Aurora A and cortical flows promote polarization and cytokinesis by inducing asymmetric ECT-2 accumulation

Katrina M Longhini and Michael Glotzer

Molecular Genetics and Cell Biology

The University of Chicago

Chicago, IL 60637

Author for correspondence: mglotzer@uchicago.edu

Abstract

In the early *C. elegans* embryo, cell polarization and cytokinesis are interrelated yet distinct processes. Here, we sought to understand a poorly understood aspect of cleavage furrow positioning. Early *C. elegans* embryos deficient in the cytokinetic regulator centralspindlin form furrows, due to an inhibitory activity that depends on aster positioning relative to the polar cortex. Polar relaxation is associated with depletion of the cortical RhoGEF, ECT-2, specifically at the posterior pole. Asymmetric ECT-2 accumulation requires intact centrosomes, Aurora A (AIR-1), and myosin-dependent cortical flows. Within a localization competent ECT-2 fragment, we identified three putative phospho-acceptor sites in the PH domain of ECT-2 that render ECT-2 responsive to inhibition by AIR-1. AIR-1 and cortical flows cooperate to promote embryo polarization. During both polarization and cytokinesis, centrosomal AIR-1 breaks symmetry via ECT-2 phosphorylation; this local inhibition of ECT-2 is amplified by myosin-driven flows that generate regional ECT-2 asymmetry. Together, these mechanisms cooperate to induce polarized assembly of cortical myosin, contributing to both embryo polarization and cytokinesis.

Introduction

The division of a single animal cell into two daughter cells is facilitated by an actomyosin-based contractile ring that generates the force that constricts the cell membrane and generates the cleavage furrow (Basant and Glotzer, 2018). Furrow positioning is dictated by the position of the mitotic spindle (Rappaport, 1985). Work in *C. elegans* has shown that distinct parts of the spindle, namely the spindle midzone and the asters, are each sufficient to induce furrowing (Dechant and Glotzer, 2003; Bringmann and Hyman, 2005). The active form of the small GTPase RhoA (C.e. RHO-1) is necessary for cleavage furrow induction in these and other contexts (Kishi et al., 1993; Jantsch-Plunger et al., 2000; Zhou and Zheng, 2013). An equatorial zone of active RhoA suffices to induce a furrow in cultured human cells (Wagner and Glotzer, 2016). Despite progress in our understanding of cytokinesis, gaps remain in our understanding of the mechanism by which asters spatially regulate RhoA activation.

The guanine nucleotide exchange factor (GEF) Ect2 (C.e. ECT-2), is the primary activator of RhoA during cytokinesis (Tatsumoto et al., 1999). This Dbl-homology family member is regulated by numerous factors. Prominent among these is the centralspindlin complex which, despite containing a subunit that contains a RhoGAP domain, plays a significant role in RhoA activation during cytokinesis. Centralspindlin both induces relief of ECT-2 auto-inhibition and promotes Ect2 recruitment to the plasma membrane (Yüce et al., 2005; Niiya et al., 2005; Zhao and Fang, 2005; Su et al., 2011; Zhang and Glotzer, 2015; Lee et al., 2018; Chen et al., 2019). Anti-parallel, bundled microtubule plus ends, kinases, and a phosphatase conspire to promote centralspindlin-dependent RhoA activation at the cell equator, midway between the two spindle poles (Yüce et al., 2005; Nishimura and Yonemura, 2006; Burkard et al., 2009; Wolfe et al., 2009; Basant et al., 2015; Hertz et al., 2016).

Despite the established role of the spindle midzone in cytokinesis in diverse metazoa, furrow formation can occur in its absence in some cell types. This is well characterized in *C. elegans* embryos where the centralspindlin-dependent and -independent furrows have been separated genetically (Dechant and Glotzer, 2003; Werner et al., 2007). Analogous phenomena operate in cultured human cells (Murthy and Wadsworth, 2008; Rodrigues et al., 2015; Chen et al., 2021; Ramkumar et al., 2021). In early *C. elegans* embryos, centralspindlin functions in parallel with NOP-1 to promote ECT-2-dependent RHO-1 activation (Fievet et al., 2012; Morton et al., 2012; Tse et al., 2012). Though it is non-essential, NOP-1 can promote formation of an incomplete furrow (Tse et al., 2012). In certain circumstances, NOP-1 suffices to induce complete furrow ingression (Canman et al., 2008; Loria et al., 2012; Zhang and Glotzer, 2015).

In addition to being genetically separable, the centralspindlin- and NOP-1-dependent pathways can be spatially separated. Whereas the position of the centralspindlin-dependent

furrow correlates with the spindle midzone (Werner et al., 2007), the position of the NOP-1 dependent furrow anti-correlates with the position of the spindle asters (Hird and White, 1993; Dechant and Glotzer, 2003; Werner et al., 2007; Tse et al., 2012). Specifically, spindle asters are associated with inhibition of contractility. This inhibitory activity has generally been ascribed to the astral microtubules and is often referred to as polar relaxation/astral relaxation (Wolpert, 1960; White and Borisy, 1983; Dechant and Glotzer, 2003; Werner et al., 2007; Mangal et al., 2018; Chapa-Y-Lazo et al., 2020). In unperturbed cells, the centralspindlin-dependent and -independent furrows align during cytokinesis.

Regional suppression of cortical contractility also occurs during polarization of the *C. elegans* zygote. After fertilization, the sperm-derived centrosome serves as a symmetry-breaking cue that inhibits cortical contractility (Goldstein and Hird, 1996; Cowan and Hyman, 2004). This reduction in cortical contractility induces anterior-directed cortical flows that advect PAR proteins, such as PAR-3 and PAR-6, to the anterior of the embryo (Munro et al., 2004). Centrosomes locally concentrate Aurora A kinase (C.e. AIR-1), which is required for centrosome-directed displacement of the RhoGEF ECT-2 from the posterior cortex during polarization (Motegi and Sugimoto, 2006; Klinkert et al., 2019; Zhao et al., 2019; Kapoor and Kotak, 2019). However, the mechanism of action of Aurora A is not known, in particular it is not known whether Aurora A regulates ECT-2 during cytokinesis.

To address these questions we quantitatively examined ECT-2 distribution during polarization and cytokinesis in early *C. elegans* embryos. We show that ECT-2 becomes asymmetrically localized coincident with the early stages of polarity establishment, as it becomes depleted posteriorly and enriched anteriorly. The degree of ECT-2 asymmetry attenuates as the embryo progresses through mitosis and increases again during anaphase, under the influence of the posterior aster of the anaphase spindle. As during polarization, ECT-2 asymmetry during anaphase requires intact centrosomes and AIR-1; the resulting asymmetric accumulation of ECT-2 has functional consequences for cytokinesis. We identify a C-terminal ECT-2 fragment that largely recapitulates the dynamic localization of ECT-2 during early embryogenesis and identify a set of putative Aurora A sites that are required for its displacement by centrosomes and Aurora A. While phosphorylation of ECT-2 by Aurora A serves as a symmetry breaking cue, ECT-2 asymmetry also requires myosin-dependent cortical flows. Our results suggest that Aurora A-triggered phosphorylation of ECT-2 induces cortical flows which redistribute ECT-2, thereby regulating polarization and cytokinesis.

Results

Cortex-associated ECT-2 is asymmetric during cytokinesis

To dissect the mechanism by which spindle asters regulate contractility, we first examined the distribution of endogenous ECT-2 during early embryogenesis. Displacement of ECT-2 from the posterior cortex during polarization has been previously described in studies utilizing an ECT-2:GFP transgene. Collectively, these works revealed that centrosomes and/or their associated microtubules induce displacement of ECT-2 during polarization (Motegi and Sugimoto, 2006; Kotak et al., 2016; Kapoor and Kotak, 2019). To further investigate the dynamics of ECT-2 localization, we engineered a mNeonGreen (mNG) tag at the C-terminus of endogenous ECT-2 (Dickinson et al., 2013). ECT-2::mNG is well tolerated (>99% embryos hatch). During both polarization and cytokinesis, we quantified the levels of cortical ECT-2 along the entire perimeter of the embryo (Figure 1, supplement 1). For each time point, we averaged cortical ECT-2 levels in the anterior 40% and the posterior 20% of the embryo, and calculated the ratio of these values as a measure of ECT-2 asymmetry. In the early zygote, the cortical pool of ECT-2 increases and becomes progressively more asymmetric (Figure 1B i-iii). Images acquired during polarity establishment confirmed the displacement of ECT-2 from a large domain in posterior cortex and a progressive increase in the anterior cortex resulting in highly asymmetric cortical ECT-2 (Figure 1A, -480 sec (relative to anaphase onset), 1B i, ii,vi). The size of the posterior domain where ECT-2 is displaced, i.e. boundary length, represents ~35% of the embryo, measured at the time point of maximal ECT-2 asymmetry. ECT-2 accumulation on the anterior cortex is accompanied by an increase in cortical myosin accumulation (Figure 1 - figure supplement 2), cortical contractility, and transient elongation of the embryo (Figure 1B ii, v). Following pronuclei meeting and centration, overall cortical ECT-2 levels fall and the embryo rounds up (Figure 1B iv, v). ECT-2::mNG remains enriched on the anterior cortex during prophase and mitosis, but it remains on the posterior cortex and slightly increases (Figure 1A, -240 sec, 1B i-vi). During anaphase, ECT-2 becomes preferentially displaced from the posterior ~30% of the embryo, becoming more asymmetric (Figure 1C, 60 secs, 1D i-iii). As furrow ingression begins, ECT-2 is not significantly enriched on the posterior cortex above its level in the cytoplasm (Figure 1C, 100 secs). A membrane probe, mCherry-PH, which detects PIP2, also exhibits anterior enrichment, though to a lesser degree than ECT-2::mNG and its levels remain stable as anaphase progresses (Figure 1 - figure supplement 3) (Hirani et al., 2019). Regional displacement of ECT-2 repeats in subsequent cell cycles; the cortical pool of ECT-2 decreases each cell cycle near spindle poles at anaphase and recovers in the subsequent cell cycles (Figure 1 - figure supplement 4). Thus, during both polarization and cytokinesis, cortical ECT-2 is highly asymmetric.

ECT-2 distribution during cytokinesis is regulated by centrosomes

Microtubule-rich asters suppress contractility in the polar regions during cytokinesis (Werner et al., 2007). Microtubule asters also contribute to ECT-2 displacement in unperturbed embryos. During spindle elongation, a correlation between spindle rocking and ECT-2 displacement is apparent: as the spindle pole more closely approaches one region of the cortex, the ECT-2 on the cortex in that region is preferentially displaced following a short (~10 sec) delay (Figure 2A).

These results suggest that cortical levels of ECT-2 are modulated by the aster, though they do not distinguish between the role of the centrosome or the astral array of microtubules. To determine whether microtubules are required, embryos were depleted of both alpha- and beta-tubulin and treated with nocodazole to depolymerize microtubules to the greatest extent possible. Despite significant depletion of tubulin and near complete depolymerization of microtubules (Figure 2B, insets), we observed strong displacement of ECT-2 from a broad region of the posterior cortex during anaphase (Figure 2B). In these embryos, the centrosomes could be identified by the residual signal from mCherry-tubulin, and the posterior displacement was centered around the position of the centrosome. ECT-2 becomes hyper-asymmetric in these embryos as compared to control embryos (Figure 2B i) and the depleted zone comprises a larger region of the embryo, as is also observed in embryos with a diminished posteriorly positioned spindle (see below).

To further examine the role of the centrosome in ECT-2 asymmetry, we depleted the centrosomal scaffold protein SPD-5. Embryos depleted of SPD-5 lack organized arrays of microtubules, though some microtubules assemble in a poorly organized manner (Hamill et al., 2002; Pelletier et al., 2004). ECT-2 asymmetry is reduced in these embryos both during polarization, as previously reported (Kapoor and Kotak, 2019), and during cytokinesis; in particular, significant ECT-2 remains on the posterior cortex (Figure 2C iii). Collectively, these studies suggest that ECT-2 asymmetry during anaphase is centrosome-directed.

The evidence presented thus far indicates that centrosomes are the primary source of an activity that induces ECT-2 displacement during anaphase. Although the embryo contains two centrosomes, one in the anterior, the other in the posterior, ECT-2 is preferentially displaced from the posterior cortex. Two classes of explanations underlie these differential responses. The centrosomes could be functionally distinct as a consequence of the disparate ages and maturity of the embedded centrioles - assuming that centrioles and their associated centrosomes undergo reproducible and stereotyped anterior-posterior positioning in the early embryo. Alternatively, the two centrosomes may be functionally equivalent, but, due to the underlying polarity of the embryo, the posterior centrosome is more closely opposed to the posterior cortex. Indeed these possibilities are not mutually exclusive. To distinguish between

these models, we depleted embryos of PAR-3, to disrupt PAR protein polarity, allowing the centrosomes to be situated equidistant to the cortices on the anterior and posterior regions of the embryo (Etemad-Moghadam et al., 1995). Upon anaphase onset, embryos depleted of PAR-3 exhibit displacement of ECT-2 from both the anterior and posterior domains (Figure 2D), indicating that both centrosomes are competent to regulate ECT-2 accumulation. Likewise, ECT-2 asymmetry is reduced in embryos depleted of PAR-2 (Figure 2 - figure supplement 1).

Cortical flows contribute to asymmetric ECT-2 during polarization and cytokinesis

During polarization, the sperm-derived centrosomes that trigger asymmetric accumulation of ECT-2 are small, while ECT-2 is broadly displaced from the posterior region of the cortex, comprising ~35% of the embryo cortex (Figure 1, -480 sec). Likewise, during cytokinesis, the zone of ECT-2 depletion is nearly as broad, and further increased when microtubules are depolymerized (Figure 2B). These observations suggest that ECT-2 asymmetry may involve processes that amplify the local centrosomal cue.

Anterior-directed, actin-dependent, cortical flows of yolk granules, cortical myosin, and anterior PAR proteins are associated with both polarization and cytokinesis (Hird and White, 1993; Munro et al., 2004). To test whether cortical flows contribute to the displacement of ECT-2 from the posterior cortex, we examined ECT-2:mNG distribution in *nop-1* mutant embryos which have attenuated cortical flows during polarization (Rose et al., 1995). In such embryos, asymmetric accumulation of ECT-2:mNG still occurs, but the extent of its asymmetry is attenuated (Figure 3A).

To more strongly disrupt cortical flows, we attenuated non-muscle myosin, NMY-2, in embryos expressing ECT-2:mNG (Munro et al., 2004). In embryos with reduced myosin-dependent flows, ECT-2:mNG asymmetry is highly attenuated, though, in 9/11 embryos, a local inhibitory effect on ECT-2:mNG accumulation in the immediate vicinity of the centrosome is detectable; these results are consistent with an earlier report (Motegi and Sugimoto, 2006) (Figure 3A and Figure 3-Supplement 1). These results suggest that cortical flows amplify the local, centrosome-induced reduction in cortical ECT-2 accumulation.

During cytokinesis, ECT-2 activation requires two factors that function in parallel to promote RHO-1 activation, the centralspindlin subunit CYK-4 and NOP-1 (Tse et al., 2012). Redistribution of ECT-2 from the posterior cortex could reflect the displacement of ECT-2 in complex with either one or both of these activators. To test each of these possibilities, we examined the distribution of ECT-2 during cytokinesis in embryos in which the functions of NOP-1 and CYK-4 are reduced, either alone or in combination. ECT-2 displacement and asymmetric accumulation was not abolished by these perturbations (Figure 3B). Thus, basal

levels of RHO-1 activation suffice to promote displacement of ECT-2 from the posterior cortex during anaphase.

Robust cortical flows are observed during cytokinesis in embryos with attenuated astral microtubules, such as nocodazole-treated embryos (Figure 2B) (Hird and White, 1993) or embryos depleted of the assembly factor ZYG-9, the ortholog of MAP215/CH-TOG (Matthews et al., 1998). In such embryos, the spindle assembles in the posterior of the embryo, close to the posterior cortex. This perturbation results in suppression of cortical contractility in the posterior cortex, strong cortical flows of myosin towards the anterior, and formation of an ectopic furrow in the anterior at the boundary between regions of high and low myosin accumulation (Werner et al., 2007). The posteriorly positioned spindle also induces a centralspindlin-dependent furrow coincident with the spindle midzone (Werner et al., 2007). To test whether enhanced cortical flows influence ECT-2 localization, we examined ECT-2::mNG in *zyg-9(b244ts)* embryos. Under these conditions, ECT-2 becomes hyper-asymmetric with a stronger reduction in the posterior cortical pool of ECT-2, an increase in the anterior cortical pool, and an expansion of the posterior domain depleted of ECT-2 (Figure 3C). As expected, the two poles of the small spindle appear to suppress ECT-2 accumulation equally. We conclude that cortical flows contribute to ECT-2 asymmetry.

Polar relaxation was proposed to result from dynein-mediated stripping of cortical myosin that generates defects in the cortical network, resulting in anterior-directed flows, contributing to polar relaxation (Chapa-Y-Lazo et al., 2020). However, asymmetric ECT-2 accumulation is not abrogated by depletion of DHC-1, the heavy chain of cytoplasmic dynein. To the contrary, DHC-1 depletion enhances ECT-2 asymmetry, akin to the situation in embryos in which microtubule assembly is strongly suppressed, which would also be predicted compromise dynein-mediated stripping of cortical myosin (Figure 2B). Thus, ECT-2 asymmetry does not require dynein-mediated mechanical disruption of the polar actomyosin network. The enhanced asymmetry in these cases may result from the close proximity of the aberrant spindle to the posterior cortex. Thus, not only are cortical flows required for asymmetric ECT-2, increases or decreases in cortical flows enhance or attenuate the extent of ECT-2 asymmetry, respectively.

To understand how cortical flows promote the ECT-2 asymmetric accumulation of ECT-2, we sought to determine whether ECT-2 itself undergoes long range flows or if ECT-2 dynamically interacts with components that undergo such flows. To that end, we performed fluorescence recovery after photobleaching (FRAP) assays on ECT-2:mNG to measure the dynamics of its cortical association. Following bleaching, ECT-2:mNG rapidly recovers with a $t_{1/2}$ of ~3 seconds; with no obvious difference detected between the anterior and posterior cortices (not

shown). We conclude that ECT-2 asymmetry is likely to reflect the dynamic association of ECT-2 with more stably associated cortical component(s) that undergo cortical flow (Figure 3D).

Although much of ECT-2 is highly dynamic, we found one context in which ECT-2 associates more stably with the cortex. In ZYG-9-deficient embryos, despite the close proximity of centrosomes, ECT-2:mNG accumulates on the furrow that forms at the posterior pole, in a centralspindlin-dependent manner. FRAP analysis of the posterior and anterior furrows indicates that in both cases the mobile fraction of ECT-2 recovers quickly, but only ~50% of ECT-2 on the posterior furrow recovers, whereas the anterior furrow fully recovers (Figure 3 - Supplement 3).

AIR-1, an Aurora A kinase, regulates ECT-2 asymmetry during cytokinesis

To further examine the origin of ECT-2 asymmetry, we examined the impact of Aurora A kinase (AIR-1) on ECT-2 distribution during cytokinesis. AIR-1 has been shown to play a central role in embryo polarization (Klinkert et al., 2019; Zhao et al., 2019; Kapoor and Kotak, 2019). Depletion of AIR-1 by RNAi also suppresses ECT-2 asymmetry during cytokinesis (Figure 4A).

Loss of ECT-2 asymmetry upon depletion of AIR-1 may result from a direct role of AIR-1 in ECT-2 localization or because the poles of the small spindles that assemble in such embryos (Hannak et al., 2001) are more distant from the cortex. To distinguish between these possibilities, we depleted AIR-1 in embryos defective for ZYG-9 function. AIR-1 depletion greatly attenuates ECT-2 asymmetry (Figure 4C), confirming that AIR-1 promotes displacement of ECT-2 from the cortex during anaphase.

Given that AIR-1 has multiple functions in centrosome maturation, microtubule stability, and embryo polarization, it is possible that the inhibition of ECT-2 asymmetry during cytokinesis by depletion of AIR-1 protein could be an indirect consequence of these defects at an earlier stage of the cell cycle (Hannak et al., 2001; Zhao et al., 2019). To rule out these possibilities, we used a chemical inhibitor of AIR-1, MLN8237 (Sumiyoshi et al., 2015). Treatment of embryos with MLN8237 at metaphase resulted in a rapid enhancement in cortical ECT-2 accumulation during cytokinesis, indicating that AIR-1 functions during anaphase to promote ECT-2 displacement from the cortex. (Figure 4, Supplement 1).

Some, but not all, of AIR-1 functions are associated with its binding partner, TPXL-1, an ortholog of Tpx2 (Ozlü et al., 2005). Tpx2 is a direct activator of Aurora A kinase activity (Zorba et al., 2014). Furthermore, TPXL-1 prominently decorates astral microtubules (Ozlü et al., 2005), which are positioned where they could conceivably modulate cortical ECT-2 accumulation. Indeed, TPXL-1 has been proposed to promote the displacement of contractile proteins from the anterior cortex of the early *C. elegans* embryo (Mangal et al., 2018). To investigate whether an AIR-1/TPXL-1 complex plays a role in ECT-2 displacement during

cytokinesis, we depleted TPXL-1 in embryos expressing ECT-2::mNG. Cortical ECT-2 accumulation is enhanced by TPXL-1 depletion, though the degree of ECT-2 asymmetry is unaffected (Figure 4B). TPXL-1 depletion results in a number of phenotypes, including reduced spindle length during metaphase (Lewellyn et al., 2010), which might indirectly impact ECT-2 accumulation by increasing the distance between spindle pole and the cortex. To distinguish between these possibilities, we examined ECT-2 localization in embryos deficient in both ZYG-9 and TPXL-1 so that the attenuated spindle assembles close to the embryo posterior. Under these conditions, we observed robust depletion of ECT-2 at the posterior pole in *zyg-9* embryos depleted of TPXL-1, but not AIR-1 (Figure 4C). We conclude that while AIR-1 is a major regulator of the asymmetric accumulation of ECT-2, the TPXL-1/AIR-1 complex does not play a central role in this process.

To further explore the function of AIR-1 in ECT-2 regulation, we sought to enhance AIR-1 activity. To that end, we depleted PPH-6 or SAPS-1, the two major subunits of the protein phosphatase 6 (Afshar et al., 2010). PPH-6 interacts with AIR-1 and is implicated in dephosphorylation of the activation loop of AIR-1 (Kotak et al., 2016). Prior work revealed that depletion of either PPH-6 or SAPS-1 blocks pseudocleavage, reduces the accumulation of cortical myosin, and attenuates the forces that drive spindle elongation forces (Afshar et al., 2010) and more recent work demonstrates that AIR-1 is epistatic to PPH-6 (Kotak et al., 2016). As expected for a negative regulator of AIR-1, SAPS-1 depletion results in an overall reduction in cortical ECT-2 (Figure 4D). Nevertheless, the cortical pool of ECT-2 remains somewhat asymmetric in SAPS-1-depleted embryos.

The C-terminus of ECT-2 recapitulates the asymmetric distribution of ECT-2

To gain insight into the mechanism by which AIR-1 regulates ECT-2 accumulation, we sought to identify a region of ECT-2 that is sufficient to recapitulate the asymmetric accumulation of ECT-2. A C-terminal fragment of ECT-2 that extends from the N-terminus of the PH domain to the C-terminus exhibits asymmetric cortical accumulation. Further deletion analysis indicates that membrane accumulation required both the PH domain and a portion of the region C-terminal to the PH domain, but not its entirety (ECT-2^{C}) (Figure 5A). The accumulation pattern of ECT-2^{C} is similar to that of endogenous ECT-2 during both polarization and cytokinesis (Figure 5D,E). This ECT-2 fragment is also recruited to the posterior, centralspindlin-dependent furrow that forms in ZYG-9-depleted embryos (not shown). AIR-1 depletion resulted in a reduction in the degree of its asymmetric accumulation (Figure 5 - Supplement 1). These data indicate that the this C-terminal fragment of ECT-2 is sufficient to respond to the inhibitory signal from the centrosomes, and that the N-terminal regulatory domain and the DH domain of ECT-2 are dispensable for asymmetric ECT-2, and its recruitment by centralspindlin.

Putative phosphorylation sites in the PH domain impact cortical accumulation of ECT-2

As AIR-1 is required for displacement of ECT-2 from the posterior cortex, we examined whether ECT-2 contains putative AIR-1 phosphorylation sites that could regulate its association with the cortex. Using a minimal Aurora consensus site [(K/R)|(K/R)(S/T)] (i.e. at least one basic residue, 1-2 residues N-terminal to a S/T residue) (Meraldi et al., 2004), we identified 5 putative sites in ECT-2^{C}. Two putative sites were located in an Alpha-fold predicted loop in the PH domain (Jumper et al., 2021), as was an additional serine residue with a near fit to the consensus sequence (RHAS^{643}) (Figure 5 B,C). We generated GFP-tagged transgenes in which either all six sites or the three sites in the PH domain loop were mutated to alanine, a non-phosphorylatable residue. As compared to ECT-2^{C}, both variants with alanine substitutions exhibited increased cortical accumulation (Figure 5 D,E). Nevertheless, these variants accumulated in an asymmetric manner. ECT-2^{C} asymmetry temporally correlated with anteriorly-directed cortical flows (Figure 5 D,E), raising the possibility that asymmetric accumulation of endogenous ECT-2 drives flows that cause asymmetry of the transgene, irrespective of its phosphorylation status.

To test whether phosphorylation regulates the displacement of ECT-2 derivatives during polarization, we depleted non-muscle myosin, NMY-2, in embryos expressing GFP:ECT-2^{C} or GFP:ECT-2^{C_3A}. In the absence of myosin-dependent flows, the degree of GFP:ECT-2^{C} asymmetry is dramatically attenuated (Figure 5 E). In 7/9 embryos, however, a local inhibitory effect in the immediate vicinity of the centrosome is detectable. In contrast, ECT-2^{C_3A} did not exhibit significant displacement near the centrosome. These results indicate that centrosome-associated AIR-1 locally inhibits cortical ECT-2 accumulation.

To test whether ECT-2 displacement from the membrane can be triggered by Aurora A, we co-expressed a derivative of AIR-1 tagged with the GFP-binding domain (GBP::mCherry::AIR-1) with fusions of GFP:ECT-2^{C}, and GFP:ECT-2^{C_3A} (Klinkert et al., 2019). Upon anaphase onset, GBP::mCherry::AIR-1 induced displacement of GFP:ECT-2^{C} (Figure 5 F). Importantly, GFP:ECT-2^{C_3A} is refractory to GBP::mCherry::AIR-1-induced displacement (Figure 5 F). These results support the hypothesis that AIR-1 directly modulates ECT-2 localization by phosphorylation of residues within a loop in the PH domain.

AIR-1 regulates centralspindlin-dependent furrowing

The AIR-1 pathway has been shown to impact centralspindlin-independent furrowing. For example, embryos deficient in centralspindlin subunits form ingressing, NOP-1-dependent furrows during cytokinesis which fail to form upon depletion of SAPS-1/PPH-6, a complex that negatively regulates AIR-1 (Afshar et al., 2010). Conversely, depletion of AIR-1 results in ectopic furrows during pseudocleavage (Kapoor and Kotak, 2019) and cytokinesis (Figure 4A).

NOP-1, the upstream-most factor in the centralspindlin-independent pathway (Tse et al., 2012), is required for these ectopic furrows.

To extend these findings, we asked whether AIR-1 impacts centralspindlin-dependent furrowing, by depleting AIR-1 in NOP-1-deficient embryos. While embryos deficient in NOP-1 furrow to completion, ~30% embryos deficient in both AIR-1 and NOP-1 ingress with markedly slower kinetics and some fail to complete ingression (Figure 6A). These results suggest that while AIR-1 primarily impacts NOP-1-directed furrowing, it also affects centralspindlin-directed furrowing. Due to the role of AIR-1 in promoting nuclear envelope breakdown (Hachet et al., 2007; Portier et al., 2007), embryos depleted of AIR-1 appear to initiate furrowing more rapidly when NEBD is used as a reference point.

Finally, we sought to confirm that the effects resulting from manipulating AIR-1 reflects the impact of this kinase on ECT-2 specifically. To that end, we generated a strain in which one of the putative phospho-acceptor residues in ECT-2, T634, was mutated to a glutamic acid. This strain could be readily maintained as a homozygote (>98% hatch rate), indicating that this allele retains significant function, although cortical accumulation of ECT-2^{T634E} is markedly reduced and less asymmetric. As previously mentioned, NOP-1 is a non-essential gene. However, whereas NOP-1-depleted embryos complete cytokinesis with only a slight delay, embryos expressing ECT-2^{T634E} and depleted of NOP-1 exhibit highly variable furrowing behavior. While a minority of such embryos complete the first division (5/12), many embryos form furrows that ingress at variable rates and to variable extents (Figure 6B). Thus, the introduction of a single phosphomimetic residue at a putative AIR-1 site in a flexible loop in the PH domain of ECT-2 profoundly affects centralspindlin-dependent furrowing.

Discussion

During cytokinesis, cleavage furrow formation is directed by a combination of a positive signal that spatially correlates with the position of the spindle midzone, and a negative signal associated with spindle poles. The positive cue is centralspindlin-dependent and is fairly well understood; far less is known about the inhibitory cue(s) associated with spindle poles. Here, we show that the inhibitory cue is associated with centrosomes that trigger a local, Aurora A kinase dependent, inhibition of cortical ECT-2 recruitment (Figure 7A). The asymmetric, posteriorly-shifted, spindle triggers an initial focal displacement of ECT-2 from the posterior cortex by Aurora A-dependent phosphorylation of the ECT-2 PH domain. Local inhibition of cortical ECT-2 recruitment triggers anterior-directed, myosin-dependent cortical flows that amplify ECT-2 asymmetric accumulation, likely generating positive feedback (Figure 7B). Inhibition of cortical accumulation of ECT-2 by Aurora A influences both centralspindlin-dependent and -independent furrowing. These results provide insights into the mechanism of generation of cortical asymmetries during polarization and cytokinesis and extends the commonalities between the two processes.

Regulated cortical association of ECT-2

In the early *C. elegans* embryo, the RHO-1 GEF ECT-2 is enriched on the cortex relative to the cytoplasm (Motegi and Sugimoto, 2006). Following an initial period of uniform cortical association, as the centrosome approaches the cortex, ECT-2 accumulation at the posterior cortex is specifically reduced, coupled with an accompanying increase in the anterior cortex leading to a asymmetric distribution of ECT-2 (Motegi and Sugimoto, 2006). The reduction of posterior ECT-2 requires AIR-1 and is triggered by the sperm-derived centrosome (Zhao et al., 2019; Kapoor and Kotak, 2019).

A C-terminal fragment of ECT-2, lacking the regulatory BRCT domains and the catalytic GEF domain, largely recapitulates the asymmetric distribution of endogenous ECT-2 throughout the early divisions. The PH domain contained in this fragment contains a sequence, predicted to form a loop, that contains several putative sites for phosphorylation by AIR-1. Mutation of these sites to non-phosphorylatable alanine residues enhances ECT-2 cortical association and renders it resistant to the inhibitory action of AIR-1; conversely, phosphomimetic mutations reduce its cortical association. We hypothesize that AIR-1 phosphorylates these sites and that the resulting negative charges inhibit ECT-2 cortical association. The flexible loop containing these putative sites is predicted to lie adjacent to the surface where phosphoinositides bind in structurally characterized PH domains (Reckel et al., 2017). The strong effect of phosphomimetic and non-phosphorylatable mutations on ECT-2 accumulation is striking, given the predicted flexibility of this loop, lending additional support to their relevance.

Our results implicating AIR-1 in polarity establishment are largely in concordance with those of Zhao et al, with the exception that these authors did not observe an effect of AIR-1 depletion on ECT-2 localization during polarity establishment (Zhao et al., 2019). In that regard, our results align with those of Kapoor and Kotak (Kapoor and Kotak, 2019). Multiple lines of evidence implicate AIR-1 in directly impacting ECT-2 localization. These include (i) changes in cortical accumulation of ECT-2 and ECT-2^{C} upon depletion of AIR-1 and SAPS-1; (ii) increase in cortical accumulation of GFP:ECT-2^{C_3A} as compared to GFP:ECT-2^{C}; (iii) susceptibility of GFP:ECT-2^{C}, but not GFP:ECT-2^{C_3A} to GBP:mCherry:AIR-1; (iv) the T634E mutation of ECT-2 attenuates its cortical accumulation and function.

During both polarization and cytokinesis, centrosomes, by virtue of their role in concentrating and activating AIR-1(Joukov et al., 2010), appear to act as cues for ECT-2 displacement. This assertion is based on several earlier studies (Klinkert et al., 2019; Kapoor and Kotak, 2019) and the observations shown here. Specifically, during both processes, ECT-2 displacement requires the core centrosomal component SPD-5, but it is not inhibited by depolymerization of microtubules and it does not require the AIR-1 activator TPXL-1 (see below). During cytokinesis, centrosomes preferentially inhibit ECT-2 cortical association in the posterior of the embryo. This difference results from the asymmetric position of the spindle that arises as a consequence of embryo polarization - inhibition of embryo polarization by depletion of PAR-3 or PAR-2 equalizes the degree of ECT-2 inhibition on the poles. This finding also rules out differential AIR-1 activity at the two centrosomes and indicates that asymmetric centrosome-cortex distance causes asymmetric ECT-2 accumulation in wild-type embryos.

To summarize our results pertaining to cytokinesis, we plotted cortical ECT-2 accumulation as a function of centrosome-cortical distance in representative embryos from a variety of experimental conditions (Figure 7A). These data demonstrate that ECT-2 accumulation and centrosome proximity inversely correlate over a range of distances up to ~20 μm (40% of embryo length). The inhibitory effect of centrosomes on ECT-2 accumulation decays rapidly in the range of 10 to 18 μm from the cortex, which correspond to the positions of the posterior and anterior centrosomes in wild-type embryos (Kemphues et al., 1988), respectively. These findings are consistent with our earlier work demonstrating that spindle elongation regulates furrowing during cytokinesis, although we had inferred that the inhibition was due to the astral microtubules, which are proximal to the cortex (Dechant and Glotzer, 2003), rather than the centrosomes which are not.

Cortical flows amplify ECT-2 asymmetry

Centrosomes and AIR-1 kinase are required for ECT-2 asymmetry during both polarity establishment and cytokinesis. During polarity establishment in myosin-depleted embryos, the zone of inhibition of ECT-2 accumulation in the posterior shrinks considerably; similar observations have reported previously (Motegi and Sugimoto, 2006). During cytokinesis, perturbations that increase the proximity of the centrosome to the cortex, such as microtubule disassembly or ZYG-9 depletion during cytokinesis, enhance cortical flows and are associated with increased anterior accumulation of ECT-2. The ability of ECT-2^{C_3A} to polarize in the presence, but not the absence of myosin, further establishes the role of flows in amplifying ECT-2 asymmetry. These results suggest that in wild-type embryos, the initial, Aurora A-dependent symmetry breaking is amplified by anterior-directed cortical flows that enhance ECT-2 asymmetry. These findings provide a mechanistic basis for the previously described requirement for myosin in polarity establishment (Guo and Kemphues, 1996). Furthermore, ECT-2 asymmetry is predicted to enhance asymmetric assembly of cortical myosin, which is predicted to accentuate cortical flows and further amplify ECT-2 asymmetry.

Long range flows result from the viscosity of the cortical actomyosin network (Mayer et al., 2010). It is unlikely that ECT-2 is advected by these flows itself, as it exchanges rapidly, on the order of seconds. Rather, we favor a model in which cortical association of ECT-2 involves interactions with cortical component(s) with sufficient cortical viscosity to be compressed by cortical flows (Mayer et al., 2010). This would explain the anterior accumulation of ECT-2 as polarization proceeds and the enhanced ECT-2 asymmetry in embryos with exaggerated flows. The PH domain of ECT-2 has been shown to bind the actomyosin binding protein anillin (Frenette et al., 2012), making it a candidate contributor to ECT-2 cortical anchoring. Further analysis of the interactions required for ECT-2 association will be a focus of future studies.

Although RhoGEF activators accelerate these flows, basal levels of myosin appear sufficient to drive the cortical flows that polarize ECT-2. For example, inactivation of NOP-1 slows, but does not eliminate, cortical flows (Rose et al., 1995), reduces the extent of ECT-2 asymmetry and a delay in the polarization of anterior PAR proteins (Tse et al., 2012). Cortical flow-mediated concentration of ECT-2 may be central to polarization of the embryo, as experimentally induced flows are sufficient to re-orient PAR protein polarity (Mittasch et al., 2018).

During anaphase, asymmetric ECT-2 accumulation is also myosin-dependent, presumably due to its role in generating cortical flows. As during polarization, basal myosin levels appear to suffice, as ECT-2 asymmetry still increases during anaphase when both NOP-1 and CYK-4 are inactivated, raising the possibility that bulk, membrane-bound ECT-2 has a low level of RhoGEF activity.

Cell cycle regulation of ECT-2 cortical localization

The temporal pattern of ECT-2 cortical accumulation is rather dynamic. ECT-2 starts out uniform and becomes progressively asymmetric through the first interphase. Subsequently, cortical ECT-2 accumulation declines throughout maintenance phase. During cytokinesis, ECT-2 is generally displaced from the cortex, but preferentially in the posterior, coincident with spindle elongation. Given the myriad events that AIR-1 regulates in the embryo (Hannak et al., 2001), the onset of ECT-2 displacement is unlikely to reflect bulk activation of AIR-1. ECT-2 displacement from the posterior requires the centrosome to approach the cortex. Unlike during polarization, the centrosome does not become directly apposed to the cortex during cytokinesis. However, the centrosome is larger at this cell cycle stage as compared to during polarization and contains significantly more AIR-1(Hannak et al., 2001). However, given that ECT-2^{C} displacement by GBP::mCherry::AIR-1 also appears cyclical, it is also possible that the susceptibility of ECT-2 to AIR-1 is cell cycle regulated. For example, Cdk1 phosphorylation of ECT-2 might inhibit its phosphorylation by AIR-1.

TPXL-1 is not required for ECT-2 asymmetry

In addition to examining the role of AIR-1 in regulating cortical association of ECT-2, we also examined the role of one of its activators, TPXL-1. TPXL-1 targets AIR-1 to astral microtubules, which are well positioned to potentially modulate cortical ECT-2. However, TPXL-1 makes a more modest contribution to ECT-2 asymmetry than does AIR-1. Additionally, the observed effect might be an indirect consequence of the role of TPXL-1 in regulating spindle assembly and positioning. Indeed, TPXL-1 is dispensable for ECT-2 asymmetry when spindle poles are close to the cortex. This finding is consistent with earlier work that revealed that several AIR-1 functions, particularly those at the centrosome, are TPXL-1 independent (Ozlü et al., 2005; Zhao et al., 2019).

A previous study demonstrated that TPXL-1 is involved in polar relaxation during cytokinesis (Mangal et al., 2018). There are several differences between that study and the present one. Here, we assayed ECT-2 accumulation in otherwise unperturbed embryos, whereas the previous study assayed accumulation of the RHO-1 effector anillin in embryos that were depleted of both myosin II and the principle RhoGAPs that inhibit RHO-1, RGA-3/4. In that context, TPXL-1 appears to specifically inhibit accumulation of anillin in the anterior cortex; anillin does not accumulate on the posterior cortex of either wild-type or TPXL-1-depleted embryos (Mangal et al., 2018). Thus, AIR-1 appears to regulate Rho-mediated contractility at multiple steps, which are differentially dependent on both TPXL-1 and NMY-2.

Centralspindlin can facilitate ECT-2 cortical association

Despite the inhibition of ECT-2 accumulation at the posterior cortex, which is even more potent when the spindle is displaced to the posterior, a furrow can form at a site adjacent to the

spindle midzone in the embryo posterior. Formation of this furrow requires centralspindlin. Despite the overall inhibition of ECT-2 cortical association in the posterior, our results suggest centralspindlin accumulates and subsequently recruits and activates ECT-2 to induce furrowing at this site.

Cortical recruitment of ECT-2 can thus occur in two modes. One mode is independent of centralspindlin, and is inhibited by AIR-1. The other mode is dependent on centralspindlin and is relatively insensitive to AIR-1 activity. This centralspindlin-dependent mode of ECT-2 accumulation correlates with a reduced mobile fraction of ECT-2 as compared to the cortex more broadly. Centralspindlin-dependent accumulation of membrane-bound ECT-2 is reminiscent of its regulation in cultured human cells. In HeLa cells, Ect2 does not constitutively associate with the cortex in human cells, due to Cdk1-mediated phosphorylation of a basic region C-terminal to the PH domain (Su et al., 2011). During anaphase in these cells, equatorial accumulation of ECT-2 requires centralspindlin (Su et al., 2011). Thus, in both systems, phosphorylation of the C-terminus of ECT-2 attenuates its membrane association. However, the kinases are distinct (Cdk1 in human cells vs AIR-1 in *C. elegans*) and the phosphorylation appears globally active in human cells, whereas it is spatially regulated in *C. elegans*.

Mechanisms of polar relaxation

The results shown here indicate that preferential inhibition of ECT-2 accumulation by centrosomal Aurora A at one spindle pole can result in a furrow forming between the two poles. This difference in ECT-2 accumulation at the polar cortices is dictated by the asymmetric position of the spindle. However, symmetric spindles can also induce centralspindlin-independent furrowing, provided the spindle elongates beyond a critical threshold (Dechant and Glotzer, 2003). The conservation of centrosomal Aurora A and ECT-2 suggests that this phenomenon could be generalizable. In mammalian cells, Aurora A may function in parallel to Cdk1 (Su et al., 2011) to antagonize membrane association of Ect2 at certain times of the cell cycle. A recent report, relying primarily on chemical inhibitors of Aurora kinases, indicates Aurora B promotes clearance of f-actin from the polar cortex (Ramkumar et al., 2021). However, these inhibitors are not highly selective for Aurora A vs Aurora B at the concentrations used in cell-based assays (de Groot et al., 2015).

Remarkably, the highly similar kinases, Aurora A and Aurora B, regulate cytokinesis in highly complementary manners. Aurora A primarily acts to inhibit contractility at cell poles through ECT-2, while Aurora B promotes contractility at the equator through centralspindlin (Basant et al., 2015).

Limitations of this study

While our data implicate AIR-1 in phosphorylation of ECT-2, we have not directly shown that AIR-1 phosphorylates ECT-2. Additionally, while mutations of these sites affect ECT-2 localization, we have not shown that these sites are phosphorylated in vivo.

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Worm Strains

Strain	Genotype
DH244	*zyg-9(b244)* II
EG6699	*ttTi5605* II; *unc-119(ed3)* III; *oxEx1578[eft-3p::GFP + Cbr-unc-119]*
GZ1232	*unc-119(ed3)* III; *isIs44[Ppie-1::gbp::mCherry::air-1 + unc-119(+)]*
JA1559	*weIs21[Ppie-1::mCherry::tub::pie-1]*
LP229	*nmy-2(cp52[NMY-2::mKate + LoxP unc-119(+) LoxP]* I; *unc-119(ed3)* III
OD70	*unc-119(ed3)* III; *ltIs44[pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)]* V
MG576	*nop-1(it142)* III; *zuIs45 [nmy-2::NMY-2::GFP + unc-119(+)]* V; *tjIs57 [pie- 1::mCherry::HIS-58; unc-119(+)]*
MG945	*ect-2(xs116[ect-2:mNeonGreen loxP SEC LoxP flagx3])* II
MG946	*ect-2(xs117[ect-2:mNeonGreen:flagx3])* II
MG961	*ect-2(xs117[ect-2:mNeonGreen:flagx3])* II; *tIs44[pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)]* V
MG980	*ect-2(xs117[ect-2:mNeonGreen:flagx3])* II; *weIs21[Ppie-1::mCherry::tub::pie-1]*
MG982	*zyg-9(b244) ect-2(xs122[ect-2:mNeonGreen:flagx3])* II; *weIs21[Ppie-1::mCherry::tub::pie-1]*
MG984	*ect-2(xs117[ect-2:mNeonGreen:flagx3])* II; *nop-1(it142)* III; *weIs21[Ppie-1::mCherry::tub::pie-1]*
MG989	*nmy-2(cp52[nmy-2::mKate])* I; *ect-2(xs117[ect-2:mNeonGreen:flagx3])* II
MG995	*xsSi53[cb-unc-119 (+) Ppie-1::gfp:ect-2(559-828aa)]* II; *unc-119(ed3)* III
MG1043	*ect-2(xs132[ect-2(*T634E*):mNeonGreen:flagx3])* II; *weIs21[Ppie-1:mCherry:tubulin:pie-1]*
MG1044	*nmy-2(cp52[nmy-2:mKate])* I; *ect-2(xs132[ect-2(*T634E*):mNeonGreen:flagx3])* II
MG1067	*xsSi78[Ppie-1:gfp:ect-2(559-828aa)(*T561A T576A S630A T634A S643A S668A*) unc-119(+)]*II*; unc-119(ed3)* III
MG1070	*xsSi53[cb-unc-119 (+) Ppie-1:gfp:ect-2(559-828aa)]II; unc-119(ed3)* III; *isIs44[Ppie-1::gbp::mCherry::air-1 + unc-119(+)]*
MG1074	*xsSi79[Ppie-1:gfp:ect-2(559-828aa(*S630A T634A S643A*) unc-119(+)]* II; *unc-119(ed3)* III
MG1078	*xsSi79[Ppie-1:gfp:ect-2(559-828aa(*S630A T634A S643A*) unc-119(+)]* II; *unc-119(ed3)* III; *isIs44[Ppie-1::gbp::mCherry::air-1 + unc-119(+)]*

Plasmids

Plasmid	Description
pDD162	pEFT-3 Cas9 + sgRNA
pDD268	mNG-C1-^SEC^3xFlag CCD
pKL226	pEFT-3 Cas9 + ECT-2 sgRNA
pKL265	ECT-2 mNG-C1-^SEC^3xFlag
pKL286	pPIE-1-GFP-ECT-2(559-828aa)-pie-1 3'utr
pKL463	pPIE-1-GFP-ECT-2(559-828aa)(T561A T576A S630A T634A S643A S668A)-pie-1 3’utr
pKL465	pPIE-1-GFP-ECT-2(559-828aa)(S630A T634A S643A)-pie-1 3'utr
pKL425	tpxl-1(50-395aa)_L4440
pKL468	tba-2+tbb-2_L4440

Materials and Methods

Plasmid and Strain Construction

MosSCI transgenic strains: *C. elegans* strains expressing GFP:ECT-2^{C} and GFP:ECT-2$^{C\text{-}3A}$, and GFP:ECT-2$^{C\text{-}6A}$ using the promoter and 3'utr elements from *pie-1*. These transgenes were integrated into ttTi5605 on Chromosome II using MosSCI (Frøkjaer-Jensen et al., 2008; Frøkjaer-Jensen et al., 2012). Plasmids were built using Gibson Assembly (Gibson et al., 2009)

CRISPR: mNeonGreen was integrated at the C-terminus of endogenous ECT-2 using CRISPR (Dickinson et al., 2015) . ECT-2^{T634E} was mutated at the endogenous locus by CRISPR using Cas9 ribonucleoprotein [IDT] complexes and a single-stranded oligodeoxynucleotide (CAGCGAGAAGAATgaAATGCAACGTCTAGCTCGTCAtGCGTCGTTTGCgAGTTTACATTC)(Paix et al., 2015; Dokshin et al., 2018).

RNAi Construction: TPXL-1(50-395aa)/L4440 was amplified from N2 lysates and inserted into L4440 between SacI and SpeI by Gibson Assembly (Gibson et al., 2009). For TBA-2+TBB-2/L4440, the TBB-2 fragment from TBB-2/L4440 (Ahringer library) was amplified and inserted into the KpnI site of TBA-2/L4440 (Ahringer library) by ligation.

RNAi

Ahringer library: *par-3, air-1, spd-5, saps-1, dhc-1, perm-1, nop-1,* and *zyg-9* (Kamath et al., 2003)

tpxl-1 [this study], *tba-2*+*tbb-2* [this study], *cyk-4*(Zhang and Glotzer, 2015),and *nmy-2* (from Ed Munro)

For all experiments, RNAi plasmids were transformed into HT115 competent cells and cultured for 6-8hrs in LB+Amp at 37C; Cultures were seeded onto NGM Amp + IPTG plates and was incubated overnight at room temperature before storage at 4C.

For *perm-1*: A PERM-1/L4440 culture was diluted [1:20] with empty vector L4440 culture (Carvalho et al., 2011). The mixture was plated on NGM Amp+IPTG plates and let sit overnight before storing at 4C; For experiments, L4 worms were transferred to plates about 16 hours prior to imaging.

For all experiments except as indicated, L4 hermaphrodites were transferred to RNAi plates for ~24hrs before imaging.

RNAi-treated embryos were phenotypically assessed for sufficient depletion. These phenotypes were assessed using Nomarski or mCherry:Tubulin. Embryos that did not show these phenotypes were excluded from analysis:

air-1(RNAi): Mitotic spindle fails to properly form; hypercontractility during polarization (Hannak et al., 2001)

cyk-4(RNAi): Loss of bundling of anti-parallel microtubules upon anaphase onset, exaggerated anaphase B (Jantsch-Plunger et al., 2000)

dhc-1(RNAi): Lack of pronuclear migration and centrosomes remained in posterior during polarization and cytokinesis (Gönczy et al., 1999)

nmy-2(RNAi)(partial): Lack of pseudocleavage and contractility during polarization (Munro et al., 2004)

nop-1(RNAi): Lack of pseudocleavage (Rose et al., 1995; Tse et al., 2012)

par-3(RNAi): Symmetric first division (Etemad-Moghadam et al., 1995)

saps-1(RNAi): Absence of spindle rocking during anaphase (Afshar et al., 2010)

spd-5(RNAi): Lack of pronuclear migration and spindle assembly failure (Hamill et al., 2002)

tba-2+tbb-2(RNAi) +nocodazole: Centrosomes remained in posterior during polarization and cytokinesis and asters did not separate during cytokinesis (Tsai and Ahringer, 2007)
tpxl-1(RNAi): Small mitotic spindle (Ozlü et al., 2005)
zyg-9(RNAi): Spindle assembles in posterior of the embryo

Imaging

Mounting:

Generally, gravid *C. elegans* were dissected in egg salts [5 mM HEPES pH 7.4, 118 mM NaCl, 40 mM KCl, 3.4 mM $MgCl_2$, 3.4 mM $CaCl_2$] and mounted on 2% agarose pads. For tubulin experiments: *C. elegans* were dissected in egg salts plus 50µg/mL nocodazole or DMSO.

For MLN8237 inhibitor experiment: After *perm-1* depletion, *C. elegans* were dissected in embryonic imaging medium [50% L-15 Leibovitz's Medium (Gibco), 10% 5mg/mL Inulin, 20% FBS (Gibco), 20% 250mM HEPES pH 7.5] on a Poly-D-Lysine (R&D Systems)-coated coverslip mounted on a slide with double-sided tape (3M) used as spacer. Additional imaging media was flowed in to fill the chamber. 50 µM MLN8237 or DMSO was flowed into the embryonic chamber at 100s post NEB while imaging.

For imaging early polarization: Worms were dissected into embryonic imaging medium [50% L-15 Leibovitz's Medium (Gibco), 10% 5mg/mL Inulin, 20% FBS, 20% 250mM HEPES, pH 7.5] on a Poly-D-Lysine (R&D Systems)-coated coverslip mounted on a slide with double-sided tape (3M) used as spacer. Additional media was flowed in to fill the chamber before imaging.

Microscopy

Time lapse recordings of embryos were acquired with a Zeiss Axioimager M1 equipped with a Yokogawa CSU-X1 spinning disk unit (Solamere) using 50mW 488nm and 561nm lasers (Coherent) and a Prime 95B camera (Photometrics). Images were taken at 63x using Micromanager or Metamorph software. ECT-2:mNG was imaged at 10s intervals during cytokinesis and at 20s intervals during polarization. NMY-2:GFP was imaged at 10s intervals during polarization and cytokinesis.

FRAP experiments were performed on a Zeiss LSM 880 equipped with AiryScan on confocal setting using a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Images were taken at 100ms intervals.

Image analysis

All embryos were processed and analyzed using FIJI. Further analysis and graphs were generated using RStudio with the following packages: tidyverse, gcookbook, rlang, mgcv, and zoo (Wickham et al., 2019).

ECT-2 Accumulation:

For each embryo, at every time point, the background and cytoplasm average intensities were measured. The embryonic membrane was identified by performing a gaussian blur on a copy of the embryo, using the FIJI magic wand to select the embryo. This area was converted to a line, which was straightened from the middle of the anterior of the embryo clockwise around the perimeter. A maximum intensity was measured at each pixel point along the line. See schematic (Figure 1, Supplement 1) for details.

Raw intensity measurements were normalized and graphed. Measurements were normalized using the background measurement and related to normalized cytoplasm intensity (Accumulation). For anterior accumulation, accumulation was averaged across 40% of the anterior of the embryo. For posterior accumulation, accumulation was averaged across 15% of the posterior of the embryo. The Anterior:Posterior Ratio was calculated as a ratio between Anterior Accumulation and Posterior Accumulation. Average Accumulation is the average membrane accumulation of the entire embryo related to normalized average cytoplasm intensity. The perimeter was measured as the length of the ROI used to identify the embryonic membrane at every time point. The perimeter was normalized to the maximum perimeter of each embryo. For Boundary Length, embryo accumulation across all positions was fitted to a regression model by GAM (Prediction Accumulation) in R Studio. The number of positions that fell below a threshold of 1.2 (for ECT-2:mNG cytokinesis and GFP:ECT-2^{C} polarization) or 1.5 (for ECT-2:mNG polarization) in the fitted curve were counted and the fraction of positions 'below threshold' was calculated.

Furrow Kinetics:

The position of the furrow tips in NMY-2:GFP or NMY-2:mKate embryos was measured at 10s intervals starting at NEBD or anaphase. The distance between the furrow tips was normalized to maximum embryo width [Furrow Width]. Furrow Width was plotted as a rolling average (period = 3) over time for individual embryos.

FRAP:

The FRAP images were analyzed using FIJI. The FRAP Analysis Script source is https://imagej.net/imagej-wiki-static/Analyze_FRAP_movies_with_a_Jython_script .

The coefficients from the fitted recovery curves of each individual embryo were averaged for each condition and the average curve was plotted.

Centrosome-Cortical Distance:

Three embryos of each treatment indicated in the legend, were chosen at random and, the time point of maximally asymmetric ECT-2 accumulation was selected for measurement. Centrosome distance to the nearest cortex was measured using the mCherry:Tubulin or Nomarski images. This distance and the average ECT-2 accumulation at the nearest cortex were plotted.

Structural analysis:

The MatchMaker function of UCSF Chimera (Pettersen et al., 2004) (https://www.cgl.ucsf.edu/chimera/) was used to align the predicted PH domain of C.e. ECT-2 (https://alphafold.com/entry/Q9U364) to 5OC7(Reckel et al., 2017).

Figure Legends

Figure 1. Cortical ECT-2 becomes asymmetric during polarization and cytokinesis

A. ECT-2 accumulation is asymmetric during polarization. Selected time points from a representative embryo expressing ECT-2:mNeonGreen (mNG) during polarity establishment. Time zero represents anaphase. Blue circles indicate pronuclei and red asterisks label the approximate locations of the centrosomes. All scale bars represent 10 μm.

B. Quantification of (i) Average Anterior:Posterior Ratio (ratio of Anterior and Posterior Membrane Accumulation (ii) Average Anterior Membrane Accumulation, (iii) Average Posterior Membrane Accumulation, (iv) Average Membrane Accumulation over the entire membrane, and (v) Normalized Perimeter of embryos over time (s). n=8; Solid line is the average of all embryos measured and the shaded ribbon represents the 95% Confidence Interval (95CI). (vi) Box plot of Boundary Length. Boundary Length is the fraction of the embryo cortex with normalized ECT-2 levels below a threshold of 1.5. This parameter is calculated at the time point of the maximum Anterior:Posterior Ratio of each embryo (see methods for details).

C. ECT-2 accumulation is asymmetric during cytokinesis. A representative embryo expressing endogenously tagged ECT-2:mNeonGreen (mNG) at the indicated time points during cytokinesis beginning at anaphase onset (0s). Asterisks label the approximate locations of the centrosomes.

D. Quantification of the (i) Average Anterior:Posterior Ratio (ii) Average Anterior Membrane Accumulation, (iii) Average Posterior Membrane Accumulation, and (iv) Average Membrane Accumulation. n=11; Solid line represents the average of all embryos and the shaded ribbon represents the 95% Confidence Interval (95CI). (v) Box plot of Boundary Length at the most asymmetric time point of each embryo.

Figure 1 Supplemental Figures

Supplement 1

Schematic describing quantitation of ECT-2 membrane accumulation. Average intensity of the background and the cytoplasm were taken at every time point in a time series of an embryo. The embryo boundaries were recognized and a 50 pixel line centered on the membrane was straightened starting from the anterior of the embryo and proceeds around the embryo perimeter clockwise through the posterior and returning to the anterior. From the straightened membrane, a 3 pixel-wide line was drawn perpendicular to the membrane and the maximum intensity was taken at each position along the line (Raw intensity). Accumulation was calculated for every position using (maximum intensity-background)/(cytoplasm-background). The measurements of anterior and posterior accumulation reflect the average accumulation in the anterior 40% or the posterior 15% of the embryo, respectively.

Supplement 2

Co-expression of ECT-2:mNG and NMY-2:mKate in a time series with select time points selected for stills. Composite includes ECT-2:mNG in green and NMY-2:mKate in magenta. Anaphase onset is set to t=0.

Supplement 3

ECT-2 is more asymmetric than a membrane marker. ECT-2:mNG; mCherry:PH embryos were imaged during cytokinesis; stills were taken from a single time lapse. Graphs show the asymmetric accumulation ratio, anterior accumulation, and posterior accumulation over time.

Supplement 4

ECT-2 accumulates in a asymmetric manner on the membrane in multi-cellular embryos Shown is an embryo with ECT-2:mNG imaged every 30 seconds for several minutes. The inset of mCherry:Tubulin was imaged concurrently. Arrows pointing to observed changes in ECT-2 accumulation. Time 0s is anaphase onset of the indicated blastomere.

Figure 2. ECT-2 asymmetry is responsive to the position of the spindle

A. A close-up view of the localization of endogenously tagged ECT-2 and tubulin during first cleavage. Images from a time-lapse series show the distribution of ECT-2:mNG and mCherry:Tubulin. Composite images are shown: ECT-2 shown in using the displayed lookup table; the tubulin signal is displayed with inverted grayscale. In the overlays, the tubulin image is advanced by 10s relative to ECT-2. Arrows highlight two sites where local accumulation of ECT-2 declines ~10 sec after the spindle approaches, as indicated by the color change in the arrows from green to red.

B. ECT-2:mNG localization in embryos under control [L4440 +DMSO] or experimental [*tba-2*+*tbb-2*(RNAi) + 50 µg/mL nocodazole] conditions. The images are stills from a time-lapse showing ECT-2:mNG localization while the insets are mCherry:Tubulin images acquired concurrently. Asterisks label the approximate locations of the asters. The graphs measure the anterior:posterior accumulation ratio, anterior accumulation, and posterior accumulation of ECT-2 relative to the cytoplasm over time starting at NEBD. The box-plot graphs posterior Boundary Length of ECT-2 at the maximum polarized time point.

C. ECT-2 localization in a *spd-5*(RNAi) or wild-type embryo with stills beginning at NEBD. The insets contain mCherry:Tubulin at the same time points. Asterisks label the approximate locations of the asters. The graphs measure the anterior:posterior accumulation ratio, anterior accumulation, and posterior accumulation of ECT-2 relative to the cytoplasm over time.

D. ECT-2 localization in a *par-3*(RNAi) or wild-type embryo with images starting at anaphase onset. Asterisks label the approximate locations of the asters. The graphs measure the anterior:posterior accumulation ratio, anterior accumulation, and posterior accumulation of ECT-2 relative to the cytoplasm over time.

Figure 2 Supplemental Figure

Supplement 1

ECT-2 localization in a *par-2*(RNAi) or L4440-treated embryo with images starting at anaphase onset. Asterisks label the approximate locations of the asters. The graphs measure the anterior:posterior accumulation ratio, anterior accumulation, and posterior accumulation of ECT-2 relative to the cytoplasm over time (anaphase onset is 0s).

Figure 3. Cortical flows contribute to cortical ECT-2 asymmetry

A. Asymmetric accumulation of ECT-2 during polarization is reduced when cortical flows are reduced. Images from a time-lapse series show ECT-2 localization during polarization in a Wild-type, *nmy-2*(RNAi), or *nop-1(it142)* embryo. The pronuculei are labeled with a blue circle and the asterisks label the approximate locations of the asters. Graphs show the anterior:posterior accumulation ratio, anterior accumulation, and posterior accumulation of ECT-2 over time. The box-plot depicts the posterior ECT-2 boundary at the time point of maximal asymmetry.

B. Asymmetric ECT-2 accumulation is independent of its activators during cytokinesis. Images are stills from time-lapse movies depicting endogenous ECT-2 localization in Wild-type, *nop-1(it142)*, *cyk-4(*RNAi), and *nop-1(it142); cyk-4*(RNAi) embryos with time 0 as anaphase onset. Asterisks label the approximate locations of the asters. Graphs measure ECT-2 accumulation relative to average cytoplasm intensity.

C. The localization of endogenously tagged ECT-2 during cytokinesis is hyper-asymmetric in *zyg-9(b244)* embryos. Images from a time-lapse series show the distribution of ECT-2:mNG in either wild-type or *zyg-9(b244)* the latter which results in a posteriorly localized small transverse spindle. Times shown are relative to anaphase onset, with the exception of NEB which is approximately ~125 sec prior to NEB. Asterisks label the approximate locations of the asters. Graphs measure ECT-2 accumulation relative to average cytoplasm intensity.

D. FRAP recovery of ECT-2 at the anterior membrane. A region of the anterior membrane was imaged and a small ROI was bleached. 25 images were taken at 100ms intervals before the region was bleached followed by 175 images were taken at 100ms intervals to document the recovery. The kymograph qualitatively shows the FRAP recovery and the graph shows fitted curves of the recovery of each embryo measured. Recovery was fitted to $y = a*(1-\exp(-b*x))$. The bold line is the average of all the embryos ($T_{1/2}$ ~ 3s).

Figure 3 Supplemental Figures

Supplement 1

Asymmetric ECT-2 accumulation is reduced in partial *nmy-2*(RNAi) depleted embryos. Asterisks label the approximate locations of the asters. Graphs measure ECT-2 accumulation relative to average cytoplasm intensity.

Supplement 2

ECT-2 asymmetry does not require dynein heavy chain function.
The localization of endogenously tagged ECT-2 during cytokinesis. Images from a time-lapse series show the distribution of ECT-2:mNG in either wild-type or embryos depleted of dynein heavy chain, DHC-1, which results in a collapsed and posteriorly localized spindle (insets depict mCherry:Tubulin). Times shown are relative to NEB. Quantitation of the accumulation of ECT-2:mNG normalized to cytoplasmic levels.

Supplement 3

FRAP recovery of the anterior and posterior furrows in a ECT-2:mNG *zyg-9*(RNAi) embryo. Shown is an image of an embryo taken after FRAP was completed. A region of the anterior furrow was bleached and a time lapse of the recovery was taken followed by a region of the posterior furrow was bleached and another time lapse was taken to document the recovery. The graph shows fitted curves the recoveries of individual embryos as well as the average fitted curve in bold. The fit curve equation $y = a*(1-\exp(-b*x))$ where the averages of a and b were used to graph both the Anterior and Posterior furrows (n=7 and 4 embryos, respectively).

Figure 4. Asymmetric ECT-2 accumulation requires AIR-1

A. The localization of endogenously tagged ECT-2 during cytokinesis. Images from a time-lapse series show the distribution of ECT-2:mNG in either wild-type or embryos depleted of Aurora A kinase, AIR-1 by RNAi. Quantitation of the accumulation of ECT-2:mNG normalized to cytoplasmic levels. Note that NEBD is delayed in AIR-1-depleted embryos resulting in contractility at an earlier time point (Hachet et al., 2007; Portier et al., 2007).

B. TPXL-1 contributes to inhibition of ECT-2 accumulation. Images from a time-lapse series show the accumulation of ECT-2:mNG in either a wild-type or *tpxl-1*(RNAi) embryo. Graphs quantify the accumulation of ECT-2 relative to cytoplasm.

C. AIR-1, but not TPXL-1, is required for inhibition of ECT-2 accumulation. ECT-2 was imaged in a *zyg-9(b244)* background with depletion of AIR-1, TPXL-1, or none. The stills represent several time points from a time lapse of a representative embryo with NEBD set as time 0. The graphs depict ECT-2 accumulation relative to the cytoplasm and the box-plot quantifies the boundary length of ECT-2 inhibition. Note that NEBD is delayed in AIR-1-depleted embryos resulting in contractility at an earlier time point (Hachet et al., 2007; Portier et al., 2007).

D. ECT-2 membrane accumulation is reduced when AIR-1 activity is increased by *saps-1*(RNAi). Images from a time-lapse series show the accumulation of ECT-2:mNG in either a wild-type or *saps-1*(RNAi) embryo with anaphase onset as time 0. Quantitation shows ECT-2 accumulation over time.

Figure 4 Supplemental Figures

Supplement 1

Addition of the AIR-1 inhibitor, MLN8237, causes an increase in cortical levels of ECT-2:mNG. Time series were taken of embryos depleted of PERM-1. At about 90s post NEBD, either DMSO or 50 μM MLN8237 in embryonic medium was flowed into the embryo chamber. In the graphs, cortical ECT-2 accumulation was quantified over time.

Figure 5. Phosphorylation of ECT-2 regulates its asymmetric cortical accumulation

A. Schematic of GFP:ECT-2 truncations tested in this study.

B. Schematic of ECT-2^{C} with locations of mutated residues.

C. Alpha Fold prediction of the ECT-2 PH domain prediction (AF-Q9U364-F1 aligned with the crystal structure of the PH domain of Bcr-Abl (PDB 5OC7).

D. Stills from representative time series of GFP:ECT-2^{C} either Wild-type, 3A, or 6A mutants and the quantitation of accumulation of each transgene with NEBD as time 0.

E. Asymmetric accumulation of GFP:ECT-2^{C} is lost in the 3A mutant when cortical flows are reduced. GFP:ECT-2^{C} was imaged in the Wild-type and 3A transgenic lines with either L4440 control or NMY-2 depleted. The graphs are split with (i) - (iii) quantifying GFP:ECT-2^{C} Wild-type accumulation over time and (iv) - (vi) quantifying GFP:ECT-$2^{C\text{-}3A}$ accumulation over time starting at NEBD. The boundary length was measured for all conditions at the time point of maximal asymmetry.

F. GFP:ECT-$2^{C\text{-}3A}$, but not GFP:ECT-$2^{C\text{-}WT}$, retains membrane accumulation during cytokinesis when co-expressed with GBP:mCherry:AIR-1. Panels show stills from embryo time series with GFP:ECT-$2^{C\text{-}WT}$ or GFP:ECT-$2^{C\text{-}3A}$ in strains co-expressing GBP:mCherry:AIR-1. The graphs reflect GFP:ECT-2^{C} membrane accumulation over time starting at anaphase onset. Accumulation on the spindle was not included in measurements.

Figure 5. Supplemental Figures

Supplement 1

GFP:ECT-2^{C} was imaged as a time series. Shown are still from either a wild-type or AIR-1-depleted embryo. Graphs depict GFP:ECT-2^{C} accumulation over time starting at NEBD. Note that NEBD is delayed in AIR-1-depleted embryos resulting in contractility at an earlier time point (Hachet et al., 2007; Portier et al., 2007).

Figure 6. AIR-1 is involved in central-spindle dependent furrowing

A. Furrowing is variable in *nop-1(it142); air-1*(RNAi) embryos. Shown are cortical maximum intensity projections 6μm deep of NMY-2:GFP; *nop-1(it142)* embryos with or without depletion of AIR-1. Furrow width as a function of time starting at NEBD in individual embryos is shown graphically using a rolling average period of 3. Note that NEBD is delayed in AIR-1-depleted embryos resulting in contractility at an earlier time point.

B. Cortical accumulation of ECT-2^{T634E} is reduced compared to wild-type and this variant affects central spindle-dependent furrowing. Embryo images are from time series of ECT-2:mNG or ECT-2^{T634E}:mNG embryos in strains co-expressing mCherry:Tubulin. (i-iii) Graphs represent ECT-2 accumulation as a function of time with anaphase onset as time 0s. (iv-v) Furrow kinetics of ECT-2:mNG or ECT-2^{T634E}:mNG in strains co-expressing NMY-2:mKate (NMY-2:mKate was used to manually mark the furrow tips to measure furrow width). Graphs represent the extent of furrow ingression (furrow width/embryo width) of individual embryos as a function of time starting at anaphase onset using a rolling average period of 3.

Figure 7. Model of local symmetry breaking of cortical ECT-2 and amplification by cortical flows

A. Summary graph of cortical ECT-2 accumulation at the time of maximal ECT-2 asymmetry as a function of proximal centrosome-cortical distance. Centrosome-cortex distance was measured using either a mCherry:Tubulin or Nomarski image. Three embryos from each treatment listed in the legend were chosen at random.

B. During both polarization and cytokinesis, centrosome-derived AIR-1 locally inhibits cortical ECT-2 accumulation, triggering symmetry breaking and generation of cortical flows directed away from the site of symmetry breaking. ECT-2 dynamically associates with components that undergo cortical flow, leading to its further depletion from the posterior cortex and its anterior accumulation. During polarity establishment, the centrosomes are small and closely apposed to the cortex, whereas during anaphase the centrosomes are large and accumulate significant AIR-1 that modulate cortical association of ECT-2. During polarization, the pseudocleavage furrow forms at the boundary between low and high ECT-2 and myosin levels; in cytokinesis, this site correlates with the centralspindlin-dependent furrow that correlates with the spindle midzone.

C. Proposed genetic pathway.

Figure 1
ECT-2 accumulates in an asymmetric manner on the membrane

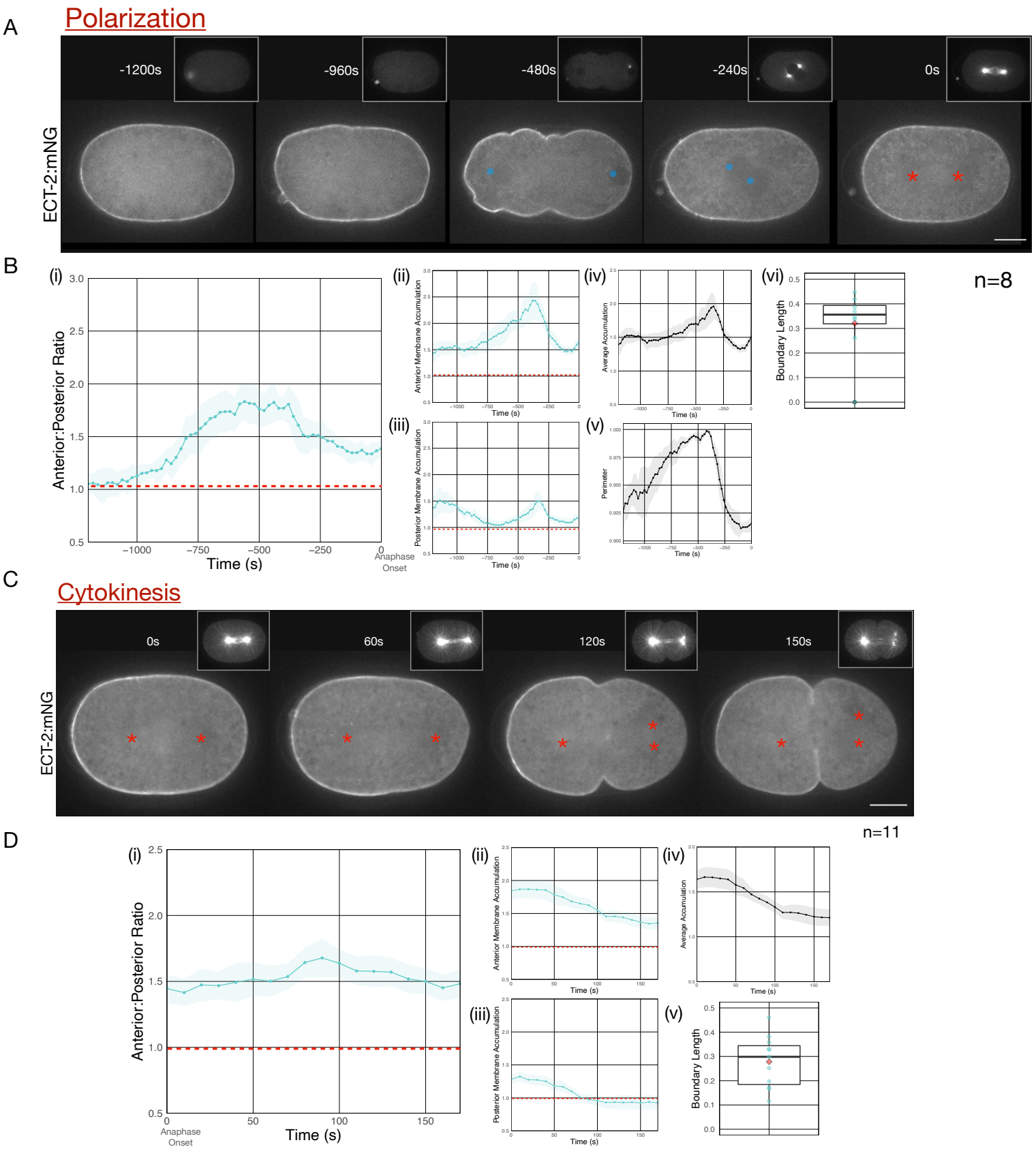
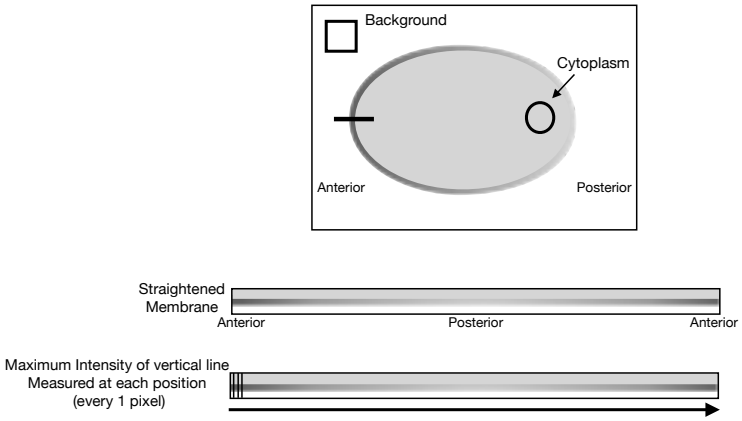


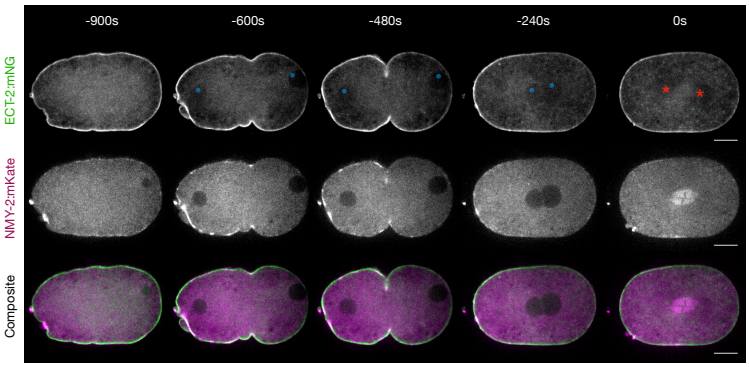
Figure 1 Supplement
ECT-2 accumulates in a polarized manner on the membrane

Supplement 1
Schematic of Quantitation

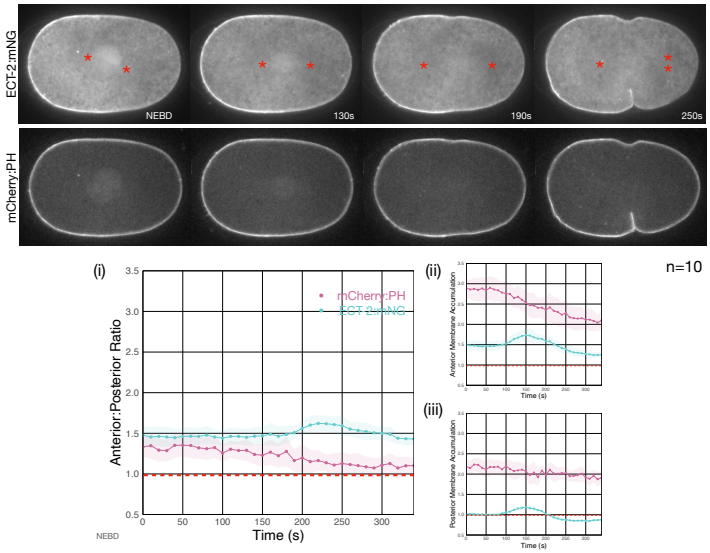


Accumulation = (Maximum Intensity - Background) / (Cytoplasm - Background)

Supplement 2
Co-expression of ECT-2:mNG and NMY-2:mKate during polarization



Supplement 3
ECT-2 is more asymmetric than a membrane marker



Supplement 4
ECT-2 accumulates asymmetrically on the membrane

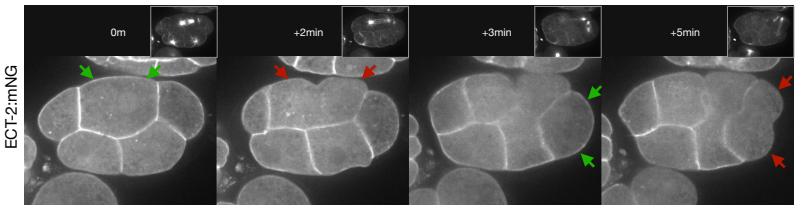
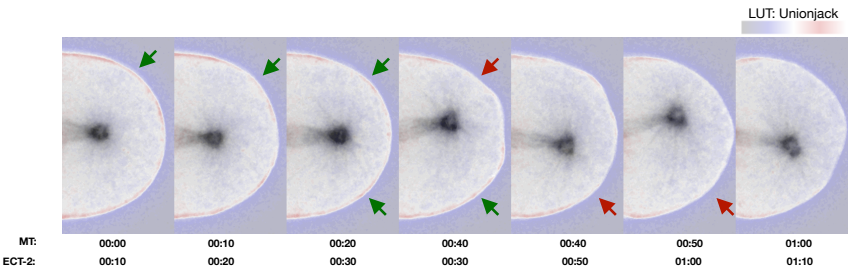
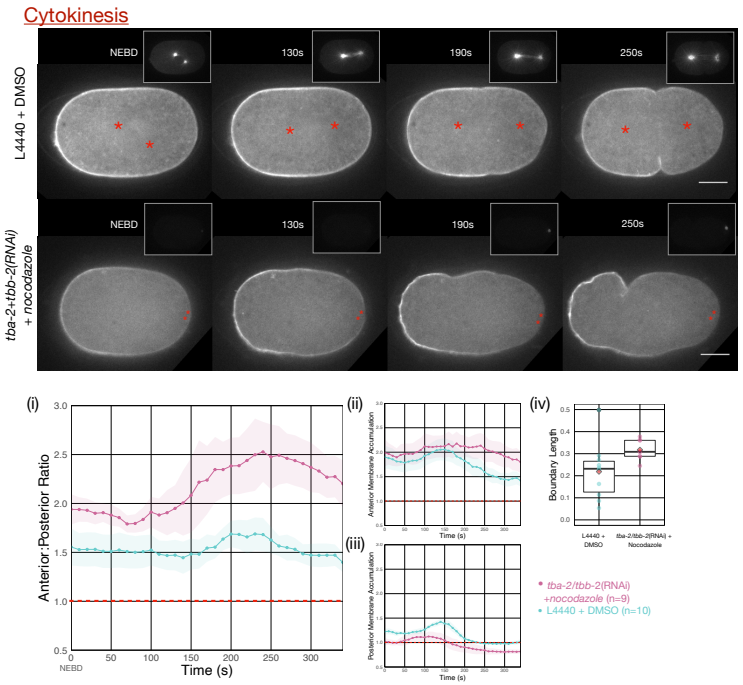


Figure 2
ECT-2 asymmetry is responsive to the position of the spindle

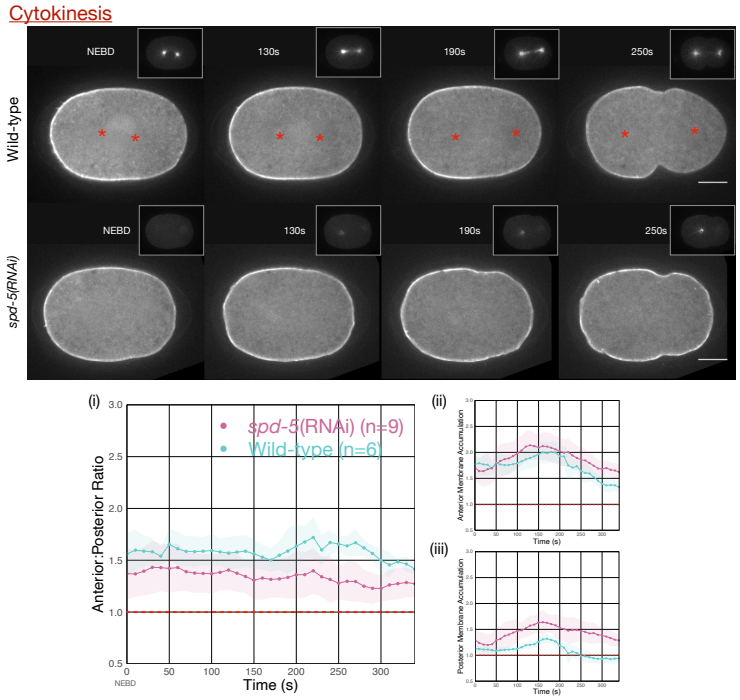
A



B



C



D

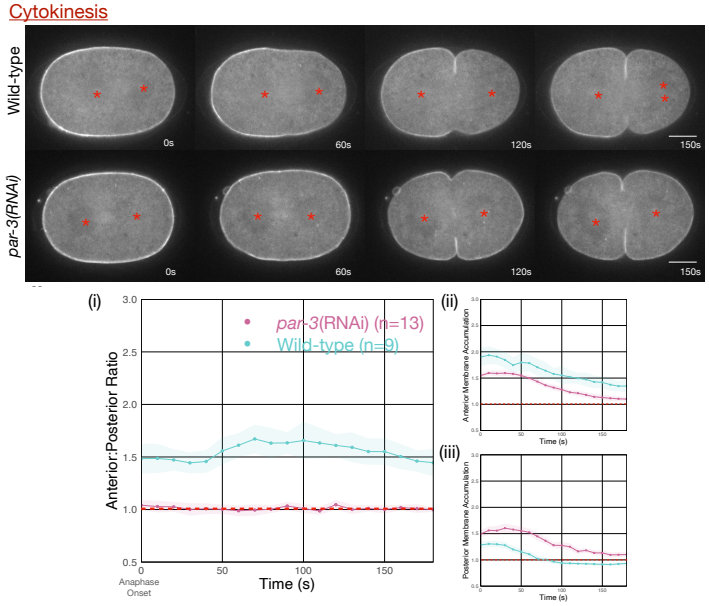


Figure 2
ECT-2 asymmetry is responsive to the position of the spindle

Supplement 1
ECT-2 asymmetry is responsive to spindle position

Cytokinesis

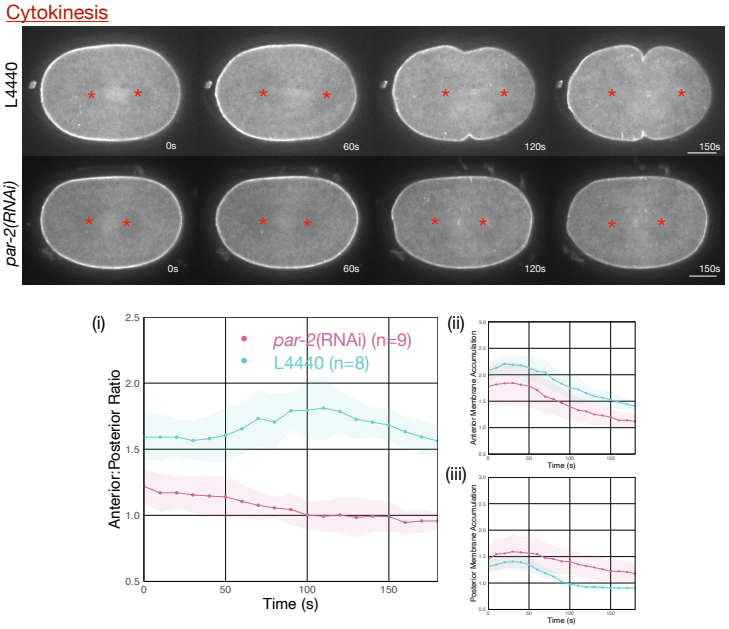


Figure 3
Cortical flows contribute to ECT-2 membrane accumulation

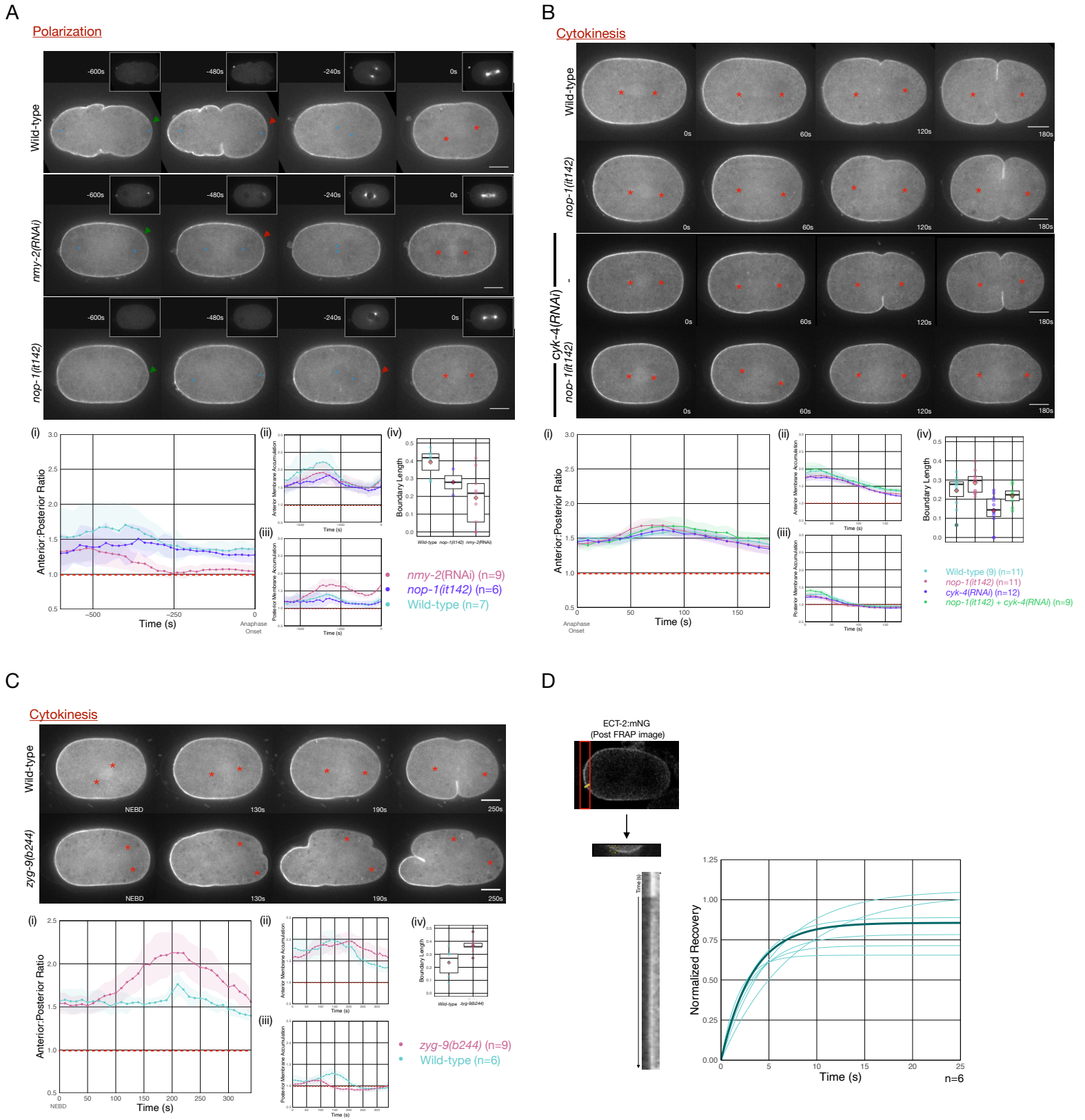
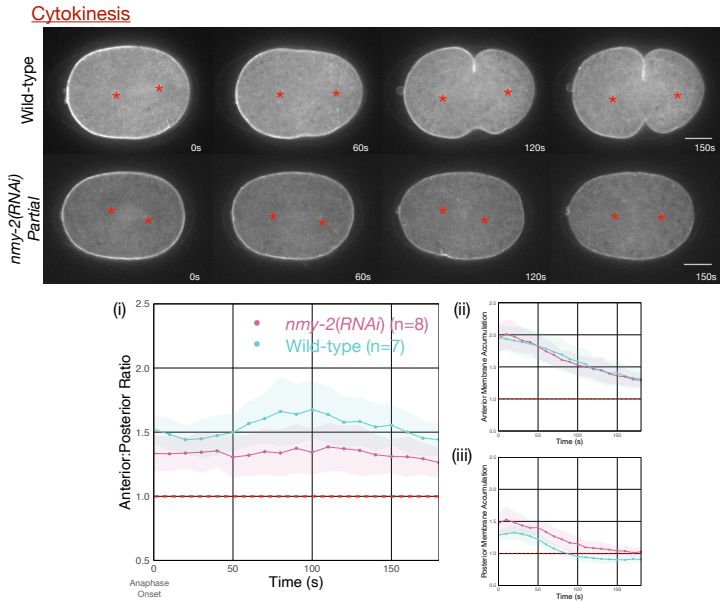
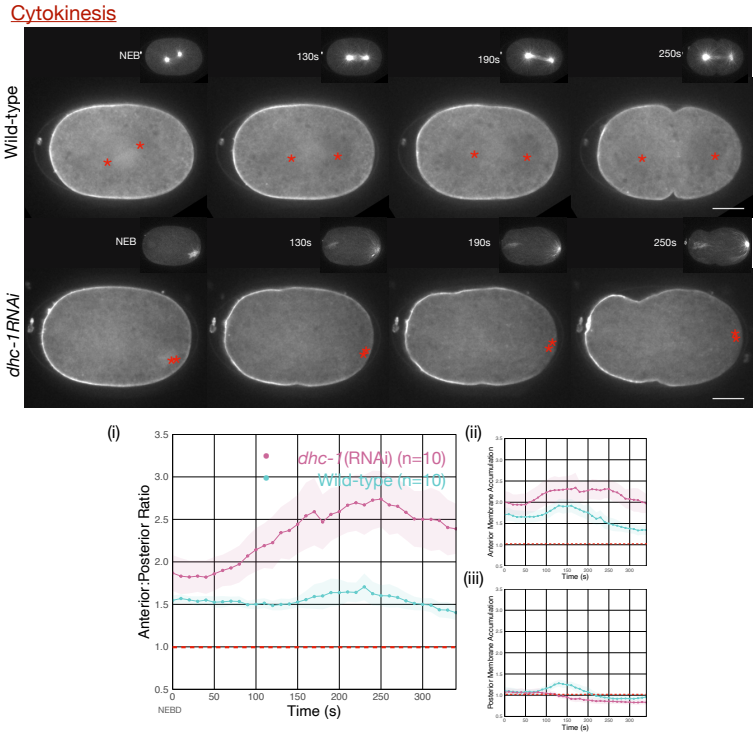


Figure 3 Supplement
Cortical flows contribute to ECT-2 membrane accumulation

Supplement 1
Asymmetric accumulation of ECT-2 is reduced in NMY-2 partially depleted embryos



Supplement 2
Asymmetric accumulation of ECT-2 does not require functional dynein



Supplement 3
ECT-2 exchange properties differ between central spindle-dependent and -independent furrows

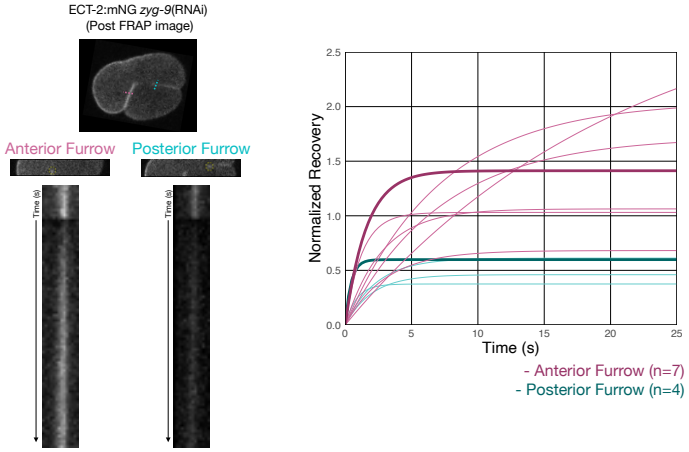


Figure 4
Asymmetric accumulation of ECT-2 requires AIR-1

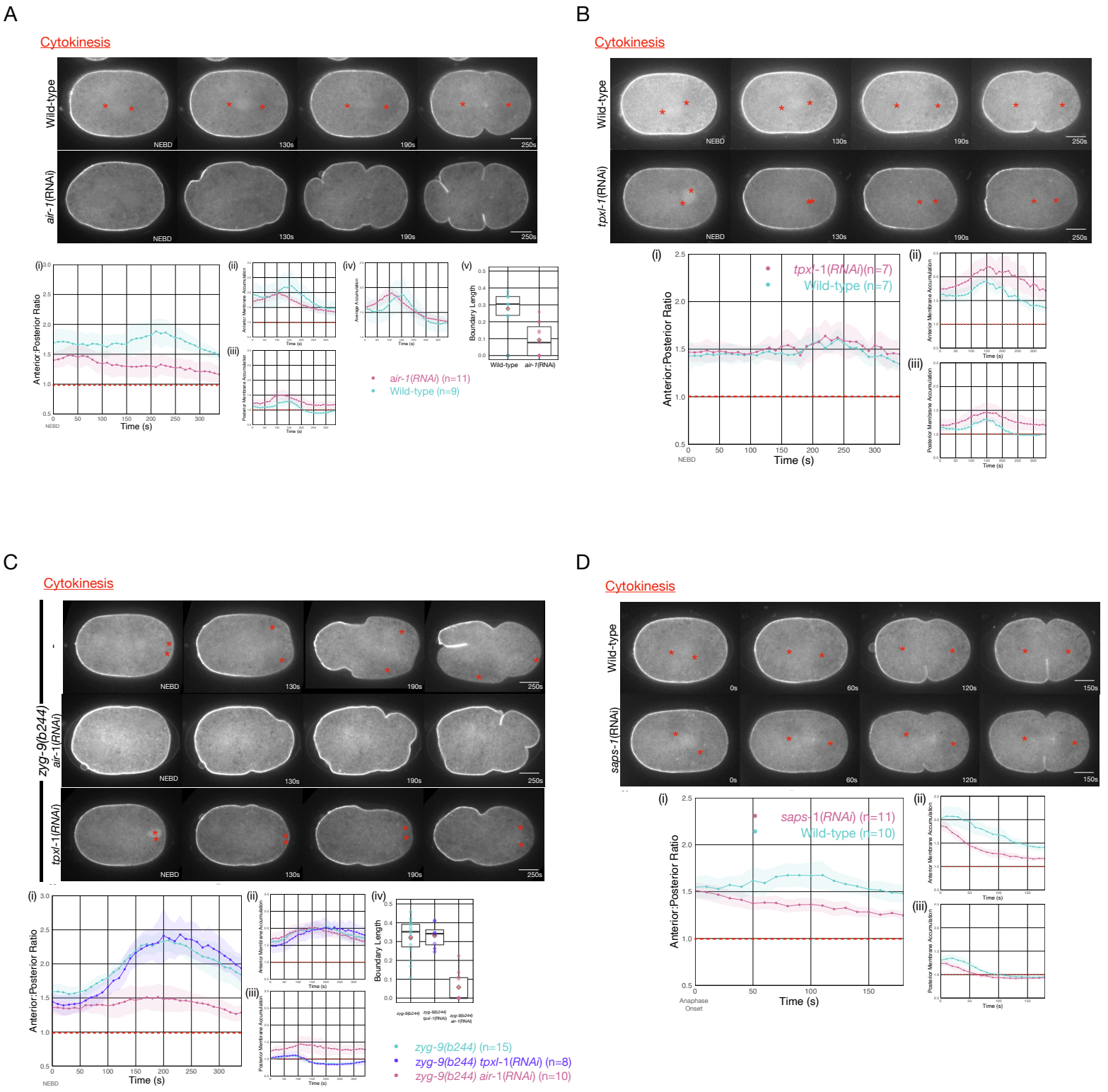


Figure 4 Supplement
Polarized accumulation of ECT-2 requires AIR-1

Supplement 1
Cortical levels of ECT-2 increase upon addition of Aurora inhibitor

Cytokinesis

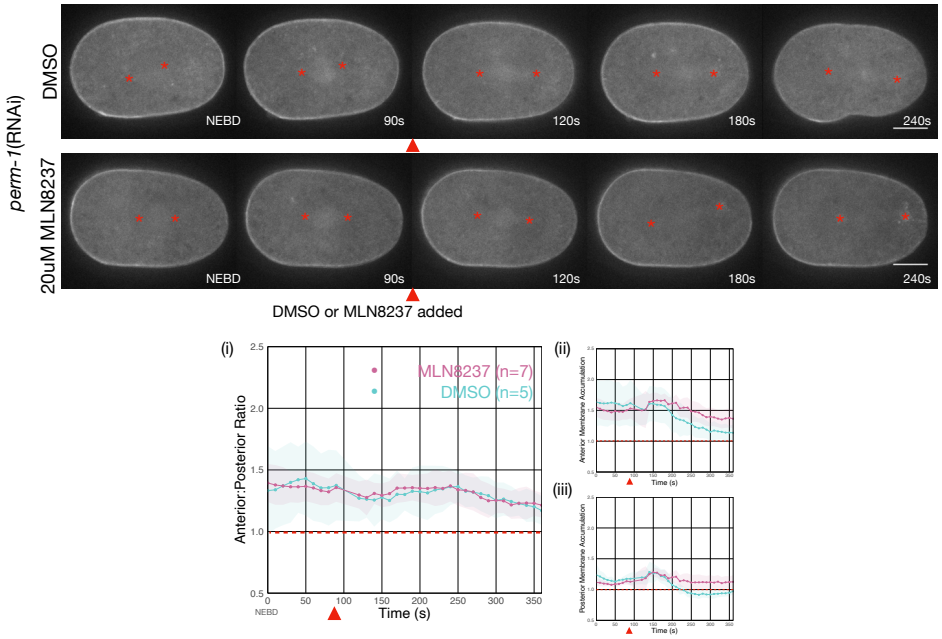
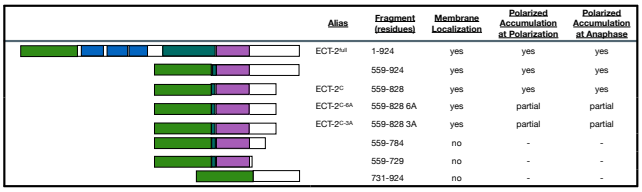


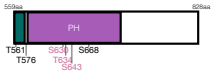
Figure 5
Phosphorylation of ECT-2 is required for accumulation at the membrane

A

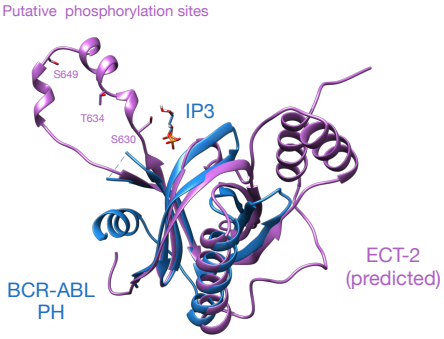


Alias	Fragment (residues)	Membrane Localization	Polarized Accumulation at Polarization	Polarized Accumulation at Anaphase
ECT-2^{full}	1-924	yes	yes	yes
	559-924	yes	yes	yes
ECT-2^{C}	559-828	yes	yes	yes
ECT-2^{C-6A}	559-828 6A	yes	partial	partial
ECT-2^{C-3A}	559-828 3A	yes	partial	partial
	559-784	no	-	-
	559-729	no	-	-
	731-924	no	-	-

B

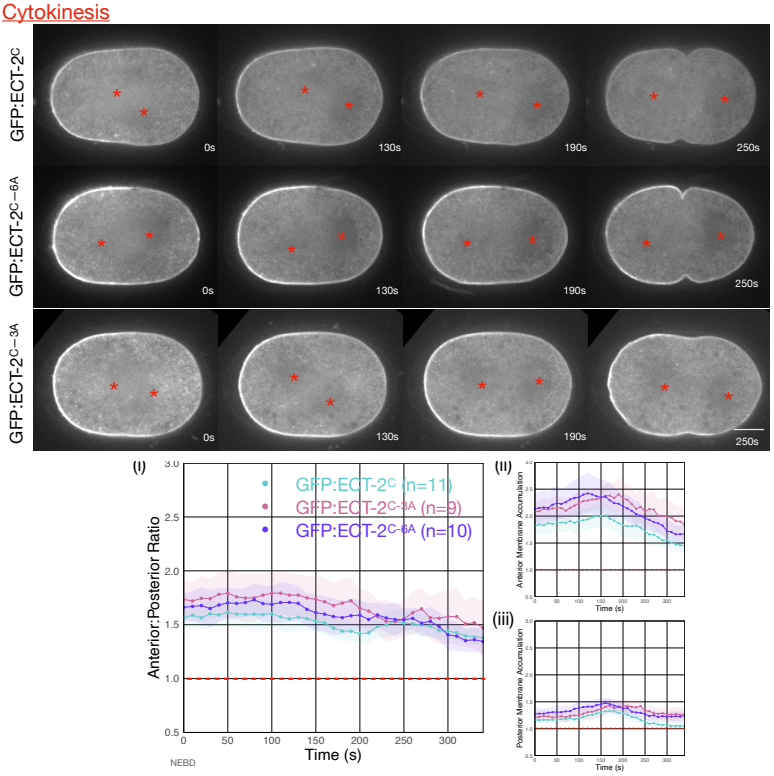


C



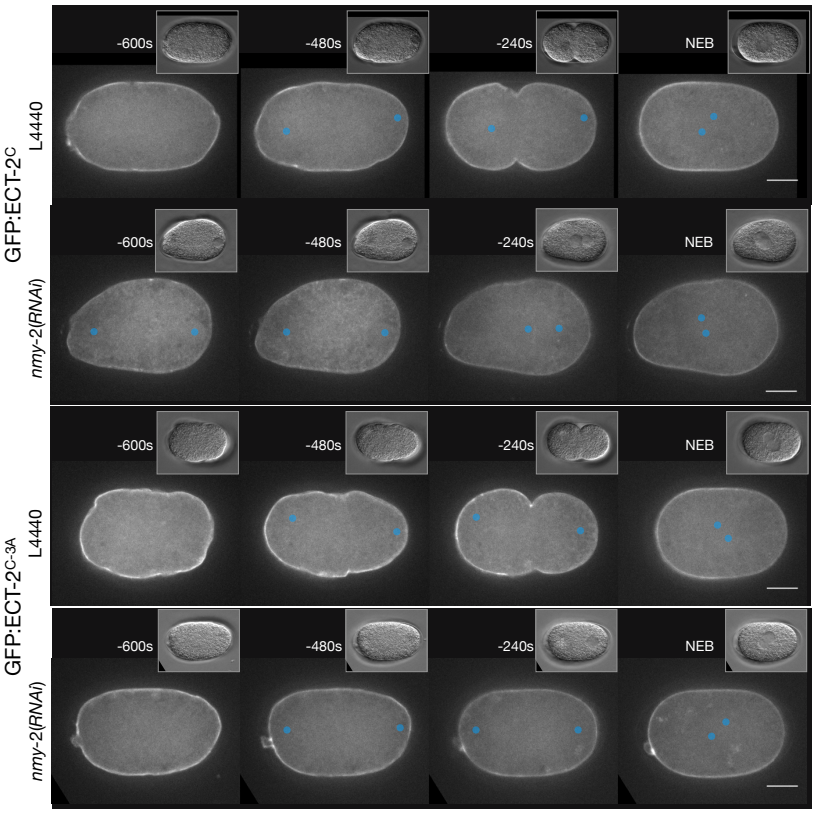
D

Cytokinesis



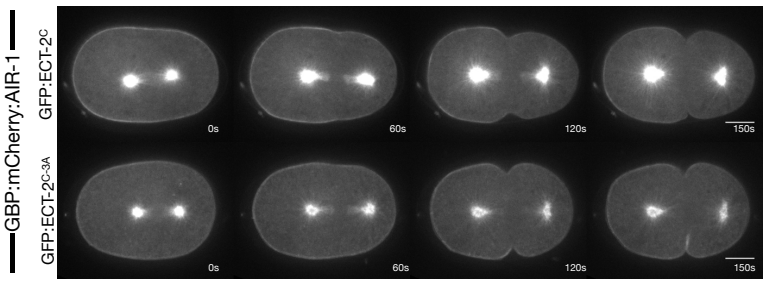
E

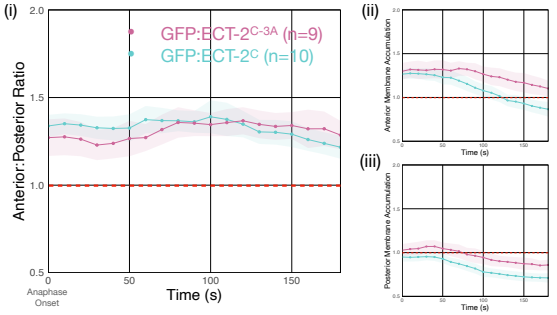
Polarization



F

Cytokinesis





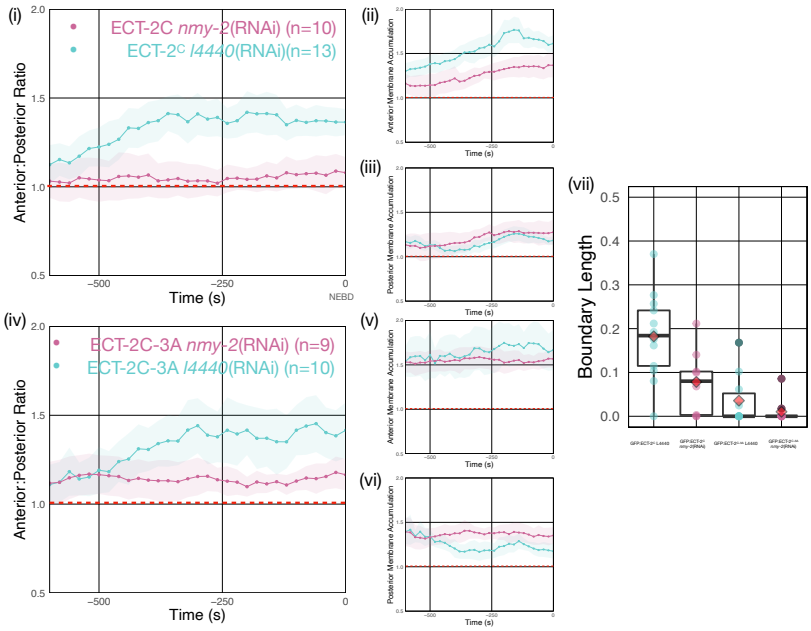


Figure 5 Supplement
Phosphorylation of ECT-2 is required for accumulation at the membrane

Supplement 1
Cortical levels of ECT-2^{C} increase when AIR-1 is depleted

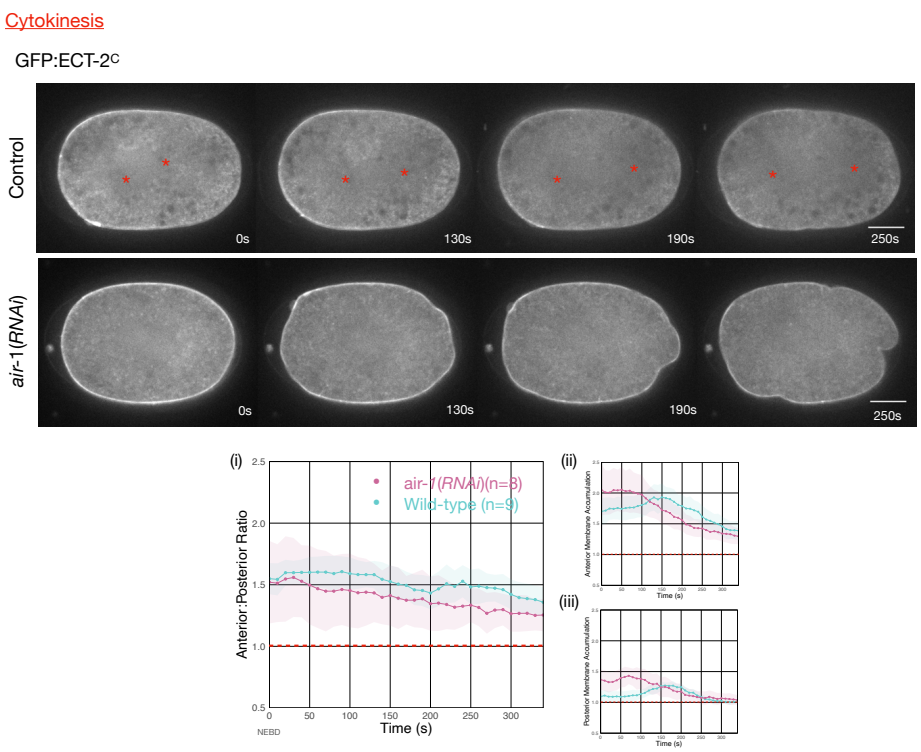
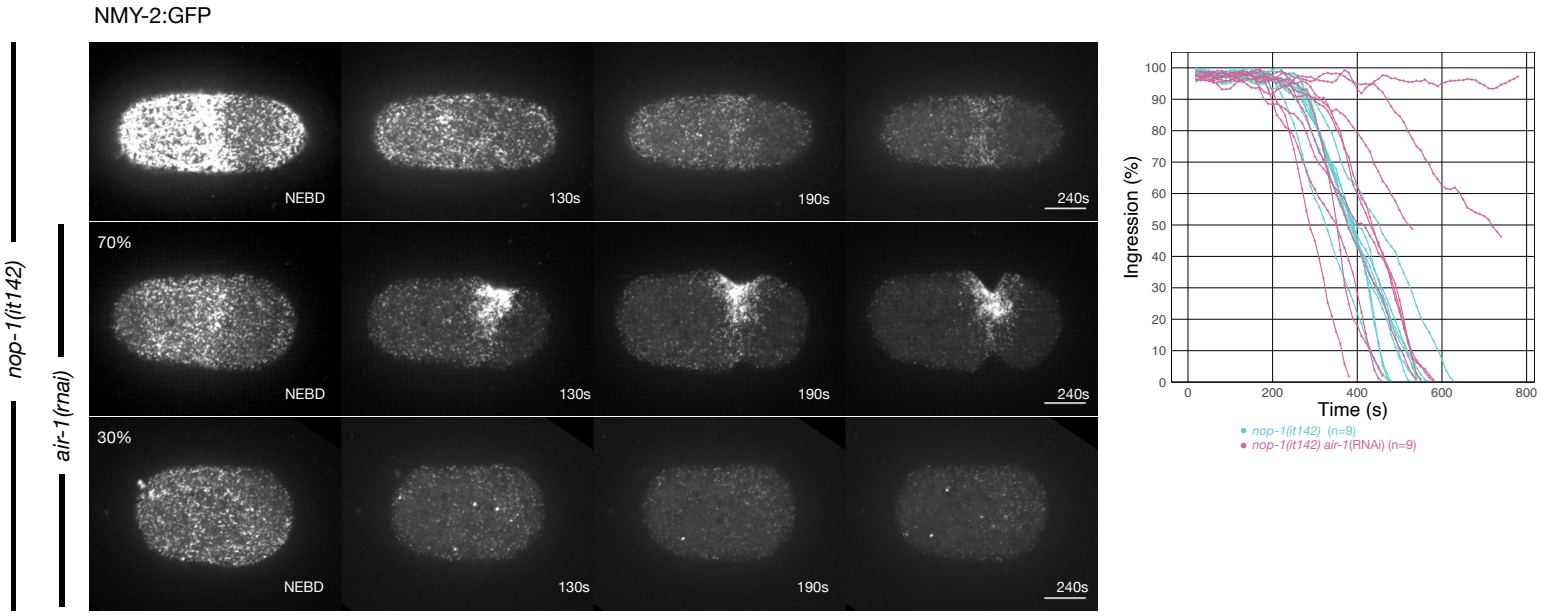


Figure 6
AIR-1 affects centralspindle-dependent furrowing

A



B

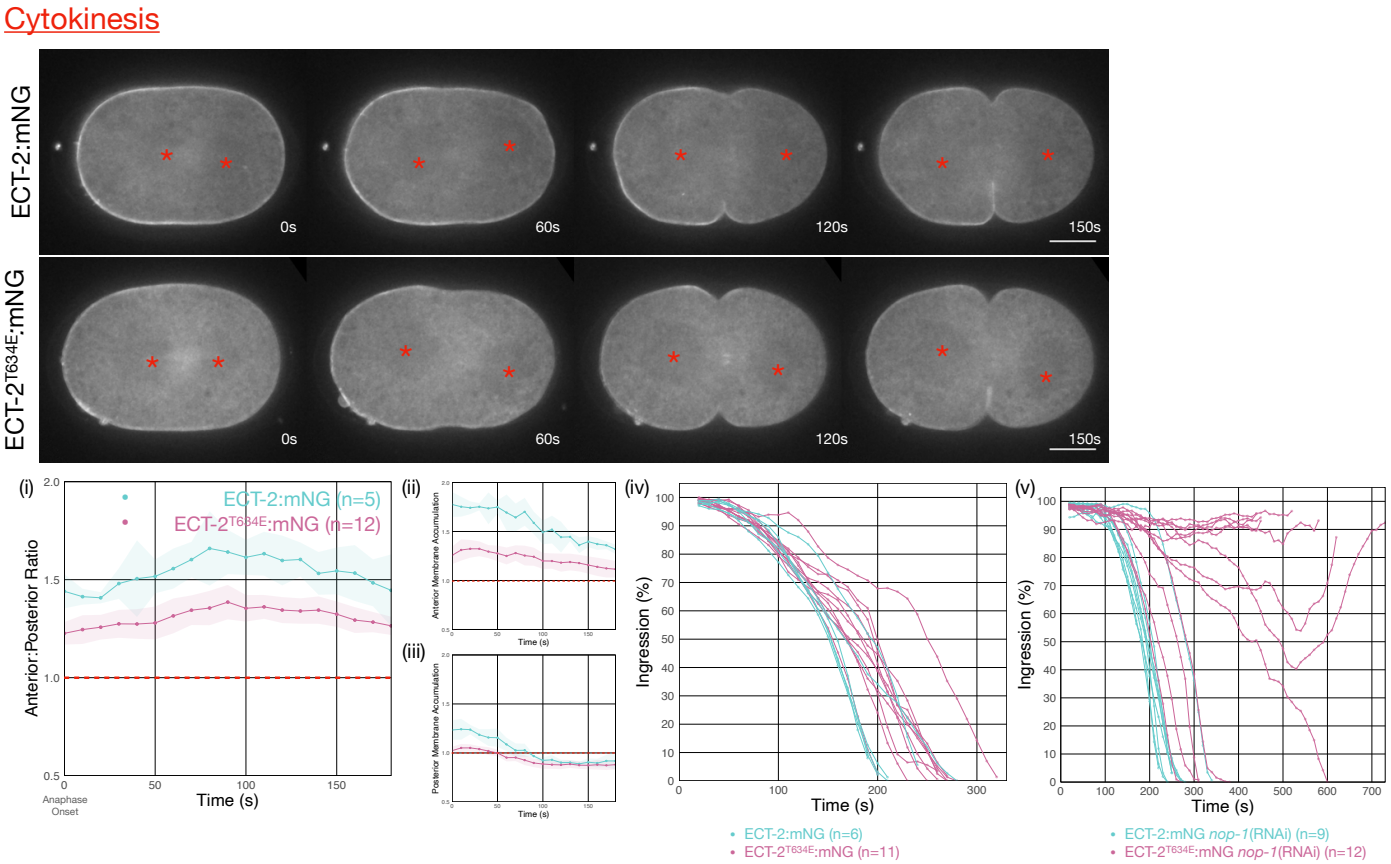
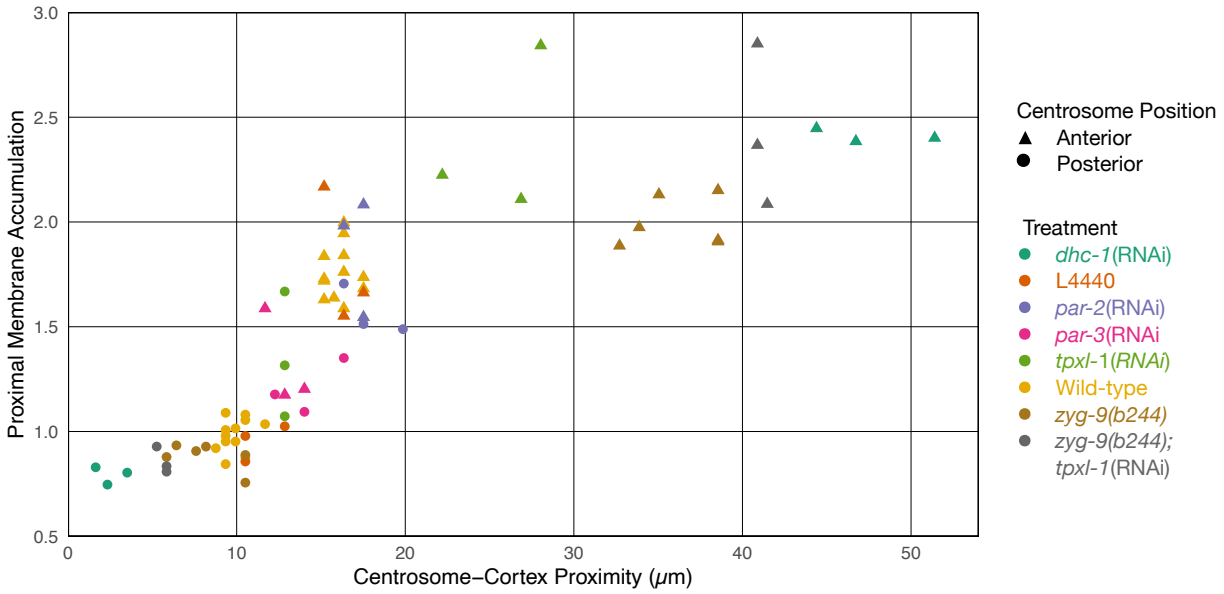


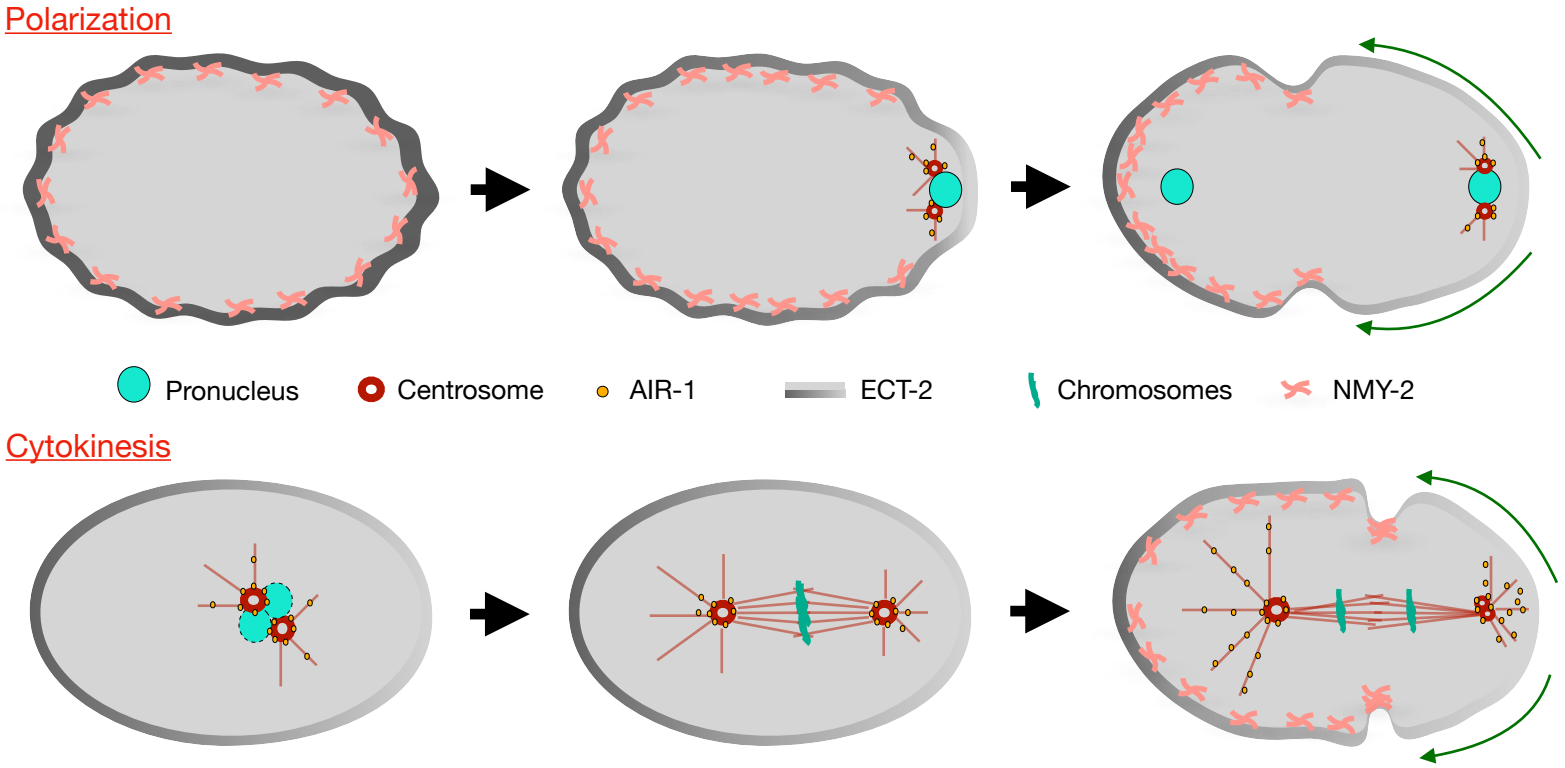
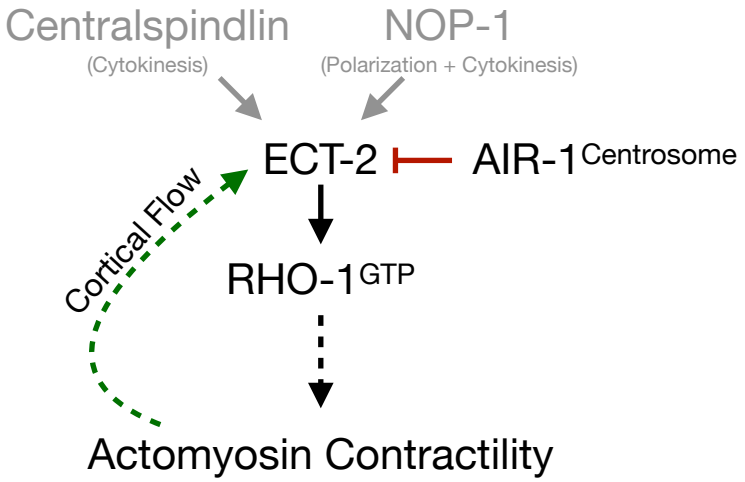
Figure 7

Model: During both polarization and cytokinesis, AIR-1 locally inhibits ECT-2 from the membrane and subsequent cortical flows amplify the inhibition

A



B



C