Supplementary Figures

Supplementary Figure 1

Injection of MOs targeted at splice sites causes alterations in processing of primary RNA transcripts. A). *smoc2*. The MO targeted the splice donor of exon 11 of *smoc2*. This gene contains 13 coding exons. Using RT-PCR primers that annealed to exons 10 and 13, a product of 400nts is present in the wild-type sample (lane 2). In the morphants (lane 3) we detected two aberrant products: (1) a lower band representing a loss of 98nt from the 3'-end of exon 11, which was then spliced to exon 12 producing a frameshift; and (2) and upper band indicating the maintenance of the entire 423bp intron 11 sequence. Zebrafish Smoc2 is normally a 440 amino acid protein and the deletion of exon 11 sequences coupled with the frameshift would delete the C-terminal 52 amino acids and replace this with 4 amino acids before a stop codon. The second form with the inclusion of intron 11 would replace the normal C-terminal 19 amino acids with 26 amino acids encoded by the intron sequence prior to a stop codon. B). *meis1*. The SPL MO targeted the exon 1 splice donor. RT-PCR primers were designed to sequences located in exons 1 and 6. These primers produced a 600nt product from wild-type zebrafish RNA (lane 1). The presence of the MO caused inclusion of the 1379 nt first intron sequence in the *meis1* transcript (lane 2). The normal Meis1 protein is nearly 400 amino acids in length. The resulting protein produced by the aberrant message would encode only 4 amino acids of

the normal Meis1 protein from exon 1, before introducing an in-frame stop-codon after an additional 34 amino acids derived from the intronic sequences. C). *wu:fb16h09*. This gene has 4 exons and the MO targeted the exon 4 splice acceptor. RT-PCR primers were designed to sequences in intron 3 and exon 4. In wild-type samples, there was no detectable product (lane 1). In contrast, a ~665 nt product was present in the morphant fish representing transcripts that still contained intron 3 sequences. Control samples indicated that intact cDNA was present in both samples as judged by detection of a β-actin RT-PCR product. Note that intron 3 is 9.8kb in length. The protein encoded by *wu:fb16h09* is normally 574 amino acids, but only 104 amino acids are encoded by the first three exons. D) *macc1*. Two independent SPL MOs were used to target this gene. Top Panel: The SPL1 MO targeted the intron 2-exon 3 splice acceptor. RT-PCR primers were designed to sequences present in exons 1 and 3 (R1). Together these produced an ~ 320nt product from wild-type zebrafish cDNA (Top, Lane 1). In the presence of SPL1 MO, an additional band was produced that included the 186nts of intron 2 (Top, Lane 2 and data not shown). This would lead to the presence of a premature termination codon after an additional 11 amino acids encoded by the intronic sequence downstream of exon 2, resulting in a protein of 51 amino acids rather than the normal predicted 854 amino acid protein. Using primers present in exons 1 and 4 (R2), a second mis-spliced product was also detected that lacked both exons 2 and 3, which would also delete the majority of the protein coding sequence (data not shown). Bottom Panel: SPL2 MO, targeted the exon 2-intron 2 splice donor. RT-PCR primers

were again present in exons 1 and 3 (R1). The ~320 nt wild-type product (lane 1) was altered to a 155 nt morphant product (lane 2), which was caused by deletion of the 168 nt exon 2 sequence. This would be expected to alter the less well-conserved N-terminus of the protein. E). *vgll4l*. The *vgll4l* SPL MO targeted the splice acceptor site of exon 5. RT-PCR primers were present in exons 3 and 5. The wild-type product was 270nts (lane 1). The presence of the SPL MO generated a new band that also contained the 114 nt intron 4 sequence (Lane 2). This would introduce an additional 38 amino acids encoded by the intron after amino acid 166 in a protein that is normally 266 amino acids in length. F) *pkdcc.* The SPL MO targeted the exon 4 splice donor. RT-PCR primers were targeted to sequences in exons 3 and 6. The wild-type product was ~400 nts (lane 1), and in the presence of the MO several additional products were generated. Sequence analysis revealed mis-spliced products that included intron 4, or included both introns 4 and 5, or skipped exon 4. These failures in splicing produced mRNAs with premature stop codons leading to >125 amino acid truncations of the conserved *pkdcc* C-terminus. M: size markers. Solid arrows, wild-type product; dotted arrows, aberrant products.

Supplementary Figure 2

Expression of *smoc2*, *meis1*, *meis2a*, and *vgll4l* at 24 and 48hrs of zebrafish development. All images are lateral views, anterior to the left. A, C, E, G are 24 hpf and B, D, F, H are 48 hpf. A, B) At 24 hpf, *smoc2* is expressed in specific brain regions as well as tail mesoderm, including somites and notochord. At 48

hpf, *smoc2* expression resolves to the brain and pharyngeal pouches. C, D) *meis1* is expressed in the hindbrain and spinal cord as well as the olfactory bulb and retina at 24hpf. Expression is also observed in the retina and branchial arches at 48hpf. E, F) *meis2a* is expressed in the midbrain, hindbrain and spinal cord as well as the olfactory bulb at 24 hpf. By 48 hpf, expression expands to include the branchial arches. G, H) At 24-48 hpf, *vgll4l* is expressed in the nose, pharyngeal pouches, lateral line, epidermis and pronephric duct. (All images taken from ZFIN with permission from Thisse et al., 2001 - Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission).

Supplementary Figure 3.

Cartilage rotation and fusion defects in *meis1* and *meis2a* morphants. A, Ventral view of viscerocranial cartilages of a 5dpf uninjected larva. A', Magnified view of A showing position of palatoquadrate (arrowhead). B, Ventral view of viscerocranial cartilages of a 5dpf *meis1* ATG morphant larva. B', Magnified view of B showing cartilage fusions in morphants as well as an apparent rotation of the palatoquadrate cartilage (arrowhead). C, Ventral view of viscerocranial cartilages of a 5pf morphant larva co-injected with both *meis1* ATG and *meis1* SPL MOs. C', Magnified view of C showing cartilage fusions in morphants and the apparent rotation of the palatoquadrate cartilage (arrowhead). D, Ventral view of viscerocranial cartilages of a 5dpf *meis2a* ATG morphant larva. D', Magnified view of D showing cartilage fusions (asterisks) in morphants. E,

Ventral view of viscerocranial cartilages that were dissected and flat-mounted from a 5dpf *meis2a* ATG morphant larva showing cartilage fusions (asterisks). Arrow indicates where a cartilage fusion was broken during dissection.

Supplementary Figure 4

Expression of *macc1*, *thsz2*, *pkdcc (LOC565254)*, *vlk*, *lix1l*, and *pcdh19* at 24 and 48hrs of zebrafish development. All images are lateral views, anterior to the left. A, C, E, G, I and K are 24 hpf and B, D, F, H, J and L are 48hpf. A, B) *macc1* expression at 24hpf is observed in the olfactory bulb, eye, midbrain-hindbrain boundary, otic vesicle, and pronephros. At 48hpf, *macc1* expression continues in the otic vesicle and becomes apparent in the roof of the stomodeum. C, D) *thsz2* is expressed in the olfactory bulb, eye and hindbrain at both 24 and 48hpf. Weak expression also occurs in the branchial arches. E, F) *pkdcc (LOC565254)* is expressed diffusely in the eye and anterior and posterior mesoderm at 24hpf and resolves to the eye, branchial arches, and the region of the forming neurocranium at 48hpf. G, H) *vlk* expression at 24hpf is observed in the eye, posterior branchial arches and fin bud. By 48hpf, expression expands to the more anterior branchial arches. I, J) Expression of *lix1l* is observed in the eye and hindbrain at 24 and 48hpf as well as more diffusely in the branchial arch region at 48hpf. K, L) *pcdh19* is expressed in all CNS structures at 24hpf and resolves to the olfactory bulb, eye, midbrain, and hindbrain at 48hpf. Abbreviations: b, brain; ba, branchial arches; e, eye; fb, fin bud; hb, hindbrain; m, mesoderm; mb, midbrain; mhb midbrain-hindbrain boundary; n,

neurocranium; o, olfactory bulb; ov, otic vesicle; pba, posterior branchial arches; pn, pronephros; s, stomodeum; sc, spinal cord.

Supplementary Figure 5.

Skeletal phenotypes obtained from other potential Class I-III morphants. A, ventral view of 5dpf skeleton of a *lix1l* ATG morphant showing inversion of the ceratohyal and loss of ceratobranchials. A', ventrolateral view of the same larva in (A). B, ventral view of a 5dpf skeleton of a *pcdh19* ATG morphant showing reduction of the ethmoid plate. B', flat mount of the neurocranium of *pcdh19* ATG morphant showing reduction of ethmoid plate and fusion of trabeculae. C and C', ventral view of a 5dpf skeleton of a *vlk* SPL morphant showing mild (C) and severe (C') loss of neural crest-derived cartilages.

Supplementary Figure 6.

Examples of morphant phenotypes maintained in the absence of p53. All images are of the morphant phenotypes seen in p53 null larvae. A, Ventral view of viscerocranial cartilages of a 5dpf *smoc2* SPL morphant showing inversion of the ceratohyal, and a reduction in ceratobranchial size and number. Figure 2 shows similar morphant phenotype obtained in wild-type larva. B, C, Compare with *macc1* morphant phenotypes obtained in wild-type larva shown in Figure 5. B, Ventral view of viscerocranial cartilages of a 5dpf *macc1* spl1 morphant larva presenting with a mild phenotype - loss of ceratobranchials, inversion of the angle between the paired ceratohyal and reduction of Meckel's cartilage. C,

Ventral view of viscerocranial cartilages of a 5dpf *macc1* SPL1 morphant presenting with a severe phenotype – almost complete aplasia of all head cartilages. D, E, Compare with *meis1* and *meis2a* morphant phenotypes obtained in wild-type larva shown in Figure 3. D, Ventral view of viscerocranial cartilages of a 5dpf *meis1* ATG morphant larva showing cartilage fusions between Meckel's and palatoquadrate cartilages and ceratohyal and ceratobranchial cartilages. E, Ventral view of viscerocranial cartilages of 5dpf *meis2a* ATG morphant larva showing cartilage fusions between paired Meckel's cartilages, Meckel's and palatoquadrate, and ceratohyal and ceratobranchial cartilages. Both *meis* morphants also show ectopic cartilages (arrowhead) and cartilage fusions (*). Abbreviations: cb, ceratobranchials; ch, ceratohyal; ep, ethmoid plate; hs, hyosymplectic; m, Meckel's; and pq, palatoquadrate.

Supplementary Figure 7.

Analysis of *Adap1* expression and function in the larval zebrafish.

A, lateral view of a 24hpf zebrafish embryo showing expression of *adap1* in the branchial arches. A', dorsolateral view of embryo in (A) showing the branchial arch expression. B, lateral view of a 48hpf zebrafish embryo showing continued expression of *adap1* in the pharyngeal endoderm. B', rostral view of embryo in (B) showing *adap1* expression around the stomodeum. C, lateral view of 24hpf uninjected embryo. D, lateral view of 24hpf larva after injection with 10ng adap1 SPL1 MO showing delayed somitogenesis and severely shortened body axis and

yolk extension. Few *adap1* morphants survive to 5dpf for analysis. E, lateral view of 5dpf *adap1* morphant showing collapse of the rostral aspect of the head and curvature of the body axis. F, ventral view of *adap1* morphant skeleton showing no changes to the cartilage structure.

Table S1. Summary of genes that were analyzed in the screen.

	Number of Genes
Total number of mouse genes selected for analysis based on expression profile	**87**
Mouse genes excluded, because they are non-coding*	**11**
Mouse genes for which we did not identify a zebrafish ortholog with available sequence data	**12**
Mouse genes that had a zebrafish ortholog	**64**
Zebrafish orthologs that were identified after the conclusion of these studies due to revision of the zebrafish genome	**9**
Zebrafish genes not targeted, because they lacked a verifiable craniofacial expression pattern	**14**
Zebrafish genes that did not have a craniofacial phenotype when targeted with one MO**	**26**
Zebrafish genes that produced a craniofacial phenotype with one MO that was not reproduced with a second MO	**7**
Zebrafish genes that produced a consistent craniofacial phenotype with two or more MOs	**8**

(*) Non-coding RNAs are often not good targets for MOs. They cannot be targeted with translation blocking MO, and detailed knowledge must be available to block processing.

(**) This includes those genes that produced early lethal phenotypes that precluded analysis of craniofacial defects (Table S2) or those for which a mouse knockout became available during the course of the screen.

Table S2. Morpholino sequences for genes that were not studied in detail.

Gene Name	Mouse Accession ID	Zebrafish Accession ID	MO sequence	CF Phenotype*
Lrba	NM_030695.2	XM_685762	TGTTTTAATCAGCTTACCAGATTGA	N
Tmem26	NM_177794	XM_001334449	ATG ATCATCGTAAAGTGAGCAGCGTCCC	N
Cxxc5	NM_133687	XM_681066	ATG CTGTCCGCCAGACATGGTCCAGCCC	N
MEGF10	NM_001001979	XM_001922489	ATG ATAGAAGAGGACCACAGGATGACAT	N
			SPL ACACGCTGCACAAAGACACAAAGCT	Y
mShisa3	NM_001033415	XM_001335257	SPL TGGCTCTGCGAACAACAGAAGTCAG	Y
Apcdd1 Drapc1	NM_133237	XM_687119 XM_002662079	SPL CAGTACAGGTGTTCTCTGACCTGGT	N
Vgll4	NM_177683	NM_213275	ACAGGTCCATTTTGGTAAAAAGCAT	N
Ecrg4 1500015O10Rik	NM_024283	NM_001017697	ATG CAGGTGAAACTTTTCAGAAAGCATG	Y
			SPL GCTGACACAAGCACTTACAGCTTCA	N
phlda2	NM_009434	NM_001020596 Zgc:110459	ATG AAATATCAGAGCCCGTCATTTTGCC	N
Adap1	NM_172723	Zgc:92360	SPL1 TCGGTCTCCGGTCTGAGTCTCCGTT	ND
			SPL2 GTCTGTGGAATTGAAACTCACTGTCG	ND
Meis2.1/Meis2b	NM_001159568	NM_130910	ATG CGTACCGTTGAGCCATCAGCATATG	N
Meis3	NM_008627	NM_131778	ATG CAACTCCTCATACCTCTTATCCATG	N
Meis4.1	NM_010789 NM_001193271	NM_131897	ATG CAGATCCTCGTACCGTTGCGCCATG	N
Scyba/Cxcl14	NM_019568	NM_131627	ATG GGCCGTACTACAGCGATTCATCCCC	N
			SPL GGTGACACTATAAACAGATGGGAAA	Y
Pid1	NM_001003948	NM_001013504	ATG CACATTCAGTTTGGTCTTCAGCATG	N
Sdpr	NM_138741	NM_001004584	ATGCGTGCTCACCATGCTACTCCTC	N
Epcam	NM_008532	NM_213175	GGCAACTAAAACCTTCATTGTGAGC	N
Pcdh19	NM_001105245 NM_001105246	NM_001127519	SPL AATTGTCTGGGTACCTGCAGTTGTA	N
Pchd20	NM_178685	XM_692151	GTAGTGCCGACTCTGCCAAGCAGTC	N
Rab25	NM_016899	NM_001008641	AGTTGTAGGCTAAATCTGTCCCCAT	N
Rps6ka6	NM_025949.2	NM_200073	GGTGTAACTAGAGGTTTATTTCTCC	ND
Capn5	NM_007602	XM_001345078	AGGATTATTAAGCGCTGACCTCAGG	N
Cpm	NM_027468	NM_001017591	ACAGGCAGAACATGAAGACCAACAT	N
Lrrc17	NM_028977	NM_205539	ACCGCAGAGCAGCCGCATGGCAAAA	N
Stmn3	NM_009133	XM_001339340	TACTGTGATGCTGAAGTTACCCTGC	N
Esrp1/Rpm35a	NM_194055	NM_001080576	ATG CCAAATAGTCGGGATTAACCGTCAT	N
Fam83b	NM_001045518	XM_691788	ATG AGTCGGATTCCATCACCGTGACCAT	N
1810019J16Rik	AK007551,	XM_001338342	ATG GGCCAGGCATAACTCACCACATTCA	ND

	BF318332, BG069889, BY028727			
A430107O13Rik	NM_001081351	XM_688609	ATG TGGACAGAGTCAAGAGACATACCAT	Y
			SPL TTCGTAAAACTCCCCCTCACCTCTT	N
Lix1	NM_025681	XM_681174	ATG CATCATCAGAATCTGCTCCAGACAT	N
Lix1l	NM_001163170	NM_001126439	ATG CTGATGGCGAAGAGACTCCATTGTC	Y
			SPL1 CACGTTGTGAACTCACGTTAAAAGA	N
			SPL2 TTCGCTCAGTAGGCTACATACCCCT	N
			SPL 3 GTTCACTGCGCCATCACAAACATAT	N
Fam70a	NM_172930	XM_001922553	CTGGCATTGTAACGTCAGGATATGC	N
Stra6	BC075657	NM_001045312	CTAAAAGACATCAAACTGACCTGAG GTTATTCACAGTTTCAGCACTCATG	N Y

Morpholinos not marked as ATG or SPL are either targeted against an ATG or associated 5' UTR sequences

*

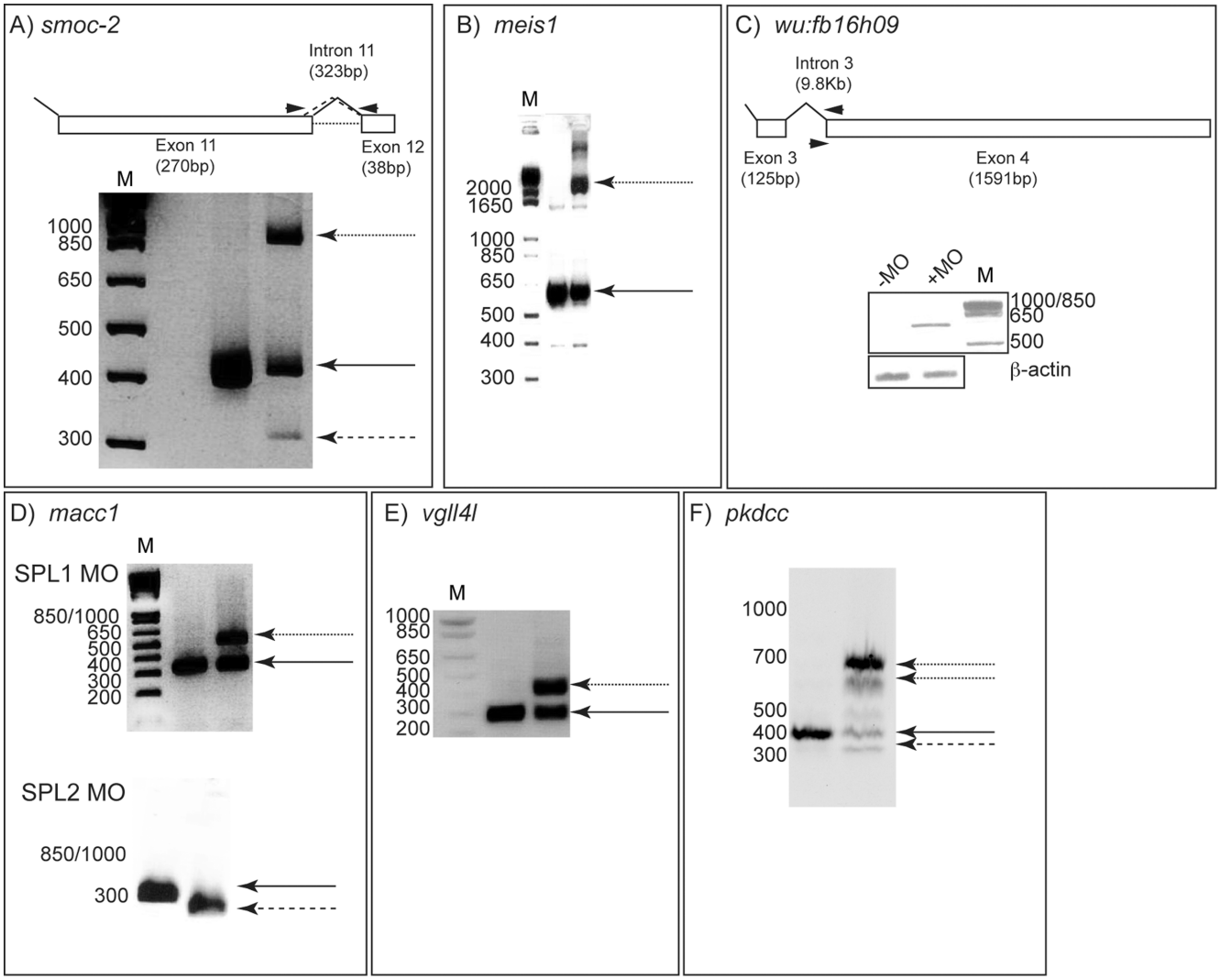
N = no consistent craniofacial phenotype at any of the MO doses tested (up to 20ng).

ND = Not determined as early embryonic lethality prevents analysis of craniofacial development.

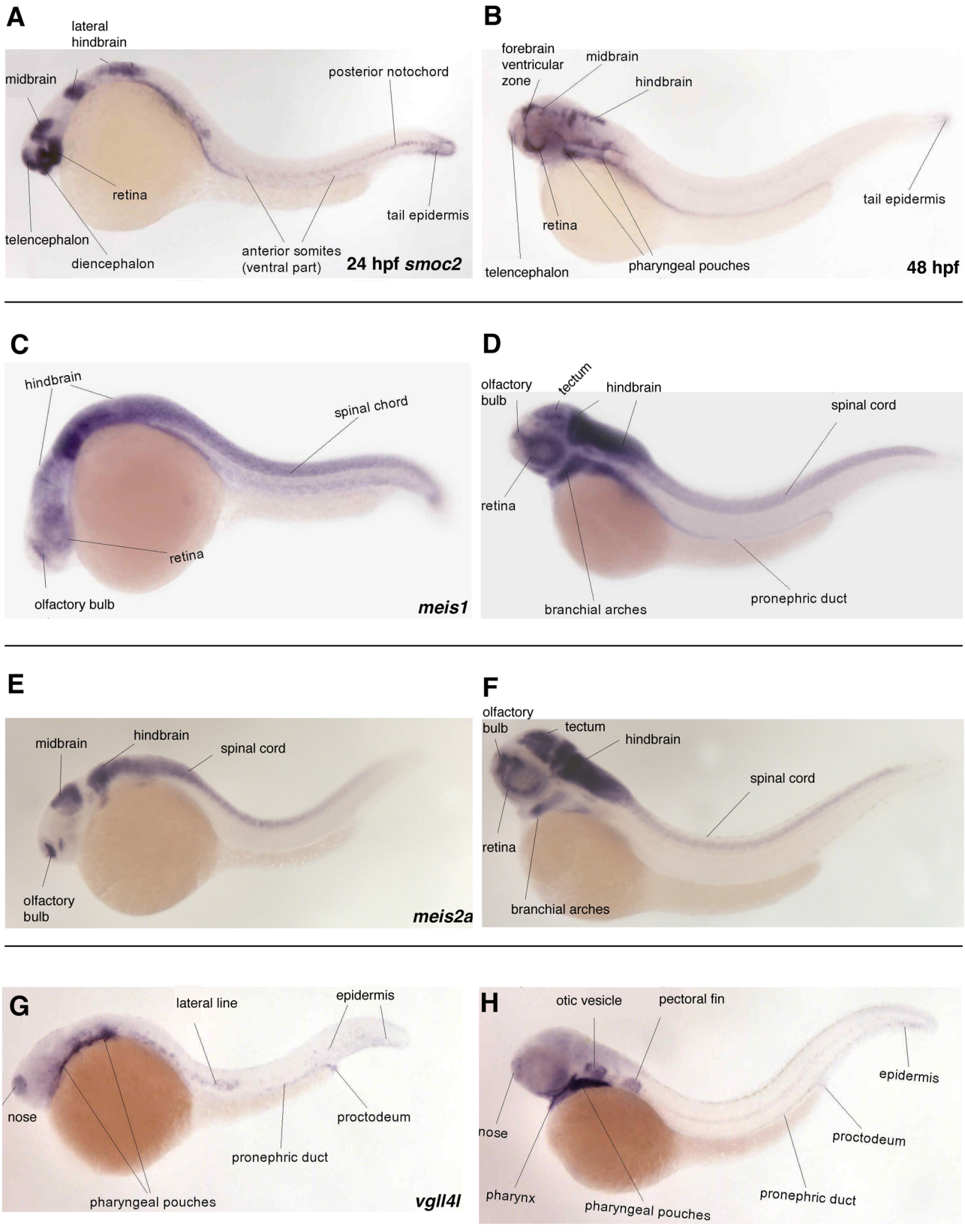
Y= some craniofacial effects. Even though we saw some facial defects in certain instances, these genes were not actively pursued further for various reasons, including a lack of reproducibility; limited phenotype; no apparent effects on splicing caused by a SPL MO; publications on gene during experimental phase of the analysis.

Supplementary Table 3. PCR Primer Sequences and PCR Product Sizes for Zebrafish In Situ Probes

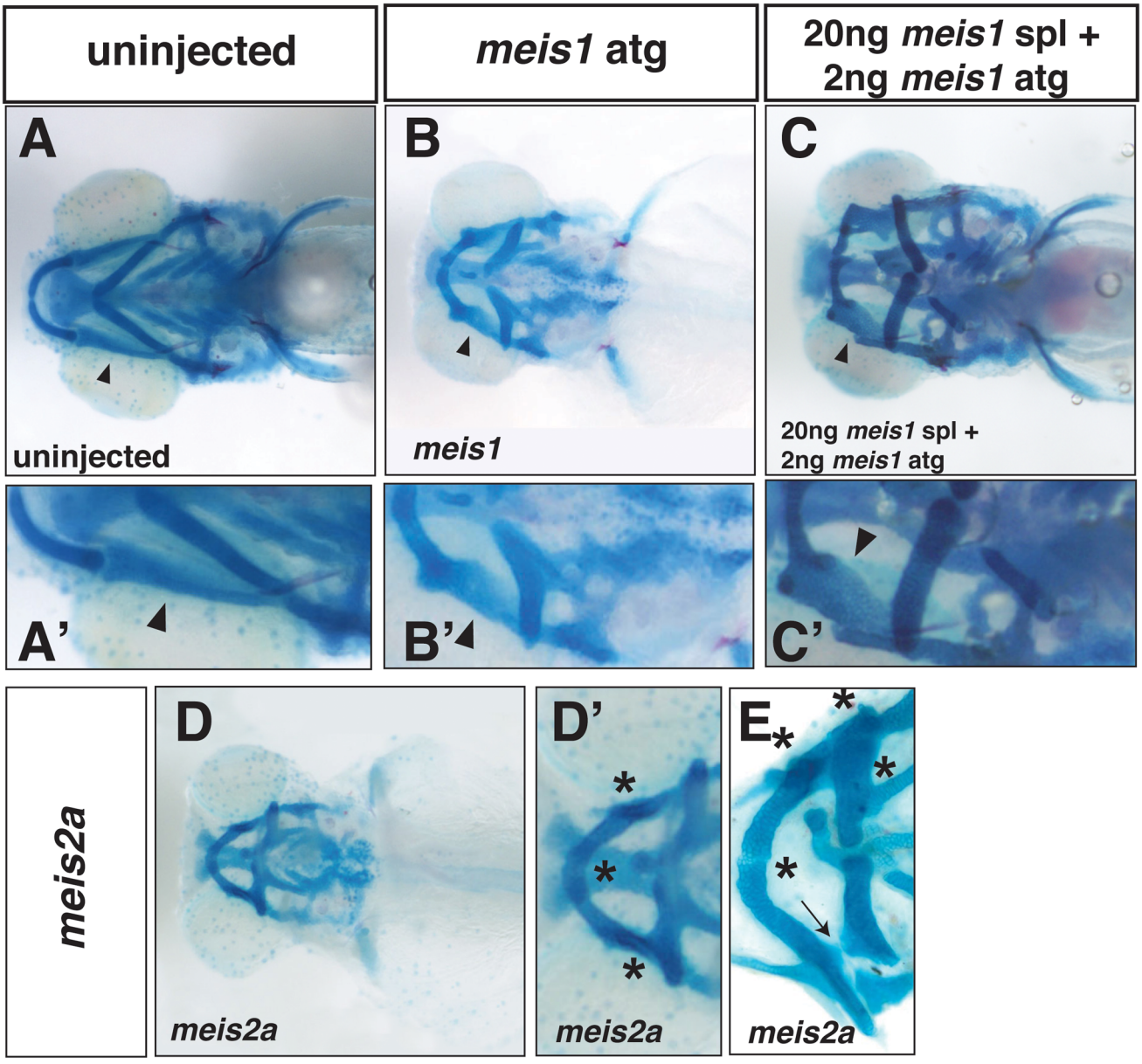
Gene/ID	Forward Primer	Reverse Primer	Approx Size (kb)	Anti-sense probe Restrict and Transcribe
wu:fb16h09 (A930038C07Rik)	5′- CAG CAT TAG CAT TGG CAT TGT GCG CGA GTA GTC -3′	5′- GGC GAG ATG GGA AAC TTG TTC TCT CTC AGA ATG -3′.	0.7	XhoI + SP6
macc1	5′- GCA ACA GCA ATA TTT CAG GGG GCA TTT CAG -3′	5′- CCA ACC CAA CTC TTC CTC GAA GAA AAC CAA TGT ACC -3′	1	NotI + SP6
pcdh19	5′- CAC CTC TTC GCT CAA CTA CTT CGA CTA CCA CCA GC -3′	5′- GCT CTG TGT ACG GGT GGA GCT GGT TAA AGC ACG ATG TCC -3′	1	NotI + SP6
pkdcc (LOC565254)	5′- GAC CTA CAC TTA AAC TTG CCA TGA CTT TCC AAA GAA GTC CG -3′	5′- GTT GCG GAG GGG AAA CTG CAG CAG GCA GTC AGA GTC C -3′	0.9	NotI + SP6
vlk	5'- ATG AAG CGG AGA AAG ATG GTG GTA GCG GCG GGC TTC TGC G -3'	5'- CGT CGT CCA GGT CGG TCA CCT TCA ACA CTC CGT CCA CC -3'	0.95	NotI + SP6
tshz2	5′- CCA GCT CAA GCA GAA TCT TAA CCA TAA GCA CAG ACC C -3′	5′- GCG GTT TCT CTA AAC CTC TAC TCA GTT CTG -3′	0.52	NotI + SP6
lix1l	5′- GAT CGT ACC CTC GAG AAA CAC TTT TGA TGA CTG CC -3′	5′- GCG TCT TGA CGG ATG CTC ATT GAA AAC AGA GTT CAT CAG GGC G -3′	0.7	HindIII +T7



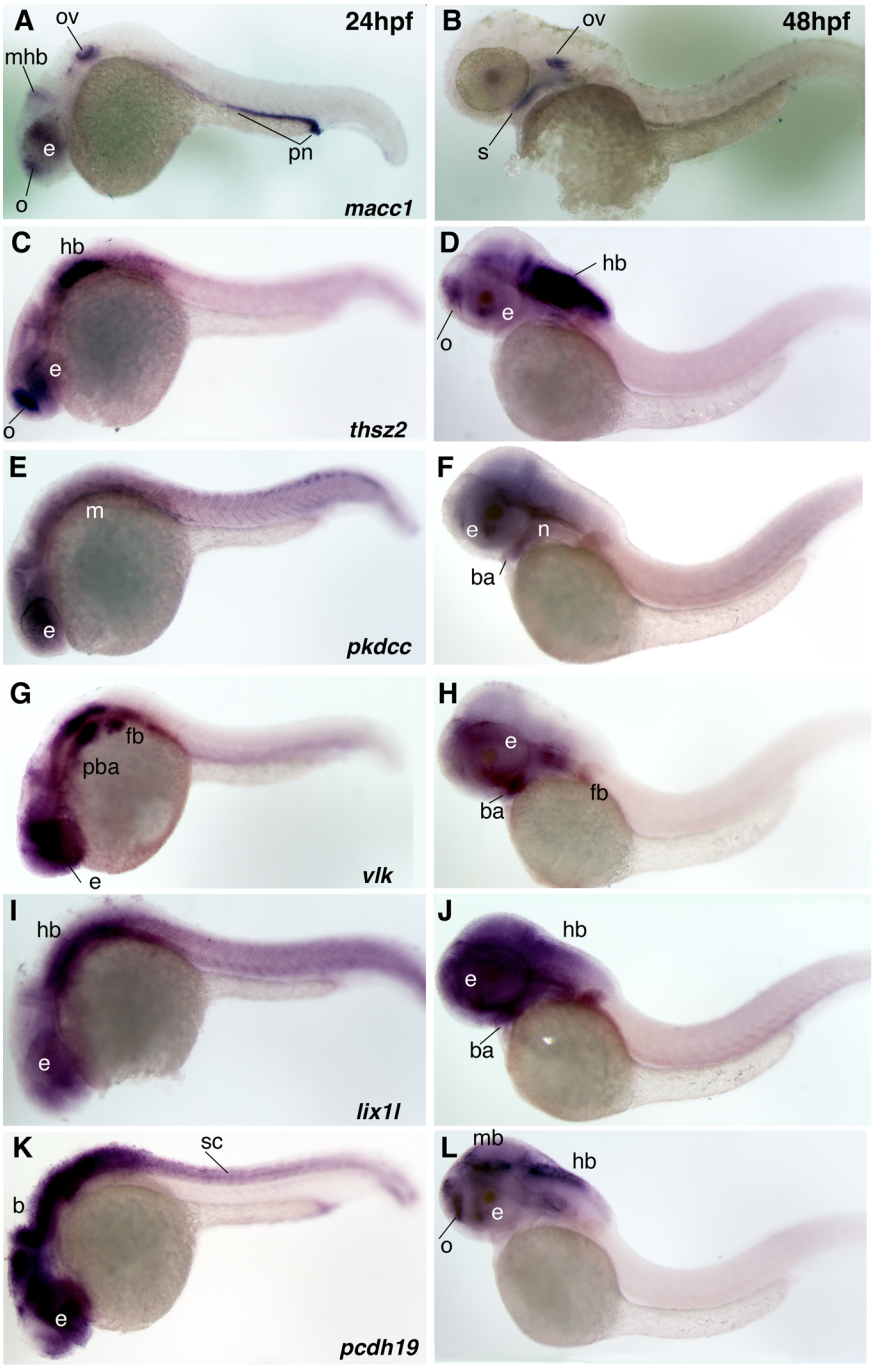
Supplementary Figure 1



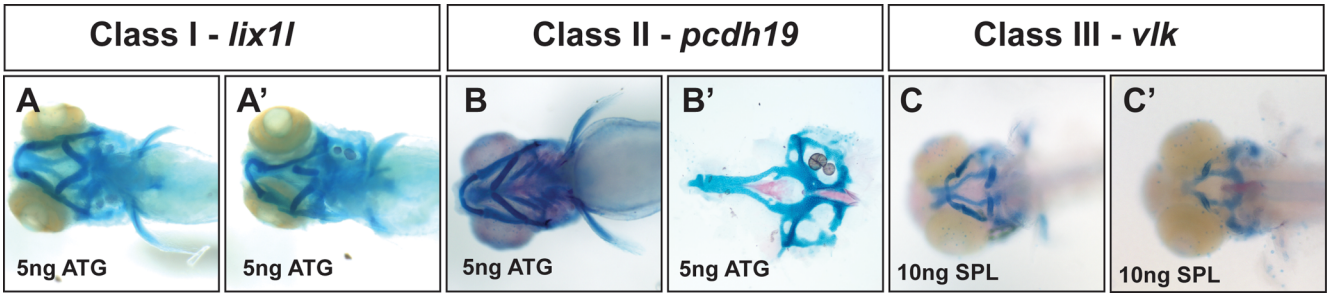
Supplementary Figure 2



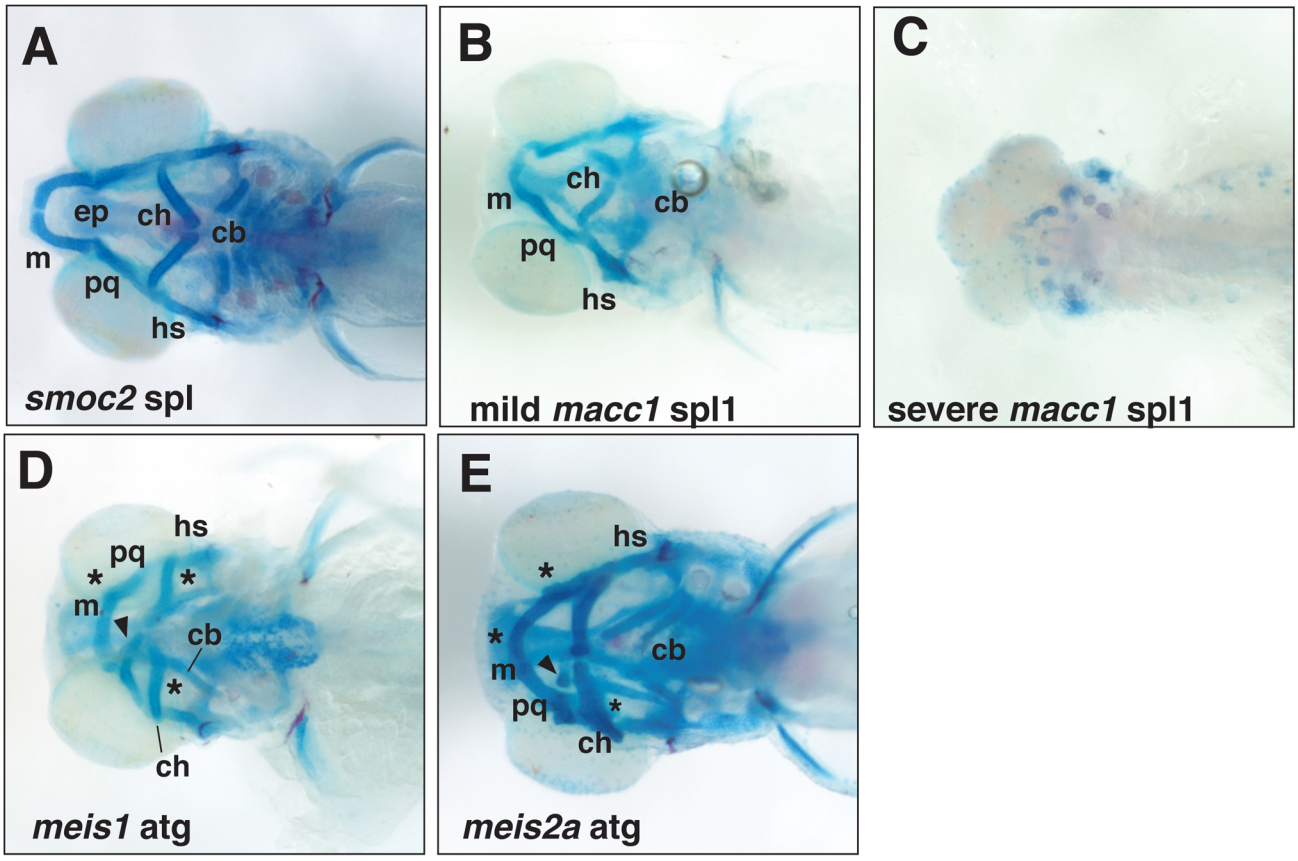
Supplementary Figure 3



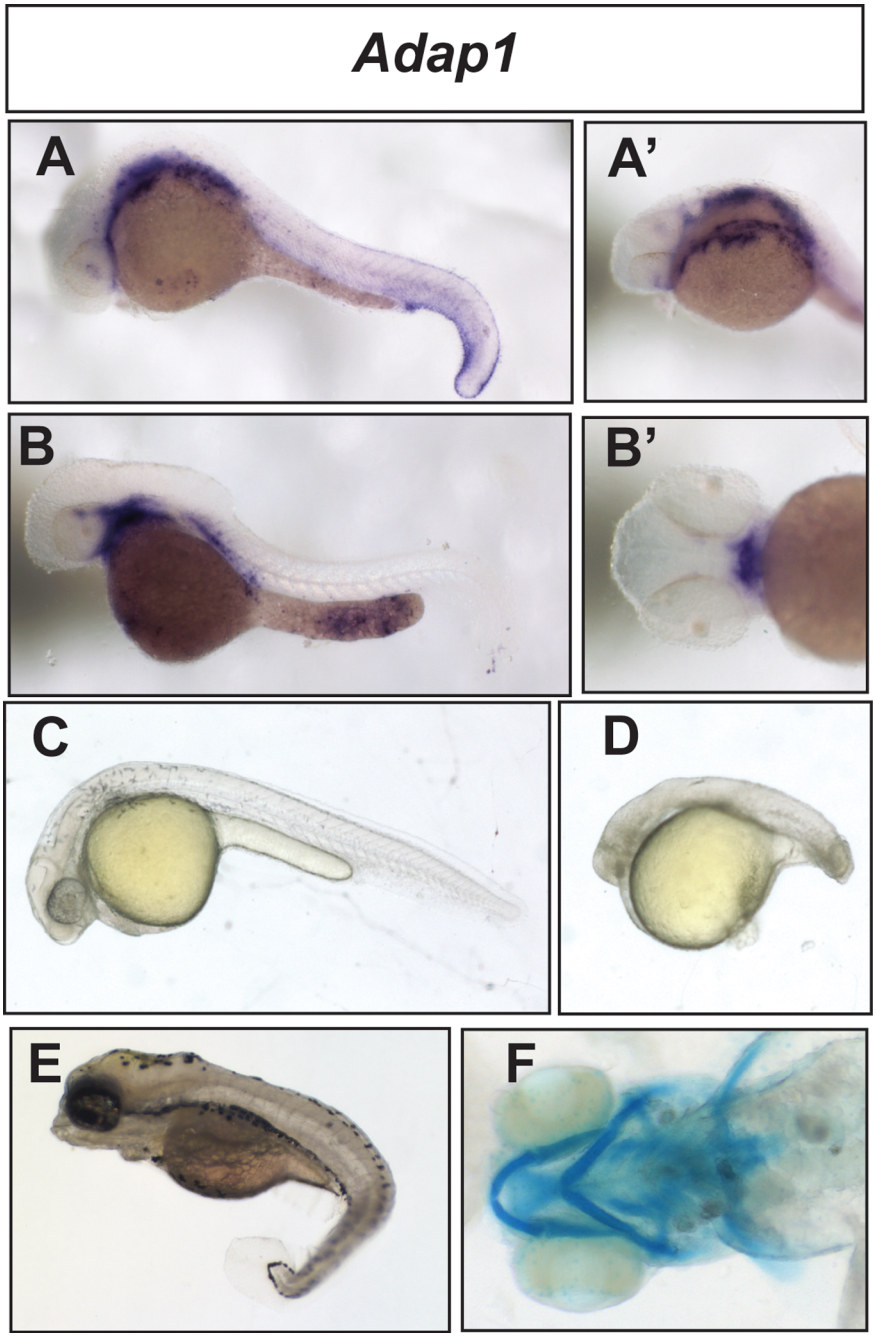
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7