

Supplement 4 (Frosk et al. 2016) – Additional Zebrafish data

Methods (not included in main text)

Transient gene suppression in zebrafish embryos

All zebrafish studies were conducted with approval from the Duke University Institutional Animal Care and Use Committee. Two splice-blocking morpholino (MO) antisense oligonucleotides targeting *cep55l* (sb1, 5'-CTGAGATAAAGCGACTAATTCACCT-3' and sb2, 5'-GTTGAGCTTTTCTTACCATCAGACT-3') were obtained from Gene Tools, LLC. To determine the optimal dose for *in vivo* complementation assays, we injected 4 ng, 5 ng, and 6 ng of MO into one-to-four cell stage zebrafish embryos obtained from natural matings of heterozygous *-1.4col1a1:egfp* transgenic adults.[15] To determine MO efficiency, we harvested embryos in Trizol (Invitrogen), extracted total RNA according to the manufacturer's instructions, and generated oligo-dT-primed cDNA with the QuantiTect reverse transcription kit (Qiagen) for use as RT-PCR template. Sanger sequencing of RT-PCR products was used to identify the precise alteration of endogenous transcript. For rescue experiments, 5 ng MO and 100 pg RNA were used either independently, or combined.

CRISPR/CAS9 genome editing of cep55l

We used the CHOPCHOP design tool to identify *cep55l* guide (g)RNA target sequences, for which one pair of oligonucleotides were synthesized and annealed.[16] Annealed oligos were ligated into the T7cas9sgRNA2 vector as described [17], and plasmids were sequence confirmed. Template vector was linearized with *Bam*HI and gRNA was transcribed with the MEGAshortscript T7 kit (Life Technologies). 100 pg of *cep55l* gRNA and 100 pg of CAS9 protein (PNA Bio) was co-injected into the cell of one-cell stage embryos. To determine targeting efficiency in F0 mutants, we harvested single embryos at 2 days post-fertilization (dpf) and 4 dpf, extracted genomic DNA, and PCR amplified a region flanking the gRNA target site. To estimate the percent mosaicism, PCR products were cloned into a TOPO-TA vector

(ThermoFisher); plasmid was prepped from multiple individual colonies (n=19-54) per embryo (n=5-8 embryos/condition); and Sanger sequenced on an ABI3730.

NGS library preparation and sequencing for CRISPR/CAS9 outcomes

Gene-specific primers were used to amplify a region flanking the gRNA target site; with tags 5'-CGCTCTCCGATCTCTG-3' and 5'-TGCTCTCCGATCTGAC-3' appended to the 5' side of the gene-specific portion for forward and reverse primers, respectively. These amplicons were used as a template for the second PCR to add the Ion Xpress barcode adapters. Equimolar concentrations from each amplicon were pooled to make the final sequencing library. The library was diluted to 100 pM and sequencing template was prepared using the Ion PGM Hi-Q OT2 Kit on the Ion One Touch 2 instrument according to manufacturer's instructions. Templated Ion Sphere Particles were enriched on an Ion One Touch ES and sequencing was performed on an Ion Torrent PGM with the Ion PGM Hi-Q Sequencing Kit. Demultiplexing of barcoded samples was performed using Torrent Suite Software 5.0.4 and reads were trimmed using Cutadapt.[18] CRISPR-Cas9 outcomes were quantified using CRISPResso computational pipeline.[19]

Automated Zebrafish Imaging

Larvae were positioned and imaged live with the Vertebrate Automated Screening Technology (VAST) platform (Union Biometrica) as described.[20] Larvae were anesthetized with 0.2 mg/mL Tricaine prior to being loaded into the sample reservoir. Dorsal and lateral images were acquired at a >70% minimum similarity from the pattern-recognition algorithm. Once recognized inside the 600 µm capillary of the VAST module on the microscope stage, larvae were rotated 180° to capture a fluorescent image of the jaw. NIH ImageJ software was used to measure the angle of the ceratohyal cartilage.

Whole mount TUNEL and immunostaining

We utilized the ApopTag rhodamine *in situ* Apoptosis Detection kit (Chemicon) as described.[21,22] For whole-mount immunostaining, embryos were fixed in Dent's solution and stained with primary antibody (anti-phospho-histone H3 Frosk et al. – Zebrafish data

(ser10)-R antibody [sc-8656-R, Santa Cruz, 1:500]; anti-acetylated tubulin [T7451, Sigma-Aldrich, 1:1000], and anti-NaK ATPase [a6F, DSHB;1:500]); and secondary detection was accomplished with Alexa Fluor goat anti-mouse IgG (A21207, Invitrogen; 1:1000) in blocking solution as described.[21,23,24] Fluorescent signals were imaged on laterally or ventrally positioned larvae and z-stacked on a Nikon AZ100 microscope, using NIS Elements AR software. We quantified staining by counting positive cells in defined regions of the head by using ImageJ software; positive cells in the eyes were removed from cell counts (TUNEL and phospho-histone H3). NIH ImageJ software was also used to measure the area of the proximal convolution or the cerebellum. Data were analyzed and graphed using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA). Holm-Šídák multiple comparison testing following one-way ANOVA was performed separately for each set of data corresponding to a phenotype. P-values less than 0.05, 0.01, 0.001, 0.0001 are summarized with one, two, three or four asterisks respectively. P-values >0.05 are indicated by “ns” (nonsignificant).

NGS library preparation and sequencing for CRISPR/CAS9 outcomes

Gene-specific primers were used to amplify a region flanking the gRNA target site; with tags 5'-CGCTCTCCGATCTCTG-3' and 5'-TGCTCTCCGATCTGAC-3' appended to the 5' side of the gene-specific portion for forward and reverse primers, respectively. These amplicons were used as a template for the second PCR to add the Ion Xpress barcode adapters. Equimolar concentrations from each amplicon were pooled to make the final sequencing library. The library was diluted to 100 pM and sequencing template was prepared using the Ion PGM Hi-Q OT2 Kit on the Ion One Touch 2 instrument according to manufacturer's instructions. Templated Ion Sphere Particles were enriched on an Ion One Touch ES and sequencing was performed on an Ion Torrent PGM with the Ion PGM Hi-Q Sequencing Kit. Demultiplexing of barcoded samples was performed using Torrent Suite Software 5.0.4 and reads were trimmed using Cutadapt.[1] CRISPR-Cas9 outcomes were quantified using CRISPResso computational pipeline.[2]

Whole mount TUNEL and immunostaining

We utilized the ApopTag rhodamine *in situ* Apoptosis Detection kit (Chemicon) as described.[3,4] For whole-mount immunostaining, embryos were fixed in Dent's solution and stained with primary antibody (anti-phospho-histone H3 (ser10)-R antibody [sc-8656-R, Santa Cruz, 1:500]; anti-acetylated tubulin [T7451, Sigma-Aldrich, 1:1000], and anti-NaK ATPase [a6F, DSHB;1:500]); and secondary detection was accomplished with Alexa Fluor goat anti-mouse IgG (A21207, Invitrogen; 1:1000) in blocking solution as described.[3,5,6] Fluorescent signals were imaged on laterally or ventrally positioned larvae and z-stacked on a Nikon AZ100 microscope, using NIS Elements AR software. We quantified staining by counting positive cells in defined regions of the head by using ImageJ software; positive cells in the eyes were removed from cell counts (TUNEL and phospho-histone H3). NIH ImageJ software was also used to measure the area of the proximal convolution or the cerebellum. Data were analyzed and graphed using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA). Holm-Šídák multiple comparison testing following one-way ANOVA was performed separately for each set of data corresponding to a phenotype. P-values less than 0.05, 0.01, 0.001, 0.0001 are summarized with one, two, three or four asterisks respectively. P-values >0.05 are indicated by "ns" (nonsignificant).

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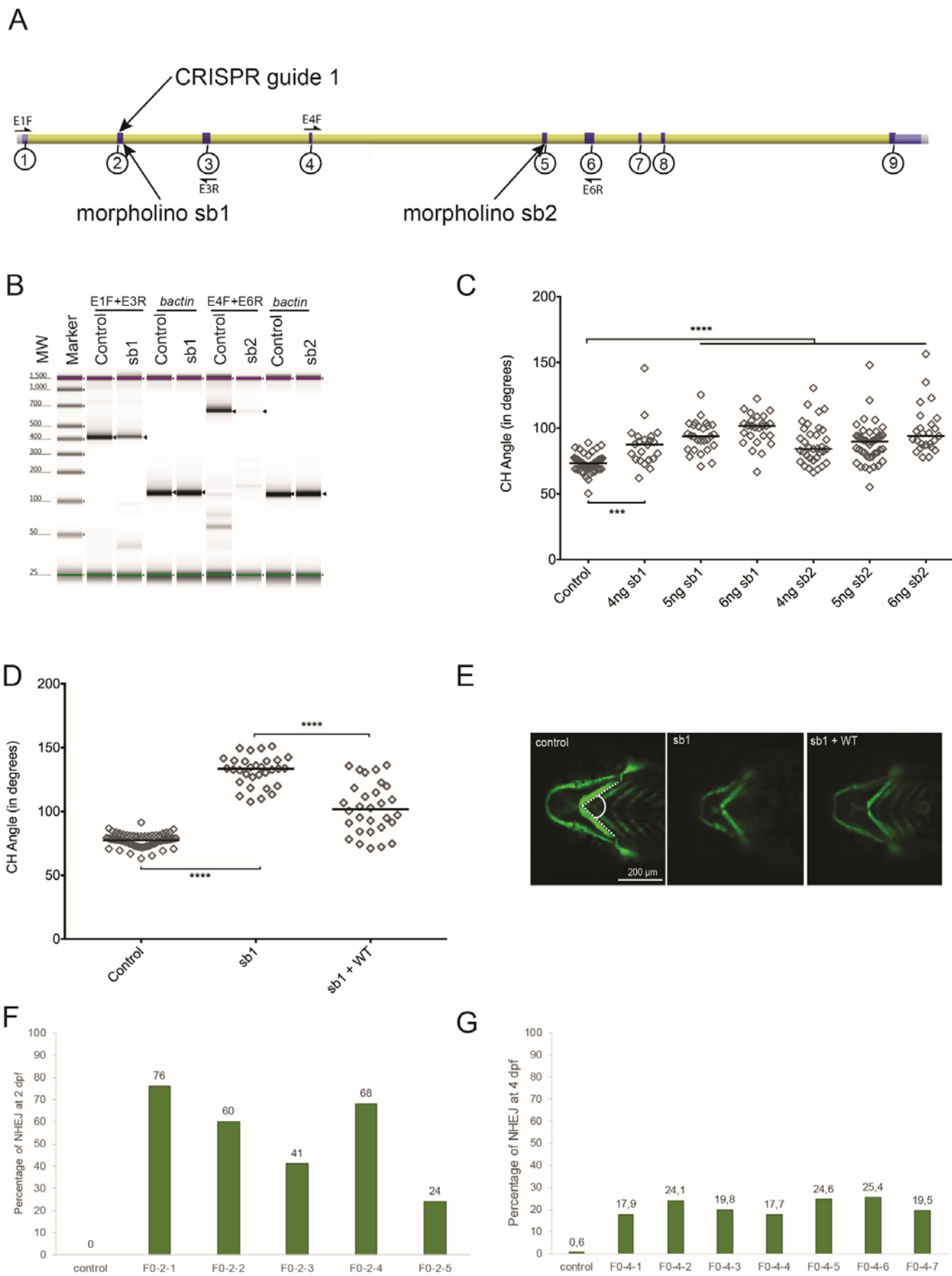
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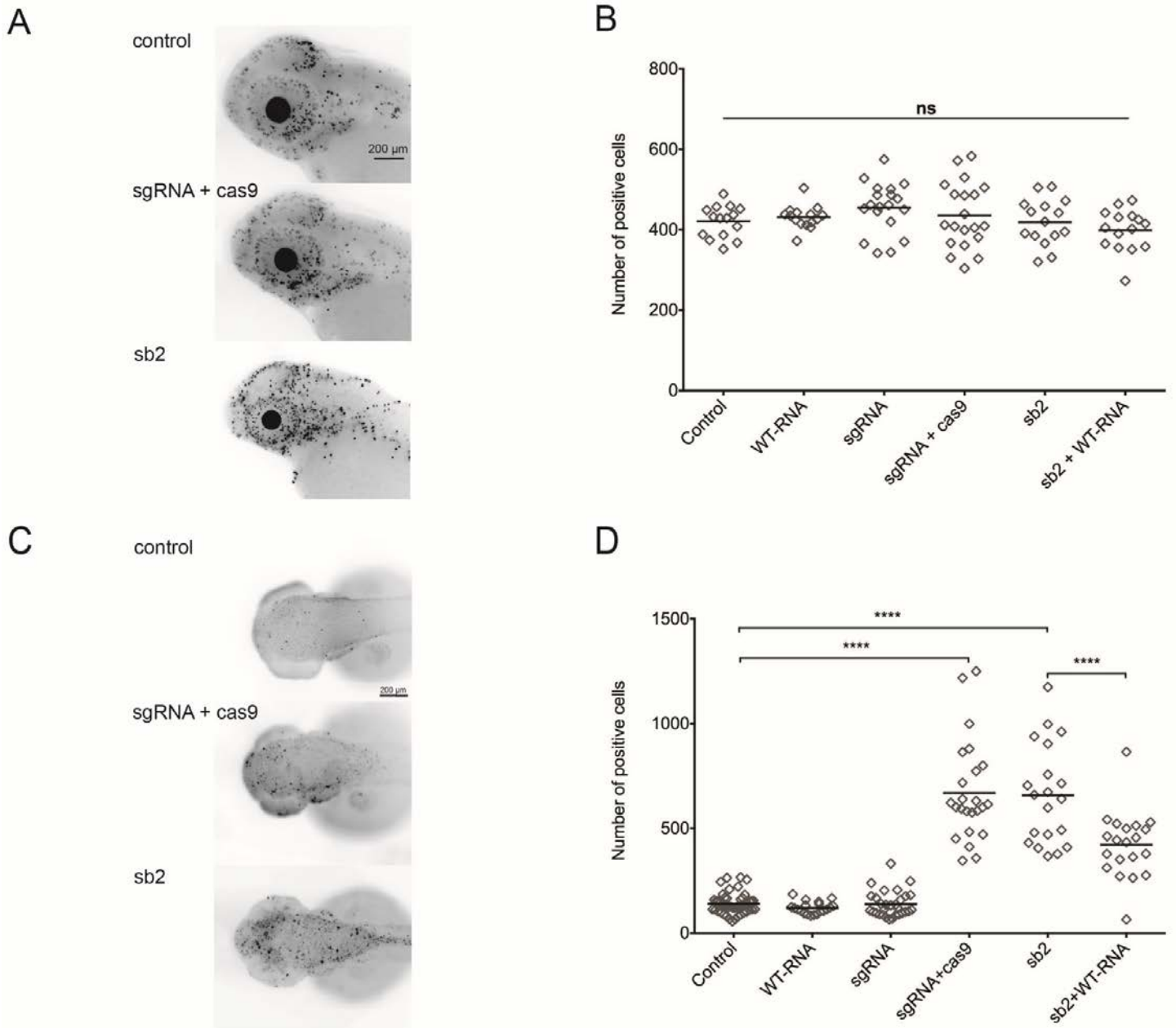
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Additional Figures



Supplementary Figure 4.1: Efficiency of morpholinos (MOs) and CRISPR sgRNA targeting *cep55l*. (A) Schematic of the *Danio rerio cep55l* locus showing the location of the two MOs and the CRISPR guide target sites. Exons are indicated with circled numbers. (B) TapeStation electrophoresis of RT-PCR products showing sb1 and sb2 morpholino efficiency. Sequencing of cloned PCR fragments showed that sb1 produces a 45 bp retention of intronic sequence, and sb2 results in deletion of 242 bp; both result in premature termination codons. Expected bands are indicated with black arrows; primer locations are indicated in panel A; *actin* was amplified to control for cDNA integrity. (C) Dose curve of sb1 and sb2 MOs. Embryo batches were injected with increasing amounts of MO, imaged live at 4 dpf, and the angle of the ceratohyal (CH) cartilage was measured (See panel E); n=22-59 embryos/injection. (D) Plot representing the effects of sb1 and sb1 + WT-RNA injections on 4 dpf *-1.4coll1a1:egfp* larvae. sb1 induces a significant increase in the CH angle compared to uninjected controls. Co-injection of WT-RNA with sb1 rescues significantly the CH angle. n= 20-63 embryos/injection, repeated. (E) Representative live ventral images of 4 dpf *-1.4coll1a1:egfp* larvae demarcate cartilage structures with the presence of GFP-positive cells. CH angle was measured as shown (dashed white lines). (F) Estimation of mosaicism in *cep55l* F0 mutant embryos harvested at 2 dpf as assessed by cloning of PCR products flanking the targeting site and Sanger sequencing. (G) Assessment of CRISPR/Cas9 genome editing efficiency using next generation sequencing of PCR products amplified from genomic DNA of control and *cep55l* F0s (n=7) harvested at 4 days post fertilization. NHEJ, non-homologous end joining; **** indicates p<0.0001.



Supplementary Figure 4.2: Apoptosis and cell cycle markers (M-phase) quantified in *cep55* morphants and F0 mutants. (A) Larvae were fixed at 2 days post fertilization and stained with rabbit IgG polyclonal p-Histone H3 (Ser 10)-R antibody to highlight the presence of dividing cells in the anterior structures of the zebrafish central nervous system. Representative lateral views of inverted fluorescent images are shown. (B) Plot represents the quantification of fluorescent signals marked by the pH3 (Ser 10)-R antibody for each injected condition. All injected conditions listed display no significant differences from one another. n=15-20 embryos/injection. (C) Larvae were fixed at 2 dpf and stained with the ApopTag rhodamine in situ Apoptosis Detection kit (Chemicon) to detect apoptotic cells. Representative dorsal views are shown (D) Plot representing the number of ApopTag rhodamine positive cells per injected condition. sgRNA + Cas9 injected embryos display an increase of apoptotic markers compared to both uninjected and sgRNA-alone conditions. n=20-58 embryos/condition, repeated. **** indicates $p < 0.0001$; ns, not significant.