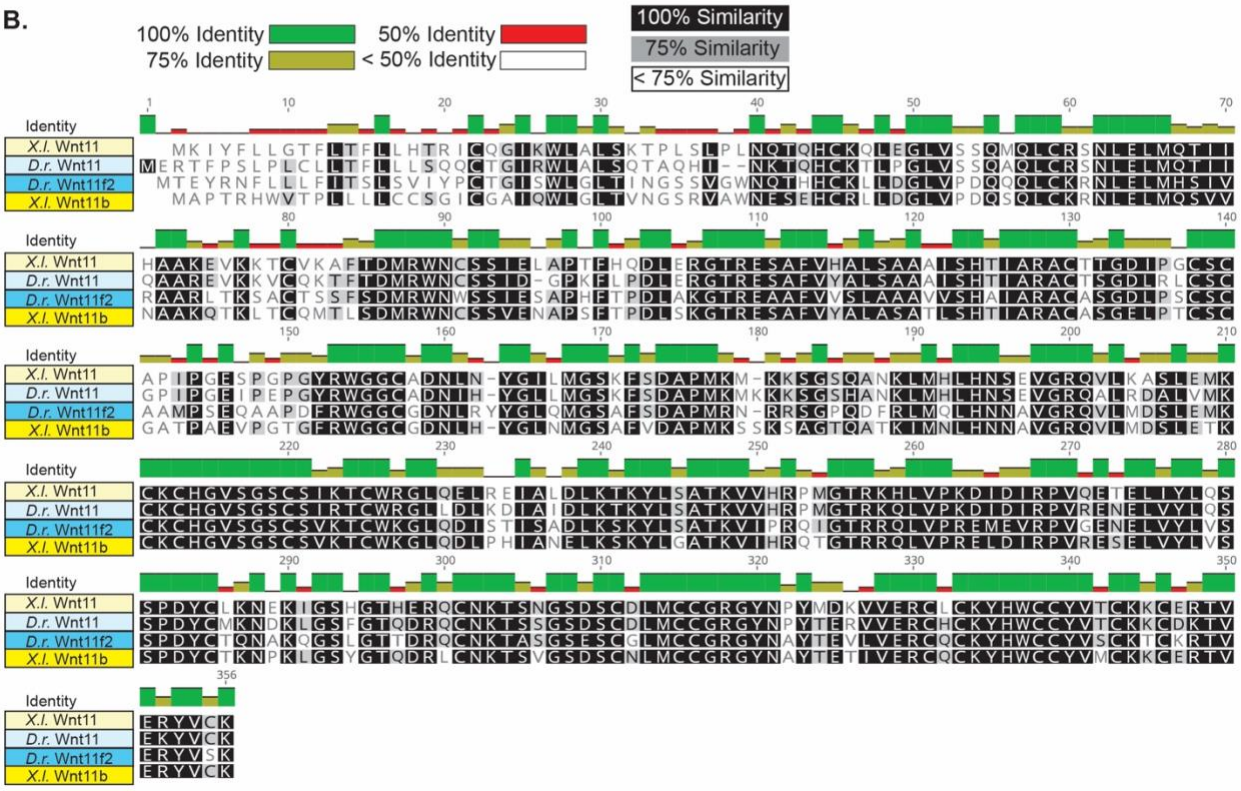
SUPPLEMENTAL MATERIALS

A.

	X.l. Wnt11	*D.r.* Wnt11	*D.r.* Wnt11f2	*X.l.* Wnt11b
X.l. Wnt11		77.6%	61.2%	63.3%
D.r. Wnt11	77.6%		60.6%	63.5%
D.r. Wnt11f2	61.2%	60.6%		69.2%
X.l. Wnt11b	63.3%	63.5%	69.2%	

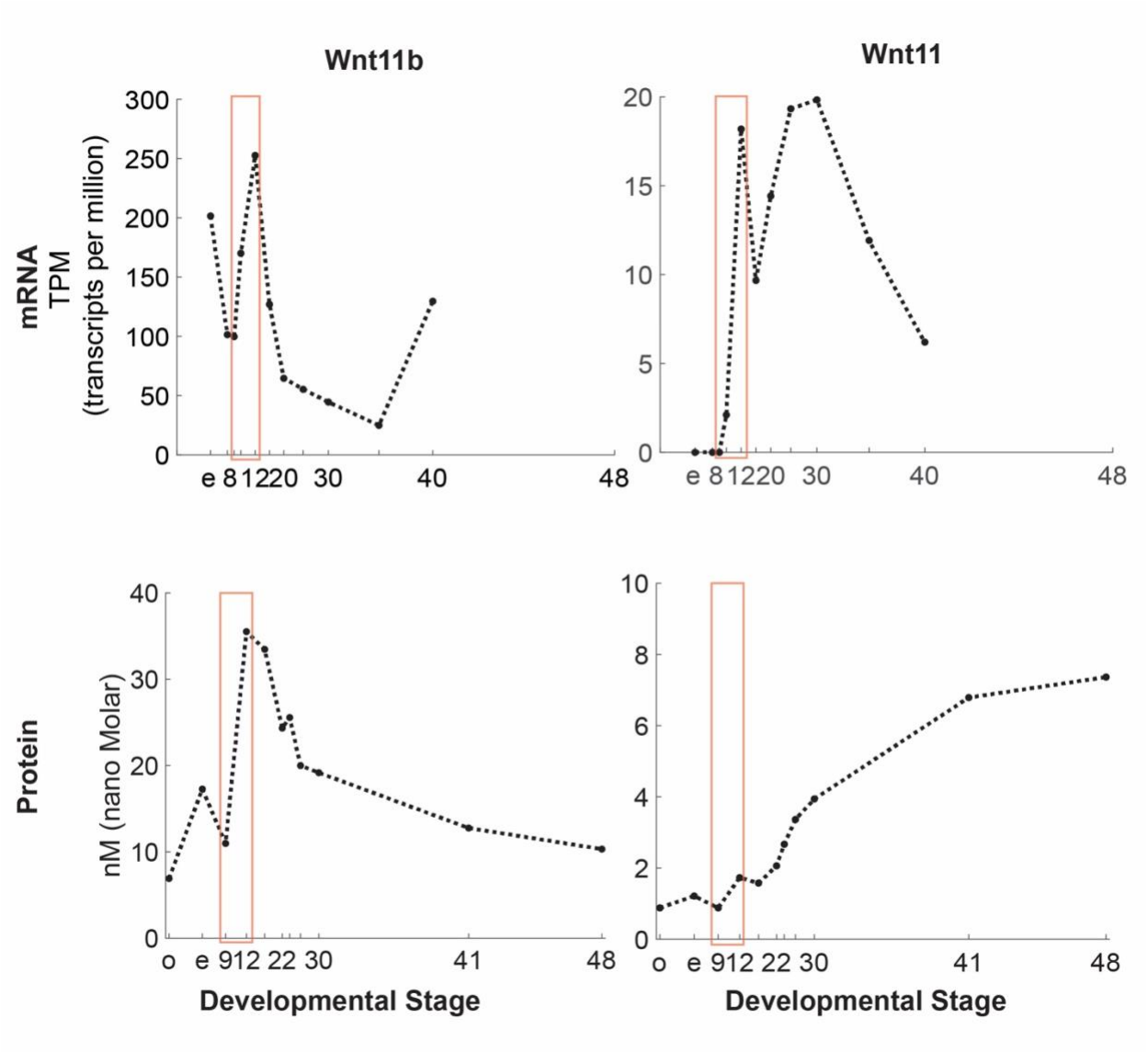
B.



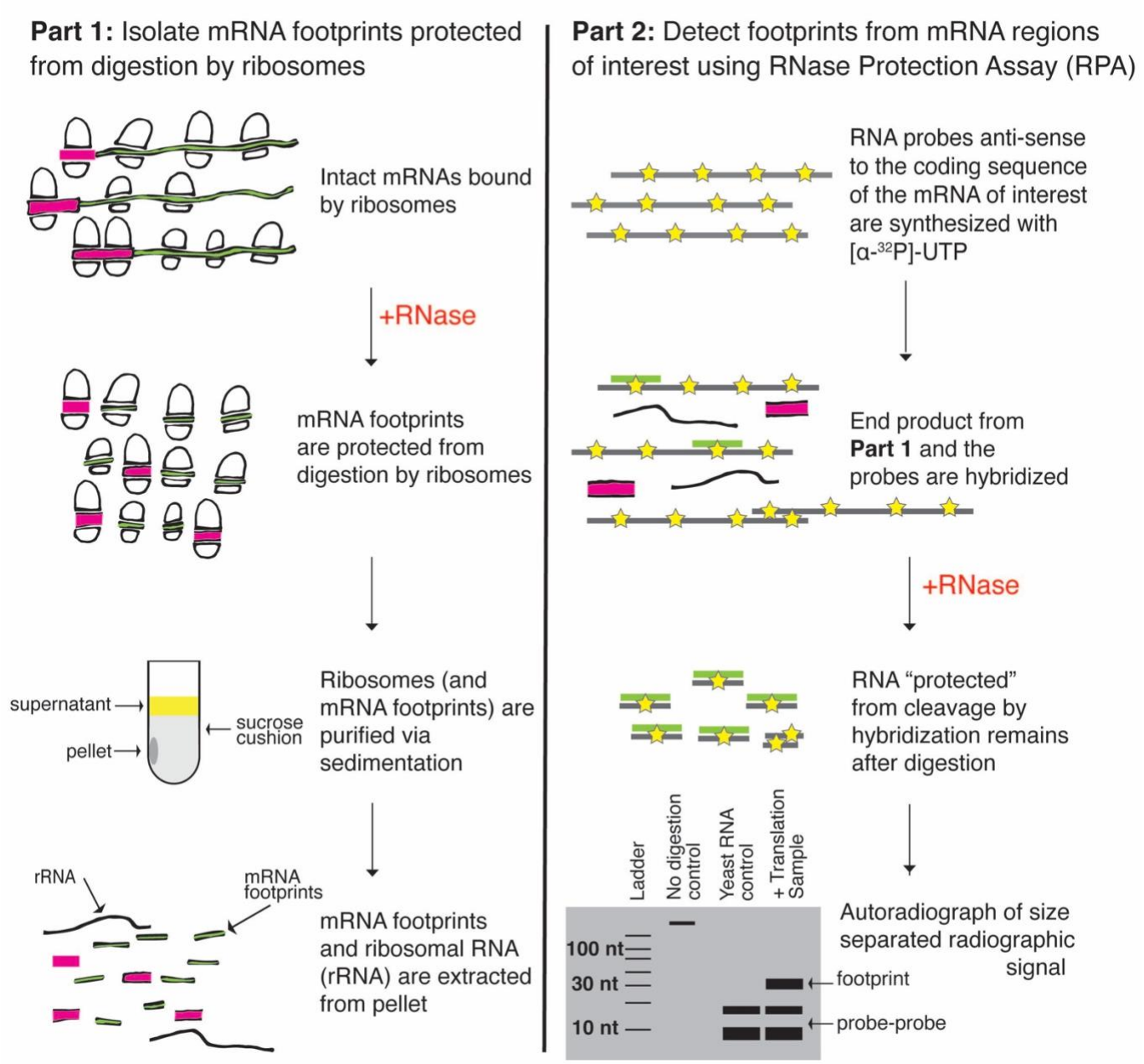
Supplemental Figure 1. The *Xenopus laevis* and *Danio rerio* (zebrafish) genomes both encode two Wnt11 family proteins.

A. *Xenopus laevis* Wnt11 and Wnt11b are more similar in protein sequence identity to their *Danio rerio* homologs (77.6%, 69.2%) than they are to each other (63.3%).

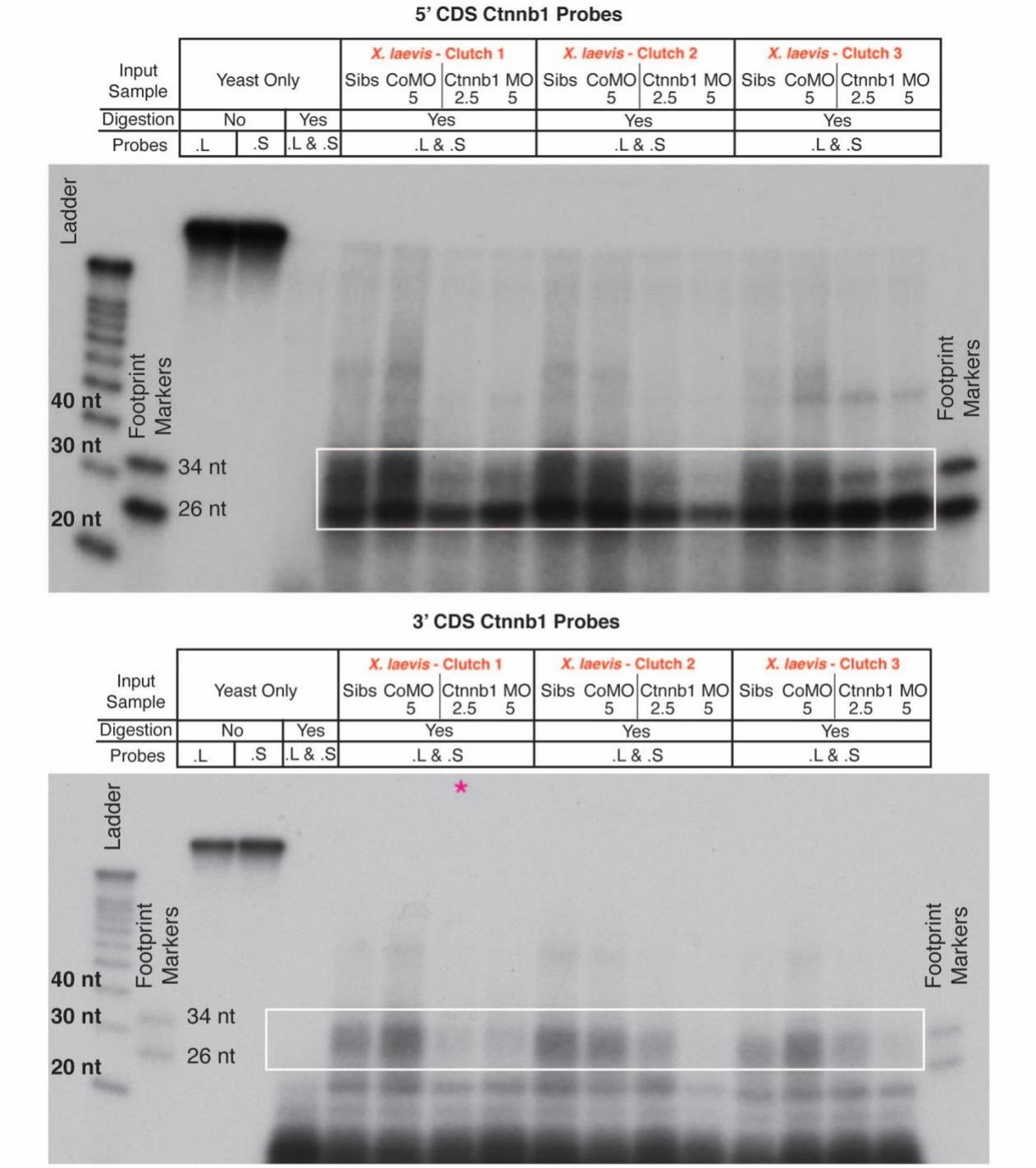
B. Multiple sequence alignment of the *Xenopus laevis* and *Danio rerio* Wnt11 family members shows that despite the closer homology between the Wnt11s and Wnt11b/Wnt11f2, there are regions of 100% similarity between all four proteins across the entire protein sequence. The first ~20 amino acids are a signal peptide that is cleaved in the forming of mature proteins. (Geneious Prime 2020; https://www.geneious.com)



Supplemental Figure 2. Recently published transcriptomics and proteomics data shows that both Wnt11b and Wnt11 increase in mRNA and protein level during gastrulation. Developmental time series transcriptomics and proteomics data both include multiple time points just before and during the gastrulation period (orange boxes; Stages 9, 10, and 12 for transcriptomics and Stages 9 and 12 for proteomics). Note the much smaller y-axis scale for Wnt11 than Wnt11b for both mRNA and protein. There are two alloalleles of Wnt11: Wnt11.L and Wnt11.S. TPM counts for both Wnt11.L and Wnt11.S are combined. The peptides measured for Wnt11 do not allow us to distinguish between Wnt11.L and Wnt11.S.



Supplemental Figure 3. Schematic explaining the modified RNase Protection Assay to assay for changes in ribosome protected mRNA fragments. Since translation blocking morpholinos are thought to decrease protein levels by perturbing translation initiation, the number of coding sequence bound ribosomes on the mRNA of interest should decrease with translation blocking morpholino treatment. To assay for the ribosome protected mRNA fragments of interest, first, stabilized polysomes are digested into monosomes with RNase. The resulting monosomes and the protected mRNA footprints are purified first by sedimentation and then by phenol-chloroform and spin-column methods. This part of the protocol is the same as the first part of the established protocol for ribosome profiling [1,2], but uses a lysis buffer very similar to one previously established for polysome profiling in Xenopus laevis [3]. Next, an RNase Protection Assay is performed with the purified RNA [4]. Briefly, the purified RNA is hybridized with radioactive probes that are synthesized antisense to a portion of the coding sequence of the mRNA of interest. More RNase is then added under conditions that promote digestion of single stranded RNA and not hybrids. The portions of the radioactive probes that are either hybridized to footprints or self-hybridized are protected from digestion. The resulting RNA is visualized after gel separation using autoradiography.

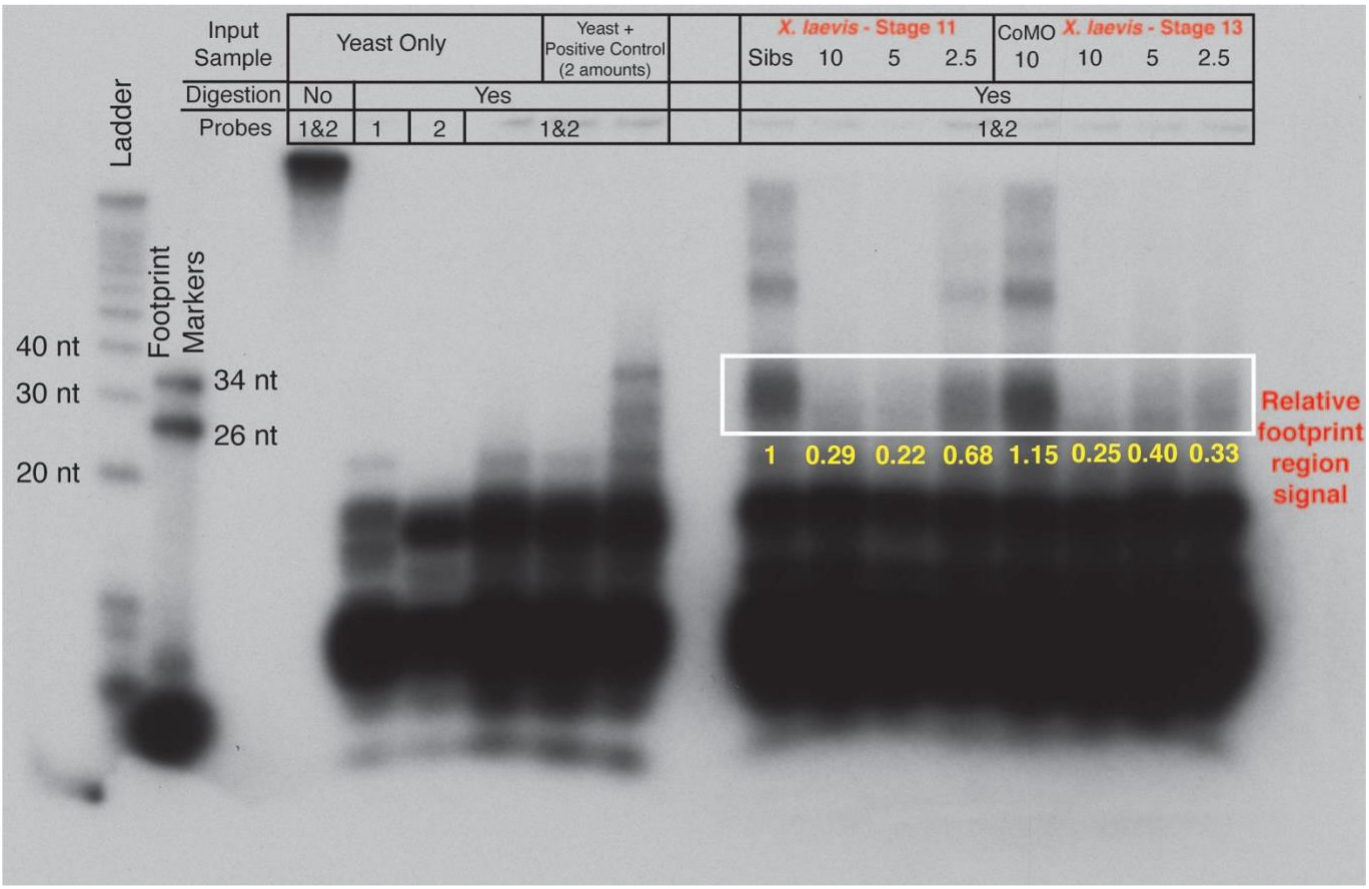


Morpholino - # picomoles	5' CDS Probe Mean Relative Signal	5' CDS Probe Relative Signal SD	5' CDS Probe Mean Relative Signal	3' CDS Probe Mean Relative Signal
CoMo - 5	1.05	0.10	1.42	0.49
Ctnnb1 Mo - 2.5	0.60	0.12	0.63	
Ctnnb1 Mo - 5	0.52	0.22	0.23	0.15

Supplemental Figure 4. Validation that the modified RPA assay confirms expected decrease in ribosomes associated with targeted mRNA using the positive control beta-catenin (Ctnnb1) morpholino.

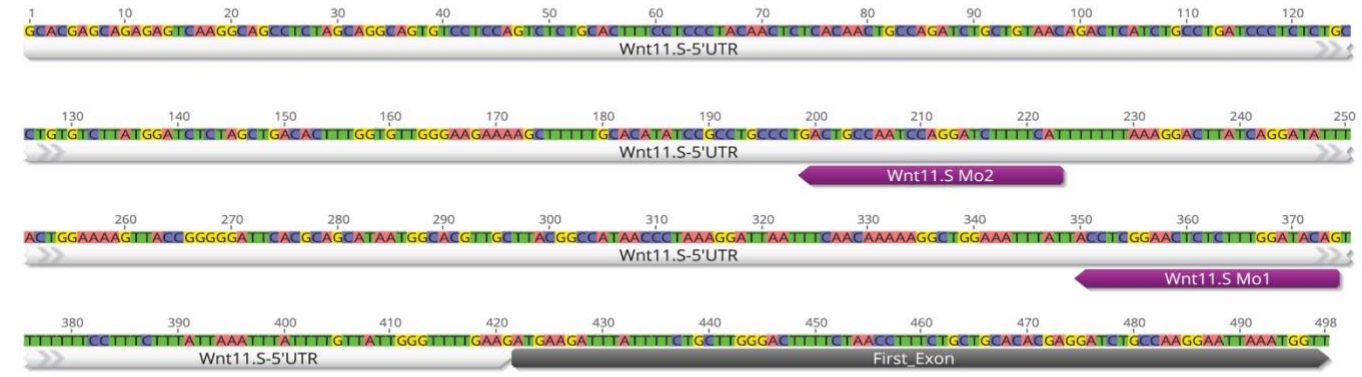
Supplemental Figure 4 (continued).

Heasman et al. showed that injection of the Ctnnb1 morpholino resulted in reduced Ctnnb1 protein levels and no decrease in mRNA levels in *Xenopus laevis* [5]. Thus, we chose to use the Ctnnb1 morpholino, which is sold by GeneTools as the *Xenopus laevis* positive control, to validate our modified RPA assay. Sets of 15 embryos were either not injected (siblings or sibs), injected with a total of 2.5 picomoles of the standard negative control morpholino, or injected with 1.25 picomoles or 2.5 picomoles of Ctnnb1 Mo and collected at Stage 10. This was repeated for a total of three biological replicates. Samples were processed to isolate RNA as described in **Supplemental Methods** with 1250 U of RNase1. 10 ug of RNA was probed with the 5' CDS Ctnnb1 probes. 15 ug of RNA was probed with the 3' CDS Ctnnb1 probes. For both sets of probes the concentration of each .L and .S probe in final hybridization reaction was 7 uM and the gels were exposed for 24 hours. The region of the film designated by the white box was quantified using ImageJ. The mean signal relative to siblings for the three repeats and the standard deviation are reported for both probes and all the experimental conditions. The red asterisk signifies that some of the sample was lost and not run on the gel.

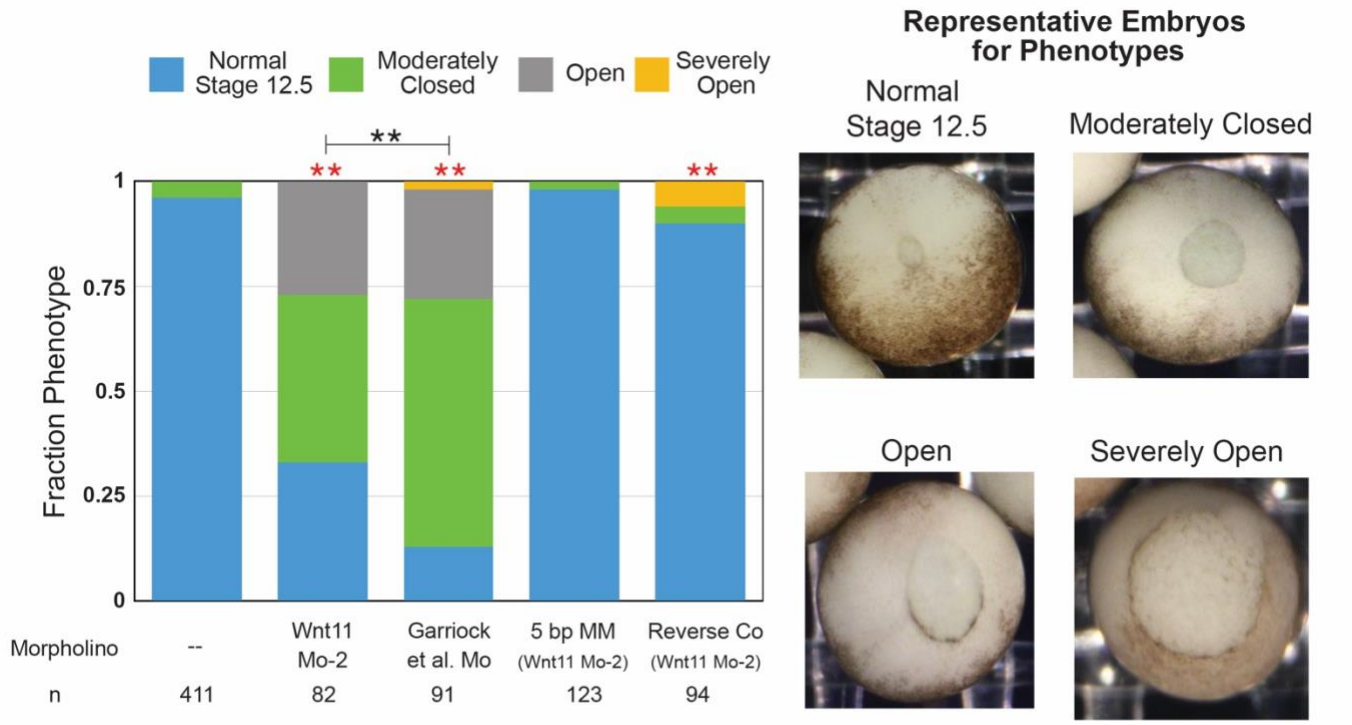


Supplemental Figure 5. Modified RPA assay shows a Wnt11b morpholino dependent decrease in Wnt11b coding sequence fragments bound by ribosomes.

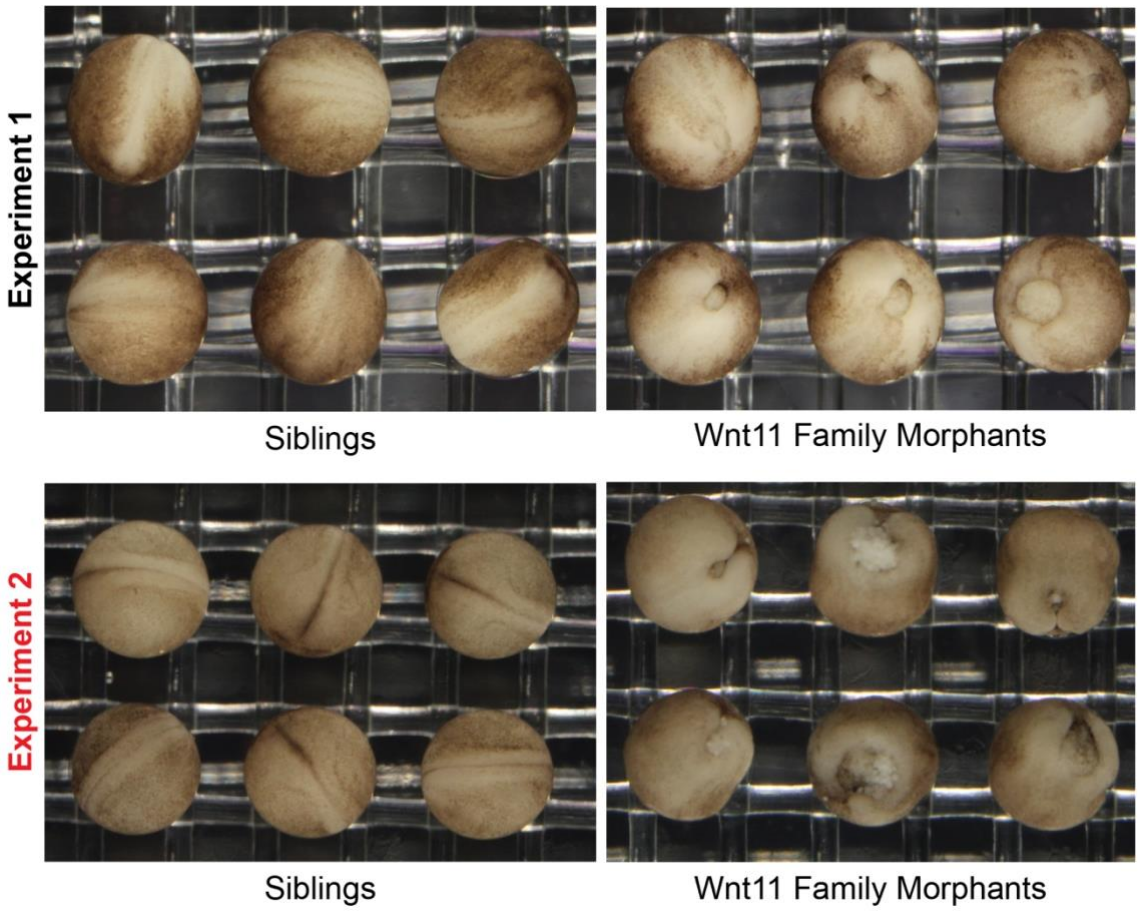
Embryos were injected as described previously with different doses of the Wnt11b morpholino (10, 5, or 2.5 picomoles) or the standard negative control morpholino (10 picomoles). Embryos were collected at Stage 11 and Stage 13. Samples were processed to isolate RNA as described in **Supplemental Methods** with 2000 U RNase1. 10 ug of input RNA was probed with anti-sense probes against two different portions of the Wnt11b mRNA coding sequence. (For the probe self-hybridization controls and the synthesized positive control, yeast RNA was added such that the total RNA amount was 10 ug.) The positive control is a synthesized 30 nt sense RNA from the Wnt11b sequence that overlaps with the probes. The two doses are $10x10^{-18}$ moles and $100x10^{-18}$ moles. At the higher dose the positive control footprints are detected. The gel was exposed for 37 hours. The region of the film designated by the white box was quantified using ImageJ and the relative signal compared to the Stage 11 sibling samples was quantified for each lane (yellow numbers). All of the Wnt11b morpholino treated samples have less signal than the sibling and negative control morpholino injected samples.



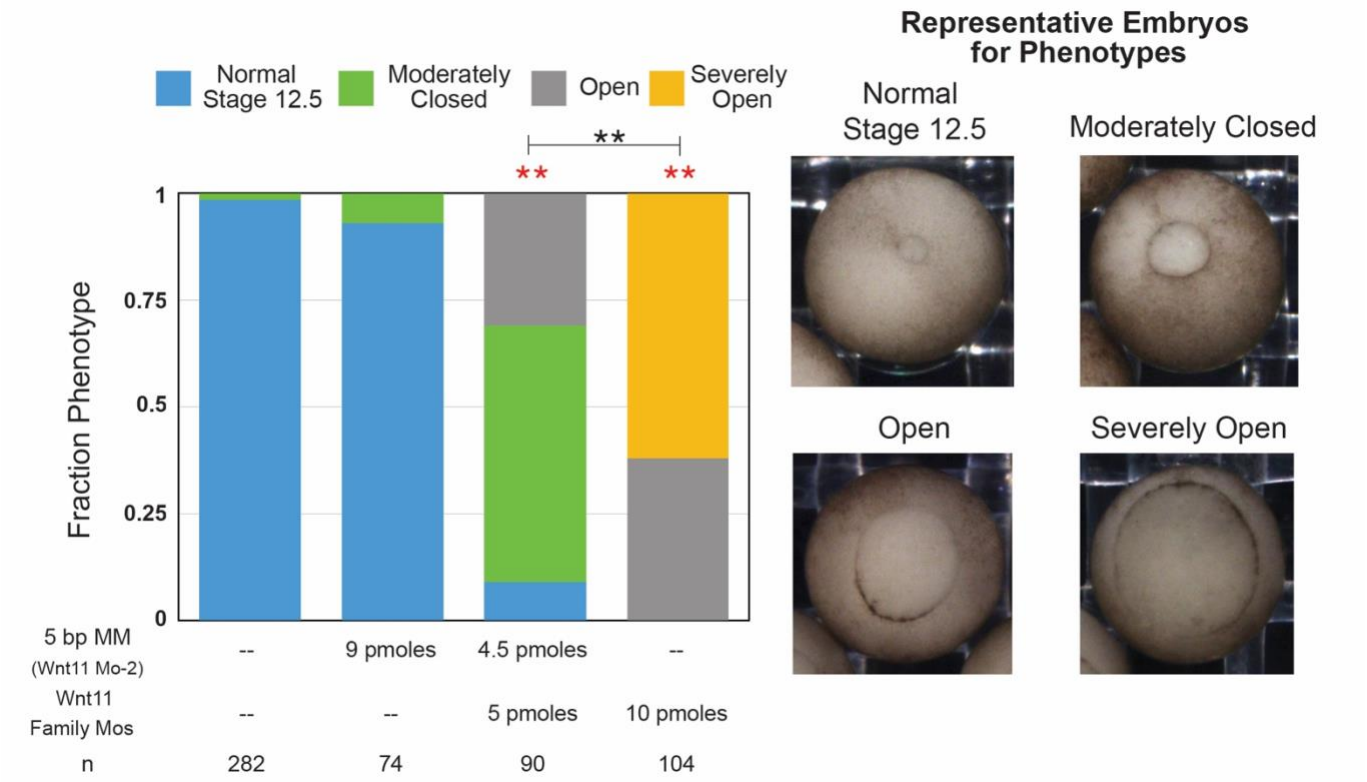
Supplemental Figure 6. The location of the targets of Wnt 11 Mo1 and Wnt11 Mo2 in the 5'UTR of Wnt11.S. (Geneious Prime 2020; https://www.geneious.com)



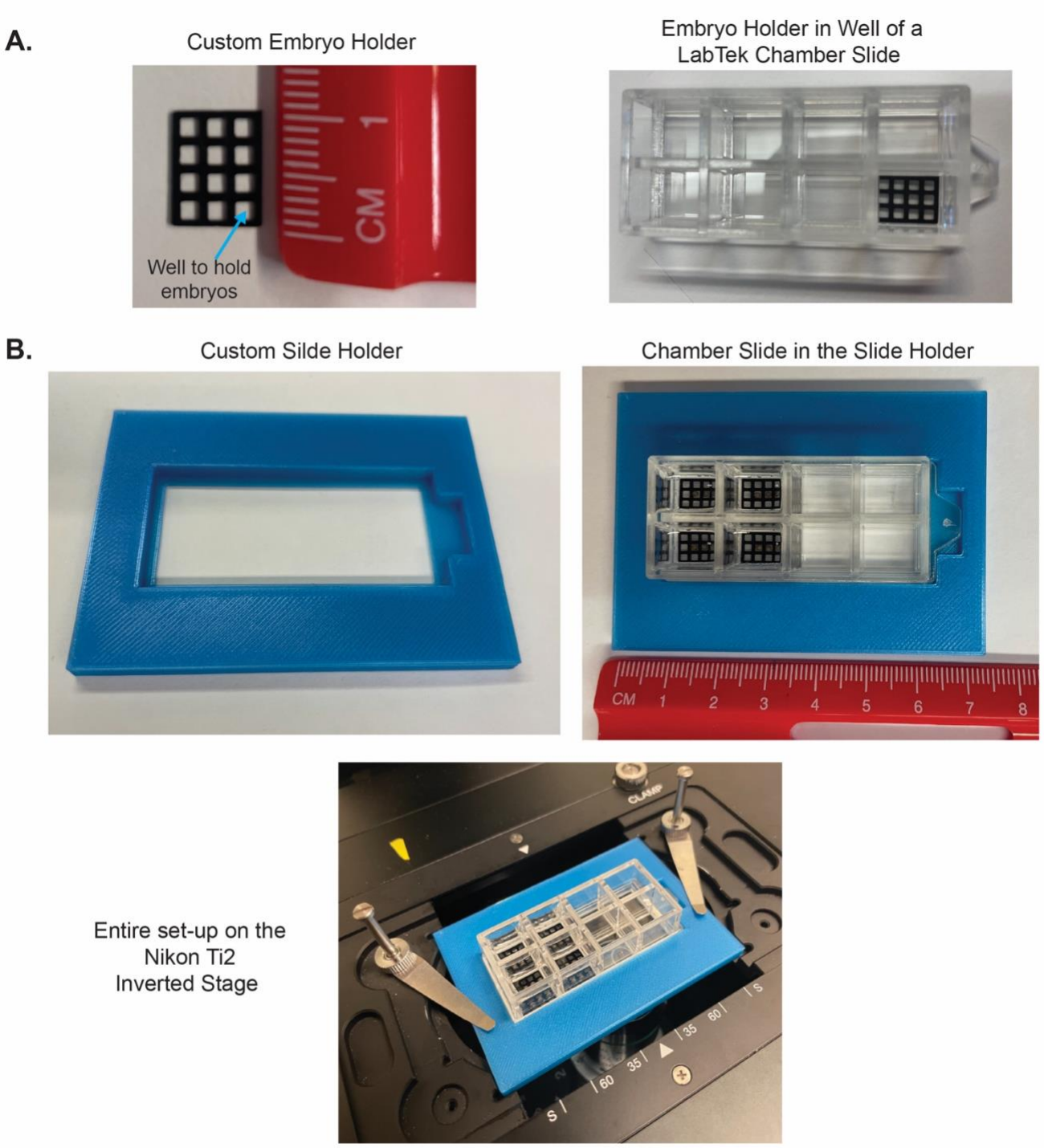
Supplemental Figure 7. Additional control morpholino experiments provide further evidence for Wnt11 morpholino phenotype specificity. For three biological replicates, we injected both blastomeres of two-cell embryos with five picomoles total of one of four different morpholinos: Wnt11 Mo-2 (introduced in this paper), the Wnt11 morpholino introduced in Garriock et al. [6,7], five base pair mismatch control morpholino for Wnt11 Mo-2 that has the same GC content as Wnt11 Mo-2, and a sequence reverse mismatch control morpholino for Wnt11 Mo-2. As expected, both the Wnt11 Mo-2 and Garriock et al. Mo injected embryos are statistically significantly different than the un-injected sibling embryos (red asterisks, $p << 0.005$). The five base-pair mismatch control morpholino is not statistically significantly different than the un-injected embryos. The reverse control morpholino is statistically significantly different that the un-injected embryos. However, ninety percent of reverse control injected embryos have the normal Stage 12.5 phenotype compared to thirty-three and thirteen percent of the on-target designed morpholinos. We ascribe the phenotype from this morpholino to be due to toxicity because it is the only morpholino in this comparison with the severely open phenotype, even though it is a small number of embryos and does not repeat over all three experiments. The phenotype results for Wnt11 Mo-2 and Garriock et al. Mo are also statistically significantly different (black asterisks, $p = 0.005$). This is consistent with the fact that the Garriock et al. Mo targets both Wnt11.L and Wnt11.S whereas the Wnt11 Mo-2 (and Wnt11 Mo-1) only target Wnt11.S.



Supplemental Figure 8. Wnt11 family morphants do not close their blastopores completely during neurulation. Images of sibling and Wnt11 family morphant embryos during neurulation from two experiments corresponding to Experiments 1 and 2 in Figure 3.



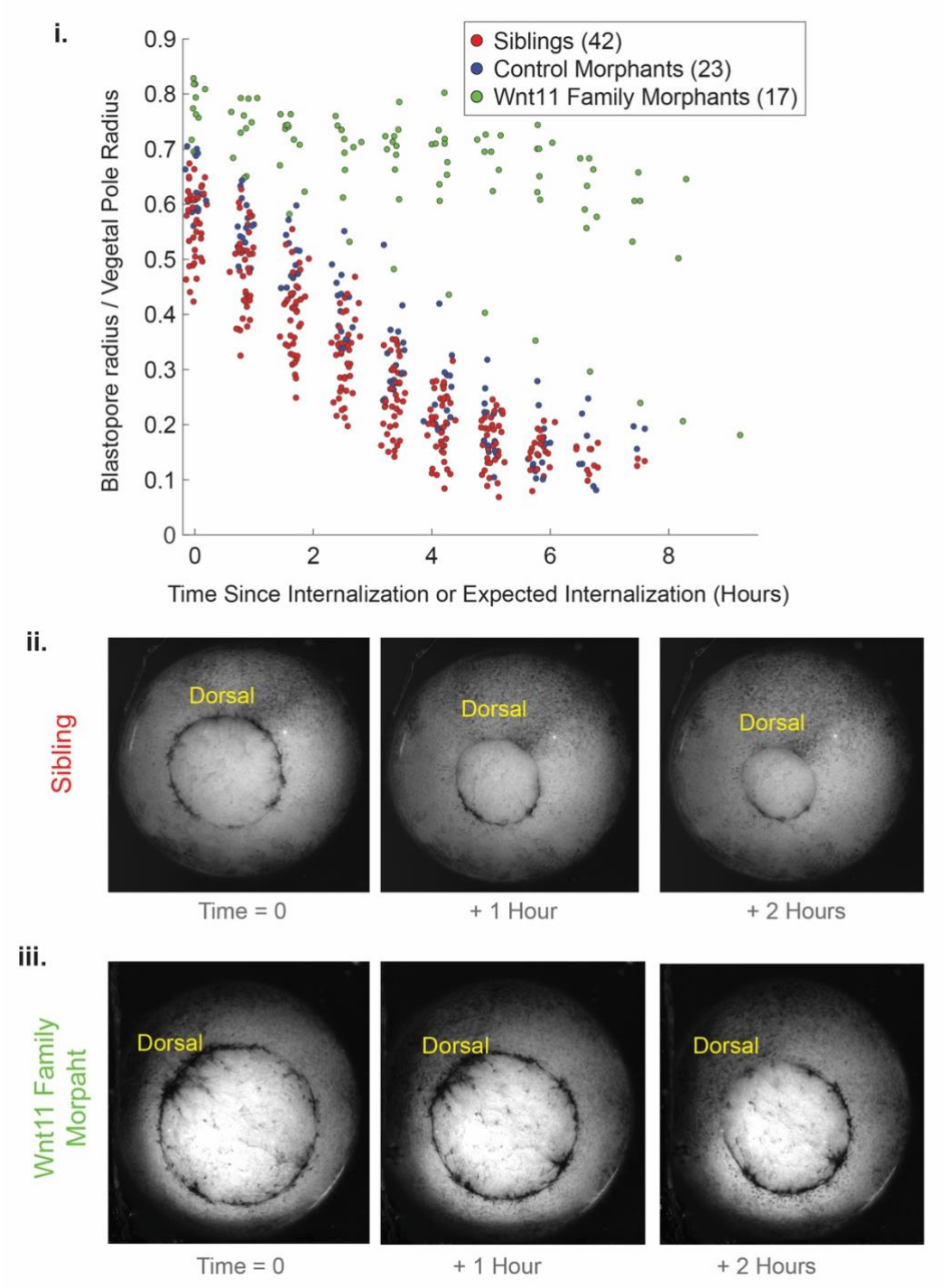
Supplemental Figure 9. Strength of the ten picomole Wnt11 family morpholino phenotype cannot be attributed to toxicity from the total morpholino amount. For three biological replicates, we injected both blastomeres of two-cell embryos with either the five base-pair mismatch control morpholino, the Wnt11 family morpholinos, or a combination of the two. The total morpholino dose was between nine and ten picomoles. The embryos injected only with the high dose of the control morpholino are not statistically significantly different than the un-injected embryos. Embryos injected with either of the Wnt11 family containing morpholinos are statistically significantly different than the un-injected embryos (red asterisks), but the embryos injected with only five picomoles of the Wnt11 family morpholinos are also statistically significantly different than the embryos injected with ten picomoles of the Wnt11 family morpholinos. The majority of the embryos injected with less of the Wnt11 family morpholinos have the moderately closed phenotype compared to the larger dose of Wnt11 family morpholinos that have the severely open phenotype. We take these results as evidence that the strength of the ten picomole Wnt11 family phenotype is not the result of morpholino toxicity effects.



Supplemental Figure 10. Custom imaging apparatus enables inverted high-resolution multiplexed imaging. More information, including files for making the custom apparatus components, can be found on GitHub.

A. Custom embryo holders were laser cut to fit inside the chambers of an eight-chamber slide. Embryos are placed inside the wells and are stable when the microscope stage moves.

B. The chamber slide is then inserted into a custom 3D printed slide holder. This slide holder is important because the flat surface of the chamber slide is too small to fit on the stage.

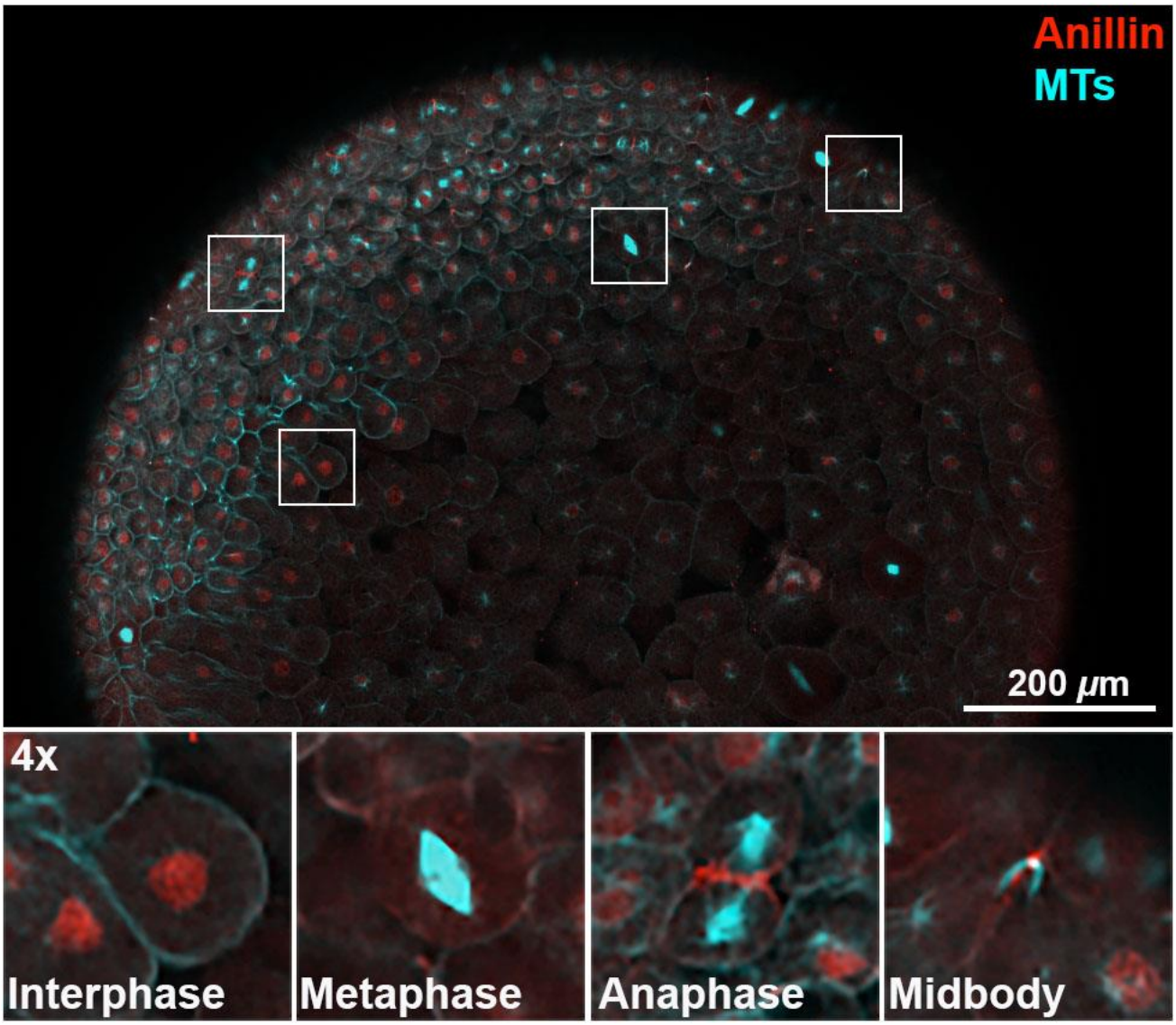


Supplemental Figure 11. Quantitative analysis of time-lapse imaging of blastopore closure after dorsal lip maturation.

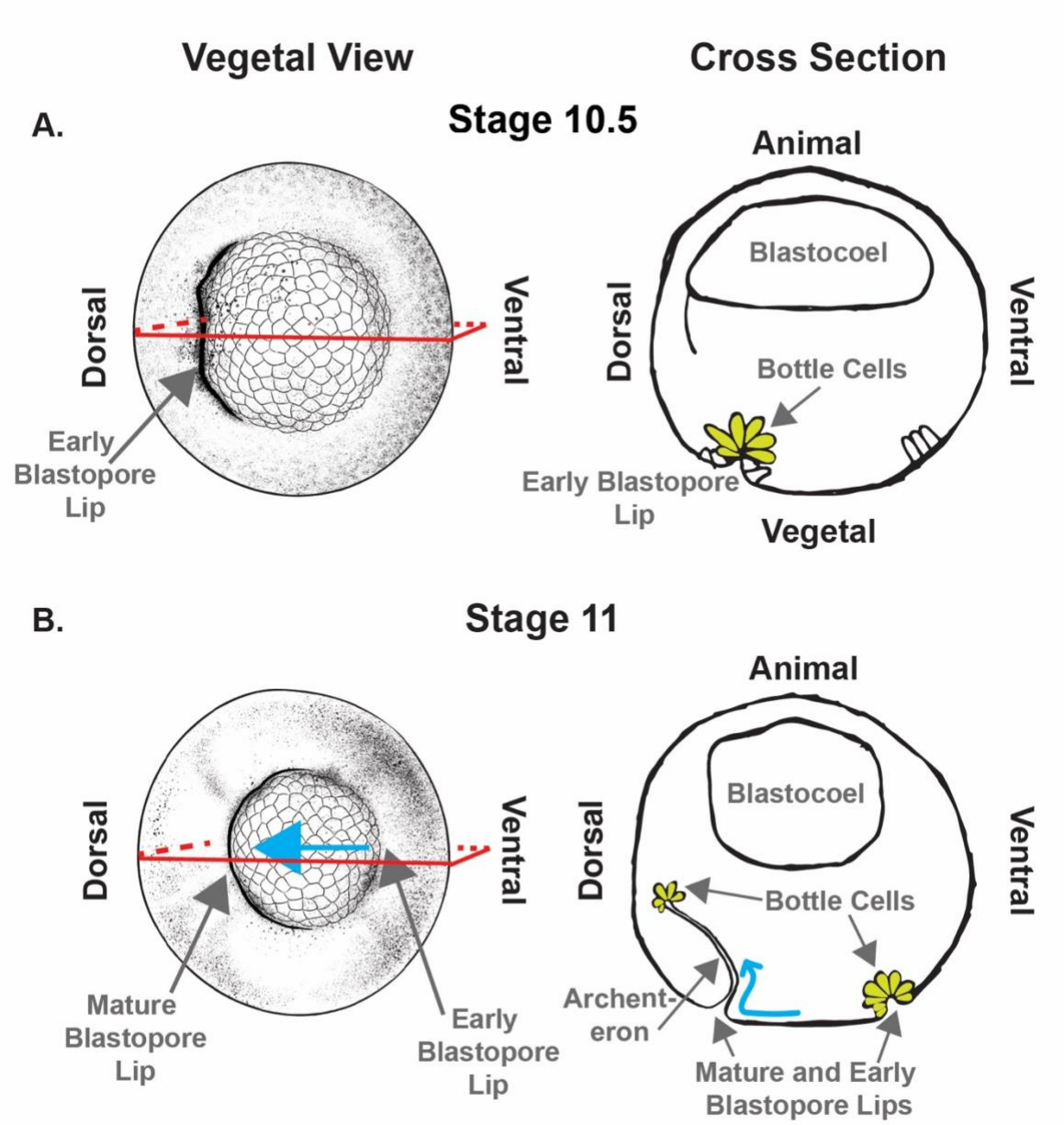
Supplemental Figure 11 (continued).

(i) Wnt11 family morphant embryos have larger blastopores at the time when the dorsal lip matures in sibling morphants and less blastopore closure during the second half of gastrulation than sibling and control morphant embryos. Quantification of the decrease in normalized blastopore radius for the sibling, control morphant, and Wnt11 family morphants during gastrulation. Quantification starts when sibling embryos form a mature blastopore lip, as determined by inspection of movie frames. For control morphant and Wnt11 family morphant embryos, quantification begins at the expected time of lip maturation as determined by the mean of the matched sibling maturation times. Wnt11 family morphants have larger blastopore radii when quantification begins and show only limited blastopore closure afterwards. In addition to Wnt11 family morphant phenotypes, control morphant embryos have, on average, larger blastopores at the expected time of lip maturation than siblings. This is consistent with the expected delay of gastrulation caused by any morpholino injection. However, the blastopores of control morphants do close over a similar time period as the sibling embryos.

(ii, iii) Still images for a sibling embryo and Wnt11 family morphant embryo at the beginning of quantification and one and two hours later. Progressive closure of the blastopore is seen in the sibling embryo. For the Wnt11 family embryo, the blastopore has formed by the expected lip maturation time, but the dorsal bottle cells are entirely visible. At the one hour timepoint the blastopore is slightly smaller, but the dorsal lip has still not matured. After two hours the dorsal vegetal material has extruded above the dorsal lip instead of being internalized.



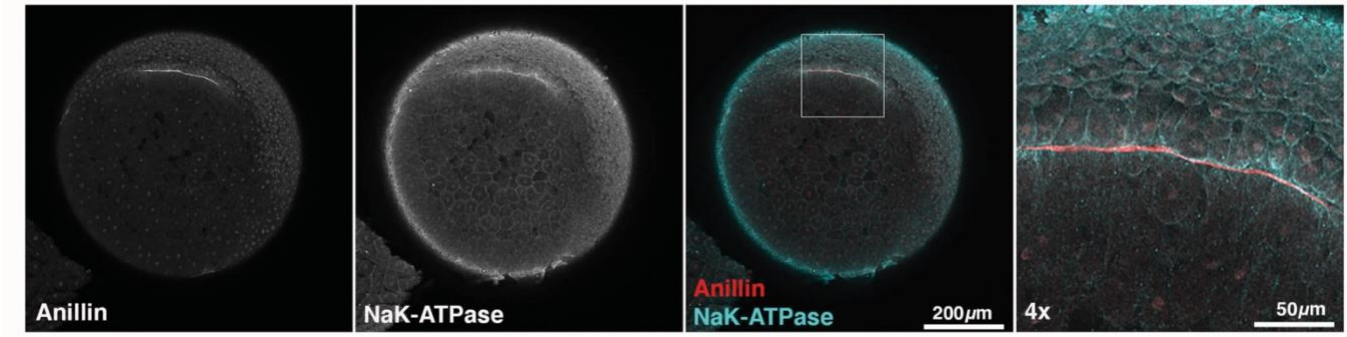
Supplemental Figure 12. Antibodies against anillin (red) and microtubules (cyan) have the expected localizations related to the cell cycle. Inset panels show examples of cells in interphase (anillin in the nucleus), metaphase (microtubules spindle), anaphase (microtubule spindles separating on either side of the anillin localized cleavage furrow), and the end of cytokinesis (anillin in the microtubule midbody structure).



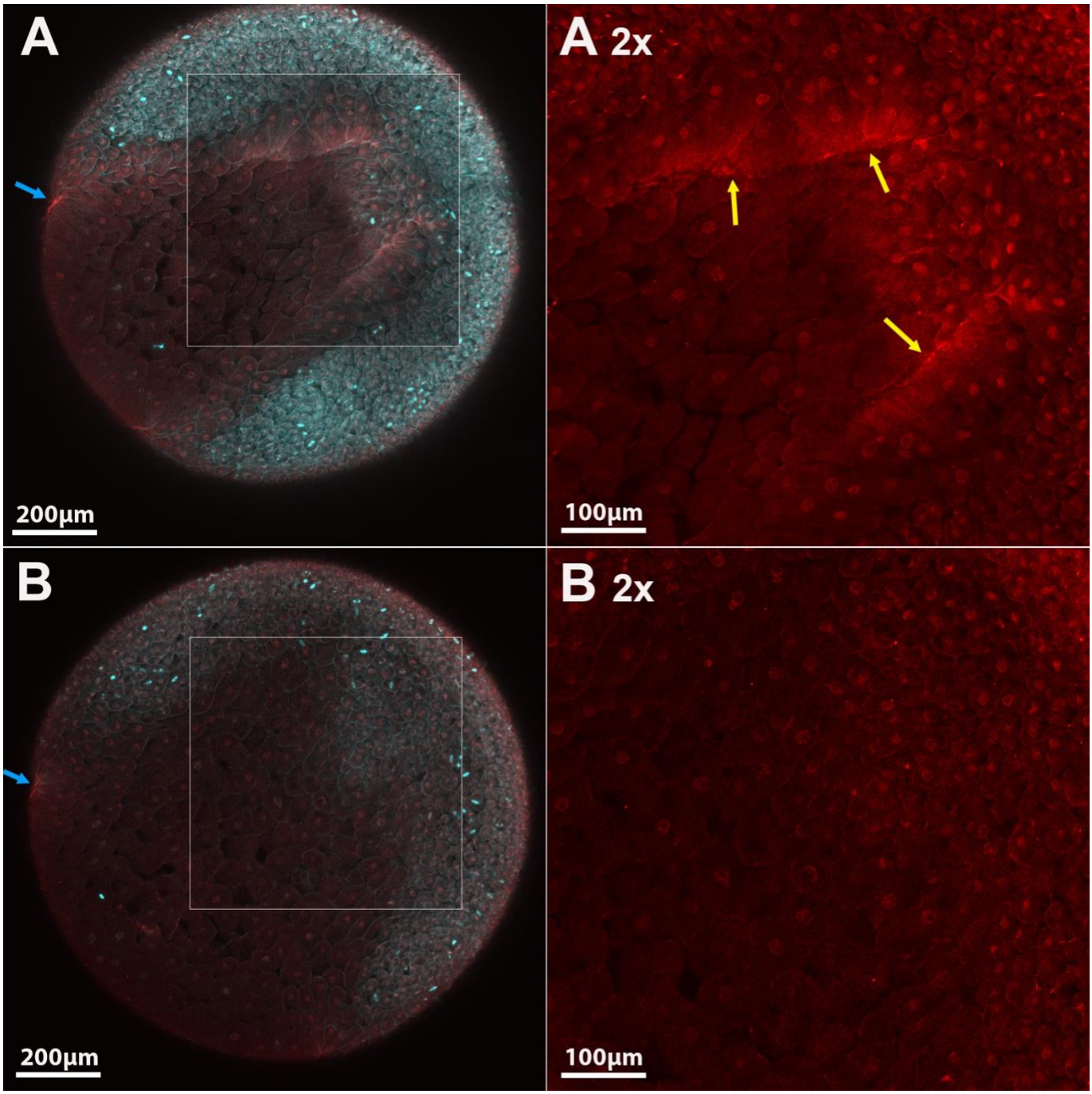
Supplemental Figure 13. Drawings and schematics showing two views of embryos at Stages 10.5 and 11. On the left, external vegetal views and on the right internal cross-section views. The schematics show the locations of the structures discussed in the main text; bottle cells, blastopore lips, and the archenteron. The red box shows the relationship of the vegetal view drawing to the cross-section schematic before a 90-degree upwards rotation.

A. At Stage 10.5 the early blastopore lip has formed on the dorsal side of the embryo. The lip is dark in color because bottle cell apices are at the lip. There are elongated pre-bottle cells where the dorsal lip will form.

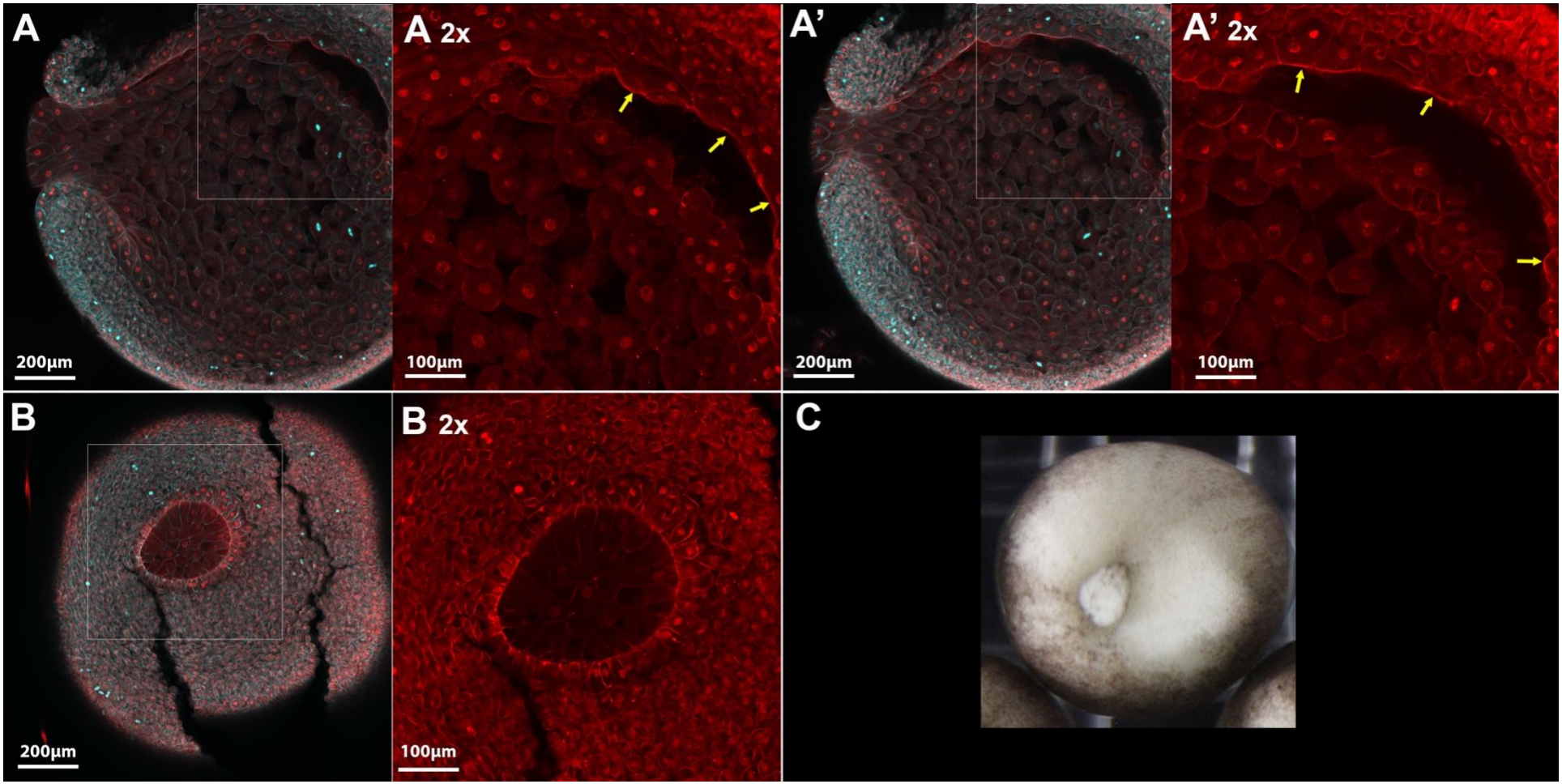
B. At Stage 11 the dorsal blastopore is mature. The external color of the mature dorsal blastopore lip has changed because the bottle cells have left the blastopore lip and moved animally. The bottle cells are at the animal extent of the archenteron which is lined by the epithelial cells that were formally above and below the early blastopore lip. Bottle cells have formed on the ventral side of the embryo resulting in an early blastopore lip. Vegetal rotation (dorsal animal movement of the vegetal endoderm; blue arrows) is occurring internally as early as Stage 10, but it is clearly visible from movies of the exterior of the embryos at Stage 11. Cross-section drawings are based on drawings by P Hausen and M Riebesell [8].



Supplemental Figure 14. Horizontal plane optical section images of whole-mount immunofluorescence against anillin (red) and NaK-ATPase (cyan). Staining against NaK-ATPase (an integral membrane protein) is seen at the membrane of many cells in the embryo. There is also moderately enhanced staining at the dorsal blastopore lip where membrane proximal anillin staining is also seen.



Supplemental Figure 15. Wnt11 family morphant embryos at Stage 11.5 still show no evidence of archenteron extension. Whole-mount immunofluorescence against anillin (red) and tubulin (cyan) of matched sibling **(A)** and Wnt11 family morphant **(B)** embryos scored in Figure 8. This set developed the furthest before fixation. In the sibling embryo, archenteron morphogenesis has proceeded past the initial upward movement. Anillin staining can still be seen in the membrane proximal location (yellow arrows). We see no evidence of this in the morphant embryo. In both embryos, anillin staining can also be seen at the small part of the blastopore lip visible in this cross-section (blue arrows).

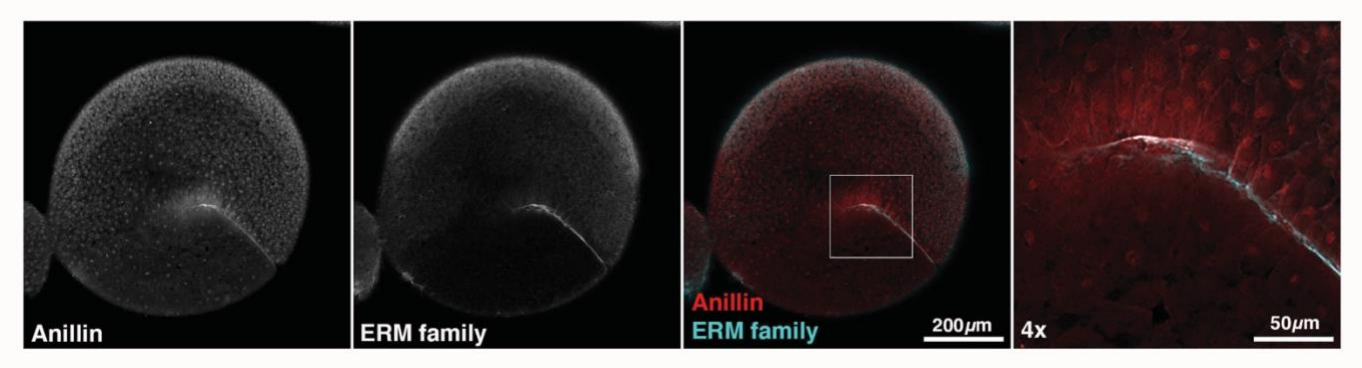

A
A 2x
A'
A' 2x
200µm
100µm
200µm
100µm
B
B 2x
C
200µm
100µm

Supplemental Figure 16. Anillin staining persists in the archenteron and blastopore through the end of gastrulation. Whole-mount immunofluorescence against anillin (red) and tubulin (cyan) of un-injected Stage 12.5 embryos.

A. Two different optical sections of the same embryo (dorsal up, blastopore left). The expanding anterior archenteron is visible on the right. The higher magnification insets show that there is membrane proximal anillin staining (yellow arrows).

B. Vegetal view of the blastopore. Anillin is enriched on the apical side of epithelial cells at the blastopore.

C. Pre-fixation external view of an embryo at the same timepoint.



Supplemental Figure 17. Horizontal plane optical section images of whole-mount immunofluorescence against anillin (red) and ERM family proteins (cyan). Staining against ERM family proteins is highly specific for cells at the blastopore, similar to anillin.

Supplemental Methods

Antibodies

Antibodies raised against the alpha subunit of ATPase, (Na (+) K(+)) were purchased from the Developmental Studies Hybridoma Bank (monoclonal antibody a5). The anti-radixin antibody was raised against full length human radixin (however likely cross reacts with ezrin and moesin) was the kind gift of William Brieher (University of Illinois at Urbana-Champaign). Alexa fluorescent secondary antibodies (Thermofisher) were used to probe for the latter two antibodies.

Modified RNase Protection Assay

Embryo manipulations

For all of the morpholino perturbations, morpholino was injected into both cells of the 2-cell stage embryos. The total amount of morpholino injected is reported in the figure legend. Morpholino concentrations were quantified according the protocol provide by Gene Tools, LLC and morpholino stocks were maintained according to best practices [9]. The oligo sequences for the standard control morpholino and Ctnnb1 morpholino are: CCTCTTACCTCAGTTACAATTTATA and TTTCAACCGTTTCCAAAGAACCAGG, respectively. Embryos were fertilized and injected at 18ºC, and embryos that were assayed during mid or late gastrulation were cultured at 18ºC. Embryos that were collected at Stage 10 were cultured at 22ºC. The solution for injection was 0.5x MMR, 4% ficoll (Ficoll PM 400, Sigma), and the embryos were rinsed into 0.1x MMR before gastrulation.

Ribosome Bound Footprint RPA – Part 1: Embryo lysis, RNase digestion, RNA purification

Fifteen embryos (occasionally a few less) were collected in a tube and flash frozen after the surrounding liquid removed. Frozen embryos were stored at -80ºC until use. Embryos were lysed by pipette titration in 150 uL of standard ice-cold lysis buffer (1% replacement NP-40, 0.5% sodium cholate, 20 mM Tris-HCl pH 7.4, 300 mM KCl, 10 mM MgCl2, 4 mM DTT, 100 ug/mL

cycloheximide and Roche complete ethylenediaminetetraacetic acid-free protease inhibitors) unless otherwise noted. Once the initial lysate was homogenous, a further 750 uL of lysis buffer was added and the entire amount was mixed by pipetting. Then a further 600 uL of lysis buffer was added, and the entire amount was mixed by inversion. Lysates were spun for 15 minutes at 12,000 x g for at 4ºC. The supernatant including the top layer of lipids was removed and added to another 1.5 mL of ice-cold lysis buffer. The final volume of lysis buffer was 3 mL. Assuming a 1 uL volume per embryo, this is a 200-fold dilution. Next, RNase1 (Ambion, cloned, 100U/uL) was added with a final concentration of 0.42 units/uL, and the tubes were incubated at room temperature with gentle shaking for 45 minutes. Upon completion SUPERaseIn RNase Inhibitor (20 U/uL) was added to a final concentration of 0.11 units/uL. Lysates were transferred to labelled and chilled 6.5 mL (16 x 64 mm) thick-walled open-top polycarbonate tubes for ultracentrifugation (355647, Beckman Coulter). The pellets were underlaid with 2.5 mL of 1 M sucrose cushion made with the same salts as the lysis buffer but with no detergents or DTT. RNase inhibitor was also added to the sucrose cushion. Lysates were spun in an MLA 80 rotor at 80,000 rpm for 4:15 hrs at 4ºC in an OptimaMax tabletop ultracentrifuge (Beckman Coulter). After centrifugation, the supernatant was removed, and the pellet RNA was extracted with the mirRNeasy kit from Qiagen according to the manufacturer's instructions. We eluted the RNA from the column with two 50 uL RNase free water elutions. We precipitated the RNA overnight with ethanol and added GlycoBlue as a carrier. We resuspended the pellets in 16 uL of RNase free water and use 1 uL to measure a 1:10 dilution by nanodrop. We usually recovered 40-50 ug of RNA total from 15 embryos.

Generation of Anti-sense probes

Probe DNA template was made from PCR amplified ~300 nucleotide regions of DNA encoding the mRNA sequence of a target gene. The 3' primer has the sequence 5'-CGC**AATTAACCCTCACTAAAGGG**AGA -3' on the 5' end of the probe sequence specific primer where the bold sequence denotes the promoter sequence for T3. NEB Q5 High-Fidelity polymerase was used for these reactions. The PCR products were gel extracted from an agarose gel, precipitated with ethanol, and resuspended in RNase free H20 to a concentration of ~ 500 ng/uL.

Radioactive anti-sense probes were made using T3 polymerase and the MAXIscript kit developed by Ambion and sold by ThermoFisher. The reaction volume was scaled down to 10 uL total. The source of radioactivity was UTP-[α-P^{32}] with "day of" concentration: 6000 Ci/mmol and 40 mCi/ml purchased from PerkinElmer (BLU507Z001MC). Taking into account the carrier UTP and adjusting for decay relative to the "day of" date, UTP-[α-P^{32}] and cold UTP were combined such that the final [UTP_{total}] = 2.5 uM and the fraction UTP-[α-P^{32}] = 0.1757. "radioactive_labeling_P32.m" is the MATLAB code for determining the volumes of radioactive and cold UTP for the reaction (https://github.com/e-vanitallie/Footprint_RPA). The transcription reaction was allowed to proceed for twelve minutes. The full reaction was run on a 6% TBE-UREA gel. The gel was exposed using film for a very short amount of time and the band with the full-length probes was cutout. The full-length probes were gel extracted overnight at 37 C. The probes were extracted in Probe Elution Buffer form the mirVANA detection assay kit. After removing the buffer from the gel piece, 2 ul of GlycoBlue was added to the buffer, and then 100% ethanol was added to precipitate the probes. The precipitation proceeded for 2 hours at -20ºC and then the probes were spun at 4ºC at 20,000xg for 20 minutes. All of the supernatant was carefully removed, and the pellets air-dried for 10 minutes. Then the pellets were resuspended without pipetting in 10 uL of RNase free H20. 1 uL of a 1:10 dilution of each probe was measured by scintillation counter to correctly determine the concentration of RNA. Using a standard curve to relate CPMs to [P^{32}] and probe length, number of Us, and fraction of UTP-[α-P^{32}], the measured probe solution was diluted with H20 so that the hybridization reactions had the appropriate concentration of probe. "radioactive_CPMtoCONC.m" is the MATLAB code for determining the correct number of CPMs per probe to add to the hybridization reactions to have the desired final concentration of probe (https://github.com/e-vanitallie/Footprint_RPA).

Ribosome Bound Footprint RPA - Part 2: Detecting footprints by hybridization

To detect ribosome protected footprints, we performed an RNase protection assay using the mirVana miRNA Detection Kit developed by Ambion and now sold by ThermoFisher. We hybridized input RNA with UTP-[α-P^{32}] labelled anti-sense probes for 14 hours at 42ºC in an incubator. The amount of input RNA was always the same and for probe-self hybridization control experiments the source of RNA was the 5 ug/uL yeast RNA included in the kit. For each set of hybridizations, no digestion controls were used to make sure that probe was not digested and yeast RNA only digestion controls were used to determine the expected signal from probe self-hybridization. The amount of probe solution to add such that the actually probe concentration was consistent across experiments was determined as described above. Hybridization solutions were prepared using master mixes that contained hybridization solution and probe as well as other shared components when appropriate. Solutions were made up with the utmost care and mixed extensively by careful vortexing. Upon adding master mix to the specific input RNA, the reactions were mixed thoroughly by pipetting. After hybridization the reactions were digested for 45 minutes at 37ºC. The RNase mix was added to the RNase digestion solution at a dilution of 1:100. The RNase was inactivated, and the reaction precipitated according the instructions. Precipitations proceeded for at least 2 hours at -20ºC, and then the reactions were pelleted at 20,000xg for 30 minutes at 4ºC. The supernatant was carefully removed and disposed of appropriately. The pellets air-dried for only 5 minutes to avoid over-drying. Then they were resuspended by flicking and vortexing (NOT pipetting) in 5 uL of the Gel Loading Buffer II included in the kit or 10 uL for the no-digestion controls. The samples were then denatured for 3 minutes at 95-100C and loaded onto a pre-run 15% TBE-urea gel. For the no-digestion controls we ran 5 uL of a 1:40 dilution of the resuspended reaction. For the digested reactions we ran the entire reaction. For ladders, see details below. The gels were wrapped in SaranWrap at exposed to Kodak BioMAX MS film contained in a high sensitivity intensifying screen for the length of time reported. The film was developed with a Kodak X-OMAT 2000A Processor with T2 X-Ray Fixer and Developer (White Mountain Imaging) and scanned for quantification.

Labeling Ladders

We used two different types of radioactively labelled size markers for visualizing the RPA results. We used the Ambion Decade Marker System sold by Thermo Fisher Scientific. We prepared the reaction mixture for labelling according to the manufacturer's instructions with ATP-[γ-P^{32}]. After calculating the specific activity of the labelled solution on the day of use, we added cold ATP such that the total concentration of the ATP in the reaction is 0.1 uM and the fraction of ATP-[γ-P^{32}] is 0.15. "radioactive_labeling_P32.m" is the MATLAB code for determining the volumes of radioactive and cold ATP for the reaction (https://github.com/e-vanitallie/Footprint_RPA). For an expected 24-hour exposure we ran 5 uL of a 1:20 dilution of the final 40 uL reaction mixture. We also used the upper and lower size marker oligoribonucleotide markers from the ribosome profiling method [2]. The sequence of the upper size marker is 5'-AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA-(Phos)-3', and the sequence of the lower size marker is ′5-AUGUACACGGAGUCGACCCAACGCGA-(Phos)-3'. These markers were also labelled with ATP-[γ-P^{32}] using the T4 phospho-nucleotide kinase and reaction buffer from New England Biolabs (NEB). The labelling reaction was 10 uL with 1 nM each of the upper and lower markers. Again, the total concentration of ATP is 0.1 uM and the fraction of radioactive ATP is 0.15. For an expected 24-hour exposure, 5 uL of 1:20 dilution of the final 20 uL reaction (after 1:1 dilution with 2x gel loading buffer) was run on the gel.

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