Science
AAAS

Supplementary Materials for

Persisted programmed cell death along the midline axis patterns ipsilaterality in gastrulation

Lisandro Maya-Ramos, Takashi Mikawa*.

*Correspondence to: takashi.mikawa@ucsf.edu

This PDF file includes:

Materials and Methods
Figs. S1 to S12
Table S1
Captions for Movies S1 to S10

Other Supplementary Materials for this manuscript include the following:

Movies S1 to S10

Materials and Methods

Experimental Design

Fertilized chicken (Gallus gallus domesticus) eggs were obtained from a local breeder (Petaluma Farms, CA) and placed in a humid incubator at 37°C until Hamburger Hamilton stage (HH) 2 (*25*). For ex-vivo manipulations, embryos were isolated in Pannett-Comptons solution [40mL of solution A, 60 mL of solution B in 1L of H2O final volume. Solution A: 121 g NaCl, 15.5 g KCl, 10.42 g CaCl 2–2H 2O, and 12.7 g MgCl 2–6H 2O in 1L of H2O final volume. Solution B: 1.88 g Na 2HPO 4, and 0.188 g NaH 2PO 4–2H 2O in 1L of H2O final volume.] under a Seizz Stemi 2000C stero microscope and cultured according to the New Culture method (*23*).

For grafting experiments, donor transgenic GFP expressing chick embryos (commercially available from Clemson University, South Carolina) and host chick embryos were incubated to stages HH 2-3. Isochronic transplantations were performed for homotopic and heterotopic grafts. Host embryos were placed in new culture, as above, while stage matched GFP-transgenic embryos isolated in Tyrodes solution [137mM NaCl, 2.7 mM KCL, 1mM MgCl2, 1.8mM CaCl2, 0.2 mM Ha2HPO4, 5.5mM D-glucose, 15mM Hepes, pH 7.4]. Homotopic grafts were done by excising a GFP-transgenic embryo epiblast area with a tungsten knife. An epiblast region of the same graft area size was removed on the same side of the host embryo. Then, the GFP graft was transferred with a capillary pipette. Grafted embryos were then lived image under temperature controlled conditions. Heterotopic grafts were performed as above, with the difference that donor GFP grafts were placed on the contralateral side of host embryos.

Microscopy

Embryos in New Culture were time-lapse imaged under temperature controlled conditions with either: Nikon Eclipse TE2000-E supported by Hamamatsu ORCA-Flash 2.8 camera or Nikon Eclipse Ti supported by ANDOR iXon camera, with a 4X, 10X and 20X objective using Nikon Elements Advance Research software V4.00.07.

For multiphoton imaging, embryos were culture with a modified version of New Culture technique, 100 µL of substrate mix of albumin and 2% agar, was added to a glass-bottom 35mm culture plate. Time-lapse images were acquired every 3-4 minutes with a Zeiss LSM 7 MP, W Plan-APOCHROMAT 2-photon microscope (using 20x/1.0 objective), equipped with a Non-descanned detector PMT, and ZEN 2012 SP2 (black) software.

Scanning electron images were acquired by following published protocols (*24*), for stages HH 3+ and 4+ embryos at the Marine Biological Laboratory, Woods Hole, MA.

Embryonic and cellular transfections

Stage HH 2 embryos were isolated in Pannett Compton Solution and placed dorsal side up on an electroporating chamber containing Tyrodes solution. DNA mix (DNA 3µL, H2O 1.5µL, FastGreen 0.5µL and 60% sucrose 0.5µL) was added to dorsal epiblast, and embryos were electroporated at 3.5 volts, 50 milliseconds pulse, 500 milliseconds intervals, 3 times using the NEPA 21 Super Electroporator Type II. Embryos were then cultured for subsequent experimentation.

For in-vitro transfections, 60-70% confluent 293T cells cultured in 6-well plates were transfected with either PCAGS-GFP or PCAGS-P35:2A:H2B-GFP using polyethylenimine (PEI). Cells were

washed with PBS and a mix of 1 mL of serum free opti-MEM media containing 2.5 µg DNA and 7.5µL PEI, previously incubated for 15 minutes, added drop-wise. Transfection was carried overnight. A final concentration of staurosporine 5µM was added for the time points of 0, 3 and 7 hours. Propidium iodide (PI) #P3566 (Life technologies, Carlsbad, California) staining, and hoechst vital dyes were added at the end of each time point for analysis.

DNA constructs and morpholinos

The pCAG plasmid (Addgene plasmid # 11150) was used as a backbone for Gibson Assembly (New England Biolabs, Ipswich, Massachusetts) cloning of: Flag:2A:H2B-GFP; Flag:2A:H2B-RFP; P35:2A:H2B-GFP, a gift from Dr. Patrick Mehlen; MMP-15 (NM_002428.2) , MMP-15:2A:mCherry, MMP-15:2A:H2B-GFP (Vector Builder, Santa Clara, California) and YFP-Sec-A5 (Addgene plasmid # 105664). pCCALL2 DN-FGFR1-IRES-EGFP, a gift from Dr. Gerard Blobbe. Membrane GFP, and membrane RFP, a gift from Dr. Orion Weiner. Translation blocking 3' Lissamine LAMA1 (NM_001199806.1) morpholino (5'-CCCATCACCATCGCCGCCAC-3') (Gene Tools, Philomath, Oregon, USA) and five-mispair sequence control (5'-CCGATCACGATCCCCCCGAC-3') and lissamine standard oligo (5'-CCTCTTACCTCAGTTACAATTTATA-3') were electroporated as indicated above.

TUNEL assay

The In Situ Cell Death Detection Kit, POD (Cat. No. 11 684 817 910) from Roche was used and the kit instructions followed with these additional modifications. Embryos were fixed in 2% glutaraldehyde in 4% pararaformaldehyde for 30 minutes at room temperature, then washed with

PBS three times for 5 minutes each. Embryos were incubated with the TUNEL reaction for 2 hours at 37°C and washed with PBS five times for 5 minutes each.The TUNEL-POD was added for 30 minutes at 37°C then rinsed with PBS. Embryos were then developed in DAB-substrate in the dark.

Immunofluorescence staining

Embryos were freshly isolated and placed on 1X PBS, then fixed in 4% paraformaldehyde in PBS for 0.5 hours at room temperature, followed by 0.5 hours of PBS washing. Blocking media, (1% BSA, 0.1% triton in PBS) was added for 1 hour at room temperature, followed by primary antibody staining. Primary antibodies were added in blocking solution overnight at room-temperature, followed by 2 hours of PBS washing. Secondary antibodies were added in blocking solution for 2 hours followed by 2 hours of PBS washing with DAPI (1:1000). Embryos were mounted dorsal side up in mounting media. Antibodies used: laminin (1:400) 3H11-S anti-mouse and fibronectin (1:400) anti-mouse B3/D6-C (Developmental Studies Hybridoma Bank, Iowa City, Iowa). MMP-15 (1:150) anti-rabbit ab15475 and Collagen IV (1:200) anti-rabbit ab6586 (Abcam, Cambridge, United Kingdom). Cleaved caspase 3 (1:150) anti-rabbit 9661S (Cell Signaling Technologies, Danvers, Massachusetts). GFP anti-rabbit (1:400) A11122, Alexa Fluor 488 (1:500) anti-rabbit A11008, Alexa Fluor 488 (1:500) anti-mouse A11001, Alexa Fluor 594 (1:500) anti-mouse A21203, Alexa Fluor 594 (1:500) anti-rabbit A11037, Alexa Fluor 647 (1:500) anti-mouse A31571 (Thermo Fisher, Waltham, Massachusetts).

In situ hybridization and HRP staining

Embryos were fixed overnight at 4°C in 4% paraformaldehyde, then washed in PBT for 30 minutes, followed by proteinase K (1:2000) in PBT treatment for 1 minute at room-temperature. Then, embryos were treated with glycine (2mg/mL) in PBT for 20 minutes and then washed with PBT for 10 minutes. Embryos were incubated at 70°C for 2 hours in pre-hybridization buffer (10mL solution 1 (1% SDS, 5X SSC, 50% formamide), 10µL of heparin (50µg/mL), 50µL yeast RNA (50µg/mL)) followed by overnight probe incubation at 70°C. Next, the probe was replaced by solution 1 and washed twice 30 minutes each. Then, washed twice with solution 3 (50% formamide and 2X SCC pH 4.5) 30 minutes each. Followed by 15 minutes of TBST washing at room temperature and blocking with 10% sheep serum in TBST for 1.5 hours at room temperature. Lastly, embryos were incubated overnight at 4°C with DIG-Fab antibody (1:2000) in blocking solution. Next, embryos were washed for 30 minutes four times with TBST at room-temperature, followed by a 10 minute wash with NTMT (2.5mL NaCl, 1mL Tris-HCl, 1ml MgCl2, 100µL Tween and 6.4mL H2O). Development of signal was done with 4.5µL NBT and 3.5µL BCIP in 1mL of NTMT. Once signal was visible, embryos were washed in PBT and stored in 4% paraformaldehyde. GFP-HRP antibody was used for GFP detection in in-situ hybridization processed embryos. Embryos were washed in PBT, then fixed in 4:1 methanol/ DMSO overnight at 4°C, then incubated for 2 hours at room-temperature with 4:1:1 methanol/ DMSO/30% H2O2 followed by 1 hour blocking at room-temperature with 1% sheep serum in TBST. The anti-GFP(HRP) antibody was added overnight in blocking media. Next, embryos were washed five times, 1 hour each, with TBST at 4°C, then washed 10 minutes with 0.05M Tris-HCl pH 7.6 followed by a 30 minute incubation in the dark with DAB in Tris-HCl pH 7.6.

Signal was developed with DAB in Tris-HCl with 0.0003% H2O2. Images were acquired on a Leica MZ16F microscope equipped with Leica DFC300 Fx camera and Leica FireCam V.3.4.1 software. Probes for ROBO1, ROBO2, SLIT1, SLIT2, FGF4 and FGF8 provided by Dr. Jeanette Hyer. Probe BMP2 provided by Dr. Tsutomu Nohno. Chordin probe provided by Dr. Thomas Jessell. Other probes, Table S1, purchased at Source Bioscience.

Propidium iodide, Alexa Fluor 488 Annexin V conjugate staining and staurosporine micro-injections

For in-ovo PI staining, 1mg/mL of PI was back loaded into a pulled glass micro-pipette and then micro-injected with an Ependorf Femtojet injection system in the space between the vitelline membrane and the dorsal epiblast. Embryos were incubated for 3-5 minutes at room temperature then imaged.

For Alexa Fluor 488 Annexin V conjugate staining (A13201 Thermo Fisher Scientific) stage HH 2 embryos were isolated placed in New Culture plate. Immediately after, using a pulled glass micro pipette, ~1.5µL of Alexa Fluor 488 Annexin V conjugate in PBS were micro-injected apically, between the vitelline membrane and the dorsal epiblast. Embryos were then time-lapsed imaged at 10X as indicated above.

For staurosporine S4400 (Sigma Aldrich, St. Louis, Missouri) injection treatment, a pulled glass micro pipette was back loaded with 0.9 µL of 0.3mM staurosporine, 0.5 µL fast green and 1.6 µL PBS, and embryos in New Culture were ventrally injected. Vehicle injections contained 0.5 µL fast green and 1.6 µL PBS and 0.9 µL of water with DMSO. All injections were recorded using a Leica MZ16 Fluorescent stereo microscope on a Jenoptik ProgMF cool digital camera.

Hoechst 33342/focal illumination PCD induction

PCD was induced by the 2Phatal protocol with the following modifications (*18*). Stage HH2/ HH3- embryos were electroporated with fluorescein-tagged LAMA1 morpholino at the PS and Flag:2A:H2B-GFP at lateral epiblast, then incubated for 1 hour at 37 °C.. Embryos were then microinjected, as indicated above, with Hoechst 33342 (ThermoFisher H3570), 0.04mg ml−1 diluted in PBS and incubated at 37 °C for 2-3 hours. The glass ring along with the vitelline membrane, was briefly taken out of the new culture plate and flipped in order to have the embryo dorsal side face the microscopic objective. Using the 10X objective a single ROI was centered on the microinjected zone and illuminated with the 405 laser for 2-3 minutes. Embryos were then time-lapse imaged .

Statistical Analysis

i. Cellular ingression

Scoring of cellular ingression was done in H2B-GFP and laminin double stained embryos. In the Imaris X64 9.2.0 software**,** first a surface mask was created using laminin as a basement membrane marker, to exclude epiblast and only analyze cells that underwent ingression. Then, the spot function was employed to count all H2B-GFP positive cells on each side of the primitive streak. Total percentage included cells from both right and left ventral sides. Left and right percentage were calculated by dividing the number of cells from that side over the sum of both sides. For the analysis of contralateral ingression in staurosporine and vehicle injected embryos, a surface mask was created on the entire contralateral side of electroporation. Then, H2B-GFP

cells were quantified inside a rectangular area measuring in length the entire mediolateral distance and in width, the size of the injection footprint. The same rectangular area was used to quantify H2B-GFP cells adjacent to the injection site. Injection and adjacent to injection site percentages were calculated by dividing the number of cells from that site over the sum of both sites. An unpaired t test was done with a two-tailed p value of <0.0001.

ii. Cellular blebbling frequency

Multiphoton time-lapse movies of membrane-GFP electroporated embryos were analyzed with Imaris X64 9.2.0 software. Movies were 2D projected in the XY plane using the MIP mode, then, using the measuring points tool, the precise location of cells undergoing blebbing was calculated (event) with respect to the PS midline. The total number of events was then plotted in a histogram by using a bin width of 50µm, all experiments (n=4) plotted on the same graph.

iii. Alexa Fluor 488 Annexin V conjugate tracking

Stage HH 2 embryos labeled as indicated below underwent time-lapse imaging acquisition. The file was then analyzed with the Imaris X64 9.2.0 software. At time zero, the spot tool was employed using the Alexa 488 channel as the source channel. Spot detection was done with an estimated XY diameter of 7.24µm and background subtraction. The spot quality was set up to 7.5%. The tracks were statistically colored to show the start position along the X axis. X axis length = 1400µm. The PS localized between 600-800µm along the X axis, with the midline residing at approximately 700µm. Y axis length = 1050µm. For the frequency distribution, the relative frequency of all spots for both X and Y axis were plotted in a histogram graph. X axis spots had a bin width of 464µm, which allowed to divide the embryo in 3 parts: left, PS, right. Y

axis spots had a bin width of 348µm, which allowed to divide the embryo in anterior, middle and posterior regions.

iv. IF staining intensity

Digital black and white cross section images from ECM (Laminin, fibronectin and collagen IV) IF stained embryos were processed in ImageJ 2.0.0-rc-541.52a. A 120 µm line was traced across the PS with the midline located at approximately 60 µm and the plot profile function used. Y axis units are arbitrary units of gray scale readings.

v. Cleaved caspase 3 number and PI-Annexin V particle count

Stage HH 2 embryos were electroporated with LAMA1 morpholino (n=3) or LAMA1 control morpholino (n=4) then IF stained for laminin and cleaved caspase 3. WT embryos (n=3) were IF stained immediately after isolation. A total of 5 different fields of view, confocal imaged at 40X, were analyzed per group. Cleaved caspase 3 number was quantified by first creating surface volumes using the specific cleaved caspase 3 channel. The precise location of cleaved caspase 3 was measured with the measurement points tool, using midline laminin as a reference point, under the ortho slicer XZ plane mode. The total number of cleaved caspase 3 numbers was plotted agains distance from the PS midline. To determine the differences in caspase 3 clusters, we compared the midline region, determined by 10µm spanning the midline, and lateral regions which were 90µm to the left and 90µm to the right of the midline. The ratios are presented in the figure.

For PI-Annexin V particle count, stages HH 3 and 4 embryos (n=3 per condition) were stained in-ovo with PI or Alexa Fluor 488 Annexin V conjugate. Images were then analyzed with the Imaris X64 9.2.0 software. The spot tool was used, with an estimated XY diameter of 2µm and

background subtraction. The spot quality was set up to 7.5%. The surface mask was then employed to isolate and quantify spots localizing to the PS only. Spots were then averaged and S.E.M reported with t test analysis done.

vi. P35 in vitro analysis

Staurosporine treatment of previously transfected 293T cells with P35:2A:H2B-GFP or control GFP and stained with PI and Hoechst vital dyes were used. For each given time point (0, 3 and 7 hours) images, taken with Zeiss Axio Vert.A1 20X objective and ZEN 2.3 blue edition software, were used to calculate the ratio of PI+, GFP+ and Hoechst+ cells over the total number of GFP+ and Hoechst+ cells in a field of view. The GFP and P35 mean ratios were 4.4 and 4.7 at t0h, 34.2 and 13 at t3h and 43.8 and 35.3 at t7h. Difference was statistically significant at t3h with p=0.0023, t test n=3.

vii. MMP-15 fluorescent activity

The MMP-15 fluorimetric assay kit (Anaspec Sensolyte 520 Cat. # 72198) was used to assess the enzymatic activity of the MMP-15:2A:mCherry molecular construct. Briefly, 293T cells were transfected using PEI as indicated above. Then trypsinized (0.25% Tryspin-EDTA 1X Gibco, Life Technologies, ref. # 25200-056). The pellet was washed twice with PBS and resuspended in 250µL of assay buffer with 0.1% triton X-100(T8787, Sigma Aldrich) and incubated for 10 minutes at 4°C. The cell suspension was then centrifuged for 10 minutes at 2500 g at 4°C and the supernatant stored at -70°C until used. Untransfected 293T cells and MMP15:2A:mCherry transfected extracts, and 1.25ng, 2.5ng rhMMP-15 were used. The GM-6001 MMP inhibitor was added at minute 50 (CC1010 Millipore Sigma).

Fig. S1

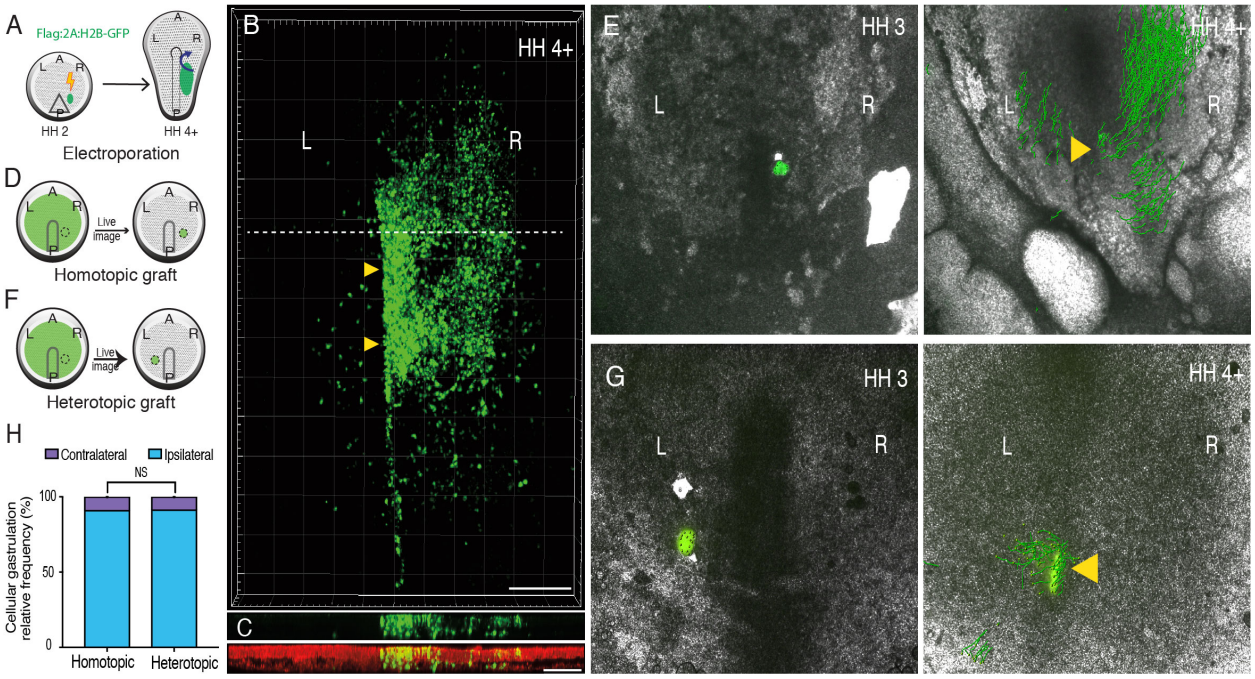

Fig. S1.
Ipsilateral gastrulation is non-cell autonomously regulated.
(**A**) Electroporation scheme of Flag:2A:H2B-GFP in the lateral epiblast. (**B**) Wholemount view of Flag:2A:H2B-GFP electroporated embryo at stage HH 4+ . Arrows point to the PS midline. Scale bar, 200 µm. (**C**) Top GFP (green), bottom GFP (green)/phalloidin (red) cross section (10µm thickness) from dotted line on (B). Scale bar, 100 µm. (**D**) Scheme of isochronic homotopic graft from transgenic GFP donor to non-GFP embryo. (**E**) (First panel) GFP graft approaching PS midline at stage HH 3. (Second panel) GFP grafted cells undergoing ipsilateral and contralateral invasion. Arrowhead points to PS midline. (**F**) Scheme of isochronic heterotopic graft from transgenic GFP donor to non-GFP embryo. (**G**) (First panel) GFP graft approaching PS midline at stage HH 3. (Second panel) GFP grafted cells undergoing ipsilateral and contralateral ingression. Arrowhead points to PS midline. (**H**) Quantification of cellular ingression relative frequency of GFP grafted cells. Homotopic 91.0±2.4% vs heterotopic 91.3±4.7%, non-statistically significant p=0.9893, n ≥ 3.

Fig. S2

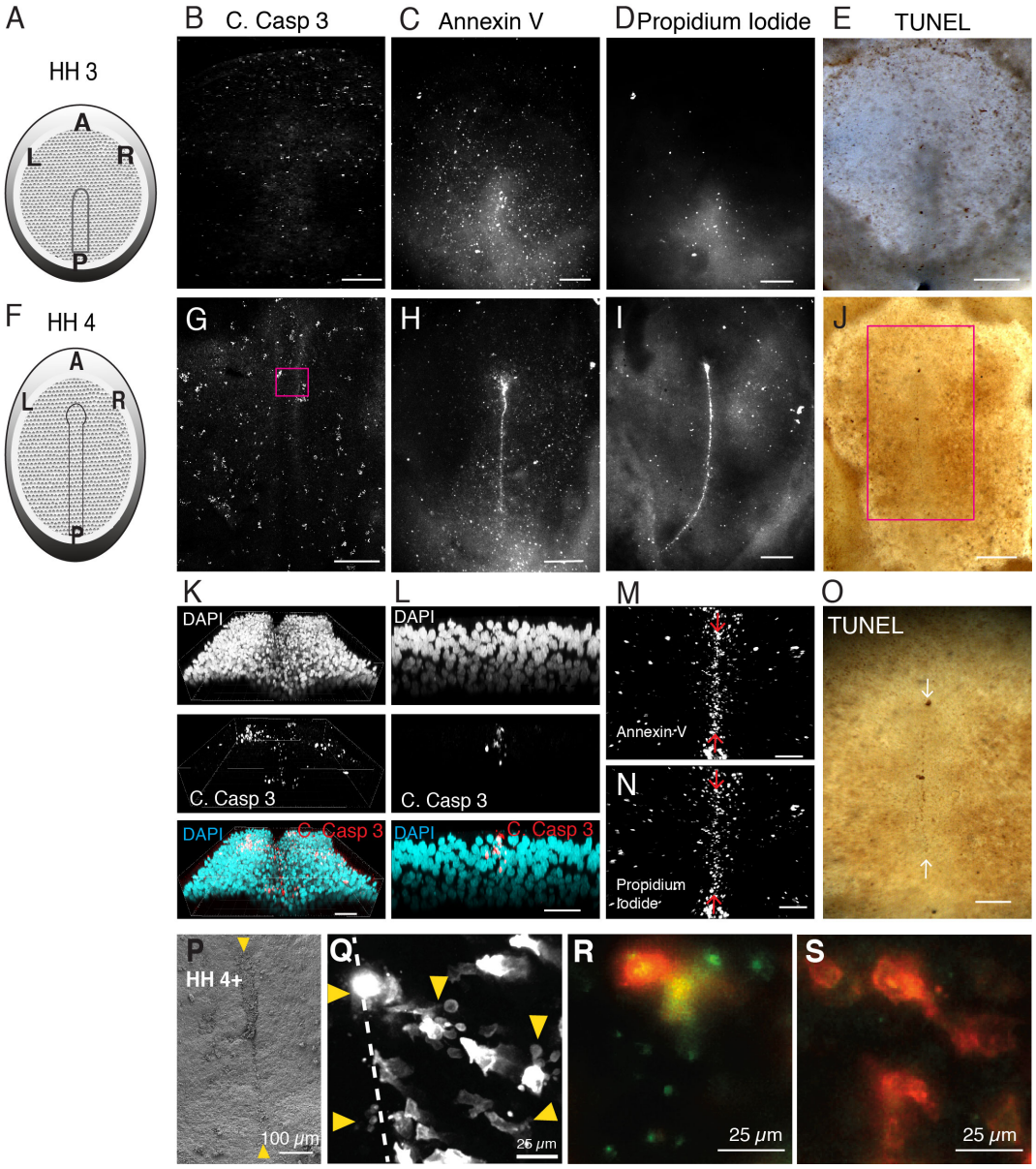

Fig. S2.

PCD markers at stage HH 3 and HH 4. (A, F) Stage HH 3 and stage HH 4 schemes. (**B, G**) Cleaved caspase 3 IF staining at stage HH 3 (B) and stage HH 4 (G). Box in G, shown at higher magnification in (K). Scale bar, 300 µm. (**C, H**) Alexa Fluor 488 Annexin V conjugate labeling at stage HH 3 (C) and stage HH 4 (H). Scale bar, 300 µm. (**D, I**) Propidium Iodide labeling at stage HH 3 (D) and stage HH 4 (I). Scale bar, 300 µm. (**E, J**) TUNEL staining at stage HH 3 (E) and stage HH 4 (J). Box in J, shown at higher magnification in (O). Scale bar, 300 µm. (**K, L**) High magnification stage HH 4 cleaved caspase 3 IF staining in 3D view (K) and cross section

(L). Scale bar, 50 µm. (**M**) High magnification stage HH 4 Alexa Fluor 488 Annexin V conjugate staining. Arrows point to PS midline. Scale bar, 50 µm. (**N**) High magnification stage HH 4 propidium iodide. Arrows point to PS midline. Scale bar, 50 µm. (**O**) High magnification stage HH 4 TUNEL staining. Arrows point to PS midline. Scale bar, 100 µm. (**P**) SEM images of persisting cellular debris along PS midline (arrowheads) at stage HH 4+. (**Q**) Membrane GFP multiphoton image at the PS midline (dotted line). Arrowheads, blebbing cells. (**R**) Blebbing cells at the PS midline region co-expressed mRFP (red) and PCD marker activated YPF-Sec-A5 (green). **(S)** as in (**R**) but negative for both blebbing and PCD marker. C casp 3, cleaved caspase 3.

Fig. S3

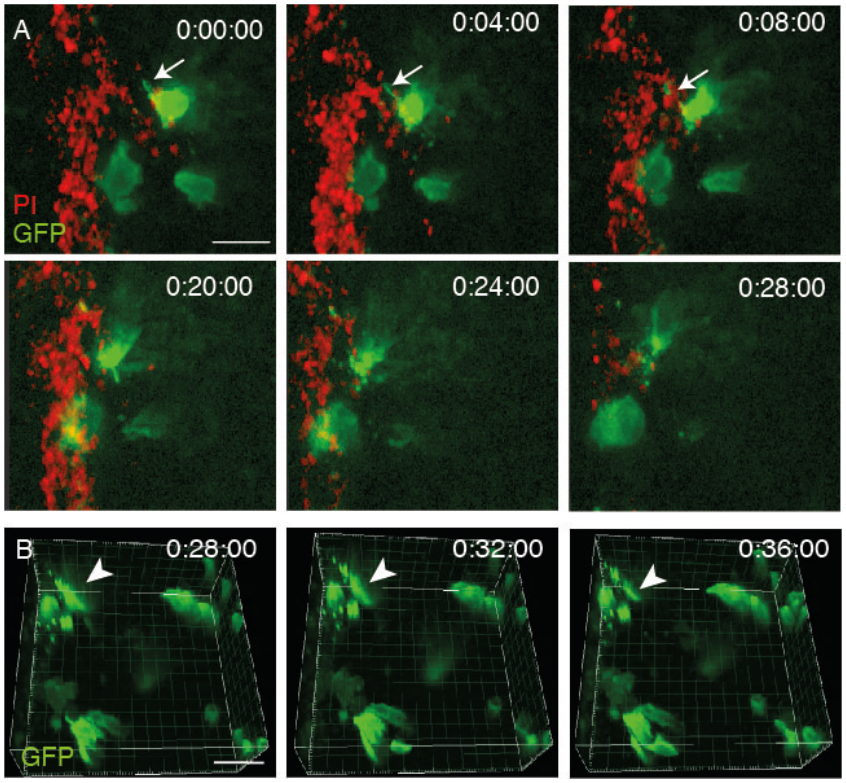

Fig. S3
Gastrulating cells contact PI+ midline before ingression. (A) Single plane multiphoton time lapse images of WT GFP+ cells and PS midline stained with propidium iodide (PI). Arrow points to cellular protrusion as cell approaches PS midline. Scale bar 15 µm. (B) 3D multiphoton time lapse images of WT GFP+ cells (same movie as in A). Arrowhead points to same cell as it undergoes ingression moving to more ventral plane. Scale bar, 30 µm.

Fig. S4

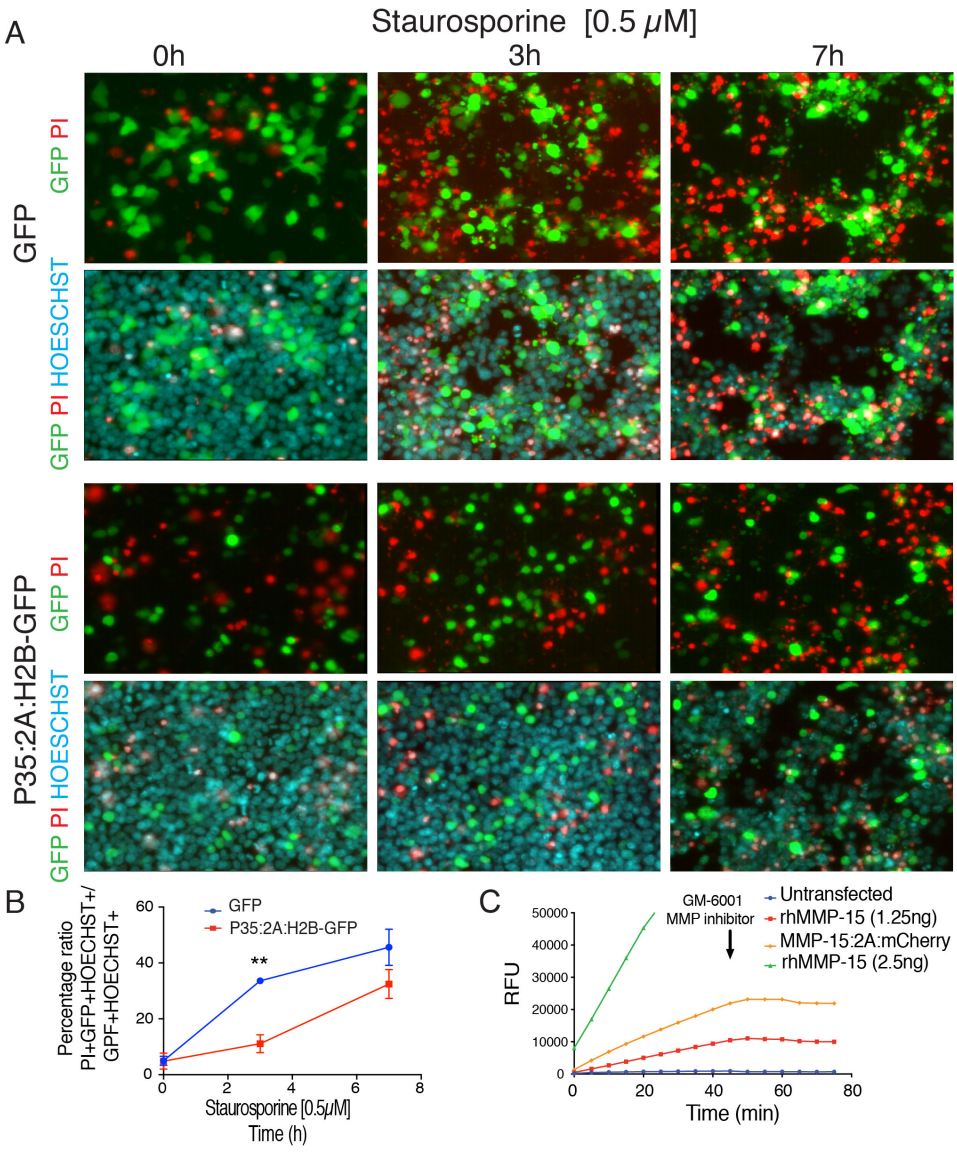

Fig. S4.

P35 and MMP-15 molecular constructs are functionally active. (**A**) Representative images of staurosporine [0.5 μM] treated 293T cells, previously transfected with PCAGS:GFP or PCAGS:P35:2A:H2B-GFP and stained with PI and Hoechst dyes. (**B**) Percentage ratio quantification of staurosporine treatment time-course for PCAGS:GFP and PCAGS:P35:2A:H2B-GFP groups. (**C**) MMP-15 Fluorometric continuous assay. MMP inhibitor added at minute 50. rhMMP-15, recombinant human MMP-15. PI, propidium iodide.

Fig. S5

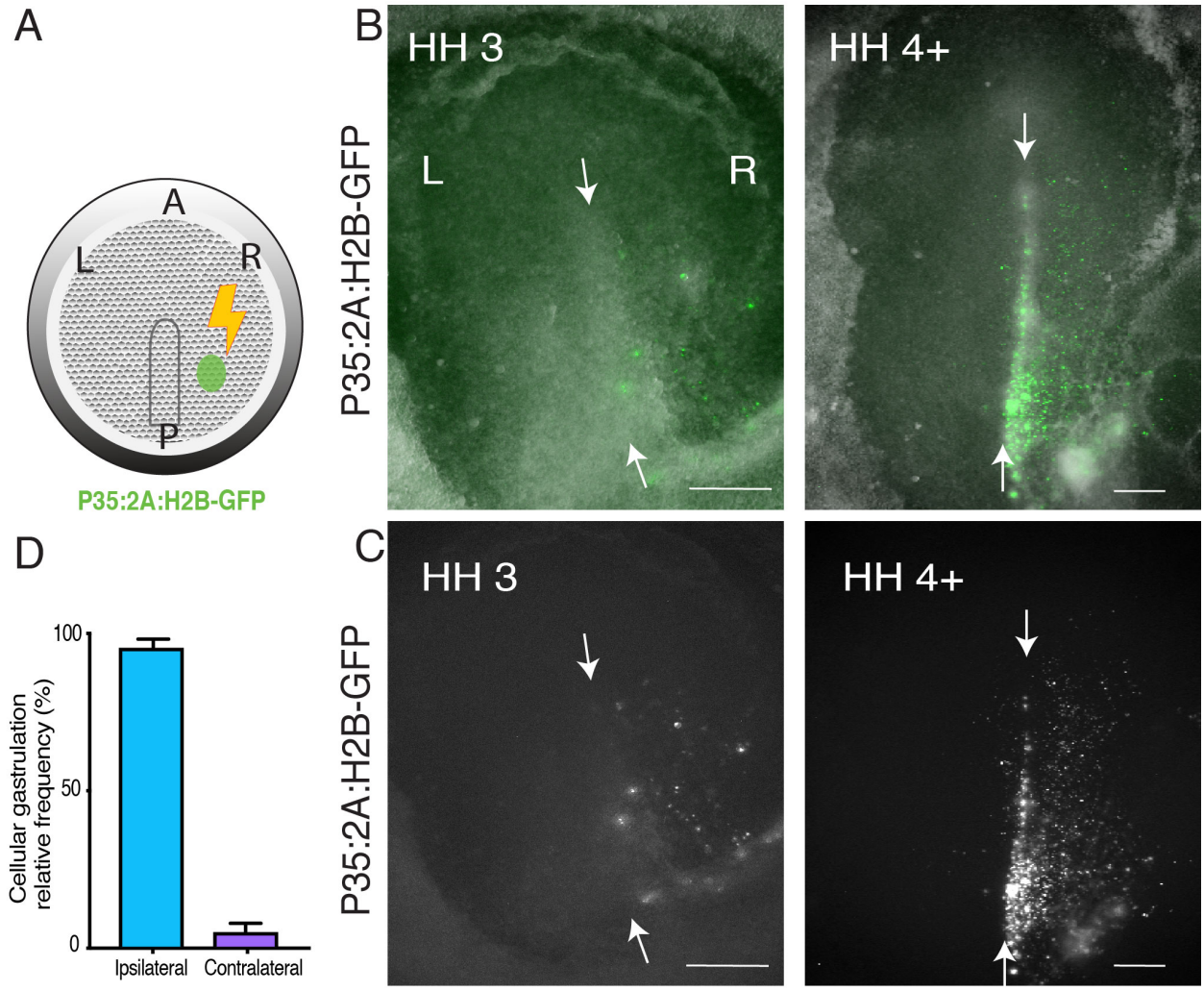

Fig. S5.
Epiblast P35 electroporation results in ipsilateral gastrulation primarily. (**A**) Electroporation scheme of P35:2A:H2B-GFP in the lateral epiblast. (**B**) Merged bright field and fluorescent images. (First panel) P35:2A:H2B-GFP cells approaching PS midline at stage HH 3. (Second panel) cells primarily undergoing ipsilateral gastrulation. Arrows point to the PS midline. Scale bar, 300 µm. (**C**) Black and white P35:2A:H2B-GFP fluorescent panels from (B). (**D**) Quantification of cellular gastrulation relative frequency. Non-statistically significant when compared to WT left and right ingression Fig. 1C. Ipsilateral ingression 95.34±1.267% $p<0.0001$ ($n = 5$).

Fig. S6

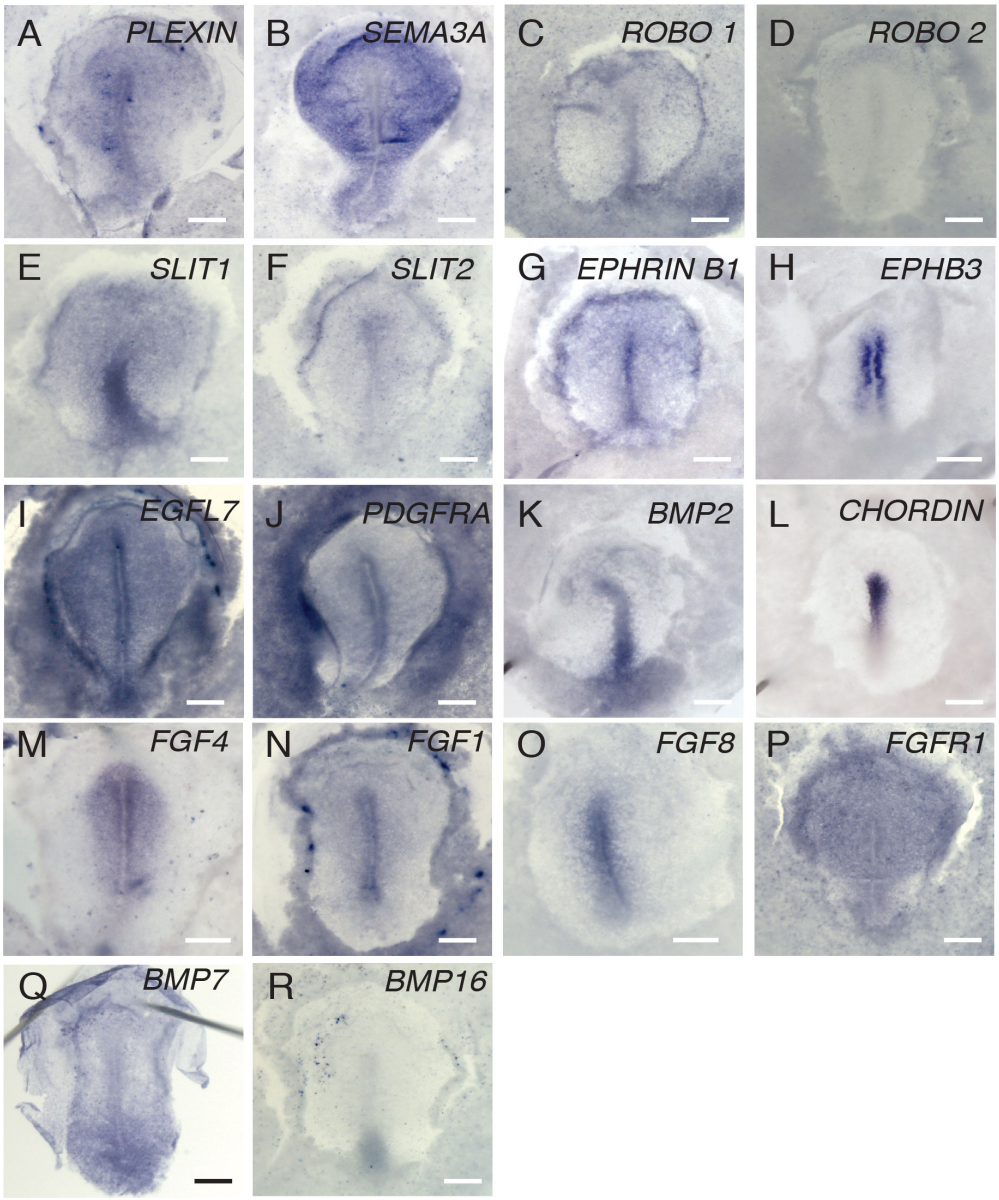

Fig. S6.
Expression of cell survival and midline central nervous system patterning factors. Stage HH 4 whole mount in situ hybridization (ISH) of (**A**) *PLEXIN* (**B**) *Semaphorin 3A* (**C**) *ROBO1* (**D**) *ROBO2* (**E**) *SLIT1* (**F**) *SLIT2* (**G**) *EPHRINB1* (**H**) *EPHB3* (**I**) *EGFL7* (**J**) *PDGFRA* (**K**) *BMP2* (**L**) *CHORDIN* (**M**) *FGF4* (**N**) *FGF1* (**O**) *FGF8* (**P**) *FGFR1* (**Q**) *BMP7* (**R**) *BMP16.* Scale bar, 400 μm.

Fig. S7

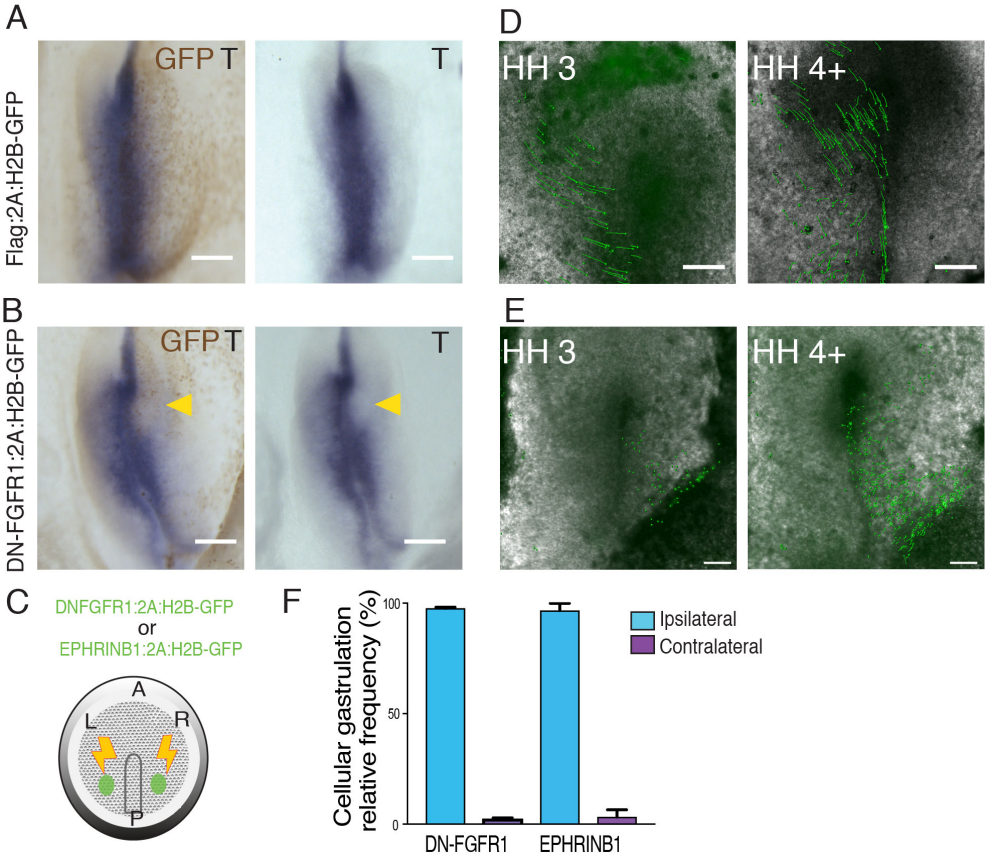

Fig. S7.
FGFR1 and EphrinB1 signaling are not required for ipsilateral gastrulation. (**A**) Representative image of whole mount ISH of *T* gene expression in Flag:2A:H2B-GFP (control) embryo. HRP-DAB used for GFP detection. Second panel, ISH of *T* gene expression alone. Scale bars, 200 µm. (**B**) Representative image of wholemount ISH of *T* gene expression in DN-FGFR1:2A:H2B-GFP embryo. HRP-DAB used for GFP detection. Second panel, ISH of *T* gene expression alone. Yellow arrowheads point to *T* signal absence in DN-FGFR1 electroporated zones. Scale bars, 300 µm. (**C**) Electroporation scheme of DN-FGFR1:2A:H2B-GFP or EphrinB1:2A:H2B-GFP at either left or right sides of PS. (**D**) (First panel) DN-FGFR1:2A:H2B-GFP cells approaching PS midline at stage HH 3. (Second panel) cells primarily undergoing ipsilateral gastrulation. Scale bar, 300 µm. (**E**) (First panel) EphrinB1:2A:H2B-GFP cells approaching PS midline at stage HH 3. (Second panel) cells primarily undergoing ipsilateral gastrulation. Scale bar, 300 µm. (**F**) Quantification of cellular gastrulation relative frequency. Non-statistically significant when compared to WT left and right ingression Fig. 1C. DNFGFR1:2A:H2B-GFP ipsilateral ingression 97.7±0.3% p=0.8959 and EphrinB1:2A:H2B-GFP 96.7±1.9% p>0.9999 ($n \geq 3$).

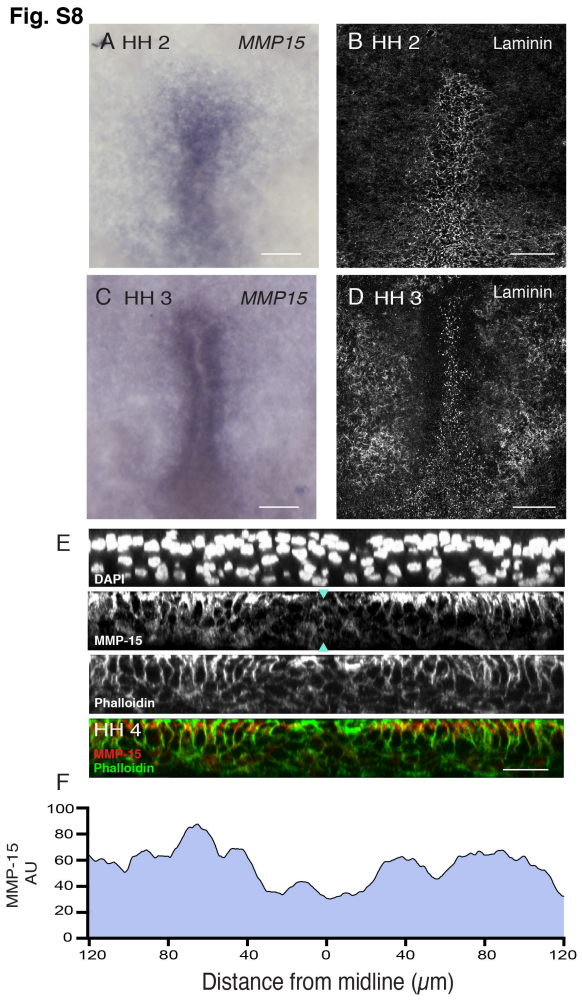

Fig. S8.
MMP-15 and laminin expression patterns. (**A, C**) *MMP15* wholemount ISH of stage HH 2 (A) and stage HH 3 (C) embryos. Scale bars, 200. (**B, D**) Laminin IF staining of stage HH 2 (B) and stage HH 3 (D) embryos. Scale bars, 200 μm. (**E**) Cross section MMP-15 IF staining. Arrowheads on MMP-15 IF staining point to PS midline. Scale bar, 30 μm. (**F**) Cross section gray scale histogram of MMP-15 (E, second panel). Y axis represents IF staining intensity. X axis represents distance (μm) from midline zone shown as 0μm. AU, arbitrary units.

Fig. S9

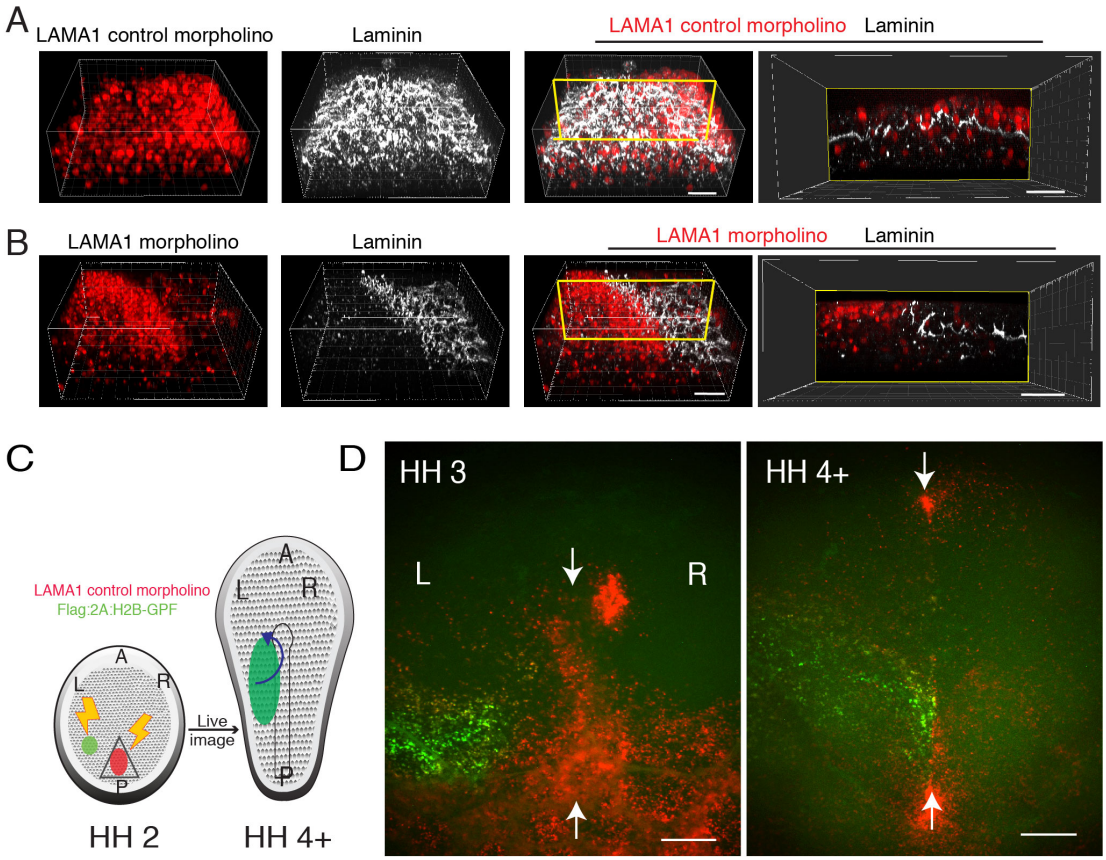

Fig. S9.
LAMA1 control morpholino does not result in laminin degradation and contralateral ingression. (**A**) High magnification of PS laminin IF staining in embryo with LAMA1 control morpholino expression. Scale bar, 50 µm. (First panel) Lissamine-tagged LAMA1 control morpholino staining. (Second panel) Laminin IF staining. (Third panel) Merged image of laminin and LAMA1 control morpholino, box shown as cross section in last panel. (**B**) High magnification of PS laminin IF staining in embryo with LAMA1 morpholino expression. Scale bar, 50 µm. (First panel) Lissamine-tagged LAMA1 morpholino staining. (Second panel) Laminin IF staining. (Third panel) Merged image of laminin and LAMA1 morpholino, box shown as cross section in last panel. (**C**) Electroporation scheme of Lisammine tagged LAMA1 control morpholino at the posterior PS and left side electroporation of PCAGS:Flag:2A:H2B-GFP. (**D**) (First panel) Flag:2A:H2B-GFP cells approaching PS midline at stage HH 3. (Second panel) cells primarily undergoing ipsilateral gastrulation. Arrows point to the PS midline. Scale bar, 300 µm.

Fig. S10

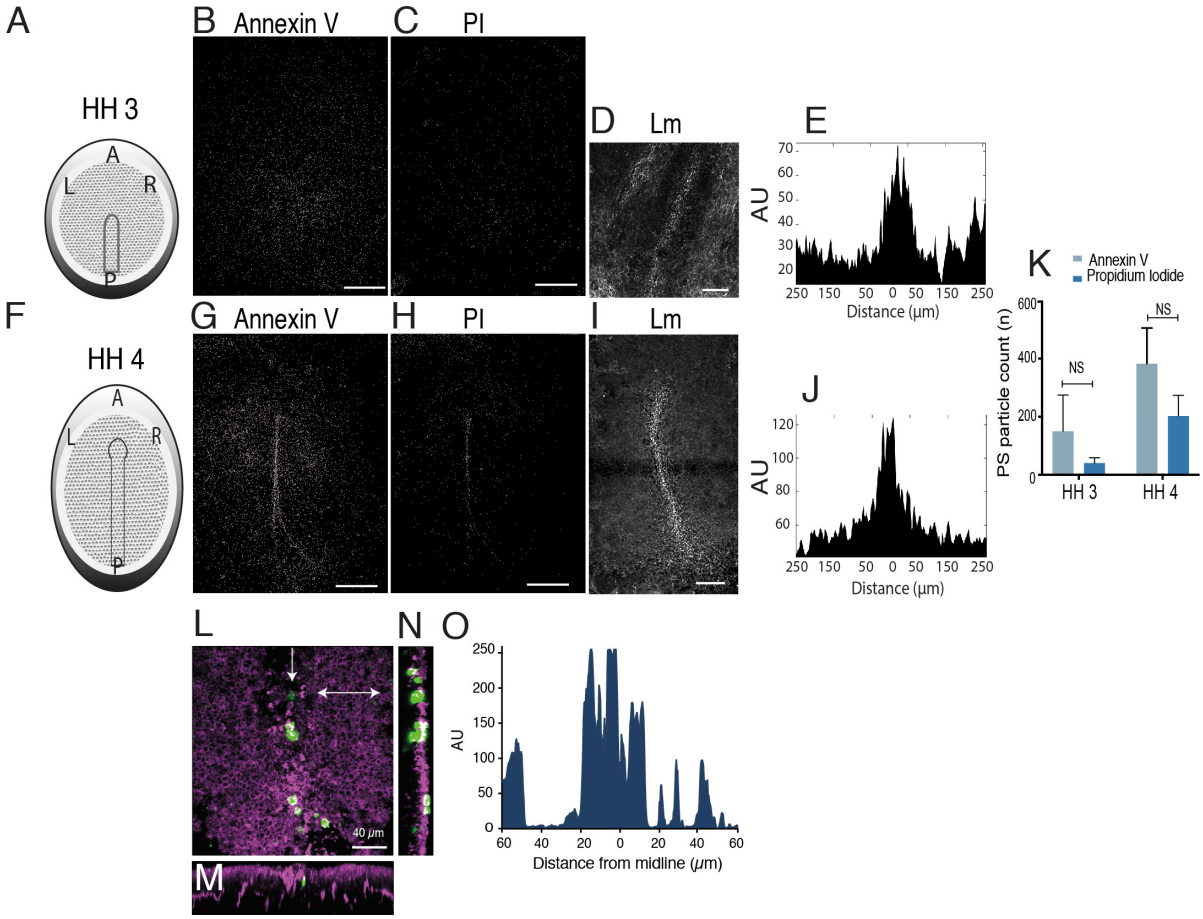

Fig. S10.
PS midline ECM enrichment precedes PS midline PCD. (**A**) Scheme of stage HH 3 embryo. (**B**) Overlay of Alexa Fluor 488 Annexin V conjugate staining stage HH 3 embryos. Fluorescent signal particles transformed to white spots with IMARIS software. Scale bar, 500 µm. (**C**) Overlay of PI stained stage HH 3 embryos. Fluorescent signal particles transformed to white spots with IMARIS software. Scale bar, 500 µm. (**D**) Laminin IF staining of stage HH 3 embryo. Scale bar, 200 µm. (**E**) Cross section gray scale histogram of (D). Y axis AU, arbitrary units, represent IF staining intensity. X axis represents distance (µm) from midline zone shown as 0µm. (**F**) Scheme of stage HH 4 embryo. (**G**) Overlay of Alexa Fluor 488 Annexin V conjugate staining stage HH 4 embryos. Fluorescent signal particles transformed to white spots with IMARIS software. Scale bar, 500 µm. (**H**) Overlay of PI stained stage HH 4 embryos. Fluorescent signal particles transformed to white spots with IMARIS software. Scale bar, 500 µm. (**I**) Laminin IF staining of stage HH 4 embryo. Scale bar, 200 µm. (**J**) Cross section gray

scale histogram of (I). Y axis represent IF staining intensity. X axis represents distance (µm) from midline shown as 0µm. AU, arbitrary units. (**K**) PS Annexin-V and PI particle count of stages HH 3 and 4 embryos. (**L**) Wholemount of P35:2A:H2B-GFP (green) electroporated embryo stained for Laminin (magenta). (**M**) As in (L), transverse section at the level indicated by double arrow. (**N**) As in (L), optical sagittal section at the level indicated by arrow. (**O**) Quantification of laminin staining intensity of (M). Arbitrary fluorescent intensity unit (AU) at various distances (µm) from midline. PI, propidium iodide. Lm, laminin.

Fig. S11

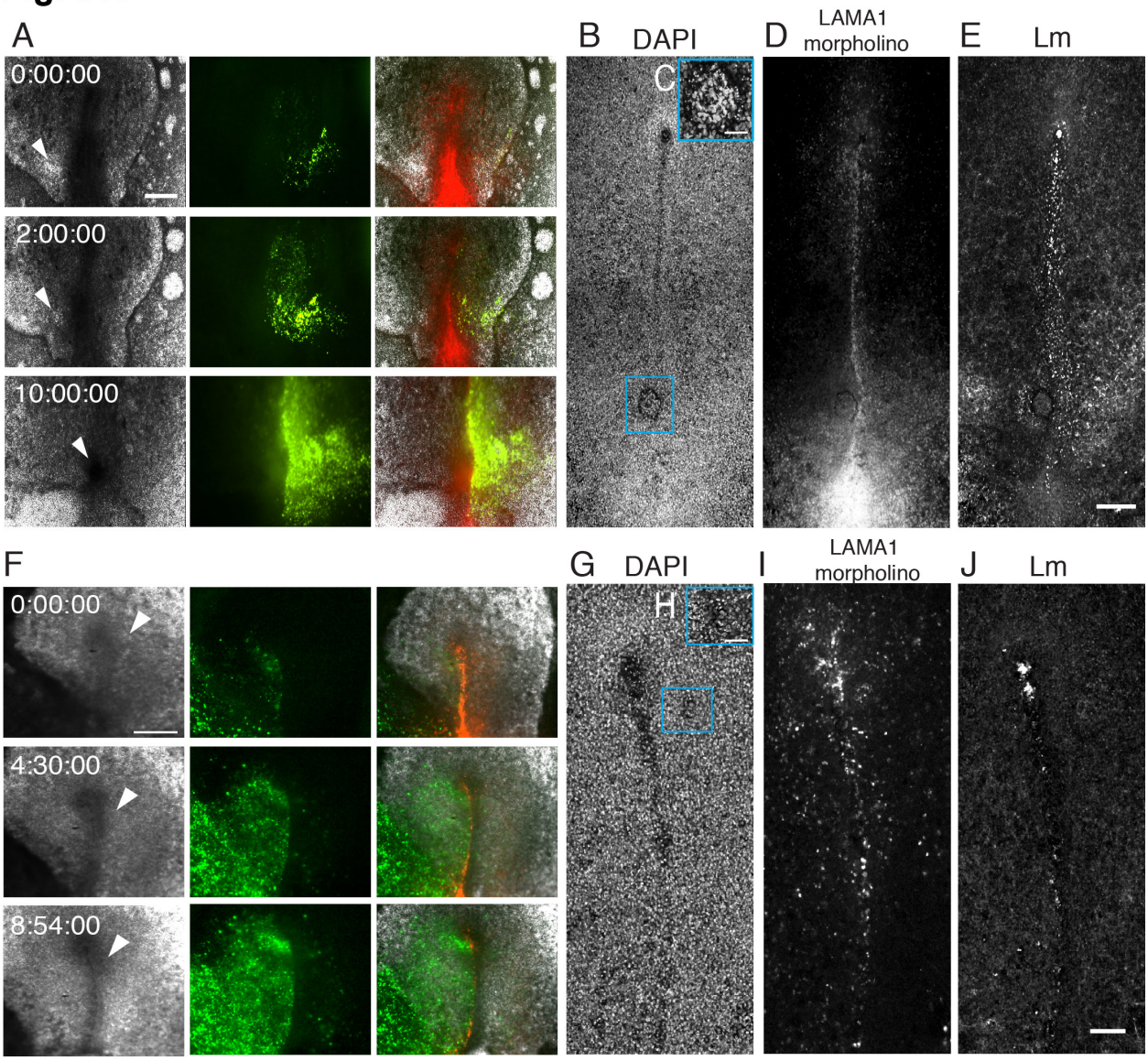

Fig. S11.
Staurosporine induced PCD rescues ipsilateral gastrulation in PS midline ECM deficient embryos. (**A**) Time-lapse images of LAMA1 morpholino (red), Flag:2A:H2B-GFP (green) electroporated embryos, microinjected with staurosporine [0.3 mM]. White arrowheads depict injection site. Scale bar, 500 µm. (**B-E**) Images from time-lapsed embryo from supplemental figure 6A. Scale bar, 200 µm. (**B**) DAPI stain, blue box encloses injection site. (**C**) High magnification DAPI stain of injection site, showing cellular debris. Scale bar, 50 µm. (**D**) LAMA1 morpholino - lissamine stain. (**E**) Laminin IF staining. (**F**) Time-lapse images of LAMA1 morpholino (red), Flag:2A:H2B-GFP (green) electroporated embryos, microinjected with staurosporine vehicle only. White arrowheads depict injection site. Scale bar, 400 µm. (**G-J**) Images from time-lapsed embryo from supplemental figure 6F. Scale bar, 100 µm. (**G**) DAPI stain, blue box encloses injection site. (**H**) High magnification DAPI stain of injection site. Scale bar, 50 µm. (**I**) LAMA1 morpholino - lissamine stain. (**J**) Laminin IF staining.

Fig. S12

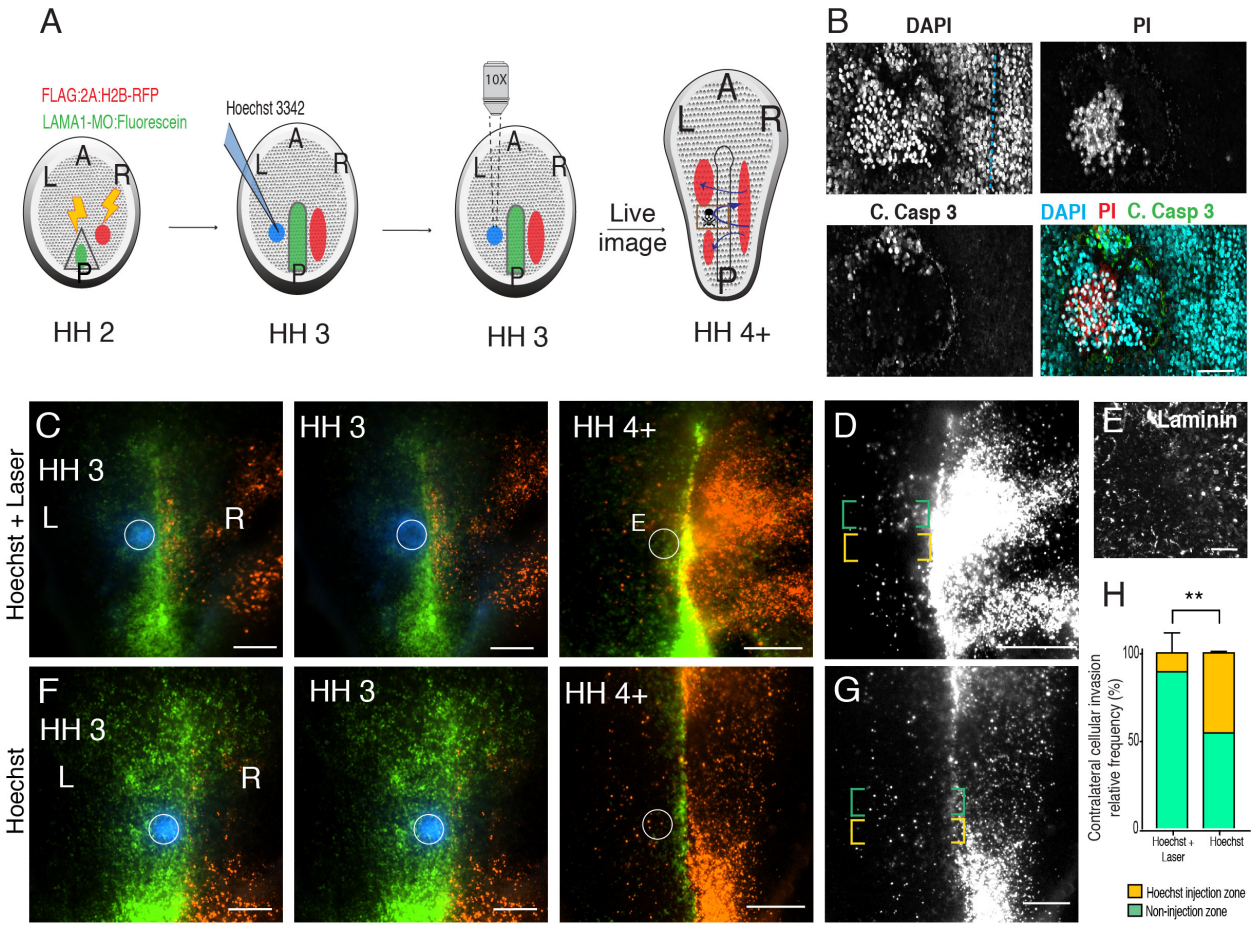

Fig. S12. Hoechst/laser induced PCD rescues ipsilateral gastrulation in in PS midline ECM deficient embryos. (**A**) Scheme of PCD induction via Hoechst 3342 and laser focal illumination. (**B**) High magnification of Hoechst/laser induced PCD showing DAPI, PI staining and cleaved caspase (C. Case 3) IF staining. Dotted line in DAPI panel shows PS midline. Scale bar, 100 µm. (**C**) (First panel) Stage HH 3 fluorescein-tagged LAMA1 morpholino at PS with Flag:2A:H2B-RFP on the right side and Hoechst 3342 injection on the left side, marked by dotted circle. (Second panel) Same embryo from first panel, after 2-3 minutes of laser focal illumination directed to Hoechst injection site. (Third panel) Stage HH 4+ embryo Flag:2A:H2B-RFP cells have invaded the contralateral side with the exception of the Hoechst 3342/laser focal illuminated site, marked by dotted circle. Scale bar, 200 µm. (**D**) Stage HH 4 embryo Flag:2A:H2B-RFP cells in white, yellow brackets show Hoechst 3342/laser focal illuminated site. Green brackets show area immediately anterior to Hoechst 3342/laser focal illuminated site. Scale bar, 200 µm. (**E**) Stage HH 4+ embryo laminin IF staining from Fig. S10 C third panel. Scale bar, 50 µm. (**F**) (First panel) Stage HH 3 fluorescein-tagged LAMA1 morpholino at PS with Flag:2A:H2B-RFP on the right side and Hoechst 3342 injection on the left side, marked by dotted circle. (Second panel) Same embryo from first panel, after 0 minutes of laser focal illumination directed to Hoechst injection site. (Third panel) Stage HH 4+ embryo Flag:2A:H2B-RFP cells have invaded the contralateral side including the Hoechst 3342 site, marked by dotted circle. Scale bar, 200 µm. (**G**) Stage HH 4+ embryo Flag:2A:H2B-RFP cells in white, yellow brackets show Hoechst 3342 site. Green brackets show area immediately anterior to Hoechst 3342 site. Scale bar, 200 µm. (**H**) Contralateral cellular ingression quantification in Hoechst 3342/laser focal illuminated and Hoechst 3342 injected embryos. In Hoechst 3342/laser focal illuminated embryos 10.67% of cells invaded the contralateral side at the injected zone compared to 89.33% of cells anterior to the injection site. In Hoechst 3342 injected only embryos, contralateral ingression was 45.40% and 54.60% at the injection site and anterior to it, respectively (p=0.0055, n=3).

Table S1.

Gene name and Clone number purchased at Source Bioscience used as probes for ISH.

Gene	Clone number
PDGFRA	Clone WTSIp6101F12203Q
EGFL7	Clone WTSIp6101J06613Q
FGF1	Clone WTSIp6101L16774Q
FGFR1	Clone WTSIp6101N18803Q
SEMA3A	Clone WTSIp6101D12687Q
PLXNB1	Clone WTSIp6101P09890Q
MMP15	Clone WTSIp6101J23460Q
BMP7	Clone WTSIp6101D05683Q
EPHB3	Clone WTSIp6101E05478Q
EFNB1	Clone WTSIp6101G131027Q

Movie S1.

Embryo bilaterally electroporated with Flag:2A:H2B-RFP (red) on the left side and Flag: 2A:H2B-GFP (pseudocolored blue) on the right side. Live imaged at 4X with an epifluorescent microscope from stage HH 3 to HH 4+. First panel with cellular tracks and second panel without tracks. Scale bar, 500 µm.

Movie S2.
Embryo electroporated with membrane-tethered GFP (pseudocolored white) and live imaged at 20X with a multiphoton microscope. An increased number of cellular blebbing occurs as cells approach the PS midline. Scale bar, 30 µm.

Movie S3.
Embryo co-electroporated with Annexin-V (YFP-Sec-A5) reporter (green) and membrane RFP. Live imaged at 20X with an epifluorescent microscope. Midline cell expressing YFP-Sec-A5 reporter and membrane RFP undergoes blebbing, as lateral cells expressing primarily RFP move towards the PS midline. Scale bar, 50 µm.

Movie S4.
Embryo apically labeled with Alexa Fluor 488 Annexin V conjugate staining and live imaged at 10X with an epifluorescent microscope. Imaging from stage HH 2 to HH 4+. Tracks were statistically colored to show the start position along the X axis. X axis length = 1400µm. The PS localized between 600-800µm along the X axis, with the midline residing at approximately 700µm. Y axis length = 1050µm. Scale bar, 100 µm.

Movie S5.

Embryo electroporated with lissamine oligo (green) at the PS and Flag:2A:H2B-GFP (red) at lateral epiblast.. Live imaged at 4X with an epifluorescent microscope from stage HH 3 to HH 4+. GFP (red) labeled WT cells undergo ipsilateral gastrulation. Scale bar, 300 µm

Movie S6.

Embryo electroporated with P35:2A:H2B-GFP at the PS and Flag:2A:H2B-RFP at lateral epiblast. Live imaged at 4X with an epifluorescent microscope from stage HH 3 to HH 4+. RFP labeled WT cells cross the P35 expressing midline to invaded the contralateral side. Scale bar, 500 µm.

Movie S7.

Embryo electroporated with lissamine-tagged LAMA1 morpholino at the PS and H2B-GFP at lateral epiblast. Live imaged at 4X with an epifluorescent microscope from stage HH 3 to HH stage 4+. GFP labeled WT cells cross the LAMA1 expressing midline to invade the contralateral side. Scale bar, 400 µm.

Movie S8.

Embryo electroporated with hMMP-15:2A:mCherry at the PS and H2B-GFP at lateral epiblast. Live imaged at 4X with an epifluorescent microscope from stage HH 3 to HH 4+. GFP labeled WT cells cross the hMMP-15 expressing midline to invaded the contralateral side. Scale bar, 200 μm.

Movie S9.

Embryo electroporated with LAMA1 morpholino at the PS and Flag:2A:H2B-GFP at lateral epiblast. PCD was locally introduced via staurosporine microinjections. Live imaged at 4X then 10X with an epifluorescent microscope from stage HH 3 to HH 4+. Staurosporine induced-PCD suppressed contralateral invasion. While contralateral migration continued in non-injected zones, at ECM-PCD deficient PS regions. Scale bar, 500 μm.

Movie S10.

Embryo electroporated with fluorescein-tagged LAMA1 morpholino at the PS and Flag: 2A:H2B-GFP at lateral epiblast. PCD was locally introduced via Hoechst microinjection and focal illumination. Live imaged at 4X from stage HH 3 to HH 4+. Induced-PCD suppressed contralateral invasion. While contralateral migration continued in non-injected zones, at ECM-PCD deficient PS regions. Scale bar, 200 μm.