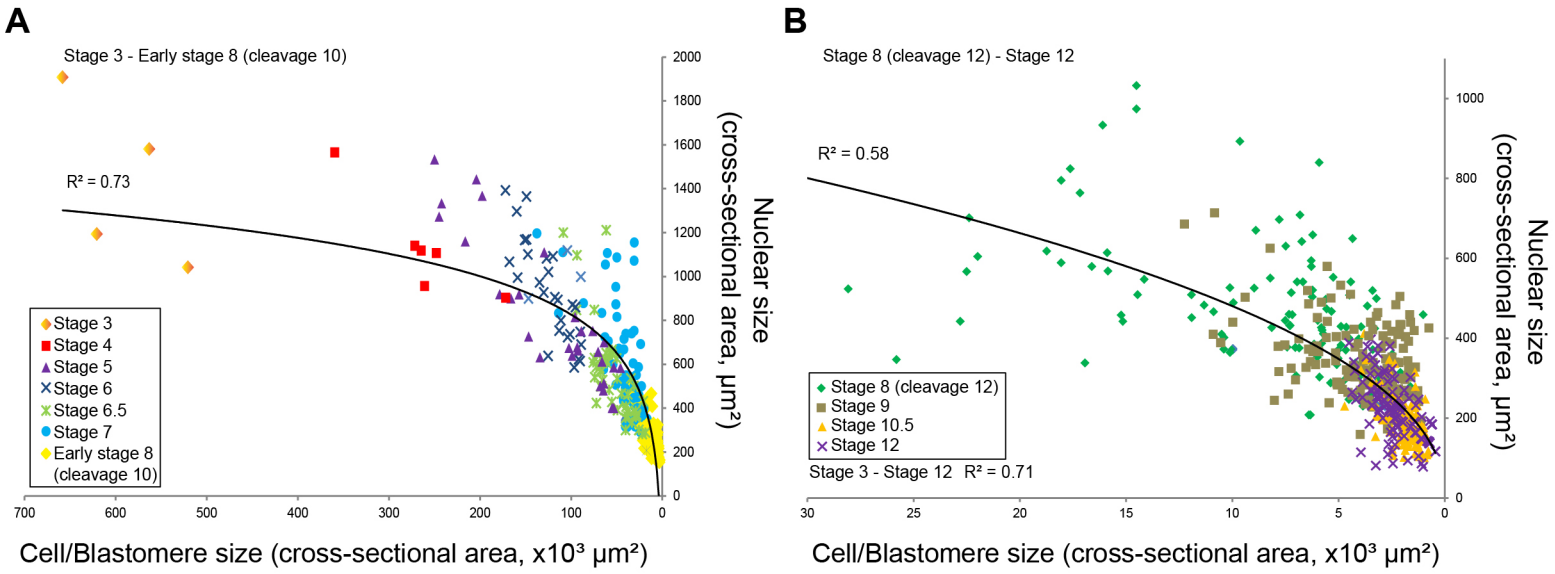
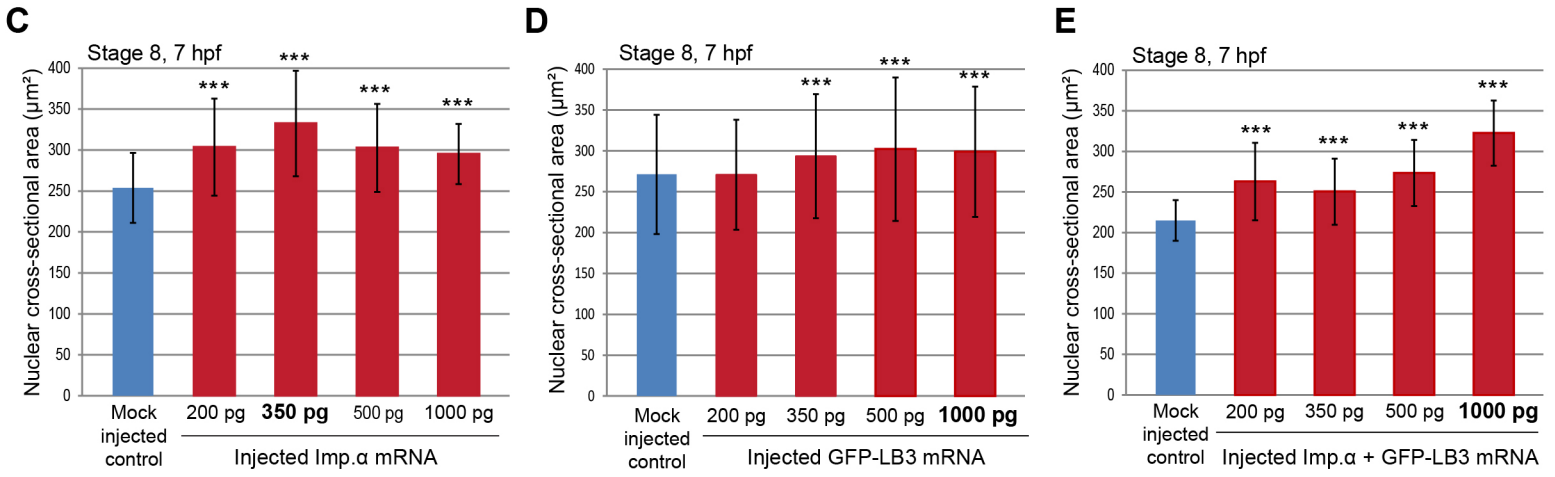
Figure S1


A
Stage 3 - Early stage 8 (cleavage 10)
R² = 0.73
Stage 3
Stage 4
Stage 5
Stage 6
Stage 6.5
Stage 7
Early stage 8 (cleavage 10)
Nuclear size (cross-sectional area, µm²)
Cell/Blastomere size (cross-sectional area, x10³ µm²)
B
Stage 8 (cleavage 12) - Stage 12
R² = 0.58
Stage 8 (cleavage 12)
Stage 9
Stage 10.5
Stage 12
Stage 3 - Stage 12 R² = 0.71
Nuclear size (cross-sectional area, µm²)
Cell/Blastomere size (cross-sectional area, x10³ µm²)


C
Stage 8, 7 hpf
Nuclear cross-sectional area (µm²)
Mock injected control
200 pg
350 pg
500 pg
1000 pg
Injected Imp.α mRNA
D
Stage 8, 7 hpf
Nuclear cross-sectional area (µm²)
Mock injected control
200 pg
350 pg
500 pg
1000 pg
Injected GFP-LB3 mRNA
E
Stage 8, 7 hpf
Nuclear cross-sectional area (µm²)
Mock injected control
200 pg
350 pg
500 pg
1000 pg
Injected Imp.α + GFP-LB3 mRNA

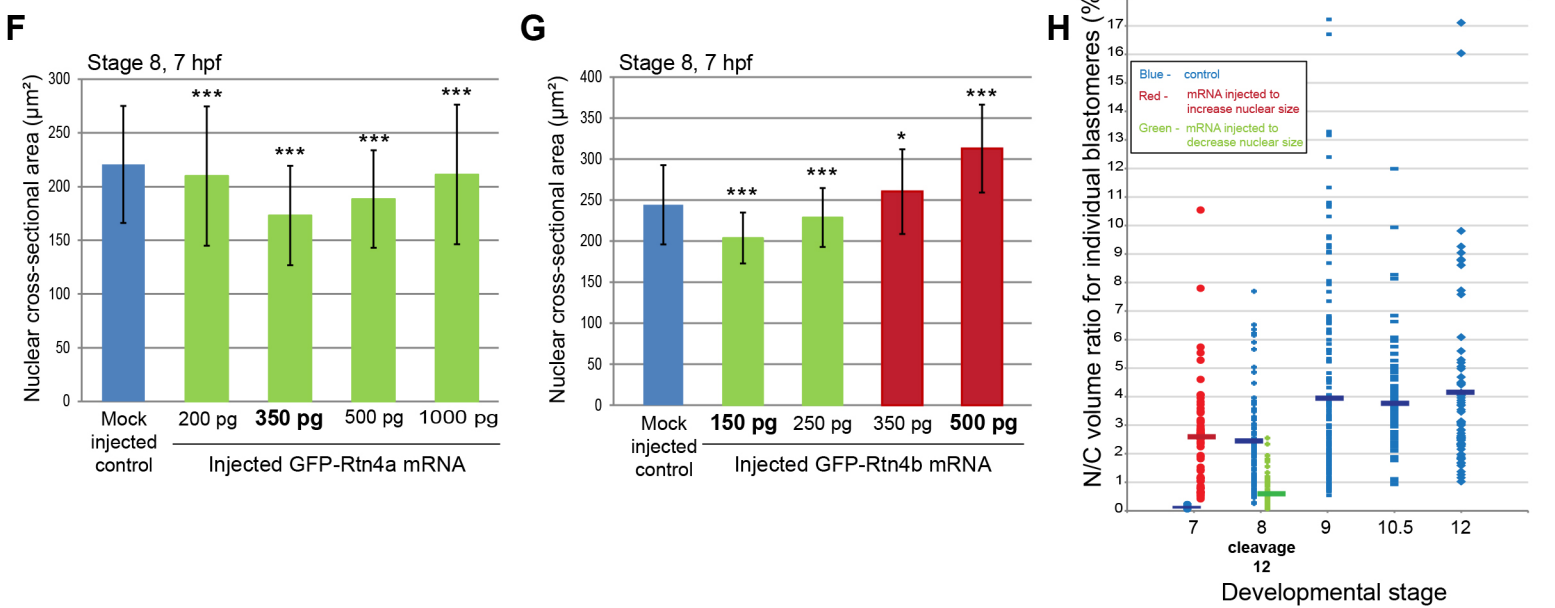

F
Stage 8, 7 hpf
Nuclear cross-sectional area (µm²)
Mock injected control
200 pg
350 pg
500 pg
1000 pg
Injected GFP-Rtn4a mRNA
G
Stage 8, 7 hpf
Nuclear cross-sectional area (µm²)
Mock injected control
150 pg
250 pg
350 pg
500 pg
Injected GFP-Rtn4b mRNA
H
Blue - control
Red - mRNA injected to increase nuclear size
Green - mRNA injected to decrease nuclear size
N/C volume ratio for individual blastomeres (%)
7
8
9
10.5
12
cleavage 12
Developmental stage

Figure S2

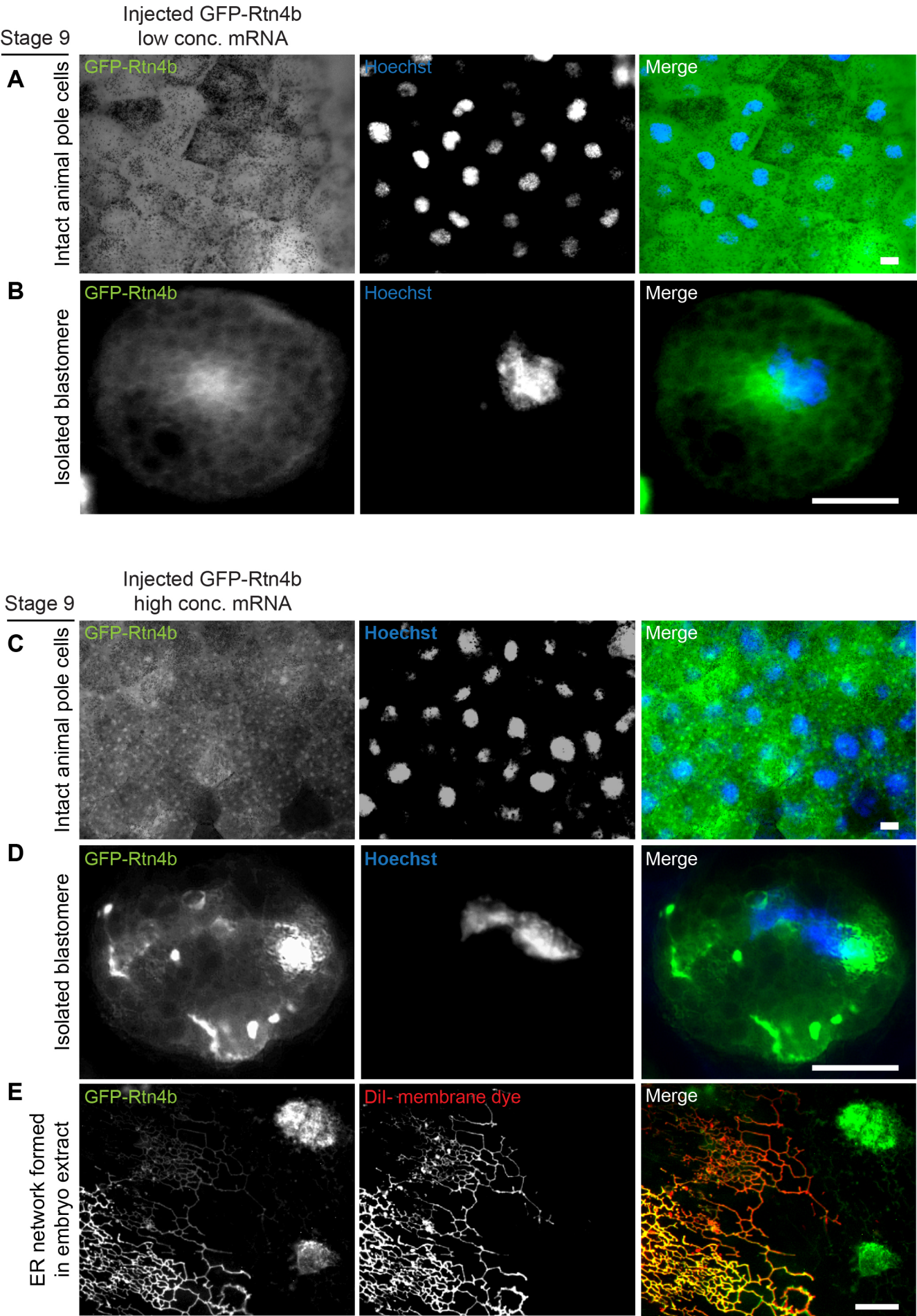

Injected GFP-Rtn4b
low conc. mRNA
Stage 9
A
Intact animal pole cells
GFP-Rtn4b
Hoechst
Merge
B
Isolated blastomere
GFP-Rtn4b
Hoechst
Merge
Injected GFP-Rtn4b
high conc. mRNA
Stage 9
C
Intact animal pole cells
GFP-Rtn4b
Hoechst
Merge
D
Isolated blastomere
GFP-Rtn4b
Hoechst
Merge
E
ER network formed
in embryo extract
GFP-Rtn4b
DiI- membrane dye
Merge

Figure S3

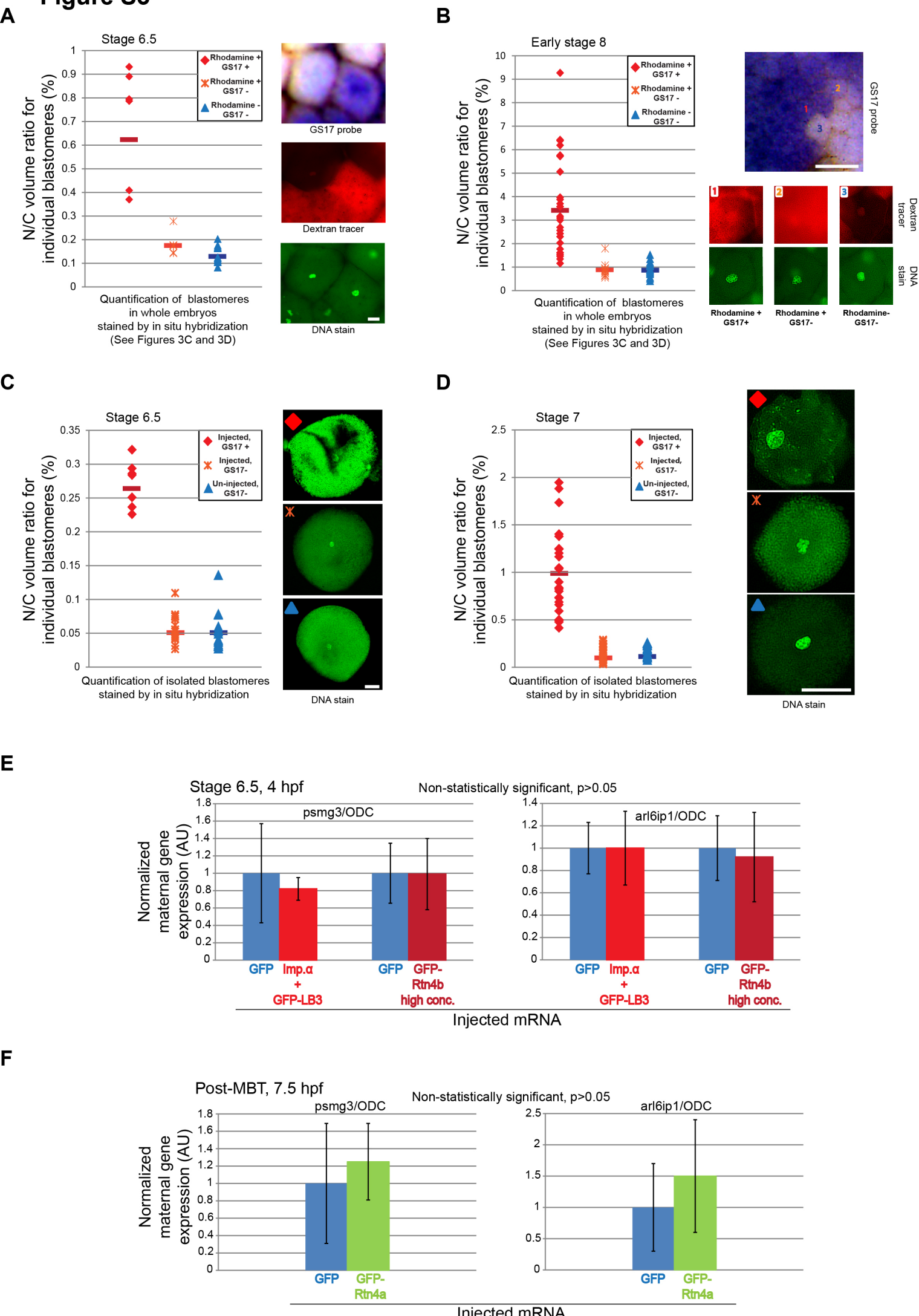
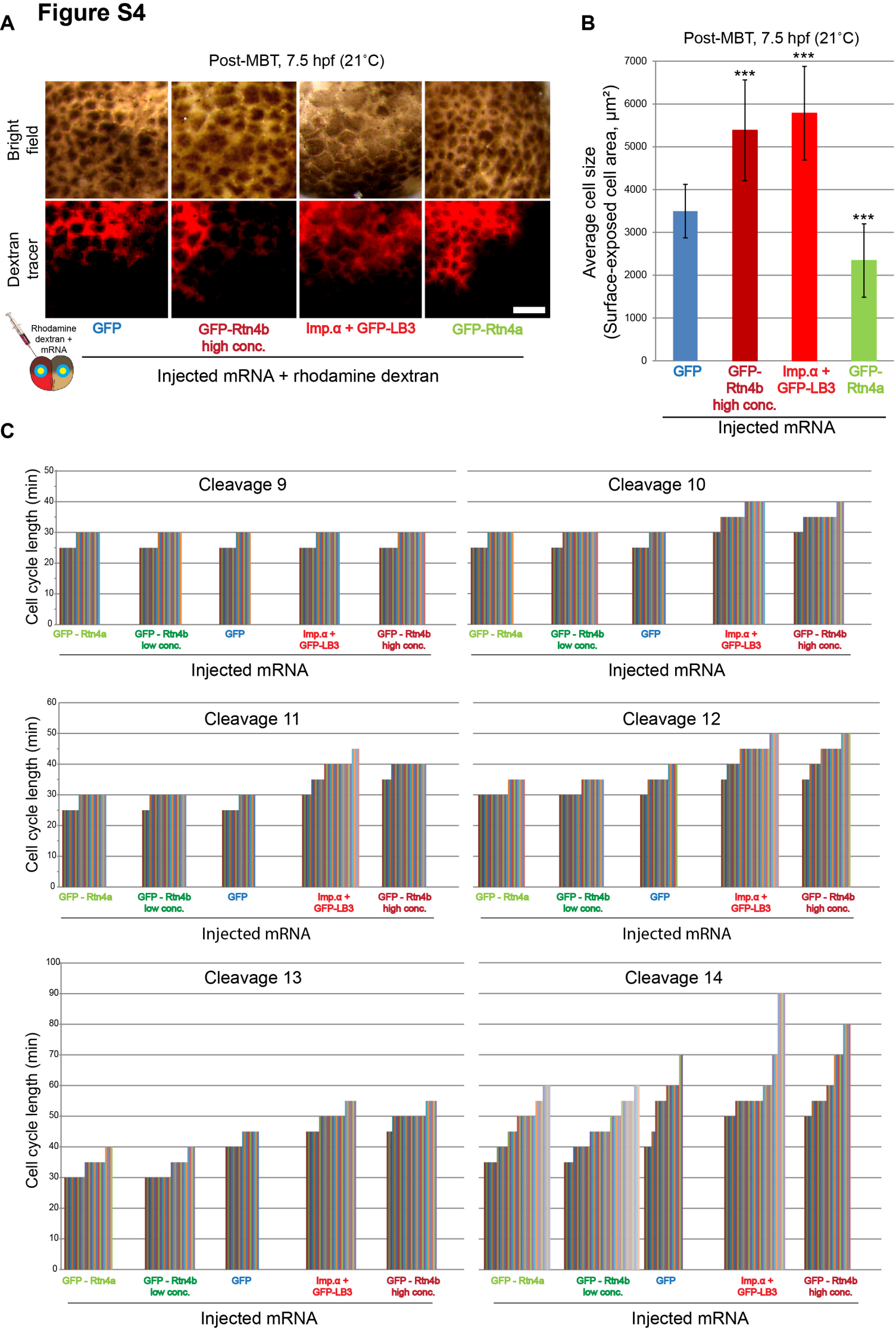

A
Stage 6.5
N/C volume ratio for individual blastomeres (%)
Rhodamine + GS17 +
Rhodamine + GS17 -
Rhodamine - GS17 -
Quantification of blastomeres in whole embryos stained by in situ hybridization (See Figures 3C and 3D)
GS17 probe
Dextran tracer
DNA stain
B
Early stage 8
N/C volume ratio for individual blastomeres (%)
Rhodamine + GS17 +
Rhodamine + GS17 -
Rhodamine - GS17 -
Quantification of blastomeres in whole embryos stained by in situ hybridization (See Figures 3C and 3D)
GS17 probe
Dextran tracer
DNA stain
Rhodamine + GS17+
Rhodamine + GS17-
Rhodamine- GS17-
C
Stage 6.5
N/C volume ratio for individual blastomeres (%)
Injected, GS17 +
Injected, GS17-
Un-injected, GS17-
Quantification of isolated blastomeres stained by in situ hybridization
DNA stain
D
Stage 7
N/C volume ratio for individual blastomeres (%)
Injected, GS17 +
Injected, GS17-
Un-injected, GS17-
Quantification of isolated blastomeres stained by in situ hybridization
DNA stain
E
Stage 6.5, 4 hpf
Non-statistically significant, p>0.05
Normalized maternal gene expression (AU)
psmg3/ODC
arl6ip1/ODC
GFP
Imp.α + GFP-LB3
GFP-Rtn4b high conc.
Injected mRNA
F
Post-MBT, 7.5 hpf
Non-statistically significant, p>0.05
Normalized maternal gene expression (AU)
psmg3/ODC
arl6ip1/ODC
GFP
GFP-Rtn4a
Injected mRNA

Figure S4


A
Post-MBT, 7.5 hpf (21˚C)
Bright field
Dextran tracer
Rhodamine dextran + mRNA
GFP
GFP-Rtn4b high conc.
Imp.α + GFP-LB3
GFP-Rtn4a
Injected mRNA + rhodamine dextran
B
Post-MBT, 7.5 hpf (21˚C)
Average cell size (Surface-exposed cell area, µm²)
***
***
***
GFP
GFP-Rtn4b high conc.
Imp.α + GFP-LB3
GFP-Rtn4a
Injected mRNA
C
Cell cycle length (min)
Cleavage 9
Cleavage 10
Cleavage 11
Cleavage 12
Cleavage 13
Cleavage 14
GFP - Rtn4a
GFP - Rtn4b low conc.
GFP
Imp.α + GFP-LB3
GFP - Rtn4b high conc.
Injected mRNA

Supplemental Figure Legends

Figure S1, Related to Figures 1 and 2. Nuclear size and cell size scale during *Xenopus* early development and titration of nuclear scaling factors alters nuclear size

(A) Raw data used to generate the plots in Figure 1 are shown, specifically nuclear versus cell cross-sectional areas for stage 3 to early stage 8 (cleavage 10). The data were fit to a power trend line and the R^2 value is shown for all data points. Embryos from three different batches of eggs were used, and for each stage, at least 10 embryos were used. Numbers of blastomeres analyzed were: Stage 3, n=4; Stage 4, n=6; Stage 5, n=28; Stage 6, n=29; Stage 6.5, n=48; Stage 7, n=86; early stage 8, n=83.

(B) Raw data used to generate the plots in Figure 1 are shown, specifically nuclear versus cell cross-sectional areas for stage 8 (cleavage 12) to stage 12. The data were fit to a power trend line and the R^2 value is shown for all data points. Embryos from three different batches of eggs were used, and for each stage, at least 10 embryos were used. Numbers of blastomeres analyzed were: Stage 8, n=112; Stage 9, n=139; Stage 10.5, n=123; Stage 12, n=138.

(C-G) One-cell embryos were microinjected with the indicated amounts of mRNAs. Nuclei were isolated from stage 8 embryos and visualized by immunofluorescence using mAb414. Nuclear cross-sectional area was quantified for at least 500 nuclei from ~100 embryos for each condition. Highlighted in bold are the amounts of injected mRNA that maximally increased or decreased nuclear size and that were used in Figures 2-4 and S2-4. Data from one representative experiment of three are shown. Error bars represent SD, *** $P<0.001$; * $P<0.05$.

(H) Nuclei were visualized in isolated blastomeres at the indicated stages by whole-mount immunocytochemistry and N/C volume ratios were quantified as in Figure 1. N/C volume ratios for individual blastomeres are plotted. N/C ratios shown in blue represent control blastomeres and were calculated from the data shown in Figure S1A-B. N/C ratios shown in red represent blastomeres with increased nuclear size (i.e. injected with importin α + GFP-LB3 or GFP-Rtn4b high concentration). N/C ratios shown in green represent blastomeres with decreased nuclear size (i.e. injected with GFP-Rtn4a or GFP-Rtn4b low concentration). Stage 7 blastomeres with increased nuclear size, n=55; stage 8 blastomeres with decreased nuclear size, n=83. Short thick horizontal lines indicate mean values for each stage.

Figure S2, Related to Figure 2. High expression levels of GFP-Rtn4b cause Rtn4 aggregation

Ectopic expression of GFP-Rtn4a and GFP-Rtn4b at lower concentrations decreased nuclear size as expected [S1] (Figure 2A-B, S1F-G). High expression levels of GFP-Rtn4b led to the formation of reticulon aggregates that we hypothesize have a dominant negative effect on ER structure resulting in a concomitant increase in nuclear size (Figure 2A-B, S1G). Studies with mammalian, yeast, and *Arabidopsis* reticulons support the idea that overexpressed reticulons aggregate and disrupt normal ER structure [S2-S4].

(A,B) One-cell embryos were microinjected with the low concentration of GFP-Rtn4b mRNA and allowed to develop to stage 9.

(A) Nuclei were stained with Hoechst and the animal pole in an intact embryo was

visualized by confocal microscopy. No Rtn4b bright foci are apparent. Scale bar, 20 μm. (B) Embryos were transferred to calcium/magnesium-free medium. Isolated blastomeres were fixed, stained with Hoechst, and visualized by confocal microscopy. No Rtn4b bright foci are apparent. Scale bar, 20 μm.

(C-E) One-cell embryos were microinjected with the high concentration of GFP-Rtn4b mRNA and allowed to develop to stage 9.

(C) Nuclei were stained with Hoechst and the animal pole in an intact embryo was visualized by confocal microscopy. Bright Rtn4b foci are visible. Scale bar, 20 μm. (D) Embryos were transferred to calcium/magnesium-free medium. Isolated blastomeres were fixed, stained with Hoechst, and visualized by confocal microscopy. Bright Rtn4b foci are visible. Scale bar, 20 μm. (E) Embryo extract was prepared as described previously [S5]. Extract was incubated for 45min at 21°C, and ER networks were visualized with CM-DiI membrane dye. The images show colocalization of GFP-Rtn4b with the tubular ER network as well as aggregates of GFP-Rtn4b not associated with membrane. Scale bar, 20 μm.

Figure S3, Related to Figure 3. Premature GS17 zygotic gene expression strongly correlates with increased N/C volume ratios, and altering the N/C volume ratio does not change maternal transcript expression levels determined by qPCR

(A,B) One blastomere of a two-cell embryo was co-injected with rhodamine-labeled dextran and mRNA to increase nuclear size (either Imp.α + GFP-LB3 or GFP-Rtn4b high concentration) in half of the embryo. Embryos were allowed to develop to stage 6.5 (A) or early stage 8 (B) and stained for GS17 by whole mount in situ hybridization (see

Figure 3A-D). Nuclei were visualized with Sytox Green, and cell and nuclear cross-sectional areas were measured. N/C volume ratios were extrapolated from these measurements for cells that received the mRNA and stained for GS17 (rhodamine+ GS17+), cells that received the mRNA and showed weak or absent GS17 staining (rhodamine+ GS17-), and cells that did not receive the mRNA and did not stain for GS17 (rhodamine- GS17-). Numbers of stage 6.5 blastomeres analyzed were: rhodamine+ GS17+, n=6; rhodamine+ GS17-, n=5; rhodamine- GS17-, n=12. Numbers of early stage 8 blastomeres analyzed were: rhodamine+ GS17+, n=28; rhodamine+ GS17-, n=13; rhodamine- GS17-, n=20. Mean values are indicated by thick horizontal lines. Scale bars, 50 μm.

(C,D) One-cell embryos were microinjected with mRNA (GFP-Rtn4b high concentration) to increase nuclear size. Blastomeres were isolated from injected and un-injected embryos at stages 6.5 and 7 and stained for GS17 by whole mount in situ hybridization. Nuclei were visualized with Sytox Green, and cell and nuclear cross-sectional areas were measured. From these data, N/C volume ratios were calculated for blastomeres from injected embryos that stained for GS17 (injected, GS17+), blastomeres from injected embryos that did not stain for GS17 (injected, GS17-), and blastomeres from un-injected embryos that did not stain for GS17 (un-injected, GS17-). Numbers of stage 6.5 isolated blastomeres analyzed were: injected, GS17+, n=7; injected, GS17-, n=13; un-injected, GS17-, n=15. Numbers of stage 7 isolated blastomeres analyzed were: injected, GS17+, n=25; injected, GS17-, n=36; un-injected, GS17-, n=16. Mean values are indicated by thick horizontal lines. Scale bars, 50 μm.

(E,F) One-cell embryos were microinjected with the indicated mRNAs to increase (E) or

decrease (F) nuclear size and allowed to develop to stage 6.5 (E) or late stage 8 (F). Total RNA was isolated from 12 embryos for each condition and converted to cDNA. Expression levels of two maternal genes (psmg3 and arl6ip1) were determined by qPCR, normalized to ODC. Gene expression levels are plotted in arbitrary units (AU) relative to GFP mRNA injected control embryos. The means from two independent experiments are shown, error bars represent SD, and all differences are not statistically significant compared to the GFP controls ($P>0.05$).

Figure S4, Related to Figure 4. **Altering the N/C volume ratio changes the timing for the onset of longer cell cycles**

(A) One blastomere of a two-cell embryo was co-injected with rhodamine-labeled dextran and the indicated mRNAs to alter nuclear size in half of the embryo. At 7.5 hpf, bright field and rhodamine fluorescence imaging were performed on the animal pole. Compared to uninjected embryo halves, cells with increased nuclear size are larger due to premature slowing of cell cycle lengths, while cells with decreased nuclear size are smaller due to additional rapid cell divisions. The scale bar is 100 μm.

(B) One-cell embryos were microinjected with the indicated mRNAs. Bright field images were acquired on the animal pole surface of late stage 8 (7.5 hpf) embryos. Cell sizes were estimated by quantifying the area of surface-exposed cells. Cells from at least four embryos were quantified for each condition. Total number of cells quantified: GFP, n=57; GFP-Rtn4b high conc., n=50; Imp.α + GFP-LB3, n=54; GFP-Rtn4a, n=66. Error bars represent SD, *** $P<0.001$.

(C) Raw data used to generate Figure 4A are shown. One-cell embryos were

microinjected with the indicated mRNAs. Bright field time-lapse imaging was performed on the animal pole at 5 min intervals. Cell cycle lengths were measured for at least four cells per embryo starting at the ninth cell division, for six embryos per condition. Each bar represents an individual cell. Data from two independent experiments are shown.

Supplemental Experimental Procedures

Plasmids and cloning

Plasmids consisting of pCS2+ containing the coding sequences for human importin α2-E (pDL17) and *X. tropicalis* GFP-LB3 (pDL19) were previously described [S5]. The coding sequence of human Rtn4b (DNASU Plasmid Repository Clone ID HsCD00081743) was cloned into pCS107-GFP3STOP (obtained from John Wallingford) generating an N-terminal GFP fusion to Rtn4b (pDL34). An SP6 promoter was cloned upstream of the human Rtn4a coding sequence in plasmid hRtn4a-GFP pAcGFP-N1 (obtained from Gia Voeltz) to generate new plasmid pDL49. For control injections, we used GFP mRNA expressed from pCS107-GFP3STOP.

Xenopus laevis embryos and microinjections

X. laevis embryos were obtained by in vitro fertilization of freshly laid *X. laevis* eggs with crushed *X. laevis* testes [S6]. Only batches with greater than 90% fertilization efficiency were used. Twenty minutes after fertilization, embryos were de-jellied in 2.5% cysteine pH 7.8 dissolved in 1/3x MMR (20x MMR = 2mM EDTA, 2M NaCl, 40mM KCl, 20 mM $MgCl_2$, 40 mM $CaCl_2$, 100 mM HEPES pH 7.8). Embryos were staged according to [S7]. All *Xenopus* procedures and studies were conducted in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Protocols were approved by the University of Wyoming Institutional Animal Care and Use Committee (Assurance # A-3216-01).

Following linearization of pCS107-GFP3STOP, pDL17, pDL19, pDL34, and pDL49, mRNA was expressed from the SP6 promoter using the mMessage mMachine kit (Ambion). Embryos at the one-cell or two-cell stage were transferred to 1/3 MMR

plus 2.5% Ficoll and injected with 10nL volumes using a PicoSpritzer III (Parker). Different amounts of mRNA were injected by varying the concentration of the mRNA stock solution (Figure S1). After 45 minutes, the buffer was changed to 1/3x MMR and embryos were allowed to develop to desired stages. Embryo extracts containing endogenous nuclei were prepared as previously described [S5].

Immunofluorescence and microscopy

Endogenous nuclei in embryo extracts were fixed and visualized with NPC antibody mAb414. Embryo extracts containing endogenous nuclei were mixed with 25 volumes of fix buffer consisting of ELB (250 mM sucrose, 50 mM potassium chloride, 2.5 mM magnesium chloride, 10 mM HEPES pH 7.8), 15% glycerol, 2.6% paraformaldehyde for 15 minutes at room temperature, layered over a 5 ml cushion (100 mM KCl, 1 mM $MgCl_2$, 100 µM $CaCl_2$, 200 mM sucrose, 25% glycerol), and centrifuged at 1000g for 15 minutes at 16°C to spin nuclei onto circular 12 mm coverslips. Coverslips were post-fixed in cold methanol, then briefly rehydrated in PBS-0.1% NP40 and blocked overnight at 4°C with PBS-3% BSA. Coverslips were incubated at room temperature for 1 hr 15 minutes each with primary antibody mAb414 (Covance; 1:1000 dilution in PBS-3% BSA) and secondary antibody DyLight 594 anti-mouse IgG (ImmunoResearch; 1:500 dilution in PBS-3% BSA). DNA was stained with 5 µg/ml Hoechst (Sigma) in PBS-0.1% NP40 for 5 minutes at room temperature. Coverslips were mounted in Vectashield (Vector Laboratories) and sealed with nail polish.

For wide-field microscopy, nuclei were visualized with an Olympus BX51 fluorescence microscope using Olympus UPLFLN 20x (N.A. 0.50, air) objective. Images were acquired with a QIClick Digital CCD Camera, Mono, 12-bit (model QIClick-F-M-12)

at room temperature using Olympus cellSens software. Cross-sectional nuclear areas were measured from original thresholded images using Metamorph software (Molecular Devices).

Whole-mount fluorescence immunocytochemistry and microscopy

For whole-mount immunocytochemistry, embryos were treated for 10 minutes at room temperature with 10 µg/ml proteinase K (Sigma) in 1/3x MMR. Vitelline membranes were removed manually and embryos were incubated in medium lacking calcium and magnesium (CMFM = 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris) to promote cell dissociation. Isolated blastomeres were fixed in Dent's fixative (80% methanol, 20% DMSO) supplemented with 30 µM digitonin (Sigma) for 2 hours at room temperature and then overnight at 4°C. Blastomeres were washed several times with 100% methanol and stored at -20°C. Blastomeres were gradually re-hydrated by consecutive 10 minute washes at room temperature in: 75% methanol, 25% dH_2O; 50% methanol, 50% dH_2O; 25% methanol, 75% PBST (PBS + 0.1% Tween 20); 100% PBST. Blastomeres were incubated in bleaching solution consisting of 1% H_20_2, 5% formamide, and 0.5x SSC (75 mM NaCl, 7.5 mM Na-citrate) for 1-2 hours at room temperature under direct light to bleach pigment granules. Blastomeres were washed twice for 1 hour each at room temperature in BBT (PBS plus 0.1% Triton X-100 and 1% BSA) and then blocked for 2 hours at room temperature in BBT plus 5% boiled donkey serum. Blastomeres were then incubated overnight at 4°C with primary antibody mAb414 (Covance; 1:250 dilution in BBT plus 5% boiled donkey serum), and then washed 3x 1 hour in BBT at room temperature followed by an overnight wash at 4°C. Blocking was performed for 2 hours in BBT plus 5% boiled donkey serum at room

temperature, and then blastomeres were incubated at 4°C overnight with DyLight 594 donkey anti-mouse secondary antibody (ImmunoResearch; 1:250 dilution in BBT plus 5% donkey serum). Blastomeres were washed in PBST and incubated with 5 µg/ml Hoechst in PBST overnight at 4°C. Cells were washed in PBST, dehydrated in methanol, and cleared in 2:1 benzyl benzoate:benzyl alcohol (BB/BA). This protocol was adapted from [S6]. Nuclei and cells were visualized as described under "Immunofluorescence and microscopy" using an Olympus PLN 10x (N.A. 0.25, air) objective. Nuclear and cellular cross-sectional areas were measured from original thresholded images using cellSens Dimension imaging software (Olympus). These measurements were then used to calculate nuclear and cell volumes. To validate this method for quantifying cell and nuclear volumes, confocal imaging was performed as described in the next paragraph.

Imaging in Figure 1A: Confocal imaging was performed on a spinning-disk confocal microscope based on an Olympus IX71 microscope stand equipped with a five line LMM5 laser launch (Spectral Applied Research) and switchable two-fiber output to facilitate imaging through either a Yokogawa CSU-X1 spinning-disk head or TIRF illuminator. Confocal images were acquired with an EM-CCD camera (ImagEM, Hamamatsu). Z-axis focus was controlled using a piezo Pi-Foc (Physik Instrumentes), and multiposition imaging was achieved using a motorized Ludl stage. An Olympus UPLSAPO 20x (N.A. 0.85, oil) objective was used. Image acquisition and all system components were controlled using Metamorph software. For stage 6 blastomeres, 100-3 µm thick z-slices were collected for each cell and 100-0.4 µm thick z-slices were collected for each nucleus. For stage 8 (cleavage 12) blastomeres, 100-1 µm thick z-

slices were collected for each cell and 100-0.25 µm thick z-slices were collected for each nucleus. Area and circumference for each slice were measured using Metamorph software (Molecular Devices). Cell and NE surface areas were calculated as the sum of the circumferences multiplied by the slice thickness. Cell and nuclear volumes were calculated as the sum of the areas multiplied by the slice thickness. For stage 6 cells and nuclei, direct volume measurements agreed within 9% of volumes extrapolated from cross-sectional areas. For stage 8 (cleavage 12) cells and nuclei, direct volume measurements agreed within 3% of volumes extrapolated from cross-sectional areas.

Whole-mount *in situ* hybridization and microscopy

One blastomere of a two-cell embryo was co-injected with mRNA and 50 ng lysine-fixable tetramethylrhodamine-labeled dextran, 70,000 MW (Invitrogen, D1818) as a fluorescent marker for the injected half. For control experiments, mRNA expressing GFP was used. For whole-mount in situ hybridization on isolated blastomeres (Figure S3C-D), one-cell embryos were microinjected with mRNA, and at the two-cell stage vitelline membranes were removed and embryos were transferred to CMFM to promote cell dissociation. At different stages, embryos or isolated blastomeres were fixed with MEMFA consisting of 1 part 10x MEMFA salts (1 M MOPS, 20 mM EGTA, 10 mM MgSO4), 1 part 37% formaldehyde, and 8 parts water for 2 hours and stored in ethanol at -20°C. Embryos were rehydrated in a methanol gradient as described for immunocytochemistry, washed in PBST, and permeabilized with 10 µg/ml proteinase K for 7 minutes. Embryos were washed in 0.1 M triethanolamine 2x5 minutes, then 2x5 minutes in 0.1 M triethanolamine supplemented with acetic anhydride (Sigma) (12.5 µl acetic anhydride to 5 ml 0.1M triethanolamine). Embryos were washed in PBST and

fixed for 20 minutes in 4% paraformaldehyde in PBST. Next, embryos were incubated for 5 hours at 60°C in hybridization buffer (50% formamide, 5x SSC, 1 mg/ml Torula RNA, 100 µg/mL heparin, 1x Denhart's, 0.1% Tween 20, 0.1% CHAPS, 10 mM EDTA). The digoxigenin-labeled anti-GS17 probe was added at 1 µg/ml (see below for preparation) and overnight hybridization was performed at 60°C. Embryos were washed 2x3 minutes in 2x SSC at 60°C, 3x20 minutes in 2x SSC at 60°C, and then 30 minutes at 37°C in 2x SSC supplemented with 20 µg/ml RNase A and 10 µg/ml RNase T_1. Embryos were washed twice in 0.2x SSC for 30 minutes at 60°C and 2x7.5 min at room temperature in 1x MAB (100 mM maleic acid, 150 mM NaCl), before blocking with MAB + 2% BMB (Boehringer Mannheim Blocking Reagent) for 2 hours at room temperature. Embryos were then incubated with anti-digoxigenin alkaline phosphatase tagged antibodies (Sigma) diluted 1:3000 in MAB + 2% BMB for 4 hours at room temperature, and washed overnight at 4°C in MAB. Alkaline phosphatase detection was performed with NBT/BCIP consisting of 4.5 mg/ml NBT (Sigma) and 3.5 mg/ml BCIP (Sigma) in alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 2 mM levamisol). After staining, embryos were fixed overnight at room temperature in MEMFA and bleached in bleaching solution under direct light (see "Whole-mount fluorescence immunocytochemistry and microscopy"). To visualize DNA, whole embryos and isolated blastomeres stained by in situ hybridization were washed 4x1 hour at room temperature in TBST (TBS + 0.1% Tween 20), incubated overnight at 4°C with Sytox Green nucleic acid stain (Life Technologies, S7020, 1:1000 dilution in TBST), and washed 4x1 hour in TBST at room temperature. To visualize nuclei, embryos and isolated blastomeres were dehydrated in methanol and cleared in BB/BA.

Embryo images were acquired with an Olympus SZX16 research fluorescence stereomicroscope, equipped with Olympus DP72 camera, 11.5x zoom microscope body, and SDFPLAPO1XPF objective.

To generate the anti-GS17 probe, we obtained a plasmid containing the full-length cDNA sequence of *X. laevis* GS17 in pCMV-SPORT6 (Open Biosystems, MXL1736-99234694). The plasmid was linearized and anti-sense probe was transcribed in vitro from the T7 promoter (NEB), with digoxigenin-11-UTP (Roche) included in the reaction. The probe was precipitated, washed, resuspended in hybridization buffer at 10 µg/ml, and stored at -80°C. This protocol was adapted from [S6].

Quantitative real-time PCR (qPCR)

For each condition, 12 embryos were lyzed and total RNA was purified from 3 embryo equivalents using the Absolutely RNA Microprep Kit (Agilent Technologies). First-strand cDNA synthesis was performed with random primers using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent technologies). qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad MyiQ cycler. PCR efficiency was verified for each primer set, and only primer pairs having amplification efficiencies within 10% of perfect were used. Each 15 µl reaction contained 10 ng cDNA and 0.5 µM of each primer. All reactions were performed in quadruplicate, at a minimum. The following "2-step amplification + melting" protocol was used: 95°C for 30 seconds; 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds; 95°C for 60 seconds; 55°C for 60 seconds; increasing at 0.5°C every 10 seconds from 55°C to 95°C. Gene expression was normalized to ODC (*Ornithine decarboxylase*) and calculated by the ΔΔCt method. Primer sequences were (5' to 3'):

BIX1.1FW	AGCACCTACTTCTCCTCCAGT
BIX1.1REV	GCTTGCTGTACTGGACTCTGT
XNR3FW	CGATGCCTCCAGTCCTACAG
XNR3REV	TCCTTGAAATTCTCTGGCTCCA
XNR5-13FW	CCTTTCACTAGGGCATGGGA
XNR5-13REV	GGTGAAGGTTCCAGTCTGTGT
PSMG3FW	CCGAGGTGGTGATCAATGGT
PSMG3REV	CTGACACAAGGGTTCCCATCT
ARL6IP1FW	GGAAGGGGACAACAGGAGTG
ARL6IP1REV	AACAACGCGATCAGTGACCA
ODCFW	CTGGAGGAAGGCTTCTCTGC
ODCREV	TGTCGCCAAGATCAGCAACA

Isolation of genomic DNA

For each condition, 12 embryos were homogenized in 360 µl of DNA extraction buffer (10 mM Tris, pH 8.0, 0.2 mM EDTA, 50 µg/ml RNase A, 0.5% SDS) and incubated at 37°C for 1 hour. Proteinase K was added at 100 µg/ml and samples were incubated at 50°C for 2 hours. Samples were extracted in one volume of phenol:chloroform:isoamyl alcohol (25:24:1). Genomic DNA was ethanol precipitated and resuspended in 60 µl 10mM Tris pH 8.5. This protocol was adapted from [S8]. 0.4 embryo equivalents of genomic DNA were run on 1.2% TAE agarose gels and visualized by staining with ethidium bromide. DNA amount was quantified using ImageJ.

Bright field time-lapse imaging of whole embryos

One-cell embryos were microinjected as already described and allowed to

develop at room temperature. Time-lapse imaging was performed with an Olympus stereomicroscope (Olympus SZX16 research fluorescence stereomicroscope, equipped with Olympus DP72 camera, 11.5x zoom microscope body, and SDFPLAPO1XPF objective) at room temperature on the animal pole starting at 3.5 hpf. Images were acquired every 5 minutes. Discontinuous light was used to illuminate embryos, controlled with a digital adjustable cycle timer (CT-1 Short Cycle Timer, Innovative Grower Corp). The number of time intervals between cell divisions was counted to determine cell cycle lengths. Division timing was measured for at least 4 cells per embryo and for each cleavage.

To monitor blastopore closure, one blastomere of a two-cell embryo was microinjected as already described. Embryos were allowed to develop at 16°C. Time lapse imaging of gastrulating embryos was performed on the vegetal pole at room temperature, as described above. Blastopore circumference was quantified using cellSens Dimension imaging software (Olympus).

Counting cell numbers in whole embryos

One-cell embryos were microinjected as already described and cultured at room temperature in CMFM. 7.5 hpf embryos with intact vitelline membranes were fixed for 2 hours at room temperature in Dent's fixative and stored at 4°C. Embryos were washed in 100% methanol, and cells from individual embryos were dispersed in 2.5 ml of methanol by gentle pipetting with a wide bore pipette tip. A 25 µl cell suspension was transferred to a small petri dish and the number of cells was counted using a stereomicroscope. Cell numbers from three samples were averaged and multiplied by the dilution factor to estimate the total number of cells per embryo.

Statistical analysis

For each immunofluorescence coverslip (Figure 2A, S1C-G), at least 500, and usually >1000, nuclei were quantified, and areas averaged. Two-tailed Student's *t*-tests assuming equal variances were performed in Excel (Microsoft) to evaluate statistical significance. The p-values, number of embryos or blastomeres analyzed, number of independent experiments, and error bars are denoted in the Figure Legends.

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