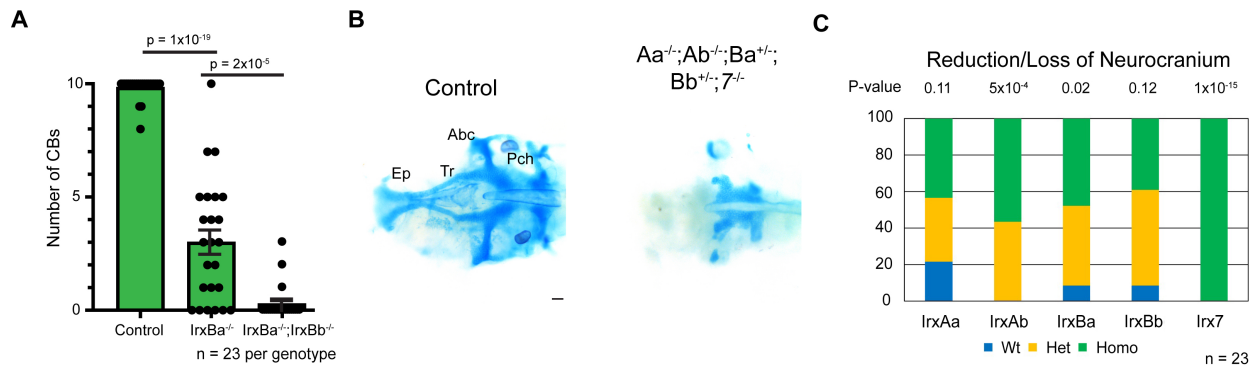
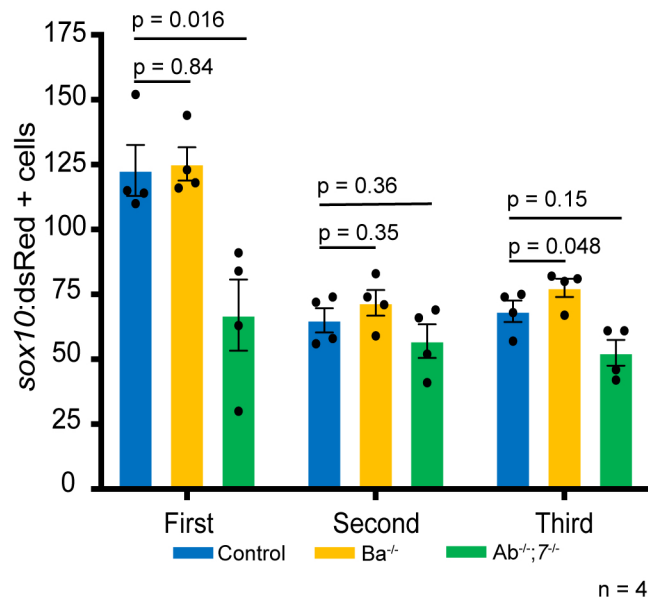


**Fig. S1. Dorsal curvature and jaw gape defects in absence of *IrxA* genes.** (A) Brightfield images show appearance of abnormal dorsal body curvature in *IrxAa<sup>-/-</sup>;IrxA<sup>b-/-</sup>* mutants between 3 and 5 dpf. (B) Differential interference contrast microscopy shows normal semicircular canals in controls and mutants. (C) *IrxAa<sup>-/-</sup>;IrxA<sup>b-/-</sup>* mutants display an abnormal jaw gape. (D) Alcian blue (cartilage) and Alizarin red (bone) staining reveals no defects in the facial skeleton of *IrxAa<sup>-/-</sup>;IrxA<sup>b-/-</sup>* mutants. Unilateral dissections of the skeletons from the first two arches are shown in lateral view. (E) Motor neurons imaged with *isl1:GFP-caax* are unaffected in mutant zebrafish. (F) Muscles imaged by phalloidin staining in control and mutant zebrafish are indistinguishable. Ch, ceratohyal; Hm, hyomandibular; M, Meckel's; Pq, palatoquadrate. Scale bars = 1 mm (A), 100  $\mu$ m (B-F).

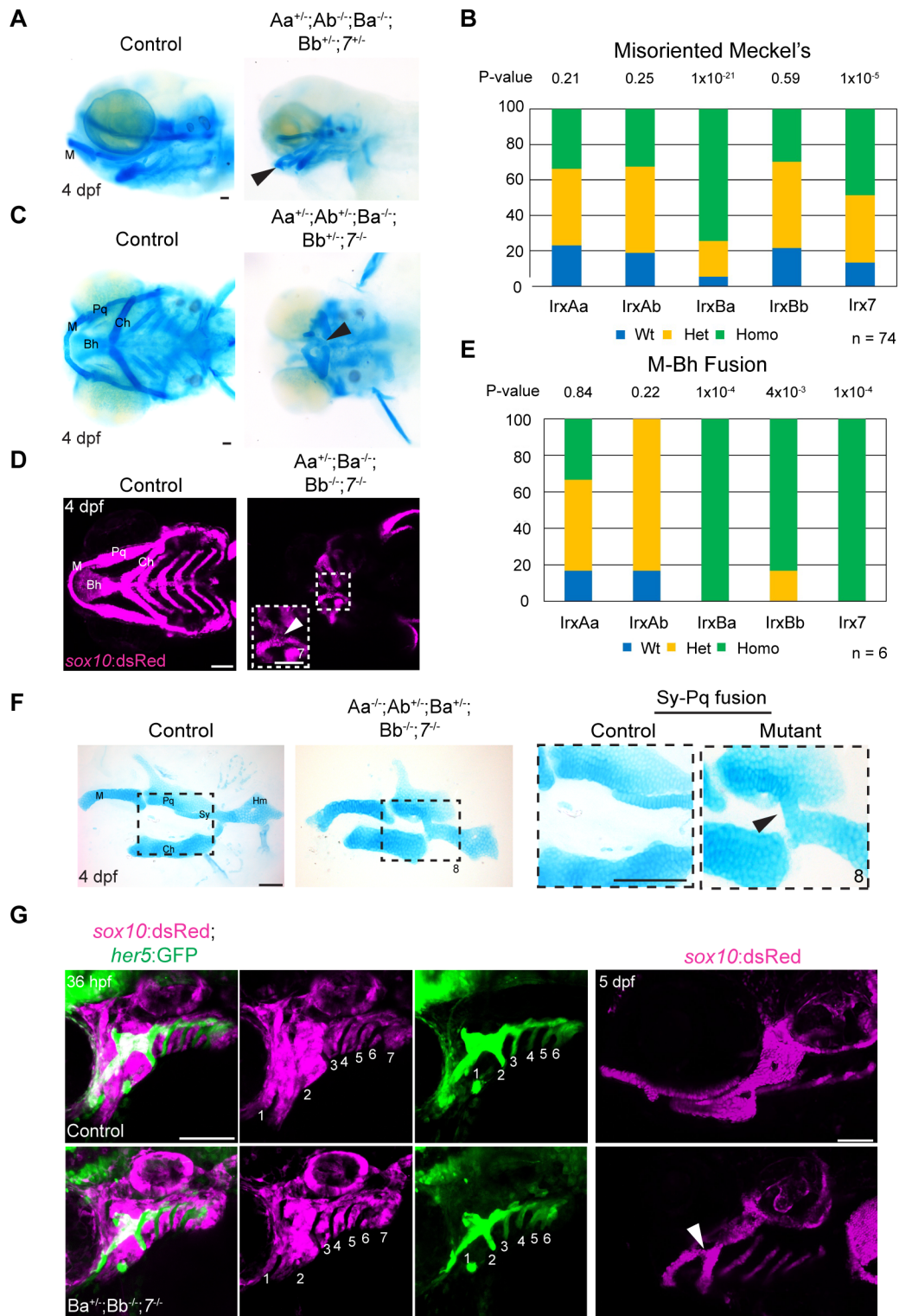


**Fig. S2. Ceratobranchial and neurocranial cartilage loss in *Irx* mutants.**

**(A)** Quantification of loss of ceratobranchial cartilages (CBs) at 5 dpf. Wild-type controls have 5 CBs per side, or 10 total. Homozygous loss of *IrxBb* exacerbates CB loss in *IrxBa* homozygous mutants. Data points represent individual embryos. P-values report t-test results, and error bars represent standard error of the mean. **(B)** Dorsal views of dissected neurocranial cartilages stained with Alcian blue at 4 dpf show severe loss of the neurocranium in combinatorial *Irx* mutants. **(C)** Summary of genotypes from a quintuple heterozygous incross that displayed reductions and/or loss of neurocranial cartilages. P-values report chi-square tests for deviation from the expected 1:2:1 wild-type:heterozygous:homozygous Mendelian ratio. Abc, anterior basicapsular commissure; Ep, ethmoid plate; Pch, parachordals; Tr, trabeculae. Scale bar = 100  $\mu$ m.



**Fig. S3. Quantification of cranial neural crest reductions in *lrx* mutants.** Quantification of the number of *sox10:dsRed*<sup>+</sup> cells within the first, second, and third neural crest streams at 16.5 hpf. Data points represent individual embryos. P-values were calculated using two-tailed non-parametric Student's t-test.

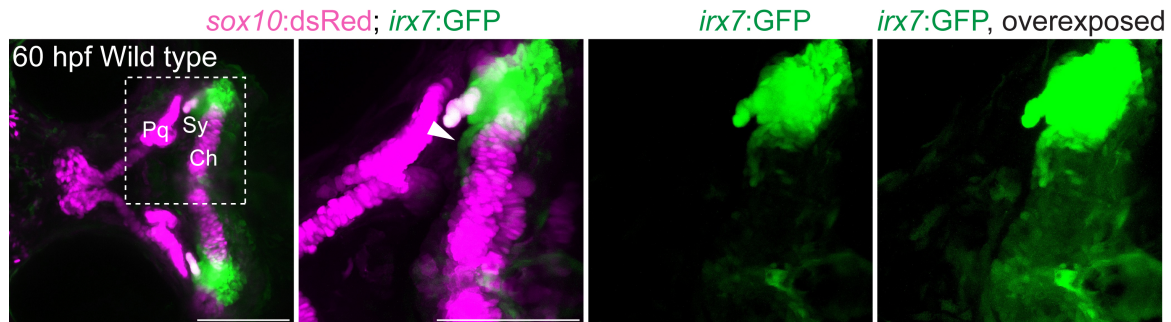


**Fig. S4. Additional facial defects in Irx mutants.**

(A) Lateral view of larval zebrafish heads stained with Alcian blue (cartilage). Arrowhead indicates misoriented Meckel's cartilage. (B) Summary of genotypes from a quintuple heterozygous incross for misoriented Meckel's cartilage. (C) Ventral view of larval zebrafish heads stained with Alcian



blue. Arrowhead indicates fusion of Meckel's cartilage with the basihyal cartilage. **(D)** Confocal images of *sox10:dsRed+* cartilages show severe reductions of facial cartilages and fusion of Meckel's and basihyal cartilages (arrowhead, inset corresponds to dashed box). **(E)** Summary of genotypes from a quintuple heterozygous incross for Meckel's-basihyal fusions. **(F)** Lateral view of dissected first and second arch cartilages shows fusion of the symplectic cartilage to the palatoquadrate in this *lrx* mutant. Dashed boxes show areas magnified at the right, and an arrowhead indicates the fusion site. **(G)** Confocal images of a wild-type control and combinatorial *lrx* mutant show a normal series of 7 pharyngeal arches containing *sox10:dsRed+* neural crest-derived cells separated by a series of 6 *her5:GFP+* endodermal pouches at 36 hpf. Re-imaging at 5 dpf (lateral view) shows inappropriate fusion of Meckel's and ceratohyal cartilages (arrowhead) in this individual mutant. P-values report chi-square tests for deviation from the expected 1:2:1 wild-type:heterozygous:homozygous Mendelian ratio. Bh, basihyal; Ch, ceratohyal; Hm, hyomandibula; M, Meckel's; Pq, palatoquadrate; Sy, symplectic; b, brain; e, endoderm. Scale bars = 100  $\mu$ m.



**Fig. S5. *irx7:GFP* expression in the ceratohyal perichondrium.**

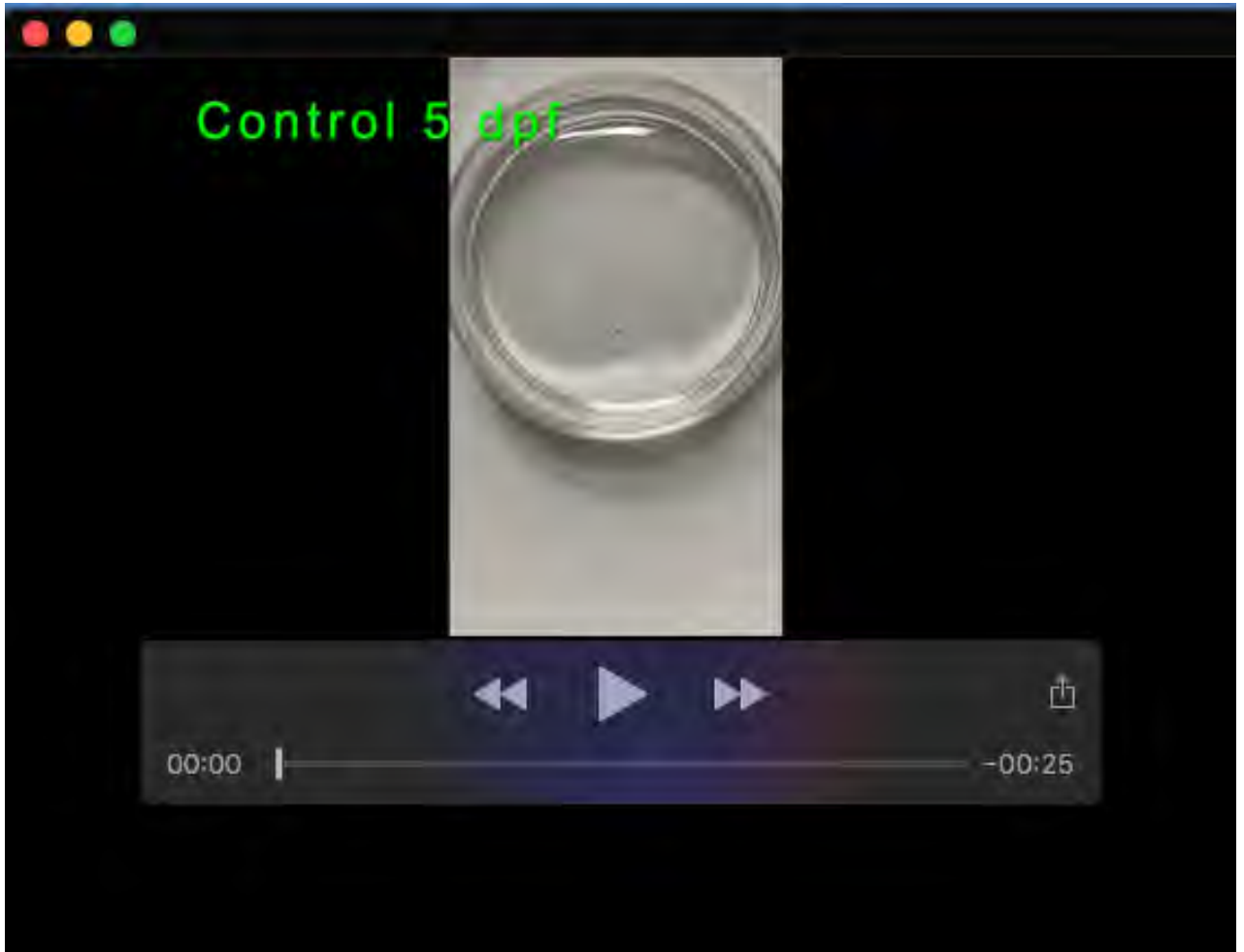
Confocal imaging of *sox10:dsRed; irx7:GFP* embryos at 60 hpf shows strong *irx7:GFP* expression around the developing hyoid joint and symplectic cartilage. Weaker signal is also detected within the perichondrium (arrowhead) of the ceratohyal, in the same position where ceratohyal to Meckel's cartilage fusions are observed in mutants. Merged and individual channels corresponding to the boxed region are magnified to the right, with an overexposed version highlighting weaker expression. Ch, ceratohyal; Pq, palatoquadrate; Sy, symplectic. Scale bar = 100  $\mu$ m.

**Table S1. List of sgRNAs and genotyping assays for *lrx* mutant alleles.****Guide RNAs**

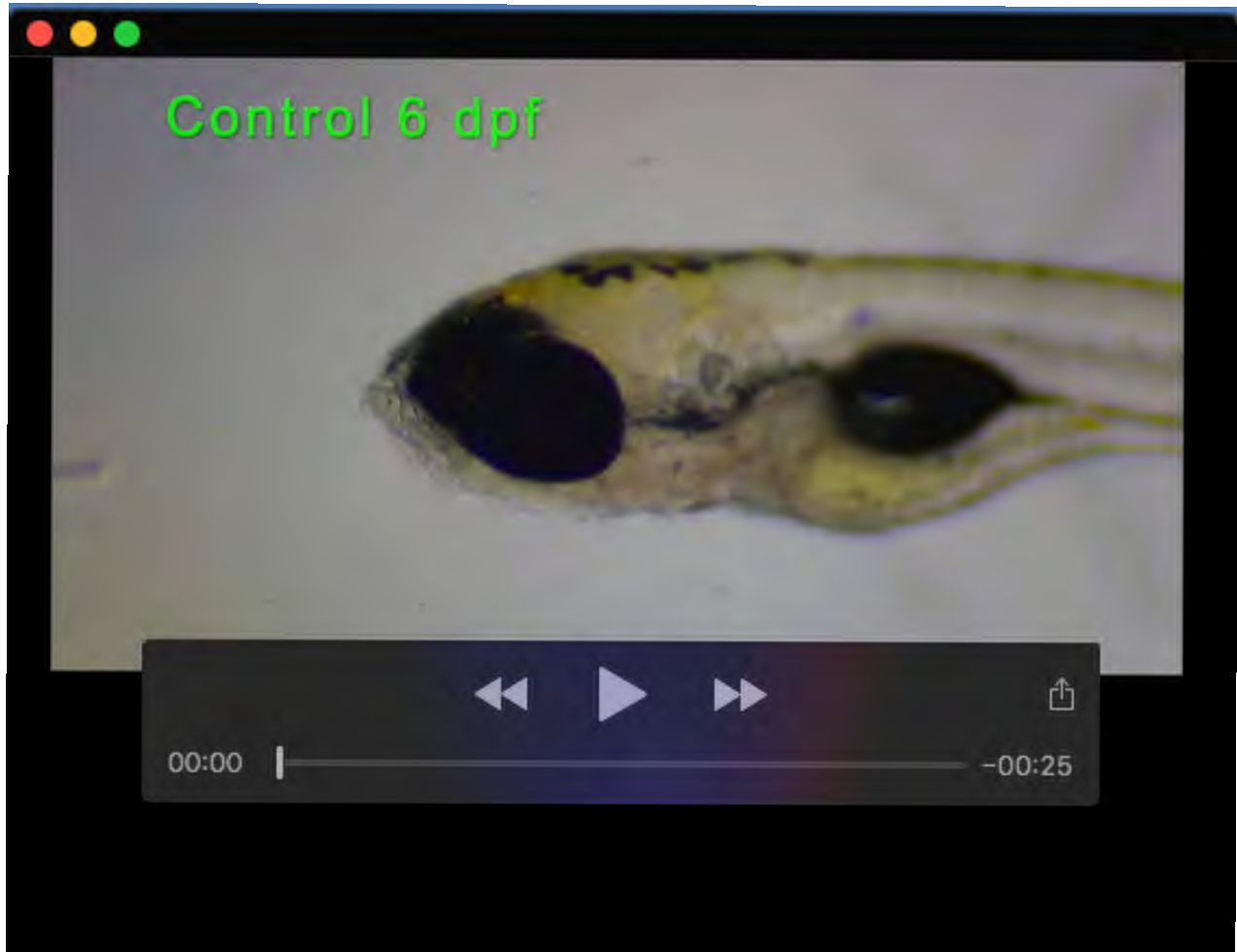
lrx1a	GGCCGCCGCTGCGGTGCCT
lrx2a	GGGTAACCGGGACTCCCGTA
lrx4a	GGGTCTGCGCCGATGAGTCC
lrx1b	GCGTGCACTGCAAATCCTGG
lrx4b	GAGTTGCCATAGACCGCAGT
lrx3a	CGCCAGGAGGAGGCTAAAGA
lrx6a	GGAGTAGATGGCGGAGGGGT
lrx3b	GGTGCTGGTCTCTCGCGGG
lrx5b	CCTGCGCGCGTTGGCGAACC

**Primers**

Gene	Forward	Reverse	Enzyme	Band Size	Digested Wild type	Mutant
lrx1a	GGGAGTTCTGACTCCGTCGT	TAAGGCAGAAAGGCGCTGTA	BsaJI	132	78, 54	Uncut
lrx2a	GAACATTTGACTGACCCCG	CGTCTTCACTTTTATTCCGC	Cvi-QI	351	236 94, 21	238,113
lrx4a	CATGACCACCAACTCTCTGACA	CGTACACCGGGCAGTACA	None	95	-	90
lrx1b	TATGGATAAAAACCGGGCGCA	CCAGGGGTCGCAGTATGAG	None	87	-	79
lrx4b	ACGAAAGTAGGCTTGTGGCC	GTAAGTGCTGCAGGAGTCGT	Acil	104	48, 56	104
lrx3a	TGCTGGCCATCATCACTAAA	ATCGATTTCTCTCGTCCT	CviKI-1	191	123,62,6	185,6
lrx5a	TGCTGGCTTTTGAGTGTTTG	ATGGCCAGCATGATCTTCTC	BsaI	311	188,123	
lrx6a	GGACCCGCTTTATCTGGTGA	GGGCAGAACTACGCCAACT	None	97	-	72
lrx3b	CCTTCTACCCATACGGACACC	CAGGCCTTCAGGGTGCTGGT	None	91	-	83
lrx5b	TCGCTTACAGTTCATCTGTGC	CACGCACAATAACACCAAAA	BssHI	502	316,186	502
lrx7	GCCTGCTTCATCAACGGGG	AAGGGATGAACTGTAGCCCG	EcoRV	176	111,65	176



**Movie 1. Abnormal circling behavior in larvae and adults with *IrxAa* and *IrxAb* loss.**



**Movie 2. Abnormal jaw gape in larvae with IrxAa and IrxAb loss.**