Anillin promotes astral microtubule-directed cortical myosin polarization

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Abbreviations
MAP   Microtubule associated protein
RhoGAP Rho family GTPase activating protein
RhoGEF Rho family guanine nucleotide exchange factor
PH    Pleckstrin Homology
AH    Anillin homology region
IAH   Central and anillin homology region
GST   Glutathione-S-transferase
His   Histidine
AP    Anterior-posterior
MTOC  Microtubule-organizing center
FRAP  Fluorescence recovery after photobleaching

Abstract
Assembly of a cytokinetic contractile ring is a form of cell polarization in which the equatorial cell cortex becomes differentiated from the polar regions. Microtubules direct cytokinetic polarization via the central spindle and astral microtubules. The mechanism of central spindle-directed furrow formation is reasonably well understood, but the aster-directed pathway is not. In aster-directed furrowing,
cytoskeletal factors accumulate to high levels at sites distal to the asters and at reduced levels at cortical sites near the asters. Here, we demonstrate that, in *C. elegans* embryos, the cytoskeletal organizing protein anillin (ANI-1) promotes the formation of an aster-directed furrow. Microtubule-directed non-muscle myosin II polarization is aberrant in embryos depleted of ANI-1. In contrast, microtubule-directed polarized ANI-1 localization is largely unaffected by myosin II depletion. Consistent with a role in the induction of cortical asymmetry, ANI-1 also contributes to the polarization of arrested oocytes. Anillin has an evolutionarily conserved capacity to associate with microtubules, providing a possible inhibitory mechanism to promote polarization of the cell cortex.

**Introduction**

Cytokinesis, the creation of two daughter cells from a single parental cell, is driven by the constriction of an actomyosin-based contractile ring that is juxtaposed to the plasma membrane. The ring defines a plane that lies perpendicular to the anaphase spindle. Assembly and constriction of the contractile ring at the correct time and place are essential for the generation of viable daughter cells. Contractile ring formation requires local activation of the small GTPase RhoA that promotes actin polymerization and activation of non-muscle myosin (reviewed in Piekny et al., 2005; Bement et al., 2006). The motor activity of non-muscle myosin slides actin filaments, thereby generating the force that drives membrane ingestion. Once the furrow ingresses extensively, a
structure termed the midbody is formed that stabilizes the ingressed furrow until the membrane undergoes abscission.

The position of the contractile ring is determined by the mitotic spindle during anaphase. Two features of the spindle control furrow positioning: the central spindle and astral microtubules (reviewed in D'Avino et al., 2005; von Dassow et al., 2009). The central spindle is composed of antiparallel microtubule bundles that lie between the segregating chromosomes during anaphase, while astral microtubules emanate radially from centrosomes at the two spindle poles (reviewed in Glotzer, 2009). Although the central spindle is required for completion of cytokinesis, cells with compromised central spindles still form ingressing cleavage furrows at a site dictated by the asters (Dechant and Glotzer, 2003). The two mechanisms for furrow induction have been extensively studied using *C. elegans* embryos (Dechant and Glotzer, 2003; Bringmann and Hyman, 2005; Werner et al., 2007; Lewellyn et al., 2010). However, there is compelling evidence that both pathways contribute to cytokinesis in a variety of metazoan cells (Alsop and Zhang, 2003; Murthy and Wadsworth, 2008; Piekny and Glotzer, 2008; von Dassow et al., 2009). Although these two mechanisms for furrow induction can function independently, they cooperate to result in more rapid induction of furrows (Dechant and Glotzer, 2003). The astral microtubule-directed pathway may play a particularly important role in large cells in which the central spindle is distant from the cell cortex.
The central spindle and astral arrays direct cleavage furrow formation via distinct molecular mechanisms. Central spindle microtubules are bundled by centralspindlin (a complex consisting of the GTPase-activating protein (RhoGAP) CYK-4/MgcRacGAP and the kinesin-6 motor protein ZEN-4/MKLP1) as well as other microtubule associated proteins (Glotzer, 2009). During anaphase, a Rho guanine nucleotide exchange factor (RhoGEF), Ect2, localizes to the antiparallel microtubules in the spindle midzone by binding to Plk1-phosphorylated HsCYK4 (Burkard et al., 2009; Wolfe et al., 2009). This interaction appears essential for activation of the exchange function of Ect2, as RhoA activation is abrogated in cells in which the interaction is prevented (Burkard et al., 2007; Petronczki et al., 2007).

When central spindle assembly is impaired and asters direct furrow formation, RhoA is globally activated in an Ect2-dependent manner leading to the global cortical recruitment of RhoA effectors (Werner et al., 2007). The accumulation of these effectors is modulated by an inhibitory cue from astral microtubules, a process known as astral relaxation. Specifically, in C. elegans embryos, a posteriorly positioned spindle locally inhibits myosin accumulation in the posterior, with the exception of a small amount of myosin that accumulates directly over the central spindle, leading to furrow formation in both the anterior and posterior (Werner et al., 2007). Therefore, cleavage furrow formation is mediated by a combination of two mechanistically distinct pathways. However,
the molecular mechanism by which cortical myosin recruitment is spatially regulated by astral microtubules is not yet known.

There are notable similarities between the aster-directed furrow and the pseudocleavage furrow that accompanies embryo polarization. *C. elegans* zygotes are not initially polarized. Sperm entry and migration of the sperm pronucleus and its associated centrosome to the nearest pole establishes the posterior (St Johnston and Ahringer, 2010). The sperm-derived centrosome is essential for polarity determination, and causes a local cessation of a cortical contractility (Cowan and Hyman, 2004). This symmetry breaking event triggers a contractile wave that results in the anterior enrichment of the initially ubiquitous anterior PAR complex and the posterior cortical accumulation of the posterior PAR complex (Munro et al., 2004). During this initial polarization stages, a pseudocleavage furrow ingresses at the anterior-posterior (AP) boundary. The genetic requirements of pseudocleavage and aster-directed furrowing overlap extensively (Werner and Glotzer, 2008; Schenk et al., 2010), suggesting similar underlying molecular mechanisms.

We sought to determine the molecular mechanism by which microtubules locally modulate cortical myosin accumulation. Previous studies have revealed that, whereas, small foci of cortical myosin accumulate in the vicinity of the astral arrays, large myosin foci only form at sites of low microtubule density (Werner et
al., 2007). Because anillin colocalizes with and mediates the formation of large myosin foci prior to pronuclear migration and cytokinesis in C. elegans embryos (Maddox et al., 2005; Werner and Glotzer, 2008), we investigated whether anillin contributes to astral relaxation.

Anillin is a multifunctional protein that becomes highly concentrated in the contractile ring (see (Piekny and Maddox, 2010) for review). Anillin organizes the cortical cytoskeleton via interactions with F-actin, myosin, RhoA, septins, and formin, and therefore acts as a cytoskeletal scaffold protein (Field and Alberts, 1995; Straight et al., 2005; Piekny and Glotzer, 2008; Watanabe et al., 2010). The C. elegans genome encodes three isoforms of anillin, ANI-1, ANI-2, and ANI-3 (Maddox et al., 2005). Whereas ANI-3 has no known function and ANI-2 contributes to the organization of the gonad, ANI-1 contributes to the organization of the cortical cytoskeleton during polarization and cytokinesis (Maddox et al., 2005). ANI-2 is present in embryos, but it only subtly modulates cortical contractility due to its inhibition by PAR-4/LKB1 (Chartier et al., 2011).

Although ANI-1-depleted C. elegans embryos complete cytokinesis, the cytokinetic furrows ingress symmetrically, whereas the furrows in control embryos are asymmetric (Maddox et al., 2007). ANI-1 is also essential for furrowing in embryos with compromised central spindles (Werner and Glotzer, 2008). Collectively, these data suggest that anillin regulates aster-directed furrow formation, however the underlying mechanism remains to be determined.
Results

**Anillin mediates aster-mediated furrowing**

Previous analysis indicated that ANI-1 primarily contributes to the central spindle independent pathway for furrow ingress in *C. elegans* embryos (Werner and Glotzer, 2008). To confirm and extend these results, we examined the contribution of anillin to the ingress of anterior and posterior furrows in *zyg-9(b244ts)* embryos. ZYG-9 is a crucial microtubule-associated protein that is responsible for rapid microtubule growth (Matthews et al., 1998; Srayko et al., 2005). Inactivation of ZYG-9 permits spatial separation of the central spindle-dependent and aster-dependent furrows in the posterior and the anterior of the *C. elegans* zygote, respectively (Fig. 1A) (Werner et al., 2007). The midzone of the posterior spindle locally induces formation of the central spindle directed furrow and the spindle asters induce an anterior biased accumulation of myosin, triggering ingress of a furrow in the anterior of the embryo. To facilitate measurement of the extent of membrane ingress, we labeled the plasma membrane with GFP::PH (from PLCδ1) (Audhya et al., 2005). Upon fertilization of *zyg-9(b244ts)* embryos at the non-permissive temperature (25 °C), migration of the female pronucleus toward the male pronucleus fails and the female and male pronuclei remain in the anterior and posterior poles, respectively (Fig. 1B, Fig1Video1). After nuclear envelope breakdown, the posterior-localized centrosomes direct spindle assembly around the male pronucleus. Upon anaphase onset, two independent furrows form in the anterior and posterior (Fig.
We measured the extent of membrane ingression during anaphase and found that anterior furrows ingress to ~40% of the width of the embryo in zyg-9(b244ts) embryos (N=10). In sharp contrast, in ani-1(RNAi);zyg-9(b244ts) embryos, anterior furrows only ingress to an average of 10% of the width of the embryos (Fig. 1B & C, Fig1Video1, N=12). Twenty-five percent of embryos lack anterior furrows altogether. Posterior furrows ingress to at least 35% of egg length, irrespective of ANI-1 depletion. In addition, we observed that pseudocleavage was abolished in both ani-1(RNAi) and ani-1(RNAi);zyg-9(b244ts) embryos, indicating effective depletion of ANI-1 (Fig 1B). These results suggest that anillin primarily contributes to induction of the anterior furrow.

**Microtubules inhibit myosin recruitment in an anillin-dependent manner**

To investigate how anillin regulates anterior furrow formation, we examined cortical myosin dynamics during the first cell division. GFP-tagged non-muscle myosin II (NMY-2::GFP) and histone H2B (GFP::HIS) were co-expressed and visualized sequentially at different focal planes to correlate cortical events with cell cycle progression. As reported previously, prior to metaphase, small NMY-2::GFP puncta are enriched in the anterior half of the embryos, forming a dense anterior cap (Fig. 2A, arrowheads) (Munro et al., 2004; Werner et al., 2007). Upon anaphase onset, the anterior cap dissipates and a morphologically distinct set of NMY-2::GFP foci accumulate and coalesce to from large foci in the equatorial region and near the anterior pole (Fig. 2A-C, Fig2Video1). High
magnification views of myosin foci in both regions reveals that small, dim foci progressively intensify over a period of ~10 s and subsequently fade with similar kinetics (Fig. 2B). In addition, neighboring foci coalesce to a single bright spot as judged by light microscopy. In contrast, in ani-1(RNAi) embryos, although NMY-2::GFP foci accumulate in the cell cortex, they fail to coalesce (Fig. 2A-C, Fig2Video1).

Next, we examined myosin recruitment in embryos devoid of organized arrays of microtubules, as a consequence of depletion of the pericentrosomal component SPD-5 (Hamill et al., 2002). In SPD-5-depleted embryos undergoing anaphase, large, bright NMY-2::GFP foci accumulate throughout the entire cell cortex (Fig. 2A & C, Fig2Video1). Formation of these large foci is dependent on anillin as they are absent from the entire cortex of spd-5(RNAi);ani-1(RNAi) embryos, in these embryos myosin accumulates in small foci throughout the cortex (Fig. 2A & C, Fig2Video1). We infer that anillin has the potential to promote myosin coalescence globally and that its activity is modulated by microtubules.

To further establish that microtubules regulate cortical myosin accumulation, we generated microtubule-depleted and microtubule-dense regions in the same embryo by depleting ZYG-9 (Fig. 1A, 2A & C). During anaphase, large cortical NMY-2::GFP foci were only observed in the anterior half of ZYG-9-depleted embryos and the small foci that accumulated in the vicinity of the posterior
spindle failed to coalesce, except immediately adjacent to the central spindle (Fig. 2A & C, Fig2Video1). Kymograph analysis reveals that, when ANI-1 is present, large, intense myosin foci accumulate at sites distal to the spindle poles (Fig. 2C).

To determine whether the inverse correlation between microtubule dense regions and myosin coalescence is anillin dependent, we compared the patterning of NMY-2::GFP foci in zyg-9(b244ts) and zyg-9(b244ts);ani-1(RNAi) embryos. NMY-2::GFP foci in the vicinity of the posterior spindle in zyg-9(b244ts) embryos are dim and fail to coalesce, comparable to the NMY-2::GFP foci in the entire cortex of zyg-9(b244ts);ani-1(RNAi) embryos (Fig. 2A & C, Fig2Video1). The similar behavior of NMY-2::GFP foci in anillin-depleted embryos and in the microtubule-dense regions of control and ZYG-9-depleted embryos suggest that astral microtubules could inhibit the coalescence of cortical myosin by negatively regulating anillin.

**Anillin recruitment is largely myosin independent**

To determine how anillin controls myosin recruitment and how it contributes to the spatial regulation of myosin recruitment, we examined the localization of anillin itself. In wild-type C. elegans embryos, the distribution of cortical anillin resembles that of cortical myosin and there is extensive colocalization (Maddox et al., 2005). Like myosin, anillin is recruited to the cortex in a polarized manner upon anaphase onset and then coalesces to form large foci in the equatorial
region and at the anterior pole (Fig. 2D, S2B & C, Fig4Video1). Depletion of the centrosomal component SPD-5 to prevent assembly of a normal mitotic spindle results in accumulation of ANI-1::GFP foci over the entire cortex as observed with myosin (Fig. 2A & D). Given the co-localization of anillin and myosin during cytokinesis and the anillin-dependent organization of cortical myosin (Maddox et al., 2005), we examined whether depletion of myosin impacts anillin localization. In embryos depleted of NMY-2 to an extent sufficient to prevent cleavage furrow formation, GFP::ANI-1 is recruited to the equatorial region upon anaphase onset (Fig. 2D). The anterior enrichment is not pronounced, but this is expected because NMY-2 depletion disrupts the asymmetric positioning of the spindle (Guo and Kemphues, 1996). To assess whether GFP::ANI-1 accumulation is modulated by microtubule density in NMY-2 depleted embryos, we co-depleted NMY-2 and ZYG-9 to induce a posterior spindle. Under these conditions, large foci of GFP::ANI-1 accumulate in a highly polarized manner, similar to control embryos depleted of ZYG-9 alone (Fig. 2D, Fig. S1). These data suggest that microtubules influence anillin localization independent of myosin and cortical contractility.

Anillin regulates myosin polarization during anaphase

To quantitatively assess whether anillin regulates the distribution of cortical myosin upon anaphase onset, we measured cortical myosin levels along the AP
axis in control embryos expressing NMY-2::GFP and GFP::HIS. We measured the average NMY-2::GFP intensity in each of ten equal sized regions along the AP axis and calculated a polarization index that reflects the differential myosin recruitment among these regions (Fig. 3A). Although myosin foci accumulate upon anaphase onset in a polarized manner in control embryos (Fig2Video1), the myosin polarization index remains constant up to 12s after anaphase onset (Fig. S2A). This lack of change is due to the persistence of an anterior myosin cap from the maintenance phase of polarization that dissipates during anaphase coincident with the appearance of post anaphase foci (Werner et al., 2007). To eliminate this anterior cap, we depleted MRCK-1, a CDC-42-dependent kinase (Kumfer et al., 2010) (Fig. 3B). The anaphase-specific accumulation of NMY-2::GFP foci is unaffected by MRCK-1 depletion, myosin is primarily recruited to the equatorial and anterior regions as in control embryos (Fig. 3B, Fig3Video1, compare to Fig. 2A, Fig2Video1). Likewise, NMY-2::GFP foci accumulate and coalesce in the anterior cortex of mrck-1(RNAi);zyg-9(RNAi) embryos (Fig. 3D, Fig3Video1). Coalescence of NMY-2::GFP foci depends upon ANI-1 in MRCK-1-depleted embryos (Fig. 3B,D Fig3Video1). In addition, anillin recruitment is largely unaffected by MRCK-1 depletion (Fig. S2B&C, FigS2Video1). Thus, MRCK-1 depletion only affects cortical myosin recruitment prior to anaphase.

We measured myosin accumulation along the AP axis in mrck-1(RNAi), mrck-1(RNAi);ani-1(RNAi), mrck-1(RNAi);zyg-9(RNAi) and mrck-1(RNAi);zyg-
9(RNAi);ani-1(RNAi) embryos (Fig. S3) and calculated polarization indices.

Within 3 s of anaphase onset, myosin is recruited to the cortex in both mrck-1(RNAi) embryos and mrck-1(RNAi);ani-1(RNAi) embryos (Fig. 3C), however, the recruitment is polarized in mrck-1(RNAi) embryos but not in mrck-1(RNAi);ani-1(RNAi) embryos (Fig. 3C). In addition, myosin accumulation is highly polarized in mrck-1(RNAi);zyg-9(RNAi) embryos, but the recruitment is less polarized in mrck-1(RNAi);zyg-9(RNAi);ani-1(RNAi) embryos (Fig. 3E). This analysis reveals that anillin contributes to the spatial regulation of cortical myosin recruitment, in addition to controlling the organization of cortical myosin foci.

To gain insight into how anillin can promote myosin accumulation, we compared the stability of myosin patches in the presence and following depletion of anillin during anaphase. Myosin foci were identified using automated particle tracking (see Supplemental Experimental Procedures). A fixed threshold was set to categorize foci into two intensity classes (dim or bright). In both control and ANI-1-depleted embryos, the majority of foci were dim and persisted for an average of 8 s (Fig. S4). Control embryos contained more bright foci than anillin-depleted embryos and these persisted for an average of three times longer than the small foci (Fig. S4). We used FRAP to examine exchange of myosin in the foci, but we did not detect appreciable recovery of fluorescence to these foci during their short lifetime in ANI-1-depleted or control embryos (data not shown). These results indicate that anillin promotes cortical myosin accumulation by promoting
myosin coalescence into large foci that do not exhibit significant exchange of myosin.

Collectively the data shown indicate that the accumulation of both myosin and anillin inversely correlates with the position of the microtubule asters. In addition, the inverse correlation between cortical anillin recruitment and microtubules is largely unaffected by myosin depletion, whereas depletion of anillin significantly impairs the polarization of myosin. Based on these data, we propose that microtubules inhibit the ability of anillin to promote myosin organization and stabilization. There are many possible underlying molecular mechanisms, but the simplest inhibitory mechanism is anillin sequestration by binding to microtubules. To investigate this possibility, we examined GFP::ANI-1 in control embryos and in embryos posteriorly localized spindles. We could occasionally, but not reproducibly, observe linear elements of anillin in the vicinity of the spindle (data not shown). To a first approximation, our inability to detect a clear association of anillin with microtubules as anillin and myosin become polarized argues against a sequestration model for inhibition of anillin function. However, this model should not be completely excluded because anillin associates with microtubules in a number of contexts (see below).

**Anillin associates with microtubules**

In the course of examining the distribution of GFP::ANI-1 in myosin-depleted embryos we observed filamentous structures that extended perpendicular from
the cortex (Fig. 4A-C, Fig4Video1). These structures appeared primarily during late anaphase/early telophase, after the initial cortical polarization, and were most frequently, though not exclusively, observed in the anterior half of the embryo. These observations indicate that GFP::ANI-1 can localize in the cytoplasm, at the cortex, and in a previously undetected subcortical site.

The linear structures pointed towards the centrosome and they were highly dynamic, suggesting that they may represent anillin bound to microtubules. Imaging with high time resolution demonstrates that these structures appear at sites with preexisting cortical GFP::ANI-1 foci that elongate, intensify and subsequently shrink to the cortex and disappear (Fig 4B and Fig4Video1 & 2). Because strong depletion of a major cytokinetic component may result in nonspecific effects, we examined whether the filamentous structures could form in embryos partially depleted of myosin and capable of forming cleavage furrows. GFP::ANI-1 filamentous structures are also frequently observed in cleavage competent nmy-2(RNAi-partial) embryos (Fig. 4A, Fig4Video1 & 2). These structures were also observed when cortical myosin levels are perturbed in embryos depleted of the regulatory light chain of myosin, MLC-4 (Fig. 4A, Fig4Video1). More infrequently, these structures could also be observed in control embryos expressing GFP::ANI-1 (Fig. 4B). These results indicate a novel localization pattern of GFP::ANI-1.
To determine whether these linear forms of anillin are microtubule-associated, we examined ANI-1 localization in embryos co-expressing GFP::ANI-1 and mCherry::Tubulin. In nmy-2(RNAi-partial) embryos, GFP::ANI-1 decorated the ends of some microtubules (Fig. 4D). Furthermore, we labeled GFP::ANI-1 and endogenous tubulin in fixed nmy-2(RNAi) embryos and found GFP::ANI-1 localized at microtubule plus ends (Fig. S5). We conclude that anillin can associate with microtubules during anaphase.

To examine whether the ability to associate with microtubules is a conserved property of anillin, we examined the localization of anillin in dividing HeLa cells. In unperturbed cells, anillin is highly concentrated at the cleavage furrow, as previously described (Oegema et al., 2000) (Fig. 4E). As taxol treatment has been previously shown to induce redistribution of several cytokinesis proteins (Hummer and Mayer, 2009), we examined anillin localization in taxol-treated anaphase cells and found that anillin redistributes from the cell cortex to spindle microtubules (Fig. 4E). Thus, microtubule association, either direct or indirect, appears to be a conserved property of anillin.

To determine if anillin binds directly to microtubules, we constructed three different N-terminal GST- and C-terminal HIS-tagged anillin fragments, expressed them and examined whether they co-sediment with microtubules (Fig. 4F). Truncated anillin (ANI-IAH), lacking the N-terminal myosin and actin binding
domains and the C-terminal PH domain, sediments with taxol-stabilized microtubules in a tubulin concentration dependent manner (Fig. 4F). Neither the proteolytic fragments of ANI-IAH (Fig. 4F), nor the other anillin constructs, nor GST alone co-sediments with microtubules (data not shown). This fragment does not quantitatively sediment, suggesting a relatively weak affinity for microtubules.

**Anillin mediates microtubule-cell cortex association during anaphase**

A recent report (Redemann et al., 2010) demonstrated plasma membrane invaginations in *C. elegans* embryos expressing the plasma membrane markers GFP::PH or mCherry::PH. Interestingly, the authors report that the abundance of these invaginations increases dramatically upon depletion of NMY-2, as we have observed for the association of GFP::ANI-1 with microtubules. Using these membrane markers, we reproduced the fine linear structures extending toward the centrosome in control cells (Fig. 5A, Fig5Video1). Co-expression of mCherry::PH and GFP::Tubulin revealed that the invaginations detected with mCherry::PH are co-linear with microtubules that approach the cell cortex (Fig. 5B). The structures observed with GFP::PH might reflect a general property of cortex-microtubule interactions. Alternatively, anillin may play a crucial role in their formation. To examine whether the GFP::PH invaginations are related to those observed with GFP::ANI-1, we simultaneously imaged GFP::ANI-1 and mCherry::PH in NMY-2 depleted embryos where the structures are abundant. Both markers could be detected in the vast majority of invaginations, but they did
not precisely overlap. The GFP::ANI-1 signal extended more internally than the mCherry::PH signal. The consistent co-occurrence of the two markers in the invagination is remarkable because whereas mCherry::PH labels the plasma membrane quite continuously, GFP::ANI-1 is localized in a highly punctate manner, suggesting that anillin could be required for their formation. We therefore measured the abundance of these structures in ANI-1-depleted embryos. As these invaginations are relatively infrequent in ani-1(RNAi) and control embryos, we quantitated the anillin dependence of these invaginations in cells depleted of NMY-2 which results in a 7-fold increase in their abundance (Fig. 5A & C, S6 Fig5Video1) (Redemann et al., 2010). The invaginations are three-fold less abundant in embryos co-depleted of ANI-1 and NMY-2 as compared to embryos depleted of NMY-2 alone (Fig. 5A & C, Fig5Video1, S6). We examined whether residual anillin might be associated with the remaining invaginations despite being significantly depleted (70%). Indeed, when both ANI-1 and NMY-2 were depleted from embryos expressing both GFP::ANI-1 and mCherry::PH, residual GFP::ANI-1 was clearly detectable in ~75% of the remaining invaginations (Fig. 5D & E). As ANI-1 promotes formation of the invaginations and the protein is present in the majority of all invaginations, we conclude that ANI-1 can link cortical factors to microtubules.
Anillin can contribute to cell polarization in meiosis

To examine whether anillin has a role in cell polarization in another context in which microtubules serve as a polarizing cue, we turned to fertilized oocytes arrested in meiosis I. Fertilized oocytes arrested in meiosis I due to inactivation of the anaphase-promoting complex (APC) exhibit polarity inversion induced by the meiotic spindle in a microtubule-dependent manner (Wallenfang and Seydoux, 2000) (Fig. S7A). Polarity inversion can be monitored by accumulation of posterior PAR proteins, such as PAR-2, on the cortex adjacent to the meiotic spindle and by asymmetric accumulation of GFP::PIE-1, a maternally deposited transcriptional repressor that segregates with the germ lineage in early embryos (Mello et al., 1996; Seydoux et al., 1996). We used depletion or mutation of APC subunits MAT-1 or EMB-27 in conjunction with depletion of ANI-1 to investigate whether anillin is involved in this model for cell polarization. GFP::PAR-2 was asymmetrically localized in ~60% (N=69) of fertilized oocytes depleted of EMB-27 alone, but only in ~30% (N=65) of fertilized oocytes depleted of both ANI-1 and EMB-27 (Fig. 6A). We next assessed whether ANI-1 contributes to the asymmetric distribution of PIE-1. Whereas GFP::PIE-1 is asymmetrically distributed in 61% of mat-1(ax161) oocytes, GFP::PIE-1 is asymmetric in 43% of mat-1(ax161);ani-1(RNAi) oocytes (Fig. 6B). Thus, anillin promotes the polarization of fertilized oocytes in response to the meiotic spindle.
These fertilized oocytes contain a meiotic spindle composed of a dense array of microtubules surrounding the maternal chromatin and few other microtubules (Fig. 6C). To examine whether anillin localizes to the meiotic spindle we imaged GFP::ANI-1 in live mat-1(ax161) oocytes immediately upon dissection. We observed GFP::ANI-1 was distinctly concentrated on the spindle (Fig. 6D). Examination of anillin truncations revealed that both N- and C-terminally truncated anillin could also associate with the microtubules of the meiotic spindle (Fig. S7B), suggesting that the primary determinant of microtubule association lies in its central region. Thus, during the microtubule-directed polarization of meiotically arrested, fertilized oocytes, ANI-1 contributes to oocyte polarization and concentrates on the meiotic spindle.

**Discussion**

Upon anaphase onset, the equatorial accumulation of cortical myosin II and the subsequent formation of a cleavage furrow is dictated by the combined action of a positive cue from the central spindle and an inhibitory cue from astral microtubules. A molecular framework for the nature of the positive cue has been described, but the molecules involved in the inhibitory cue remain obscure. Here, we have demonstrated that the inhibition of cortical myosin recruitment by astral microtubules in the early C. elegans embryo involves the cytoskeletal scaffold protein anillin which has the capacity to bind to microtubules.
**Anillin contributes to cell polarization**

During anaphase, cortical myosin accumulates in a highly polarized manner. In particular, cortical myosin inversely correlates with the proximity of microtubules to the cell cortex. Repositioning of the spindle redirects the polarized accumulation of myosin, strengthening this inverse correlation (Werner et al., 2007). In regions where microtubules are less dense – or in the entire embryo if centrosome assembly is prevented – myosin coalesces into larger foci that persist for longer than small myosin foci. This coalescence of myosin into larger foci requires anillin as does the polarization accumulation of myosin. Like myosin, the cortical recruitment of anillin is polarized. Whereas myosin polarization requires anillin, anillin can accumulate in a polarized manner in cells depleted of myosin. We propose that the polarized accumulation of anillin promotes the polarized recruitment of myosin.

Not only does anillin promote the formation of the aster-directed furrow, it also contributes to the ability of the meiotic spindle to direct asymmetric accumulation of PAR-2 and PIE-1 in arrested oocytes. However, anillin is unlikely to be the sole mediator of either astral inhibition or oocyte polarization. Anterior-directed myosin flows are detectable in ANI-1 depleted embryos, indicating residual asymmetry. Likewise, anillin depletion does not abrogate PAR-2 recruitment to the cortex adjacent to the meiotic spindle.

The polarized accumulation of myosin that facilitates cytokinesis during anaphase shares several properties with the polarized accumulation of myosin
that facilitates embryo polarization upon fertilization. The dynamics of cortical
myosin are similar during these two processes and there is a common
requirement for several proteins. In addition to ANI-1, these include the protein
phosphatase PPH-6 and its associated subunit SAPS-1, as well as wild-type
levels of the formin, CYK-1 and the RhoGEF ECT2 (Werner et al., 2007; Werner
and Glotzer, 2008; Afshar et al., 2010; Zonies et al., 2010). One apparent
difference between these processes is the initial cue that leads to either the
anterior accumulation of myosin during polarization or the equatorial
accumulation of myosin during cytokinesis. In the latter case, microtubule asters
provide the positional information, whereas in the former case the process is
initiated by the sperm centrosome in a microtubule-independent manner (Cowan
and Hyman, 2004; Sonneville and Gonczy, 2004), although there is some
evidence that the centrosome cue can involve microtubules (Tsai and Ahringer,
2007). Irrespective of the mechanism of symmetry breaking, microtubule-
mediated inhibition of anillin could further enhance differential myosin recruitment
and promote the formation of an deeply ingressing furrow.

Anillin associates with microtubules
Our results indicate that microtubules inhibit cortical recruitment of anillin in
microtubule-rich regions of the embryo. From our global depletion studies we
infer that regional inhibition of anillin would result in regional inhibition of myosin
accumulation. What molecular mechanism could mediate the regional inhibition
of cortical anillin recruitment? There are a number of possible mechanisms including, but not limited to, local sequestration by binding to microtubules, microtubule-directed post-translational modification, or local inhibition of a critical co-factor. Although our results do not discriminate between these possible mechanisms, we note that local sequestration of anillin is the simplest model and we have also demonstrated that anillin has a conserved capacity to associate with microtubules which is a prerequisite for the sequestration model.

When we examined anillin localization in non-muscle myosin-depleted embryos, we observed intense anillin-labeling of a subset of microtubules during late anaphase. These labeled microtubules are also associated with invaginations of the plasma membrane. These invaginations are far less abundant when anillin is depleted and the remaining invaginations are highly enriched in residual anillin, suggesting that anillin is required to link microtubules to the membrane, rather than being a passive component. Anillin also concentrates on the meiotic spindle in arrested oocytes. An interaction between anillin and microtubules is also detectable in human cells. Direct binding of anillin to microtubules can be reconstituted with purified components, however, additional factors may participate in vivo. Collectively, these diverse observations indicate that anillin associates with microtubules, either directly or indirectly.
Indeed, anillin has been previously shown to associate with microtubules \textit{in vitro}. \textit{Drosophila} anillin was found in an early proteomics screen for proteins that bind to both f-actin and taxol-stabilized microtubules (Sisson et al., 2000). More recently, in \textit{Drosophila} cells treated with actin depolymerizing agents, anillin was observed to form extended filamentous structures some of which could associate with the extreme plus ends of microtubules (D'Avino et al., 2008; Hickson and O'Farrell, 2008). The binding of anillin to stabilized microtubules may well explain the recent finding that taxol treatment during anaphase Rankin and Wordeman, 2010 induces the remarkable cortical oscillations that are a distinguishing feature of anillin-depleted cells (Straight et al., 2005; Zhao and Fang, 2005; Piekny and Glotzer, 2008). Thus, although anillin is a prominent cortical component, it can also associate with microtubules in \textit{C. elegans}, \textit{Drosophila}, and mammalian cells under certain conditions.

Given the conserved capacity of anillin to associate with microtubules and the observation that anillin recruitment anti-correlates with microtubule density, we speculate that microtubules could sequester anillin and prevent its recruitment to the cortex where it organizes and stabilizes myosin. In this model, microtubule and cortex recruitment are predicted to be mutually exclusive, which is consistent with the finding that depletion of myosin enhances the association of anillin with microtubules. However, it has proven difficult to detect the association of anillin with microtubules during early anaphase as myosin become polarized.
association of soluble anillin with the large number of astral microtubules may result in only a weak enrichment over background, as opposed to the bright labeling that results from the large multisubunit particles that associate with microtubule tips at the cell cortex. However, anillin can be easily and reproducibly detected concentrating on the dense microtubules of the meiotic spindle, indicating that cortical enrichment is not a prerequisite for microtubule association.

Further evidence for anillin sequestration could come measurements of the rate of anillin diffusion in microtubule-rich and depleted regions of the embryo. Proof that this mechanism is responsible for astral relaxation will require the generation of an anillin variant that is specifically defective in microtubule association.

**Materials and Methods**

**Strains**

*C. elegans* strains (listed in Table S1) were maintained using standard procedures on NGM plates. Some nematode strains were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health (NIH) National Center for Research Resources (NCRR).

**RNA Interference (RNAi)**

All the RNAi constructs were obtained from the RNAi feeding library of Ahringer and colleagues (Kamath et al., 2003). All RNAi experiments were performed using the feeding method as described (Timmons and Fire, 1998). Bacteria
cultures were grown in LB and 200μL were seeded on NGM plate containing 100 μg/mL ampicillin and 1 mM IPTG and kept at RT for 8 hr. For double and triple RNAi feeding experiments, bacteria cultures were mixed in equal ratio according to cell density as measured by absorbance at 600 nm. Young L4 hermaphrodites were picked onto the plates for feeding at 25 °C at least 24 hr prior to examination.

Confocal Microscopy
To prepare slides for imaging of C. elegans embryos, gravid hermaphrodites were dissected in egg salt buffer on coverslips, mounted on 2.5% agarose pads and sealed with Vaseline. Embryos are imaged with a 63X/1.4 numerical aperture oil-immersion lens on a Zeiss Axiovert 200M equipped with a Yokogawa CSU-10 spinning disk unit (McBain) and illuminated with 50 mW 473 nm and 25 mW 561 nm lasers (Cobolt). Images were captured on a Cascade 512B EM-CCD camera (Photometrics) controlled by MetaMorph (Molecular Devices). Image processing was performed with ImageJ.

For the live imaging of embryos expressing GFP::PH in Fig. 1, we acquired GFP and Nomarski images at the embryo center in every 5 s with 250 ms exposure time. For Fig. 2 and 3, we imaged live embryos expressing NMY-2::GFP; GFP::HIS continuously with five cortical planes spanning 2 μm and a single central plane. GFP exposure times were 250 ms. For the montage analysis in Fig. 2C, we selected a region in 220 x 30 pixels along the AP axis of the embryos.
from every fifth time point and generated montages by MetaMorph and processed in ImageJ. In Fig. 4A, we imaged live embryos continuously with eleven cortical planes spanning 5 μm and a single central plane. GFP exposure time was 200 ms and mCherry was 250 ms. In Fig. 4B, we imaged live embryos continuously around the center with three frames (0.5 μm increments, 1 μm in total) for GFP and a single plane for mCherry. GFP exposure time was 500 ms and 250 ms for mCherry. For imaging the embryos expressing GFP::PH; mCherry::HIS in Fig. 5, we acquired three planes spanning 1 μm in the center for GFP and a single central plane for mCherry in every 5 s. In Fig. 5D, we continuously acquired a 1 s GFP image followed by a 1.5 s mCherry image at a central focal plane.

To prepare slides for imaging of meiotically arrested C. elegans oocytes in Fig. 6, hermaphrodites were dissected in M9 buffer on coverslips, mounted on 2.5% agarose pads and sealed with Vaseline. We acquired five central planes spanning 5 μm for both GFP (2 s exposure) and mCherry (500 ms exposure), a single plane was selected and presented.

All Z-stacks were projected with a maximum-intensity algorithm. Resulting time-lapse projections were assembled into movies by MetaMorph and ImageJ. To make movies of single time-point 3-D rotation and 3-D projection over time for the myosin-depleted embryos expressing GFP::ANI-1 in Fig. 4C, the resultant Z
stacks were converted to 8 bit TIFFs with ImageJ and further processed with Osirix.

**Epifluorescence Microscopy**

To prepare slides for imaging of meiotically arrested *C. elegans* oocytes in Fig. 6A & E, gravid hermaphrodites were dissected in M9 buffer on coverslips, mounted on glass slides with Vaseline on the corners to prevent over compression and sealed with Vaseline. Fertilized oocytes are imaged with a 40X/0.75 numerical aperture oil-immersion lens on a Zeiss Axiolmager M1 microscope. Images were captured on a Cascade 1K EM-CCD camera (Photometrics) controlled by MetaMorph (Molecular Devices). Image processing was performed with ImageJ. We acquired all images with single plane around the center of the oocytes. Both GFP and mCherry exposure times were 500 ms. For MAT-1 depleted embryos expressing GFP::Tubulin in Fig. 6 C,D, we stained the embryos with DAPI in M9 buffer (1:1000) for 5 min to visualize chromatin before imaging.

**Cell culture, Drug Treatment and Immunostaining**

To perform drug treatment, HeLa cells were cultured on 6-well culture plates to 80% confluence. 2.5 μM S-trityl-l-cysteine (STC) was added and incubated for 6 hr. Then, 10 μM Taxol was added. 10 min later 22.5 μM Purvalanol was added and incubated for another 30 min. The cells were then fixed in methanol at -20 °C for 30 min and washed with PBST. The fixed cells were further subjected to
blocking solution (PBS with 5% goat serum) for an hour. Mixture of anillin antisera (rabbit) at 1:200 and mouse anti-DM1α antibodies at 1:500 were used as primary antibodies, followed by detection with rabbit Alexa-488 and mouse Alexa-568. Cells were mounted in fluoromount G (Southern Biotechnology) and imaged by the epifluorescence microscopy system described above.

**Recombinant Protein Expression and Purification**

Truncated anillin constructs, ANI-AH, ANI-I and ANI-IAH, were amplified and inserted into the pGEX-TEV vector and expressed in BL21 (DE3) RIL cells. Protein expression was induced by the addition of 0.4 mM IPTG at 25 °C for 7-8 hr. Bacteria were harvested and resuspended in 10 mM HEPES (pH7.7), 50 mM NaCl, 1 mM EGTA, 1 mM MgCl2, 0.1% Triton-X 100, 1 mM DTT, 0.1 mM ATP, 10 mg/ml leupeptin/pepstatin, and 1 mM phenylmethylsulfonylfluoride containing 0.5 mg/ml lysozyme prior to sonication. Lysates were centrifuged in a JA.20 Beckman rotor at 18,000 rpm at 4 °C for 20 min. Prewashed glutathione-Agarose beads were added to the cleared lysates and incubated at 4 °C for 2 hr with mixing. Following washes, proteins were eluted with 20 mM glutathione in HEPES buffer. The purified recombinant proteins were stored in aliquots at -80 °C.

**Sedimentation Assay and SDS-PAGE**

To perform the tubulin sedimentation assay, 0.1 M GTP and 0.25 M MgCl2 were added to recycled tubulin and centrifuged at 80,000 rpm for 10 min at 4°C. Then the precleared recycled tubulin was polymerized in 2 mM GTP, 24% Glycerol in
1X BRB-80 buffer and 40 \( \text{M} \) Taxol for 30min. Purified recombinant proteins were added to the taxol-stabilized microtubules and incubated for 45 min at room temperature with gentle mixing. 20 \( \mu l \) of total 100 \( \mu l \) reaction mixtures was sampled “total”. The remaining reaction mixture was loaded onto the top of a 48% sucrose cushion and centrifuged at 40,000 rpm for 10 min. 30 \( \mu l \) of supernatant was collected “supernatant”. The pellet was washed twice with 1X BRB-80 buffer and resuspended in 80 \( \mu l \) 1X BRB-80 buffer “pellet” fraction. Equal amounts of total, supernatant and pellet fractions were loaded into 8% SDS-PAGE gel. The resulting gel was stained in coomassie blue.

**Image Quantification**

For measurements of the extent of the membrane ingression in ZYG-9 mutant embryos expressing GFP::PH (Fig. 1), the images with maximum membrane ingression were selected manually in ImageJ. For embryos with anterior furrows, we used ImageJ to measure the distance between the two furrow tips. For embryos without anterior furrows, we measured the distance between the anterior cortices at 1/3 egg length (Fig. 1). The ingression percentage was calculated by the equation as shown in Fig 1C.

To measure the myosin intensity upon anaphase onset in the embryos as shown in Fig. 3, we equally divided the AP axis of the embryos into ten equal regions in each projected images (5 planes spanning 2 \( \mu m \)) and measured the average
intensities in each region by MetaMorph. The data was exported to Excel for further analysis. Polarization index was calculated by the equation shown in Fig. 3A.

For measurement of the number of invaginations in embryos expressing GFP::PH (Fig. 5), we first adjusted the contrast of the projected images (3 planes spanning 1 μm) in ImageJ to reduce the background and enhance the GFP signals, and then made binary images. Then we used ABSnake in ImageJ to outline the inner cortex and reduced the size of the outline to 97%. After that, we pasted the scaled outlines as a line into the binary images and executed a line scan. The intensity data were further analyzed in Excel and counted the number of intensity peaks. Each intensity peak represents a single invagination. Peaks with less than 2 pixel width were designated as noise and excluded from the measurement.

To count the number of embryos with symmetric/asymmetric distribution of GFP::PAR-2 and PIE-1::GFP in meiotically arrested oocytes (Fig. 6A & B), we selected the oocytes with condensed chromatin and classified the GFP distribution manually.

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References


**Figure 1** Anillin is required for aster-mediated furrow ingression. (A) Schematic depicting the position of the spindle in a wild-type embryo and a ZYG-9-depleted embryo with reduced aster size. (B) Images from a time-lapse sequences of the first division of *zyg-9*(b244ts) and *ani-1*(RNAi);*zyg-9*(b244ts) embryos. *zyg-9*(b244ts) mutant embryos form pseudocleavage furrows (black arrows) and anterior (white arrowheads) and posterior furrows (black arrowheads) during anaphase, whereas *ani-1*(RNAi);*zyg-9*(b244ts) embryos only form posterior furrows (black arrowheads) during anaphase. (C) The maximal extent of anterior furrow formation was quantitated using GFP::PH as a membrane marker as diagrammed. Error bars in the bar chart represent ± SEM. ** represents p<0.01. All images are arranged with anterior to the left, 10 µm scale bars.
Figure 2 Microtubules control the distribution of cortical myosin. (A) Selected images from time-lapse sequences of embryos expressing NMY-2::GFP at the indicated cell cycle phases. Myosin images are maximum intensity projections of 5 planes spanning 2 µm. Time relative to anaphase onset is shown and the insets at top right show the chromatin imaged with GFP::HIS. (B) Detailed view of the behavior of representative foci (represented by the color coding in A) in control and ani-1(RNAi) embryos. (C) Kymograph analysis of the first 42 s after anaphase onset demonstrating the relative position of the myosin foci (green) and the chromatin (red). The approximate positions of the centrosomes, inferred by the position of the chromatin are indicated by arrows at the bottom. (D) Selected images from time-lapse sequences of embryos expressing GFP::ANI-1 at the indicated cell cycle phases. The insets at top right show the chromatin imaged with mCherry::HIS. 10 µm (A, C & D) and 2 µm (B) scale bars.
Figure 3 Anillin promotes the polarized accumulation of myosin. (A) Schematic and equation used to calculate a polarization index. (B) The distribution of cortical NMY-2::GFP in embryos depleted of MRCK-1 or both MRCK-1 and ANI-1 are shown during metaphase and anaphase. Cell cycle timing was determined by GFP::HIS (insets, top right). All images are projections of 5 planes spanning 2 μm. (C) Quantification of the GFP intensity in embryos acquired as in (B). (D) As in (B), but the embryos were additionally depleted of ZYG-9 to misposition the spindle in the posterior. (E) Quantification of the GFP intensity in embryos acquired as in (D). Error bars represent ± SEM; Single asterisk represents p<0.05; double asterisk represents p<0.01 from paired t-tests. Scale bars represent 10 μm.
Figure 4 Anillin associates with microtubules. (A) Selected images from time-lapse sequences of embryos expressing GFP::ANI-1 (projections of 11 planes spanning 5 μm) at the indicated cell cycle phases. Time relative to anaphase onset is shown and the insets show the chromatin imaged with mCherry:HIS. In embryos partially (cytokinesis-competent) or severely depleted of NMY-2 or the myosin regulatory light chain, MLC-4, radially distributed linear structures are observed near the cortex (red arrowheads). (B) Embryos like those in (A) were imaged at central planes (projection of 3 planes spanning 1 μm). Linear structures are also observed with this imaging modality (shown with red asterisks). High magnification views of the yellow box region demonstrating the
origin of linear structures at high time resolution. Dashed line indicates frames shown at top and bottom rows. (C) Z-stacks were collected halfway through an embryo strongly depleted of NMY-2 and expressing GFP::ANI-1. Maximum intensity projections at the indicated times are shown as are 3-D reconstructions of the data set from two difference perspectives, from the side and end on view from the anterior, as indicated in the schematics. (D) Embryo partially depleted of NMY-2 and expressing GFP::ANI-1 (green) and mCherry::Tubulin (red) were imaged at a central plane (projection of 3 planes spanning 1 µm). The linear GFP::ANI-1 structures were collinear with the microtubules. Arrowheads indicate the linear structures. (E) Immunofluorescence of control or taxol (10 µM) treated HeLa cells during anaphase revealing the distribution of anillin (green) and microtubules (red). (F) Schematic of three ANI-1 expression constructs and a coomassie blue stained SDS-PAGE of the microtubule co-sedimentation assays. T: Total fraction; S: Supernatant fraction after ultracentrifugation; P: Pellet fraction after ultracentrifugation. Arrow on the right indicates full length ANI-IAH, arrowhead indicates taxol-stabilized microtubules. Scale bars represent 10 µm.
Figure 5 Anillin mediates microtubule-plasma membrane cortical interactions. (A) Selected images from time-lapse sequences of the central plane of embryos expressing GFP::PH (projection of 3 planes spanning 1 μm) at the indicated time relative to anaphase onset is shown (insets show the chromatin as detected with mCherry::HIS). Small, local, transient cortical invaginations (indicated by red asterisks) are observed in control embryos and some ANI-1 depleted embryos. (B) Dual color confocal imaging of mCherry::PH and GFP::Tubulin. Boxed
regions are shown below the embryos at high magnification. (C) Total number of
cortical invaginations from t=0 s to 180 s as embryos in (A). (D) Selected images
from timelapse sequences of embryos expressing GFP::ANI-1 and mCherry::PH
following depletion of NMY-2 alone or a both NMY-2 and ANI-1. Cortical
invaginations are indicated by red asterisks. GFP::ANI-1 accumulated to the
equatorial cortex in NMY-2-depleted embryos (white arrowheads) but not in
embryos depleted of both NMY-2 and ANI-1. Timelapse sequences of the
indicated (*) invaginations are shown at high magnification. (E) Invaginations
detected by mCherry::PH were scored for the presence of GFP::ANI-1 in
embryos as shown in (D). Error bars are ±SEM, double asterisks indicate p <
0.05 by paired t-test. Scale bars represent 10 μm.
Figure 6  Anillin concentrates on meiotic spindles and contributes to cell polarization.  (A) GFP::PAR-2 distribution in control and ANI-1 depleted oocytes arrested in meiosis I due to EMB-27 depletion.  (B) PIE-1::GFP distribution in control and ANI-1 depleted *mat-1(ax161)* oocytes arrested at the restrictive temperature in meiosis I.  (C) Overview of the microtubule cytoskeleton in meiotically arrested oocytes.  MAT-1 depleted, GFP::Tubulin expressing oocytes were stained with DAPI in M9 buffer.  (D) GFP::ANI-1 distribution in fertilized oocytes defective for the APC/C due to depletion of *mat-1(ax161)* conditional mutation.  Scale bars represent 10 μm.
Supplemental Experimental Procedures
Myosin foci intensity and residence time measurement

To measure the maximum intensity and residence times of NMY-2::GFP foci upon anaphase onset, we imaged the embryos as in Fig. 2 and tracked each focus using the Particle detector & Tracker Plugin to ImageJ (Sbalzarini and Koumoutsakos, 2005) (radius: 5, cutoff: 0, percentile: 0.5%, link range: 1). Foci with maximum intensities higher than 3 were classified as bright foci, others were counted as dim foci.

Embryo immunostaining

To detect the localization of microtubules and GFP::ANI-1 in myosin-depleted embryos (Fig. S5), we froze the hermaphrodites between a poly-lysine coated slide (Sigma) and coverslip in liquid nitrogen. To crack the eggshells, we removed the coverslip quickly under low temperature and then fixed the embryos in methanol and rehydrated in ethanol. Then the embryos were blocked in PBS with 5% goat serum for 1 hr. Mixture of rabbit anti-GFP antibodies at 1:400 and mouse anti-DM1α at 1:100 were used as primary antibodies staining, followed by rabbit Alexa-488 and mouse Alexa-568 staining. Embryos were mounted in fluoromount G (Southern Biotechnology) and imaged by the confocal spinning disk system described above.
Supplemental Figure Legends

Figure S1
Myosin depletion does not significantly alter anillin polarization in embryos with posterior spindles. (A) Schematic and equation used to calculate the polarization index. (B) Quantification of extent of polarized recruitment of GFP::ANI-1 in embryos depleted of ZYG-9 alone or depleted of both ZYG-9 and NMY-2. Error bars represent ± SEM.

Figure S2
MRCK-1 depletion blocks the formation of the polarity cap of NMY-2::GFP and does not significantly alter anillin recruitment during anaphase. (A) Quantification of the GFP intensity in NMY-2::GFP control embryos and MRCK-1-depleted embryos. (B) The distribution of cortical GFP::ANI-1 in control and MRCK-1-depleted embryos are shown during metaphase and anaphase. Cell cycle timing was determined by mCherry::HIS (insets, top right). All images are projections of 5 planes spanning 2 μm. (C) Quantification of the degree of polarization of GFP::ANI-1 in control embryos and MRCK-1-depleted embryos. Error bars represent ± SEM; Single asterisk represents p<0.05; double asterisk represents p<0.01 from paired t-tests. 10 μm scale bar.

Figure S3
Anillin promotes the polarized accumulation of myosin. (A) Schematic and equation used to calculate normalized myosin intensity profiles. (B-E) The distribution of cortical NMY-2::GFP are quantified in embryos depleted of MRCK-
1 (B), MRCK-1 and ANI-1 (C), MRCK-1 and ZYG-9 (D) and MRCK-1, ZYG-9 and ANI-1 (E). In ANI-1-depleted embryos, there is only slightly more NMY-2::GFP recruitment in the equatorial region (region 6 and 7) and anterior pole (region 2) as compared to the remaining regions. In addition, the intensity gradient along the AP axis is greatly reduced in mrck-1(RNAi);zyg-9(RNAi);ani-1(RNAi) embryos as compared to mrck-1(RNAi);zyg-9(RNAi) embryos. (F-G) The distribution of NMY-2::GFP from 4.5 s to 7.5 s after anaphase onset calculated as in (B-E).

**Figure S4**
Depletion of anillin reduces the fraction of bright NMY-2::GFP foci. Fraction of dim (A) and bright (B) NMY-2::GFP foci in control and ani-1(RNAi) embryos. The percentage of foci with bright GFP signals is significantly reduced in ANI-1-depleted embryos. (C and D) Lifetime of NMY-2::GFP foci in control embryos (C) and ani-1(RNAi) embryos (D). No significant difference on the lifetime of bright and dim NMY-2::GFP foci in control and ANI-1-depleted embryos.

**Figure S5**
Anillin associates with microtubules. Embryos expressing GFP::ANI-1 were fixed and immunostained to detect endogenous tubulin (red) and GFP::ANI-1 (green). The boxed regions are shown at higher magnification below.

**Figure S6**
Quantitative analysis of the appearance of cortical invaginations. At 30 s intervals after anaphase onset (t=0) in embryos of the indicated genotypes
expressing GFP::PH; mCherry::HIS were quantified as described in materials and methods. Error bars are ±SEM.

**Figure S7**

Anillin concentrates on meiotic spindles. (A) GFP::ANI-1 distribution in APC/C defective fertilized oocytes (due to depletion of EMB-27 by RNAi or the *mat-1(ax161)* mutation). (B) The distribution of GFP::ANI-1(ΔCT) and GFP::ANI-1(ΔNT) in meiotically-arrested fertilized oocytes.
Supplemental videos

**Fig1Video1** DIC microscopy of zyg-9(b244ts) and ani-1(RNAi);zyg-9(b244ts) embryos expressing GFP::PH.

**Fig2Video1** Color combined images of confocal images of cortical NMY-2::GFP (Green, maximum intensity projection of 5 planes spanning 2 μm) and GFP::HIS (Red, single plane) in control, spd-5(RNAi), zyg-9(RNAi), ani-1(RNAi), spd-5(RNAi);ani-1(RNAi) and zyg-9(RNAi);ani-1(RNAi) embryos. Images were acquired every 1.5 seconds and played back at 10 frames per second.

**Fig3Video1** Color combined images of confocal images of cortical NMY-2::GFP (Green, maximum intensity projection of 5 planes spanning 2 μm) and GFP::HIS (Red, single plane) in control, spd-5(RNAi), zyg-9(RNAi), ani-1(RNAi), spd-5(RNAi);ani-1(RNAi) and zyg-9(RNAi);ani-1(RNAi) embryos. Images were acquired every 1.5 seconds and played back at 10 frames per second.

**Fig4Video1** Color combined images of confocal images of cortical GFP::ANI-1 (Green, maximum intensity projection of 11 planes spanning 5 μm) and mCherry::HIS (Red, single plane) in control, nmy-2(RNAi), nmy-2(RNAi) partial, and mlc-4(RNAi) [top row and bottom right]. Also shown are color combined images of confocal images of GFP::ANI-1 (Green, maximum intensity projection of 3 central planes spanning 1 μm) and mCherry::HIS (Red, single plane); asterisks indicate linear concentrations of GFP::ANI-1. Images were acquired
every 2.45 s and played back at 10 frames per second.

**Fig4Video2** 3D visualization over time of GFP::ANI-1 in *nmy-2(RNAi) partial* and *nmy-2(RNAi)* embryos. Same embryos as in Fig4Video1. 3-D rotation of GFP::ANI-1 in *nmy-2(RNAi) partial* and *nmy-2(RNAi)* embryos. Same embryos as in Fig4Video1.

**Fig5Video1** Color combined images of confocal images of GFP::PH (Green, maximum intensity projection of 3 central planes spanning 1 μm) and mCherry::HIS (Red, single plane) in control, *ani-1(RNAi)*, *nmy-2(RNAi)*, and *ani-1(RNAi); nmy-2(RNAi)* embryos. Asterisks indicate membrane invaginations. Images were acquired every 5 s and played back at 10 frames per second.

**FigS2Video1** Color combined images of confocal images of cortical GFP::ANI-1 (Green, maximum intensity projection of 5 planes spanning 2 μm) and mCherry::HIS (Red, single plane) in control, *mrck-1(RNAi)* and *ani-1(RNAi)* embryos. Images were acquired every 1.5 s and played back at 10 frames per second.