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# The Survivin-like *C. elegans* BIR-1 Protein Acts with the Aurora-like Kinase AIR-2 to Affect Chromosomes and the Spindle Midzone

**Elizabeth K. Speliotes,* Anthony Uren,†§ David Vaux,† and H. Robert Horvitz*‡**
*Howard Hughes Medical Institute
Department of Biology
Building 68 Room 425
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
†The Walter and Eliza Hall Institute of Medical Research
Post Office Royal Melbourne Hospital
Victoria 3050
Australia

## Summary

**Baculoviral IAP repeat proteins (BIRPs) may affect cell death, cell division, and tumorigenesis. The *C. elegans* BIRP BIR-1 was localized to chromosomes and to the spindle midzone. Embryos and fertilized oocytes lacking BIR-1 had defects in chromosome behavior, spindle midzone formation, and cytokinesis. We observed indistinguishable defects in fertilized oocytes and embryos lacking the Aurora-like kinase AIR-2. AIR-2 was not present on chromosomes in the absence of BIR-1. Histone H3 phosphorylation and HCP-1 staining, which marks kinetochores, were reduced in the absence of either BIR-1 or AIR-2. We propose that BIR-1 localizes AIR-2 to chromosomes and perhaps to the spindle midzone, where AIR-2 phosphorylates proteins that affect chromosome behavior and spindle midzone organization. The human BIRP survivin, which is upregulated in tumors, could partially substitute for BIR-1 in *C. elegans*. Deregulation of *bir-1* promotes changes in ploidy, suggesting that similar deregulation of mammalian BIRPs may contribute to tumorigenesis.**

## Introduction

The separation and segregation of sister chromatids prior to cytokinesis must be tightly regulated to ensure that newly forming cells and gametes receive appropriate numbers of chromosomes. Defects in chromosome segregation leading to changes in ploidy may result in cellular and organismal death (Takahashi et al., 1994) or in cellular transformation and tumorigenesis (Zou et al., 1999).

Cytological, genetic, and biochemical studies have begun to elucidate the mechanisms by which sister chromatids separate and segregate to opposite poles. At the metaphase/anaphase transition, activation of the anaphase promoting complex (APC) leads to sister chromatid separation through the degradation of proteins that promote sister chromatid cohesion (reviewed by Nasmyth, 1999). The separated chromatids then segregate along kinetochore microtubules to opposite poles with the help of motor proteins (Hoyt and Geiser, 1996). Spindle checkpoints ensure that only one member of each pair of sister chromatids moves to a particular pole by preventing the activation of the APC until all chromosomes are aligned at the metaphase plate and are connected to microtubules at their kinetochores (reviewed by Amon, 1999). Between the segregating sister chromatids in anaphase, a tubulin-containing structure called the spindle midzone forms. Proteins that localize to the spindle midzone may affect cytokinesis by specifying the site of formation of the cytokinetic furrow or by promoting furrow ingression or resolution (reviewed by Glotzer, 1997). Following cytokinesis, the cytokinetic remnant, known as the midbody in mammals, remains at the site where the cytokinetic furrow divides the cell at the spindle midzone.

Phosphorylation and dephosphorylation events are critical for the cytoskeletal and chromosomal changes that occur during mitosis. The dissolution of the nuclear membrane, condensation of chromosomes, duplication of the spindle poles, and cytokinesis have been correlated with the phosphorylation of putative effectors, including lamins, histones, Cut12p, and myosin II, respectively (Satterwhite et al., 1992; Bridge et al., 1998; Collas, 1999; Wei et al., 1999). The Cdc2, Polo, and Aurora families of kinases, which are found at multiple sites during the cell cycle, may mediate some of these phosphorylations (reviewed by Glover et al., 1998; Giet and Prigent, 1999; Ohi and Gould, 1999). The localization of these kinases to particular sites may be important for positioning them near their substrates. The identification of molecules required for the localization of mitotic kinases may help establish how the spatial and temporal events of mitosis are coordinated.

Baculoviral inhibitor-of-apoptosis (IAP) repeat (BIR) proteins (BIRPs) were first identified in studies of baculoviruses by their ability to prevent apoptosis (Crook et al., 1993). Since then, many nonviral BIRPs have been identified in organisms ranging from yeast to humans (reviewed by Uren et al., 1998). Members of the BIRP protein family are defined by the presence of one to three N-terminal BIR motifs (BIRs); their C termini are variable (Uren et al., 1998). Many BIRPs can suppress apoptosis when overexpressed, and the BIRs are necessary for this protective function. The normal functions of the cellular members of this family and how these functions relate to the ability of this family of proteins when overexpressed to protect against programmed cell death are only beginning to be explored.

Recent findings suggest that some BIRPs may have roles in cell division. *S. cerevisiae* and *S. pombe* lacking *bir* genes die during meiosis and mitosis, respectively (Rajagopalan and Balasubramanian, 1999; Uren et al., 1999), interference with *C. elegans bir-1* leads to defects in cytokinesis (Fraser et al., 1999), and interference with expression of the human BIRP survivin leads to the formation of polyploid cells (Li et al., 1999). Survivin, which is upregulated in many tumors (Ambrosini et al., 1997), localizes to the mitotic spindle and midbody (Li et al., 1998), consistent with its playing a role in cell division. How BIRPs affect cell division is unclear. To better understand the normal functions of BIRPs, we characterized *C. elegans* BIR-1.

‡To whom correspondence should be addressed (e-mail: horvitz@mit.edu).
§Present address: Genentech, Inc., 1 DNA Way, MS#40, South San Francisco, California 94080.

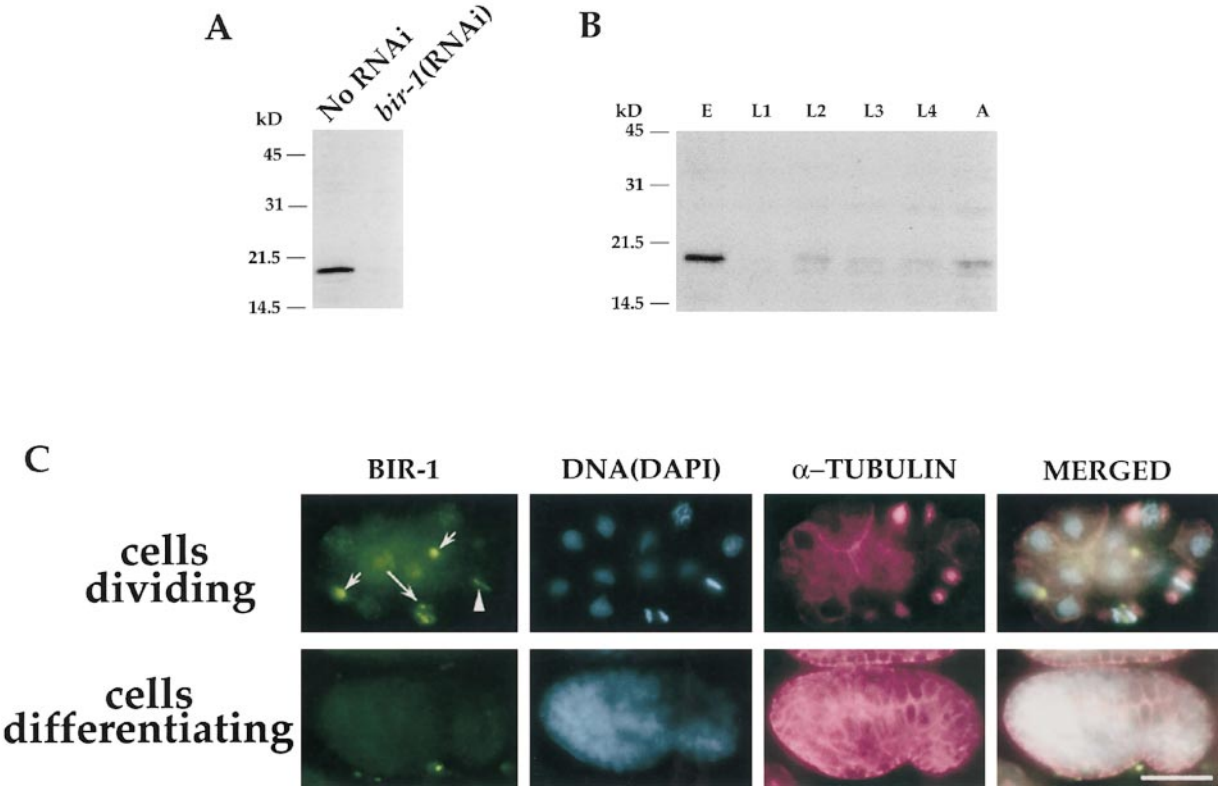


Figure 1. BIR-1 Expression Correlates with Cell Divisions

(A) Total protein extracts from wild-type No RNAi or *bir-1*(RNAi) embryos were stained with affinity-purified anti-BIR-1 antibody.

(B) Protein extracts from synchronized wild-type worm populations were immunoblotted and probed with affinity-purified anti-BIR-1 antibody. E, embryos; L1, L2, L3, and L4, larval stages 1, 2, 3, and 4; and A, adults.

(C) Fixed embryos were stained with anti-BIR-1 or anti-α-tubulin antibodies to visualize BIR-1 (green) and α-tubulin (red), respectively, and counterstained with DAPI to visualize DNA (blue). The top row shows an early embryo in which cells were dividing. Arrows point to BIR-1-positive structures. The bottom row shows an older embryo (~500 cells) in which cells were starting to differentiate and little BIR-1 staining was detectable. Scale bar, 20 μm.

## Results

### *C. elegans* BIR-1 Is Most Similar to Human Survivin

We and others (Fraser et al., 1999) independently isolated cDNA clones for *C. elegans bir-1*. BIR-1 encodes a 155 amino acid (aa) protein that is most similar in size and structure to human survivin (Fraser et al., 1999).

### BIR-1 Expression Correlates with Cell Division

To determine when and where BIR-1 is expressed during development, we generated antibodies against BIR-1, which we used for Western blot analysis and whole-mount staining. Our affinity-purified anti-BIR-1 antibodies recognized a single band of about 18 kDa; this band was absent in wild-type embryos depleted of BIR-1 using RNA-mediated gene interference (RNAi) (Fire et al., 1998), confirming that the 18 kDa band was BIR-1 (Figure 1A).

We probed total protein extracts from embryos, synchronized larvae and adults with affinity-purified anti-BIR-1 antibodies, and found that BIR-1 expression was highest in embryos, in which there is substantial cell division, and lowest in L1 larvae that were growth and cell-division arrested by food deprivation (Figure 1B). BIR-1 was present at low levels in growing L2, L3, and L4 larvae as well as in adults, in which only the germline proliferates (Figure 1B). Immunostaining of fixed embryos with affinity-purified anti-BIR-1 antibodies revealed that BIR-1 was present in dividing cells at multiple sites (Figure 1C, top row). Little BIR-1 immunoreactivity was detected in differentiating cells (Figure 1C, bottom row). All BIR-1 staining was eliminated following *bir-1* RNAi, further confirming the specificity of the antibodies (data not shown). These findings suggest that BIR-1 is expressed in dividing cells.

### BIR-1 Localizes to Chromosomes and to the Spindle Midzone during Mitosis and Meiosis

To characterize the subcellular localization of BIR-1 in dividing cells, we focused on the first embryonic division, in which mitotic structures are largest (Figure 2A). BIR-1 immunoreactivity was first detected on prometaphase chromosomes (Figure 2A, PROPHASE) and persisted on these chromosomes at the metaphase plate (Figure 2A, METAPHASE). BIR-1 staining overlapped with DNA rather than with the kinetochore, as determined by triple-staining with DAPI and antibodies against BIR-1 and HCP-1 (a kinetochore component (Moore et al., 1999) (data not shown). During anaphase, BIR-1 was present both on chromosomes and the spindle midzone (Figure 2A, ANAPHASE). During telophase,

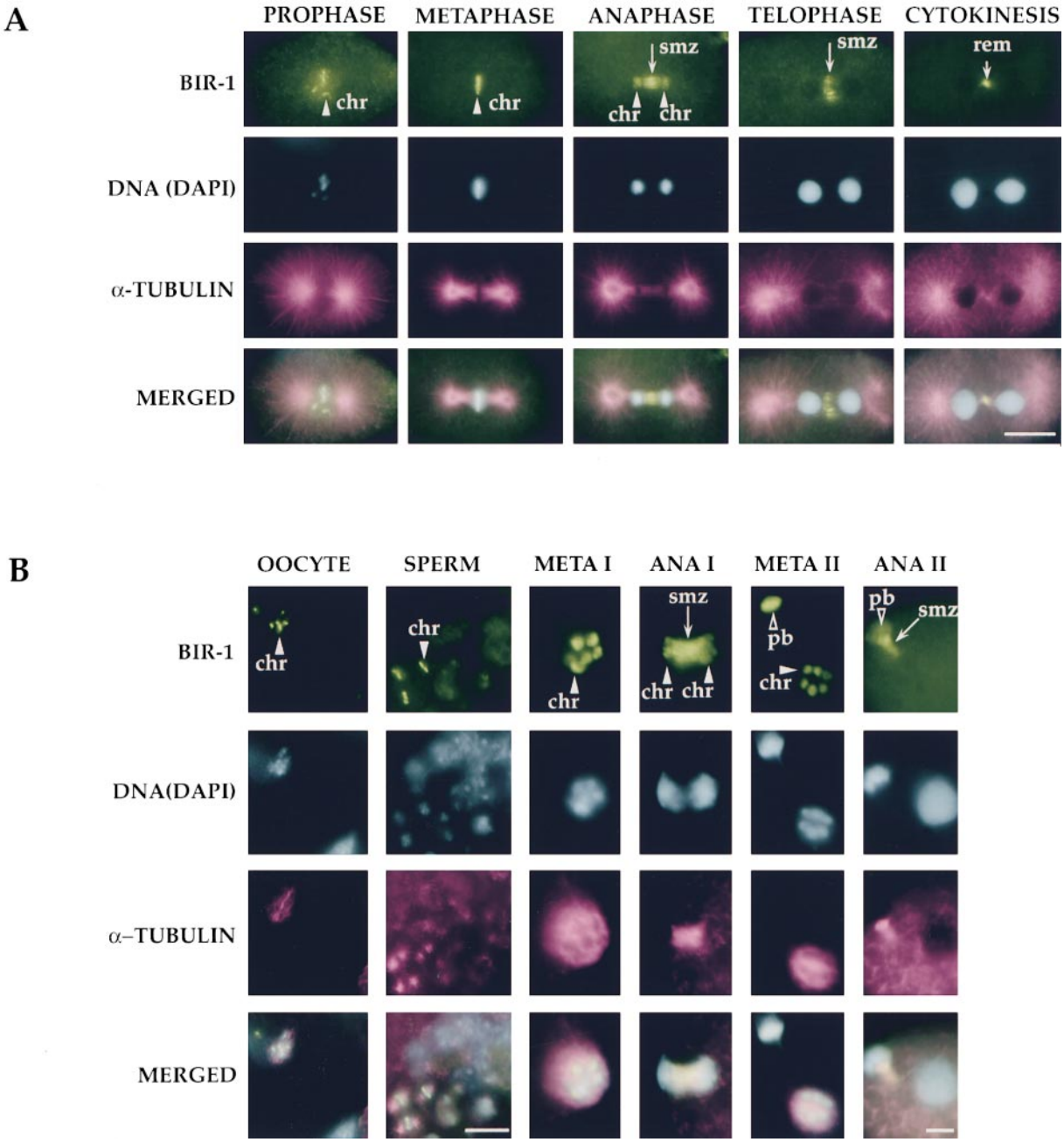


Figure 2. BIR-1 Localizes to Chromosomes and to the Spindle Midzone during Mitosis and Meiosis

Wild-type fertilized oocytes, embryos, and worms were stained with anti-BIR-1 antibody, anti-α-tubulin antibody, and DAPI to visualize BIR-1 (green), α-tubulin (red), and DNA (blue), respectively.

(A) One-cell embryos in different stages of mitosis.

(B) Oocyte most proximal to spermatheca (OOCYTE) and spermatocytes in hermaphrodite gonad (SPERM). Female meiosis occurring at the anterior of the fertilized oocyte: metaphase I (META I), anaphase I (ANA I), metaphase II (META II), or anaphase II (ANA II). Arrows indicate BIR-1-positive structures. Closed arrowheads, chromosomes (chr); long arrows, spindle midzone (smz); short arrow, cytokinetic remnant (rem); open arrowheads, polar bodies (pb). Mitosis, SPERM (and OOCYTE) scale bars, 20 μm. Meiosis scale bar, 5 μm.

BIR-1 immunoreactivity was no longer detectable on decondensing DNA but was still present on the spindle midzone (Figure 2A, TELOPHASE). Following cytokinesis, BIR-1 staining persisted at the cytokinetic remnant (Figure 2A, CYTOKINESIS). We observed a similar pattern of immunoreactivity within cells during subsequent divisions (data not shown).

BIR-1 was also present during meiosis (Figure 2B). *C. elegans* oocytes are arrested at diakinesis of meiosis I until meiotic maturation and nuclear envelope breakdown are initiated in the oocyte adjacent to the spermatheca. Oocytes ovulate by entering the spermatheca, where sperm is stored, and become fertilized. Female meiotic divisions proceed in the anterior of the fertilized

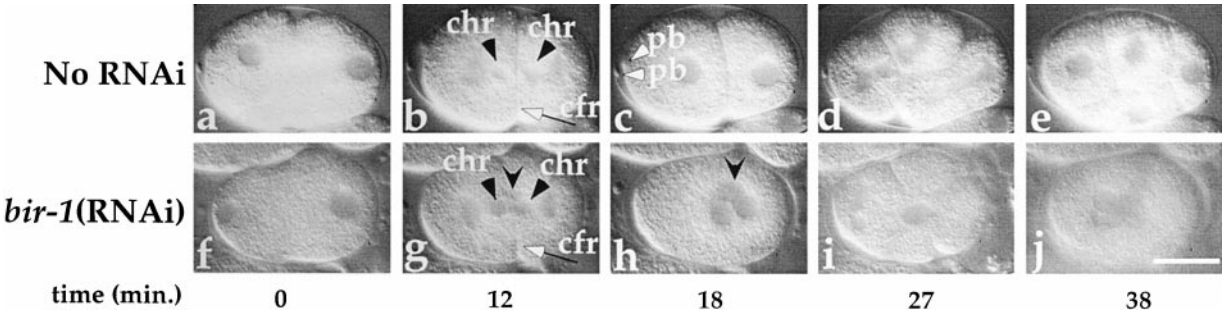


Figure 3. *bir-1*(RNAi) Embryos Have Defects in Cytokinesis and Chromosome Segregation and Lack External Polar Bodies

Fertilized oocytes were dissected from uninjected or *bir-1* dsRNA injected (*bir-1*(RNAi)) hermaphrodites and their development visualized over time using Nomarski optics. (A, B, C, D, and E) Wild-type (No RNAi). (F, G, H, I, and J) *bir-1*(RNAi). Time 0, pseudocleavage. Arrows, cytokinetic furrow (cfr); white arrowheads, polar bodies (pb); black arrowheads, chromosomes; indented arrow, nonsegregating chromosomal mass. Scale bar, 20 μm.

oocyte, while sperm chromosomes remain condensed in the posterior. As homologs and then sister chromatids separate, two sequential cytokinetic furrows divide the cell at the spindle midzone that forms between segregating chromosomes. These divisions expel the DNA that will not contribute to the maternal pronucleus as two polar bodies at the anterior of the fertilized oocyte. Upon completion of meiosis II, the oocyte and sperm DNA decondense to form the maternal and paternal pronuclei, respectively, and the single paternally derived centrosome duplicates. The pronuclei migrate to meet each other, and during this migration chromosomes begin to condense. The pronuclei meet, merge to form the prophase nucleus of the one-cell embryo, and the first mitotic division ensues.

In hermaphrodite oocytes, BIR-1 first became visible on the condensed chromosomes of the oocyte most proximal to the spermatheca (Figure 2B, OOCYTE). BIR-1 localization to chromosomes in this oocyte required sperm in the spermatheca and either preceded or was concomitant with nuclear envelope breakdown (data not shown). BIR-1 was also associated with meiotic chromosomes in spermatocytes (Figure 2B, SPERM) but was not associated with DNA in mature sperm or condensed paternal DNA following fertilization (data not shown).

Following fertilization, BIR-1 remained localized to condensed maternal chromosomes during metaphase I (Figure 2B, META I) and was found associated with chromosomes as well as the spindle midzone during anaphase I (Figure 2B, ANA I). During metaphase II, BIR-1 could be seen associated with polar body DNA and the sister chromatids (Figure 2B, META II). BIR-1 was again associated with both polar body DNA and the spindle midzone during anaphase II (Figure 2B, ANA II).

The localization of BIR-1 to chromosomes and the spindle midzone suggested that BIR-1 may function in chromosomal and spindle processes during meiosis and mitosis.

### Fertilized Oocytes and Embryos Lacking BIR-1 Display Chromosomal and Spindle Defects

To determine whether BIR-1 is functionally important for chromosomal and spindle processes, we used RNAi to block *bir-1* function and, using Nomarski differential interference contrast optics, found that *bir-1*(RNAi) embryos lacked polar bodies and had a defect in the completion of cytokinesis during mitosis (Figure 3H). Relative to spindle assembly, which was normal, the cytokinetic furrow initiated at the correct place and time (Figures 3B and 3G), but after ingressing to a great extent then regressed, leaving multiple nuclei within one cytoplasm (Figure 3H). These findings corroborate the observations of Fraser et al. (1999). In addition, we observed that in some *bir-1*(RNAi) embryos as chromosomes segregated, a separate nonsegregating presumably chromosomal body was present in the center of the embryo (Figure 3G). In some embryos, distinct chromosome separation did not occur (data not shown). Even though *bir-1*(RNAi) embryos did not complete cytokinesis, they initiated multiple rounds of abortive cell division (Figures 3I and 3J) and ultimately arrested as highly polyploid mostly single-cell embryos. When some cellularization occurred, the resulting cells had variable amounts of DNA and sometimes none at all, as determined by staining with DAPI (data not shown). We also observed defects in cytokinesis during female meiotic divisions (data not shown).

Because cytokinesis defects may result from abnormalities in astral or spindle midzone microtubules, the cytokinetic furrow (reviewed by Glotzer, 1997) or chromosome segregation (by creating a physical barrier that blocks furrow completion [Mullins and Biesele, 1977]), we histologically examined these structures and processes in *bir-1*(RNAi) fertilized eggs and one-cell embryos. We staged and compared wild-type and *bir-1* (RNAi) fertilized oocytes and embryos during meiosis, pronuclear formation, and mitosis (Figure 4; data not shown) (for staging criteria see Experimental Procedures).

Aspects of both meiosis and mitosis were abnormal in the absence of BIR-1 (Figure 4; Table 1). Germ cells progressed through the pachytene and diakenesis stages of meiosis, and apparently normal oocytes formed. In *bir-1*(RNAi) fertilized oocytes, meiotic maturation occurred normally, since at metaphase I a nuclear envelope (as determined using an anti-nucleoporin antibody) was not present (data not shown). Even though chromosomes appeared properly paired and condensed, *bir-1*(RNAi) fertilized oocytes had defects in the alignment of paired homologs during meiosis I (Figures 4A and 4B). Furthermore, separation of some if not all homologs and sister chromatids did occur, but segregation of these chromosomes and formation of the spindle midzone were severely compromised in *bir-1*(RNAi) fertilized oocytes (Figures 4D and 4E). Paternal meiosis to form sperm, which occurs during larval development

prior to the time we injected double-stranded (ds) *bir-1* RNA, was unaffected (data not shown). Furthermore, in *bir-1*(RNAi) fertilized oocytes, polar bodies were not formed and all chromosomes decondensed and became part of the maternal pronucleus (Figures 4G and 4H). In *bir-1*(RNAi) fertilized oocytes in which the maternal and paternal pronuclei were near each other, the paternal chromosomes were always condensed while the maternal chromosomes were often not condensed (data not shown). By contrast, in wild-type fertilized oocytes maternal and paternal chromosomes condense at the same time.

During mitosis, *bir-1*(RNAi) one-cell embryos displayed multiple defects in chromosome behavior and spindle organization. Chromosomes often failed to condense (six, presumably paternal, chromosomes always condensed, whereas maternal chromosomes often condensed late if at all relative to the paternal chromosomes) (Figures 4J, 4K, 4M, 4N, 4P, 4Q, 4V, and 4W); align at the metaphase plate (Figures 5M and 5N); and separate/segregate (Figures 4P, 4Q, 4S, and 4T). When chromosome separation and segregation occurred normally, the spindle midzone either was not formed or was so severely disorganized that it was not detectable by staining with anti-$\alpha$-tubulin antibodies or with antibodies against any of three proteins that normally localize to the spindle midzone, the kinesin-like-protein ZEN-4/MKLP1 (Powers et al., 1998; Raich et al., 1998) (Figures 4V and 4W), the Aurora-like-kinase AIR-2 (Schumacher et al., 1998), or the Polo-like kinase, PLK-1 (Chase et al., 2000) (data not shown).

Using phalloidin, we found that actin appeared to be present on the cytokinetic furrows during mitosis (data not shown), suggesting that at least one component of the cytokinetic furrow was intact.

### *air-2*(RNAi) and *bir-1*(RNAi) Fertilized Oocytes and Embryos Display Similar Defects

We histologically compared *cyk-1* (Swan et al., 1998), *zen-4* (Raich et al., 1998), and *air-2*(RNAi) (Schumacher et al., 1998; Woollard and Hodgkin, 1999) embryos with *bir-1*(RNAi) embryos, since these embryos all have defects in cytokinesis and polar body extrusion visible with Nomarski optics. Chromosome alignment, separation/segregation, and condensation were normal in *cyk-1* and *zen-4* fertilized oocytes and embryos during female meiotic divisions and the first embryonic mitosis (data not shown). *zen-4* but not *cyk-1* embryos were defective in the formation or organization of the middle part of the spindle midzone, as has been described (Powers et al., 1998; Raich et al., 1998). This defect was less severe than the complete absence of the spindle midzone that we observed in *bir-1*(RNAi) embryos (see above).

By contrast, we discovered that interference with the function of the Aurora-like kinase *air-2* leads to defects indistinguishable from those seen following interference with *bir-1* function (Figure 4; Table 1). Interestingly, the temporal and spatial localization of AIR-2 (Schumacher et al., 1998) appears to be identical to that of BIR-1. These observations suggest that BIR-1 and AIR-2 may act together during meiosis and mitosis.

### AIR-2 Is Not Present on Chromosomes in the Absence of BIR-1

We stained for BIR-1 in *air-2*(RNAi) fertilized oocytes and embryos and for AIR-2 in *bir-1*(RNAi) fertilized oocytes and embryos. While BIR-1 was localized to chromosomes in the absence of AIR-2 (Figure 5A), AIR-2 was not localized to chromosomes in the absence of BIR-1 (Figure 5B). This lack of AIR-2 localization to chromosomes was at least partially specific, because another kinase, the Polo-like kinase PLK-1, was still localized to chromosomes in the absence of BIR-1 (Figure 5C). We could not evaluate the presence of BIR-1 and AIR-2 on the spindle midzone, since in the absence of either AIR-2 or BIR-1 this structure was either not formed or so severely disorganized that it was not visible with any spindle midzone markers.

### Histone H3 Phosphorylation and HCP-1 Staining Require BIR-1 and AIR-2

Since chromosome condensation, alignment, and segregation require histone H3 phosphorylation (Wei et al., 1999) and intact kinetochores, we stained wild-type, *bir-1*(RNAi), and *air-2*(RNAi) fertilized oocytes for phosphorylated histone H3 and for HCP-1, a component of the kinetochore (Moore et al., 1999) (Figure 6). We found that both histone H3 phosphorylation and HCP-1 staining were reduced and often absent in *bir-1*(RNAi) and *air-2*(RNAi) fertilized oocytes. Staining for HCP-3, a histone H3-like protein that marks centromeric DNA (Buchwitz et al., 1999), was not affected in *bir-1*(RNAi) and *air-2*(RNAi) fertilized oocytes, suggesting that the reduction in phosphohistone H3 and HCP-1 staining was specific for these epitopes.

### The Cytokinesis Defect of *bir-1*(RNAi) Embryos Is Partially Rescued by Human Survivin

We tested whether human survivin (Ambrosini et al., 1997), the BIRP most similar to BIR-1, could functionally substitute for BIR-1. We coexpressed human survivin and the green fluorescent protein (GFP) (to mark transgenic embryos) in *bir-1*(RNAi) embryos. We counted the number of cells in arrested transgenic *bir-1*(RNAi) embryos, and found that *survivin*-transgenic embryos arrested with more cells on average than did embryos transgenic for GFP alone (Figures 7A and 7B) (p value = 0.0001). Two *survivin*-transgenic embryos had more than 20 cells. These embryos were severely disorganized and arrested before morphogenesis (data not shown). *Survivin*-transgenic *bir-1*(RNAi) embryos that had survivin eliminated using RNAi arrested with as many cells on average as GFP transgenic embryos (Figures 7A and 7C) (p value = 0.08). Thus, survivin reduced the cytokinesis defect seen in *bir-1*(RNAi) embryos, albeit slightly, confirming similar observations reported by Fraser et al. (1999).

Since many BIRPs can inhibit programmed cell death when overexpressed (reviewed by LaCasse et al., 1998), it is possible that BIRPs normally function to prevent cell death. We tested whether the cytokinesis defect seen in *bir-1*(RNAi) embryos could be prevented by blocking programmed cell death. We injected *ced-3*(*n717*) and *ced-4*(*n1162*) animals, in which little or no programmed cell death occurs (Ellis and Horvitz, 1986), with *bir-1* dsRNA and found that the cytokinesis defect of *bir-1*(RNAi) embryos was not affected by preventing programmed cell death (Figures 7D, 7E, and 7F) (p value for N2 vs *ced-3* = 0.10; p value for N2 vs *ced-4* = 0.08).

## Discussion

### BIR-1 May Localize AIR-2

We propose that BIR-1 acts together with AIR-2 to affect chromosome alignment, separation/segregation, condensation, spindle midzone formation, and cytokinesis.

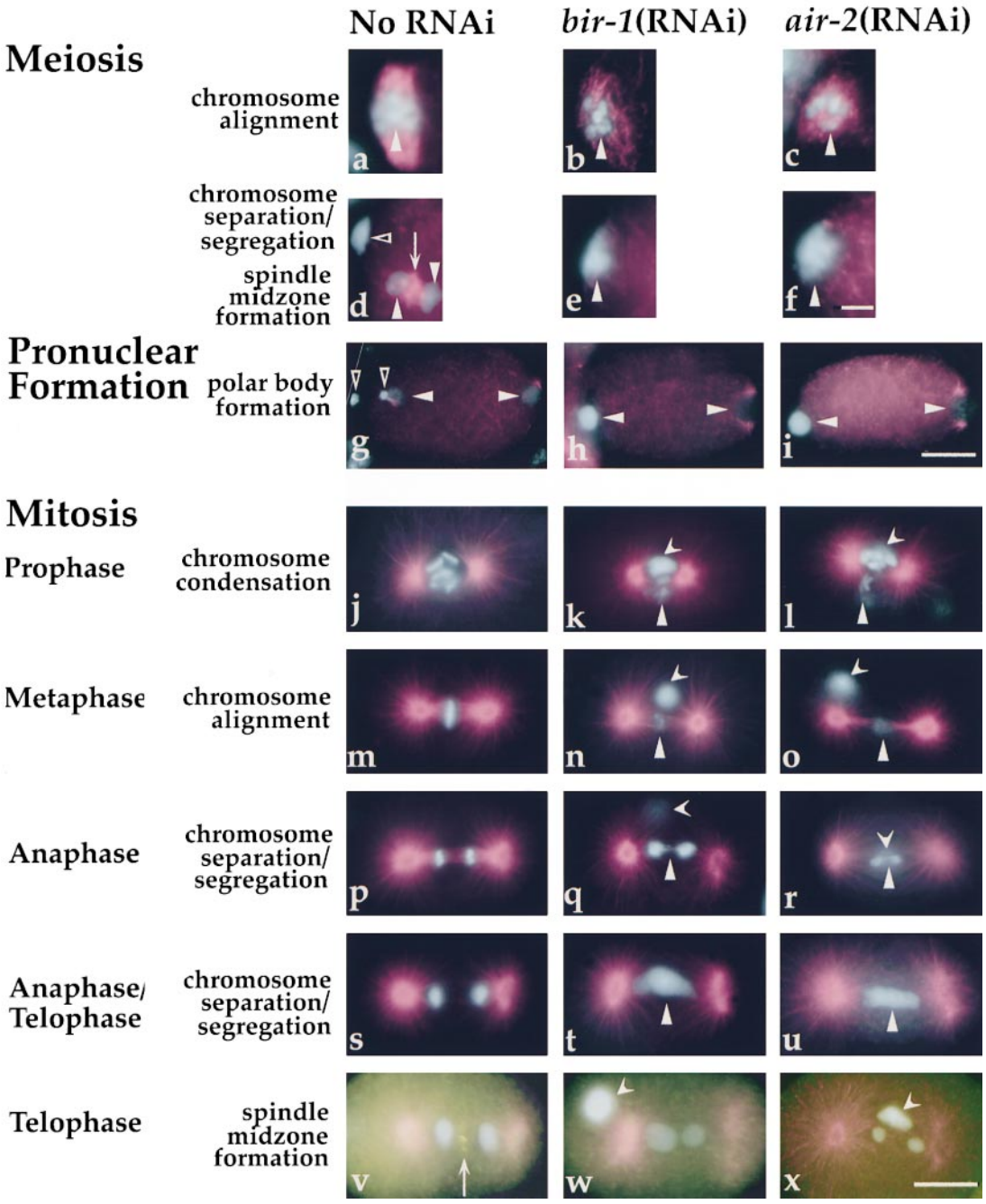


Figure 4. *bir-1*(RNAi) and *air-2*(RNAi) Fertilized Oocytes and Embryos Have Phenotypically Indistinguishable Defects in Chromosome Behavior and Spindle Midzone Formation

Fertilized oocytes and embryos from uninjected mothers and from mothers injected with *bir-1* or *air-2* dsRNA were stained with anti-α-tubulin antibody and DAPI (blue) to visualize tubulin (red) and DNA (blue), respectively, and the images merged. Anterior, left; posterior, right. (A, D, G, J, M, P, S, and V) Wild-type (no RNAi). (B, E, H, K, N, Q, T, and W) *bir-1*(RNAi). (C, F, I, L, O, R, U, and X) *air-2*(RNAi).

(A) Anterior pole of normal fertilized oocyte in metaphase of meiosis I. Paired homologs (solid arrowhead) are aligned in the meiotic spindle.

(B and C) Anterior pole of *bir-1*(RNAi) and *air-2*(RNAi) fertilized oocytes in metaphase of meiosis I. Chromosomes (solid arrowhead) are dispersed throughout the meiotic spindle.

(D) Anterior pole of normal fertilized oocyte in anaphase of meiosis II. Sister chromatids (solid arrowheads) have separated and segregated, and the spindle midzone (arrow) has formed between them. Polar body DNA formed during meiosis I has been extruded (open arrowhead).

(E and F) Anterior poles of *bir-1*(RNAi) and *air-2(*RNAi) fertilized oocytes in anaphase I, metaphase II, or anaphase II (staging was not possible, since the normal morphological markers were not present). Maternal chromosomes (arrowhead) are aggregated at the anterior pole and have not been extruded. No spindle midzone is visible. Paternal chromosomes, located in the posterior of the fertilized oocyte, were condensed and are not shown in (A)–(F).

(G) Normal postmeiotic fertilized oocyte showing two polar bodies (open arrowheads) and the maternal (anterior) and paternal (posterior) pronuclei (solid arrowheads), both with decondensed DNA.

Table 1. *bir-1*(RNAi) and *air-2*(RNAi) Fertilized Oocytes and Embryos Display Similar Defects[a]

| | No RNAi | *bir-1*(RNAi) | *air-2*(RNAi) |
|---|---|---|---|
| Meiosis | | | |
| Chromosome alignment[b] | 0/13 (0%) | 7/8 (88%) | 4/4 (100%) |
| Chromosome separation/segregation and/or spindle midzone formation[c] | 0/52 (0%) | 28/30 (93%) | 16/16 (100%) |
| Pronuclear formation | | | |
| Maternal pronucleus polyploidy[d] | 0/7 (0%) | 44/44 (100%) | 22/22 (100%) |
| Mitosis | | | |
| Chromosome condensation[e] | 0/23 (0%) | 49/67 (73%) | 12/20 (60%) |
| Chromosome alignment[f] | 0/2 (0%) | 4/4 (100%) | 2/2 (100%) |
| Chromosome separation/segregation[g] | 0/6 (0%) | 19/38 (50%) | 10/11 (91%) |
| Spindle midzone formation[h] | 0/6 (0%) | 30/30 (100%) | 7/7 (100%) |

[a] Randomly selected *bir-1*(RNAi) and *air-2*(RNAi) embryos were stained with anti-α-tubulin antibodies and DAPI to visualize α-tubulin and DNA, respectively. Embryos were also stained with anti-BIR-1, anti-AIR-2, anti-ZEN-4, or anti-PLK-1 antibodies to help visualize the central spindle or chromosomes. The number of animals abnormal for a process over the number of animals scored for such a defect are shown. Staging was determined as in Experimental Procedures.
[b] Only fertilized oocytes with separating homologs were scored.
[c] Only fertilized oocytes in anaphase I, metaphase II, or anaphase II were scored.
[d] All fertilized oocytes at the pronuclear stage were scored.
[e] All one-cell embryos were scored.
[f] Only one-cell embryos in metaphase were scored.
[g] Only one-cell embryos in anaphase or telophase were scored.
[h] Only one-cell embryos in anaphase or telophase that had normal or mild (chromatin bridges) chromosome separation/segregation defects were scored.

First, *bir-1*(RNAi) and *air-2*(RNAi) fertilized oocytes and embryos displayed indistinguishable chromosomal and spindle defects. Second, BIR-1 and AIR-2 were localized spatially and temporally to the same structures. Third, AIR-2 was not present on chromosomes in the absence of BIR-1; this defect in AIR-2 localization preceded and could potentially have caused the chromosomal and spindle defects we observed in the absence of BIR-1 or AIR-2.

AIR-2 is a member of the Aurora-family of serine-threonine kinases, which are involved in a variety of mitotic processes. Aurora-like kinases at centrosomes control spindle pole body duplication and bipolar spindle formation, those on chromosomes affect chromosome segregation, and those on the spindle midzone affect cytokinesis (reviewed by Giet and Prigent, 1999). The functions of these kinases presumably depend on their being properly positioned near their targets at the right time during the cell cycle. We propose that BIR-1 helps localize AIR-2 to chromosomes and perhaps to the spindle midzone where AIR-2 phosphorylates local targets (which might include BIR-1 itself) required for chromosomal and spindle processes. BIR-1 may help recruit, tether, or protect AIR-2 from degradation at these sites. The structure of BIRPs is consistent with our hypothesis that these proteins may serve as adaptors in complexes. The BIRs of BIRPs have been shown to interact with many proteins and are usually connected to variable C termini (reviewed by LaCasse et al., 1998; Uren et al., 1998)). The C termini of BIRPs contain other interaction motifs, such as RING fingers, CARD domains, or coiled-coil regions that may play roles in protein-protein, protein-DNA, or protein-RNA interactions (Saurin et al., 1996; Li et al., 1998; Uren et al., 1998).

### BIR-1 Is Required for Chromosome Alignment, Separation/Segregation, and Condensation

How can the absence of BIR-1 or AIR-2 result in multiple defects in chromosome behavior? One possibility is that BIR-1 and AIR-2 affect properties of the chromatin onto which multiprotein complexes that mediate chromosome condensation, alignment, and segregation assemble. Phosphorylation of histone H3 has been correlated with and may mediate chromosome condensation (Wei et al., 1999), perhaps by helping to dock proteins that condense chromosomes, such as the condensin complex (Hirano, 1999). Similarly, kinetochores, which are multiprotein complexes that connect chromosomes to the meiotic and mitotic spindles, assemble on chromatin and are necessary for proper chromosome alignment and segregation. BIR-1 and AIR-2 promote phosphorylation of histone H3 and localization of the kinetochore

(H and I) *bir-1*(RNAi) and *air-2*(RNAi) postmeiotic fertilized oocytes. No polar bodies have formed, and a polyploid maternal (anterior) and normal paternal pronucleus (posterior) are visible (solid arrowheads).
(J–X) One-cell embryos at different stages of mitosis. (J–L) Prophase. (J) Chromosomes are normally condensed. (K and L) Some chromosomes (indented arrowhead) are decondensed (also seen in N, O, Q, R, W, and X), whereas others appear to be condensed normally (arrowhead). (M–O) Metaphase. (M) Chromosomes normally aligned at the metaphase plate. (N and O) Condensed chromosomes are not aligned at the metaphase plate (arrowheads). (P–R) Anaphase. (P) Normal chromosome separation/segregation. (Q and R) Chromatin bridges (arrowheads) connect segregating chromosomes. (S–U) Late anaphase or telophase. (S) Normal chromosome separation/segregation. (T and U) Severe defects in chromosome separation/segregation; chromosomes (arrowheads) remain near the midzone. (V–X) Telophase. These one-cell embryos were stained as above as well as with anti-ZEN-4/MKLP1 to visualize the spindle midzone (green). (V) Normal spindle midzone (arrow) (W and X) No spindle midzone is visible. Scale bars: (A)–(F), 5 μm, (G)–(X), 20 μm.

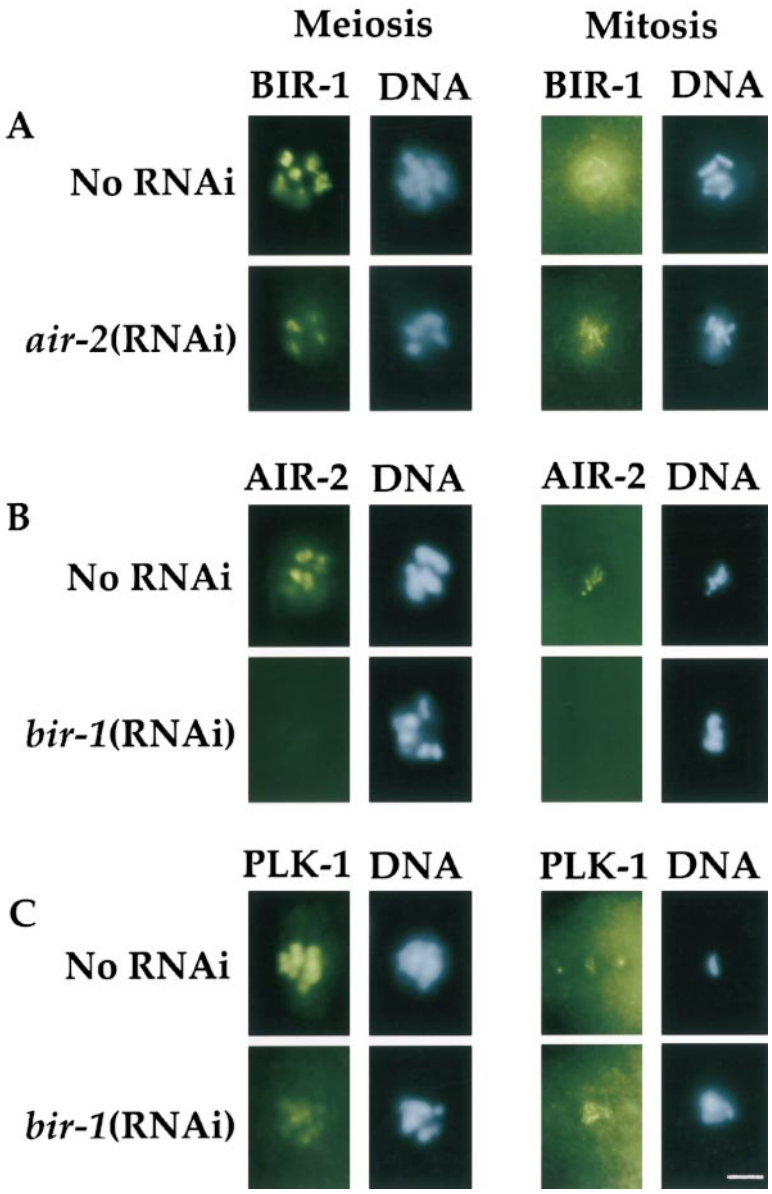


Figure 5. AIR-2 Is Not Present on Chromosomes in the Absence of BIR-1

Fertilized oocytes and embryos from uninjected (No RNAi), *bir-1* (*bir-1*(RNAi)) and *air-2* (*air-2*(RNAi)) dsRNA injected mothers were stained with anti-BIR-1 (A), anti-AIR-2 (B), or anti-PLK-1 (C) antibodies to visualize AIR-2, BIR-1, and PLK-1, respectively (green), and counterstained with DAPI to visualize DNA (blue). Meiosis scale bar, 5 μm. Mitosis scale bar, 10 μm.

component HCP-1, suggesting molecular mechanisms for their effects on chromosome condensation and alignment/segregation, respectively. An alternative and not mutually exclusive possibility is that BIR-1 and AIR-2 affect the phosphorylation of multiple distinct proteins that are involved in chromosome behavior. In support of this possibility, *S. cerevisiae* BIR-1 interacts in a yeast two-hybrid assay with Ndc10p (Yoon and Carbon, 1999), a core kinetochore component that is also an in vitro substrate for Ipl1p kinase (Biggins et al., 1999), the Aurora-like kinase of *S. cerevisiae*. Interestingly, chromosome condensation during meiotic prophase was unaffected in the absence of BIR-1 or AIR-2, suggesting that phosphorylation of histone H3 may not be necessary for all types of chromosome condensation. The apparent defect in separation of chromosomes may be a consequence of either a role of BIR-1 in chromosome separation or a lack of spindle forces acting to separate chromosomes because of poor microtubule/kinetochore interactions. Further work will be needed to distinguish these possibilities.

**BIR-1 Is Required for Completion of Cytokinesis**

Our histological analysis suggests that the cytokinetic furrow may not resolve because the presence of unsegregated chromosomes in the absence of BIR-1 causes a physical barrier to furrow resolution, or alternatively, because an organized spindle midzone may be required for the completion of cytokinesis. However, the presence of chromatin at the cytokinetic furrow in *C. elegans* embryos does not necessarily prevent cytokinesis (O'Connell et al., 1998), whereas the absence of an organized spindle midzone does lead to a late defect in furrow resolution (O'Connell et al., 1998; Powers et al., 1998; Raich et al., 1998). The spindle midzone may be important for the delivery of molecules that stabilize the advancing furrow and/or are required for the completion

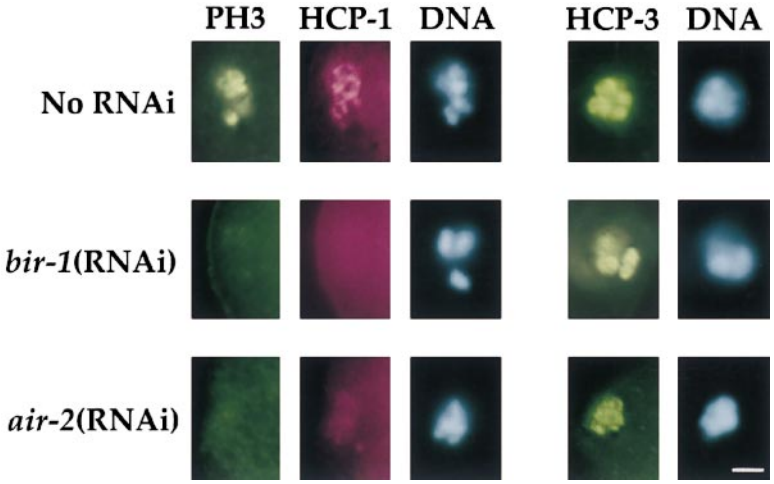


Figure 6. Histone H3 Phosphorylation and HCP-1 Staining Are Reduced in the Absence of BIR-1 and AIR-2

Fertilized oocytes from uninjected (No RNAi), *bir-1* (*bir-1*(RNAi)) and *air-2* (*air-2*(RNAi)) dsRNA-injected mothers were stained with anti-phosphohistone H3 (PH3) and anti-HCP-1 antibodies or with anti-HCP-3 antibody to visualize phosphorylated histone H3 (green), HCP-1 (red), and HCP-3 (green), respectively, and counterstained with DAPI to visualize DNA (blue). Fertilized oocytes in metaphase I of meiosis are shown. Scale bar, 5 μm.

of cytokinesis. For example, in plants Golgi-derived vesicles are transported along microtubules to the equatorial region, where they fuse to form a cell plate that ultimately connects with the plasma membrane to form the two daughter cells (Staehelin and Hepler, 1996). Kinesin-like proteins present on the spindle midzone not only may help transport these vesicles but also may help to bundle the microtubules that form the spindle itself (Powers et al., 1998; Raich et al., 1998). As kinesin-like proteins have been shown to be regulated by phosphorylation (Liao et al., 1994) and are targets of Aurora-like kinases (Giet et al., 1999), it is possible that BIR-1 acts with AIR-2 to affect the activity of kinesin-like proteins to mediate bundling of the midbody microtubules and transport of vesicles/proteins along these microtubules. Alternatively, BIR-1 and AIR-2 by affecting chromatin properties may affect the binding of spindle midzone microtubules to chromosomes; defects in this process could result in poor spindle midzone formation or organization.

Our findings do not support the hypothesis (Cao and Wang, 1996) that the spindle midzone is the site from which signals that control the site of furrow formation and ingression of the furrow arise since these processes

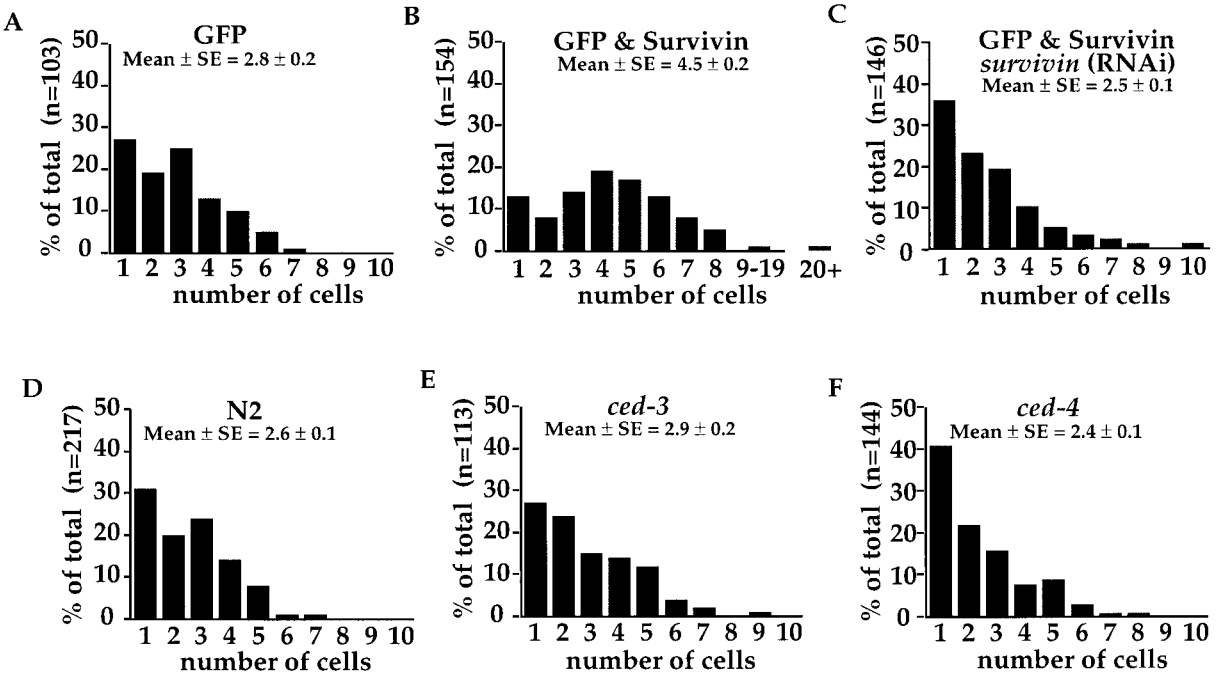


Figure 7. Human Survivin, but Not Blocking Programmed Cell Death, Partially Rescues the Cytokinesis Defect of *bir-1*(RNAi) Embryos

(A, B, and C) Animals that had been injected with *bir-1* dsRNA (A and B) or *bir-1* dsRNA and *survivin* dsRNA (C) and were producing affected embryos were reinjected with green fluorescent protein (GFP) driven by the *bir-1* promoter (A) or with both GFP and *survivin* driven by the *bir-1* promoter (B and C). The number of cells in arrested GFP-fluorescing embryos was scored. (D, E, and F) *bir-1* dsRNA was injected into wild-type (N2), *ced-3*, and *ced-4* animals. The number of cells in arrested embryos was scored. Mean number of cells ± standard error (SE) shown.

occur even in the absence of a spindle midzone in early *C. elegans* embryos.

### BIR-1 May Couple Chromosome Separation to Cytokinesis

The presence of BIR-1 on both chromosomes and the spindle midzone is similar to the localization of some mammalian "chromosomal passenger proteins" (Choo, 1997). Embryos that lack one of these passenger proteins, the inner centromere protein (INCEP), have severe defects in chromosome alignment, chromosome segregation, and midbody formation (Cutts et al., 1999). These defects are similar to those we observed in *bir-1*(RNAi) and *air-2*(RNAi) embryos. Interestingly, dominant-negative INCEP constructs that interfere with the transfer of the protein to the spindle midzone result in cytokinesis defects (Mackay et al., 1998) similar to those we observed in *bir-1*(RNAi) and *air-2*(RNAi) embryos. These findings suggest that proteins such as INCEPs, BIR-1, and AIR-2, which are present on chromosomes and later appear on the spindle midzone, may serve to couple chromosome separation with cytokinesis.

### *bir-1* and *air-2* Define a Distinct Class of Cell Cycle Genes

The localizations of BIR-1 and AIR-2 as well as the defects associated with loss of BIR-1 and AIR-2 are similar to each other but not similar to the localization or loss-of-function phenotypes of many other proteins that function in cell cycle related processes, including proteins that act in cytokinesis (Raich et al., 1998; Swan et al., 1998), in meiotic and mitotic spindle function (Clark-Maguire and Mains, 1994a, 1994b; Lorson et al., 2000), in kinetochore function (Moore et al., 1999), in mitotic spindle checkpoint control (Kitagawa and Rose, 1999), in the regulation of cyclin-dependent kinase inhibitor degradation (Feng et al., 1999), and in cell cycle progression (Boxem et al., 1999). Thus, *bir-1* and *air-2* define a distinct class of *C. elegans* cell cycle genes.

### Some BIR-1 Functions May Be Evolutionarily Conserved with Other BIRPs

The expression of survivin, but not the inhibition of cell death, partially rescued the cytokinesis defect of *bir-1*(RNAi) embryos, suggesting that BIR-1 and survivin share an evolutionarily conserved function in affecting chromosomal or spindle processes that is separate from any function in affecting cell death. BIR-1 and survivin are localized to analogous structures in their respective species: the cytokinetic remnant (our data) and the midbody (Li et al., 1998). For these reasons, we propose that survivin and BIR-1 affect similar processes that include but may not be limited to cytokinesis. Indeed, interference with survivin expression can lead to the formation of polyploid cells (Li et al., 1999) similar to those we observed in *bir-1*(RNAi) embryos. Furthermore, both *bir-1*(RNAi) embryos and yeast *bir* mutants (Rajagopalan and Balasubramanian, 1999; Uren et al., 1999; Yoon and Carbon, 1999) have defects in chromosome condensation and segregation, suggesting that a loss of BIRPs may contribute to aneuploidy.

### Survivin and Other BIR-1-Like Mammalian BIRPs May Contribute to Tumorigenesis by Affecting Aurora-Like Kinase Function

Overexpression of *C. elegans air-2* or of *S. pombe* Pbh1p/Bir1p results in defects in chromosome segregation (Rajagopalan and Balasubramanian, 1999; Woollard and Hodgkin, 1999), suggesting that levels of Aurora-like kinases and BIRPs may need to be carefully regulated to prevent aneuploidy. Aneuploidy may contribute to tumorigenesis (Zou et al., 1999). Interestingly, overexpression of human *aurora2* transforms Rat-1 cells in vitro, and these cells can form tumors in nude mice. Even though no functional connections have been made between Aurora-like kinases and BIRPs in other organisms, given our findings that human survivin can partially substitute for BIR-1 in worms and that BIR-1 localizes AIR-2 we suggest that such connections may exist. Indeed, in the literature we noted that *S. cerevisiae* Bir1p interacts with Ndc10p (Yoon and Carbon, 1999), a substrate for Ipl1p kinase (Biggins et al., 1999), the Aurora-like kinase homolog in *S. cerevisiae*. Also, *survivin*, like *aurora2* (Bischoff et al., 1998), is upregulated in some tumors (Ambrosini et al., 1997), and human aurora2 and aurora1 are found on centrosomal and midbody microtubules, respectively (Bischoff et al., 1998), precisely where human survivin is located (Li et al., 1998). We propose that human survivin may affect chromosomal and spindle processes and perhaps tumorigenesis via an evolutionarily conserved interaction with Aurora-like kinases. Learning how BIR-1 mediates its functions with AIR-2 in *C. elegans* not only may help elucidate the role of other BIRPs in chromosomal and spindle processes but also may help reveal how BIRPs such as survivin contribute to tumorigenesis.

## Experimental Procedures

### Strains

*C. elegans* strains were cultured using standard techniques (Brenner, 1974). All experiments were done using the wild-type Bristol strain N2 (Brenner, 1974) unless otherwise noted. The following mutant strains were used: *ced-3*(*n717*) (Ellis and Horvitz, 1986), *ced-4*(*n1162*) (Ellis and Horvitz, 1986), *cyk-1*(*t1611*) (Swan et al., 1998), and *zen-4*(*or153*ts) (B. Bowerman, personal communication).

### Isolation of *bir-1* cDNA

Fragments from the *bir-1* gene as predicted by Genefinder (*C. elegans* Sequencing Consortium, 1998) were amplified by PCR and used to probe $1 \times 10^6$ clones of a mixed-stage cDNA library cloned into UniZAP XR phage vector (Stratagene). The DNA sequences of clones were determined using an automated ABI 373A DNA sequencer (ABI Biosystems).

### BIR-1 Antibodies

We raised two separate polyclonal antibodies in New Zealand White Rabbits against a full-length BIR-1-GST fusion protein. Both antibodies gave the same results. Six month bleeds were used. Postimmune sera were affinity purified against HIS-BIR-1 fusion protein immobilized on nitrocellulose, eluted with 1 ml of 100 mM glycine HCl (pH 2.5), and used for staining.

### Western Analysis

For antibody-specificity Western analysis, animals that were or were not soaked in *bir-1* dsRNA (see below) were lysed to release embryos, which were used for blotting. For developmental Western analysis, embryos were obtained by lysing wild-type gravid hermaphrodites and synchronized by hatching in S-medium without food to obtain L1 growth-arrested larvae (Wood et al., 1988 [Wood

and the community of *C. elegans* researchers]). Synchronized L1 growth-arrested larvae were transferred to plates with bacteria and allowed to resume growth. Animals were staged by examining gonad development, harvested, and analyzed using SDS-PAGE. SDS-PAGE gels were stained with Coomassie blue to standardize loading, or samples were transferred to nitrocellulose and protein levels gauged by Ponceau staining followed by immunoblotting by standard procedures. BIR-1 protein was visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Amersham). Affinity-purified anti-BIR-1 antibody was used at 1:20 or 1:50 dilutions.

**Immunohistochemistry**

For most antibody staining, animals were processed and stained as follows. Gravid hermaphrodites were dissected in M9 (Wood et al., 1988) on poly-L-lysine coated slides, overlaid with a coverslip, and frozen. Coverslips were removed and slides were immersed in −20°C methanol for 10 min. Tissue was blocked with 10% normal serum (NS) in AbA (Finney and Ruvkun, 1990) (NS-AbA) for 1–2 hr at room temperature and then incubated in NS-AbA with primary antibody overnight at 4°C. Slides were washed four times for 15 min with 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween-20 (TBST) at room temperature, incubated in NS-AbA with secondary antibody for 2 hr at room temperature, washed with TBST three times for 15 min at room temperature, and incubated with 1 μg/ml 4, 6-diamino-2-phenylindole (DAPI) in AbB (Finney and Ruvkun, 1990) to visualize DNA. Samples were air dried, mounted in Vectashield (Vector Labs), and a coverslip overlaid. For staining with anti-HCP-1, anti-HCP-3, and anti-phosphorylated H3 antibodies, animals were processed and stained as described by Moore et al. (1999). Alexa568 phalloidin (Molecular Probes) was used to visualize actin by staining according to the manufacturer's instructions.

Primary antibodies were used at the following dilutions: affinity-purified anti-BIR-1 rabbit polyclonal (1:25); affinity-purified rabbit anti-AIR-2 rabbit polyclonal (1:50) (gift from J. Schumacher and P. Donovan); affinity-purified anti-ZEN-4/MKLP1 rabbit polyclonal (1:25) (gift from W. Raich and J. Hardin); anti-PLK-1 rabbit polyclonal (1:200) (gift from D. Chase and A. Golden); anti-HCP-1 mouse monoclonal (1:10) (gift from L. Moore and M. Roth); anti-HCP-3 rabbit polyclonal (1:200) (gift from B. Buchwitz and S. Henikoff); anti-phosphohistone H3 (1:50) (Upstate Biotechnologies); anti-α-tubulin DMIA mouse monoclonal (Sigma) (1:100); anti-nucleoporin Mab414 mouse monoclonal (Babco) (1:10); and anti-α-tubulin rat monoclonal (Harlan Sera-Lab) (1:50). Anti-α-tubulin and anti-HCP-1 antibodies were visualized using Texas Red while all other primary antibodies were visualized with FITC-conjugated secondary antibodies, respectively. Secondary FITC- or Texas Red-conjugated antibodies (Cappel Research and Jackson Immunoresearch Laboratories) were used at 1:100 dilutions.

Criteria for staging of fertilized oocytes and embryos were as follows. Female meiosis: metaphase I, presence of condensed bivalents; anaphase I, homologs had all separated, or the anaphase spindle was present or both were present; metaphase II, condensed paired sister chromatids were present with tubulin around them; anaphase II, separating sister chromatids were present or the anaphase spindle was present or both were present. At all stages during female meiotic divisions, sperm DNA was condensed. Pronuclear formation: paternal DNA (which was identified by its associated centrosome[s]) was decondensing. One-cell embryos: prophase, centrosomes were round and kinetochore microtubules were absent; metaphase, centrosomes were round and kinetochore microtubules were present; anaphase, centrosomes were round, kinetochore microtubules were present, and separating DNA was present; telophase, the posterior centrosome was flat.

**RNA-Mediated Gene Interference**

Full-length *bir-1* and *air-2* cDNAs in pBluescript vectors were amplified using PCR to include flanking T3 and T7 promoters, and the PCR mixture was used to make sense and antisense RNA with T3 and T7 RNA polymerase (Stratagene). Equal amounts of sense and antisense RNA were mixed to a final concentration of 1 mg/ml, annealed, and injected into the gonads of young adult hermaphrodites (Mello et al., 1991) for all histology and phenotypic analysis experiments. Embryos produced 15–18 hr after injection were examined for defects. For Western analysis, L3-L4 staged animals were soaked in *bir-1* dsRNA at a concentration of 1 mg/ml in 0.2 M sucrose and 0.1 M PBS with lipofectin reagent (0.067 μg/ml, Gibco BRL) for 36 hr at 20°C. Survivin rescue experiments were performed as follows: animals were injected with *bir-1* dsRNA or *bir-1* and *survivin* dsRNA and were checked ~18 hr later to confirm that they were producing affected embryos. These animals were then reinjected with buffer, with GFP driven by the *bir-1* promoter, or with both GFP and *survivin* being driven by the *bir-1* promoter, and embryos scored 15–24 hr after they were laid for GFP fluorescence and cellularization. For the cell-death experiments, N2, *ced-3* (*n717*), or *ced-4*(*n1162*) animals were injected with *bir-1* dsRNA, and embryos produced by mothers 15–18 hr after injection were scored for cellularization about 18 hr after they were laid. Statistical analyses were done using the Mann-Whitney test of significance in Statview.

**Microscopy and Image Acquisition**

We used a Zeiss Axioplan II microscope for Nomarski differential interference contrast optics and immunofluorescence microscopy. Images were acquired using Sensys cooled CCD camera (Photometrics), followed by image analysis and computational deconvolution with Openlabs software (Improvision). Images were colored and merged using Adobe Photoshop.

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