Developmental Cell

Supplemental Information

A Temporal Window for Signal Activation Dictates the Dimensions of a Nodal Signaling Domain

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Supplemental Figures

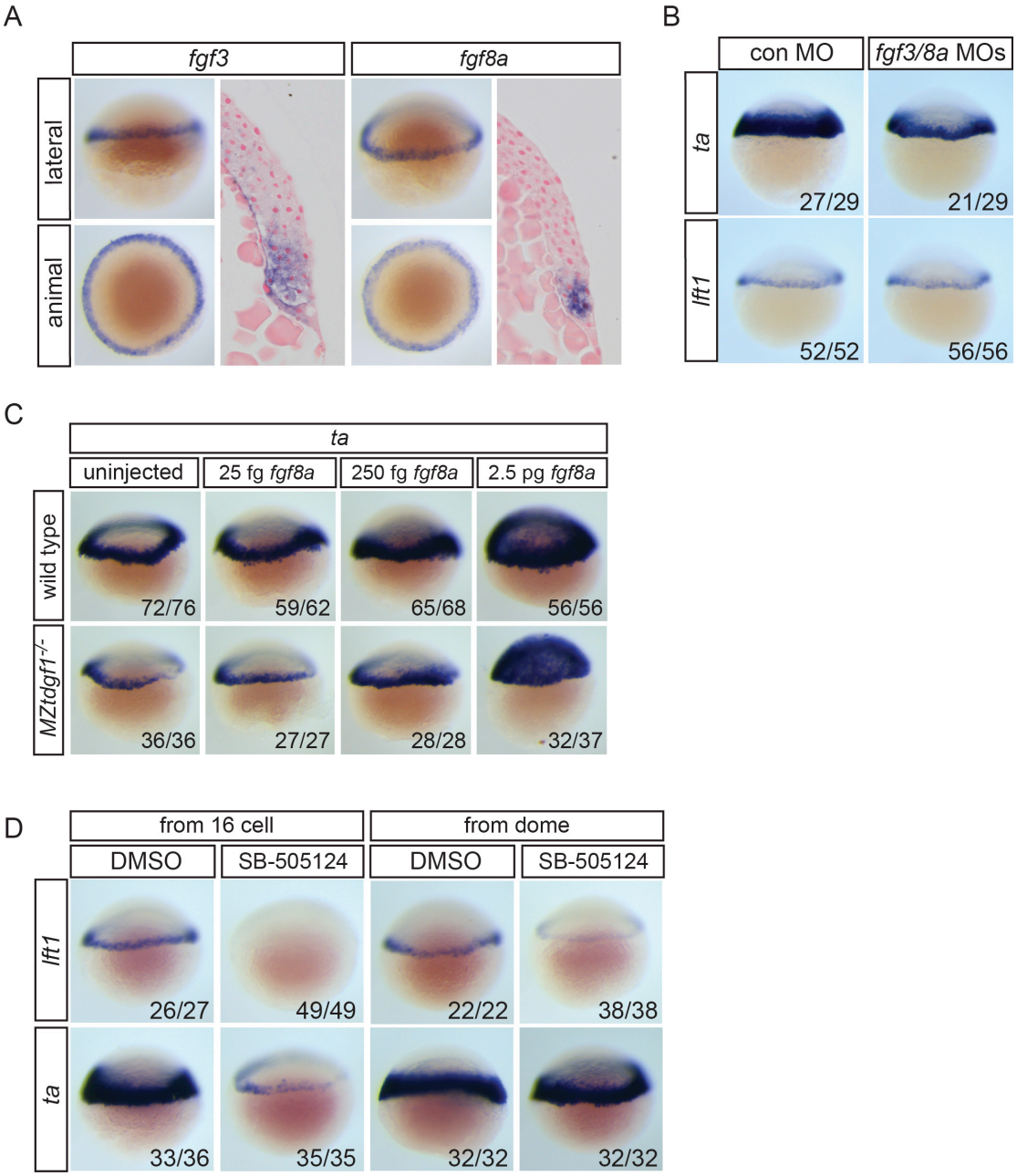

Figure S1, relates to Figure 1. Fgf signaling inhibition and Nodal signaling
(A) Expression of *fgf3* and *fgf8a* in 40% epiboly embryos in the margin shown as WISH and sections.
(B) *ta* and *lft1* expression in 40% epiboly embryos injected with control MO or a combination of *fgf3* and *fgf8a* MOs.
(C) *ta* expression in wild type and maternal-zygotic (MZ) *tdgf1*$^{-/-}$ mutants. *fgf8a* mRNA (25 fg, 250 fg or 2.5 pg) was injected into wild type or MZ*tdgf1*$^{-/-}$ mutant embryos, which were assayed by WISH for *ta* expression at 40% epiboly. *ta* is induced equally well in both backgrounds with 2.5 pg *fgf8a* mRNA.
(D) *lft1* and *ta* expression following inhibition of Nodal signaling with SB-505124 from the 16-cell or dome stage. When inhibited from dome stage, *lft1* expression is severely reduced, whereas *ta* expression is unaffected.

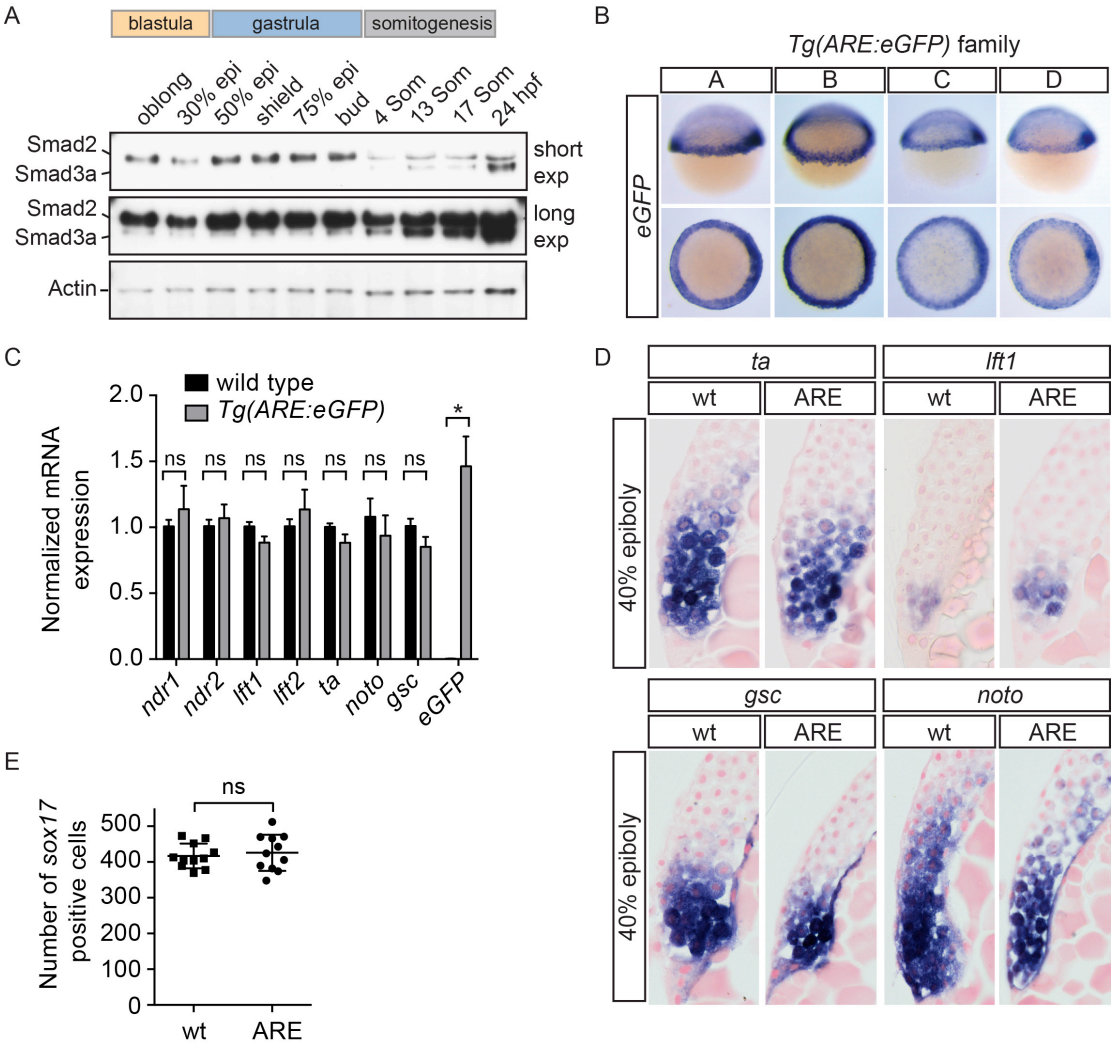

Figure S2, relates to Figure 2. Generation of the *Tg(ARE:eGFP)* transgenic line

(A) Western blot for Smad2 and Smad3a/b during development. Smad3a/b are not expressed at appreciable levels until somitogenesis stages, whereas Smad2 is expressed at all stages shown. A long and short exposure (exp) of the same blot is shown. Actin is a loading control.

(B) WISH for *eGFP* in four *Tg(ARE:eGFP)* lines obtained from independent founders (labeled A–D). Note that individual lines differ slightly in strength of staining and background.

(C) qPCR on pooled 40% epiboly wild type and *Tg(ARE:eGFP)* embryos for the indicated genes normalized to *eef1a1l1*. Means ± SEM are shown (* t-test: $P < 0.05$, n=5). ns, not significant. No significant differences were found for any of the genes tested, except *eGFP*.

(D) Sections of WISH-stained 40% epiboly embryos. No differences were found in the extent of *ta*, *lft1, gsc* or *noto* (*flh*) staining between wild type and *Tg(ARE:eGFP)* embryos. Note that lateral sections are shown for *ta* and *lft1*, whilst dorsal sections are shown for *gsc* and *noto*.

(E) Comparison of the number of *sox17* positive cells in 75% epiboly wild type and *Tg(ARE:eGFP)* embryos. No significant difference was found between the number of endodermal cells between the two lines using a Mann-Whitney U test.

In (D) and (E) wt, wild type; ARE, *Tg(ARE:eGFP)*.

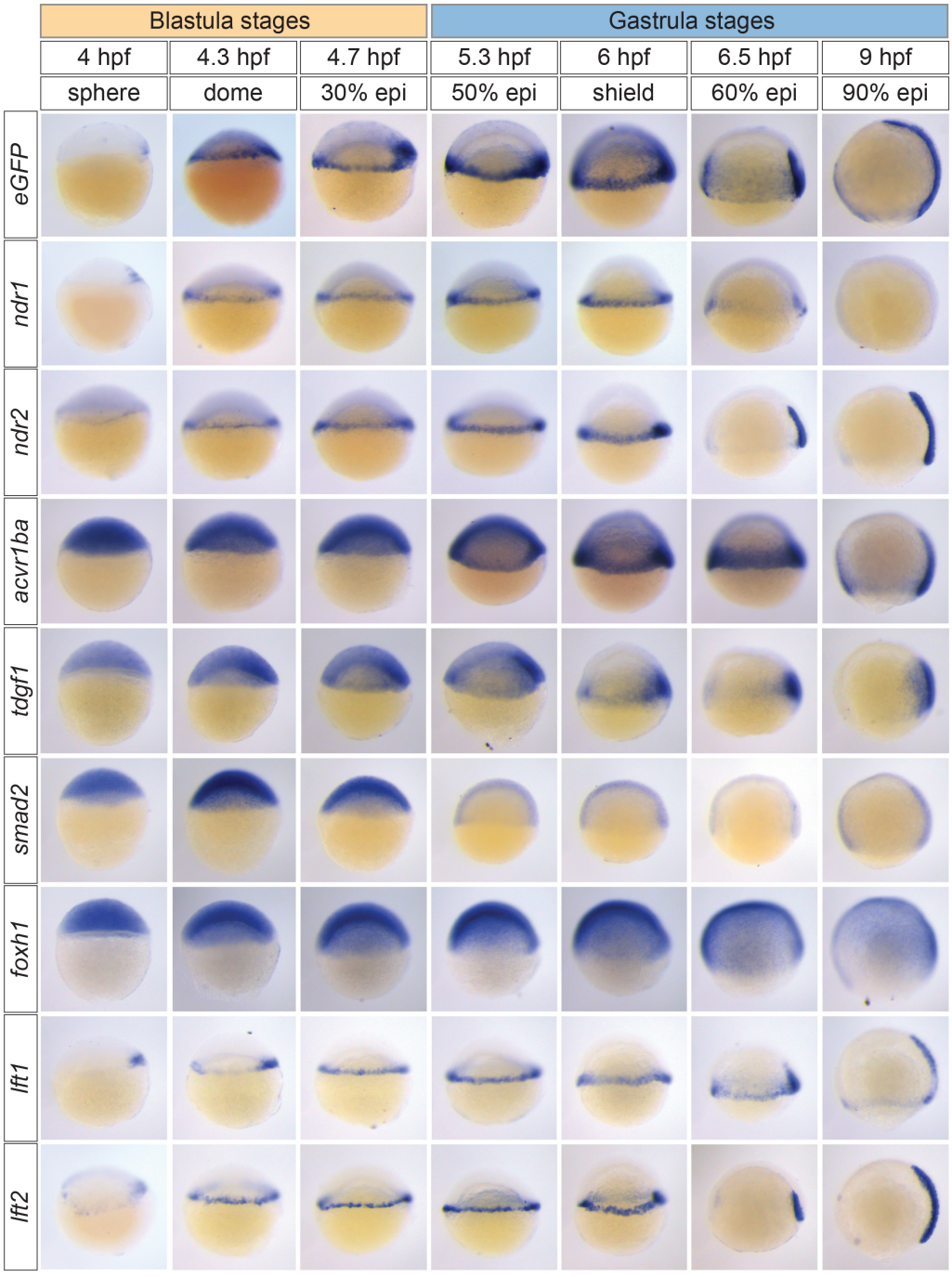

Figure S3, relates to Figure 2. Comparison of Nodal signaling activation in *Tg(ARE:eGFP)* embryos and expression of core components of the Nodal signaling pathway

WISH for *eGFP*, *ndr1* (*sqt*), *ndr2 (cyc)*, *acvr1ba (tar-a)*, *tdgf1 (oep)*, *smad2*, *foxh1 (sur)*, *lft1* and *lft2* in blastula and gastrula stage *Tg(ARE:eGFP)* embryos.

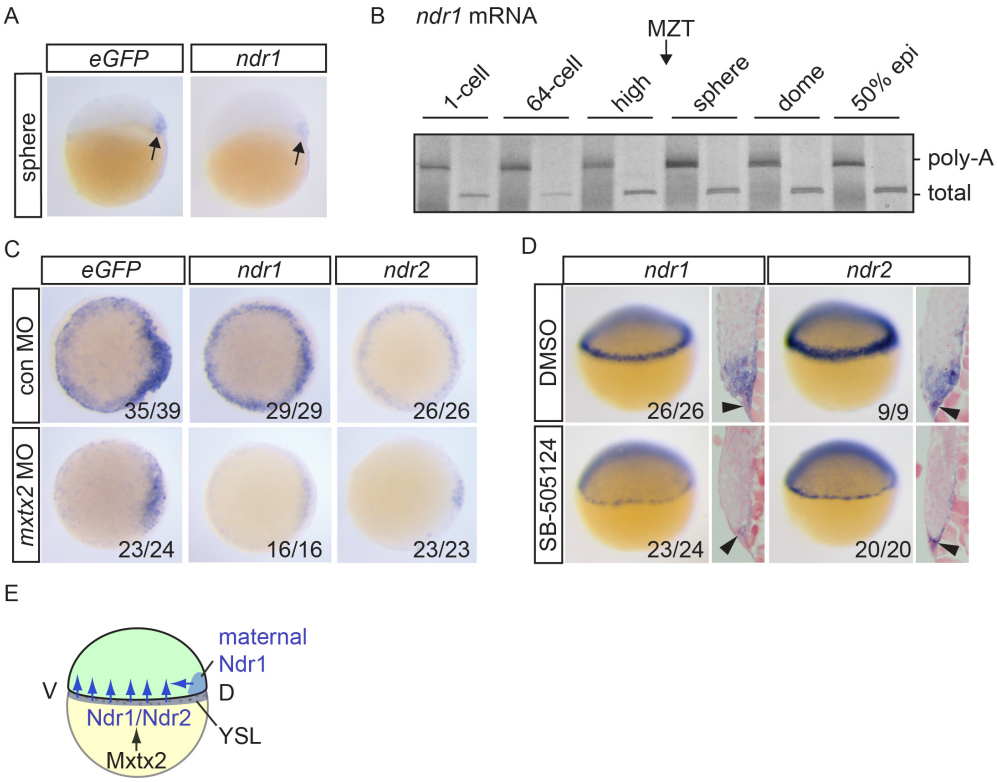

Figure S4, relates to Figure 2. Initiation of Nodal signaling in *Tg(ARE:eGFP)* embryos
(A) *eGFP* and *ndr1* expression in sphere stage embryos. Arrows indicate dorsal expression domains.
(B) ePAT for *ndr1* mRNA. A silver-stained, non-denaturing polyacrylamide gel is shown indicating total and polyadenylated mRNA. Poly-A, polyadenylated; MZT, maternal to zygotic transition.
(C) *eGFP, ndr1* and *ndr2* expression in dome stage *Tg(ARE:eGFP)* embryos injected with control (con) or *mxtx2* MOs. Animal views are shown.
(D) Lateral views and sections of 30-40% epiboly embryos treated with DMSO or SB-505124 at the 32-cell stage and stained for *ndr1* and *ndr2* expression. Black arrowheads indicate expression in the YSL.
(E) Schematic representation showing how Nodal signaling is initiated in zebrafish embryos. For details, see text.

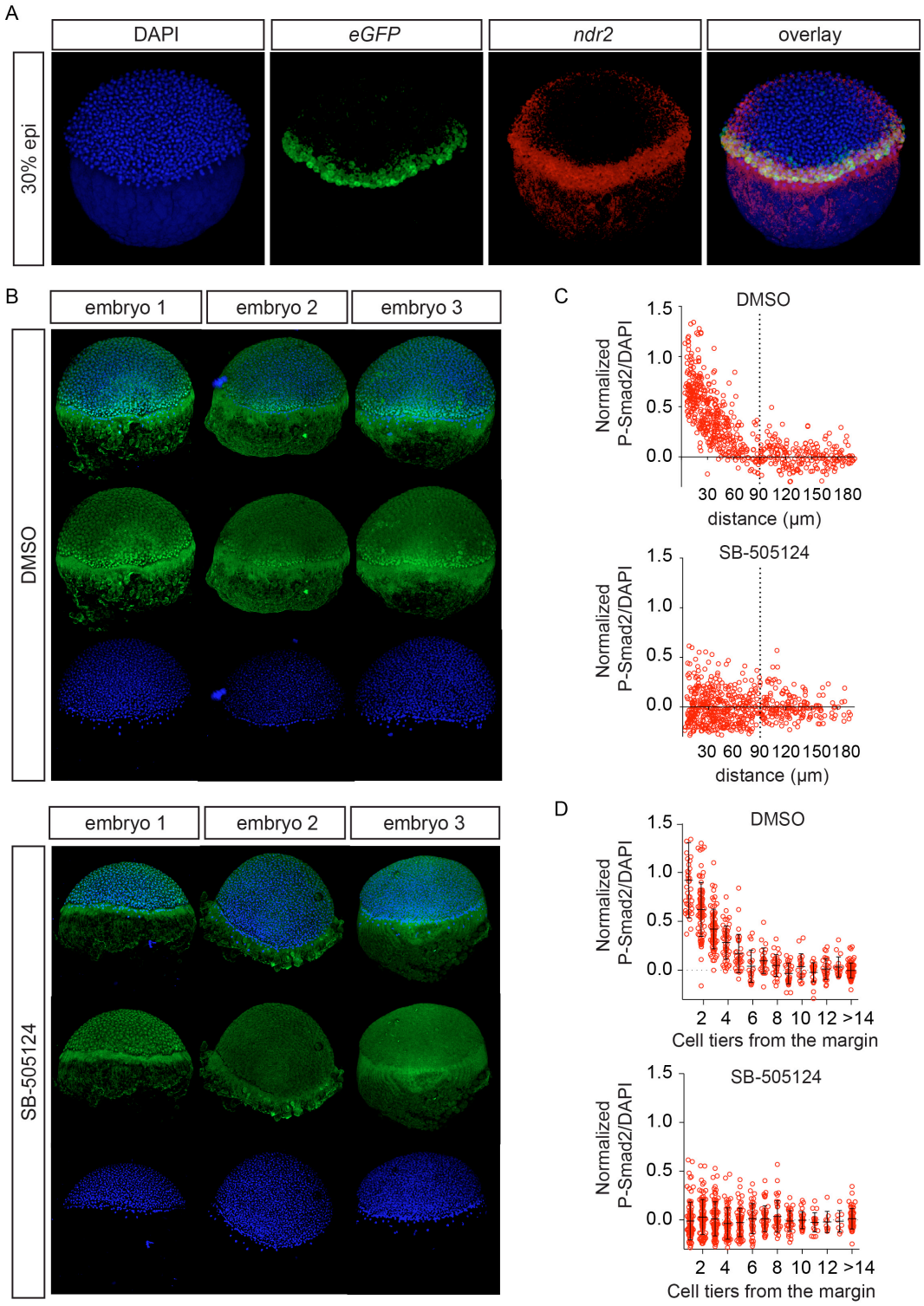

Figure S5, relates to Figure 3. Extent of Nodal signaling in the margin
(A) Double fluorescent WISH for *eGFP* and *ndr2* in 30% epiboly (epi) embryos.
(B) Z-projections of whole mount immunofluorescence for P-Smad2 in 50% epiboly embryos either treated with DMSO or SB-505124, which were used for quantification of Nodal signaling in the margin shown in (C) and (D). DAPI was used as a counter-stain. Note that embryo 3 in both cases are those shown in Figure 1A stained with P-Erk, which was performed as a double whole mount immunofluorescence with the P-Smad2.

(C) Quantification of P-Smad2 staining intensity in DMSO- and SB-505124-treated embryos depicted in (B). Intensities are expressed as P-Smad2/DAPI ratios as a function of distance to the border of the margin. Distance is plotted on the x-axis and the dotted line indicates 90 μm, which corresponds to around six cell tiers.
(D) As in (C) but with data binned in 15 μm bins. The black horizontal line represents the average intensity for each bin ± SD.

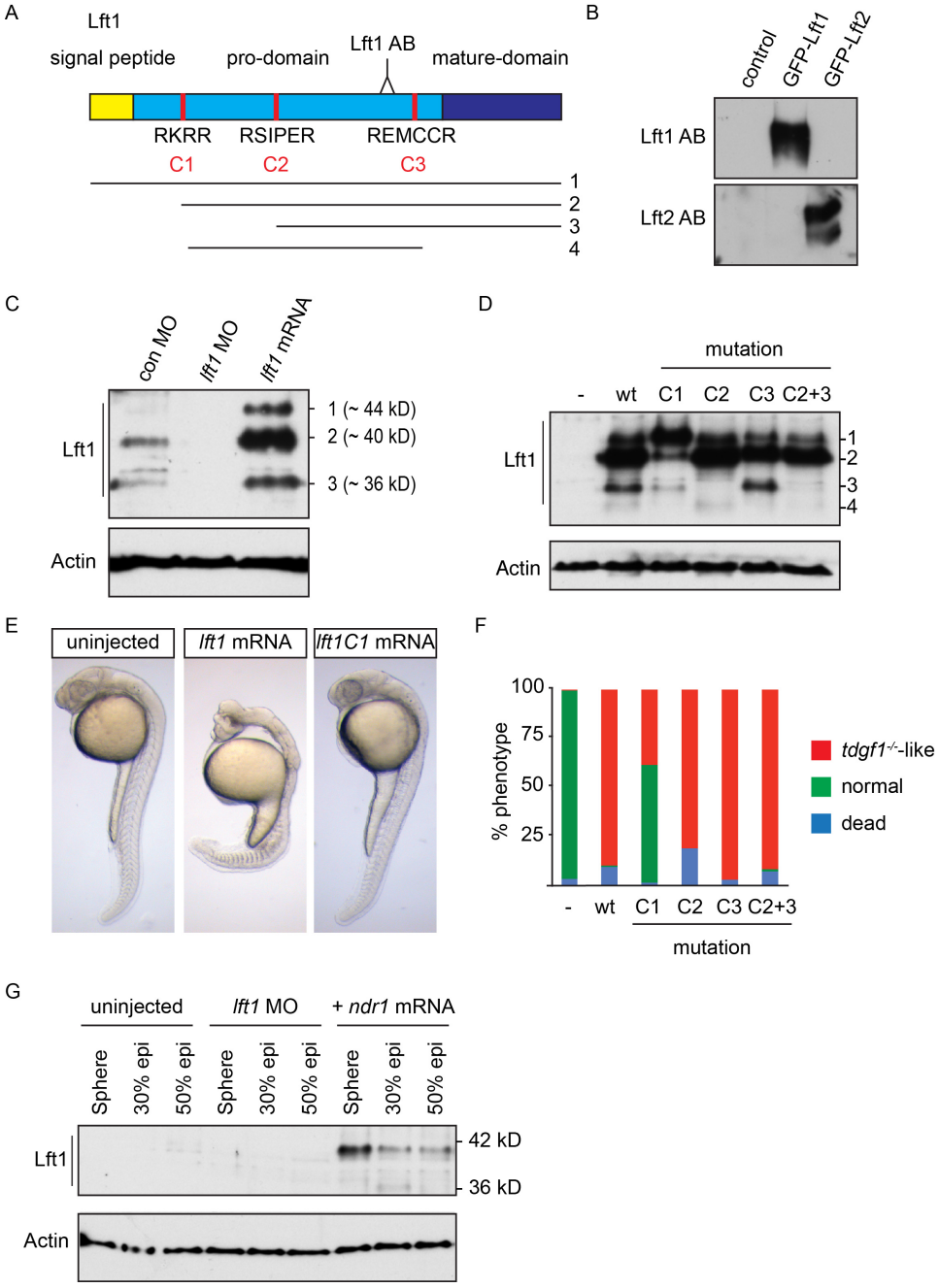

Figure S6, relates to Figure 4. The Lft1 antibody recognizes the 40 kD cleaved and active Lft1 protein

(A) Schematic representation of the zebrafish Lft1 protein. The Lft1 epitope is located in the pro-domain (light blue), just upstream of the mature ligand domain (dark blue). The Lft1 protein contains a signal peptide (gold) and three cleavage sites (C1-C3, red). The cleavage products (1–4) detected by SDS-PAGE are shown. AB, antibody

(B) Western blot for in vitro reticulocyte-translated Lft1 and Lft2 using the cognate antibodies. Note that the Lft1 antibody recognizes GFP-Lft1, but not GFP-Lft2, and vice versa.

(C) Western blot for endogenous and overexpressed Lft1 in pooled 50% epiboly embryo lysates. Overexpressed Lft1 runs as three bands at ~44, ~40, ~36 kD. The second band (~40

kD) corresponds to the main visible endogenous band. These bands are all absent when Lft1 is knocked down using a *lft1* MO.
(D) Western blot for wild type (wt) and mutant overexpressed Lft1 proteins in pooled zebrafish embryos. C1-C3 corresponds to mutated cleavage sites depicted in (A). An additional product (4) is seen in this experiment which results from cleavage at C1 and C3. Actin is a loading control. We conclude that the ~40 kD band arises from cleavage at C1.
(E) Phenotype in 24 hpf wt embryos either uninjected or injected with 5 pg *lft1* or *lft1C1* mutated mRNA. Note that mutating the C1 site leads to rescue of the *lft1* phenotype.
(F) Quantification of phenotypes of 24 hpf embryos, injected with 5 pg wild type or mutant *lft1* mRNAs. Assays were performed on at least 50 embryos.
(G) Western blot for Lft1 in wild type embryos either uninjected or injected with *lft1* MOs or *ndr1* mRNA. Note that both the 40 kD and 36 kD bands are visible in *ndr1*-injected embryos and that the 40 kD band is barely visible in 50% epiboly uninjected embryos.

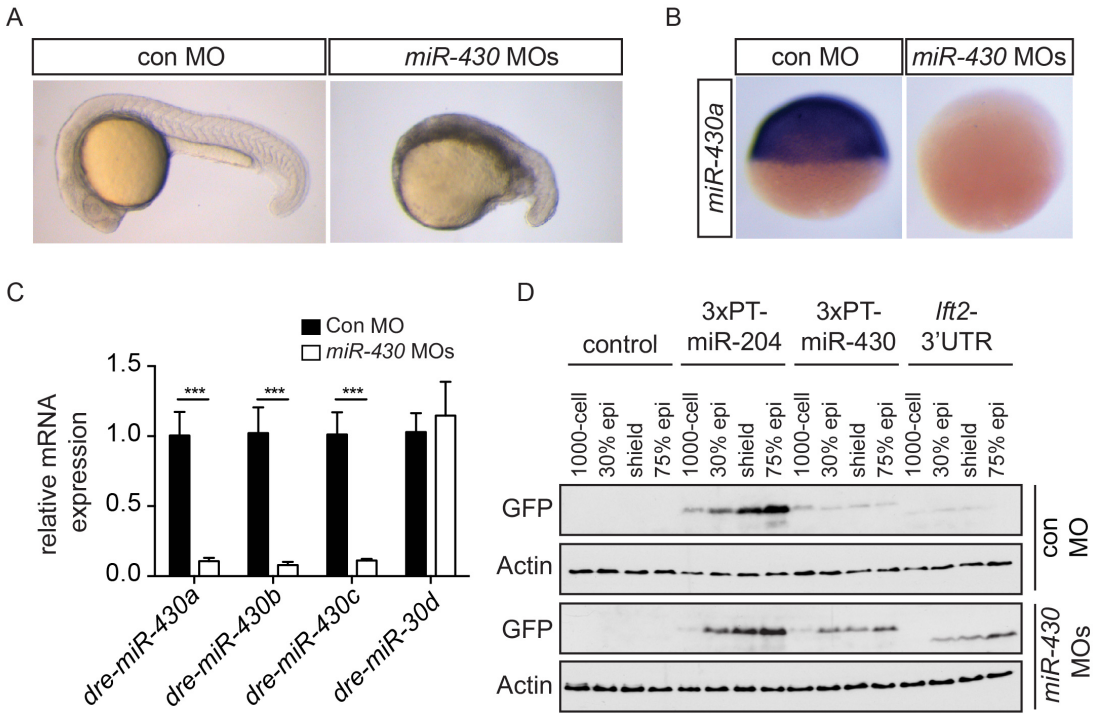

Figure S7, relates to Figure 5. Specificity of the *miR-430* morpholinos
(A) Phenotype of control (con) and *miR-430* morpholino-injected embryos at 22 hpf.
(B) WISH for *miR-430a* with an LNA probe after injection of a mix of *miR-430* or control MOs.
(C) qPCR for *miR-430a*, *b* and *c* after injection of control MOs (black bars) or *miR-430* MOs (white bars). *miR-30d* was not affected by *miR-430* morpholino injection. The data shown are means normalized to control MO ± SD (*** p-value < 0.001, t-test, n=6)
(D) Western blot for GFP on pooled embryo lysates from uninjected embryos or embryos injected with 50 pg mRNA encoding GFP reporter constructs containing 3'UTRs for *miR-204* (3xPT-miR-204), *miR-430* (3xPT-miR-430) or the *lft2* 3'UTR with either control MO or with *miR-430* MOs. *miR-430* MOs lead to an increase in translation of the 3xPT-miR-430 and *lft2*-3'UTR reporter constructs. Note that the GFP in reporters 3xPT-miR-204 and 3xPT-miR-430 migrates more slowly than that in the *lft2* 3'UTR reporter due to a membrane tethering tag. Actin is a loading control.

Supplemental Experimental Procedures

Fish husbandry, transgenesis and staging of embryos

Wild type, *tdgf1*^{tz257}, and *Tg(ARE:eGFP)* zebrafish lines were maintained as previously described (Westerfield, 2000). The *Tg(ARE:eGFP)* transgenic lines were generated by injecting one-cell stage embryos with 60 pg pT2KXGΔin-AREγActin-eGFP plasmid and 50 pg capped *Tol2* transposase mRNA (Kawakami, 2007). Founders for the AREγActin-eGFP transgene were identified by outcrossing injected adult zebrafish to wild type and screening their progeny for *eGFP* expression in the heart at 30 hpf by fluorescence. Fish from eight different founders were raised. Experiments described here use progeny from four different founders. All embryos were carefully staged according to morphological features (Kimmel et al., 1995) to avoid effects caused by general delays in development. In all figures where embryos were manipulated, numbers of embryos showing the representative phenotype out of the total number of embryos assayed are given.

Bead implantations

Bead implantations were performed as previously described with modifications (Picker et al., 2009). Polybead polystyrene 45 μm microspheres (Polysciences, 07314) were washed in ethanol, dried at 50°C and resuspended in 10 μl 100 μg/ml NODAL and incubated for 2 hrs at room temperature. *Tg(ARE:eGFP)* embryos were dechorionated with forceps and individually mounted on 2% agarose injection plates in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). At the 1000-cell stage, beads were inserted into the blastoderm using a blunt needle and embryos were allowed to develop up to ring stage before fixing in 4% paraformaldehyde (PFA).

Morpholino injections

Morpholinos (MOs) (Gene Tools LLC) were diluted in water and injected at the 1-2 cell stage at concentrations ranging from 2 to 6 ng (Ramel and Hill, 2013). The following MOs were used:

lefty1: 5' - GAAGTCATCTTTTCAAGGTGCAGGA - 3' (Agathon et al., 2001); *lefty2*: 5' - AGCTGGATGAACAGAGCCAT - 3' (Agathon et al., 2001);

foxh1: 5' - TGCTTTGTCATGCTGATGTAGTGGG - 3' (Pei et al., 2007);

mxtx2: 5'- CATTGAGTATTTTGCAGCTCTCTTG - 3' (Bruce et al., 2005);

fgf3: 5'- CATTGTGGCATGGCGGGATGTCGGC - 3' (Maves et al., 2002);

fgf8a: 5'- GAGTCTCATGTTTATAGCCTCAGTA - 3' (Kawakami et al., 2005);

dre-miR-430a: 5'- ACTACCCCAACAAATAGCACTTACC - 3';

dre-miR-430b: 5'- TCTACCCCAACTTGATAGCACTTTC - 3';

dre-miR-430c: 5'- ACTACCCCAAAGAGAAGCACTTATG - 3';

control MO: 5'- CCTCTTACCTCAGTTACAATTTATA - 3'.

Plasmids and mRNA Generation

All oligonucleotides used for cloning are listed in the Table below. To generate the pT2KXGΔin-AREγActin-eGFP plasmid, a fragment corresponding to the three AREs and the γActin minimal promoter was PCR amplified from ARE_3-Luciferase (Pierreux et al., 2000) and was cloned into pT2KXGΔin (Urasaki et al., 2006). The pCS2-Fgf8a plasmid was generated by PCR amplifying the *fgf8a* ORF with oligos extended with BamH1 and EcoR1 restriction sites and then cloned into pCS2+. The pGEMT-pri-miR430 and pGEMT-oep plasmids were generated by PCR amplification and cloning into pGEMT (Promega). For 3'UTR reporter assays, the pFTX4KeGFPC1-DrLft2-3'UTR plasmid was generated by PCR amplifying the *lft2*-3'UTR from a 30% epiboly zebrafish cDNA library, which was

cloned into pFTX4KeGFPC1 (Harding et al., 2014). pCS2+3xPT-miR-204 and pCS+3xPT-miR-430 were as previously described (Giraldez et al., 2005). Wild type *lft1* was PCR amplified and cloned into pCS2+. Point mutations in putative proprotein cleavage sites were introduced by PCR and plasmids were all verified by sequencing. For *in vitro* translation of Lft1/2, pCS2-lft1-gfp and pCS2-lft2-gfp were used (Muller et al., 2012). The following additional plasmids were used for mRNA injections: pXFD (XdnFgfR) (Amaya et al., 1991) (injected at a concentration of 500 pg/embryo), pCS2+ndr1 (Feldman et al., 1998) (injected at a concentration of 10 pg/embryo). mRNA was synthesized and microinjections were performed as previously described (Ramel and Hill, 2013).

Oligonucleotides used for cloning

Oligo name	**Sequence 5' – 3'**	**For plasmid**
ARE_XhoI_fw	CTTGCTCGAGGGTACCCCCCACCCTTGC	pT2KXGΔIN-AREγActin-EGFP
ARE_AgeI_rv	CTTGACCGGTCGACGGATCCCCGTCCACTG	pT2KXGΔIN-AREγActin-EGFP
fgf8a_BamH1_fw	CCGGGGATCCAACATGAGACTCATACCTTC	pCS2+-FGF8A
fgf8a_ EcoRI_rv	CCGGGAATTCTCAACGCTCTCCTGAGTAGC	pCS2+-FGF8A
pre-miR-430a_fw	CTATCGGTACCCTCACAAAGGCA	pGEMt_miR-430_ISH
pre-miR-430b_rv	CTACCCCAACTTGATAGCACTTT	pGEMt_miR-430_ISH
lft2_3UTR_fw_BamH1	TGACGGATCCCAGTGTGGTGTCGAATAGTTTGCTC	pFTX4KeGFPC1-DrLft2-3'UTR
lft2-3UTR_rv SpeI	CATGACTAGTTAAAATTAAGCTACTTACTTTATTT	pFTX4KeGFPC1-DrLft2-3'UTR
lft1_fw1_ClaI	CAGGATCCCATCGATGCCACCATGACTTC	pCS2+Lft1 + cleavage mutants
lft1_rv1_Xho1	CGCGCTCGAGCTTATACAACTGAAATATTGTCCATTGCGCATCC	pCS2+Lft1 + cleavage mutants
lft1_mut1_fw	AACTTCACCACTCA**G**GAAAACGCCGCTCGC	pCS2+lft1-mutant-C1
lft1_mut1_rv	GCGAGCGGCGTTTTC**C**TGAGTGGTGAAGTT	pCS2+lft1-mutant-C1
lft1_mut2_fw	ACACAAG**G**GCTCCATACCGGAGAG	pCS2+lft1-mutant-C2
lft1_mut2_rv	CTCTCCGGTATGGAGC**C**CTTGTG	pCS2+lft1-mutant-C2
lft1_mut3_fw	CAAAGAC**G**GTGAAATGTGCTGCAGGG	pCS2+lft1-mutant-C3
lft1_mut3_rv	CCCTGCAGCACATTTCAC**C**GTCTT	pCS2+lft1-mutant-C3

	TG	
tdgf1_fw	GAACACGCAAACGCCGCAAC	pGEMt-oep-ISH
tdgf1_rv	CACTCGAGCTACAGCAGGCGGT	pGEMt-oep-ISH

The letters in red indicate mutated residues.

Lefty antibodies and immunoblotting

Peptides CVHFTTQDPDDNTLGKPELVLYTLN and PELVLYTLDLDEYGSQGNC were used to generate rabbit antisera against Lft1 and Lft2 respectively. The antibodies were affinity purified using commercially available columns, coupled to the peptides according to the manufacturer's instructions (Thermo Scientific, 44999). For blotting of endogenous Lft1, 10 embryos were manually de-yolked without disrupting the blastoderm and snap frozen in 10 µl E3 medium. Lysates were prepared and immunoblotting was performed as previously described (Batut et al., 2007). The following additional antibodies were used: anti–phosphylated-Smad2 (Millipore, 04-953), anti-Smad2 (Cell Signaling Technology, 3103), anti-Smad3 (also detects zebrafish Smad2) (Abcam, ab28379), anti-phosphorylated-Erk (Sigma M8159), anti-Erk (Santa Cruz, sc-94), anti-GFP (Roche, 11814460001), anti-MCM6 (Santa Cruz Biotechnology, sc-9843) and anti-Actin (Sigma, A3853). All experiments were performed at least in triplicate. The quantifications of protein expression in Figure 4F were performed using ImageJ.

WISH and sectioning

Whole mount in situ hybridization (WISH) and double fluorescent WISH were performed and imaged as previously described (Ramel and Hill, 2013). For a full list of plasmids and oligos that were used to generate riboprobes, see the Tables below. All WISH experiments were performed at least in duplicate and unless stated otherwise in the figure legend, lateral views are shown with dorsal to the right. For sectioning, embryos were embedded in

paraffin, sectioned at 8 μm and counterstained with Nuclear Fast Red for 5 min (Vector laboratories, H3403). Quantification of *ta* staining in Figure 1 was performed blind and the statistical difference assessed using a Mann-Whitney U test. For expression analysis of mature *miR-430a* and *miR-430b*, locked nucleic acid (LNA) probes (Exiqon), digoxigenin (DIG) labeled as previously described (Harding et al., 2014), were used for WISH. The sequences of the probes were: *dre-miR-430a*: 5'- CTACCCCAACAAATAGCACTTA -3'; *dre-miR-430b*: 5'- CTACCCCAACTTGATAGCACTTT -3'. The hybridization temperature was optimized and found to be 55°C for both probes. To quantitate the number of *sox17*-positive cells, 75% epiboly embryos were stained by WISH for *sox17*, after which the blastoderm was dissected from the yolk and opened up along the dorsal midline to count the number of individual cells under a stereomicroscope. The statistical difference was assessed using a Mann-Whitney U test.

WISH probes generated from plasmids.

Target gene	**Plasmid**	**reference**	**Digest/ RNA polymerase**
ta	pBKS-ntl-a	(Schulte-Merker et al., 1992)	Xho1/T7
fscn1a	pBS-fscn1a	Thisse et al., 2001 ZFIN online publication	BamH1/T7
lft2	pAD-gal4-Lft2	(Bisgrove et al., 1999)	MluI/T7
ndr1	pBSK-squint	(Rebagliati et al., 1998a)	BamH1/T7
ndr2	pBSK-cyclops	(Rebagliati et al., 1998b)	Not1/T7
eGFP	pBSK-eGFP	(Narayanan et al., 2011)	BamH1/T7
pri-miR-430	pGEMt-miR-430-ISH	n/a	Not1/T7
tdgf1	pGEMt-oep-ISH	n/a	Not1/T7
smad2	pCRII-Smad2_ISH	(Dick et al., 2000)	BamH1/T7
noto/*flh*	pBSK-flh	(Talbot et al., 1995)	EcoR1/T7
sox3	pBUT2-Sox3	(Dee et al., 2008)	XbaI/T7
fgf3	pBSK-fgf3	(Kiefer et al., 1996)	BamH1/T7
fgf8a	pBSK-fgf8a (cb110)	Thisse et al., 2001 ZFIN online publication	Not1/T7
sox17	pBSK-sox17	(Alexander and Stainier, 1999)	NcoI/Sp6

gsc	pBS-gsc	(Stachel et al., 1993)	EcoR1/T7

Oligonucleotides for WISH probes generated by PCR

Oligo name	**Sequence 5' – 3'**	**RNA polymerase**
acvr1ba_fw	TCCCTCGGGTCAGCCTGGTT	T7
acvr1ba_T7_rv	TAATACGACTCACTATAGGAGGCCGAAGCCTCTG GTCACA	T7
foxh1_fw	GTCCACAGGGATGGCGCAGG	T7
foxh1_T7_rv	TAATACGACTCACTATAGGAAGCGCATGCTTGGA GGGGC	T7
lft1-ISH_fw	TGCCTCCTTTGCGCAGCACT	T7
lft1-ISH_T7_rv	TAATACGACTCACTATAGGCGGCAGCCGCCTTTA CACCT	T7

Northern blotting, ePAT and qPCR

Detection of miRNAs by northern blotting was performed as described (Harding et al., 2014) using 16 µg of total RNA and ^{32}P-radiolabeled complementary LNA probes. Detection of miRNAs by qPCR was as described (Harding et al., 2014). ePAT was performed as previously described (Janicke et al., 2012) except that for the gene-specific PCR amplification, random-primed cDNA was used. Samples were separated on 8% non-denaturing polyacrylamide gels, which were silver stained (Pierreux et al., 2000). Sequences of oligonucleotides used in these assays are listed in the Table below.

Oligonucleotides used in ePAT experiments

ePAT_Anchor	GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT
ePAT_anchor _rv	AGCTCCGCGGCCGCG
ndr1_ePAT_fw	TTGCAGAATGCGGCTGCCACTGA
ndr1_GS_rv	TTACAGATAAGGCAAACACGCAAAGC
lft1_ePAT_fw	AGGAAATGCGCGGTCGTCGAG
lft1_GS_rv	ACAACAAACCCGTGCTATATGCTC
lft2_ePAT_fw	AGGATGCAAGCAGCCTAAACG
lft2_GS_rv	AAGTGCTCAGTGGGGATTTGGG

For qPCR, mRNA was extracted using Trizol (Life Technologies, 15596-026), reverse transcribed with AffinityScript (Agilent, 600559), diluted 1:20 and measured with Express Sybr Green ER (Invitrogen, 11784). Calculations were performed using the ddCT method and were normalized to levels of *eef1a1l1,* and then to the values obtained in untreated cells. Experiments were performed at least in triplicate and statistical differences were assessed using t-tests with a 95% confidence interval. All oligonucleotides used for qPCR assays are listed in the Table below.

Oligonucleotides used in qPCR experiments

Oligo name	**Sequence 5' – 3'**	**target**
eef1a1l1_fw	TGCTGTGCGTGACATGAGGCAG	*eukaryotic translation elongation factor 1 alpha 1, like 1*
eef1a1l1_rv	CCGCAACCTTTGGAACGGTGT	*eukaryotic translation elongation factor 1 alpha 1, like 1*
odc1_fw	ACACTATGACGGCTTGCACCG	*ornithine decarboxylase 1*
odc1_rv	CCCACTGACTGCACGATCTGG	*ornithine decarboxylase 1*
eGFP_fw	TTCAGCCGCTACCCCGACCA	*enhanced GFP*
eGFP_rv	ACCAGGGTGTCGCCCTCGAA	*enhanced GFP*
lft1_fw	GCTGGTGCTTTACACACTCAACCT	*lefty1*
lft1_rv	GTTCCCTGCAGCACATTTCACG	*lefty1*
lft2_fw	GGACATGGGCGCACCAGAACT	*lefty2*
lft2_rv	TACCCGGCCGGCTCGATGAT	*lefty2*
ndr1_fw	CTCCGTCTTGAGCCTCGTCG	*nodal-related 1*
ndr1_rv	TCGCTGGACGTCATCGCTTG	*nodal-related 1*
ndr2_fw	AATGCATACCGGTGCGAGGG	*nodal-related 2*
ndr2_rv	GCAGGAACACGACTGGGGTG	*nodal-related 2*
ta_fw	AAGACGCGGAGTTGTGGACC	*T, brachyury homolog a*
ta_rv	ACTGGCTCTGAGCACGGGAA	*T, brachyury homolog a*
fscn1a_fw	ACTGCCTCTACCAAGTCTGC	*fascin actin-bundling protein 1a*
fscn1a_rv	ATTTTCCATTGGTGGCACGC	*fascin actin-bundling protein 1a*
dre-miR-430a	TAAGTGCTATTTGTTGGGGTAG	*dre-miR-430a*
dre-miR-430b	AAAGTGCTATCAAGTTGGGGTAG	*dre-miR-430b*
dre-miR-430c	TAAGTGCTTCTCTTTGGGGTAG	*dre-miR-430c*
dre-miR-30d	TGTAAACATCCCCGACTGGAAG	*dre-miR-30d*
miR_universal_rv	GCGAGCACAGAATTAATACGACTCAC	*reverse for all miRNA PCRs*

gsc_fw	CAAGAGAACAACTGGCACGA	*goosecoid*
gsc_rv	TCCTCTGACGACCTTTT	*goosecoid*
noto_fw	TCTCAGGAGAACTCCAAGAGTC	*notochord homeobox*
noto_rv	GCTGCCCTTGGAACTCAGAT	*notochord homeobox*

Supplemental References

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