

Supplemental Materials

Molecular Biology of the Cell

Hyland *et al.*

SUPPLEMENTAL FIGURE 1: CRISPR primer design and on-/off-target analyses of the *cx43^{lh10}* guide RNAs. (A) The gRNA target sequences within the Cx43 cDNA sequence are highlighted, with gRNA 1 (targeting amino acid 256) in dark red, and gRNA 2 (targeting amino acid 289) in light blue. PAM sequences are underlined and shown in light green. Vertical purple bars represent the double-strand breaks (DSBs) generated by Cas9 as verified by *cx43^{lh10}* genomic sequencing (shown in Supplemental Figure 3C). First and last amino acid codons of the zf *cx43* gene are highlighted in red. (B) gRNA analyses generated by ChopChop design tool for each gRNA target sequence in the 5'-3' direction (gRNA 1: top, gRNA 2: bottom). Each gRNA exhibits an optimal GC content (desired range is 45-65%), has a high efficiency score of ~60, contains only 1 target site in the zebrafish genome in the *cx43* gene (MM0), and exhibits no additional off-target sites even when 1, 2, or 3 mismatches are allowed (MM1 to MM3). (C) DNA sequence alignment of zf *cx40.8* (accession number GDQH01030906.1) and zf *cx43* genes (accession number AF035481.1) shows specificity of gRNAs (shown in red) and PCR-verification primers (shown in blue). PCR primers were used for DNA sequencing. First and last amino acid codons of the zf *cx43* gene are highlighted in red.

SUPPLEMENTAL FIGURE 2: Generation of the *cx43^{lh10}* zebrafish line. (A) Schematic illustrating the steps required to generate the homozygote zebrafish line bearing deletion of amino acid residues 256-289 in their C-terminal domain of the Cx43 protein (designated *cx43^{lh10}*). One-cell stage embryos were injected with CRISPR components. CRISPR/Cas9 mutagenesis generated the deletion in one of the two *cx43* alleles in some of the injected embryos. If mutagenesis does not occur at the one-cell stage, but only later, mosaic embryos result (not all cells of the embryo contain a copy of the mutant allele) (see B). Mosaic adults were outcrossed to WT zebrafish for selection of germline transmission mutants and generation of truly heterozygous fish (all cells contain one copy of the mutant allele). Heterozygous adults were intercrossed for generation of homozygous *cx43^{lh10}*

zebrafish (all cells contain 2 copies of the mutant allele) (see C) (B) Representative agarose gel showing the PCR verification of CRISPR/Cas9 injected zebrafish embryos at 24hpf. The WT allele is represented by a ~550bp fragment, whereas the *cx43^{h10}* allele is represented by a shorter, ~450bp fragment. Mutated embryos are highlighted with a red arrow. (C) Representative agarose gel showing the PCR verification of a heterozygous intercross. WT (not mutated), heterozygote and homozygote mutations (labeled with blue, green, and purple arrows, respectively) follow the mendelian distribution of 1 : 2 : 1.

SUPPLEMENTAL FIGURE 3: Verification of the amino acid residue 256-289 deletion. (A) Schematic of the coding sequence of the zf *cx43* gene with codons and corresponding amino acids in the mutated region shown. Area highlighted in gray corresponds to the deleted nucleotides and corresponding amino acids. gRNAs are shown in red, PAM sequences are shown in cursive. PCR/sequencing primers are shown in blue. First and last amino acid codons of the zf *cx43* gene are highlighted in red. (B) Alignment of WT and mutant zf Cx43 amino acid sequences showing the deleted region. First and last amino acid are highlighted in red. (C) Original chromatograms of sequencing reads of WT *cx43* and homozygote mutant *cx43* fish verifying the established deletion in the *cx43^{h10}* fish.

A

Cx43 cDNA sequence **PAM Sequences** **gRNA 1** **gRNA 2** | DSB generated by Cas9

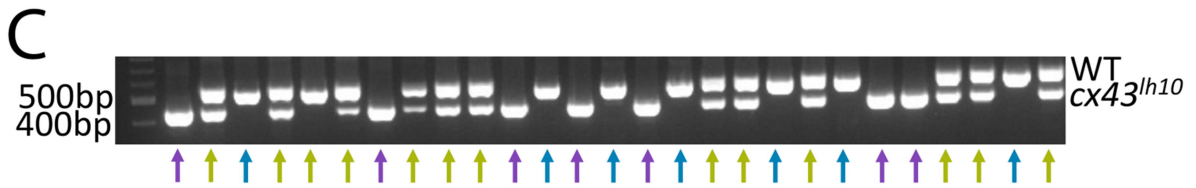
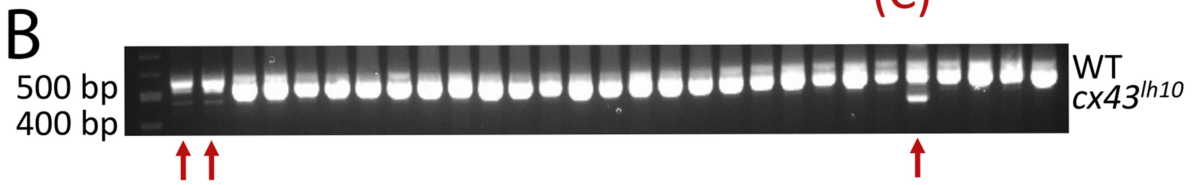
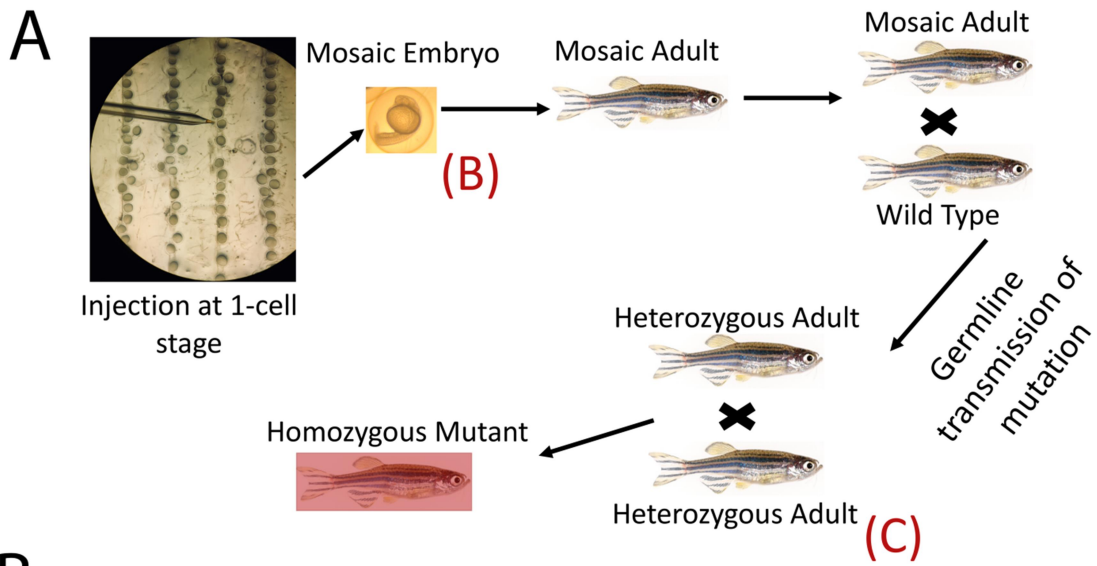
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 CTTTGG**CCCA**CGCCGAAGGA**ACTGTCTA**CGACCAAAATACGGTACTACAATGGTTGCTCTCCACCAACTGCA
 CCGCTCTACCAATGTACCT**CCAGGCTACAACTGGCCACCGG**CGAAAGGACCAACTTTCGCCCAATTACAA
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 CACATGCACAAGCCTTCGACTACCTGATGATACACATGAGCAAGAAGCTGACGCCAGGCGATGAGTTGCAGCCATTGGCG
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 381

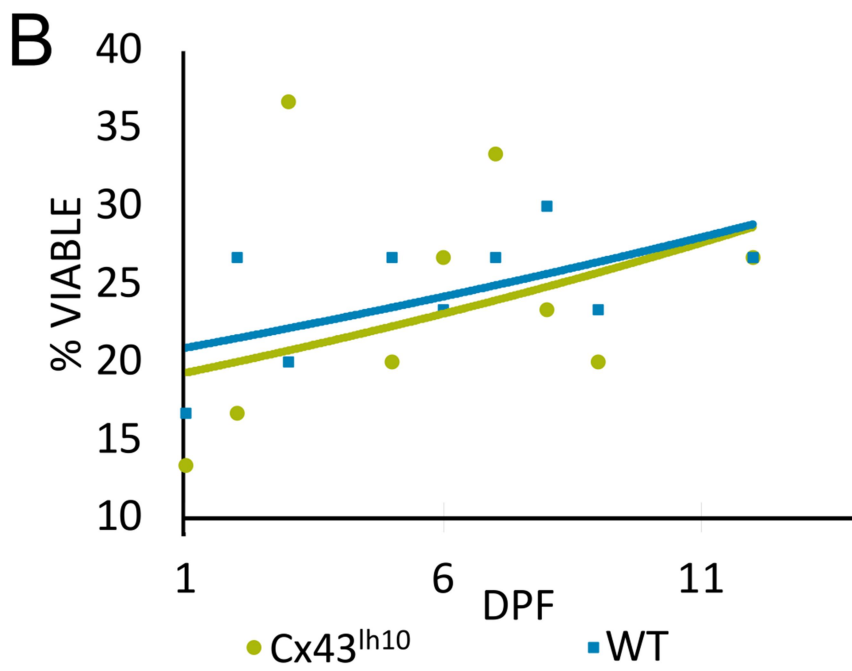
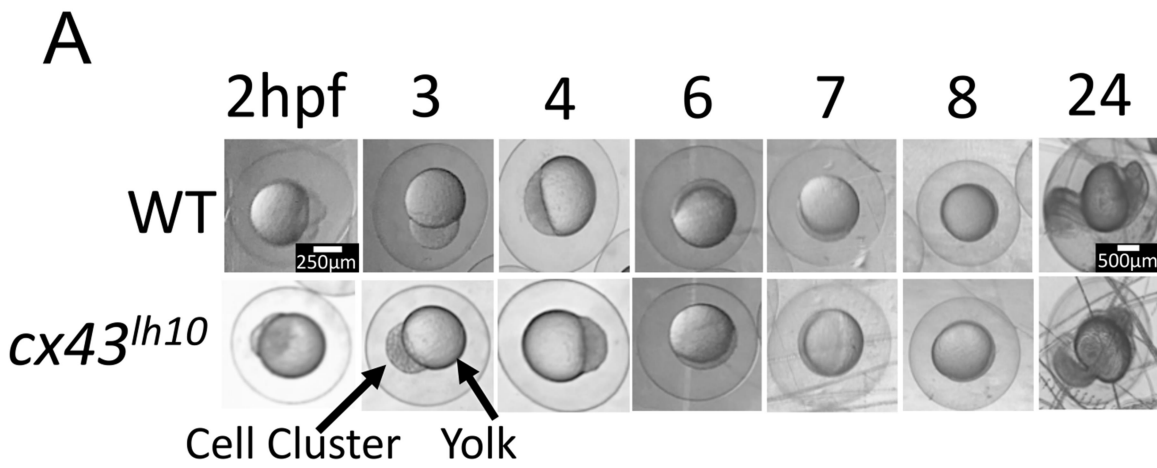
B

gRNA Sequence (including downstream PAM)	Strand	GC Content (%)	MM0	MM1	MM2	MM3	Efficiency
TAGACAGTTCCTCGCGTGGG	-	55	1	0	0	0	59.55
CCAGGCTACAACTGGCCACGG	+	60	1	0	0	0	60.32

C

zf cx40.8 1 ATGGGTGACTGGAGCGCACTGGGAAACTTCTTGACAAGGTCCAGGCTACTCCACTGCT 60
 zf cx43 1 ATGGGTGACTGGAGTGCCTTGGGAAGGCTTCTTGACAAGGTCCAGGCTACTCCAGGCC 60
 61 GGAGGCAAGTCTGGCTCTCGCTCTTTCATCTCCGGATCCTGGGTGTTGGGACGGCGG 120
 61 GGAGGGAAGGCTGGCTCTCTGTGCTTTCATCTCCGGATCCTTGTTCGGGAACAGCA 120
 121 GTGGAGTCCGCTGGGAGACGAGCAGTCCGCTTCAAAATGCAACACGCTGCAACCTGGA 180
 121 GTGGAAATCGGCTGGGGTACGAGCAGTCACTTCAAGTCAATACCCAGCAGCTGGT 180
 181 TGTGAGACGCTGTCTATGATAGTCTTCCCATCTCCACGCTGGCTTCTGGGTGCTG 240
 181 TGCGAAGTGTCTGCTATGACAAATCGTCCCATCTCGCACGTGCGCTTCTGGGTGCTT 240
 241 CAGATTATTTGTGCTCATGCGGACCTCTTATATCTCAGCCATGTGGTGTCTCTATG 300
 241 CAGATCATCTCTGCTGTCACGCGGACGCTCTTACTCTGGCGCATGTCTTACTCTGATG 300
 301 AACAAAGGAGGAGAACTGAA--TAAAAAGAGGACAACTACGAGACATCCAAAGCAA 357
 301 CGAAAGGAGGAGAACTCAACCTAAAGAAGAGG--AGCTGAAGGCCGTGCAAGACGAC 357
 358 GCGGAGATGTGGACGTCTCTGCGC---AAAATCGAAGCAGGAAGTTCAAGTACGGA 414
 358 GCGCGCACGTTGA---GCTCCATCTCAAGAAATCGAGCTCAACAAGTTAAGCATGGC 414
 415 TTGGAGGATCCAGGGAAGATCAAGATGAGGGGAGGATATTTACACGTATATAGTACG 474
 415 CTAGAGGACACGGCAAGCTGAAGATGAAGGTAGCCTGCTGCGCACCTACATCTCAGC 474
 475 ATCGTGTGAAGTCCGTATTTGAAATGTCTTCCCTTTTAAACAGTGGCATCTTTACGGA 534
 475 ATCATTTTCAAGTCCATCTGTGAGGTGCTTCTCTGCTCATCAATGTTACTCTACGGC 534
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 654 CTTCGCTTTTGTCTAACATCATCGAGCTCTTCTACGTGCTCTTCAAACGAATCAAGGA 713
 714 CCAAATAGGGAGTCTGAGAAGA-----AATTGACAGTGCCTGCAATATCAAGCCCTGT 768
 714 CCGGTCA--AAAGCCGACAAACACACAGTTTCCACTG---GCACCTTGAAGCCCAAG 768
 769 CCGAGGAATCTGTC--CGGC---TATGATATTACAATGACTGCT-----CGGCC 813
 769 CCGAAGGAATCTGTCAGACCAAAATACGCTACTACAATGGTTGCTCTCACCAACTGCA 828
 814 CCGTCT--CCAA-----ATCTAGGCTACAATCTAGACACTGTGATAAATCCAACCTCC 864
 829 CCGTCTCACCAATGTACCTCTCAGGCTACAACCTGGCCACCGGCGAAAGGACCAACTCT 888
 865 TCTGATAATTACGACAGCAGGCTAATGAGCAGAACTGGACTAATTACAGCACAGAACAG 924
 889 TGCCGCAATTACAAACAGCAGGCTAATGAGCAGAAATGGGCCAACTACAGCACAGAACAG 948
 925 AACAGTGGGTCA·CCAGTAGTCGAGCAGCAGTGCAGCCAGGCCGGATGATCTTGACATC 1062
 949 AATCGCTTGGGCCA·CCAGCAGCCGATGAGCAGTGCAGCGAGGCTGATGACCTGGACGTC 1143





1. Quantitative RT-PCR Results

<i>Gene</i>	<i>Average C_T</i>	<i>ΔC_T (gene of interest-keratin)</i>	<i>ΔΔC_T (ΔC_{T(exp)} - ΔC_{T(control)})</i>	<i>Fold Difference</i>
Cx43	21.41 ± 0.25	6.75 ± 0.15	-0.26 ± 0.12	1.05 (0.80-1.25)
Rep 1	21.63	6.60	-0.32	1.25
Rep 2	21.47	6.75	-0.33	0.80
Rep 3	21.13	6.91	-0.12	1.10
VEGF	25.34 ± 0.45	10.47 ± 0.09	0.70 ± 0.16	0.62 (0.57-0.71)
Rep 1	25.60	10.56	0.50	0.71
Rep 2	25.18	10.46	0.78	0.58
Rep 3	24.61	10.39	0.81	0.57

The ΔC_T value is determined by subtracting the average C_T value of a housekeeping gene (keratin) from the average gene C_T value. The standard deviation of the difference is calculated from the standard deviations of the gene and keratin values using the comparative method. The calculation of the $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value. This is a subtraction of an arbitrary constant, so the deviation of the $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. The range given for a gene relative to WT is determined by evaluating the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T -s$, where s = standard deviation of the $\Delta\Delta C_T$ value. Three biological replicates (Rep 1-3) were performed for all comparisons.