Supplemental Information

Figure S1, related to Figure 2.

A. Single-plane confocal images of wild-type and *pkc-3 (RNAi)* embryos expressing GFP::PAR-1 or Dendra::MEX-5. Note that in *pkc-3(RNAi)* embryos, PAR-1 localizes throughout the cortex and cytoplasm and Dendra^{R}::MEX-5 is evenly distributed throughout the cytoplasm. PAR-1 also localizes on centrosomes (bright dots) as reported previously (Gönczy et al., 2001).

B. Single-plane confocal images of zygotes of the indicated genotypes immunostained with antibodies against PKC-3 (Aono et al., 2004) and PAR-1 (Guo and Kemphues, 1995) or expressing Dendra::MEX-5. PKC-3 is enriched on the anterior cortex in all genotypes.

C. Quantification of the GFP::PAR-1 concentration gradient in wild-type and *par-2 (RNAi)* zygotes at pronuclear centration (pronuclei have met at the center of the embryo) and after NEBD. GFP::PAR-1 concentration was determined for multiple embryos (between 7 and 15) at regular positions along the long axis. Obtaining accurate scale measurements is complicated by the fact that GFP::PAR-1 levels approach auto-fluorescence levels in the anterior cytoplasm. To correct for the background signal from auto-fluorescence, auto-fluorescence was measured in embryos not expressing GFP (n = 8) and subtracted from GFP::PAR-1 values. GFP::PAR-1 levels are expressed as the mean concentration relative to the concentration at 2.5% embryo length

(normalized to 1 for each embryo). Values between 45% and 65% embryo length were omitted because of signal distortion caused by accumulation of GFP::PAR-1 on centrosomes in this region. Error bars represent SEM.

D. Quantification of endogenous PAR-1 levels in wild-type embryos. Ten mitotic stage embryos were stained with anti-PAR-1 antibody (Guo and Kemphues, 1995) and imaged at the midplane of the cell. Fluorescence levels are expressed as the mean concentration relative to the concentration at 2.5% embryo-length (normalized to 1 for each embryo). Values between 35% and 55% embryo-length were omitted because of signal distortion caused by the presence of pronuclei and the accumulation of PAR-1 on centrosomes. Unlike the GFP::PAR-1 quantification in panel C, background cytoplasmic staining (Guo and Kemphues, 1995) has not been subtracted from these values. This likely results in the apparently weaker enrichment of endogenous PAR-1 in the posterior cytoplasm relative to GFP::PAR-1. In an additional 10 out of 12 embryos (not included in this quantification), PAR-1 levels were higher in the posterior cytoplasm compared to the anterior cytoplasm.

E. Western blot analysis of PAR-1 in wild-type, *par-1(it51)* and *par-1(b274)* worms. Extracts from approximately 130 hermaphrodites were separated on SDS-PAGE gels and probed with anti-PAR-1 (Guo and Kemphues, 1995) and anti-tubulin antibodies (mouse anti-tubulin monoclonal DM1A, Sigma-Aldrich). Position of molecular weight markers are indicated on the left. *par-1(b274)* worms lack full length PAR-1, and express instead a truncated form (~86 kDa) at 14% of wild-type levels.

F. Concentration ratio of anterior to posterior Dendra^{R}::MEX-5 in wild-type and *par-2(RNAi)* embryos at pronuclear centration and following NEBD. Note that Dendra^{R}::MEX-5 asymmetry at pronuclear centration is reduced in *par-2(RNAi)* embryos compared to wild-type and weakens further following NEBD. This is consistent with the weaker PAR-1 gradient that forms by pronuclear meeting in *par-2(RNAi)* embryos and its decay after NEBD (Panel C).

Figure S2, related to Figure 3.

A. Western blot demonstrating the specificity of the anti-MEX-5 (pS404) and anti-MEX-5 (pS458) phosphospecific antibodies: Western blot analysis of samples from *in vitro* kinase reactions using fusion proteins partially purified from *E. coli*. MBP::MEX-5 fusion proteins containing the indicated substitutions were incubated with or without MBP::PAR-1 (aa1-492, T325E). Samples were separated by SDS PAGE and probed with antibodies against MEX-5, MEX-5 (pS404), and MEX-5 (pS458).

B. Confocal micrographs of GFP::PAR-1 localization in wild-type and *let-92 (RNAi)* embryos. GFP::PAR-1 segregates to the posterior of *let-92 (RNAi)* embryos as it does in wild-type.

C. Cortical PAR-1 domain expressed as a percentage of total embryo length in wild-type (WT) and *let-92 (RNAi)* embryos. The variability in PAR-1 domain size in *let-92(RNAi)* embryos is due to the occasional embryo with an off-axis GFP::PAR-1 domain, but PAR-1 was asymmetric in all embryos examined.

Figure S3, related to Figure 4. The gradient formed by MEX-5 (C286S,C292S,C331S,C337S) depends on endogenous MEX-5 and MEX-6.

A. Ratio of anterior to posterior concentration of Dendra^{R}::MEX-5 and Dendra^{R}::MEX-5 (C286S,C292S,C331S,C337S) (denoted CC-SS in graph) in wild-type embryos and *mex-5(zu199); mex-6(RNAi)* embryos at NEBD. C286S, C292S,C331S, C337S are mutations that replace the first two zinc-coordinating cysteines in each finger and are predicted to disrupt folding of the fingers and RNA binding (Lai et al., 2002). Unlike other MEX-5 fusions in this study, which are driven by the *mex-5* promoter, these fusions were driven by the weaker *pie-1* promoter. We attempted to generate Dendra::MEX-5(C286S,C292S,C331S,C337S) driven by the higher expressing *mex-5* promoter, but were not able to recover lines raising the possibility that this fusion is toxic at higher expression levels. As in Tenlen et al., 2008, we were able to recover lines driven by the weaker *pie-1* promoter and found that Dendra^{R}::MEX-5 (C286S,C292S,C331S,C337S) formed a gradient similar to that seen with wild-type Dendra^{R}::MEX-5. As shown here, however, the Dendra^{R}::MEX-5 (C286S,C292S,C331S,C337S) gradient is dependent on endogenous MEX-5 and its homologue MEX-6. In *mex-5(zu199); mex-6(RNAi)* zygotes, Dendra^{R}:MEX-5(C286S,C292S,C331S,C337S) formed at most a weak gradient.

B. Apparent diffusion coefficients of Dendra^{R}::MEX-5 mutants measured at NEBD (after polarization) in the presence of endogenous MEX-5 and MEX-6. Dendra^{R}::MEX-5 (C286S,C292S,C331S,C337S) diffuses approximately twice as fast as Dendra^{R}::MEX-5, consistent with a defect in

anchoring. We conclude that the RNA binding domain of MEX-5 restricts mobility, and that interactions among MEX-5 and MEX-6 molecules may also influence mobility.

Figure S4, related to Figure 5.

A. Representative UV traces and western blots following sucrose gradient fractionation of whole worm extracts with or without RNAseA treatment from transgenic worms expressing Dendra::MEX-5. UV traces were generated by flowing gradients through a UV detector (described in the Methods) during fraction collection. The position of polysomes, 40S, 60S, and 80S ribosomal subunits are indicated. Note that the relatively mild RNAse treatment eliminates polysomes but preserves 80S subunits. Photoshop was used to crop out lanes containing protein standards from the western blots.

B. Comparison of apparent MEX-5 diffusion coefficients determined using photoconversion of Dendra^{R}::MEX-5 or using FCS on GFP::MEX-5 and fitting the spectra to one or two-component models. For two component FCS models, population diffusion coefficients were calculated as the weighted average of fast and slow-diffusing components. One-component FCS models yielded diffusion coefficients that were significantly lower than those determined experimentally with Dendra^{R}::MEX-5. In contrast, two component models fit the Dendra^{R}::MEX-5 values well. The Dendra^{R}::MEX-5 diffusion coefficients are also presented in Figures 2C and 2D.

C. Box and whiskers plot of diffusion coefficients of GFP::MEX-5 complexes as calculated by FCS analysis. The boxes contain the 25th to 75th percentile and the whiskers contain 10-90th percentile. Data points outside the 10-90th percentile are plotted as individual points. Note that in all measurements, both fast and slow components were detected, with ~100 fold difference in diffusion. The mean percentage of each component is indicated below the graph and is also presented in Figure 5B. 3-component models (analyzed between time-lags 7.2μsec and 3.35 sec as used for 1 and 2 component models) yielded a similar range of average diffusion coefficient values (anterior = .038, 0.41, and 5.15 μm^2/sec; posterior = 0.028, 0.45 and 8.04 μm^2/sec). A 3-component model analyzed between the time-lags 16μsec and 184msec as used by Daniels et al., 2010 again yielded a similar range of average diffusion coefficients (anterior = .032, 0.48, and 5.59 μm^2/sec; posterior = 0.030, 0.60 and 9.51 μm^2/sec). In all cases, the slowest-diffusing component was no less than 35% of total in the posterior cytoplasm and 43% of total in the anterior cytoplasm. We conclude that including the slow component is important when constructing models of MEX-5 diffusion.

Figure S5, Related to Figure 6. Unsteady-state analysis of cytoplasmic PAR-1 model.

A. Schematic of the cytoplasmic PAR-1 model using the base parameters. In this model, the transitions of MEX-5 between the phosphorylated, fast diffusing form (depicted by a green circle) and the dephosphorylated, slow

diffusing form (depicted by a red circle) is controlled by PAR-1 and PP2A. PAR-1 activity is in an anterior/low to posterior/high linear cytoplasmic gradient. Phosphatase activity is uniform in the cytoplasm. Because k_{phos} is uniform, the mean time spent in the fast state (τ_{fast}) and the root-mean-squared distance traveled by the fast (l_{fast}) species are uniform throughout the cell. In contrast, the mean time spent in the slow state (τ_{slow}) and the root-mean-squared distance traveled by the slow species (l_{slow}) varies along the A/P axis. The length of the embryo (L) is 50 μm and the values for l_{fast}, τ_{fast}, l_{slow}, and τ_{slow} at midpoint of the cell (X=0.5) are shown. See the Extended Experimental Procedures for a discussion of the contribution of different parameters to the cytoplasmic PAR-1 model.

B. Graphs showing the results of a 600 second simulation of the cytoplasmic PAR-1 model with the base set of parameters: cytoplasmic PAR-1 activity gradient (K_{kin} = 0.02-0.11s^{-1}), uniform phosphatase activity (k_{phos} = 0.1s^{-1}), D_{Slow} = 0.07 μm^2/sec, and D_{Fast} = 5.0 μm^2/sec. The concentrations of the slow MEX-5 species (C_a, left graph), the fast MEX-5 species (C_b, middle graph) and total MEX-5 (C_t, right graph) are plotted against Time (in seconds) and Distance along the anterior/posterior axis (anterior, x = 0 μm; posterior, x = 50 μm). Concentration is coded by a rainbow scale in which red represents maximum and blue represents minimum concentration for each species within each simulation. At t=0, 70% of MEX-5 is in the slow state and 30% of MEX-5 is in the fast state.

C. The effect of increasing phosphatase activity 10-fold (k_{phos} = 1 s^{-1}).

D. The effect of decreasing phosphatase activity 10-fold (k_{phos} = 0.01 s^{-1}).

E. The effect of increasing kinase activity 10-fold (k_{kin} = 0.2-1.1 s^{-1}).

F. The effect of decreasing kinase activity 10-fold (k_{kin} = 0.002-0.011 s^{-1}).

G. The effect of increasing kinase and phosphatase activity 10-fold (k_{kin} = 0.2-1.1 $s^{-1,}$ k_{phos} = 1 s^{-1}).

H. The effect of decreasing kinase and phosphatase activity 10-fold (k_{kin} = 0.002-0.011 $s^{-1,}$ k_{phos} = 0.01 s^{-1}). Note that this simulation was run for 6000 seconds because of the slow reaction kinetics.

I. The effect of decreasing kinase and phosphatase activity 100-fold (k_{kin} = 0.0002-0.0011 $s^{-1,}$ k_{phos} = 0.001 s^{-1}). Note that this simulation was run for 6000 seconds because of the slow dynamics.

J. The effect of increasing the diffusivity of the slow MEX-5 species 10-fold (D_{slow} = 0.7 μm^2/sec).

K. The effect of decreasing the diffusivity of the slow MEX-5 species 10-fold (D_{slow} = 0.007 μm^2/sec).

L. The effect of increasing the diffusivity of the fast MEX-5 species 10-fold (D_{fast} = 50 μm^2/sec).

M. The effect of decreasing the diffusivity of the fast MEX-5 species 10-fold (D_{fast} = 0.5 μm^2/sec).

Figure S6, Related to Figure 6. Unsteady-state analysis of cortical PAR-1 model.

A. Schematic of the cortical PAR-1 model. As in the cytoplasmic PAR-1 model, the transition of MEX-5 between the phosphorylated, fast diffusing form (depicted by a green circle) and the dephosphorylated, slow diffusing form (depicted by a red circle) is controlled by PAR-1 and PP2A. Phosphatase activity is uniform in the cytoplasm. PAR-1 activity is restricted to the posterior cortex and is assumed to be instantaneous in order to maximize its potential affect on MEX-5. See the Extended Experimental Procedures for a discussion of the contribution of different parameters to the cortical PAR-1 model.

B. Cortical PAR-1 model with $k_{phos} = 0.1\ s^{-1}$. Note that in this and all subsequent panels in this Figure, the simulation was run for 7200 seconds because of the slow dynamics.

C. The effect of decreasing phosphatase activity 10-fold ($k_{phos} = 0.01\ s^{-1}$).

D. The effect of decreasing phosphatase activity 1000-fold ($k_{phos} = 0.0001\ s^{-1}$).

E. The effect of increasing diffusivity of the slow MEX-5 species 10-fold ($D_{slow} = 0.7\ \mu m^2/sec$).

F. The effect of decreasing diffusivity of the slow MEX-5 species 10-fold ($D_{slow} = 0.007\ \mu m^2/sec$).

Table S1. Transgenic strains used in this study.

Strain	Description of Transgene	Genotype	Reference
JH2802	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR - mex-5 3'UTR	*unc-119(ed3); axIs1950[pEG584]*	This Study
JH2804	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(S404A) - mex-5 3'UTR	*unc-119(ed3); axIs1951 [pEG585]*	This Study
JH2721	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(aa1-355) - mex-5 3'UTR	*unc-119(ed3); axIs1952 [pEG610]*	This Study
JH2891	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(aa1-245) - mex-5 3'UTR	*unc-119(ed3); axIs1953 [pEG628]*	This Study
JH2892	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(aa246-468) - mex-5 3'UTR	*unc-119(ed3); axIs1954 [pEG634]*	This Study
JH2889	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(S458A) - mex-5 3'UTR	*unc-119(ed3); axIs1955 [pEG587]*	This Study
JH2890	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(R274E, K318E) - mex-5 3'UTR	*unc-119(ed3); axIs1956 [pEG593]*	This Study
JH2905	mex-5 prom::Dendra2/TEV/Speptide::MEX-5RR(M288E, F294N, Y343E, F339N) - mex-5 3'UTR	*unc-119(ed3); axIs1956 [pEG670]*	This Study
JH2549	pie-1 prom – Dendra2/TEV/Speptide/MEX-5 - pie-1 3'UTR	*unc-119(ed3); axIs1957 [pEG345]*	This Study
JH2553	pie-1 prom – Dendra2/TEV/Speptide/MEX-5(aa345-468) - pie-1 3'UTR	*unc-119(ed3); axIs1958 [pEG408]*	This Study
JH2560	pie-1 prom – Dendra2/TEV/Speptide - pie-1 3'UTR	*unc-119(ed3); axIs1959 [pEG412]*	This Study
JH2636	pie-1 prom – Dendra2/TEV/Speptide/MEX-5(C286S, C292S, C331S, C337S) - pie-1 3'UTR	*unc-119(ed3); axIs1960 [pEG474]*	This Study
JH2593	pie-1 prom – Dendra2/TEV/Speptide/MEX-5(S404A) - pie-1 3'UTR	*unc-119(ed3); axIs1961 [pEG476]*	This Study
JH2550	pie-1 prom – Dendra2/TEV/Speptide/MEX-5(S458A) - pie-1 3'UTR	*unc-119(ed3); axIs1962 [pEG389]*	This Study
JH2607	pie-1 prom – Dendra2/TEV/Speptide/MEX-5(S404A, S458A) - pie-1 3'UTR	*unc-119(ed3); axIs1963 [pEG494]*	This Study
JH2800	mex-5 prom::GFP/TEV/FLAG::MEX-5::mex-5 3'UTR	*unc-119(ed3); axIs1964 [pEG607]*	This Study
JH2801	mex-5 prom::GFP/TEV/FLAG::MEX-5::mex-5 3'UTR	*unc-119(ed3); axIs1965 [pEG608]*	This Study
JH2078	pie-1 prom-LAP::MEX-5::pie-1 3'UTR	*unc-119(ed3); axIs1504[pCG2]*	Gallo, 2010
JH1734	pie-1 prom-GFP::PAR-1::pie-1 3'UTR	*unc-119(ed3); axIs1245[pAC2.01]*	This Study

Abbreviations:

Prom; promoter

3'UTR; 3' untranslated region

:: ; Gateway recombination sites

TEV ; TEV protease cleavage site

RR ; RNAi Resistant version of MEX-5 in which exon 2 was recoded such that endogenous MEX-5 and MEX-6 could be depleted with RNAi constructs targeting exon 2.

Note that all transgene plasmids include the wild-type *unc-119* gene as a selection marker.

Extended Experimental Procedures

Transgenics and worm strains

Dendra::MEX-5 constructs were constructed as follows. A 4.4 kb *mex-5* promoter fragment based on (Tenlen et al., 2008) was cloned into pDONR201 P4P1R (Invitrogen). Dendra2/TEV/S-peptide (Gallo et al., 2010) was cloned into pDONR201. A MEX-5 genomic fragment from the start ATG through 648bp of 3'UTR was cloned into pDONR201 P2PR3. Exon 2 of *mex-5* was recoded to be RNAi-resistant (GenScript) so as to allow depletion of endogenous MEX-5/6 without depletion of the transgene. These constructs were assembled into pCG150 using three-way Gateway system (LR reaction) (Invitrogen) (Merritt et al., 2008). Mutations were made by recombinant PCR and all inserts were sequenced verified. The wild-type Dendra::MEX-5 transgene rescued *mex-5(RNAi)* to 97% viability (n=244) and *mex-5/6(RNAi)* to 58% viability (n=136). GFP::MEX-5 transgenes were constructed in the same way as Dendra::MEX-5 constructs except a pDONR201-GFP/TEV/FLAG entry clone was used and wild-type sequence of MEX-5 exon 2 was used. MEX-5 transgenes driven by the *pie-1* promoter and *pie-1* 3'UTR were constructed by cloning the *mex-5* cDNA as a

SpeI fragment downstream of a Dendra2/TEV/S-peptide tag derived into pIC26 LAP tag (Cheeseman et al., 2004). GFP::PAR-1 was constructed by cloning PAR-1 cDNA into pDONR201 and then recombining into pID3.01, a Gateway destination vector containing the *pie-1* promoter, GFP, and *pie-1* 3'UTR. The GFP::PAR-1 transgene rescues the embryonic lethality of *par-1(it32)*: GFP::PAR-1; *par-1(it32)* hermaphrodites are self-fertile and can be maintained over several generations. GFP::PAR-1 localization patterns were the same in wild-type and *par-1(it32)* backgrounds. Transgenic lines were generated by microparticle bombardment (Praitis et al., 2001) and are listed in Table S1. The following mutant strains were used in this study: *par-1(it51) rol-4(sc8)/DnT1*, *par-1(b274) rol-4(sc8)/DnT1* (Guo and Kemphues, 1995), *mex-6(pk440); mex-5(zu199) unc-30(e191)/nT1* (Schubert et al., 2000). To identify the mutation in *par-1(b274),* PAR-1 exons were PCR amplified and sequenced as PCR products from wild-type, *par-1 (it51)*, and *par-1(b274)* homozygous worms.

Live microscopy

Embryos were dissected in egg salts on a coverslip and inverted onto a 3% agarose pad. Spinning disk confocal images were collected on a Zeiss Axio Imager.Z1 microscope with a 63X 1.4 NA oil immersion objective and collected on a QuantEM 512SC camera (Photometrics). The microscope was controlled by the Slidebook software package (Intelligent Imaging Innovations, Inc.). Fluorescence intensities were determined from a single midplane images (the plane in which the pronuclei are in focus) and corrected for background by

subtracting signal outside the embryo (Image J). Anterior/posterior concentration ratios were determined by dividing the average fluorescence intensity in the anterior cytoplasm by the average fluorescence intensity in the posterior cytoplasm (anterior/posterior boundary was defined as 50% embryo-length). GFP::PAR-1 quantification was also corrected for embryo autofluorescence (which fluoresces in the same channel as GFP) by subtracting signal averaged from eight wild-type non-transgenic zygotes.

Antibodies and immunofluorescence

For immunofluorescence, gravid hermaphrodites were dissected in M9 media on 0.1% Poly-L-lysine-coated slides, placed under a coverslip, frozen on dry ice-chilled aluminum blocks, and freeze-cracked by removing the coverslips. For PAR-1 and PKC-3 staining, slides were incubated in -20°C methanol for 15 minutes, -20°C acetone for 10 minutes, and blocked in PBT + 0.1% BSA for 30 minutes. For MEX-5 staining, slides were fixed in -20°C methanol for 2 minutes and 2% paraformaldehyde for 20 minutes at 24°C, then blocked in PBT + 0.1% BSA for 30 minutes. Antibody dilutions were as follows: 1:100 rat anti-PKC-3 (Aono et al., 2004), 1:50 rabbit anti-PAR-1 (Guo and Kemphues, 1995), 1:100 guinea pig anti-MEX-5 (this study). For western blotting, anti-PAR-1 antibody (Guo and Kemphues, 1995) was diluted 1:1000 and anti-MEX-5 antibody was diluted 1:500.

Anti-MEX-5 antibodies were raised in 2 guinea pigs immunized with the peptide RMSHDDQDYDQDVIPEDYKKKC (Covance). Phospho-specific antibodies were

generated by Bethyl laboratories in rabbits using the peptides RNVAG(pS)MMCLSN (MEX-5 pS404) and CSTKWT(pS)EENLG (MEX-5 pS458).

Determination of Dendra^{R}::MEX-5 diffusion coefficients

Dendra::MEX-5 was photoconverted using either an 800msec pulse of 405nm laser controlled by a Mosaic Digital Illumination System (Photonic Instruments, INC) or 3 second pulse of light from a EXFO X-cite120 metal halide epifluorescence light source passed through a thin slit in a single layer of aluminum foil placed in front of a DAPI filter cube. 2 exposures prior and 15 exposures following photoconversion were collected (every 1.05 or 1.8 seconds). For all experiments except Figure 1D and Figure 1E "short axis", Dendra::MEX-5 was photoactivated in a stripe along the long axis positioned at the middle of the cell. For Figure 1E "short axis", Dendra::MEX-5 was photoactivated at ~50% embryo-length in a line perpendicular to the anterior/posterior axis. Time lapse images were analyzed in ImageJ (NIH). For Figure 1E intensity was averaged at the indicated positions in a 10 by 120 pixel box drawn perpendicular to the photoactivation stripe. For all other figures, intensity was averaged in a 20 by 120 pixel box positioned perpendicular to the photoactivation stripe at 25% and 75% embryo-length. Intensity values were fit to Gaussian distributions for each time point (GraphPad Prism). The standard deviation of the Gaussian was converted to variance in microns and plotted versus time. The slope of the linear regression = $2D_c$ (Berg, 1993). Error bars are standard error of the mean from a

minimum of 5 embryos. For Figure 1E, values along the long axis were derived from 14 embryos before pronuclear formation, 12 embryos at pronuclear formation, and 5 embryos at embryos at NEBD. Values along the short axis were derived from 4 embryos before pronuclear formation, 11 embryos at pronuclear formation and 11 embryos at NEBD. For all other figures, apparent diffusion coefficients were derived form at least 5 embryos.

RNAi and Latrunculin A treatment

RNAi was induced by feeding worms bacteria expressing double-stranded RNA as in (Timmons and Fire, 1998). For PAR-1 depletion prior to immunoprecipitation, worms were synchronized as L1s and then plated as L4s on bacteria expressing double-stranded PAR-1 RNA for 28 hours at 25°C. Latrunculin A (Sigma) treatment was performed as in (Severson and Bowerman, 2003), except that worms were fed *F08F8.2(RNAi*) bacteria for 24hrs at 25°C to increase egg shell permeability (Redemann et al., 2010).

Recombinant protein expression

Gateway cloning was used to clone full length MEX-5 and PAR-1(1-492) PCR products into pDONR201 (Invitrogen). pDONR constructs were recombined into the destination vector pJP1.09 (Stitzel et al., 2006). MBP-fusion proteins were expressed in *E. coli* strain CAG-456 overnight at 16°C following induction with 400μM isopropyl-β-D-1-thiogalactopyranoside (IPTG). MBP:MEX-5 cultures were supplemented with 0.1mM $ZnCl_2$ at the time of induction. Bacterial pellets

were sonicated in cold column buffer (20mM Tris 7.4, 500mM NaCl, 0.1mM $ZnCl_2$, 10% glycerol, 1mM DTT for MBP:MEX-5 and 20mM Tris 7.4, 500mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT for MBP:PAR-1(aa1492, T325E)) and centrifuged for 30 minutes at ~20,000g. Lysate supernatants were bound in batch to amylose resin (New England Biolabs), washed extensively and eluted with column buffer containing 10mM maltose. Partially purified proteins were aliquoted and stored at -80°C.

Kinase and dephosphorylation assays

Isotopic kinase assays were performed by incubating MBP:MEX-5 with MBP:PAR-1(aa1-492, T325E) in 20 mM Tris (7.4),150mM NaCl, 10μM ATP, and 2.5μCi [^{32}P] ATP (NEN) for 30 minutes at 30°C. Reactions were terminated by addition of NuPAGE LDS sample buffer (Invitrogen) and heating for 10 minutes at 70°C. For cold kinase and dephosphorylation assays, MBP:MEX-5 was incubated in 20 mM Tris (7.4),150mM NaCl, 100μM ATP with MBP:PAR-1(aa1-492, T325E) at 30°C and samples were taken at the indicated time points. The kinase reaction was terminated after 120 minutes with 20 nM staurosporine (Sigma). Dephosphorylation assays began with the addition of 0.25 μL embryonic extract with or without pretreatment with 200nM okadaic acid. Each sample was run on two separate SDS-PAGE gels and processed for Western blot using pS404 and pS458 antibodies. Embryonic extracts were prepared from embryos sonicated in equal volume 20mM Tris (7.4), 150mM NaCl, and 0.05%

NP-40 supplemented with Complete protease inhibitors (Roche) and stored in small aliquots at -80°C.

Sucrose gradient fractionation

Sucrose gradient fractionation was adapted from Hundley et al 2008 (Hundley et al., 2008). Young adults (grown from synchronized L1s at 25°C on NEP plates seeded with NA22 bacteria) were washed 3 times in M9 and 2 times in lysis buffer (20mM Hepes (pH 7.4), 5mM $MgCl_2$, 10mM KCl, 1mM $ZnCl_2$, 1mM EGTA, 10% glycerol). Worms were then incubated for 5 minutes in 3 volumes of lysis buffer supplemented with 0.5 mg/mL cycloheximide, 0.2 mg/mL heparin, Complete protease inhibitors (Roche) and PhosStop phosphatase inhibitors (Roche) for 5 minutes and frozen in liquid nitrogen. Worms were ground with a mortar and pestle chilled with liquid nitrogen and stored at -80°C. 1 gram of worm powder was lysed in 1mL Lysis buffer supplemented with protease and phosphatase inhibitors, 0.5mM DTT, 0.2 mg/mL Heparin, 80U/mL RNAseOut (Invitrogen), and 0.5 mg/mL cycloheximide by sonication. Lysates were clarified by centrifugation at 4°C for 10 minutes at 22,000g. 200μL of lysate was fractionated on a 10.5mL linear 10%-45% sucrose gradients (poured with a BioComp Gradient Master in 14X89mm polyallomar tubes (Beckmann #331372)) by centrifugation at 39,000rpm for 3 hours in a SW41 rotor. Gradients were passed through a UV detector (ISCO UA-6 UV) by pumping Fluorinert FC-40 (Sigma) below the gradients and 700μL fractions were collected. 30μL were analyzed by western blot using anti-Dendra antibody diluted 1:10,000 (Axxora).

Immunoprecipitations

Lysates were prepared as for sucrose fractionation except lysis buffer was 20mM Hepes 7.4, 150mM NaCl, 5mM $MgCl_2$, 1mM $ZnCl_2$, 1mM EGTA, 0.5mM DTT, 0.05% NP-40. pS404 antibody was coupled to ProteinG Dynabeads and used to immunoprecipitate from 1mL of adult worm lysate 4°C overnight, washed 4 times with 1 mL lysis buffer and eluted in NuPAGE LDS sample buffer (Invitrogen) for 10 minutes at 70°C.

Fluorescence Correlation Spectroscopy

Prior to imaging, GFP::MEX-5 expression was reduced by treating at least 50 worms with a dilution series of GFP(RNAi) bacteria diluted from 1:10 to 1:200 in L4440 (empty vector) RNAi bacteria. Worms expressing optimal levels of GFP were indentified empirically each day. Embryos were dissected in egg salts on a coverslip, inverted into a Fluoro-dish culture dish (World Precision Instruments) and immersed in egg salts. Imaging was performed on a Zeiss LSM 510 Confocal microscope equipped with a Confocor 3 FCS module using a 40X water immersion objective. Ten 10-second scans were collected at both 30 and 70% embryo-length in the same embryo. The first scan at each position was discarded to avoid photo-bleaching artifacts. Autocorrelation curves were analyzed between time-lags 7.2μs and 3.35 seconds using one, two or three component 3-dimensional models within the Zeiss Confocor 3 software package. The slow-diffusing species accounts for more than 35% of total MEX-5 when our

data is fit to a 3-component model using the parameters used in Daniels et al., 2008 (Daniels et al., 2010).

Mathematical Modeling of the MEX-5 gradient

The model is similar to that described in Lipkow and Odde (2008), with the following modifications. We assume that the kinase is distributed in a linearly increasing concentration along the AP-axis starting at the left boundary of a rectangular cell at x=0 (i.e. the anterior end of the embryo), and the phosphatase is distributed uniformly throughout the cytoplasm over 0<x<L. In this case where the kinase acts in the cytoplasm, the governing equations are given by

$$\frac{\partial c_A}{\partial t} = D_A \frac{\partial^2 c_A}{\partial x^2} + k_{phos} c_B - k_{kin}(x) c_A$$

for the dephosphorylated, slow-diffusing form of MEX-5 (here designated as “A”) and

$$\frac{\partial c_B}{\partial t} = D_B \frac{\partial^2 c_B}{\partial x^2} - k_{phos} c_B + k_{kin}(x) c_A$$

for the phosphorylated, fast-diffusing form of MEX-5 (here designated as “B”), where D_A and D_B are the diffusion coefficients of A and B, respectively, c_A and c_B are the molar concentrations of A and B, respectively, and k_{phos} and $k_{kin}(x)$ are

the first-order phosphatase and kinase rate constants, respectively. At steady-state, these equations become

$$0 = D_A \frac{\partial^2 c_A}{\partial x^2} + k_{phos} c_B - k_{kin}(x) c_A \quad (1)$$

and

$$0 = D_B \frac{\partial^2 c_B}{\partial x^2} - k_{phos} c_B + k_{kin}(x) c_A \quad (2)$$

.

The kinase activity gradient in the cytoplasm is assumed to be a linear function of position in the embryo given by

$$k_{kin}(x) = b + mx$$

where m (units: $\mu m^{-1}\ s^{-1}$) and b (units: s^{-1}) are the slope and intercept of the linear gradient. For the cytoplasmic PAR-1 only model (i.e. the base model), no flux boundary conditions were imposed for both A and B at both x=0 and x=L, so that $\left.\frac{\partial c_A}{\partial x}\right|_{x=0} = \left.\frac{\partial c_B}{\partial x}\right|_{x=0} = \left.\frac{\partial c_A}{\partial x}\right|_{x=L} = \left.\frac{\partial c_B}{\partial x}\right|_{x=L} = 0.$

In the case of PAR-1 acting at the posterior cortex, we modified the boundary

conditions at x=L such that

$$-D_A \left.\frac{\partial c_A}{\partial x}\right|_{x=L} = k_{kin,boundary} c_A(L) \tag{3}$$

and

$$-D_B \left.\frac{\partial c_B}{\partial x}\right|_{x=L} = -k_{kin,boundary} c_A(L) \tag{4}$$

where $k_{kin,boundary}$ is the first-order rate constant for the heterogeneous kinase reaction at the right boundary at x=L (set to 3 μm/s, sufficiently high to enforce $c_A(L)\approx 0$). In addition, for the cortical kinase model, $k_{kin}(x)=0$. At any point in the system, the total protein concentration, $c_T(x)$, is given by

$$c_T(x) = c_A(x) + c_B(x) \; . \tag{5}$$

The initial condition was assumed to be uniformly 70% A (slow) and 30% B (fast), with a total uniform concentration (arbitrary) of 3 μM.

The steady-state governing equations can be recast into a dimensionless form

$$0 = \alpha_A Y_A'' + Y_B - \beta(X) Y_A$$

and

$$0 = \alpha_B Y_B'' - Y_B + \beta(X) Y_A$$

where $Y_A = c_A/c_{T0}$, $Y_B = c_B/c_{T0}$, X=x/L, and

$$\alpha_A = \frac{D_A}{k_{phos} L^2}$$

$$\alpha_B = \frac{D_B}{k_{phos} L^2}$$

$$\beta(X) = \frac{b + mx}{k_{phos}} = \frac{b + mXL}{k_{phos}} \quad .$$

These dimensionless parameters help define regimes in which the cytoplasmic gradient model will work. First, for the cytoplasmic model to yield an appreciable gradient, the slow species must be much slower than the fast species, i.e.

$$\alpha_B >> \alpha_A$$

In this case, the relatively rapid diffusion of B means that gradients of B will be relatively weak, i.e. that $Y_B \approx$ constant. Thus, the two differential equations

become a single equation, given by the following equation, subject to the mass conservation constraint

$$\int_0^1 (Y_A + Y_B) dX = 1.$$

For the gradient of the slow species of A to be appreciable, the dimensionless parameter α_A must be small, i.e.

$$\alpha_A << 1$$

As α_A approaches unity, the gradient becomes weaker. Using the base parameter set, we obtain $\alpha_A = 2.8 \times 10^{-4}$, well below unity as required (see supplementary section for parameter sensitivity analysis). In some contexts, such as chemical reaction engineering and in previous modeling of intracellular signaling gradients, this dimensionless parameter is referred to as a Thiele modulus (Froment and Bischoff, 1990; Meyers et al., 2006). The Thiele modulus is a dimensionless number that scales the relative rates of reaction and diffusion given by

$$\Phi_A = \frac{1}{\sqrt{\alpha_A}} \quad .$$

When the Thiele modulus is less than unity (or equivalently when α_A is greater than unity), then the gradient is weak.

The dimensionless quantity, β, gives the relative rate of the kinase and phosphatase rates. Since β depends on position, it is really a variable rather than a parameter. However, it is useful to consider its value at the boundaries, i.e. at X=0 and at X=1. Experimentally, $\beta(1)$ is constrained to be ~1 so that the fast and slow species are at about equal concentrations in the posterior cortical region, while $\beta(0) < \beta(1)$ so that the kinase rate increases with X along the AP axis. For our base parameter set, $\beta(1)=1.1$ and $\beta(0)=0.2$, and their ratio is $\gamma=\beta(1)/\beta(0)=5.5$, where γ is a dimensionless parameter that quantifies the steepness of the kinase gradient. When $\gamma=1$, then there is no gradient in kinase activity; increasing values of γ reflect the increasing steepness of the kinase gradient in the cytoplasm. Additional discussion of the model, including the parametric determinants of the rate of approach to steady-state are included in the supplementary material.

Steady-state gradient and unsteady-state dynamics of MEX-5 protein concentration gradient models

Note: In this discussion, the fast-diffusing form of MEX-5 is designated “A” and the slow diffusing form is designated “B”.

Time and length scales of the model

Using the base set of parameters, we can define a number of dimensional quantities that describe the relationships between space and time in the model. First, molecules in the slow state will diffuse slowly until phosphorylated by the kinase. In the cytoplasmic model, the rate of phosphorylation varies with the position in the cell. At the extreme ends of the cell (X = 0 anterior-most position, X = 1 posterior-most position), the mean time spent in the slow state is given by:

$$\tau_{slow}(X=0) = 1/k_{kin}(0) = 1/(0.02s^{-1}) = 50s$$

$$\tau_{slow}(X=1) = 1/k_{kin}(1) = 1/(0.11s^{-1}) = 9s \quad .$$

At an intermediate point between these two extremes, for example at the equator where X=x/L=0.5, we obtain:

$$\tau_{slow}(X=0.5) = 1/k_{kin}(0.5) = 1/(0.065s^{-1}) = 15s$$

For the reverse transition, the mean time spent in the fast state for all X is given by:

$$\tau_{fast} = 1/k_{phos} = 1/(0.1s^{-1}) = 10s$$

(this equation is valid for all X, since the rate of dephosphorylation is constant throughout the cytoplasm).

Using the diffusion coefficients, we can then compute the mean distance traveled in the fast and slow states. The root-mean-squared (r.m.s.) distance traveled by molecules in the slow state also depends on position. For simplicity as an intermediate case, let us use the value again at the cell midpoint (X=0.5),

$$l_{slow}(0.5) = \sqrt{2D_A / k_{kin}(0.5)} = \sqrt{2(0.07\,\mu m^2/s) / 0.065 s^{-1}} = 1.5\,\mu m$$

and the r.m.s. distance traveled by fast molecules is

$$l_{fast} = \sqrt{2D_B / k_{phos}} = \sqrt{2(5.0\,\mu m^2/s) / 0.1 s^{-1}} = 10\,\mu m \quad .$$

Thus, in the base case, a molecule will not typically be able to diffuse the entire length of the cell during one fast phase. Rather, it will switch multiple times to move from one end to the other, or even to move e.g. from X=3L/4 to X=L/4, a distance of L/2=25 µm as depicted in Figure S6A.

Because a molecule will switch multiple times, it is appropriate to approximate the diffusion process by a single effective diffusion coefficient defined here as

$$D_{eff} = fD_A + (1-f)D_B$$

where f is the fraction of time spent in the slow state (i.e. a duty cycle for MEX-5), which is given by

$$f = \frac{\tau_{slow}(0.5)}{\tau_{slow}(0.5) + \tau_{fast}} \quad .$$

This effective diffusion coefficient can then be used to calculate the time to travel half the length of cell (i.e. for a molecule to move from the midpoint of the posterior cytoplasm to the midpoint of the anterior cytoplasm), which is

$$\tau = \frac{\left(L/2\right)^2}{2D_{eff}} \quad .$$

In the case of an initially uniform concentration profile, τ approximates the mean time for a molecule to move from posterior cytoplasm to anterior cytoplasm, and thereby begin to establish the emerging steady-state gradient along the AP axis.

For the base parameter set, we estimate

$$f = \frac{15s}{15s + 10s} = 0.6$$

meaning that near the equator, molecules spend about 60% of their time in the slow state, in agreement with experiment. From this value we can calculate the effective diffusion coefficient as

$$D_{eff} = (0.6)(0.07\,\mu m^2/s) + (0.4)(5\,\mu m^2/s) = 2\,\mu m^2/s$$

in agreement with experiment. Therefore, the time scale of gradient formation in the case of the base parameter set is

$$\tau = \frac{(50\,\mu m/2)^2}{2(2\,\mu m^2/s)} = 160 s \qquad ,$$

which is in good agreement with experiment and with the base case unsteady-state simulation. Therefore, the cytoplasmic model with the base parameter set explains the rapid formation of the gradient over a few minutes, as shown in Figure S5B.

Parameter sensitivity analysis: Cytoplasmic model

To explore the extent of valid parameter space in the cytoplasmic model, we conducted a sensitivity analysis. In each perturbation described below, we first discuss the *a priori* expectation based on the time and length scales defined above.

Varying k_{phos} alone (Figure S5C and S5D)

If k_{phos} were increased 10-fold, then molecules would spend relatively little time in the fast state compared to the base case. In this case

$$\tau_{fast} = 1s$$

and the majority of the time will be spent in the slow state so that

$$f = \frac{15s}{15s + 1s} = 0.94 \quad .$$

This will slow the effective diffusion so that

$$D_{eff} = (0.94)(0.07\,\mu m^2/s) + (0.06)(5.0\,\mu m^2/s) = 0.37\,\mu m^2/s$$

and the corresponding approach to steady-state will take on the order of

$$\tau = \frac{(50\,\mu m/2)^2}{2(0.37\,\mu m^2/s)} = 840s \quad .$$

Thus, a gradient will form, but will take longer as shown in Figure S5C. The gradient is slightly weaker than the base case because the entire model hinges on the two diffusion coefficients being different. If they are the same, then no gradient forms at all. The larger the disparity between the two diffusion coefficients, the larger the gradient will be.

If k_{phos} were decreased 10-fold, then

$$\tau_{fast} = 100s$$

and the majority of the time will be spent in the fast state so that

$$f = \frac{15s}{15s + 100s} = 0.13 \text{ .}$$

This will speed up the effective diffusion so that

$$D_{eff} = (0.13)\left(0.07\,\mu m^2/s\right) + (0.87)\left(5.0\,\mu m^2/s\right) = 4.4\,\mu m^2/s$$

and the corresponding approach to steady-state will take on the order of

$$\tau = \frac{(50\,\mu m/2)^2}{2\left(4.4\,\mu m^2/s\right)} = 70s \qquad .$$

In this case, the gradient forms quickly, but will be relatively flat due to the large fraction in the fast state and the long distances that fast molecules diffuse, which in this case is,

$$l_{fast} = 30\mu m \quad .$$

Therefore, this case fails due to the lack of a gradient, as shown in Figure S5D.

Varying k_{kin} alone (Figure S5E and S5F)

This case is identical to varying k_{phos} alone, except that increasing k_{kin} results in the same outcome as decreasing k_{phos}, and vice versa. Simulations confirm this interpretation, as shown in Figure S5E and S5F.

Varying k_{phos} and k_{kin} coordinately (Figure S5G-S5I)

If k_{phos} and k_{kin} are varied coordinately, then the relative fractions of fast and slow are constant, and so the effective diffusion coefficient will not vary. For example, if both k_{phos} and k_{kin} are increased 10-fold, then neither the steady-state gradient nor the dynamic approach to steady-state should change. As shown in Figure S5G, simulations confirm this reasoning. However, if k_{phos} and k_{kin} are both decreased by 10-fold, then the times spent in each of the two states is 10-fold greater so that

$$\tau_{slow}(X=0.5)=150s$$

and

$$\tau_{fast}=100s$$

As a result, molecules will cover greater distances than in the base case,

$$l_{slow}(0.5) = 4.7\mu m$$

and

$$l_{fast} = 32\mu m$$

meaning that the assumption regarding effective diffusion, i.e. that molecules switch multiple times as they diffuse from end of the cell to the other, is starting to break down. As a result, the gradient should start to weaken, which is observed to be the case as shown in Figure S5H (note: the time scale was increased 10-fold to 6000 seconds because of the slow dynamics). If k_{phos} and k_{kin} are both decreased 100-fold, the effect becomes even more pronounced, and now even the slow molecule will diffuse over appreciable distances due to the slow kinase kinetics. In this case,

$$\tau_{slow}(X = 0.5) = 1500s$$

and

$$\tau_{fast} = 1000s$$

As a result, molecules will cover distances approaching, or exceeding, the length of the embryo (L=50 μm),

$$l_{slow}(0.5) = 15\mu m$$

and

$$l_{fast} = 100\mu m \quad .$$

Not only does this effectively destroy the gradient, it also takes a very long time for the gradient to form, since the kinetics are limiting. In this case the approach to steady-state will take >1000 s (17 min), far longer than observed experimentally. These dynamics were confirmed in simulation, as shown in Figure S5I (note: the time scale was increased 10-fold to 6000 s because of the slow dynamics).

Varying D_A and D_B (Figure S5J-S5L)

The only remaining parameters in the cytoplasmic model are the diffusion coefficients, D_A and D_B. As mentioned at above, the values of D_A and D_B must be quite different from each other in order for the gradient to form at all.

We first consider the effect of varying the slow diffusion coefficient, D_A. If D_A were increased 10-fold, then the effective diffusion coefficient will change only modestly,

$$D_{eff} = (0.6)(0.7\,\mu m^2/s) + (0.4)(5\,\mu m^2/s) = 2.4\,\mu m^2/s$$

which is an increase of only 20%. Note that in the regime where the model works, i.e. where $D_A << D_B$, the effective diffusion coefficient is dominated by the fraction of time spent in the slow state, f, and the fast diffusion coefficient, D_B,

$$D_{eff} \approx (1-f)D_B \qquad D_A << D_B$$

Thus, the gradient should be slightly weaker due to the increased slow diffusion coefficient, but the approach to the steady-state should be on the same time scale as the base case, as confirmed in Figure S5J.

In the opposite case of a 10-fold lower value, the gradient should be steeper, due to the increased disparity between the fast and slow diffusion coefficients. However, the effect should only be slight because even in the base case, the disparity is already large: i.e. $D_B/D_A = 70$. Increasing the ratio further to $D_B/D_A = 700$, as in the case of decreasing D_A 10-fold, should increase the steepness of the gradient only modestly. The effect on dynamics in also modest, as the

effective diffusion coefficient is insensitive to the slow diffusion coefficient. In this case,

$$D_{eff} = (0.6)(0.007\,\mu m^2/s) + (0.4)(5\,\mu m^2/s) = 2.0\,\mu m^2/s \quad .$$

Thus, the model is insensitive to 10-fold changes in the slow diffusion coefficient, D_A. These behaviors are documented in Figures S5J and S5K.

Increasing the fast diffusion coefficient by 10-fold will only serve to steepen the gradient, although again this will only increase an already large disparity between fast and slow diffusion coefficients and the effect should be modest. A stronger effect will be in the unsteady-state dynamics, which should proceed according to

$$D_{eff} = (0.6)(0.07\,\mu m^2/s) + (0.4)(50.0\,\mu m^2/s) = 20\,\mu m^2/s$$

and the corresponding approach to steady-state will take on the order of

$$\tau = \frac{(50\,\mu m/2)^2}{2(20\,\mu m^2/s)} = 16s \quad .$$

However, it is important to note that this time scale is approaching the time-scale of the slow state (τ_{slow}= 15 s), so that the approach to steady-state will be limited by both the kinase reaction and subsequent diffusion in the fast state.

Decreasing D_B by 10-fold will slightly weaken the gradient, due to the modest lessening of the disparity between the fast and slow diffusion coefficients as discussed above for varying D_A. However, the approach to steady-state should slow significantly due to dependence of the effective diffusion coefficient on the slow diffusion coefficient. Quantitatively, D_{eff} is reduced to

$$D_{eff} = (0.6)(0.07\,\mu m^2/s) + (0.4)(0.5\,\mu m^2/s) = 0.24\,\mu m^2/s$$

so that

$$\tau = \frac{(50\,\mu m/2)^2}{2(0.24\,\mu m^2/s)} = 1300 s \quad .$$

Thus, the gradient will be weak and slow to reach steady-state. The effect of changing D_B is shown in Figures S5L and S5M, and the effects predicted are observed in the simulation.

Parameter sensitivity analysis: Cortical model (Figure S6A-S6F)

The steady-state behavior of the cortical model has been discussed extensively in Lipkow and Odde (2008). In the present study, two assumptions were altered

from those in the previous study: 1) the kinase was moved from the left boundary to the right boundary, and 2) the kinase generates the fast state instead of the slow state. However, these changes do not fundamentally alter the model, only the nomenclature. Fundamentally, the cortical model is invalidated by the observation that elimination of cortical PAR-1 does not eliminate the MEX-5 gradient (Figure 2D). Nevertheless, for completeness we consider some of the quantitative aspects of the cortical model, and point out additional shortcomings of this model. It is important to note that the fundamental idea of the original model still underlies the cytoplasmic model to explain the MEX-5 gradient: spatially segregated kinase-phosphatase reactions will generate a *steady-state total protein concentration gradient of their substrate*, provided the diffusion coefficients of the phosphorylated and dephosphorylated species differ appreciably.

For the cortical model to work in the case of MEX-5, the kinase reaction at the right boundary (X=1), needs to be rapid, so that the concentration of the slow species at the boundary, $c_A(X=1)$, is nearly zero. This creates an AP gradient in the slow species, as observed experimentally. As the fast species is generated at X=1, it rapidly diffuses away to create a nearly uniform concentration of B within the cytoplasm, as depicted in Figure S6A. Summed together, the two gradients form a total protein concentration gradient as described previously in Lipkow and Odde.

If we use the base case value for k_{phos} from the cytoplasmic model, k_{phos}=0.1 s^{-1}, and constrain D_A and D_B to be the experimentally obtained values (0.07 $\mu m^2/s$ and 5.0 $\mu m^2/s$, respectively), then the resulting steady-state total protein concentration gradient is appreciable, although only near the posterior pole at X=1 (Figure S6B). The gradient length can be increased to extend further into the anterior cytoplasm (X<0.5) by decreasing the rate of the phosphatase reaction. For example, if k_{phos}=0.01 s^{-1}, then better agreement is achieved with the observed steady-state gradient extending over the length of the embryo (Figure S6C). While this gradient is reasonable, it requires that almost all of the protein be in the slow state, which is inconsistent with experimental observation.

To approximate the observed relative ratios of slow:fast (~2:1 in anterior cytoplasm, ~1:1 in posterior cytoplasm), the phosphatase rate needs to be very slow, comparable to the kinase rate. The kinase rate is in turn diffusion-limited by the time it takes for molecules in the slow state to diffuse to the right boundary at X=1. As shown in Fig. S6D, when the phosphatase rate is decreased to k_{phos}=0.0001 s^{-1}, we find reasonable ratios of slow:fast.

While the cortical model can give rise to the observed total protein gradient, the time scale of approach to steady-state is limited by the rate at which the slow molecules can diffuse to the right boundary. Using a minimum gradient length of L/4 (i.e. the distance from X=3L/4, the midpoint of the posterior cytoplasm, to the right boundary at X=1), we obtain a time scale for approach to steady-state of

$$\tau = \frac{(L/4)^2}{2(D_A)} = \frac{(50\,\mu m/4)^2}{2(0.07\,\mu m^2/s)} = 1100 s \quad .$$

Note that the rate of approach to steady-state should be insensitive to the phosphatase rate constant, k_{phos}, and instead will depend on the slow diffusion coefficient, D_A, which we confirmed occurs in the simulations as shown in Figure S6E and S6F.

Thus, the cortical model has the following difficulties: 1) It cannot account for the experimentally observed MEX-5 gradient that develops in the absence of cortical PAR-1, and 2) requires a slower approach to steady-state than observed experimentally. In particular, it predicts a half-time for gradient formation (~1000 s = ~17 min) that occurs ~5 minutes after NEBD. By contrast, the cytoplasmic model accounts for both of these observations, and all other observations so far as described above.

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