Inventory of Supplemental Information

Figure S1.

(A, A') Comparison of patterns of normal expression for *adam13*, *efnb1* and *efnb2* at gastrulation demonstrates degree of tissue overlap. These data provide baseline expression information in support of Figures 1-4.

(B, C) *In situ* hybridization of *adam13* at early premigratory and late migratory CNC stages showing expression persists in the CNC beyond gastrula stages.

(D) Schematic of head cartilage tissues and key to scoring of cartilage defects reported in Figure 1D.

(E, F) Effects of ADAM13 MO on *snail2* and *twist* expression at migratory CNC stages extend and complement early stage CNC induction data (Figure 1E, F).

(G, H) Late ADAM13 KD phenotypes rescued with wild-type ADAM13. Deficiencies in *snail2* expression at migratory CNC stages and head cartilage structures evident in ADAM13 morphants (Figures 1D, S1E) are largely restored; extends also the data presented in Figure 1G.

Figure S2.

(A, B) Evidence for membrane-bound "stubs" of EfnB1 and EfnB2, respectively, generated by ADAM13 proteolytic activity consistent with presence of EfnB2 ectodomain shedding in HEK293T cells (Figure 2A-C).

(C) ADAM13 KD leads to accumulation of HA-tagged EfnB1 expressed in embryos, consistent with observed accumulation of endogenous EfnB1/B2 in embryos (Figure 2D, E).

(D) Control experiment demonstrating specificity of C18 antibody used in Figure 2D for EfnB1/B2 but not EfnB3.

Figure S3.

(A, B) ADAM13 MO dependent late-stage defects in *snail2* expression and head cartilage formation, respectively, are rescued by the same EphB1ΔC and Xdsh constructs used to restore early-stage *snail2* expression (Figure 3B).

(C) Ectopic overexpression of EphB1ΔC causes anterior expansion of *snail2* as predicted from MO 13-3 rescue experiments (Figure 3B), and is consistent with the role of forward EfnB signaling in antagonizing canonical Wnt signaling (Figure 4D).

(D) Inhibition of canonical Wnt signaling by both EfnB1ΔC and wild-type EfnB1 in TOP/FOPFLASH assays, which supports the role of forward EfnB signaling in antagonizing canonical Wnt signaling (Figures 3A-D, 4D).

(E) TOP/FLOPFLASH assays of embryo lysates demonstrate that EphB1ΔC enhances canonical Wnt signaling while EfnB1 overexpression attenuates the affects of Wnt8 overexpression. These data support the conclusions of TOP/FOPFLASH experiments in transfected cells (Figure 3D), axis duplication assays (Figure 3C) and the *snail2* expression analyses in early stage embryos (Figures 3A, B and S3C).

Figure S4.

(A) Specificity of MO 13-3 knockdown of *snail2* in CNC demonstrated in whole embryos. Expression of *snail2* is reduced in the presumptive CNC but not in the midline; accompanies RT-PCR data in Figure 4A.

(B, C) Late stage expression of the CNC marker *twist* and deficiencies in head cartilage following ADAM13 KD, respectively, are partially restored by Snail2 overexpression; consistent with restoration of *sox9* expression (and CNC induction) by Snail2 in early-stage embryos deficient in ADAM13 (Figure 4B).

EXTENDED EXPERIMENTAL PROCEDURES

Plasmids and Antibodies.

DNA constructs for *X. laevis* Efns B1, B2 and EphB1ΔC were provided by Dr. Ira Daar; *X. laevis* EfnB3 construct was provided by Dr. Andre Brandli. Wild-type and mutant *Xdsh* constructs were provided by Dr. Mungo Marsden. Constructs used in secondary axis induction and Topflash assays, including pCS2+-*wnt8* (with the 3'-UTR deleted), pTopflash, pFopflash, pCMV-*β-gal*, pCS2+-*GFP*, and pCS2+-*wnt3a* were provided by Dr. Barry Gumbiner. The 9E10 anti-myc antibody and the anti-EfnB1/B2 (C-18) antibody were from Santa Cruz, the 12CA5 anti-HA antibody was from Abcam, and the HRP-conjugated anti-β-actin antibody was from Sigma. The anti-ADAM13 antibody (Abcam ab39158), originally produced to recognize the cytoplasmic tail of *X. laevis* ADAM13, was predicted to cross-react with *X. tropicalis* ADAM13 based on sequence homology.

Cloning and Construct Generation.

The following *X. tropicalis* clones were identified by bioinformatics and purchased from the indicated sources: *snail2* (Geneservice; clone ID: Ttba075D05), *sox9* (Open Biosystems; IMAGE clone ID: 7711846), *twist* (Geneservice; clone ID: Ttba039M23), *efnb1* (Open Biosystems; IMAGE clone ID: 7657923), and *efnb2* (Open Biosystems; IMAGE clone ID: 7629902). All clones were confirmed by DNA sequencing.

X. tropicalis orthologues of *X. laevis adam13* and mammalian *adams 12* and *19* were identified by blasting against JGI *X. tropicalis* genome database, and the predicted sequences were used to design primers for RT-PCR to obtain partial clones. 5'- and 3'-RACE was then performed using BD Smart RACE kit (BD Biosciences), and full-length cDNA sequences were assembled into pCR2.1.

Constructs encoding myc_6- or HA-tagged proteins were generated by subcloning into pCS2+ vectors modified to append corresponding epitopes. For myc- or HA-tagged ADAMs and myc-tagged Efns, the corresponding epitopes were attached to the C-terminus. For HA-tagged Efns, the epitope was inserted several residues downstream of the predicted signal peptide cleavage site. Rescue constructs were made by introducing sense mutations downstream of the translational start codon in corresponding myc-tagged expression constructs using a QuickChange mutagenesis kit (Stratagene).

Injections.

X. tropicalis embryos were obtained by *in vitro* fertilization and injected using a PLI-100 microinjector (Medical Systems) as described (Ogino et al., 2006). Alexa Fluor 555 dextran (Invitrogen) was co-injected as lineage tracer. Embryos were cultured in 0.1x MBS to desired stages and sorted according to the injected side using a Zeiss Lumar epifluorescence stereomicroscope before being fixed for in situ hybridization or for phenotype scoring.

TOP/FOPFLASH reporter assays.

For cell-based TOP/FOPFLASH assays (Figures 3D and S3C), HEK293T cells were cultured in 24-well plates and transfected with the following amount of plasmids (per well): 30 ng pTopflash or pFopflash, 50 ng pCMV-*β-gal*, 50 ng pCS2+-*wnt3a*, 50,

100 or 200 ng pCS2+-*efnb1* or pCS2+-*efnb1ΔC*, 200 ng pCS2+-*adam13* or pCS2+-*adam13ΔPM*. Total DNA transfected was normalized to 500 ng per well using pCS2+-*GFP*. For *in vivo* TOP/FOPFLASH assays (Figure S3E), 4-cell stage embryos were injected in all four blastomeres with 2.5 pg pTopflash or pFopflash, together with transcripts encoding EphB1(ΔC) (200 pg), Wnt8 (5 pg), or Wnt8 with EfnB1 (500 pg), and raised to stage ~11. To prepare whole embryo extracts, 5 embryos were homogenized in 50 µl cold 50 mM Tris-HCl (pH 7.5), extracted with 50 µl freon, and centrifuged for 20 min at 12,000 g at 4°C. Supernatants were collected, and the volume was brought up to 250 µl with the reporter lysis buffer provided with the luciferase assay kit (Promega). The samples were freeze-thawed and luciferase activity was measured as described in Experimental Procedures. Results are presented as ratios of TOPFLASH vs. FOPFLASH luciferase activity (both were normalized for protein concentration as determined by $O.D._{280}$), and the value calculated for embryos injected with pTop/Fopflash only was set to 1.

Quantitative RT-PCR.

Quantitative RT-PCR was performed by the Biomolecular Research Facility at the University of Virginia. Reverse transcription was carried out using the High Capacity RNA-to-cDNA kit (Applied Biosystems), and real-time PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The oligo sequences designed for *snail2* are: CACTTCAACTCGGCCAAAAAG (forward), GGGTACCGCTCATACAGGAATG (reverse), and TGGACAACCATACAGTGATCATCTCC (TaqMan probe). The oligo sequences designed for *β-actin* are: GCACCCCGTGCTGTTTTC (forward), ACGTAGCTATCTTTCTGTCCCATTC (reverse), and TGGTCGCCCAAGACATCAGGG (TaqMan probe).

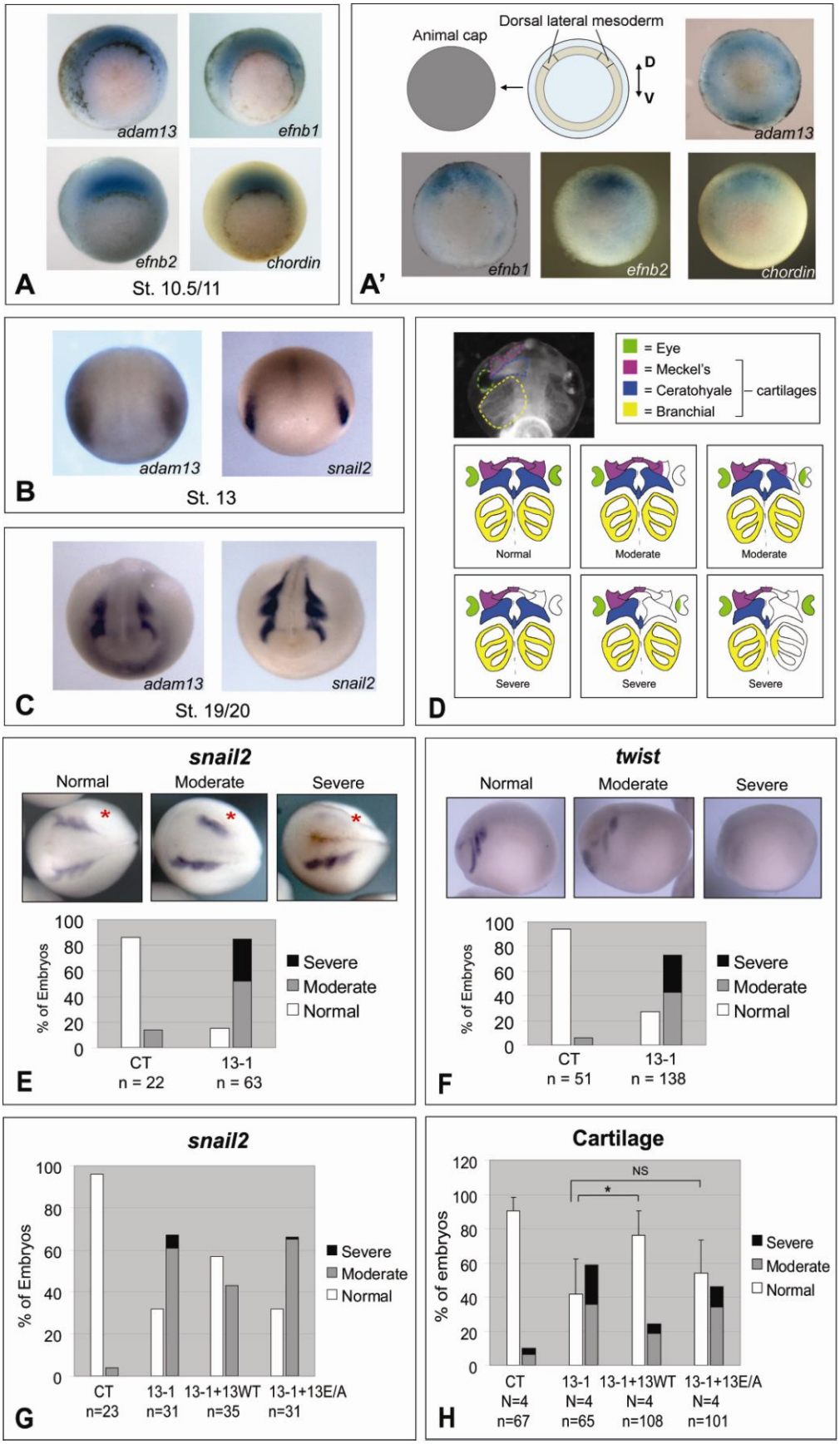
adam13
efnb1
efnb2
chordin
A
St. 10.5/11
Animal cap
Dorsal lateral mesoderm
D
V
A'
B
St. 13
C
St. 19/20
= Eye
= Meckel's
= Ceratohyale
= Branchial
cartilages
Normal
Moderate
Severe
D
snail2
Normal
Moderate
Severe
% of Embryos
CT
n = 22
13-1
n = 63
E
twist
n = 51
n = 138
F
G
CT
n=23
13-1
n=31
13-1+13WT
n=35
13-1+13E/A
n=31
Cartilage
% of embryos
NS
*
N=4
n=67
n=65
n=108
n=101
H

Figure S1, related to Figure 1. ADAM13 metalloproteinase activity is required for CNC induction in *X. tropicalis* embryos.

(A-C) Developmental expression of *adam13*, *efnb1* and *efnb2* by *in situ* hybridization. (A and A') Expression of *adam13* overlaps with *efnb1* and *efnb2* in dorsal mesoderm during early to mid gastrulation (stage 10.5/11). *In situ* hybridization of whole embryos (A; vegetal pole views with dorsal at the top) and bisected embryos with animal cap removed as shown in the cartoon (A'; animal pole views with dorsal at the top) was carried out using BCIP as the substrate for alkaline phosphatase (signals in blue/turquoise). *chordin* was included to show the position of dorsal-most mesoderm. (B and C) Expression of *adam13* overlaps with *snail2* in newly formed and migrating CNC (stages 13 and 19/20, respectively). Whole-mount *in situ* hybridization was carried out using BM purple as the substrate for alkaline phosphatase (signals in dark blue/purple). All embryos are shown in anterior views with dorsal at the top.

(D) Scoring system for craniofacial defects. Bright field image of a normal stage ~46 embryo at top, ventral view; color-coded dashed lines indicate the location of tissue structures used in scoring phenotypes. Scoring was established by comparing tissues on the MO-injected side of each embryo with the un-injected side. Bottom panels represent examples of the color-coded "scoring template" (adapted from Sadaghiani and Thiebaud 1987) used to record cartilage phenotype for each embryo, with the injected side shown on the right; colors correspond to key at top right. Cartilage phenotypes were scored as severe when one or more cartilage structures (i.e., Meckel's, Ceratohyle, Branchial) were absent. Abnormal morphology and/or partial loss of one or more of these structures was scored as "moderate". Defects in both eye and cartilage were frequently noted in individual embryos but not always; phenotypes were also observed to segregate independently.

(E and F) ADAM13 MO knocks down CNC markers at later stages. Four-cell stage embryos were injected in one anterior-dorsal blastomere with MO 13-1 (3 ng), and processed for *in situ* hybridization at stage ~19 for *snail2* (E) and at stage ~22 for *twist* (F). One example of each phenotype is shown in the upper panels, and results of multiple independent experiments are graphed in the lower panel. Red asterisks in E denote the injected side; in F only the injected side is shown.

(G and H) Later defects in *snail2* expression and CNC morphology caused by ADAM13 MO can be rescued by wild-type ADAM13 but not the E/A mutant. One anterior-dorsal blastomere of 8-cell stage embryos was injected with the indicated MO (1.5 ng) and rescue RNA (25 pg each). Embryos were processed for *in situ* hybridization for *snail2* at stage 19/20 (G), or scored for head cartilage defects at stage ~46 (H). See Figures S1D and S1E, and Experimental Procedures for details on phenotype scoring and statistics. Error bars represent standard deviations calculated for 4 independent experiments. *: $P = 0.03$; NS: not significant ($P = 0.40$).

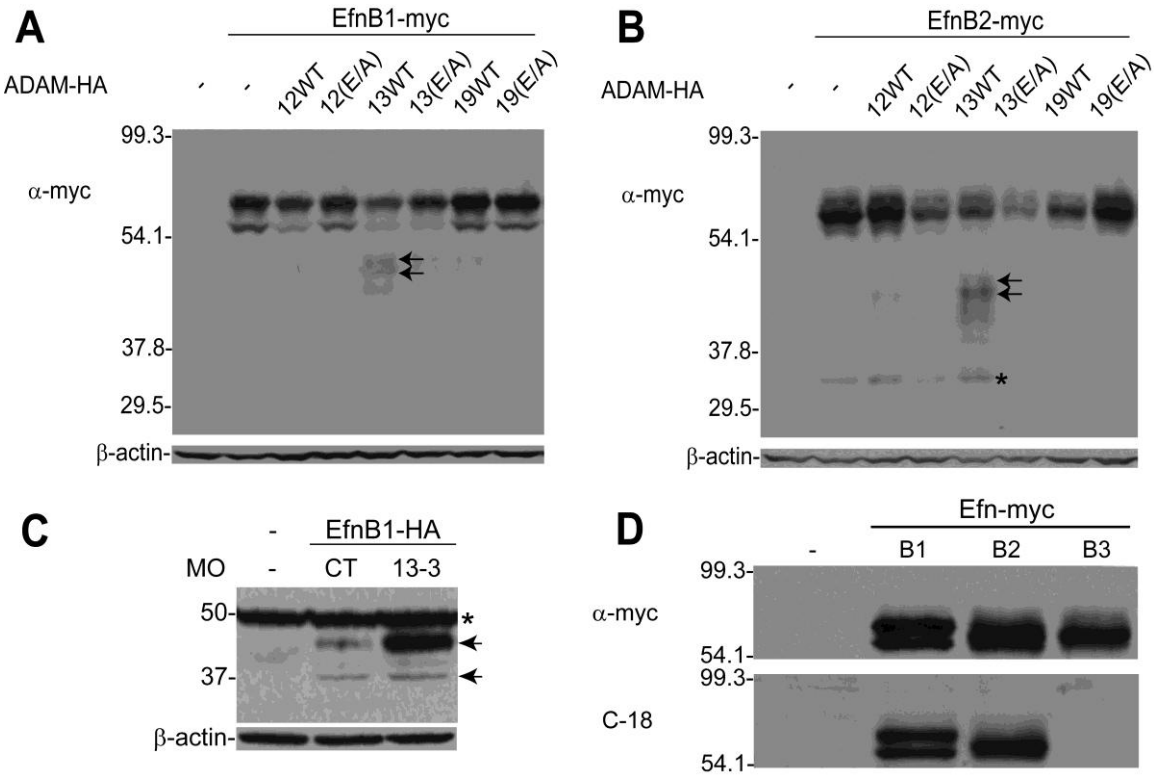

Figure S2, related to Figure 2. Cleavage of Efns B1 and B2 by ADAM13.

(A and B) Cleavage of Efns B1 and B2 (with a cytoplasmic myc epitope) by ADAM13 in HEK293T cells generates membrane-bound "stubs". Cultured HEK293T cells were transfected with DNA constructs (0.5 μg each) expressing the indicated Efn (with a C-terminal myc_6 tag) and either wild-type or the E/A mutant of different *Xenopus* ADAMs (with a C-terminal HA tag), as described in Experimental Procedures. Western blots of whole cell lysates were carried out using an anti-myc antibody, and membranes were stripped and re-blotted for β-actin. Arrows indicate the membrane-bound cleavage products ("stubs") generated by ADAM13, and asterisk indicates a cleavage product of EfnB2 generated by an endogenous protease in HEK293T cells. The myc-tagged EfnBs appear larger than the endogenous proteins (as seen in Figure 2D) because of the size of the myc_6 epitope (~10 kDa).

(C) ADAM13 MO protects ectopically expressed EfnB1 in *X. tropicalis* embryos. One blastomere of 2-cell stage embryos was injected with the indicated MO (6 ng) and RNA encoding HA-tagged EfnB1 (500 pg). Embryos were allowed to develop to stage ~15 and Western blot of whole-embryo lysates was carried out using an anti-HA antibody. Arrows indicate intact EfnB1 (the presence of multiple bands may be due to different post-translational modifications), and asterisk indicates a nonspecific band.

(D) A control showing that the C-18 antibody recognizes *Xenopus* Efns B1 and B2, but not B3. Cultured HEK293T cells were transfected with DNA constructs expressing the indicated myc-tagged Efns (0.5 μg each) as described in Experimental Procedures. Western blot of whole cell lysates was carried out using anti-myc antibody (upper panel). The membrane was stripped and re-blotted with the C-18 antibody (lower panel).

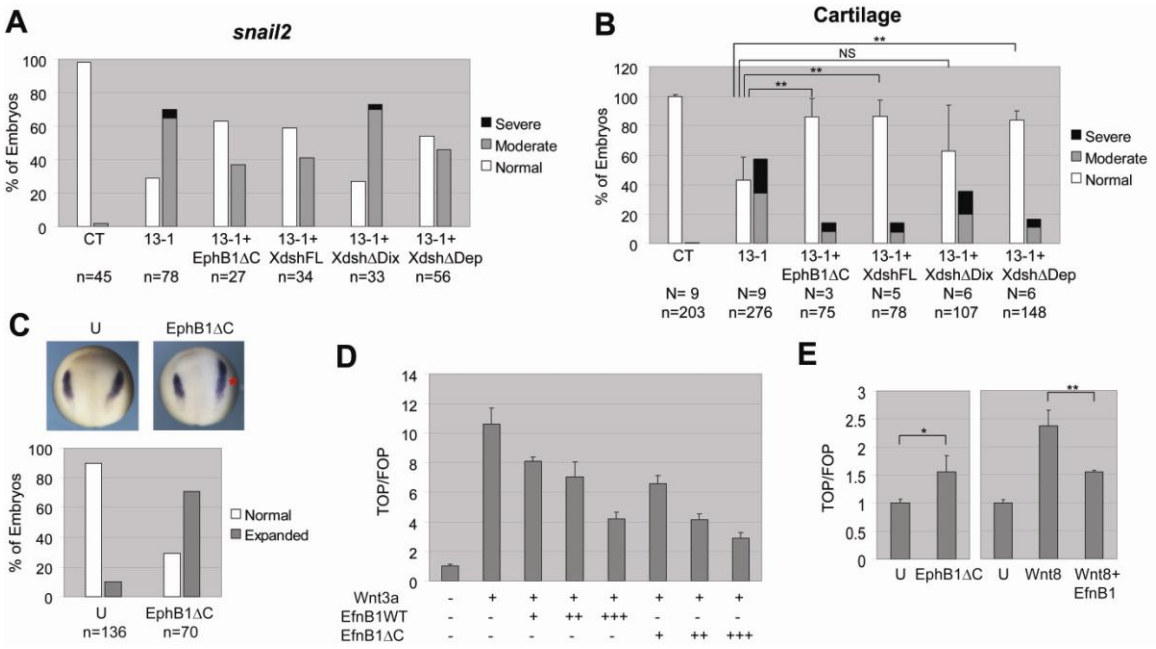

Figure S3, related to Figure 3. EfnB and Wnt signaling mediates ADAM13 function in CNC induction.

(A and B) Later defects in *snail2* expression and CNC morphology caused by ADAM13 MO can be rescued by EphB1ΔC, full-length Xdsh and XdshΔDep, but not by XdshΔDix. One anterior-dorsal blastomere of 8-cell stage embryos was injected with the indicated MO (1.5 ng) and RNA (100 pg each). Embryos were processed for *in situ* hybridization for *snail2* at stage 19/20 (A), or scored for head cartilage defects at stage ~46 (B). See Figures S1D and S1E, and Experimental Procedures for details on phenotype scoring and statistics. Error bars represent standard deviations calculated for multiple (N) independent experiments. **: P = 0.002, P < 0.001, and P < 0.001 for rescues with EphB1ΔC, XdshFL and XdshΔDep, respectively; NS: not significant (P = 0.12).

(C) Ectopic expression of EphB1(ΔC) causes an anterior expansion of *snail2* expression domain. Embryos were injected at 4-cell stage in one anterior-dorsal blastomere with RNA encoding EphB1(ΔC) (400 pg), and processed for *in situ* hybridization at stage 12.5/13 for *snail2*. The injected side is denoted with a red asterisk. U, uninjected.

(D) The EfnB1ΔC mutant mimics wild-type EfnB1 in inhibition of canonical Wnt signaling. TOP/FOPFLASH assays were carried out in HEK293T cells and Wnt activity was calculated as described in Figure 3D legend and Experimental Procedures. A representative experiment performed in triplicate is shown here (error bars represent standard deviations).

(E) Effects of EphB1(ΔC) and EfnB1 on canonical Wnt signaling in gastrula stage *X. tropicalis* embryos, as shown by *in vivo* TOP/FOPFLASH assays. Four-cell stage embryos were injected in all four blastomeres with the indicated RNA together with the pTopflash or pFopflash plasmid, and Wnt activity was measured as described in Supplemental Experimental Procedures. Error bars represent standard deviations calculated for 3 independent experiments. *: P = 0.03; **: P = 0.007.

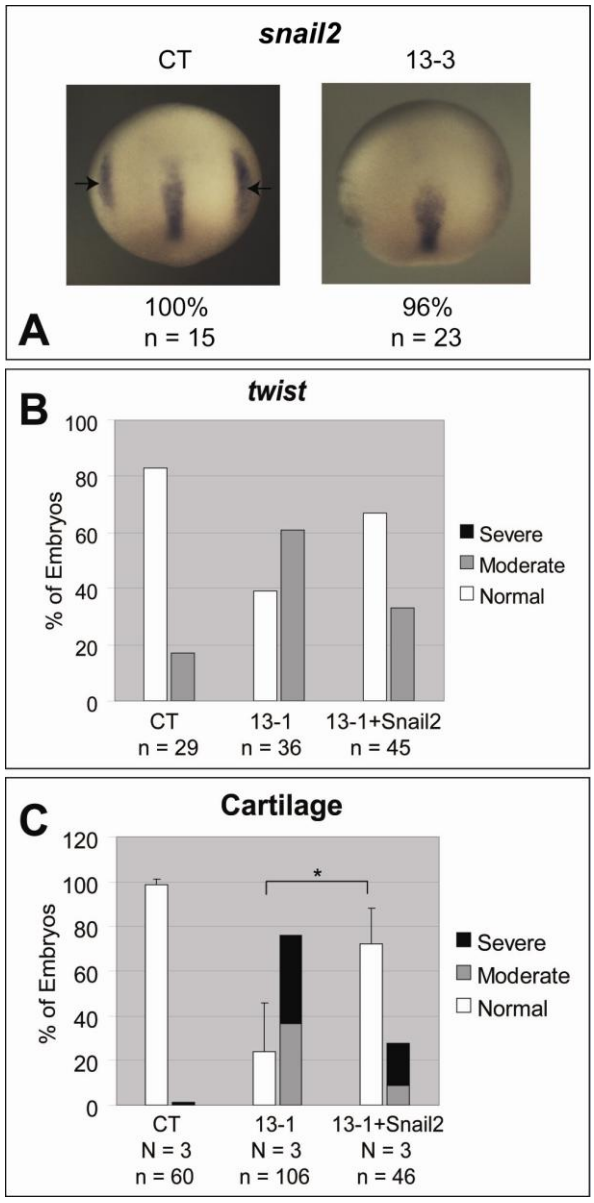

Figure S4, related to Figure 4. ADAM13 regulates CNC induction through controlling the level of Snail2. (A) *Snail2* expression is specifically inhibited at the anterolateral border of the future neural plate (as indicated by the arrows) in late gastrula stage 13-3 morphants. Two-cell stage embryos were injected in each blastomere with 6 ng MO 13-3, and *in situ* hybridization was carried out for *snail2*. Embryos were subjected to prolonged color development until the midline staining was apparent. (B and C) Later defects in *twist* expression and CNC morphology caused by ADAM13 MO can be rescued by exogenous Snail2. One anterior-dorsal blastomere of 8-cell stage embryos was injected with the indicated MO (1.5 ng) and RNA encoding Snail2 (100 pg). Embryos were processed for *in situ* hybridization for *twist* at stage ~22 (B), or scored for head cartilage defects at stage ~46 (C). See Figure S1D and S1F, and Experimental Procedures for details on phenotype scoring and statistics. Error bars represent standard deviations calculated for 3 independent experiments. *: P = 0.04.