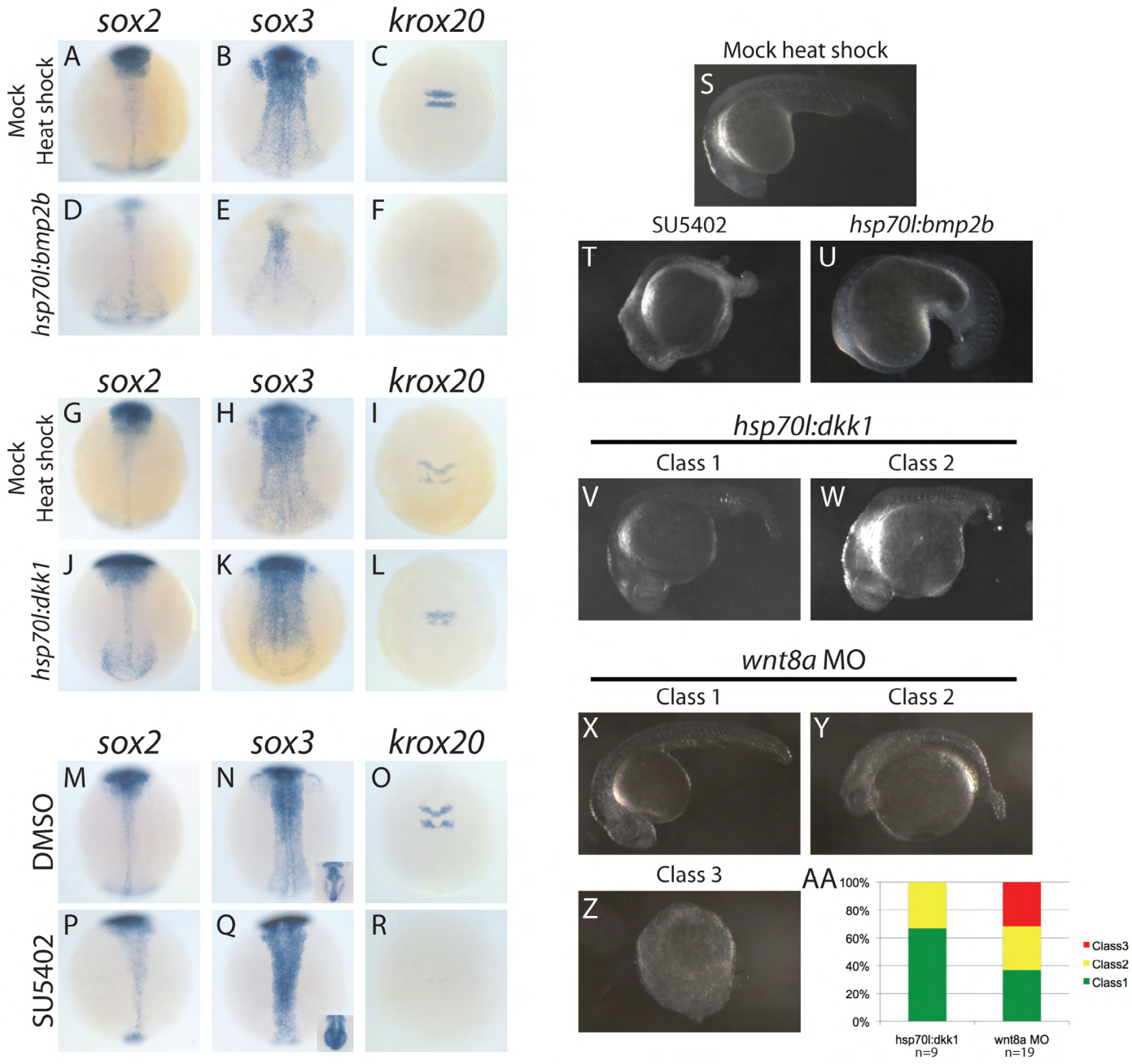


Fig. S1. The effects of heatshock-induced *bmp2b* and *dkk1* overexpression, and SU5402 treatment on *sox2*, *sox3* and *krox20* expression and embryo phenotype. (**A-F**) Embryos carrying the *hsp70l:bmp2b* transgene were heatshocked at shield stage and fixed at 12hpf. *bmp2b* overexpression led to a decrease in the expression of *sox2* (100%, *n*=8) (D versus A), *sox3* (80%, *n*=10) (E versus B) and *krox20* (100%, *n*=10) (F versus C). (**G-L**) Embryos carrying the *hsp70l:dkk1* transgene were heatshocked at shield stage and fixed at 12 hpf. *dkk1* overexpression led to a slight decrease in *sox2* expression and a shortening of its posterior expression domain (54%, *n*=13) (J versus G), a slight decrease intensity of the anterior aspect of *sox3* expression with little change in intensity in the posterior along with a shortening of its posterior expression domain (100%, *n*=12) (K versus H) and a decrease in the extent of *krox20* expression (100%, *n*=17) (L versus I). (**M-R**) Embryos were treated with 90 µM SU5402 starting at 7 hpf and fixed at 12 hpf. SU5402 treatment led to a decrease in the extent and intensity of *sox2* expression (P versus M) (100%, *n*=14) and an almost complete loss of *krox20* expression (R versus O) (100%, *n*=16). SU5402 treatment caused an increase in the level of *sox3* mRNA in the anterior neural plate, but the expression pattern was less organized (Q versus N, insets) (100%, *n*=16). (**S-Z**) The 18 hpf phenotypes of embryos undergoing various treatments are shown. The phenotype of embryos treated with SU5402 is similar to the *fgf8a* mutant phenotype (Brand et al., 1996), including a bulge in the midbrain primordium and defects in mesoderm development (T versus S). Embryos overexpressing *bmp2b* exhibit strong ventralization (U versus S). Embryos overexpressing *dkk1* or injected with *wnt8a* MO exhibit anterior-posterior patterning defects consistent with a loss of Wnt signaling (V-Z versus S). We split embryos injected with *wnt8a* MO or overexpressing *dkk1* into three classes of increasingly severe Wnt phenotypes (V-Z). (**AA**) The proportion of embryos from each treatment in each class is shown. In general, embryos injected with *wnt8a* MO had more severe phenotypes then those overexpressing *dkk1* after a shield-stage heatshock.

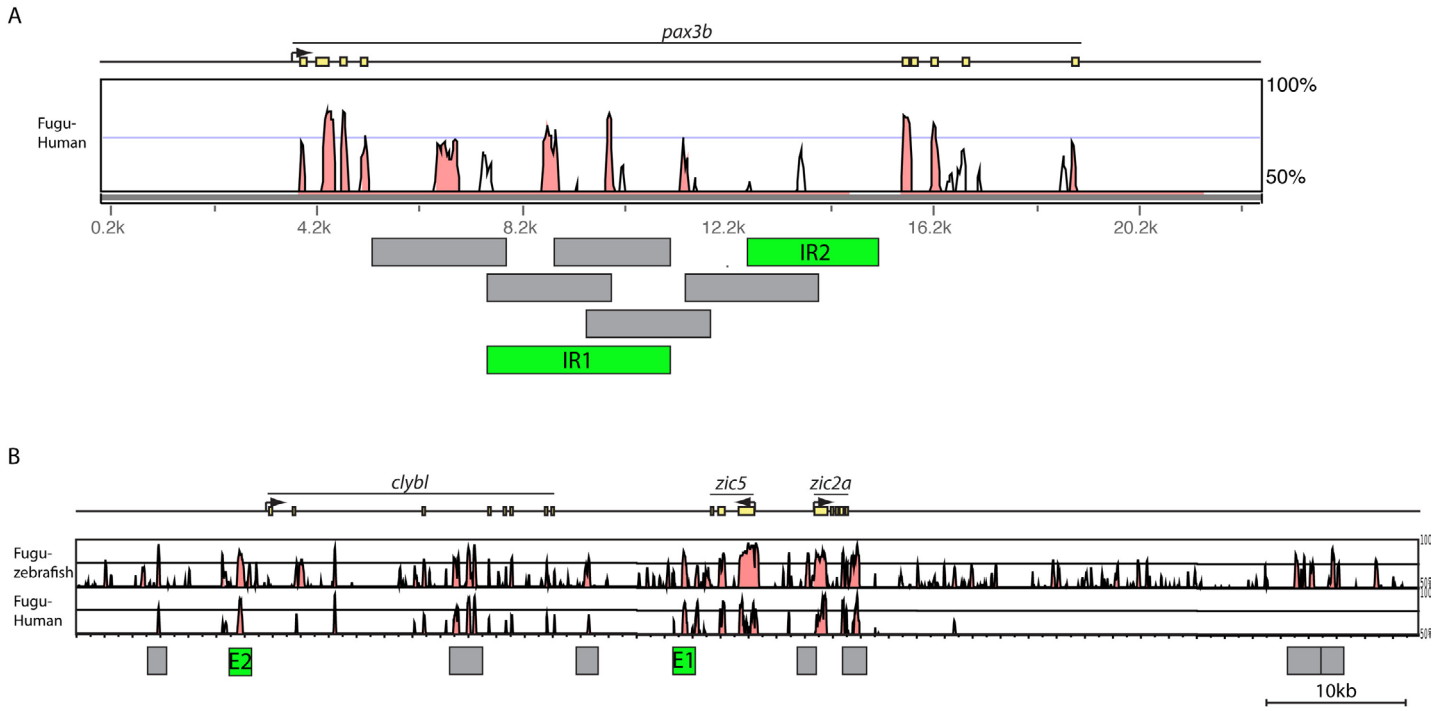


Fig. 2. Enhancer search strategy. (**A**) The genomic region of *Fugu pax3b* is shown with the level of sequence identity between human and *Fugu* plotted below. Intron 4 of *pax3b* is well conserved between human and *Fugu* so we tested overlapping regions from across intron 4 for enhancer activity. The regions tested are shown as boxes under the Vista plot. Regions in green drove robust neural plate border expression and regions in gray did not. Homologs of *pax3b* IR1 and IR2 are easily identifiable in its close paralog *pax3a* and the *pax3a* enhancers drove more robust gene expression, so we focused our studies on *pax3a* IR1 and IR2. (**B**) The genomic region of *Fugu zic2a* and *zic5* is shown with the level of sequence identity between zebrafish and *Fugu*, and human and *Fugu* plotted below. We tested the enhancer activity of nine regions from the *zic2a/zic5* locus which had greater than 75% *Fugu*-zebrafish sequence identity. The locations of these regions are shown as boxes under the Vista plot. The green boxes indicate regions that drive neural plate border gene expression, the gray boxes indicate regions that do not. Homologs of *zic2a/zic5* E1 and E2 are identifiable in the *zic3/zic6* locus. We focused on the *zic3/zic6* enhancers in this study because *zic3/zic6* regulation is less well studied than *zic2a/zic5* regulation.

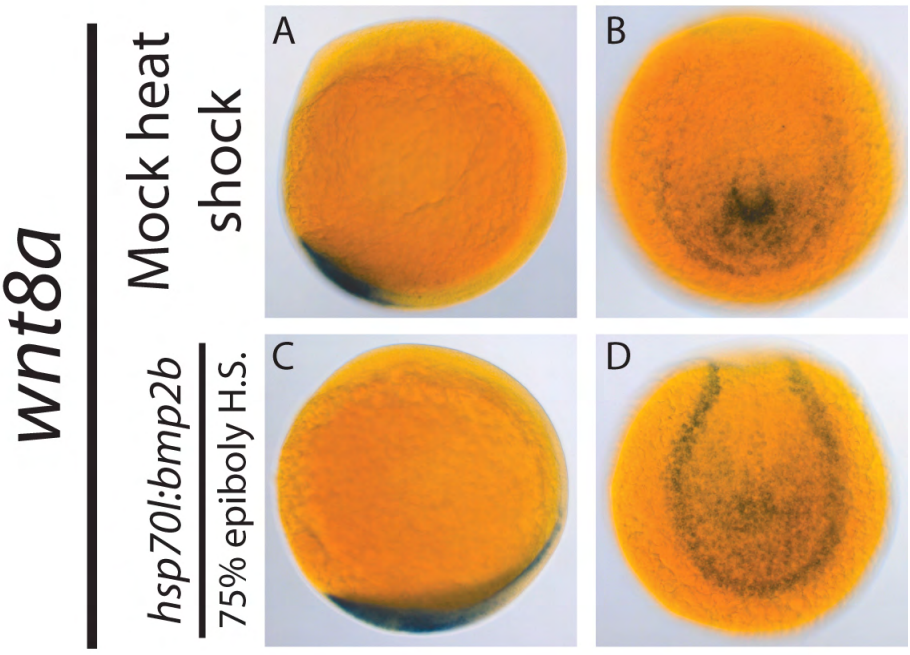


Fig. S3. *bmp2b* overexpression leads to an upregulation of *wnt8a*. *hsp70l:bmp2b* embryos were heatshocked at 75% epiboly, fixed at six somites and stained for *wnt8a* mRNA. Embryos overexpressing *bmp2b* had an increased level of *wnt8a* expression in the tail ectoderm relative to mock heatshocked embryos (100%, 14 of 14) (C,D versus A,B). A and C are lateral views with dorsal to the right; B and D are posterior dorsal views.

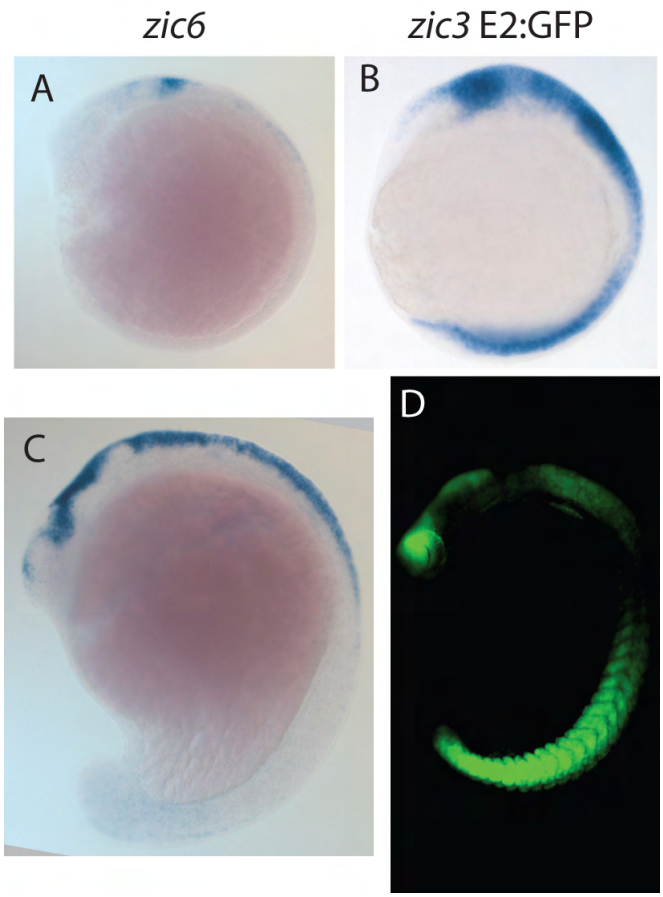


Fig. S4. *zic6* is expressed in a similar pattern to *zic3* E2-driven GFP in the extoderm. (**A**,**B**) *zic6* expression (A) and GFP expression in *zic3* E2:GFP embryos (B) at 12 hpf. (**C**,**D**) *zic6* expression (C) and GFP expression in *zic3* E2:GFP embryos (D) at 24 hpf. All pictures are lateral views with dorsal towards the right.

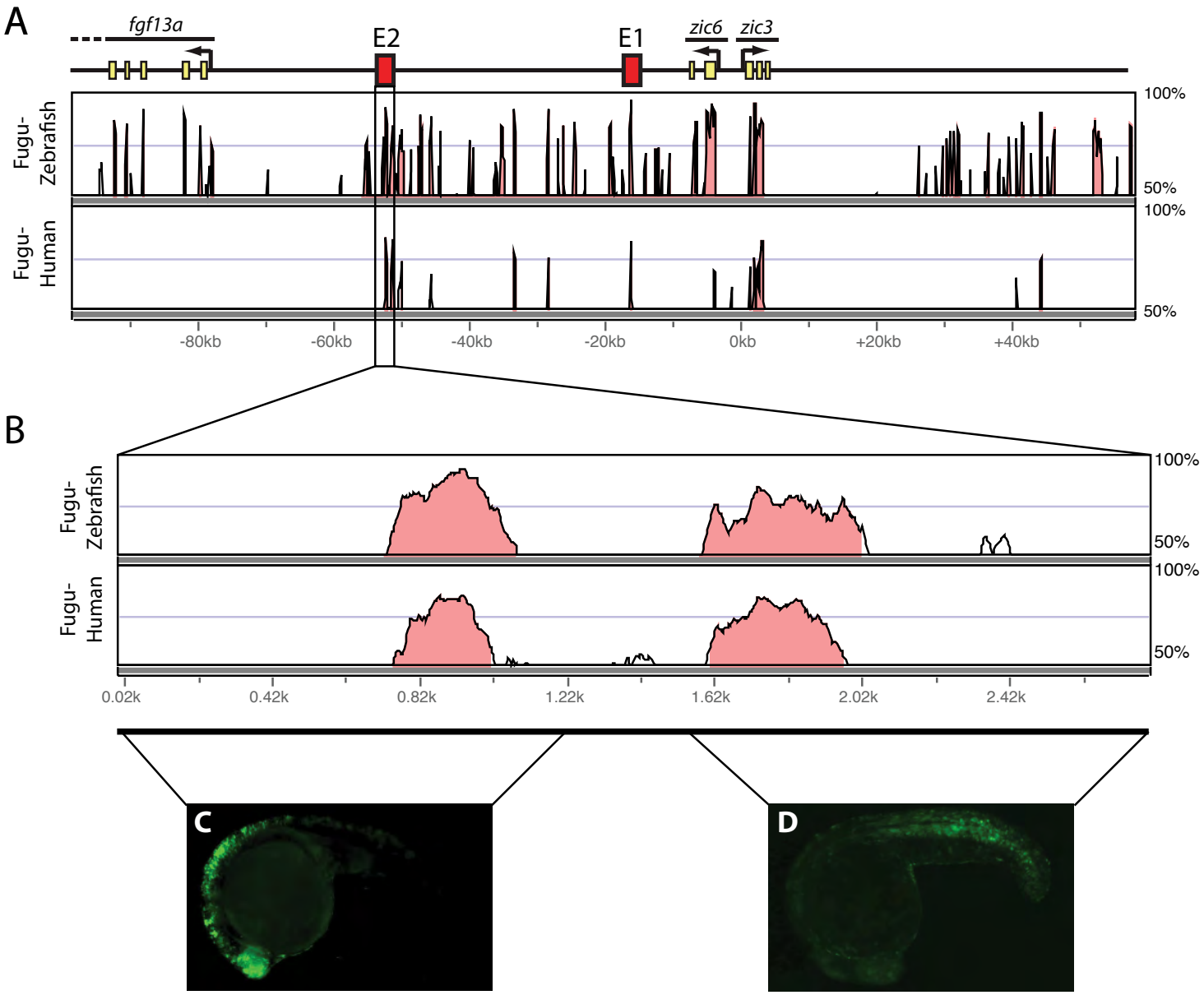
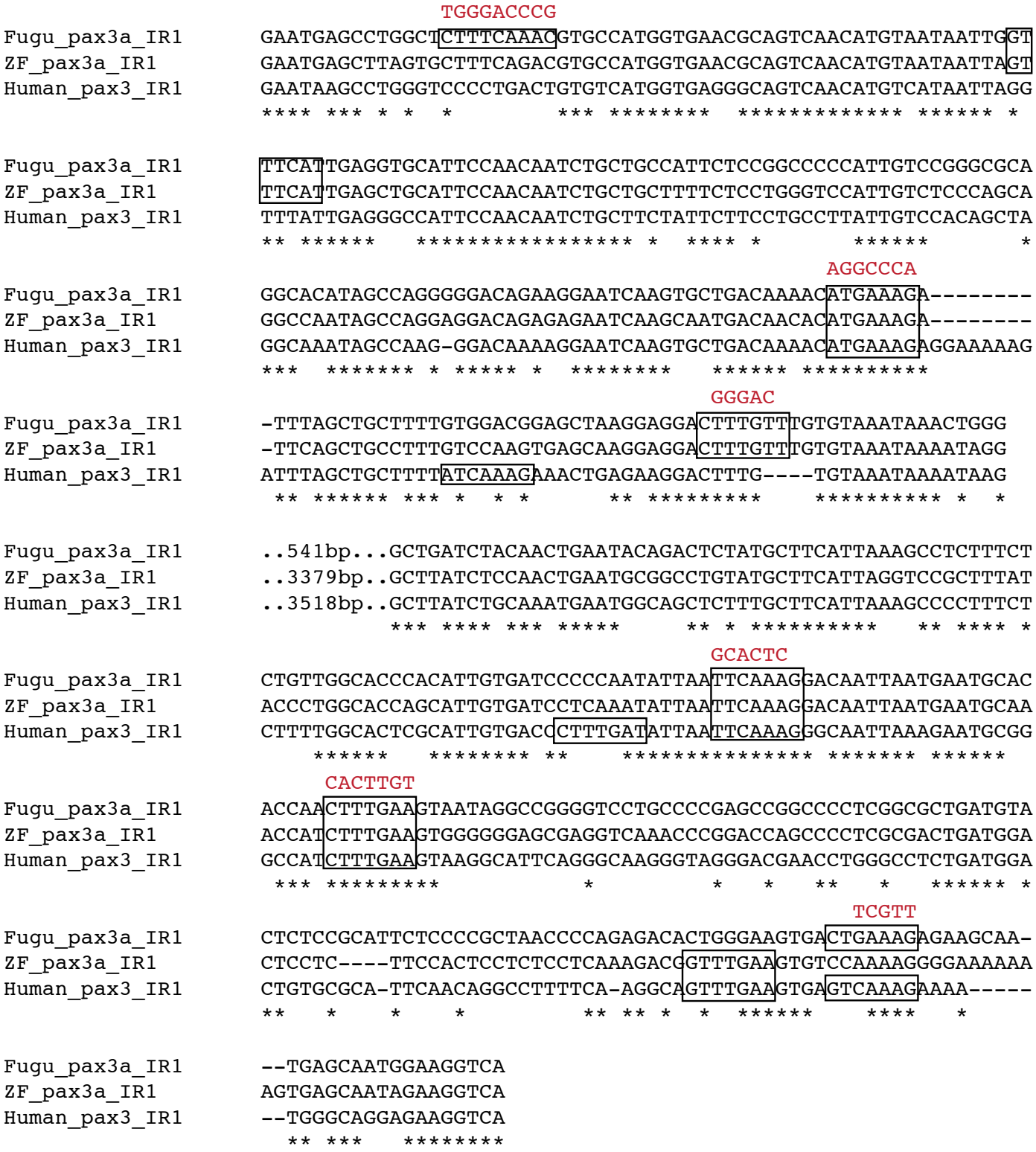


Fig. S5. The neural plate border and mesodermal enhancer activities of *zic3* E2 are separable. (**A**) The positions of *zic3* E1 and E2 are shown with the level of sequence identity between *Fugu* and zebrafish and *Fugu* and human underneath. (**B**) The Vista plot comparing *Fugu*, zebrafish and human *zic3* E2 regions indicates that there are two regions of high sequence identity within the enhancer. The 5′ and 3′ regions of *zic3* E2 were separately placed upstream of the mouse Fos promoter and GFP, and their activities were analyzed in transiently transgenic embryos. (**C**,**D**) The 5′ region of E2 drives dorsal neural tube expression (C) and the 3′ region drives mesodermal GFP expression (D). C,D are lateral views with dorsal upwards and anterior towards the left.

A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ZF_pax3a_IR1 GAATGAGCTTAGTGCTTTCAGACGTGCCATGGTGAACGCAGTCAACATGTAATAATTAGT
Human_pax3_IR1 GAATAAGCCTGGGTCCCCTGACTGTGTCATGGTGAGGGCAGTCAACATGTCATAATTAGG
Fugu_pax3a_IR1 TTCATTGAGGTGCATTCCAACAATCTGCTGCCATTCTCCGGCCCCCATTGTCCGGGCGCA
ZF_pax3a_IR1 TTCATTGAGCTGCATTCCAACAATCTGCTGCTTTTCTCCTGGGTCCATTGTCTCCCAGCA
Human_pax3_IR1 TTTATTGAGGGCCATTCCAACAATCTGCTTCTATTCTTCCTGCCTTATTGTCCACAGCTA
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ZF_pax3a_IR1 -TTCAGCTGCCTTTGTCCAAGTGAGCAAGGAGGACTTTGTTTGTGTAAATAAAATAGG
Human_pax3_IR1 ATTTAGCTGCTTTTATCAAAGAAACTGAGAAGGACTTTG----TGTAAATAAAATAAG
Fugu_pax3a_IR1 ..541bp...GCTGATCTACAACTGAATACAGACTCTATGCTTCATTAAAGCCTCTTTCT
ZF_pax3a_IR1 ..3379bp..GCTTATCTCCAACTGAATGCGGCCTGTATGCTTCATTAGGTCCGCTTTAT
Human_pax3_IR1 ..3518bp..GCTTATCTGCAAATGAATGGCAGCTCTTTGCTTCATTAAAGCCCCTTTCT
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ZF_pax3a_IR1 ACCATCTTTGAAGTGGGGGGAGCGAGGTCAAACCCGGACCAGCCCCTCGCGACTGATGGA
Human_pax3_IR1 GCCATCTTTGAAGTAAGGCATTCAGGGCAAGGGTAGGGACGAACCTGGGCCTCTGATGGA
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Fugu_pax3a_IR1 --TGAGCAATGGAAGGTCA
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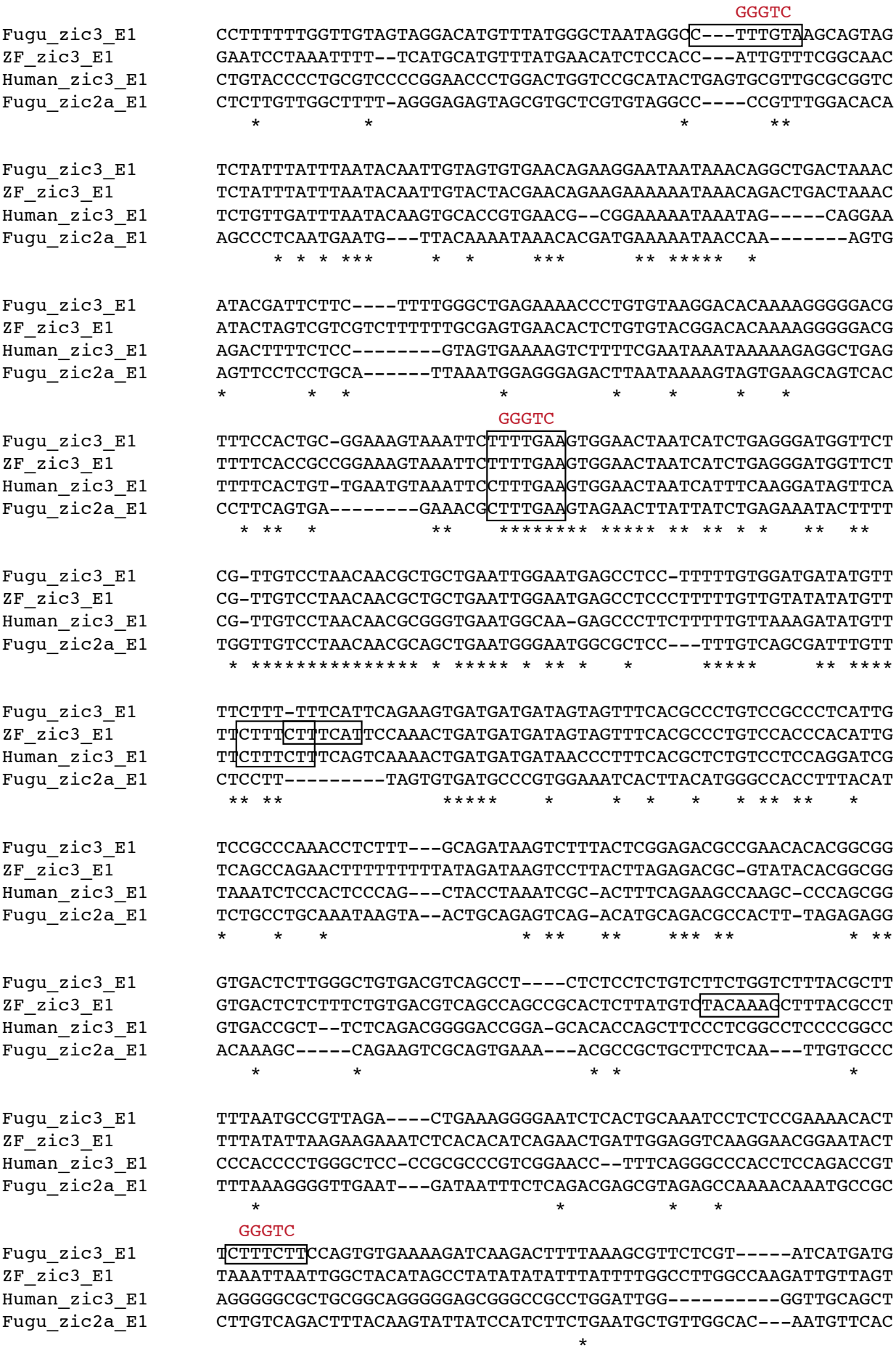
Human_pax3_IR1 --TGGGCAGGAGAAGGTCA

B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Human_zic3_E1 CTGTACCCCTGCGTCCCCGGAACCCTGGACTGGTCCGCATACTGAGTGCGTTGCGCGGTC
Fugu_zic2a_E1 CTCTTGTTGGCTTTT-AGGGAGAGTAGCGTGCTCGTGTAGGCC----CCGTTTGGACACA
GGGTC
Fugu_zic3_E1 TTTCCACTGC-GGAAAGTAAATTCTTTTGAAGTGGAACTAATCATCTGAGGGATGGTTCT
ZF_zic3_E1 TTTTCACCGCCGGAAAGTAAATTCTTTTGAAGTGGAACTAATCATCTGAGGGATGGTTCT
Human_zic3_E1 TTTTCACTGT-TGAATGTAAATTCCTTTGAAGTGGAACTAATCATTTCAAGGATAGTTCA
Fugu_zic2a_E1 CCTTCAGTGA--------GAAACGCTTTGAAGTAGAACTTATTATCTGAGAAATACTTTT
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C

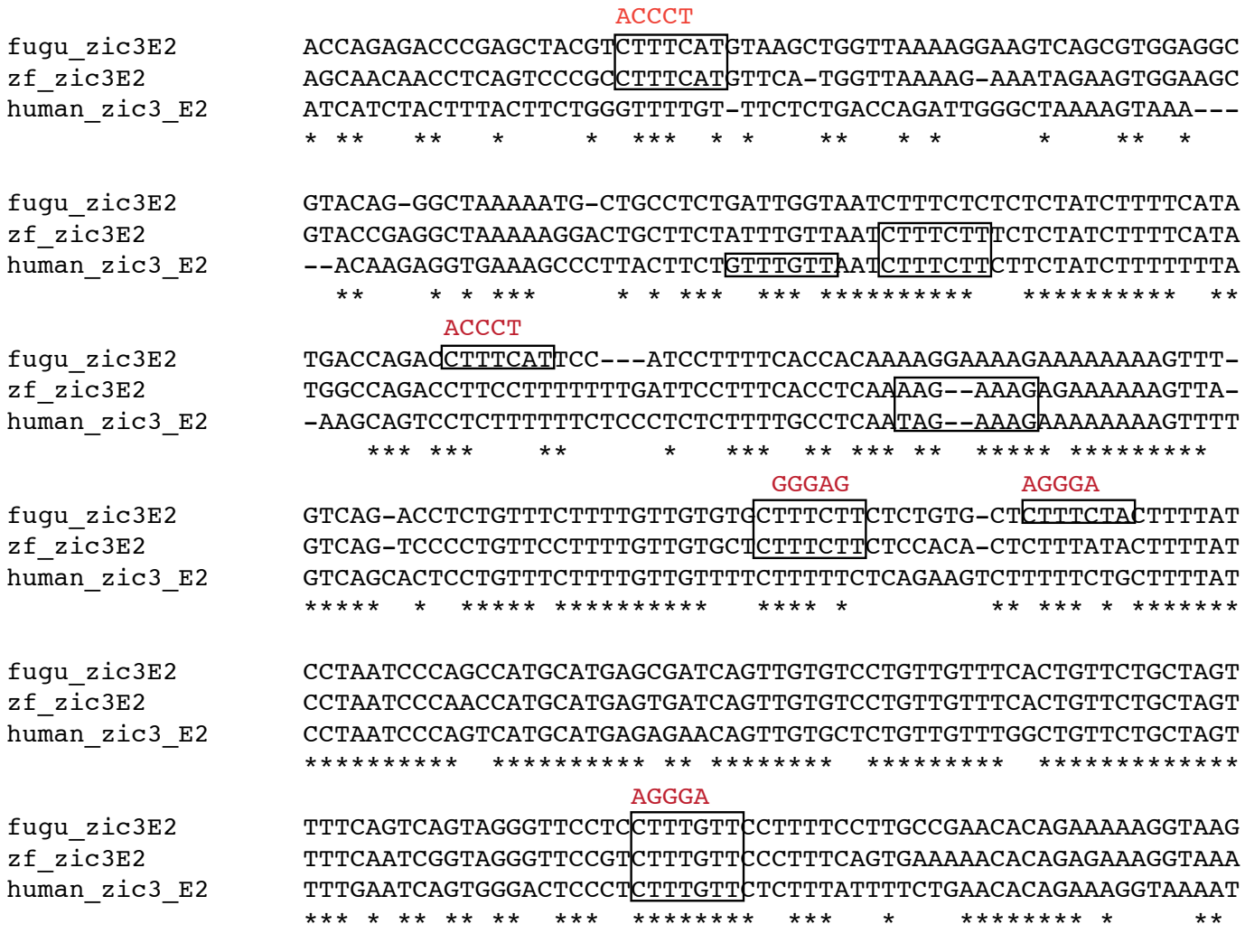

Fig. S6. Sequences of putative Tcf/Lef binding sites in *pax3a* IR1, *zic3* E1 and *zic3* E2. (**A**) The alignment of the *Fugu*, zebrafish and human sequences for two highly conserved regions in *pax3a* IR1 are shown with putative Tcf/Lef-binding sites outlined. The mutations made in Fig. 6 are shown in red above the alignment. (**B**) The alignment of a highly conserved region of *Fugu*, zebrafish and human *zic3* E1 along with the sequence of paralogous the *zic2a* D5 region described by Nyholm et al. (Nyholm et al., 2007). Putative Tcf/Lef sites are outlined and the mutations made in Fig. 8 are shown in red above the alignment. (**C**) The alignment of a highly conserved region of *Fugu*, zebrafish and human *zic3* E2. Putative Tcf/Lef sites are outlined and the mutations made in Fig. 8 are shown in red above the alignment.

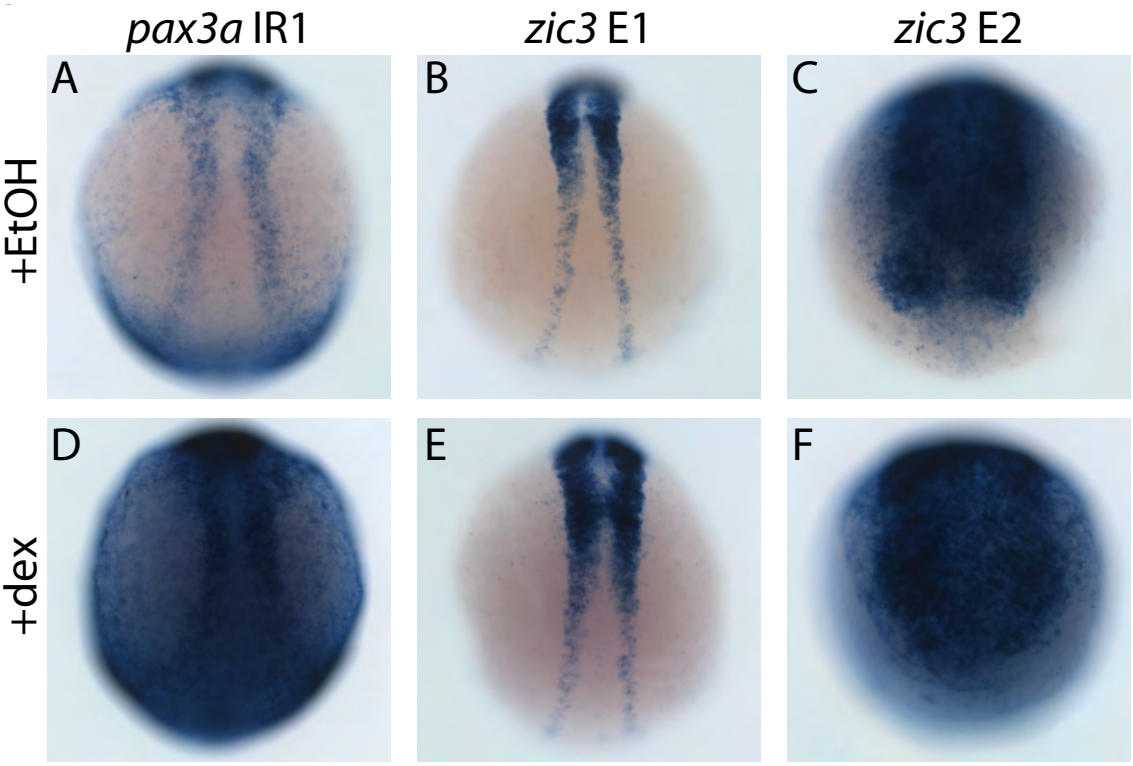

Fig. S7. Constitutively active Lef1 activates *pax3a* IR1, *zic3* E1 and *zic3* E2 activity in the absence of cycloheximide. Embryos from the *pax3a* IR1, *zic3* E1 and *zic3* E2 transgenic lines were injected with glucocorticoid receptor-tagged Lef1-β-catenin and treated with ethanol (A-C) or dexamethasone (D-F) beginning at tailbud stage. *pax3a* IR1 exhibited an increase in enhancer activity upon induction of Lef1-β-catinin (D versus. A) (79%, *n*=19) as did *zic3* E1 (E versus B) (45%, *n*=11) and E2 (F versus C) (100%, *n*=11).

Table S1. Primer sequences

Primer name	Sequence	Notes
pax3a IR1 forward	ggggACAAgTTTgTACAAAAAAgCAggCTCCTATATGTTGTCGCAGCAGTAGAGG	Used for amplifying pax3a IR1 from genomic DNA
pax3a IR1 reverse	ggggACCACTTTgTACAAgAAAgCTgggTCATCACCGACCCCGCAACAAACTCCC	
pax3a IR2 forward	ggggACAAgTTTgTACAAAAAAgCAggCTCTGTCACCTAGCCGCTCCGCAGTGG	Used for amplifying pax3a IR2 from genomic DNA
pax3a IR2 reverse	ggggACCACTTTgTACAAgAAAgCTgggTCAGTGCGATGGGCATAAAGGGAGC	
zic3 E1 forward	ggggACAAgTTTgTACAAAAAAgCAggCTGGGCTACCGCTGGTGTATCCTGG	Used for amplifying zic3 E1 from genomic DNA
zic3 E1 reverse	ggggACCACTTTgTACAAgAAAgCTgggTCGGACAAGCAGAAAGGCTGACAG	
zic3 E2 forward	ggggACAAgTTTgTACAAAAAAgCAggCTCAGGGTTGACCTGGCCTTATCACC	Used for amplifying zic3 E2 from genomic DNA
zic3 E2 reverse	ggggACCACTTTgTACAAgAAAgCTgggTCCAACACAGGCTAGGTTCTACC	
pax3a IR1 mTcf1 for	CCCGgtgccatggtgaacgcag	Used for mutating putative Tcf/Lef binding sites in the pax3a IR1 enhancer. mTcf2 and mTcf3 were used together and mTcf4 and mTcf5 were used together.
pax3a IR1 mTcf1 rev	TCCCAagccaggctcattcaatttgac	
pax3a IR1 mTcf3 for	gagctaaggaggacGGGACttgtgtaaataaactgggttacc	
pax3a IR1 mTcf2 rev	cgtccacaaaagcagctaaatTGGGCCTgttttgtcagcacttgattcc	
pax3a IR1 mTcf5 for	atgcacaccaacacttgtgtaataggccggggtcctgcc	
pax3a IR1 mTcf4 rev	tcattaattgtccgagtgcttaatattgggggatcacaatgtg	
pax3a IR1 mTcf6 for	gacactgggaagtgacttcgttagaagcaatgagcaatgg	
pax3a IR1 mTcf6 rev	ccattgctcattgcttctaacgaagtcacttcccagtgtc	
zic3 E1 mTcf1 for	cttgatcttttcacactggaGACCCgaagtgttttcggagagg	Used for mutating putative Tcf/Lef binding sites out of zic3 E1.
zic3 E1 mTcf1 rev	cctctccgaaaacacttcGGGTCtccagtgtgaaaagatcaag	
zic3 E1 mTcf2 for	cagatgattagttccactGACCCagaatttactttccgcag	
zic3 E1 mTcf2 rev	ctgcggaaagtaaattctGGGTCagtggaactaatcatctg	
zic3 E1 mTcf3 for	gtattaaataaatagactactgcttGACCCggcctattagcccataaac	
zic3 E1 mTcf3 rev	gtttatgggctaataggccGGGTCaagcagtagtctatttatttaatac	
zic3 E2 mTcf1 for	GAGACCCGAGCTACGTaccctATGTAAGCTGGTTAAAAGG	Used for mutating putative Tcf/Lef binding sites out of zic3 E2. mTcf3 and mTcf4 were used together.
zic3 E2 mTcf1 rev	CCTTTTAACCAGCTTACATagggtACGTAGCTCGGGTCTC	
zic3 E2 mTcf2 for	ctttTCATATGACCAGACaccctATTCCATCCTTTTcacc	
zic3 E2 mTcf2 rev	ggtgAAAAGGATGGAATagggtGTCTGGTCATATGAaaag	
zic3 E2 mTcf4 for	TGCTagggaTACTTTTATCCTAATCCCAGC	
zic3 E2 mTcf3 rev	CAGAGActcccGCAcacaacaaaagaaacagagg	
zic3 E2 mTcf5 for	GTCAGTAGGGTTCCTCagggaTTCCTTTTCCTTGCCGAACAC	
zic3 E2 mTcf5 rev	GTGTTCGGCAAGGAAAAGGAAtccctGAGGAACCCTACTGAC	