Thank you for submitting your article "Aurora A and cortical flows promote polarization and cytokinesis by inducing asymmetric ECT-2 accumulation" for consideration by eLife. Your article has been reviewed by 3 peer reviewers at Review Commons and an eLife referee, and the evaluation at eLife has been overseen by a Reviewing Editor (Mohan Balasubramanian) and Anna Akhmanova as the Senior Editor.

Based on the previous reviews and the revisions, the manuscript has been improved but there are some remaining issues that need to be addressed, as outlined below:

The eLife expert raised some points which are transmitted verbatim below. I have read them carefully. I think the vast majority of points can be addressed by rewriting and providing explanations or toning down some of the conclusions. Also, the referee has asked that you provide some more experimental details, statistical methods, and additional citations.

Points arising from your response letter and revision:

1. *In a revised manuscript, I am not convinced with their interpretation of the phenotype of air-1(RNAi);par-2(RNAi) zygotes. Given that either single par-2(RNAi) or single air-1(RNAi) abolished the anterior-enriched distribution of ECT-2::mNG (Fig. 4 supplement 2), this data simply indicates the indispensable roles of both PAR-2 and AIR-1 in ECT-2 asymmetry, but they cannot conclude that ECT-2 asymmetry in air-1(RNAi) condition is due to PAR-2-dependent weaker cortical flows. Indeed, the anterior/the posterior ratio of ECT-2::mNG in air-1(RNAi) zygotes shown in Fig. 4 supplement 2 is very close to 1.0 throughout mitosis, which is quite different from that in Fig. 4A. This discrepancy should be addressed in the final manuscript.*

   These PAR-2 related experiments were not included in the first version of the manuscript, but they were added in response to the Review Commons referees. As is well documented in several papers (PMIDs 30801250, 30861375, 31636075, 31155349), AIR-1 depletion does not eliminate cortical reorganization, and PAR-2 accumulates, aberrantly, to the cortex in when AIR-1 is depleted. A reproducible accumulation of lateral ECT-2 is observed during mitosis in AIR-1 depleted embryos; which is associated with ectopic furrowing (see point 11, below). While this increase in ECT-2 is visible in the images, it is not surfaced by our standard quantification method which focuses on the anterior and posterior domains. Given this and the established interplay between AIR-1 and PAR-2, we felt it appropriate to include an analysis of ECT-2 localization in embryos deficient in both AIR-1 and PAR-2.

   The referee states, “they cannot conclude that ECT-2 asymmetry in air-1(RNAi) condition is due to PAR-2-dependent weaker cortical flows.” We do not discuss PAR-2 in the context of cortical flows in the manuscript. The only sentences in the manuscript that explicitly discuss PAR-2 state ”Likewise, ECT-2 asymmetry during cytokinesis is reduced in embryos depleted of PAR-2.” and “The residual asymmetry in ECT-2 accumulation in AIR-1 depleted embryos is further reduced by co-depletion of PAR-2.”

   While there are differences in these datasets, we consider them minor; when the ratios are plotted together only 4/35 timepoints show any degree of statistically significant difference. The pattern of anterior accumulation is virtually identical in the two experiments, but the experiment in the supplemental figure shows a slightly stronger ECT-2 accumulation in the posterior. While the images were processed in the same manner, the two figures were generated with strains with distinct markers: while both figures contain the same

ECT-2:mNG allele, in Figure 4A it is paired with with mCh:Tub while Figure 4 supp 2 is paired with NMY-2:mKate, as the two figures were addressing different aspects of the phenotype.

2. *The authors' response "While cytokinesis generally involves an equatorial contractile ring, furrow formation can be driven by an asymmetric - i.e. non-equatorial - accumulation of actomyosin. This behavior is exemplified during pseudocleavage during which the entire anterior cortex is enriched for actomyosin and the posterior is depleted of myosin (Figure 1 Supplement 2). Several published studies provide evidence that the asymmetric pattern of myosin accumulation contributes to cytokinesis (PMID 22918944, 17669650)."*
The role of switching off the cortical flow from the P-to-A alone mode to the bidirectional mode in cytokinetic furrow formation has been reported in many papers (PMID: 27719759, 29963981, 32497213, etc.) as mentioned above. A simple unidirectional asymmetry is not sufficient in discussing the spatial regulation of cytokinesis.

   We regret that the context of our answer was apparently not sufficiently clear. The original referee asked “However, how does asymmetric Ect2 localization result in more active Ect2 at the cell equator, which is required for the formation of the active RhoA zone? Would we not expect an accumulation of Ect2 at the cell equator, or if that is not the case more active Ect2 at the equator versus the poles?”
   Our initial response was narrowly targeted to this question: “while cytokinesis **generally** involves an **equatorial contractile ring**, furrow formation **can be** driven by an asymmetric - i.e. non-equatorial - accumulation of actomyosin.” Our response implies that while bidirectional mode is the norm, there are cases in which a unilateral mode is sufficient (see bolded words in the response). The pseudocleavage furrow is a prime example of this behavior.

3. *The authors' response "However, as seen in e.g. ZYG-9 depleted embryos, ECT-2 is recruited to the posterior cortex in a centralspindlin-dependent manner"*
I don't understand the logic here. Has this been directly tested by, for example, depletion of ZEN-4 or CYK-4 in zyg-9(b244) mutant embryos? In Figure 3C (zyg-9(b244)), no particular enrichment of ECT-2 was observed at the posterior furrow, which is formed by centralspindlin.

   The underlying logic is that the posteriorly positioned, bipolar spindle induces the cortical accumulation of centralspindlin on the adjacent cortex. This pool of centralspindlin apparently recruits ECT-2 from the cytoplasm, despite the strong inhibitory activity of AIR-1 in this region. We infer that this binding may reflect a distinct mode of ECT-2 accumulation, as it appears impervious to AIR-1 activity. Indeed, FRAP experiments indicate this pool of ECT-2 has a larger immobile fraction than the cortical pool of ECT-2 elsewhere in the embryo.

   In figure 3D of Werner et al, 2007, we demonstrated that the posterior furrow that forms in embryos with posterior furrows requires centralspindlin. In that particular experiment, MEL-26 depletion was used to induce the spindle to assemble in the posterior. In subsequent unpublished work, we have found that the posterior furrow similarly requires centralspindlin when ZYG-9 depletion is used to reposition the spindle (both MEL-26 and ZYG-9 enhance spindle assembly, albeit via distinct molecular mechanisms).

Additionally, in Tse et al, 2012, we showed that the posterior furrow is largely independent of NOP-1 and that embryos depleted of both NOP-1 and CYK-4 fail to furrow altogether.

ECT-2 is readily detected on the posterior furrow once they ingress further than the time point shown in Figure 3C, as shown in Figure 3 Supplement 4.

4. *"In contrast, the Gomez-Cavasos paper (PMID 32619481) shows in figure S2 that the PH domain is required for cortical localization of ECT-2; this paper does not focus extensively on the cortical accumulation of ECT-2"*
I think Gomez-Cavasos should also be cited as these provide complementary information as to the role of the PH domain.

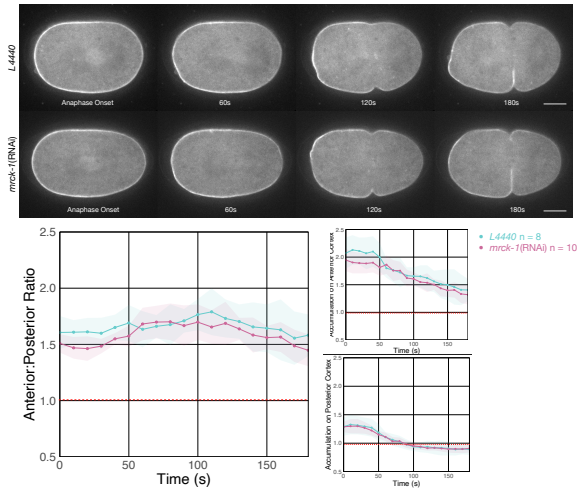
   In this case, the focus of this section is which parts of ECT-2 are **sufficient** for membrane recruitment of ECT-2. That PH domains contribute to membrane accumulation of GEFs has been extensively documented (eg PMID 17007612 from 2006). For example, the PH and the C terminal regions of HsEct2 are required for its membrane accumulation (PMID 27926870). It would not be appropriate to cite Gomez-Cavasos, without citing these papers. The requirement for a PH domain in cortical association does not seem significant enough to warrant a summary of the literature, particularly since membrane binding is the canonical function of PH domains. The somewhat surprising result was that the PH domain was not sufficient, as previously shown by Chan and Nance, which is cited.

In addition to the above, please answer either by rewriting or with experiments the points raised by the eLife referee below. As I read it, some experiments on Ect2 localization are required to firmly test your models. All other points raised might involve a balanced discussion of various observations and raising the limitations of the work.

eLife referee comments verbatim:

Asymmetric actomyosin contractility plays key roles in various cellular activities. In C. elegans embryos, both the post-fertilization polarity establishment and cytokinesis depend on this process, which is known to be under the control of Rho GTPase. In this manuscript, the authors studied the molecular mechanism of symmetry breaking, focusing on ECT-2 RhoGEF, the crucial upstream activator of Rho, and its regulation by AIR-1, Aurora A kinase. Although the roles of these molecules both in polarity establishment and cytokinesis have already been reported, it remains unclear whether and how AIR-1 might regulate the activity of ECT-2.

By a combination of genetic manipulation and high-quality quantitive microscopy, the authors compared various perturbations and concluded that centrosomes and the cortical flow driven by



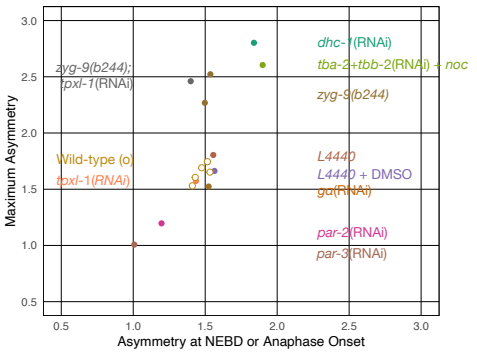
Depletion of MRCK-1 does not affect the distribution of cortical ECT-2 during anaphase. MRCK-1 depletion was confirmed by observing the loss of the cortical cap of co-expressed NMY-2:mKate during metaphase.

actomyosin network play roles in the asymmetric cortical localization of ECT-2 while astral microtubules and TPXL-1, a conserved Aurora A regulator that recruits AIR-1 to the astral microtubules, are not essential for the ECT-2 asymmetry during cytokinesis in contrary to the previous reports. Then, the authors tested the hypothesis that AIR-1 induces cortical asymmetry by directly phosphorylating ECT-2 and presented the in vivo phenotypes of the ECT-2 constructs with mutations at the putative phosphorylation sites, which were consistent with their hypothesis.

This referee does not mention that we unambiguously demonstrate that AIR-1 plays an important role in generating cortical asymmetry during cytokinesis. In particular, we show that the ability of centrosomes to inhibit cortical contractility during anaphase depends on AIR-1. Furthermore, we show that AIR-1 is specifically required to induce the displacement of ECT-2 from the cortex during both polarization and cytokinesis. We identify sites on ECT-2 that when mutated to non-phosphorylatable residues increase ECT-2 membrane accumulation one of which, which when mutated to a phosphomimetic residue is sufficient to reduce the cortical accumulation of ECT-2. Further we show that a non-phosphorylatable substitution of these sites impacts the response of ECT-2 C-terminal fragments to an AIR-1:GBP fusion protein. Finally, although AIR-1 clearly regulates the bulk flow of cortical myosin during anaphase, this pool of myosin is largely dependent on a non-essential protein called NOP-1. Given the evolutionary novelty of NOP-1 we further showed that a putative AIR-1 phosphosite also regulates the ability of the conserved cytokinetic regulator centralspindlin to induce cytokinesis.

5. *(Influence of the cortical asymmetry at the mitotic entry)*
   There is a flaw in their interpretation of the results of various perturbations. Their model in Figure 7B cytokinesis depicts that NMY-2 is absent at the cell cortex during earlier stages of mitosis (pro~meta). This is not precise and misleading. In normal embryos, even after the pseudo-furrowing settles, NMY-2 doesn't disappear from the cell cortex and, importantly, is kept anteriorly enriched though at slightly lower intensity in smaller patches than in the earlier phase (eg. Tse et al 2012 PMID:22918944, Fig 3). Actin filaments also remain more enriched in the anterior cortex than in the posterior cortex. This is a clear difference from the post-fertilization polarity establishment, in which uniform distribution of the actomyosin network is maintained until the entry of sperm breaks the symmetry.
   During the early stages of mitosis, the cortical flow is suspended due to the inhibitory activity of CDK1. The onset of anaphase cancels this inhibition and triggers the contraction of the actomyosin network. If the symmetry of cortical actomyosin is already broken as in the normal embryos, even uniform activation of actomyosin throughout the cell will result in a cortical flow towards the region with a denser actomyosin network (= the anterior cap). If the cortical symmetry is not broken for some reason, it needs to be broken to cause the cortical flow. In theory, the target of symmetry breakage can be any component of the contractile actomyosin network.

   While we dispute that there is a flaw in our interpretations, we do not dispute that NMY-2 does not disappear from the cortex during polarity



Summary figure comparing the average degree of ECT-2 asymmetry at NEBD/Anaphase Onset vs the maximal asymmetry of ECT-2 during anaphase. These values are not correlated.

maintenance, and have revised the model schematic in Figure 7B accordingly. However, our results indicate that this pool of myosin does not play a role in the organization of myosin upon anaphase onset and we have seen no evidence that this anterior cap is sufficient to direct anterior-directed cortical flows. As previously shown (PMID 21737681), polarized myosin clusters assemble normally during anaphase in MRCK-1 depleted embryos. MRCK-1 is known to be required for cortical myosin accumulation during the establishment phase (Fig 6, PMID 19923324).

Notably, the anterior cap of myosin during polarity maintenance (i.e. during mitosis) is qualitatively different from that during interphase. Myosin clusters during maintenance phase are far smaller and less clustered than those present during establishment phase or anaphase. Likewise the organization of actin filaments are quite different during these stages. During maintenance phase Arp-2/3 branched filaments predominate whereas a significant pool of unbranched Formin nucleated filaments assemble during establishment phase and anaphase. These distinct pools of myosin and actin appear to exhibit distinctly different abilities to induce contractions. To substantiate this assertion, we examined the asymmetry of ECT-2 distribution during cytokinesis in control and MRCK-1 depleted embryos. As indicated in the accompanying figure, MRCK-1 depletion has no significant impact on the distribution of ECT-2 during cytokinesis.

This result was predictable. As shown in Figure 2 Supplement 2, although Gα-depleted embryos enter anaphase with asymmetric ECT-2 (and there is no evidence to suggest that Gα-depletion perturbs the myosin cap during mitosis). Despite this preexisting asymmetry, as anaphase initiates, these embryos do not exhibit potent anterior-directed flows that lead to increasingly asymmetric ECT-2, rather ECT-2 becomes progressively more symmetric as anaphase proceeds. The progressive symmetrization of these embryos likely results from the symmetric position of the spindle and its reduced elongation during anaphase. These changes to spindle length and position result in the anterior and posterior centrosomes lying equidistant - and rather distal from the anterior and posterior cortices.

The referees comment “In theory, the target of symmetry breakage can be any component of the contractile actomyosin network” seems to suggest that symmetry breaking is a one time event and that the strength and duration of this symmetry breaking is immaterial. However, as the results of our study shows, dramatically exemplified in Figure 3C and summarized in Figure 7A, the nature of the symmetry breaking events during anaphase have dramatically different consequences on cortical ECT-2 and myosin depending upon the position of centrosomes relative to the cortex. Indeed, figure 2A provides an example of how cortical ECT-2 changes acutely in response to the position of the posterior aster as the spindle rocks. To emphasize this point, we added this sentence to the section of the discussion that focuses on cortical flows, “Further, we speculate that centrosomal AIR-1 not only breaks symmetry, but that this regulation of ECT-2 by centrosomal AIR-1 continues throughout anaphase.”

6. *The authors observed attenuated ECT-2 asymmetry during cytokinesis in the embryos depleted of SPD-5 (Figure 2C), PAR-3 (Figure 2D), PAR-2 (Figure 2 Supplement 1), NMY-2 (Figure 2 Supplement 1), and AIR-1 (Figure 4A, Figure 4 Supplement 2). In all these cases, the cortical ECT-2 at NEB was found more symmetric (A:P ratio at NEB =1.3, 1.0, 1.2, 1.3, and 1.1, respectively) than the normal embryos (1.4~1.6). In almost all the cases where ECT-2 was asymmetric at NEB (or metaphase), with Galpha(RNAi) as an exception, ECT-2*

asymmetry during cytokinesis was normal or enhanced (Tubulin(RNAi)+nocodazole, nop-1(it142), zyg-9(b244), dhc-1(RNAi), tpxl-1(RNAi), tpxl-1(RNAi);zyg-9(b244), and saps-1(RNAi)). There is a simple and strong correlation between the ECT-2 asymmetry upon mitotic entry and the ECT-2 asymmetry during cytokinesis.

Partial overlap in the requirements for polarization and asymmetric cytokinesis is expected, as these processes rely on a shared machinery. Additionally the degree of asymmetric accumulation of ECT-2 during anaphase depends on the asymmetric positioning of centrosomes, which depend on embryo polarization. In addition, there are exceptions to the correlation the referee cites, and they are highly informative. Gα - depleted embryos polarize normally and enter anaphase with control levels of ECT-2 asymmetry but, upon anaphase onset the degree of the asymmetry of ECT-2 declines. Conversely, the extent of ECT-2 asymmetry prior to anaphase onset in ZYG-9 depleted embryos is similar to wild-type yet during anaphase, ZYG-9-depleted embryos exhibit radically more highly asymmetric ECT-2 during anaphase than control embryos. Contrary to the assertion of the referee, ECT-2 asymmetry during mitosis and its asymmetry during cytokinesis are not well correlated.

7. *The authors challenge the roles of astral microtubules and dynein in the polar relaxation during cytokinesis based on the observations of the cortical flow in the embryos in which microtubules and spindles are drastically messed up (Tubulin(RNAi)+nocodazole, zyg-9(b244), dhc-1(RNAi)). However, starting with the asymmetric actomyosin network that was successfully established after the fertilization, the anterior-directed cortical flow should occur spontaneously upon reactivation of the contractility following the anaphase onset even without any additional cue. The ECT-2 asymmetry during cytokinesis in these embryos can just be reflecting the fact that these treatments didn't completely disrupt the post-fertilization cortical polarity. Indeed, the ECT-2 asymmetry at the mitotic entry in embryos in Tub(RNAi) +noc and dhc-1(RNAi) embryos was more intense than in the normal embryos (A:P ratio at NEB = 1.9 and 1.8, respectively).*

   See the responses to points 6 and 8 above. Furthermore, “drastically messed up” it is not an accurate description of the spindles in ZYG-9 depleted embryos. These spindles exhibit normal bipolarity and morphology and they segregate chromosomes normally. These spindles are small and, due to a combination of the position of sperm entry and the bias in cortical forces, they assemble close to the posterior cortex.
   Were dynein (or microtubules) required for polar relaxation during anaphase, it is reasonable to expect that dynein depletion (or MT disassembly) would result in a **reduction** in polar relaxation during anaphase, whereas the results show that dynein depletion results in a dramatic **increase** in polar relaxation.

8. *The role of switching of the cortical flow from the P-to-A alone mode to the bidirectional mode in cytokinetic furrow formation has been reported in many papers (PMID: 27719759, 29963981, 32497213, etc.). In this sense, the influence of the anterior centrosome/aster on the anterior cortex is crucial for the spatial control of cytokinesis. This must be a rationale for Mangal 2018 (PMID:29311228) to focus on the role of TPXL-1 in the clearing of anillin from the anterior cortex. Acceleration of furrow formation in correlation with the anterior shift of the anterior centrosome/aster has also been reported (PMID: 32497213). No such test has been performed for ECT-2 localization in this manuscript.*

   We concur that the anterior centrosome regulates cortical behavior, including controlling ECT-2 localization. However, because the posterior centrosome is closer to the posterior

cortex than their anterior counterparts, the equatorial directed myosin-dependent cortical flows are more pronounced in the posterior domain than in the anterior domain (e.g. Fig. 3C). Indeed, the dramatic differences in ECT-2 and NMY-2 localization in ZYG-9 depleted embryos (Figure 3C) is likely to result from the combination of the close proximity of both centrosomes to the posterior cortex and the absence of the anterior centrosome from the anterior domain.
It is important, furthermore, to not only consider the flows of myosin foci that appear, but also the rate at which such foci assemble in the anterior and posterior domains. Two domains can exhibit similar rate of flows, but if the foci are smaller and less numerous in one domain than the other, the net accumulation of myosin in the two domains can differ dramatically.
Importantly, this manuscript focuses on the mechanisms that regulate both the assembly of myosin foci downstream of RHO-1 activation and the subsequent flows (centrosomal AIR-1 inhibiting ECT-2 accumulation), the finding that ECT-2 localization depends upon these flows, and the proposal that asymmetric ECT-2 could function to sustain these flows. These findings extend our current understanding of the mechanism and is consistent with all the data in the literature to our knowledge.

9. *(Phosphorylation of ECT-2 by AIR-1)*
Although the authors claim the role of centrosomes based on the spatial correlation, no direct evidence has been provided for the positive role of centrosomes in these embryos. For example, if the ECT-2 asymmetry during anaphase is regulated by centrosomes, disruption of the centrosomes after anaphase onset should disrupt the cortical ECT-2 asymmetry, which is turning over in seconds. In this sense, treatment with MLN8237 (Figure 4-Supplement 1) at metaphase is highly interesting. A caveat here is that MLN8237 is not really specific to Aurora A. It also inhibits Aurora B-INCENP at Ki = 27 nM (just 5~27-fold larger than the Ki for Aurora A) (de Groot et al. 2015). The concentration of MLN8237 used (20 uM) is 700x higher than the Ki for Aurora B-INCENP. The phenotype might be due to the inhibition of Aurora B. Indeed, the ECT-2 signal at the midzone, which is likely to depend on centralspindlin and Aurora B, was lost by the MLN8237 treatment. A mild delay in the removal of ECT-2 from the posterior cortex might have been caused by the inhibition of Aurora B/AIR-2 in addition to or instead of AIR-1.

   We demonstrate that ECT-2 polarization during cytokinesis depends upon the core centrosomal component SPD-5, though it does not require the astral MTs that emanate from centrosomes.
   We have shown the involvement of AIR-1 in ECT-2 regulation by both depletion of AIR-1 and its regulator SAPS-1 and a gain of function approach using GBP-AIR-1.
   The dramatic difference in ECT-2 localization in embryos deficient in ZYG-9 and AIR-1 as compared to embryos deficient in ZYG-9 and TPXL-1 further supports models in which the centrosomal, I.e. not the astral, pool of AIR-1 is relevant (TPXL-1 is required for AIR-1 to associate with astral microtubules).
   The experiments with MLN8237 were merely included to complement the AIR-1 depletion studies and to show that acute inhibition can impact ECT-2 accumulation in otherwise unperturbed embryos. We have no basis to we assert, nor do we assert that these treatments fully inhibit AIR-1 activity. We also do not rule out some affect on AIR-2.

10. *Point mutations at the phosphorylation sites on ECT-2 are expected to compensate for the above issue of specificity and strengthen the author's theory of direct regulation of ECT-2 by*

AIR-1. However, as the authors admit in the "Limitations of this study" section, evidence for the in vivo phosphorylation of the putative sites is missing. In addition, it has not been tested whether AIR-1 can phosphorylate these sites. The Glotzer group has revealed key phospho-regulatory mechanisms in cytokinesis (PMID: 15282614, 15854913, 17488623, 19468300). In these works, they showed both the in vivo phosphorylation of the putative phospho-acceptor sites and the in vitro phosphorylation by a protein kinase as well as the in vivo phenotypes of the point mutants of the phosphorylation sites. Currently, what we can conclude from the data in Figures 5 and 6B is that some mutations in a loop in the ECT-2 PH domain mildly affect the cortical association of ECT-2. The gap between this and the phosphorylation of these sites by AIR-1 is huge.

The mutational studies on ECT-2 must be considered in context of the loss of function studies of AIR-1 (reduction in AIR-1 activity resulting in increased ECT-2 accumulation; reduction in SAPS-1 activity resulting in increase in AIR-1 activity and an decrease in ECT-2 accumulation. Furthermore, these residues affect the response of an GFP-ECT-2 fusion protein to a GBP-AIR-1 fusion protein). While we would very much like to study the dynamic phosphorylation of ECT-2 in vivo (it is likely highly regulated in space and time) at this moment there are no tools available suitable to do so. The majority of the in vivo phosphorylation studies the referee mentions were performed in bulk lysates from human cells which can be synchronized; this is not possible with C. elegans embryos. Further, in the singular case where in vivo phosphorylation was shown in nematode embryos, a phospho-specific antibody was used to label a localized pool of ZEN-4; in this case, ECT-2 phosphorylation by AIR-1 triggers its delocalization. Demonstration of ECT-2 phosphorylation by AIR-1 in vitro would be nice, but neither a positive result nor a negative result would be particularly informative as kinases can be promiscuous in vitro and we can not rule out a requirement for other factors.

11. *Figure 6 tests AIR-1 depletion in nop-1(it142) mutant embryos (A) and the phenotype of the endogenous T634E mutation of ect-2 (B). Is 'Figure 6. AIR-1 is involved in central-spindle-dependent furrowing' (page 42) or 'AIR-1 affects centralspindlin-dependent furrowing' (page 56) an appropriate title for this figure? The dependency on centralspindlin or the central spindle has not been directly tested. Although synthetic defects in NOP-1 and centralspindlin suppress furrow formation, this doesn't necessarily mean that all the residual cortical activity in nop-1(it142) embryos relies on centralspindlin or the central spindle. In nop-1(it142) embryos, the NMY-2 cortical accumulation is globally weakened in comparison with the wild-type embryos. Additional depletion of AIR-1 might promote furrowing by facilitating the cortical recruitment of ECT-2 or prevent cytokinesis by suppressing the aster/centrosome-dependent pathway, which may or may not be dependent on centralspindlin. By the way, how does ECT-2 behave in these embryos?*

    Furrow formation in wild-type embryos involves both NOP-1 and centralspindlin. Whereas depletion of NOP-1 has a dramatic effect on global cortical myosin, it has a limited effect on formation of the contractile ring, which ingresses to completion with near normal kinetics. Conversely, embryos deficient in centralspindlin subunits (CYK-4 or ZEN-4) form slowly ingressing furrows that only partially ingress. Embryos deficient in both NOP-1 and CYK-4 fail to form furrows. Thus, these two genes function in parallel pathways upstream of ECT-2. These findings are well established in the literature and also shown in figure 3B (PMID 22918944, 26252513, 32619481).

That said, as the referee indicates, depletion of AIR-1 increases cortical ECT-2 and induces a modest degree of hyper-contractility. For example, as shown in Figure 4A, AIR-1 depleted one-cell embryos form multiple furrows during anaphase. Formally, AIR-1 depletion could result in bypass suppression in embryos deficient in either CYK-4 and/or NOP-1. However, this hypercontractility depends upon NOP-1 during pseudocleavage (fig 5E PMID 31636075) and during cytokinesis as embryos deficient in both AIR-1 and NOP-1 form a single furrow which is positioned at approximate midplane of the spindle (fig 6A), suggesting it is centralspindlin directed. Indeed, it was precisely because AIR-1 depletion primarily enhances NOP-1 dependent contractility, we thought it would be relevant to examine whether AIR-1 has some impact of centralspindlin-directed furrowing. This was the underlying logic for the experiments shown in figure 7.

Nevertheless, to formally rule out the possibility of bypass suppression, we examined furrowing in embryos deficient in AIR-1, NOP-1, and CYK-4. Embryos deficient in all three factors (nop-1(it142); air-1(RNAi); cyk-4(RNAi)) fail to form furrows during anaphase (and pseudocleavage) (100% of embryos, n=8). The pattern of ECT-2 accumulation…Such embryos exhibit the membrane invaginations characteristic of embryos with weakened cortex (PMID 20808841). There were no significant differences in ECT-2 accumulation in (nop-1(it142); air-1(RNAi); cyk-4(RNAi) embryos as compared to air-1(RNAi) embryos. This sentence was added to the results to reflect these findings, “To test whether AIR-1 depletion does not bypass the requirement for CYK-4 and NOP-1 in ECT-2 activation, we depleted both AIR-1 and CYK-4 in *nop-1(it142)* embryos. These triply deficient embryos fail to furrow during anaphase (100%, n=8).”

Given the facts above, the finding that AIR-1 depletion and a phosphomimetic substitution of T634 in ECT-2 affects furrowing behavior in NOP-1 deficient embryos is consistent with AIR-1 involvement in centralspindlin-dependent furrowing and ‘AIR-1 affecting central-spindlin-dependent furrowing’.

We thank the referee for pointing out the title on page 42, it has been revised to “AIR-1 is involved in centralspindlin-dependent furrowing.”

Regarding the inactivation of NOP-1 having only a very modest effect on cortical ECT-2 (figure 3B); depletion of AIR-1 results in a significant increase in the accumulation of ECT-2 on the posterior cortex (Figure 4B), and the phosphomimetic substitution of T634 in ECT-2 reduces its cortical accumulation.

Recommendation for authors:

12. *The feedback loop from myosin to the biochemically upstream regulator RhoGEF is highly intriguing. Focusing on the mechanisms for this phenomenon might be more fruitful than spending time trying to obtain evidence for the phosphorylation on the sites that are not conserved during evolution and only weakly match with the consensus for the Aurora phosphorylation.*

    This suggestion is beyond the scope of the current manuscript.

13. (page 17) "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, indicating that bulk, cortical ECT-2 has a low level of RhoGEF activity." Difficult to

understand the logical structure due to repeated "as".

Thank you for pointing out this confusing sentence. We have revised it as follows:

"During cytokinesis, basal myosin levels appear to be sufficient to promote asymmetric ECT-2 accumulation, as ECT-2 asymmetry increases during anaphase even when both NOP-1 and CYK-4 are attenuated. We infer that bulk, cortical ECT-2 has a low level of RhoGEF activity."

Other points:

14. *(page 3) "Despite progress in our understanding of cytokinesis, gaps remain in our understanding of the mechanism by which asters spatially regulate RhoA activation." This is correct. However, considering the feedback loop shown by this work (dependence of ECT-2 asymmetry on NMY-2), this might be misleading. The point of regulation of the cortical contraction could be anywhere in the loop (RhoGEF, RhoGAP, Rho, formin, Rho-kinase, myosin phosphatase, myosin-II, actin, actin-bundling proteins, ...).*

    We have changed the statement to *"Despite progress in our understanding of cytokinesis, gaps remain in our understanding of the mechanism by which asters spatially regulate actomyosin contractility."*

15. *(page 25 method of measuring Boundary Length) "ECT-2:mNG accumulation across all positions along the perimeter of the embryo was fitted to a regression model by GAM (Prediction Accumulation) in R Studio." It is not clear what the 'regression model' was and what the "GAM (Prediction Accumulation) in R Studio". Provide the precise (mathematical) description of the model and the proper reference to the method as well as the R source code.*
    We have added the following text to the methods section "ECT-2:mNG accumulation across all positions along the perimeter of the embryo was fitted to a regression model by Generalized Additive Model (GAM) (Prediction Accumulation) in R Studio using the mgcv:gam function."
    For additional information the reviewer could refer to https://www.rdocumentation.org/packages/mgcv/versions/1.8-41/topics/gam

16. *(page 26 statistical test) It reads that t-test was performed between treatments at every time point. I am not sure whether this is an appropriate approach. Data from different time points from a time series are correlated. It is not clear how this should be reflected in handling the issue of multiple comparisons. Can't we get advice from an expert on statistics?*

    While we are not experts on statistics, we believe that t-test is appropriate. Though data from different time points are correlated, the tests do not involve comparisons of data from neighboring time points. Rather we compare the data at a given time point between conditions. Regarding multiple comparisons, in each case, experimental results are compared to controls, no multiple comparisons are performed (ie we do not compare two

different experimental treatments to each other). Finally, in each case, we provide 95% confidence intervals which provide a direct indication of the experimental noise.

17. *(Figure Supplement 1 and Figure 5F) Average Accumulation (same as 'Anterior:Posterior Ratio'?) is only shown. Isn't it better to include 'Accumulation on Anterior Cortex' and 'Accumulation on Posterior Cortex' as well for consistency with the other figures?*

    Average accumulation is shown in cases where we focus on temporal regulation, as opposed to the spatial regulation. For example, in Figure 1Bi we show average accumulation to demonstrate the overall changes in ECT-2 accumulation during the cell cycle. This is also the case in Figure 4 Supplement 1, where we measure the overall changes in ECT-2:mNG in response to global AIR-1 inhibition with a chemical inhibitor. This is also the case in Figure 5F, where we track the overall accumulation of GFP:ECT-2^{C} and GFP:ECT-$2^{C\text{-}3A}$,in the presence of GBP:AIR-1, which globally colocalizes with these C-terminal fragments of ECT-2.