Supplementary Materials and Methods

Morpholino sequences

Two independent morpholino oligonucleotides (MO; GeneTools, Inc.) were designed to bind to the 5' UTR region near the translation start site of *Xenopus laevis sim2* mRNA (#1), or to the intron 1 – exon 2 boundary (splice-blocking MO; #2):

MO #1 5' – CGTTCTTGGATTTCTCCTTCATGTC – 3'

MO #2: 5' – CCTAATCCTGGATTGCAAAAATGGA – 3'

A standard control MO targeting a splice mutant of human β-globulin (5' – CCTCTTACCTCAGTTACAATTTATA – 3'), and a MO previously demonstrated to effectively and specifically target *Xenopus laevis pitx2c* (Davis et al., 2017) were also used.

Morpholino-resistant *sim2* mRNA

The morpholino-resistant version of *sim2* mRNA was obtained using wild type, full-length *Xenopus sim2* cDNA as a template and the following PCR primers:

5' – GGCGGATCCGACATGAAAGAAAAGAGCAAAAATGCAGCGAAAACGCGCAGAGAG – 3'

5' – GGCTCTAGAGTCCTAATATCACCTTCCGTTGG – 3'.

The upstream primer has seven conservative nucleotide changes within the first seven amino acids, preserving the WT amino acid sequence. The resulting amplification product was cloned into pCS2, and transcribed using the mMessage Machine SP6 kit.

List of Antibodies

- anti-integrin [Developmental Studies Hybridoma Bank (DSHB), created by the NICHD of the NIH and maintained at The University of Iowa; 8C8-c, 1:1000]
- anti-β-catenin (Santa Cruz, sc-7199, 1:100)
- anti-α-tubulin (Sigma, T9026, 1:1000)
- anti-E-cadherin (DSHB 5D3, 1:200)
- anti-GFP (ThermoFisher, A6455 1:1000)
- anti-GAPDH (Millipore AB2302, 1:1000)
- anti-phospho-histone H3 (Millipore, 06-570, 1:100)

- Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, A11029; 1:2000)
- Alexa 488-conjugated goat anti-rabbit IgG (ThermoFisher A11034; 1:2000)
- Alexa 546-conjugated donkey anti-mouse IgG (ThermoFisher A31570; 1:2000)
- Alexa 546-conjugated goat anti-rabbit IgG (Invitrogen, A11035; 1:2000)
- Alexa 647-conjugated Donkey anti-mouse IgG (Invitrogen, A31571 1:2000)
- HRP-conjugated donkey anti-rabbit IgG (ThermoFisher A16035, 1;10,000)

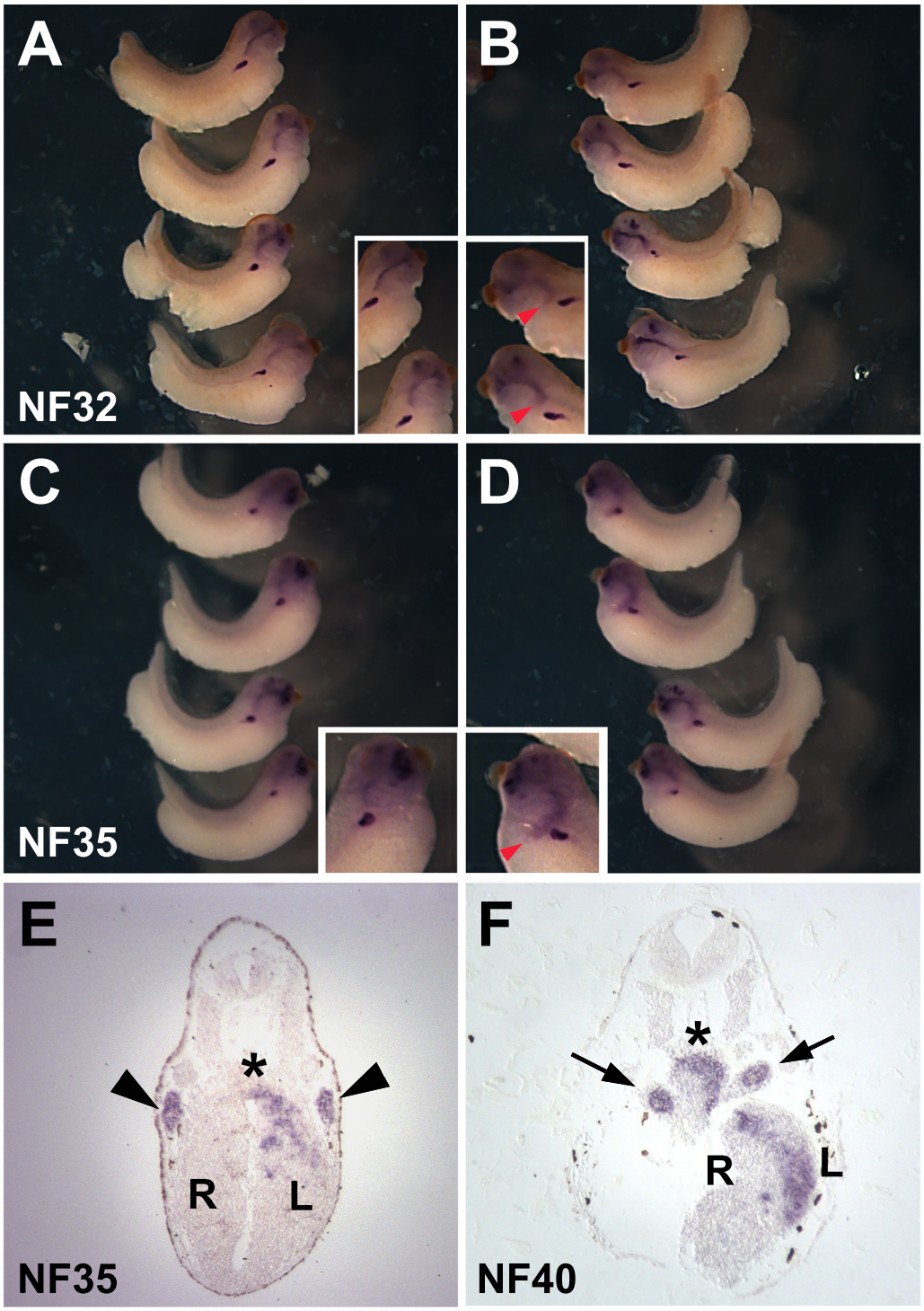

Fig. S1. *Sim2* is expressed in both symmetrical and asymmetrical patterns in different tissues. The expression of *Xenopus sim2* was determined by RNA *in situ* hybridization on whole embryos (A-B, NF32; C-D, NF35) or on sections through the foregut (E, NF35; F, NF40). In A-D, each embryo is shown in right (A,C) and left (B, D) views; the insets show higher magnification views of bilateral expression in craniofacial structures, with red arrowheads indicating asymmetric *sim2* expression, which first becomes visible in the prospective stomach region of the left foregut. In sections (E-F), stomach-specific expression is visible only in the left (L) wall of the stomach; the right (R) side is largely devoid of appreciable *sim2* expression. Asterisks indicate expression in the dorsal midline of the foregut. Arrowheads in E indicate bilateral expression in both left and right pronephric tubules (NF35), while arrows in F indicate expression in both left and right lung buds (NF40).

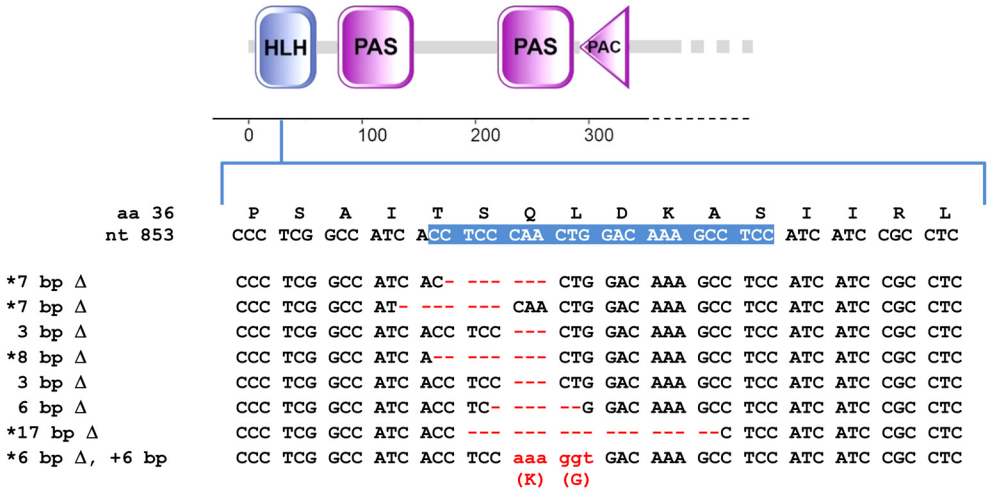

Fig. S2. Indels produced by *sim2* CRISPR. A gRNA targeting the conserved HLH domain of *Xenopus laevis sim2* (top) was injected into 1-cell *Xenopus* embryos along with *Cas9* mRNA. Pooled genomic DNA from 10 neurulae was PCR amplified using exon 1-specific primers. Sequencing of a subset of individual clones validated the presence of deleterious mutations (red) corresponding with the *sim2* gRNA target sequence (highlighted in blue). Of these, 62.5% (5/8; asterisks, *) are expected to generate null alleles (frameshifts) and/or compromise function (non-conservative amino acid substitution, e.g., Q to K).

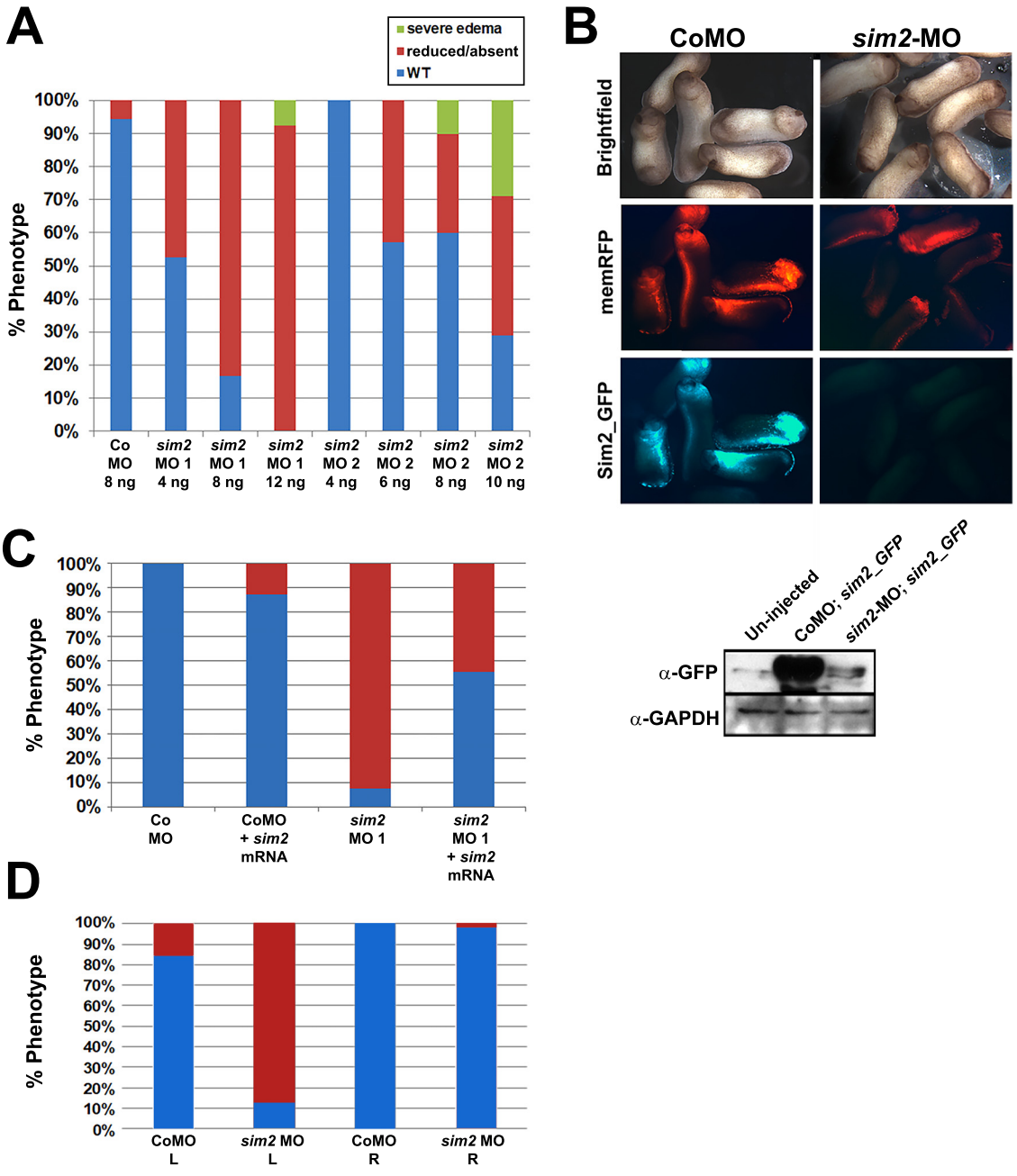

Fig. S3. Specificity and efficacy of the *sim2* morpholino

A) The graph indicates the frequency (percentage) of embryos in which the greater curvature of the stomach was normal (WT, blue) or reduced/absent (red), as elicited by injection of different concentrations of Control MO (CoMO), *sim2*-MO #1 (targeting the ATG start site of *Xenopus sim2* mRNA) or *sim2*-MO #2 (targeting the boundary between intron 1 and exon 2 of *Xenopus sim2*); n= 20 embryos, on average (range 7-38), per condition. As expected, some embryos began to exhibit evidence of non-specific toxicity (severe edema, green) at higher concentrations. The two independent morpholinos displayed similar results, but MO #2 had lower efficacy and higher toxicity, so MO #1 was used for further experiments.

B) *Xenopus* embryos (8-cell stage) were injected with CoMO or *sim2*-MO plus mRNA encoding membrane-tethered Red Fluorescent Protein (memRFP), and mRNA encoding GFP fused with the *sim2* #1 MO target sequence (*sim2*_GFP), and cultured until NF24. Translation of memRFP (red), which does not contain the *sim2*-MO target sequence, remains unaffected by the presence of CoMO or *sim2*-MO. In contrast, while embryos injected with CoMO are able to effectively translate the *sim2*_GFP mRNA, as indicated by the GFP fluorescence (green), translation of this protein is completely knocked down in embryos injected with *sim2*-MO (as indicated by the lack of GFP fluorescence), demonstrating the efficacy of the *sim2*-MO reagent. Western blotting also confirms drastic reduction of GFP protein levels in extracts from *sim2c*-MO- but not CoMO-injected embryos. GAPDH, loading control.

C) Frequency (percentage) of embryos in which the greater curvature of the stomach is normal or reduced/absent when CoMO or *sim2*-MO #1 are co-injected with exogenous, MO-resistant *sim2* mRNA (n= 5-13 per condition). The frequency of *sim2*-MO induced stomach phenotypes was partially rescued (i.e., reduced) by the presence of *sim2* mRNA, confirming the specificity of the MO reagent.

D) Frequency (percentage) of embryos in which the greater curvature of the stomach is normal or reduced/absent when CoMO or *sim2*-MO #1 are targeted to the left (L) versus right (R) side of the stomach (n=3-16 per condition, 3 trials); right side injections have no effect on stomach curvature.

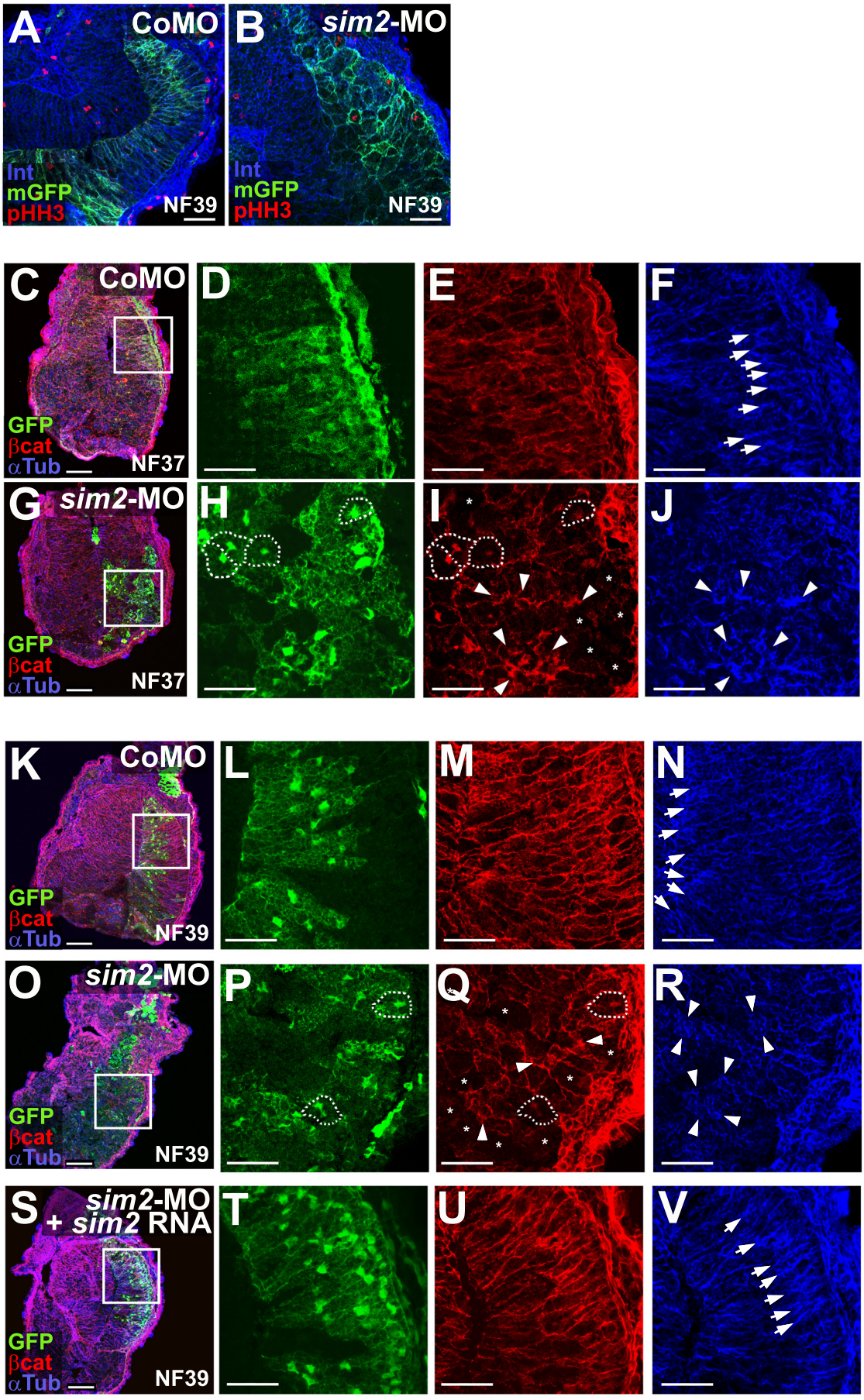

Fig. S4. The *sim2*-MO induced cellular phenotype is independent of proliferation, precedes stomach curvature, and is specific to Sim2 function

A-B) *Xenopus* embryos were injected with mRNA encoding membrane-localized GFP (mGFP) and either control morpholino (CoMO; A) or *sim2* morpholino (*sim2*-MO; B), targeting the left stomach wall. Sections through the stomach (NF39) were stained to reveal Integrin (Int; blue), phoshphohistone H3 (pHH3; mitotic cells; red) and mGFP (green), showing that some *sim2*-MO injected (green) cells are also pHH3-positive; thus, Sim2 activity is not required for endoderm cells to remain proliferative.

C-J) *Xenopus* embryos were injected with mRNA encoding nuclear GFP (GFP) and either control morpholino (CoMO; C-F) or *sim2* morpholino (*sim2*-MO; G-J), targeting the left stomach wall, and harvested several hours prior to stomach curvature (NF37). Sections through the still-straight stomach were stained to reveal nuclear GFP (green; C-D, G-H), β–catenin (βcat, red; C, E, G, I), and alpha-tubulin (αTub, blue; C, F, G, J). The boxed areas in C and G are shown in magnified view in D-F and H-J, respectively. In the left wall of CoMO stomachs, endoderm cells are columnar, with consistently membrane-localized βcat (E) and apicobasally polarized microtubules (arrows, F). In contrast, endoderm cells in *sim2*-deficient stomachs are already rounded (dotted outlines in H and I), with irregularly distributed βcat (I; arrowheads, abnormal accumulation; asterisks, low/absent levels; dotted outlines, nuclear localization), and sparse or randomly-oriented (arrowheads, J) microtubules.

K-V) *Xenopus* embryos were injected with mRNA encoding nuclear GFP plus CoMO (K-N), *sim2*-MO (O-R), or *sim2*-MO plus a MO-resistant mRNA encoding *Xenopus sim2* (S-V) targeting the left stomach wall. Sections through the stomach (NF39) were stained to reveal nuclear GFP (green; K, L, O, P, S, T), β–catenin (βcat, red; K, M, O, Q, S, U), and alpha-tubulin (αTub, blue; K, N, O, R, S, V). The boxed regions in K, O and S are shown in magnified view in L-N, P-R, and T-V, respectively. In the left wall of control stomachs, CoMO-injected endoderm cells are columnar, with consistently membrane localized βcat (M) and apicobasally polarized microtubules (arrows, N). As expected, endoderm cells in *sim2*-deficient (*sim2*-MO injected) stomachs are rounded (dotted outlines in P and Q), with irregularly distributed βcat (Q; arrowheads, abnormal accumulation; asterisks, low/absent levels; dotted outlines, nuclear localization), and sparse or randomly-oriented (arrowheads, R) microtubules. In contrast, consistent membrane-localized βcat (U) and apicobasally polarized microtubules (arrows, V) are restored when *sim2* mRNA is co-injected with the *sim2*-MO, confirming the cellular effects of the morpholino reagent are specific to loss of Sim2 function. Scale bars for A, B, C, G, K, O, and S = 100 μm; scale bars for all other panels = 50 μm.

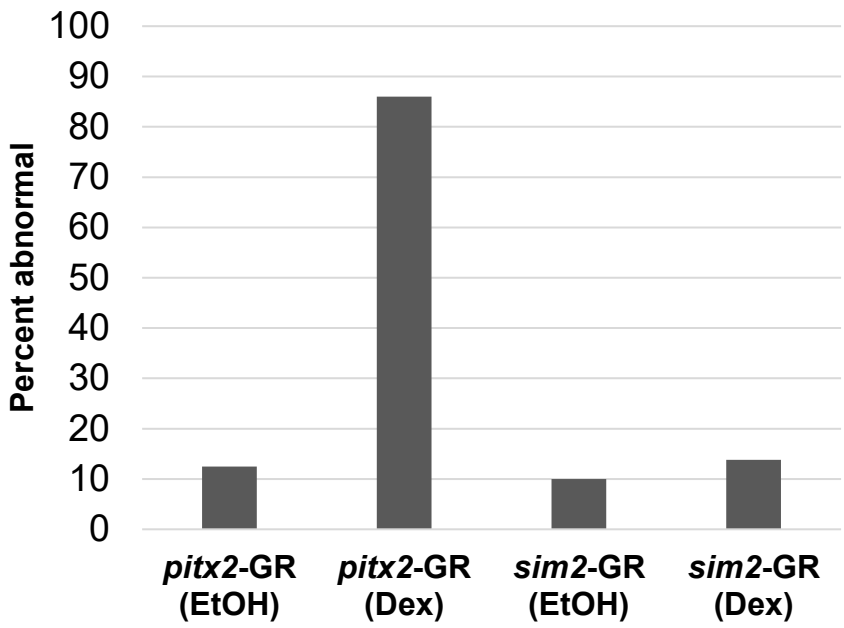

Fig. S5. Over-expression of *sim2* does not alter stomach curvature. *Xenopus* embryos were injected with mRNA encoding a dexamethasone-inducible *pitx2* construct (*pitx2*-GR) or a dexamethasone-inducible *sim2* construct (*sim2*-GR) on the right side and exposed to ethanol control (EtOH) or dexamethasone (Dex). The graph indicates the frequency (average percentage) of embryos in which the greater curvature of the stomach was reduced/absent, as elicited by injection of *pitx2*-GR (n=4-21 per condition, 2 trials) or *sim2*-GR (n=7-16 per conditions, 4 trials). While right side induction of *pitx2*-GR by Dex elicited a high percentage of abnormal stomach phenotypes compared to controls, ectopic induction of *sim2*-GR had no effect on stomach curvature.

Table S1. Genes with left or right enriched expression (q-value ≤0.05) during at least one stage of stomach morphogenesis

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