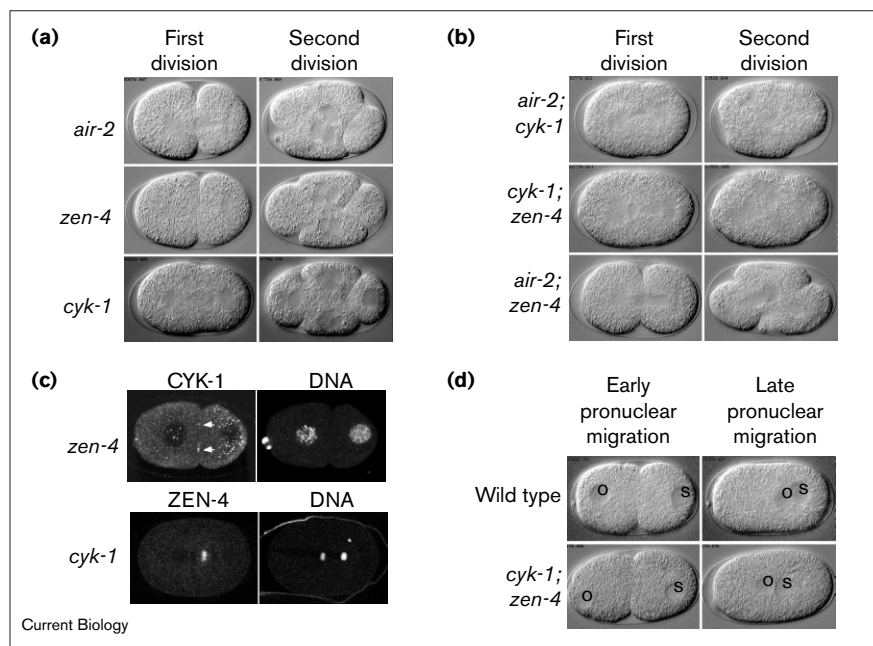
AIR-2 interacts directly with ZEN-4 *in vitro*

Consistent with AIR-2 and ZEN-4 acting together in a pathway, we found that they could interact directly *in vitro* (Figure 7a). When a bacterially expressed fusion protein between glutathione-S-transferase and AIR-2 (GST–AIR-2) was mixed with [35S]methionine-labeled, *in vitro* translated ZEN-4 (35S–ZEN-4), both proteins could be immunoprecipitated with antibodies to AIR-2; 35S–ZEN-4 alone could not be immunoprecipitated by either the AIR-2 antibody or by GST beads, suggesting that ZEN-4 and AIR-2 interact specifically (Figure 7a). Thus, our genetic, immunocytochemical and biochemical studies all suggest that AIR-2 acts in cytokinesis through ZEN-4.

Discussion

Two models have been proposed to explain the role of AIR-2 in cytokinesis. AIR-2 may act directly in cytokinesis, possibly through the localization of ZEN-4 [12].

Figure 6

CYK-1 and ZEN-4 may function in parallel pathways that interact during cytokinesis. **(a)** Extensive cleavage furrows formed during the second attempt at cytokinesis in *cyk-1*, *zen-4* and *air-2* single mutant embryos. Note that *cyk-1(or36)* mutant embryos fail to form cleavage furrows during the first cell cycle. **(b)** Cleavage furrows ingressed extensively in *air-2; zen-4* double mutant embryos, consistent with inactivating two components of a single pathway. In contrast, furrows failed to ingress in *air-2; cyk-1* and *cyk-1; zen-4* embryos, suggesting that CYK-1 may function in a parallel pathway. **(c)** CYK-1 protein localized at the leading edge of the cleavage furrow in *zen-4(or153ts)* mutant embryos fixed at the restrictive temperature (arrowheads), as occurs in the wild type. In addition to the cleavage-furrow staining, the CYK-1 antibody also non-specifically recognizes P granules [19]. ZEN-4 protein localized normally to the spindle interzone in *cyk-1(or36)* mutant embryos. **(d)** Following fertilization, the oocyte pronucleus (o) migrates to meet the sperm pronucleus (s) in the posterior of wild-type embryos. In contrast, pronuclei frequently met in the center of *cyk-1; zen-4* double mutant embryos. During pronuclear migration, a transient furrow (the

pseudocleavage furrow) ingressed between the pronuclei. This furrow always regressed, and

could be seen in wild-type embryos, as well as in some *cyk-1; zen-4* double mutant embryos.

Alternatively, the cytokinesis defects in *air-2* embryos may result indirectly from the failure to segregate DNA [13]. Here, we have established that AIR-*2* is required at metaphase or early anaphase for ZEN-4 localization, whereas ZEN-4 first acts late in cytokinesis. Mutations in either gene enhanced the cytokinesis defects in *cyk-1* mutant embryos. Because defects in DNA segregation were not sufficient to disrupt cytokinesis in other mutants, we conclude that AIR-*2* and ZEN-4 function sequentially in a linear pathway that is specifically required for the completion of cytokinesis.
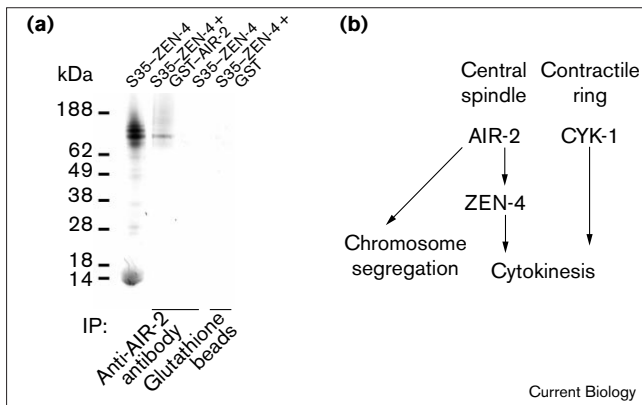
Table 1

Extent of cleavage furrow ingression in *C. elegans* embryos.

| | First division* | | Second division† | |
Genotype	< 1/3 embryonic diameter	> 1/3 embryonic diameter	Weak or absent furrows	Deep furrows, one or both ends
Wild type		10/10		10/10
cyk-1(or36)	6/6			6/6
zen-4(or153ts)		6/6		6/6
zen-4(RNAi)		8/8		8/8
air-2(or207ts)		6/6		7/7
air-2(RNAi)		8/8		8/8
cyk-1(or36); zen-4(or153ts)	9/9		20/21	1/21
cyk-1(or36); zen-4(RNAi)	3/3		2/3	1/3
cyk-1(or36); air-2(or207ts)	13/13		14/14	
cyk-1(or36); air-2(RNAi)	8/8		8/8	
air-2(or207ts); zen-4(or153ts)		6/6		5/5
air-2(RNAi); zen-4(RNAi)		3/3	1/6	5/6

The first two attempts at cytokinesis were observed by timelapse video microscopy using differential interference contrast (DIC) optics. *The extent of cleavage furrow ingression during the first division was determined as the ratio of the embryonic diameter in the plane of the cleavage furrow to the total diameter of the embryo. †A tetrapolar spindle was established during mitosis of the second cell cycle in mutant embryos. Cleavage furrows were specified at positions that bisect each spindle arm. In single mutant embryos, the posterior-most furrows almost invariably ingressed extensively through the cytoplasm. A variable amount of furrowing occurred at the anterior end of the embryo, ranging in extent from small membrane dimples in some embryos to deep furrows in others. In double mutant embryos, the extent of furrowing was reduced at both ends of the embryo.

Figure 7

(a) ZEN-4 and AIR-2 interact *in vitro*. Lane 1, ZEN-4 translated *in vitro* in the presence of [^{35}S]methionine (S35–ZEN-4; the amount in lane 1 is 1:100 of that used for the binding assays); lane 2, 100 ng GST–AIR-2 (purified from *Escherichia coli* by glutathione binding) mixed with 10 μl of the ZEN-4 *in vitro* translation reaction; lane 3, S35–ZEN-4 alone (no GST–AIR-2); lane 4, S35–ZEN-4 plus GST (purified from *E. coli* on glutathione beads). Binding reactions were placed at 30°C for 60 min. Anti-AIR-2 antibody was added to the reactions in lanes 2 and 3, then pulled down with protein A–sepharose. Glutathione beads were added to the reaction in lane 4. Beads were washed three times in 1% NP40, 150 mM NaCl, 50 mM Tris pH 8.0, resuspended in 2× SDS–PAGE loading buffer, boiled for 3 min, and loaded onto a 10% NuPAGE gel (Novex). The gel was processed by fluorography and exposed to X-ray film for 18 h. **(b)** Model for cytokinesis pathways in the *C. elegans* embryo. The contractile ring component CYK-1 functions in parallel to a mitotic spindle pathway that includes AIR-2 and ZEN-4. In addition to a role in cytokinesis, AIR-2 also functions in DNA segregation.

ZEN-4 is required late in cytokinesis, and to maintain cell separation during the next cell cycle

Our analysis of ZEN-4 suggests a role for this MKLP late in cytokinesis, after cleavage furrows have already ingressed extensively. In contrast, other MKLPs are required earlier in mitosis. For example, *Drosophila* Pav is required for the formation of the contractile ring in embryonic cytokinesis [8], and perturbing MKLP-1 function by antibody injection results in metaphase arrest in sea urchin embryos and PtK1 kidney epithelial cells [22,23]. As *cyk-1; zen-4* double mutants failed to form furrows early in cytokinesis, it is possible that ZEN-4 has additional early functions that are dispensable in embryos with wild-type CYK-1.

Surprisingly, cleavage furrows regressed frequently when *zen-4(or153ts)* embryos were shifted to the restrictive temperature at the two-cell stage. Following the completion of cytokinesis, ZEN-4 persists between sister blastomeres in a spot that may be a remnant of the spindle midbody [10,11]. Perhaps ZEN-4 functions at this site to stabilize ingressed furrows until the plasma membranes surrounding daughter cells are completely separated. The lengthy requirement for ZEN-4 may indicate that the final membrane fusion and termination events occur relatively late

in the subsequent cell cycle. Alternatively, it is possible that the completion of cytokinesis at the permissive temperature in *zen-4(or153ts)* embryos takes longer than in wild-type embryos.

AIR-2 performs dual functions in the first cell cycle

Our analysis of the temperature-sensitive *air-2* allele *or207ts* suggests that AIR-2 is required during metaphase or early anaphase for ZEN-4 localization and chromosome segregation, but is no longer required during cytokinesis. Because ZEN-4 localization and chromosome segregation occur at approximately the same time, we have been unable to use temperature shifts to determine whether the ZEN-4 localization defect alone is sufficient to disrupt cytokinesis in *air-2* mutants. Nevertheless, ZEN-4 localization and cytokinesis occurred normally in *hcp-3(RNAi)* embryos, even though segregation failed. Thus, proper chromosome segregation is not required for the completion of cytokinesis. While it is possible that the segregation defects in *hcp-3* and *air-2* mutant embryos are somehow different, we do not favor this model because *hcp-3; zen-4* double mutant embryos strongly resembled *air-2* mutant embryos in their segregation defects. We conclude that AIR-2 acts in DNA segregation, and independently in cytokinesis.

It remains possible that AIR-2 is required for formation of a normal interzone, which may be essential for ZEN-4 localization. Because ZEN-4 is itself necessary to organize the central spindle, it has not been possible to address this issue. Nevertheless, mislocalization of ZEN-4 alone can account for the disorganization of the central spindle in *air-2* mutants. Furthermore, the observation that AIR-2 can bind ZEN-4 *in vitro* suggests that AIR-2 may regulate ZEN-4 localization through a direct interaction, and not indirectly through the mitotic spindle midzone.

In the accompanying article, Kaitna *et al.* report the requirement for a *C. elegans* inner centromere protein (INCENP), called ICP-1, in cytokinesis [24]. ICP-1 interacted directly with AIR-2, and was required for the localization of AIR-2 to chromosomes and the mitotic spindle. Interestingly, a fusion protein between ZEN-4 and the green fluorescent protein (ZEN-4–GFP) localized transiently to the mitotic spindle in *icp-1(RNAi)* embryos, suggesting that ICP-1 is dispensable for the initial localization of ZEN-4. In contrast, we have never detected ZEN-4 at the interzone of *air-2(RNAi)* or *air-2(or207ts)* mutant embryos, and our upshift experiments suggest that AIR-2 is not required to maintain ZEN-4 localization later in the cell cycle. Perhaps transient localization of ZEN-4 is difficult to detect in fixed embryos. Additionally, AIR-2 may function to maintain ZEN-4 at the central spindle, but the brief (~2–3 minute) incubation at the restrictive temperature may not be sufficient to inactivate AIR-2 in our upshift experiments. Nevertheless, 6/9 embryos shifted to the restrictive temperature

2–3 minutes before the metaphase to anaphase transition failed to segregate DNA (Figure 3d), suggesting that AIR-2 activity is severely compromised under similar conditions. Alternatively, transient ZEN-4 localization may result from overexpression of ZEN-4–GFP, or from incomplete elimination of AIR-2 from the interzone of *icp-1(RNAi)* embryos. Although these issues remain unresolved, AIR-2 is clearly a key regulator of ZEN-4 localization during cytokinesis.

Aurora-like kinases function in multiple mitotic processes

In contrast to AIR-2, most Aurora-like kinases described to date appear to be dispensable for cytokinesis. *Drosophila* Aurora and *Xenopus* Eg2 are required for centrosome separation and formation of a bipolar spindle [25,26], whereas *S. cerevisiae* Ipl1p is required for chromosome segregation and bipolar spindle assembly [27,28]. Finally, a second *C. elegans* Aurora homolog, AIR-1, is required for formation of a normal spindle and the proper partitioning of developmental factors [29]. The mammalian Aurora-like kinase AIM-1, however, is required specifically for cytokinesis [14], and additional homologs are present in the *Drosophila* and *Xenopus* genomes for which functions are not yet known [15]. Thus, studies of AIR-2 and AIM-1 may have revealed a conserved requirement for Aurora-like kinases in cytokinesis.

As we have proposed for AIR-2 and ZEN-4, Aurora-like kinases may function through kinesins in other processes. The Aurora-like kinase Eg2 and the *bimC*-related kinesin Eg5 are both required for centrosome separation and formation of a bipolar mitotic spindle in *Xenopus* egg extracts [26], and Eg2 can bind to and phosphorylate Eg5 *in vitro* [30]. Furthermore, the Cin8p kinesin is dispensable in budding yeast, but becomes essential in the absence of Ipl1p [31]. Thus, Aurora-like kinases may regulate multiple mitotic processes by targeting different kinesins.

CYK-1 may function in parallel to an AIR-2/ZEN-4 pathway during cytokinesis

Consistent with AIR-2 acting through ZEN-4, *air-2; zen-4* double mutants had late cytokinesis defects like those in either single mutant. In contrast, both *air-2; cyk-1* and *cyk-1; zen-4* double mutants exhibited enhanced cytokinesis defects. Thus, AIR-2 and ZEN-4 appear to act in a linear pathway, in parallel to CYK-1 (Figure 7b). This interpretation is supported by our findings that CYK-1 and ZEN-4 localized independently, and that *zen-4(or153ts)* behaved like a null allele at the restrictive temperature. Because cytokinesis failed in *air-2; cyk-1* double mutant embryos well before daughter nuclei reformed, this synergistic phenotype probably does not result from defects in DNA segregation, but rather from specific requirements for AIR-2 and ZEN-4 in cytokinesis. Moreover, these results suggest that ZEN-4 may act early in cytokinesis, as does Pav in *Drosophila* embryos. These early ZEN-4 functions were, however, dispensable in embryos with wild-type CYK-1.

ZEN-4 and the Rho GTPase-activating protein (Rho GAP) CYK-4 localize at the central spindle in an interdependent process [32], and both are required for its formation. Although Rho GAPs and FH proteins act antagonistically in other systems, inactivation of CYK-4 enhanced the defects in *cyk-1* mutant embryos (A.F.S., unpublished data). Thus, CYK-4 may act positively in a pathway with AIR-2 and ZEN-4. Perhaps this pathway is required for formation of a normal central spindle, which becomes essential for furrow ingression in embryos when contractile ring function is weakened by mutations in *cyk-1*.

The role of the mitotic spindle during embryonic cytokinesis in *C. elegans*

While CYK-1 may be required for the assembly or function of the contractile ring, the role of an AIR-2/ZEN-4 pathway early in cytokinesis is less clear. One possibility is that CYK-1 functions in a contractile ring pathway that provides the force for cytokinesis, whereas ZEN-4 functions in a second process such as membrane secretion [33–35]. In this model, ZEN-4 might deliver vesicles to the cleavage furrow, where they could then fuse with the plasma membrane during cytokinesis [35]. Alternatively, although CYK-1 localization is independent of ZEN-4, the localization or activity of other contractile ring components might require AIR-2 and ZEN-4, or an intact spindle midzone. Perhaps, a functionally compromised contractile ring can assemble in *cyk-1* or *zen-4* single mutant embryos, whereas in double mutants its assembly or function is further reduced such that cleavage furrows fail completely to ingress. As additional genes required for cytokinesis are identified, these models can be tested by further genetic and molecular studies.

Conclusions

We have established that the Aurora/Ipl1p-related kinase AIR-2 is required for cytokinesis, a role that is independent of its function in DNA segregation. Because AIR-2 is required for ZEN-4 localization at the same time that it is required for cytokinesis, we conclude that AIR-2 functions in cytokinesis through the localization of ZEN-4. In contrast to Pav and MKLP-1, ZEN-4 is first required late in cytokinesis. Nevertheless, ZEN-4 may have additional functions early in cytokinesis that are dispensable in embryos that have wild-type CYK-1. Finally, we suggest that AIR-2 and ZEN-4 interact directly, and in parallel to a contractile ring pathway that includes CYK-1.

Materials and methods

Strains and alleles

C. elegans culture was performed as described [36]; N2 Bristol was used as the wild-type strain. The following alleles and balancer chromosomes were used. Linkage group I (LGI): *air-2(or207ts), let-603(h289)* and *unc-13(e51)*; LGIII: *lon-1(e185), cyk-1(or36), unc-36(e251)* and *qC1(inversion balancer: dpy-19, glp-1)*; LGIV: *dpy-13(e184sd), zen-4(or153ts), zen-4(w35), unc-8(n491sd)* and *him-8(e1489)*. The temperature-sensitive alleles *air-2(or207ts)* and *zen-4(or153ts)* were

maintained by growing homozygous animals at 15°C. For observation of the mutant phenotype, L4 larvae were raised overnight at 25°C. Embryos produced the next day showed fully penetrant mutant phenotypes.

Isolation and cloning of new alleles of air-2 and zen-4

The *or207ts* and *or153ts* alleles were identified in a screen for temperature-sensitive embryonic-lethal mutations [16]. Standard linkage group and three factor mapping techniques were used to position the mutations in these alleles at approximately +0.5 map units (mu) on chromosome I and +2.7 mu on chromosome IV, respectively. Based on the map positions of these mutations and their associated phenotypes, we identified *air-2* and *zen-4* as candidate genes. Gene identity was tested by complementation. The *or207ts* allele failed to complement *air-2(h289),* formerly known as *let-603* [13]; *or153ts* failed to complement *w35,* a null allele of *zen-4* [10]. To confirm gene identity, we sequenced the *air-2* gene in *or207ts* worms and the *zen-4* gene in *or153ts*. Each region was amplified from genomic DNA by PCR, and sequenced by the University of Oregon DNA Sequencing Facility, using an ABI 377 Prism automated fluorescent sequencer. Clones from two independent PCR reactions were sequenced for each gene, and compared with sequences from *lin-2* worms, the parental strain used for mutagenesis. Codon 265 of the *air-2* gene was mutated from CCC to CTC in *or207ts*, resulting in a P to L substitution in subdomain XI of the predicted kinase domain. Codon 520 of the *zen-4* gene was altered from GAC to AAC in *or153ts*, and codon 735 was altered from GAT to AAT. These mutations both resulted in D to N substitutions; one in a region of the ZEN-4 protein predicted to form a coiled coil, and a second in a carboxy-terminal region unique to the MKLP-1 family of kinesins. It will require rescue assays using transgenes with single lesions to determine whether both mutations contribute to the temperature-sensitive phenotype of *or153ts*. Both alleles resulted in 100% penetrant embryonic lethality at 25°C (*or153ts*, $n = 179$; *or207ts*, $n = 180$). At 15°C, 96% of *or153ts* embryos hatched ($n = 154$), whereas 68% of *or207ts* embryos hatched ($n = 70$). Embryos produced by *or153ts/w35* mothers, or by *or153ts* mothers injected with *zen-4* double-stranded RNA (dsRNA) were indistinguishable from *or153ts* mutant embryos. Thus, *or153ts* behaves like a null allele at the restrictive temperature. The *or207ts* allele is likely a hypomorphic allele of *air-2*, as many *or207ts* mutant embryos extruded polar bodies at the restrictive temperature. However, the cytokinesis defects in *or207ts* embryos were identical to those in embryos produced by *or207ts* mothers injected with *air-2* dsRNA. Thus, the late defect observed in *or207ts* embryos may represent the null phenotype for cytokinesis.

RNAi

Double-stranded RNA was produced from cDNA clones (kindly provided by Yuji Kohara) and injected by standard methods [37] at a needle concentration of at least 1 μg/ml. The following clones were used: *zen-4,* yk391b3; *air-2,* yk483g8; *cyk-1,* yk471h10; topoisomerase II, yk117e10; *hcp-3,* yk283b9. Embryos were harvested 12–24 h after injection.

Immunofluorescence and microscopic analysis

Embryos obtained by cutting open gravid hermaphrodites in distilled water in a watchglass were transferred by mouth pipette to a polylysine-coated slide. The eggshell was permeabilized by the freeze-crack method [38], and embryos were fixed for 15 min in room-temperature methanol. Slides were incubated for 30 min in blocking solution [39], then incubated with antibodies for at least 6 h at 4°C. The following antibodies and dilutions were used: rabbit anti-CeMKLP1, diluted 1:2000 [11] (kindly provided by Susan Strome); rabbit anti-AIR-2, diluted 1:250 [12]; rabbit anti-CYK-1, diluted 1:100 [19]; and a monoclonal anti-tubulin antibody (clone DM1α; Sigma), diluted 1:250. DNA was labeled with 0.2 μM TOTO3 (Molecular Probes). All fluorescent images were obtained on a BioRad MRC 1024 confocal microscope.

For immunofluorescence of temperature-sensitive mutants, homozygous L4 mothers were raised overnight at 15°C or 25°C. The following day, embryos were processed for immunofluorescence as described above, except for the following changes: worms were cut open in distilled water pre-equilibrated to 15°C or 25°C; a variable-temperature, thermoelectric peltier heat pump (Cambion) was used to maintain the temperature of the slide before the freeze-crack step; and slides were snap frozen in liquid nitrogen before fixation. For timelapse video microscopic analysis, embryos were obtained by cutting open gravid hermaphrodites in M9 buffer. Embryos were mounted on a 3% agarose cushion, or on a polylysine coverslip inverted over a drop of M9 in a depression slide. DIC images were captured at 10 sec intervals using a Dage MTI VE1000 digital camera under the control of Scion Image software, and stored on CD-ROM media.

Temperature-shift experiments

For upshift experiments, *air-2(or207ts)* or *zen-4(or153ts)* homozygous mutant worms were cut open in a watchglass containing 15°C M9. A single pronuclear stage embryo was mounted on a 3% agarose cushion, and observed under high power on a Zeiss Stemi SV11 dissecting scope. A peltier block equipped with an observation hole (Cambion) was used to maintain the stage temperature at 15°C. When the embryo reached the desired stage, it was rapidly transferred to a compound microscope in a room at approximately 25°C, and the phenotype recorded by DIC timelapse microscopy. For downshift experiments, L4 hermaphrodites were raised overnight at 25°C. The following morning, single pronuclear stage embryos were mounted on agarose cushions and observed on a compound microscope at approximately 25°C. At the desired stages, embryos were rapidly chilled on a peltier block maintained at 15°C and observed on a dissecting scope during the first attempt at cytokinesis.

ZEN-4 and AIR-2 proteins cycled rapidly between active and inactive forms following temperature shifts in these mutants. When *zen-4(or153ts)* mutant embryos were shifted to the restrictive temperature early in the two-cell stage, cleavage furrows began to regress within 1 min. When embryos were shifted to the permissive temperature approximately 1 min before cleavage furrows met in the center of the embryo, cytokinesis was completed normally. Similarly, upshifting *air-2(or207ts)* embryos 1–2 min before the metaphase–anaphase transition frequently prevented DNA segregation. Therefore, we believe our temporal studies of ZEN-4 and AIR-2 accurately indicate when each protein is required.

In vitro *binding assay*

The entire AIR-2 cDNA was cloned in-frame with GST in the pGEX vector (Pharmacia) and transformed into BL21 *E. coli*. The GST–AIR-2 fusion protein was expressed and purified on glutathione–sepharose as directed by the manufacturer (Pharmacia). The ZEN-4 protein was labeled with [^{35}S]Met by *in vitro* translation of a ZEN-4 cDNA using the TNT coupled transcription and translation kit (Promega). Approximately 100 ng of the GST–AIR-2 fusion protein were mixed with 10 μl of the ZEN-4 translation reaction and incubated in 100 μl binding buffer (150 mM NaCl, 100 mM Tris pH 8.0, 0.25% NP40) at 30°C for 1 h. The GST–AIR-2 binding complex was immunoprecipitated by addition of the anti-AIR-2 antibody and protein A–sepharose (4°C, 60 min). Protein A immune complexes were pelleted and washed three times in 150 mM NaCl, 100 mM Tris pH 8.0, 1% NP40. The samples were boiled for 3 min in 1 × SDS–PAGE loading buffer and resolved by electrophoresis through a 10% NuPAGE gel (Novex). Gels were fixed and enhanced by soaking in 1M sodium salicylate for 20 min, then dried and exposed to X-ray film. Control binding reactions included incubation of the ZEN-4 *in vitro* translation product with purified GST and isolating the GST moiety and bound proteins with glutathione–sepharose (with washes as above). GST–AIR-2–ZEN-4 binding reactions were also incubated with protein A–sepharose in the absence of antibody.

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