Coupling between cytoplasmic concentration gradients through local control of protein mobility in the Caenorhabditis elegans zygote

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ABSTRACT Cell polarity is characterized by the asymmetric distribution of factors at the cell cortex and in the cytoplasm. Although mechanisms that establish cortical asymmetries have been characterized, less is known about how persistent cytoplasmic asymmetries are generated. During the asymmetric division of the Caenorhabditis elegans zygote, the PAR proteins orchestrate the segregation of the cytoplasmic RNA-binding proteins MEX-5/6 to the anterior cytoplasm and PIE-1, POS-1, and MEX-1 to the posterior cytoplasm. In this study, we find that MEX-5/6 control the segregation of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 by locally increasing their mobility in the anterior cytoplasm. Remarkably, PIE-1, POS-1, and MEX-1 form gradients with distinct strengths, which correlates with differences in their responsiveness to MEX-5/6. We show that MEX-5/6 act downstream of the polarity regulators PAR-1 and PAR-3 and in a concentration-dependent manner to increase the mobility of GFP::PIE-1. These findings suggest that the MEX-5/6 concentration gradients are directly coupled to the establishment of posterior-rich PIE-1, POS-1, and MEX-1 concentration gradients via the formation of anterior-fast, posterior-slow mobility gradients.

INTRODUCTION Cell polarity is fundamental to the biology of most cells and is characterized by the asymmetric distribution of factors at the cell cortex and in the cytoplasm. The PAR (partitioning defective) proteins are broadly conserved polarity regulators that concentrate at the cortex of polarized cells and control the segregation of both cortical and cytoplasmic factors (Kemphues, 2000; Goldstein and Macara, 2007; Nance and Zallen, 2011). Although mechanisms by which the PAR proteins establish cortical asymmetries have been characterized, relatively little is known about how they control the formation of precise and stable cytoplasmic asymmetries.

The Caenorhabditis elegans zygote provides a powerful system in which to characterize the mechanisms that generate cytoplasmic asymmetries. Upon the completion of meiosis, the zygote initiates an ~10 min polarization process, during which a collection of maternally deposited cytoplasmic factors are partitioned along the anterior/posterior (A/P) axis. The similar tandem CCCH zinc finger (TZF) RNA-binding proteins MEX-5 and MEX-6 (MEX-5/6 hereafter) redistribute to form anterior-high, posterior-low cytoplasmic concentration gradients (Schubert et al., 2000; Tenlen et al., 2008; Daniels et al., 2010; Griffin et al., 2011). Concurrently a number of cytoplasmic factors, collectively called the germ plasm, become enriched in the posterior cytoplasm (Updike and Strome, 2010; Wang and Seydoux, 2013). The germ plasm includes nonmembranous ribonucleotide organelles called P granules, as well as three TZF RNA-binding proteins, PIE-1, POS-1, and MEX-1, which concentrate diffusely in the posterior cytoplasm and weakly on P granules (Strome and Wood, 1982; Mello et al., 1996; Guedes and Priess, 1997; Tenenhaus et al., 1998; Tabara et al., 1999; Ogura et al., 2003; Updike and Strome, 2010; Wang and Seydoux, 2013). The zygote divides ~20 min after the completion of meiosis, leading to the preferential inheritance of MEX-5/6 by the anterior daughter cell and the germ plasm by the posterior daughter cell. As a result of the asymmetric inheritance of these factors, the anterior and posterior blastomeres adopt distinct somatic and germline identities, respectively (Sulston et al., 1983; Rose and Kemphues, 1998; Wang and Seydoux, 2013).
The partitioning of factors in the zygotic cytoplasm is controlled by the PAR proteins. During polarity establishment, the anterior PARs (the PDZ proteins PAR-3, PAR-6, and the aPKC kinase PKC-3) become enriched at the anterior cortex, and the posterior PARs (the kinase PAR-1 and the RING-finger protein PAR-2) become enriched at the posterior cortex (Etamad-Moghadam et al., 1995; Guo and Kemphues, 1995; Boyd et al., 1996; Watts et al., 1996; Tabuse et al., 1998). MEX-5/6, PIE-1, POS-1, and MEX-1 are symmetrically distributed in par-mutant embryos (Tenenhaus et al., 1998; Schubert et al., 2000; Cuenca et al., 2003; Ogura et al., 2003). Studies of fluorescently tagged MEX-5 have shown that differential mobility along the A/P axis underlies the segregation of MEX-5 to the anterior cytoplasm. PAR-1 phosphorylates MEX-5 and acts to increase MEX-5 mobility in the posterior cytoplasm (Tenlen et al., 2008; Griffin et al., 2011). Uniformly distributed PP2A phosphatase counteracts the increase in MEX-5 mobility by PAR-1, resulting in relatively low mobility of MEX-5 in the anterior cytoplasm (Schlaitz et al., 2007; Griffin et al., 2011). As a consequence of the differential in MEX-5 mobility along the A/P axis, MEX-5 is preferentially retained in the anterior cytoplasm, forming a persistent threefold concentration gradient that spans the A/P axis (Tenlen et al., 2008; Daniels et al., 2010; Griffin et al., 2011).

MEX-5/6 are required downstream of PAR polarity to control PIE-1, POS-1, and MEX-1 segregation. In mex-5/6 mutant embryos, PIE-1, POS-1, and MEX-1 remain symmetrically distributed even though most mex-5/6 mutant embryos establish polarized PAR domains (Schubert et al., 2000; Cuenca et al., 2003). MEX-5's role in segregating PIE-1 and POS-1 depends on MEX-5 phosphorylation by the kinase MBK-2, which is activated at the completion of meiosis (Pellettieri and Seydoux, 2003; Quintin et al., 2003; Pang et al., 2004; Nishi et al., 2008). The diffusion of GFP::PIE-1 is significantly slower in the posterior cytoplasm than in the anterior cytoplasm, indicating that, like MEX-5, differential mobility along the A/P axis likely underlies PIE-1 segregation (Daniels et al., 2009). It is not known whether the segregation of POS-1 and MEX-1 similarly results from local control of their mobility. Furthermore, it is not known how the PAR proteins and MEX-5/6 regulate the dynamics of PIE-1, POS-1 and MEX-1 in order to control their segregation.

We have analyzed the segregation of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 in the C. elegans zygote. We find that GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 form posterior-rich concentration gradients that are established at distinct rates and have distinct strengths. All three proteins diffuse more slowly in the posterior cytoplasm than in the anterior cytoplasm, and their differential mobility along the A/P axis correlates with their respective concentration gradients. We find that MEX-5/6 act downstream of PAR-1 and PAR-3 and in a concentration-dependent manner to increase the mobility of GFP::PIE-1. These results support a model in which the MEX-5/6 concentration gradients are directly coupled to the formation of the PIE-1 concentration gradient via the formation of a PIE-1 diffusion gradient.

RESULTS

Quantification of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 segregation

To analyze the dynamics underlying the segregation of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1, we first quantified their localization using time-lapse spinning-disk confocal microscopy. Before the onset of polarization, each protein is symmetrically distributed along the A/P axis (Figure 1A). Beginning with the onset of polarization, each protein is symmetrically distributed along the A/P axis (Figure 1A). Beginning with the onset of polarization,
the concentrations of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 progressively decrease in the anterior cytoplasm as they increase in the posterior cytoplasm, consistent with previous analysis of GFP::PIE-1 (Figure 1B; Reese et al., 2000; Daniels et al., 2009). GFP::PIE-1 and GFP::POS-1 respectively reach half-maximal enrichment in the posterior 4.5 min ($n = 5$, SEM $= 0.38$) and 4.6 min ($n = 5$, SEM $= 0.13$) after the onset of polarization and reach maximal enrichment after ~9 min. The accumulation of GFP::MEX-1 in the posterior cytoplasm occurs significantly more slowly than the accumulation of either GFP::PIE-1 or GFP::POS-1. GFP::MEX-1 reaches half-maximal enrichment after 6.3 min ($n = 5$, SEM $= 0.39$) and continues to enrich in the posterior cytoplasm through nuclear envelope breakdown (NEBD) ~11 min after onset of polarization (Figure 1B).

To characterize the shape and amplitude of their respective gradients, we next quantified the concentration of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 along the A/P axis at NEBD using line scan analysis. The concentration of each protein increases gradually between ~30 and ~90% embryo length (Figure 1C). The gradient formed by transgenic GFP::PIE-1 has a maximal amplitude of 4.8-fold and is very similar to the gradient formed by PIE-1 tagged at the endogenous locus with green fluorescent protein (GFP; Figure 1C and Supplemental Figure S1, A and B; Kim et al., 2014). The GFP::POS-1 and GFP::MEX-1 gradients are weaker than the GFP::PIE-1 gradient, with maximal amplitudes of three- and 1.8-fold, respectively (Figure 1C). The differences in gradient strength correlate with significant differences in the posterior enrichment of each protein. The ratio of the mean concentration in the posterior relative to the mean concentration in the anterior cytoplasm is 2.52 for GFP::PIE-1, 1.93 for GFP::POS-1, and 1.39 for GFP::MEX-1 (Supplemental Figure S2A). In contrast, GFP::MEX-3, which is an RNA-binding protein enriched on P granules but otherwise symmetrically distributed in the cytoplasm, has a posterior enrichment of only 1.05 (Figure 1, A and C, and Supplemental Figure S2A; Draper et al., 1996). Taken together, these data indicate that all three proteins begin to segregate to the posterior at the onset of polarization, but their rate of segregation and the strength of their respective gradients differ.

**Diffusion of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1**

We next used fluorescence correlation spectroscopy (FCS) to estimate the apparent diffusion rate of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 in the anterior and posterior cytoplasm of polarized zygotes. FCS is an imaging technique in which the apparent diffusion coefficient of a fluorescently tagged protein can be estimated from analysis of fluorescence fluctuation patterns (Schwille et al., 1999). We first estimated the apparent diffusion coefficient of two symmetrically distributed proteins, GFP and GFP::MEX-3. As expected and consistent with previous findings, the mobility of GFP is similar in the anterior and posterior cytoplasm (Figure 2 and Supplemental Table S1; Tenlen et al., 2008; Daniels et al., 2010). Similarly, the diffusion of GFP::MEX-3 is uniform along the A/P axis, although it is significantly lower than the apparent diffusion rate of GFP (Figure 2 and Supplemental Table S1). We next used FCS to estimate the diffusivity of GFP::PIE-1 and found that, consistent with previous FRAP and FCS analysis of GFP::PIE-1, the apparent diffusion rate of GFP::PIE-1 is significantly slower in the posterior than in the anterior cytoplasm (Figure 2 and Supplemental Table S1; Daniels et al., 2009). We find similar results for PIE-1 tagged with GFP at the endogenous locus (Supplemental Figure S1C; Kim et al., 2014). At intermediate positions along the A/P axis, the apparent diffusion of GFP::PIE-1 is intermediate relative to the anterior and posterior cytoplasm, suggesting that there is a continuous gradient of GFP::PIE-1 mobility along the A/P axis (Supplemental Figure S1, D and E).

We next characterized the mobility of GFP::POS-1 and GFP::MEX-1 in the anterior and posterior cytoplasm at NEBD. Like GFP::PIE-1, both GFP::POS-1 and GFP::MEX-1 are less mobile in the posterior cytoplasm than in the anterior cytoplasm. Interestingly, the apparent diffusion rates of GFP::MEX-1 and GFP::POS-1 in the anterior cytoplasm are significantly lower than that of GFP::PIE-1, resulting in a smaller differential in the mobility of GFP::MEX-1 and of GFP::POS-1 along the A/P axis (Figure 2 and Supplemental Table S1). The mobility of GFP::POS-1 is slightly higher than GFP::PIE-1 in the posterior cytoplasm, which also contributes to its smaller differential in diffusivity (Figure 2 and Supplemental Table S1). Therefore, the differentials in diffusivity along the A/P axis correlate with the strength of the respective concentration gradients such that proteins exhibiting a larger differential in mobility (e.g., GFP::PIE-1) form a stronger concentration gradient than proteins with a smaller differential in mobility (e.g., GFP::POS-1). Furthermore, the relatively low mobility of GFP::MEX-1 in the anterior cytoplasm likely limits the rate at which GFP::MEX-1 partitions to the posterior cytoplasm, resulting in the relatively slow rate of GFP::MEX-1 gradient formation (Figure 1B).

Because PIE-1 concentrates on P granules and in the cytoplasm surrounding P granules, we next sought to determine the extent to which enrichment on P granules contributes to the segregation of PIE-1 (Mello et al., 1996; Tenenhaus et al., 1998). PGL-1, PGL-3, GLH-1, and GLH-4 are core P granule components, and pgl-1;pgl-3;glh-1;glh-4(RNAi) results in dispersal of P granules (Updike et al., 2014). We find that in pgl-1;pgl-3;glh-1;glh-4(RNAi) embryos, GFP::PIE-1 is not concentrated in P granule–like foci and yet forms a gradient similar to control RNA interference (RNAi) embryos.
MEX-5/6 act to increase the mobility of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1

MEX-5/6 form anterior-high, posterior-low concentration gradients and are required for the segregation of PIE-1, POS-1, and MEX-1 to the posterior cytoplasm (Schubert et al., 2000; Cuenca et al., 2003). Therefore we next characterized the role of MEX-5/6 in the regulation of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 mobility. For all three proteins, depletion of MEX-5/6 levels by RNAi results in uniform diffusivity, primarily due to significant decreases in their mobility in the anterior cytoplasm (Figure 2 and Supplemental Table S1). The mobility of GFP::PIE-1 and GFP::MEX-1 in mex-5/6(RNAi) embryos is similar to their mobility in the posterior cytoplasm of wild-type embryos (Figure 2 and Supplemental Table S1). In contrast, the mobility of GFP::POS-1 is slightly lower in mex-5/6(RNAi) embryos than in the posterior of wild-type embryos, suggesting that GFP::POS-1 mobility is sensitive to the relatively low concentration of MEX-5/6 in the posterior cytoplasm of wild-type embryos (Figure 2 and Supplemental Table S1). These data indicate that MEX-5/6 act to increase the mobility of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1, particularly in the anterior cytoplasm, where MEX-5/6 concentrations are high.

MEX-5/6 act downstream of PAR-1 and PAR-3 to increase GFP::PIE-1 mobility

The partitioning of factors in the zygotic cytoplasm is controlled by the PAR proteins. For example, in par-1 and par-3 mutant embryos, MEX-5/6 and PIE-1 are symmetrically distributed throughout the cytoplasm (Terenhaus et al., 1998; Schubert et al., 2000; Cuenca et al., 2003). Therefore we next characterized the role of PAR-1 and PAR-3 in the regulation of GFP::PIE-1 mobility. The apparent diffusion rate of GFP::PIE-1 is uniform in par-3(it71) and par-1(it51) mutant embryos and is significantly higher than in mex-5/6(RNAi) embryos (Figure 3 and Supplemental Table S2; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Tenenhaus et al., 1998). In mex-5/6(RNAi);par-1(it51) and mex-5/6(RNAi);par-3(it71) embryos, the apparent diffusion rate of GFP::PIE-1 is similar to that in mex-5/6(RNAi) embryos (Figure 3 and Supplemental Table S2). Therefore mex-5/6 are epistatic to par-1 and par-3 with respect to the control of GFP::PIE-1 mobility, suggesting that MEX-5/6 function downstream of PAR-1 and PAR-3 to increase GFP::PIE-1 mobility.

We noted that the apparent diffusion of GFP::PIE-1 is significantly higher in par-1(it51) embryos than in par-3(it71) embryos (Figure 3 and Supplemental Table S2). Depletion of PAR-1 in par-3(it71) increases GFP::PIE-1 mobility, suggesting that PAR-1 functions downstream of PAR-3 in the control of GFP::PIE-1 mobility (Figure 3 and Supplemental Table S2). MEX-5 expression levels are similar in par-1 and par-3 mutant worms, suggesting that the difference in GFP::PIE-1 mobility may reflect differences in MEX-5 activity rather than MEX-5 expression levels (Supplemental Figure S3). This interpretation is consistent with the previous finding that PAR-1 acts downstream of PAR-3 to regulate MEX-5/6 activity with respect to the control of PIE-1 nuclear localization (Cuenca et al., 2003). PAR-1 phosphorylates MEX-5 on two residues near the C-terminus, and we speculate that this phosphorylation may reduce MEX-5’s ability to stimulate increased GFP::PIE-1 mobility (Tenlen et al., 2008; Griffin et al., 2011).

MEX-5 controls GFP::PIE-1 mobility in a concentration-dependent manner

The preceding results are consistent with a model in which the PAR proteins function to establish the MEX-5/6 concentration gradients, which in turn function in a concentration-dependent manner to generate a gradient in the diffusivity of GFP::PIE-1 along the A/P axis. This model predicts that GFP::PIE-1 mobility should be sensitive to the concentration of MEX-5/6 independent of the activity of the PAR proteins. To test this prediction, we used partial MEX-5/6 RNAi to deplete MEX-5/6 levels to various extents in par-1(it51) embryos (schematized in Figure 4A). In parallel, we analyzed GFP::PIE-1 diffusion by FCS and MEX-5 levels by quantitative Western blot analysis. We were not able to monitor MEX-6 levels because there is not an antibody that specifically recognizes MEX-6. We find that as MEX-5/6 levels are depleted, the apparent diffusion coefficient of GFP::PIE-1 progressively decreases, reaching a minimum when MEX-5/6 levels are depleted to ~25% of the levels in par-1(it51) worms (Figure 4, B–D). These data indicate that GFP::PIE-1 mobility is controlled by the concentration of MEX-5/6 and suggest a direct coupling between the MEX-5/6 concentration gradients and the formation of the GFP::PIE-1 diffusion gradient.

**DISCUSSION**

Taking together this and previous studies, we propose the following working model connecting cortical PAR polarity to the partitioning of cytoplasmic CCCH-finger proteins. The posterior enrichment of PAR-1 kinase results in a differential in MEX-5 (and presumably MEX-6) diffusivity along the A/P axis that leads to the formation of anterior-rich MEX-5/6 concentration gradients (Tenlen et al., 2008; Daniels et al., 2010; Griffin et al., 2011). In turn, MEX-5/6 act in a concentration-dependent manner to increase the mobility of PIE-1, resulting in a gradient in PIE-1 mobility along the A/P axis. As a consequence, PIE-1 is preferentially retained in the posterior cytoplasm and forms a persistent, posterior-rich concentration gradient.
may result from their association with cytoplasmic RNA. Consistent with this possibility, mutations predicted to reduce the affinity of MEX-5 for RNA increase MEX-5 mobility and flatten the MEX-5 gradient (Griffin et al., 2011). In addition, mutations predicted to cause misfolding of the second PIE-1 zinc-finger RNA-binding domain prevent the segregation of GFP::PIE-1 (Reese et al., 2000). It has been shown that, in vitro, MEX-5 can compete POS-1 off of a common target mRNA (Oldenbroek et al., 2013). Therefore one possibility is that MEX-5/6 competes with PIE-1, POS-1, and MEX-1 for common transcripts that retard their diffusion, thus promoting their “release” into a free-diffusing state. The apparent difference in the responsiveness of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 to MEX-5/6 may result from their association with cytoplasmic RNA. Consistent with this possibility, mutations predicted to reduce the affinity of MEX-5 for RNA increase MEX-5 mobility and flatten the MEX-5 gradient (Griffin et al., 2011). In addition, mutations predicted to cause misfolding of the second PIE-1 zinc-finger RNA-binding domain prevent the segregation of GFP::PIE-1 (Reese et al., 2000). It has been shown that, in vitro, MEX-5 can compete POS-1 off of a common target mRNA (Oldenbroek et al., 2013). Therefore one possibility is that MEX-5/6 competes with PIE-1, POS-1, and MEX-1 for common transcripts that retard their diffusion, thus promoting their “release” into a free-diffusing state. The apparent difference in the responsiveness of GFP::PIE-1, GFP::POS-1, and GFP::MEX-1 to MEX-5/6 may
reflect how easily these proteins can be dissociated from slow-diffusing complexes by MEX-5/6. We consider it unlikely that MEX-5/6 act solely to control the partitioning of an unknown regulator of GFP::PIE-1 mobility, because if this were the case, we would not predict that modulating MEX-5/6 levels in a nonpolarized embryo (such as a par-1 mutant embryo) would alter GFP::PIE-1 mobility.

MEX-5/6 contribute to the disassembly of P granules in the anterior cytoplasm and have been proposed to control the saturation concentration at which P granule components phase separate (Schubert et al., 2000; Brangwynne et al., 2009; Gallo et al., 2010). One intriguing possibility is that MEX-5/6 act through a common mechanism to contribute to P granule disassembly and to increase PIE-1, POS-1, and MEX-1 mobility. P granule components assemble into micrometer-sized foci in part due to the propensity of P granule proteins to aggregate through intrinsically disordered domains and/or high-velocity interactions (Hanazawa et al., 2011, Updike et al., 2014; Wang et al., 2014; Elbaum-Garinlke et al., 2015). PIE-1, POS-1, and MEX-1 may be less prone to assemble/aggregation than other P granule components, which may explain why they from diffuse cytoplasmic gradients rather than discrete foci in the posterior cytoplasm.

For cytoplasmic concentration gradients to persist, the dissipation of gradients by random diffusion must be overcome (Elowitz et al., 1999; Lipkov and Odde, 2008). In the case of morphogens, local protein production coupled with protein movement and degradation can generate gradients that form over the course of hours and span hundreds of micrometers (Wartlick et al., 2009; Muller et al., 2013). These mechanisms are unlikely to generate gradients at intracellular length scales due to the homogenizing effects of random diffusion (Howard, 2012). The work presented here, along with recent theoretical and experimental studies, supports an emerging model in which local modulation of protein mobility provides a robust and rapid mechanism by which cytoplasmic concentration gradients can be established at cellular length scales (Lipkov and Odde, 2008; Daniels et al., 2009; Griffin et al., 2011; Kiekebusch et al., 2012).

MATERIALS AND METHODS
Worm strains
All worms were maintained at 25°C on nematode growth medium (NGM) plates and OP50 bacteria unless otherwise noted. The following transgenic strains were used in this study: JH2015, GFP::PIE-1 (unc-119(ed3); axes1462[pCM4.08]; Merritt et al., 2008); JH2214, GFP::MEX-3 (unc-119(ed3); axes1602[pCM4.38]; Merritt et al., 2008); JH1766, GFP::POS-1 (unc-119(ed3); axes1266[pMS4.03]; Stitzel et al., 2007); and JH1743, GFP::MEX-1 (unc-119(ed3); axes1251, a gift of G. Seydoux, Johns Hopkins University School of Medicine, Baltimore, MD). All four transgenic strains express N-terminal GFP fusion proteins at similar levels under the control of the pie-1 promoter and their own 3’ untranslated region (UTR), with the exception of GFP::POS-1, which is controlled by the PIE-1 3’ UTR. Strains with PIE-1 tagged at its endogenous locus were WM329 (PIE-1(ne4300[gfp::PIE-1])) and WM330 (PIE-1(ne4301[pie-1::GFP])); Kim et al., 2014. The mutant strains used in this study were KK292 (par-1(it51) rol-4(sc80)Dnt1), Guo and Kemphues, 1995; KK571 (lon-1(e185) par-3(it71)/qC1 dpy-19(e1259) gfp-1(q339)/lii); Cheng et al., 1995; and JJ1244 (mex-6(pk440) II; unc-30(e191) mex-5(zu199) IV/nT1 [IV];V); Schubert et al., 2000.

RNAi
RNAi depletions were performed using the feeding method with plasmids derived from L4440 (Timmons and Fire, 1998). HT115 bacteria (110 μl) transformed with an RNAi feeding plasmid were spread on NGM plates containing 1 mM isopropyl-β-D-thiogalactoside and 25 μg/ml carbenicillin. Plates were incubated overnight at 22°C. L4 worms were then grown on these RNAi plates for 24 h at 25°C, except for par-1 (RNAi), which was incubated for 28 h. MEX-5 (pEG656) and MEX-6 (pEG658) RNAi constructs target the second exons of MEX-5 and MEX-6, respectively. The PAR-1 RNAi construct (pEG793) targets the full-length PAR-1 coding sequence. pDU49 was used to simultaneously deplete GLH-1, GLH-4, PGL-1, and PGL-3 (gift of D. Updike, MDI Biological Laboratory, Bar Harbor, ME; Updike et al., 2014). For partial depletion of MEX-5/6 by RNAi, MEX-5 and MEX-6 RNAi bacteria were mixed and diluted 1:2.5, 1:5, and 1:7.5 and 1:10 with HT115 bacteria transformed with L4440 (empty RNAi feeding vector) and 1:40 dilution of GFP RNAi bacteria (in order to reduce expression of the GFP::PIE-1 transgene for FCS analysis).

Western blots
For quantification of MEX-5 levels after partial depletion by RNAi, 90 worms from each RNAi treatment were washed twice in egg salts, suspended in 45 μl of egg salts, flash frozen on dry ice, and stored at ~80°C. Samples were thawed, and 15 μl of 4× Laemmli Sample Buffer (Bio-Rad, Hercules, CA) containing 5 mM phenylmethylsulfonyl fluoride (PMSF) was added. Samples were sonicated for 90 s in a Branson 1510 sonicatoring water bath. After sonication, 15 μl of dithiothreitol (DTT) was added, making a final concentration of 200 mM for DTT and 1 mM for PMSF. Samples were boiled at 95°C for 5 min, centrifuged at 13,000 rpm for 1 min, and loaded onto Any kD Mini-PROTEAN TGX Gel (Bio-Rad). Extracts from the equivalent of 14.4 (two experiments) or 10 (one experiment) worms treated with various strengths of mex-5/6-, GFP/(RNAi) were loaded per lane. To generate a standard curve for the MEX-5 and tubulin Western blot signals, a range of extract volumes from adult par-1(it51) worms (between 3.6 and 18 worm equivalents for two experiments and 2.5 and 12.5 worm equivalents for one experiment) were loaded, and the chemiluminescence intensities of MEX-5 and tubulin were plotted against the number of par-1 (nt51) worm equivalents loaded in each lane. The background signal for the MEX-5 antibody was determined using extract from JJ1244 worms (mex-6(pk440) II; unc-30(e191) mex-5(zu199) IV/nT1 [IV];V). Curves were fitted to polynomial functions with $R^2 > 0.985$ in Excel (Microsoft, Redmond, WA). For all experiments, the signal from the MEX-5/6 RNAi samples was within the range of the standard curve. The relative concentration of MEX-5 was determined by normalizing to tubulin and averaged between duplicate blots. Proteins were transferred to Immobilon-P polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA) and probed first with guinea pig anti-MEX-5 (Griffin et al., 2011) at a 1:1000 dilution. These blots were stripped using Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) and probed with mouse monoclonal anti-α-tubulin antibody DM1A (Sigma-Aldrich, St. Louis, MO) at 1:2500 dilution. Peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:20,000 dilution. All antibodies were diluted in phosphate-buffered saline/Tween-20 (0.1%) with 5% milk. Blots were developed with the Clarity Western ECL Substrate (Bio-Rad) and imaged with the ChemiDoc XRS system (Bio-Rad).

Live microscopy and image analysis
For gradient quantification (Figure 1), worms were dissected and embryos were imaged in M9 buffer on a 3% agarose pad. Images for gradient quantification (Figure 1) were obtained under the following conditions: 530 nm excitation and 580 nm emission wavelengths with a 10x objective (Zeiss). Worms were imaged in M9 buffer on a 3% agarose pad. Images were obtained under the following conditions: 530 nm excitation and 580 nm emission wavelengths with a 10x objective (Zeiss).

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with a Zeiss Plan-Apochromat 63x/1.4 numerical aperture (NA) oil immersion objective, an Evolve 512 x 512 electron-multiplying charge-coupled device camera (Photometrics, Tucson, AZ), a 488-nm, 50-mW solid-state laser, and a CSU-X1 spinning disk (Yokogawa, Tokyo, Japan). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). For all acquisitions, the camera intensification was 300, and the camera gain was 1. Images in Figure 1A are from time-lapse acquisitions in which images were captured at 15 s intervals with the 488-nm laser set at 60% power and 600 ms exposures. Time-lapse images quantified in Figure 1B were collected every 20 s using 200 ms exposures and 488 nm laser set at 45% power. Images quantified in Figure 1C were captured at the cell midplane using 488-nm laser set at 60% power and 1 s exposures.

To quantify the mean concentration in the anterior and posterior cytoplasm (Figure 1B), the mean fluorescence intensities in the anterior half, the posterior half, and throughout the zygote were determined at each time point. Background signals (outside the embryo) were subtracted from the embryonic values. Fluorescence intensities were normalized to the initial total value for each embryo. Movies were aligned temporally relative to pronuclear meeting. For line scan analysis (Figure 1C), fluorescence intensities were averaged for 15 pixels perpendicular to the A/P axis using ImageJ (National Institutes of Health, Bethesda, MD). Background signals (outside the embryo) were subtracted from the embryonic values. The fluorescence intensities along the A/P axis were normalized to the average of the first 10 pixels on the anterior end. To average the fluorescence values from multiple embryos that differ slightly in length, the signal at 201 regularly spaced points along the A/P axis (correlating to every 0.5% embryo length) was estimated using linear interpolation with the interp1 function in Matlab (version R2013a; MathWorks, Natick, MA).

Fluorescence correlation spectroscopy
To reduce GFP expression levels to facilitate FCS analysis, 60 worms were fed with GFP RNAi bacteria diluted at 1:40 in L4440 empty vector bacteria for 24 h at 25°C. Embryos were dissected from young adults and imaged in M9 on a 3% agarose pad. FCS was performed on a Nikon A1 laser scanning confocal microscope using young adults and imaged in M9 on a 3% agarose pad. FCS was quantified in Figure 1C were captured at the cell midplane using a 488-nm laser set at 60% power and 600 ms exposures. Time-lapse acquisitions in which images were captured at 15 s intervals with the 488-nm laser set at 60% power and 600 ms exposures. Time-lapse images quantified in Figure 1B were collected every 20 s using 200 ms exposures and 488 nm laser set at 45% power. Images quantified in Figure 1C were captured at the cell midplane using 488-nm laser set at 60% power and 1 s exposures.

To quantify the mean concentration in the anterior and posterior cytoplasm (Figure 1B), the mean fluorescence intensities in the anterior half, the posterior half, and throughout the zygote were determined at each time point. Background signals (outside the embryo) were subtracted from the embryonic values. Fluorescence intensities were normalized to the initial total value for each embryo. Movies were aligned temporally relative to pronuclear meeting. For line scan analysis (Figure 1C), fluorescence intensities were averaged for 15 pixels perpendicular to the A/P axis using ImageJ (National Institutes of Health, Bethesda, MD). Background signals (outside the embryo) were subtracted from the embryonic values. The fluorescence intensities along the A/P axis were normalized to the average of the first 10 pixels on the anterior end. To average the fluorescence values from multiple embryos that differ slightly in length, the signal at 201 regularly spaced points along the A/P axis (correlating to every 0.5% embryo length) was estimated using linear interpolation with the interp1 function in Matlab (version R2013a; MathWorks, Natick, MA).

Fluorescence correlation spectroscopy
To reduce GFP expression levels to facilitate FCS analysis, 60 worms were fed with GFP RNAi bacteria diluted at 1:40 in L4440 empty vector bacteria for 24 h at 25°C. Embryos were dissected from young adults and imaged in M9 on a 3% agarose pad. FCS was performed on a Nikon A1 laser scanning confocal microscope using a 60X/1.27 NA water immersion objective (Plan Apo IR; Nikon, Melville, NY). A pulsed 485-nm laser at 20-MHz repetition frequency (PDL800-D; PicoQuant GmbH, Berlin, Germany) was used, and autocorrelation curves were obtained with afterpulsing suppression in SymPhoTime (PicoQuant). The pinhole was set to 1 Airy unit. The microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations). This microscope was controlled with the SlideBook software package (Intelligent Imaging Innovations).

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REFERENCES
Boyd L, Guo S, Levitan D, Stinchcomb DT, Kemphues KJ (1996). PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans embryos. Development 122, 3075–3084.
Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharaikhani J, Jülicher F, Hyman AA (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. Science 324, 1729–1732.
Cheng NN, Kirby CM, Kemphues KJ (1995). Control of cleavage spindle orientation in Caenorhabditis elegans: the role of the genes par-2 and par-3. Genetics 139, 549–559.
Cuenca AA, Schetter A, Aceto D, Kemphues K, Seydoux G (2003). Polarization of the C. elegans zygote proceeds via distinct establishment and maintenance phases. Development 130, 1255–1265.
Daniels BR, Dobrowsky TM, Perkins EM, Sun SX, Wirtz D (2010). MEX-5 enrichment in the C. elegans early embryo mediated by differential diffusion. Development 137, 2579–2585.
Daniels BR, Perkins EM, Dobrowsky TM, Sun SX, Wirtz D (2009). Asymmetric enrichment of PIE-1 in the Caenorhabditis elegans zygotes mediated by binary counterdiffusion. J Cell Biol 184, 473–479.
Drapor BW, Mello CC, Bowerman B, Hardin J, Priess JR (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell 83, 743–752.
Gallo CM, Wang JT, Motefi F, Seydoux G (2010). Cytoplasmic partitioning of P granule components is not required to specify the germ line in C. elegans. Science 330, 1685–1689.

Goldstein B, Macara IG (2007). The PAR proteins: fundamental players in animal cell polarization. Dev Cell 13, 609–622.

Griffin EE, Odde DJ, Seydoux G (2011). Regulation of the MEX-5 gradient by a spatially segregated kinase/phosphatase cycle. Cell 146, 955–968.

Guedes S, Priess JR (1997). The C. elegans MEX-1 protein is present in germ line blastomers and is a P granule component. Development 124, 731–739.

Guo S, Kemphues KJ (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81, 611–620.

Hanazawa M, Yonetani M, Sugimoto A (2011). PGL proteins self associate and bind RNPs to mediate germ granule assembly in C. elegans. J Cell Biol 192, 929–937.

Howard M (2012). How to build a robust intracellular concentration gradient. Trends Cell Biol 22, 311–317.

Kemphues K (2000). PARsing embryonic polarity. Cell 101, 345–348.

Kiekebusch D, Michie KA, Essen LO, Löwe J, Thanbichler M (2012). Localized dimerization and nuclear binding drive gradient formation by the bacterial cell division inhibitor Mip2. Mol Cell 46, 245–259.

Kim H, Ishidate T, Ghanta KS, Seth M, Conte D, Ji, Shirayama M, Mello CC (2014). A Co-CRISPR strategy for efficient genome editing in Cae norhabditis elegans. Genetics 197, 1069–1080.

Lipkov K, Odde DJ (2008). Model for protein concentration gradients in the cytoplasm. Cell Mol Bioeng 1, 84–92.

Mello CC, Schubert C, Draper B, Zhang W, Lobel R, Priess JR (1996). The PIE-1 protein and germ line specification in C. elegans embryos. Nature 382, 710–712.

Merritt C, Rasoloson D, Ko D, Seydoux G (2008). Three UTIs are the primary regulators of gene expression in the C. elegans germ line. Curr Biol 18, 1767–1772.

Muller P, Rogers KW, Yu SR, Brand M, Schier AF (2013). Morphogen transport. Development 140, 1621–1638.

Nance J, Zallen JA (2011). Elaborating polarity: PAR proteins and the cytoskeleton. Development 138, 799–809.

Nishi Y, Rogers E, Robertson SM, Lin R (2008). Polo kinases regulate C. elegans embryonic polarity via binding to DYRK2-primed MEX-5 and MEX-6. Development 135, 687–697.

Ogura K, Kishimoto N, Mitani S, Gengyo-Ando K, Kohara Y (2003). Translational control of maternal glp-1 mRNA by POS-1 and its interacting protein SPN-4 in Caenorhabditis elegans. Development 130, 2495–2503.

Oldenbroek M, Robertson SM, Guven-Oztan T, Spike C, Greenstein D, Lin R (2013). Regulation of maternal Wnt mRNA translation in C. elegans embryos. Development 140, 4614–4623.

Pang KM, Ishidate T, Nakamura K, Shirayama M, Trzepacz C, Schubert CM, Priess JR, Mello CC (2004). The minibrain kinase homolog, mbk-2, is required for spindle positioning and asymmetric cell division in early C. elegans embryos. Dev Bio 265, 127–139.

Pellettiere J, Reinke V, Kim SK, Seydoux G (2003). Coordinate activation of maternal protein degradation during the egg-to-embryo transition in C. elegans. Dev Cell 5, 451–462.

Quintin S, Mains PE, Zinke A, Hyman AA (2003). The mbk-2 kinase is required for inactivation of MEI-1/katanin in the one-cell Caenorhabditis elegans embryo. EMBO Rep 4, 1175–1181.

Reese KJ, Dunn MA, Waddle JA, Seydoux G (2000). Asymmetric segregation of PIE-1 in C. elegans is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. Mol Cell 6, 445–455.

Rose LS, Kemphues KJ (1998). Early patterning of the C. elegans embryo. Annu Rev Genet 32, 521–545.

Schlaht A-L, Srayko M, Dammermann A, Quintin S, Wielsch N, MacLeod I, de Robillard Q, Zinke A, Yates JR 3rd, Muller-Reichert T, et al. (2007). The C. elegans RSA complex localizes protein phosphatase 2A to centrosomes and regulates mitotic spindle assembly. Cell 128, 115–127.

Schubert CM, Lin R, de Vries CJ, Plasterk RH, Priess JR (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Mol Cell 5, 671–682.

Schwille P, Haupts U, Maiti S, Webb WW (1999). Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation. Biophys J 77, 2251–2265.

Stitziel ML, Cheng KC, Seydoux G (2007). Regulation of MBK-2/Dyrk kinase by dynamic cortical anchoring during the oocyte-to-zygote transition. Curr Biol 17, 1545–1554.

Strome S, Wood WB (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans. Proc Nat Acad Sci USA 79, 1558–1562.

Stulson JE, Schierenberg E, White JG, Thomson JN (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Bio 100, 64–119.

Tabara H, Hill RJ, Mello CC, Priess JR, Kohara Y (1999). pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans. Development 126, 1–11.

Tabuse Y, Irimi Y, Pino F, Kemphues KJ, Miwa J, Ohno S (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. Development 125, 3607–3614.

Tennenhaus C, Schubert C, Seydoux G (1998). Genetic requirements for PIE-1 localization and inhibition of gene expression in the embryonic germ lineage of Caenorhabditis elegans. Dev Bio 200, 212–224.

Tenlen JR, Molk JN, London N, Page BD, Priess JR (2008). MEX-5 asymmetry in one-cell C. elegans embryos requires PAR-4 and PAR-1-dependent phosphorylation. Development 135, 3665–3675.

Timmons L, Fire A (1998). Specific interference by ingested dsRNA. Nature 395, 854.

Updike DL, Hatchey SJ, Kreher J, Strome S (2011). P granules extend the nuclear pore environment in the C. elegans germ line. J Cell Biol 192, 939–948.

Updike DL, Knutson AK, Egelhofer TA, Campbell AC, Strome S (2014). Germ-granule components prevent somatic development in the C. elegans germline. Curr Biol 24, 970–975.

Updike D, Strome S (2010). P granule assembly and function in Caenorhabditis elegans germ cells. J Androl 31, 53–60.

Wang JT, Seydoux G (2013). Germ cell specification. Adv Exp Med Biol 757, 17–39.

Wang JT, Smith J, Chen BC, Schmidt H, Rasoloson D, Paix A, Lambrus BG, Calidas D, Betzig E, Seydoux G (2014). Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in C. elegans. Elife 3, e04591.

Warlick O, Kicheva A, Gonzalez-Gaitan M (2009). Morphogen gradient formation. Cold Spring Harb Perspect Biol 1, a001255.

Watts JL, Etemad-Moghadam B, Guo S, Boyd L, Draper BW, Mello CC, Priess JR, Kemphues KJ (1996). par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. Development 122, 3133–3140.