Supplementary materials

Table S1. Summary of expression information of Eph and ephrin genes in zebrafish embryos[a].

Genes	Expression during gastrulation		Expression in DFCs	
	Analyzed previously	Analyzed in this study	Reported previously	Results from this study
Epha2a	Yes (Kudoh et al., 2001)	Not analyzed	Not in DFCs	Not in DFCs
Epha2b	Yes (Kudoh et al., 2001)	Yes	Not determined	Not in DFCs
epha3	Yes (Oates et al., 1999)	Not analyzed[b]	Not determined	
epha4a	Yes (Thisse and Thisse, 2005)	Yes	Not determined	Not in DFCs
Epha4b	Not analyzed	Yes		Not in DFCs
epha6	Not analyzed	Not analyzed[c]		
epha7	Yes (Taneja et al., 1996)	Yes	Not determined	Not in DFCs
epha8	Not analyzed	Not analyzed[c]		
ephb1	Not analyzed	Not analyzed[c]		
Ephb2a	Not determined (Veien et al., 2008)	Yes	Not Determined	Not in DFCs
Ephb2b	Not analyzed	Yes		Not in DFCs
ephbB3a	Yes (Thisse and Thisse, 2005)	Yes	Not determined	Low in DFCs
Ephb3b	Yes (Challa and Chatti, 2013)	Failed cloning	Not determined	
Ephb4a	Yes (Thisse et al., 2001)	Yes	Not determined	Not in DFCs
Ephb4b	Yes (Thisse et al., 2001)	Yes	Not determined	High in DFCs
ephb6	Not analyzed	Not analyzed[c]		
Efna1a	Yes ((Brown et al., 2008)	Yes	Not determined	Not in DFCs
Efna1b	Yes (Thisse and Thisse, 2004)	Yes	Not determined	Not in DFCs
efna2	Not detected (Thisse and Thisse, 2005)	Not analyzed	Not detected	

efna3a	Not analyzed	Not analyzed[c]		
Efna3b	Not detected (Thisse and Thisse, 2005)	Not analyzed	Not detected	
efna5a	Not analyzed	Yes		Not in DFCs
efna5b	Not detected (Thisse et al., 2001)	Yes	Not detected	Not in DFCs
efnb1	Not detected (Thisse and Thisse, 2005)	Not analyzed	Not detected	
efnb2a	Yes (Thisse et al., 2001)	Yes	Not Determined	Not in DFCs
efnb2b	Not determined (Chan et al., 2001)	Yes	Not Determined	Not in DFCs
Efnb3a	Not analyzed	Not analyzed[c]		
efnb3b	Not detected (Thisse and Thisse, 2005)	Yes	Not detected	Not in DFCs

[a]Genes whose transcripts are previously reported to be absent during gastrulation were usually not analyzed in this study.

[b]The sequence of this gene could not be found in NCBI database.

[c]These genes were annotated after the completion of this study and are not analyzed in this study.

References

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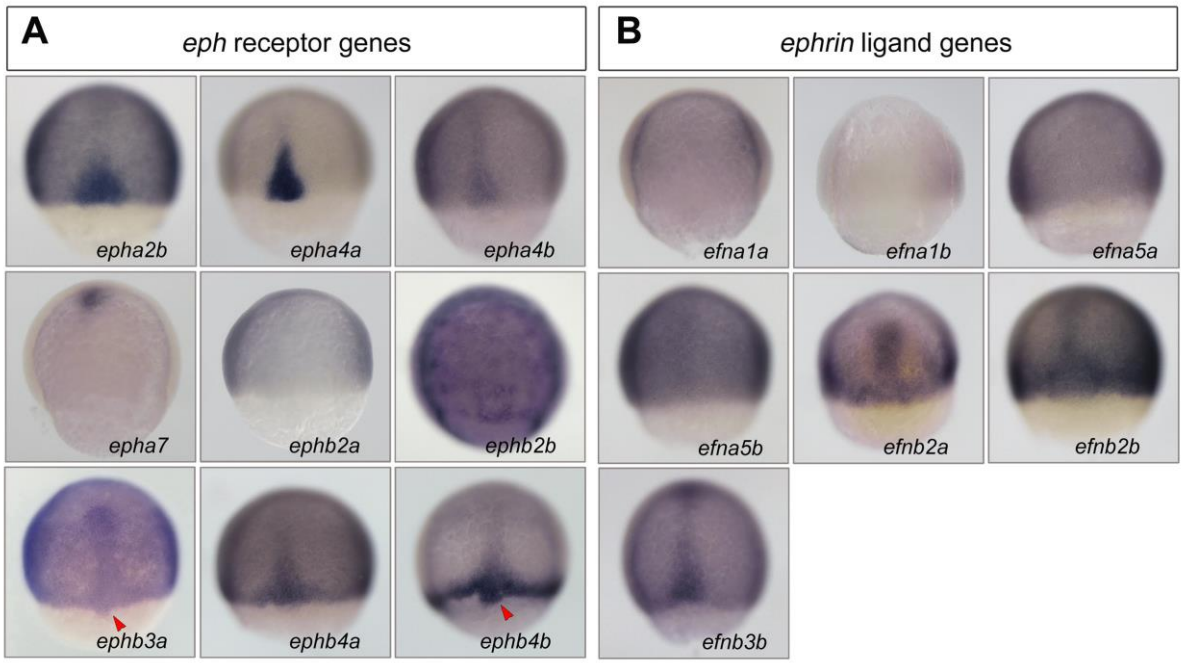


Fig. S1. Expression patterns of Eph receptor and ligand genes in zebrafish gastrulas. Wild-type embryos were collected around 75% ES and probed with indicated gene probes. All embryos were dorsally viewed with animal pole to the top. Arrowheads indicated DFCs.

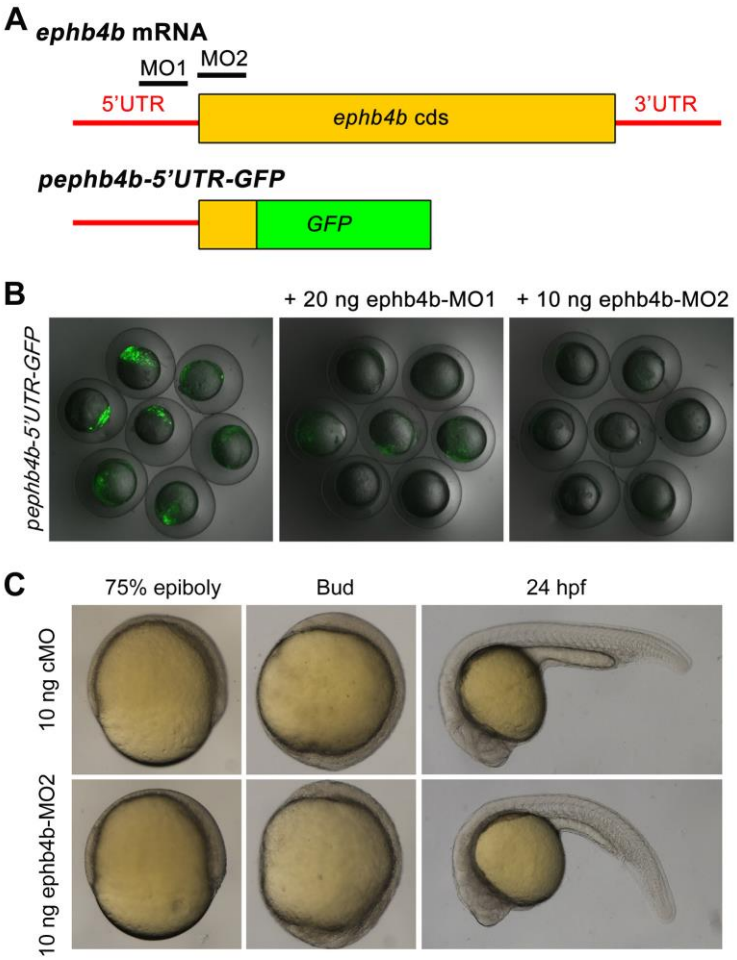


Fig. S2. Effectiveness test of *ephb4b* morpholinos. (A) Illustration of *ephb4b* mRNA and reporter structures. The approximate positions of two morpholinos, MO1 and MO2, were indicated. (B) Effect of MO injection on the reporter expression. 75 pg of the reporter plasmid *pephb4b-5'UTR-GFP* was injected alone or together with 20 ng ephb4b-MO1 or 10 ng ephb4b-MO2 into one-cell stage embryos. The injected embryos were observed at midgastrulation stages for GFP expression under a dissect fluorescence microscope. (C) Effect of ephb4b-MO2 on development. Embryos at the one-cell stage were injected with 10 ng ephb4b-MO2 or 10 ng cMO (standard control MO). The live embryos were observed at later stages as indicated. All embryos were orientated laterally. No severe abnormalities were found in ephb4b-MO2 injected embryos.

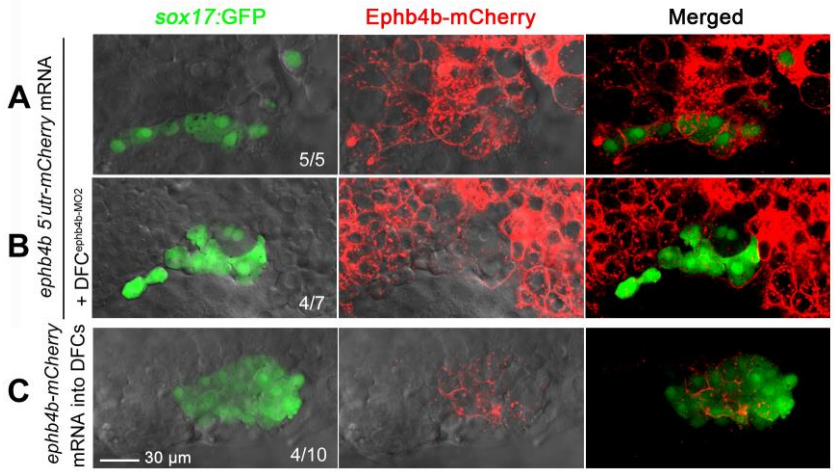


Fig. S3. Efficiency of mRNA and morpholino injection at midblastulation stages. (A,B) Injection of 250 pg *ephb4b 5'utr-mCherry* fusion mRNA, which contained 5' untranslated sequence including the ephb4b-MO2 binding site, into one-cell *Tg(sox17:GFP)* transgenic embryos led to global expression of mCherry including DFCs. When *Tg(sox17:GFP)* embryos were first injected with 250 pg *ephb4b 5'utr-mCherry* mRNA at the one-cell stage and then injected with 10 ng ephb4b-MO2 at the 512-cell stage, mCherry in GFP-positive DFCs was drastically reduced while it was retained in surrounding cells, suggesting that ephb4b-MO2 was restricted to DFCs. (C) Injection of 400 pg *ephb4b-mCherry* mRNA, which contains full-length *ephb4b* coding sequence, into 256-cell to 512-cell *Tg(sox17:GFP)* embryos led to mCherry expression specifically in DFCs. All of injected embryos were observed by confocal microscopy focusing on the DFC region at the 75% epiboly stage. The ratio of embryos with representative pattern was indicated. Scale bar: 30 μm.

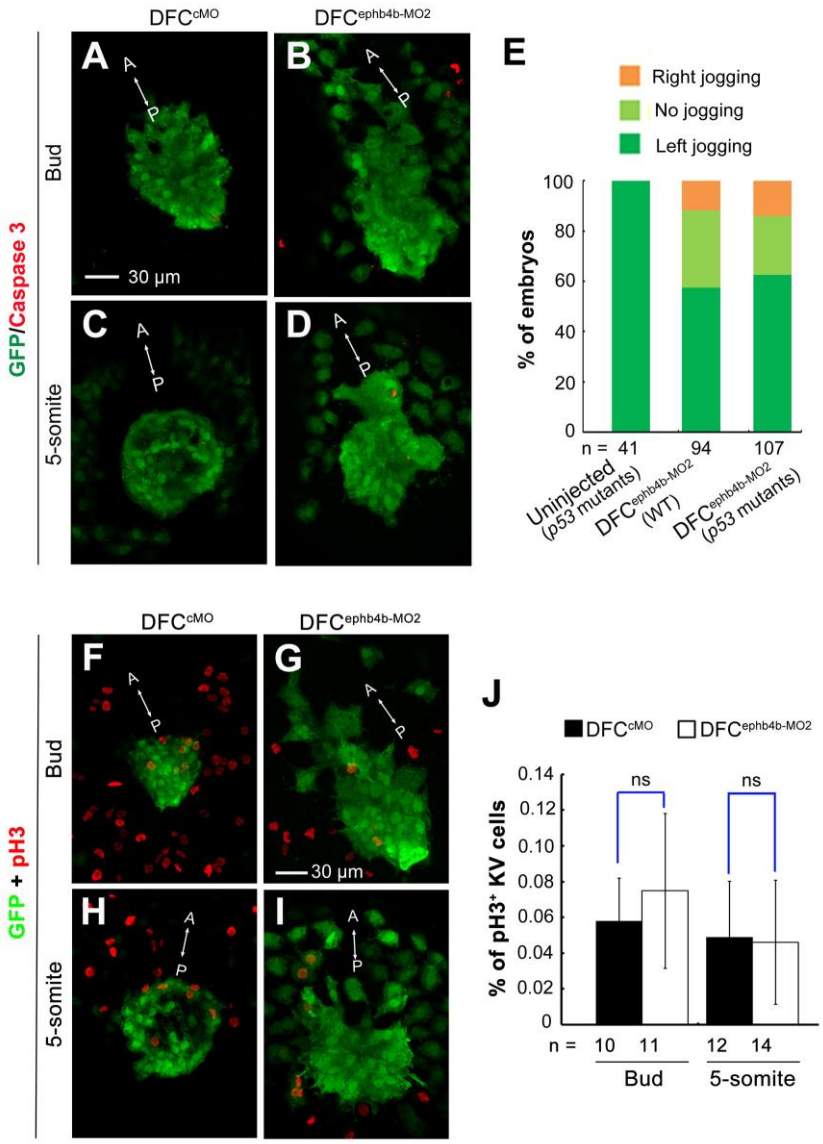


Fig. S4. Effect of *ephb4b* knockdown on DFCs clustering and KV are unrelated to apoptosis or defective cell proliferation. (A-D) Effect of *ephb4b* knockdown on apoptosis of DFCs and KV cells. 10 ng cMO or ephb4b-MO2 was injected into the yolk of *Tg (sox17: GFP)* transgenic embryos at the 512-cell stage and harvested at the bud stage (A,B) and the 5-somite stage (C,D) for immunostaining using anti-GFP and anti-(active) Caspase 3 antibodies. The signal of active Caspase 3 was hardly detected in KV of either wild-type embryos (A,C) or *ephb4b* morphants (B,D). (E) Effect of *ephb4b* knockdown on heart jogging in wild-type or *tp53*$^{-/-}$ mutant embryos. 10 ng ephb4b-MO2 was injected into wild-type (WT) or *p53*$^{-/-}$ mutant embryos at the one-cell stage. The embryos were collected at 28 hpf for probing *cmlc2* expression in the heart. The ratio of embryos for each category of heart jogging was calculated. (F-J) Effect of *ephb4b* knockdown on DFCs and KV cells proliferation. 10 ng

cMO or ephb4b-MO2 was injected into the yolk of *Tg (sox17: GFP)* transgenic embryos at the 512-cell stage and harvested at the bud stage (F,G) and the 5-somite stage (H,I) for immunostaining using anti-GFP and anti-pH3 antibodies. The immunostained embryos were observed by confocal microscopy. The anterior (A)-posterior (P) axis was indicated. The proportion of pH3-positive KV cells was calculated (J). n, the number of observed embryos. ns, statistically non-significant at $P > 0.05\%$. Scale bar: 30 μm.

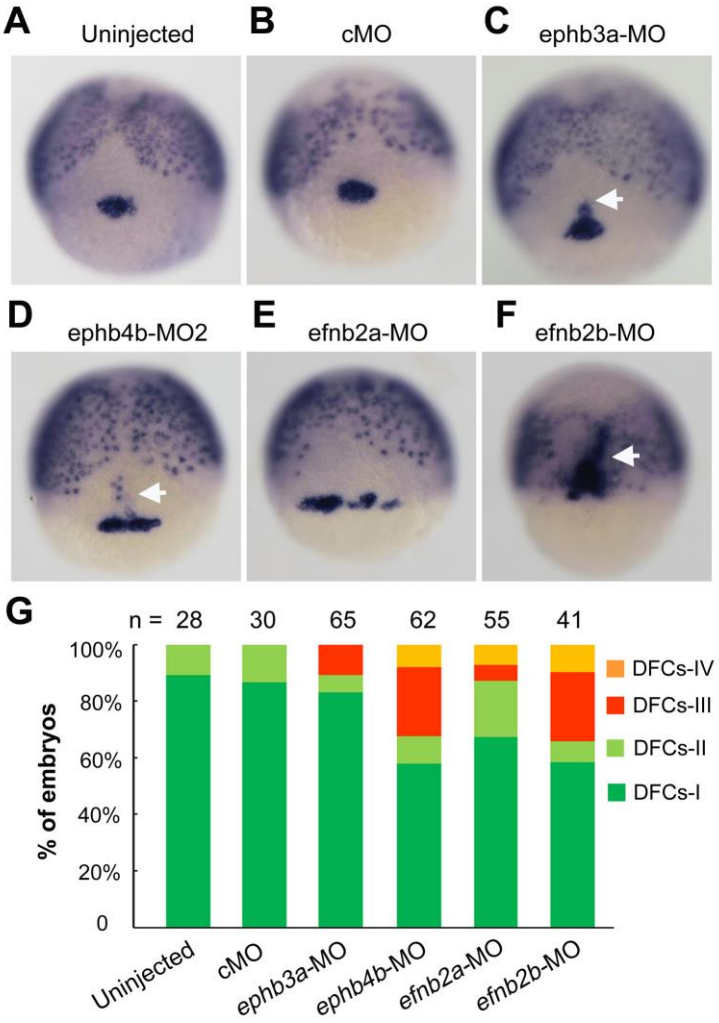


Fig. S5. Comparison of knockdown effects of different *eph*/*ephrin* genes on DFCs aggregation. (A-F) Representative DFCs patterns in morphants. Embryos were injected with individual morpholinos at the one-cell stage and fixed at the 75% epiboly stage for probing *sox17* expression. Embryos were dorsally viewed. White arrows indicated presumably involuted DFCs-derived cells. MO doses: cMO, 10 ng; ephb3a-MO, 10 ng; ephb4b-MO2, 10 ng; efnb2a-MO, 10 ng; efnb2b-MO, 7 ng. (G)The ratios of embryos with different DFCs patterns (See Fig. 2N) were shown. n, the number of observed embryos.

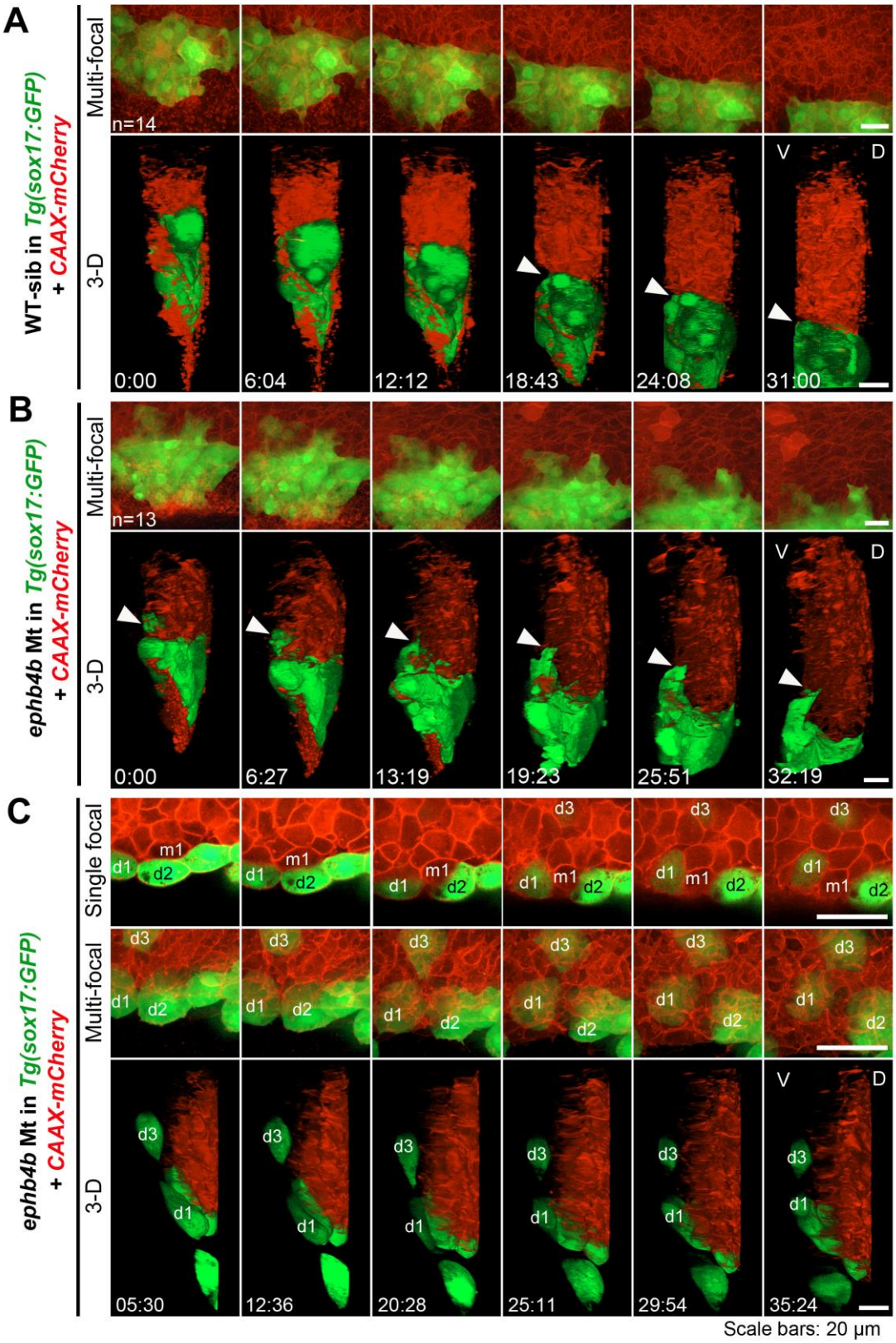


Fig. S6. DFCs involute or migrate away from the original cluster in *ephb4b* mutants.

Embryos derived from crosses of *ephb4b*$^{+/-}$*;Tg(sox17:GFP)* fish were injected with

CAAX-mCherry mRNA at the one-cell stage to label cell membrane and observed by confocal microscopy from 60% ES to about 80% ES. At the end of observation, each embryo was genotyped. (A) Time-lapse dynamic observation of DFCs (green) in a WT sibling embryo in a time window indicated. Upper, multi-focal plane image series in dorsal view; lower, 3-D reconstituted image series in lateral view (same for (B) and (C)). The interface between DFCs and axial mesodermal precursors was indicated by an arrowhead. (B) Time-lapse dynamic observation of DFCs in a mutant embryo. The arrowhead indicated an involuting DFC. (C) Time-lapse dynamic observation of DFCs in a mutant embryo showing run-away and involution of DFCs. Three recognizable DFCs (d1-d3) and one axial mesodermal precursor were labeled if possible. Note that the cell m1 could not be recognized in the shown multi-focal and 3-D images. The cells d1 and d2 was eventually separated by m1. Also see Movie S3 and S4.

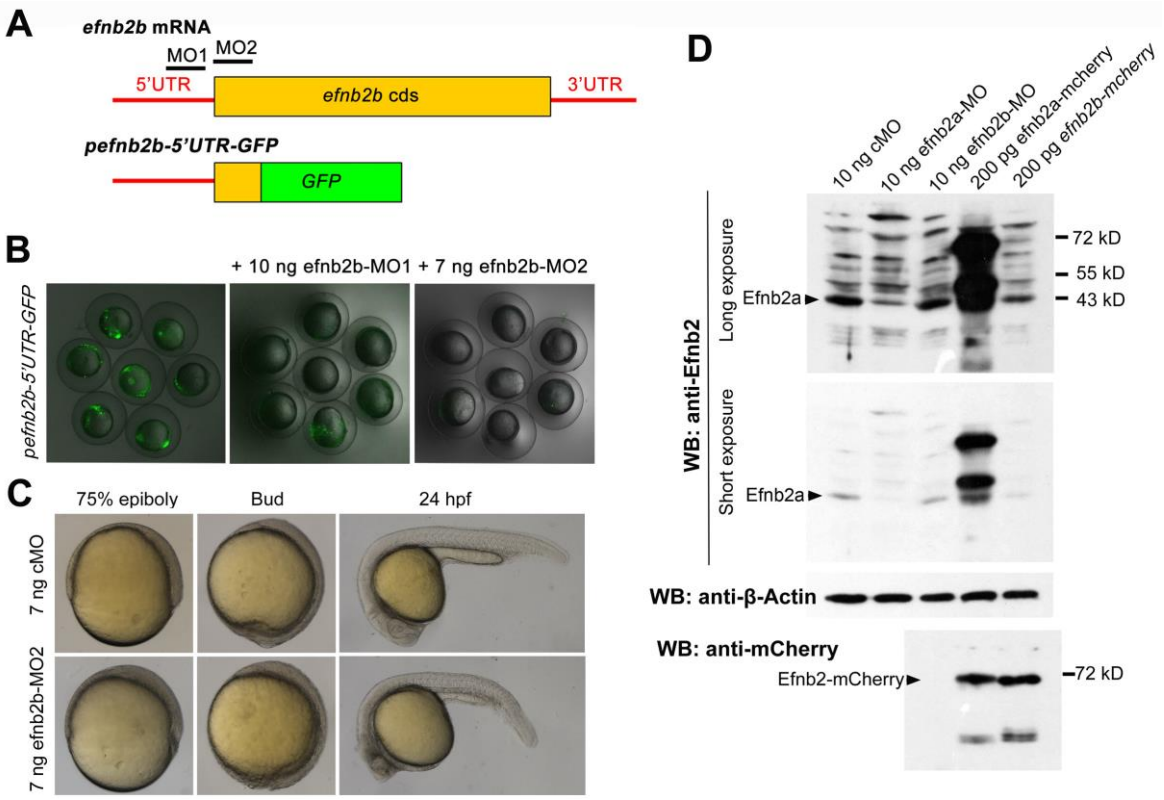


Fig. S7. Effectiveness test of *efnb2b* and *efnb2a* morpholinos. (A) Illustration of *efnb2b mRNA* and reporter structures. The approximate positions of two morpholinos, MO1 and MO2, were indicated. (B) Effect of MO injection on the reporter expression. 75 pg of the reporter plasmid *pefnb2b-5'UTR-GFP* was injected alone or together with 10 ng efnb2b-MO1 or 7 ng efnb2b-MO2 into one-cell stage embryos. The injected embryos were observed at midgastrulation stages for GFP expression under a dissect fluorescence microscope. (C) Effect of efnb2b-MO2 on development. Embryos at the one-cell stage were injected with 7 ng efnb2b-MO2 or 7 ng cMO. The live embryos were observed at later stages as indicated. All embryos were orientated laterally. Embryos injected with efnb2b-MO developed slower than those injected with cMO. (D) Effect of *efnb2a* knockdown on Efnb2a protein level. Embryos were injected with a morpholino or mRNA species at the one-cell stage and harvested at the 60% epiboly stage for Western blotting using different antibodies. Note that anti-Efnb2 appears to only recognize Efnb2a protein.

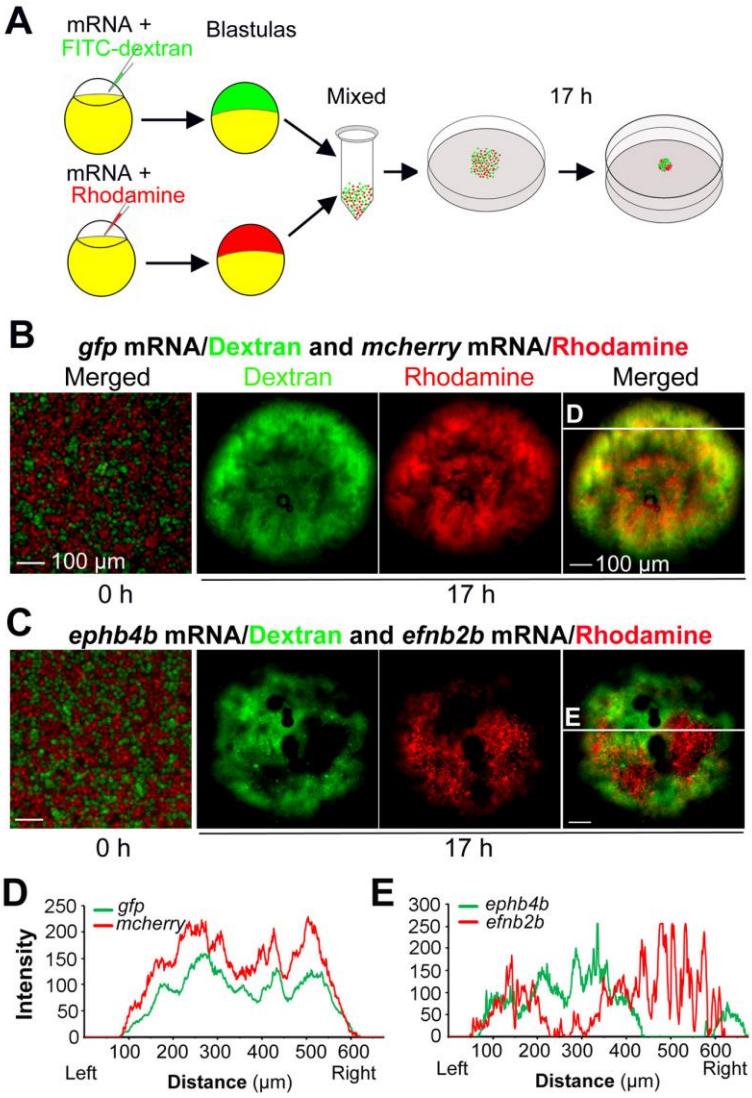


Fig. S8. Ephb4b-expressing cells and efnb2b-expressing cells are mutually repulsive. (A) Scheme of hanging drop cell culture experiment. (B-E) Distribution of differently treated cells in hanging culture. Embryos were injected with indicated mRNA species and dye. For each mix, 5 drops were individually incubated. And three independent experiments were performed. The showed were examples. Differently labeled cells were well intermingled at the initial time-point (0 h) of incubation. The red and green fluorescence intensities at different positions along the line shown in (B) and (C) were depicted in (D) and (E) respectively. Note that two curves in the control group were in parallel while two curves representing *ephb4b*- or *efnb2b*-expressing cells were mutually exclusive. Scale bars: 100 μm.

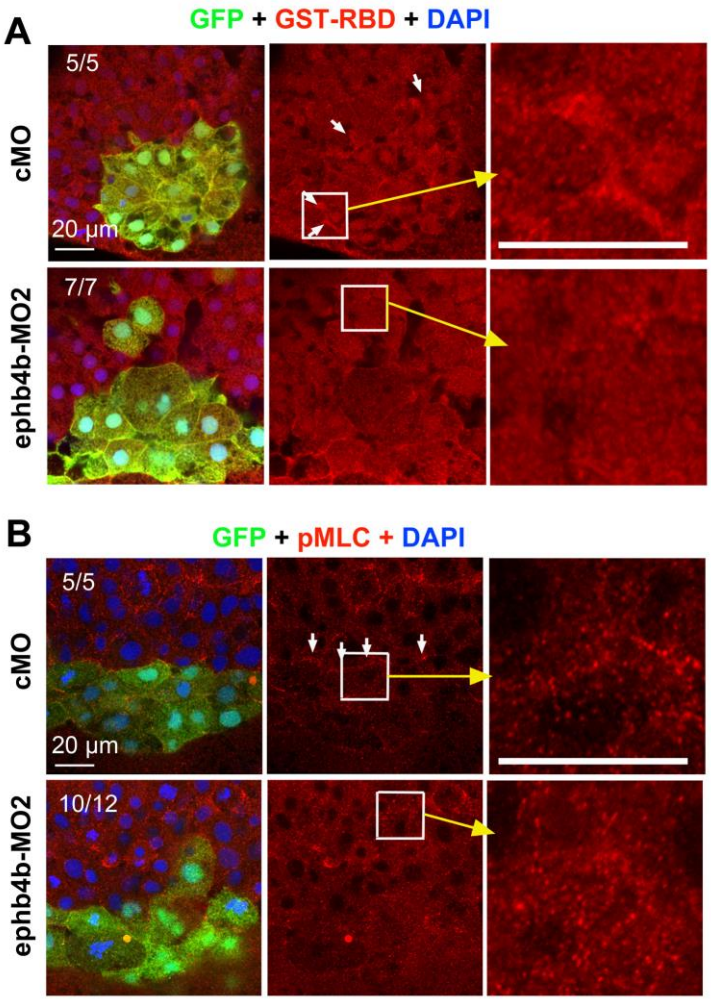
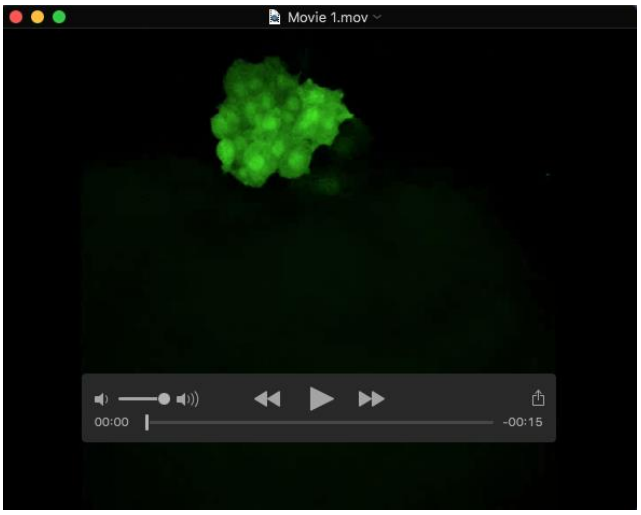
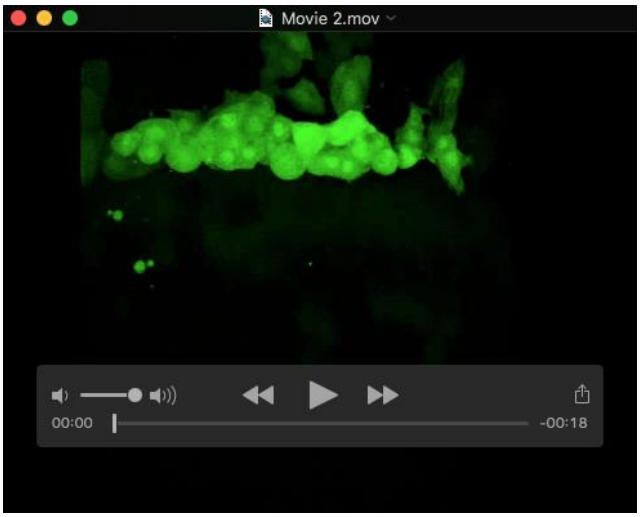


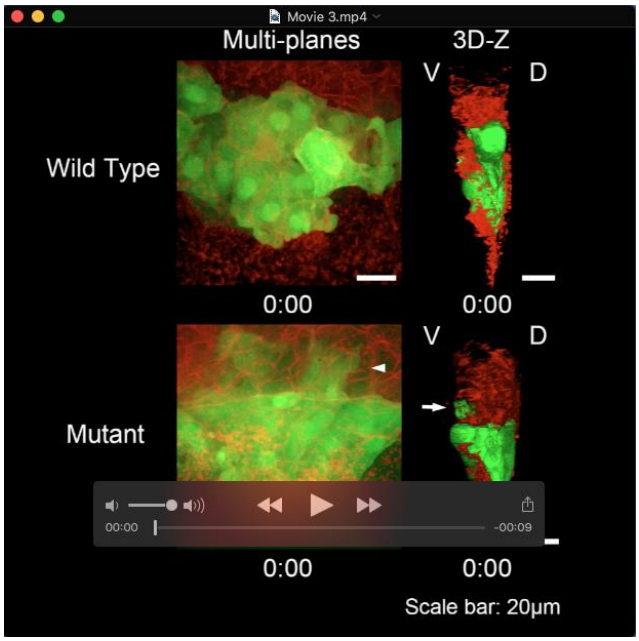
Fig. S9. *ephb4b* requirement for RhoA activation and MLC2 phosphorylation at the DFCs boundary. (A) *ephb4b* requirement for RhoA activation at the DFCs boundary. 100 pg *GST-rhotekin-RBD* mRNA was injected into *Tg(sox17:GFP)* embryos at the one-cell stage to label active RhoA (GTP-RhoA), followed by injection with 10 ng cMO or ephb4b-MO2 injection. Embryos were collected at 75% ES for immunofluorescence staining. GST-Rhotekin-RBD (red) protein was enriched at the DFCs (green) boundary (indicated by arrows) in control embryos but not in *ephb4b* morphants. The ratio of embryos with the representative pattern was indicated. (B) *ephb4b* requirement for MLC2 phosphorylation at the DFCs boundary. *Tg(sox17:GFP)* embryos were injected with 10 cMO or ephb4b-MO2 at the one-cell stage and collected at 75% ES for immunofluorescence with anti-p-MLC2 (red) and anti-GFP antibodies.



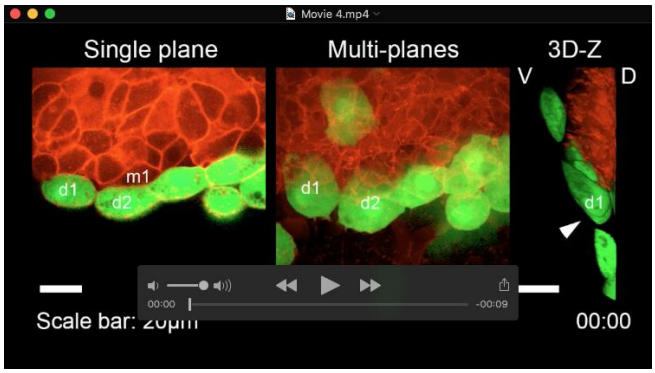
Movie 1. Vegetal/posterior movement of DFCs in a control embryo during gastrulation. *Tg(sox17:GFP)* transgenic embryos were injected with 10 ng cMO at the 512-cell stage. A live embryo was embedded and observed dorsally starting at the 60% epiboly stage by confocal microscopy with a focus on the DFCs region. Each frame consisted of 30 planes from z-stacks.



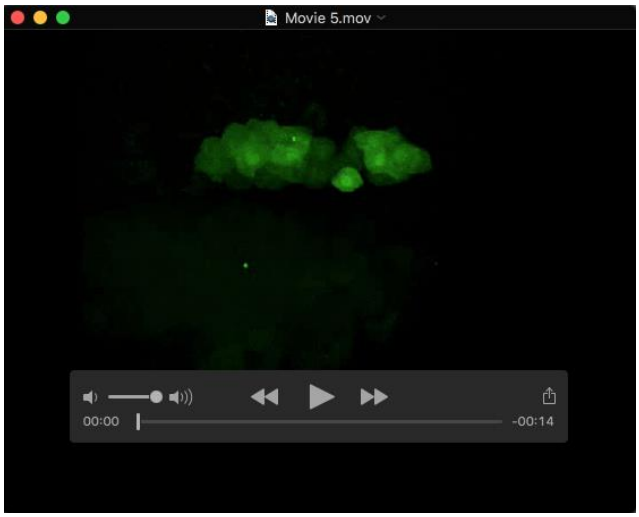
Movie 2. Vegetal/posterior movement of DFCs in an ephb4b-MO2 injected embryo during gastrulation. *Tg(sox17:GFP)* transgenic embryos were injected with 10 ng ephb4b-MO2 at the 512-cell stage. A live embryo was embedded and observed dorsally starting at the 60% epiboly stage by confocal microscopy with a focus on the DFCs region. Each frame consisted of 30 planes from z-stacks.



Movie 3. Migration of DFCs in *ephb4b* mutant and WT sibling embryos during gastrulation. Embryos derived from crosses between *ephb4b*$^{+/-}$*;Tg(sox17:GFP)* fish were used and the cell membrane was labeled by CAAX-mCherry (red). Live embryos were embedded and observed dorsally starting at the 60% epiboly stage. The left and right panels showed dorsal and later views of DFCs (green) respectively. Time-points were indicated with starting time as zero. Also see Fig. S6.

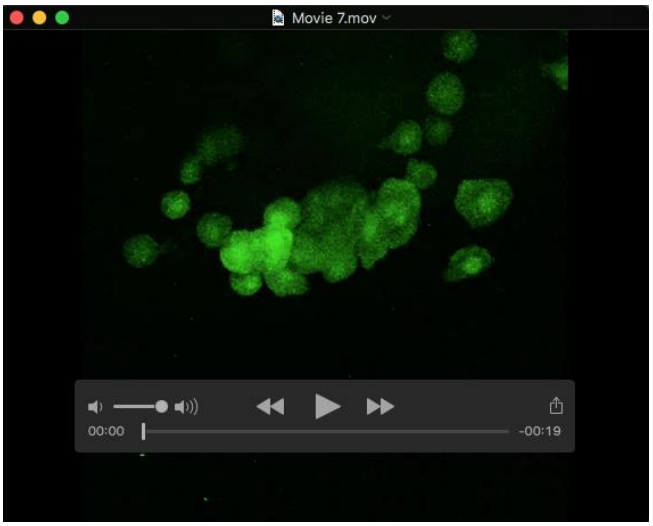


Movie 4. Abnormal migration of DFCs in an *ephb4b* mutant embryo. The embryo derived from crosses between *ephb4b*$^{+/-}$*;Tg(sox17:GFP)* fish. The left and middle window showed dorsal views of DFCs (green) and right window showed the lateral view. Time-points were indicated with starting time as zero. Note that the DFC d1 was involuting and d1 and2 was separated by the axial mesodermal cell m1.

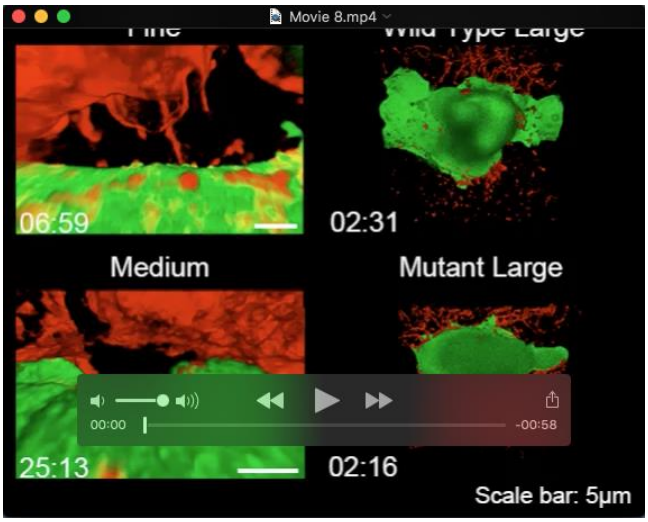


Movie 5. Migration of DFCs in control mRNA-injected embryo during gastrulation. *Tg(sox17:GFP)* transgenic embryos were injected with 250 pg *CAAX-mcherry* mRNA at the one-cell stage. A live embryo was embedded and observed dorsally starting at the 60% epiboly stage by confocal microscopy with a focus on the DFCs region. Each frame consisted of 30 planes from z-stacks.

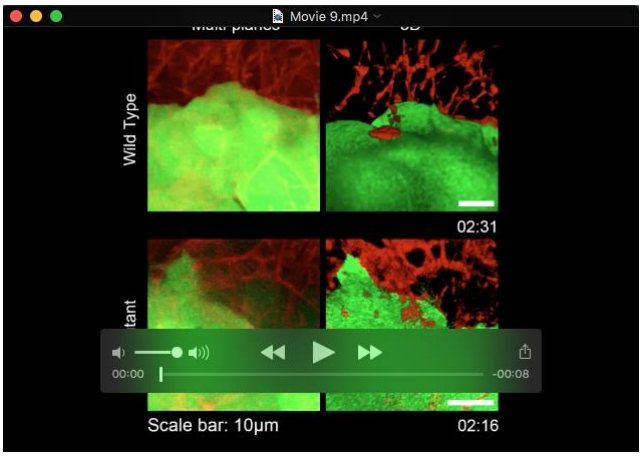
Movie 6. Migration of DFCs in an embryo overexpressing *efnb2b-mcherry* during gastrulation. *Tg(sox17:GFP)* transgenic embryos were injected with 250 pg *efnb2b-mcherry* mRNA at the one-cell stage. A live embryo was embedded and observed dorsally starting at the 60% epiboly stage by confocal microscopy with a focus on the DFCs region. Each frame consisted of 30 planes from z-stacks.



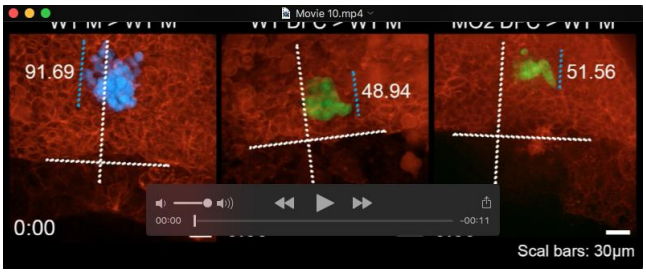
Movie 7. Migration of DFCs in an embryo overexpressing *s-efnb2b-mcherry* during gastrulation. *Tg(sox17:GFP)* transgenic embryos were injected with 250 pg *s-efnb2b-mcherry* mRNA at the one-cell stage. A live embryo was embedded and observed dorsally starting at the 60% epiboly stage by confocal microscopy with a focus on the DFCs region. Each frame consisted of 30 planes from z-stacks.



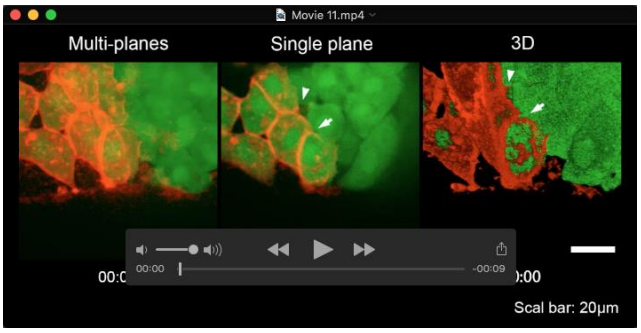
Movie 8. 3-D animation of membrane protrusion processes of DFCs. DFCs and axial mesodermal precursors were labeled by GFP and membrane-anchored mCherry respectively. Each window represented one protrusion of DFCs reconstituted from planes at five time-points as shown in Fig. 6A-D.



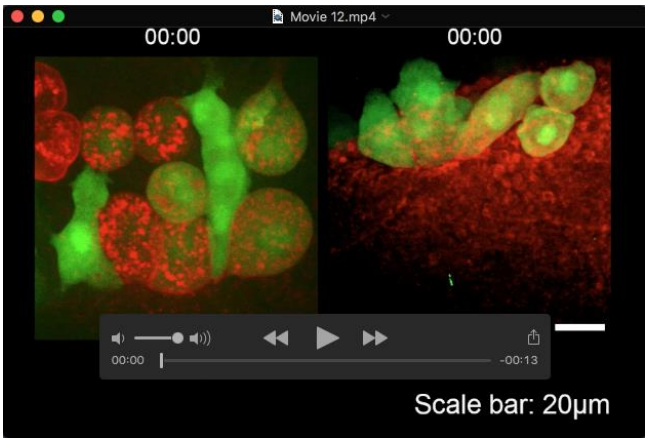
Movie 9. Example of dynamics of large protrusions of DFCs in *ephb4b* mutant and WT sibling embryos. DFCs and axial mesodermal precursors were labeled by GFP and membrane-anchored mCherry respectively. Left panel, multi-focal plane movies; right panel, 3-D reconstituted animation.



Movie 10. Migration behavior of transplanted cells in host embryos. Cerulean-labeled donor cells taken from lateral blastodermal margin were involuting and migrating towards the animal pole when transplanted into lateral margin of host embryo (left window). GFP-labeled donor DFCs did not involute and remained clustering when transplanted into lateral margin of a host embryo (middle window). GFP-labeled donor DFCs derived from an *ephb4b* morphant were migrating towards the animal pole (right window). The bottom broken lines indicated the leading edge of the blastodermal margin. The long vertical line indicated the animal-pole/vegetal pole axis.



Movie 11. Behavior of CAAX-mCherry-expressing DFCs during gastrulation. Several DFCs expressing CAAX-mCherry (red) stayed together with other DFCs (green). The same embryo was viewed dorsally in all windows.



Movie 12. Behavior of efnb2b-mCherry-expressing DFCs during gastrulation. Two embryos with different levels of efnb2b-mCherry were dorsally viewed. Note that DFCs without efnb2b-mCherry actively extended membrane protrusions to efnb2b-mCherry-positive DFCs and the protrusions were retracted once touched the membrane of the latter.