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*^{lh10} 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*^{lh10} fish.

A

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

1

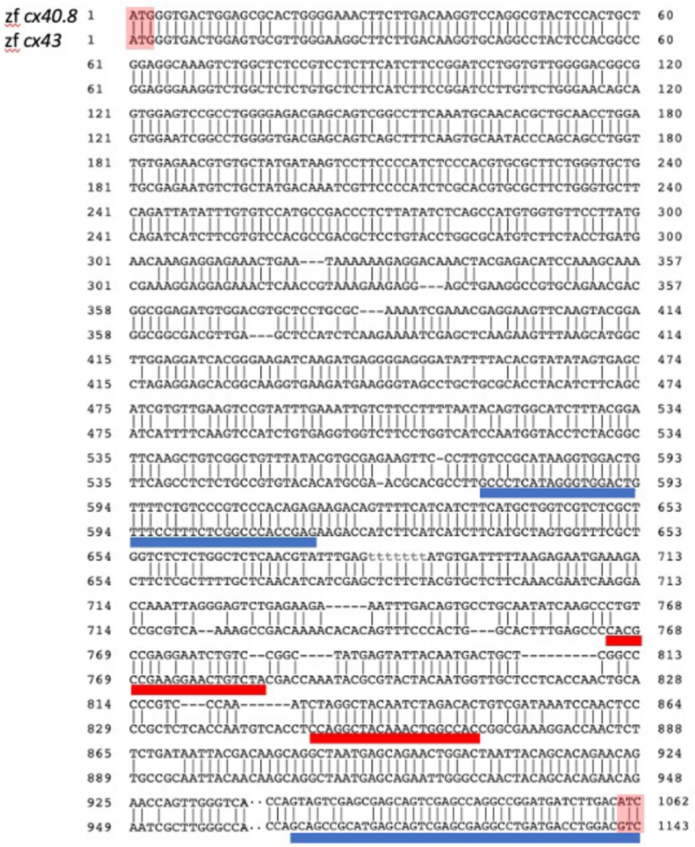
*ATG*GGTGACTGGAGTGCGTTGGGAAGGCTTCTTGACAAGGTGCAGGCCTACTCCACGGCCGGAGGGAAGGTCTGGCTCTCTG
TGCTCTTCATCTTCCGGATCCTTGTTCTGGGAACAGCAGTGGAATCGGCCTGGGGTGACGAGCAGTCAGCTTTCAAGTGCAATA
CCCAGCAGCCTGGTTGCGAGAATGTCTGCTATGACAAATCGTTCCCCATCTCGCACGTGCGCTTCTGGGTGCTTCAGATCATCTT
CGTGTCCACGCCGACGCTCCTGTACCTGGCGCATGTCTTCTACCTGATGCGAAAGGAGGAGAAACTCAACCGTAAAGAAGAGG
AGCTGAAGGCCGTGCAGAACGACGGCGGCGACGTTGAGCTCCATCTCAAGAAAATCGAGCTCAAGAAGTTTAAGCATGGCCT
AGAGGAGCACGGCAAGGTGAAGATGAAGGGTAGCCTGCTGCGCACCTACATCTTCAGCATCATTTTCAAGTCCATCTGTGAGG
TGGTCTTCCTGGTCATCCAATGGTACCTCTACGGCTTCAGCCTCTCTGCCGTGTACACATGCGAACGCACGCCTTGCCCTCATAG
GGTGGACTGTTTCCTTTCTCGGCCCACCGAGAAGACCATCTTCATCATCTTCATGCTAGTGGTTTCGCTCTTCTCGCTTTTGCTCA
ACATCATCGAGCTCTTCTACGTGCTCTTCAAACGAATCAAGGACCGCGTCAAAAGCCGACAAAACACACAGTTTCCCACTGGCA
CTTTGAG**CCCC**A**CGCCGAAGGAACTGTCTA**CGACCAAATACGCGTACTACAATGGTTGCTCCTCACCAACTGCA
CCGCTCTCACCAATGTCACCT**CCAGGCTACAAACTGGCC**A**CGG**CGAAAGGACCAACTCTTGCCGCAATTACAA
CAAGCAGGCTAATGAGCAGAATTGGGCCAACTACAGCACAGAACAGAATCGCTTGGGCCAGAATGGCAGCACCATCTCCAATT
CACATGCACAAGCCTTCGACTACCCTGATGATACACATGAGCACAAGAAACTGACGCCAGGGCATGAGTTGCAGCCATTGGCG
TTGATAGATGCACGGCCGTGCAGCCGTGCCAGCAGCCGCATGAGCAGTCGAGCGAGGCCTGATGACCTGGAC*GTC*

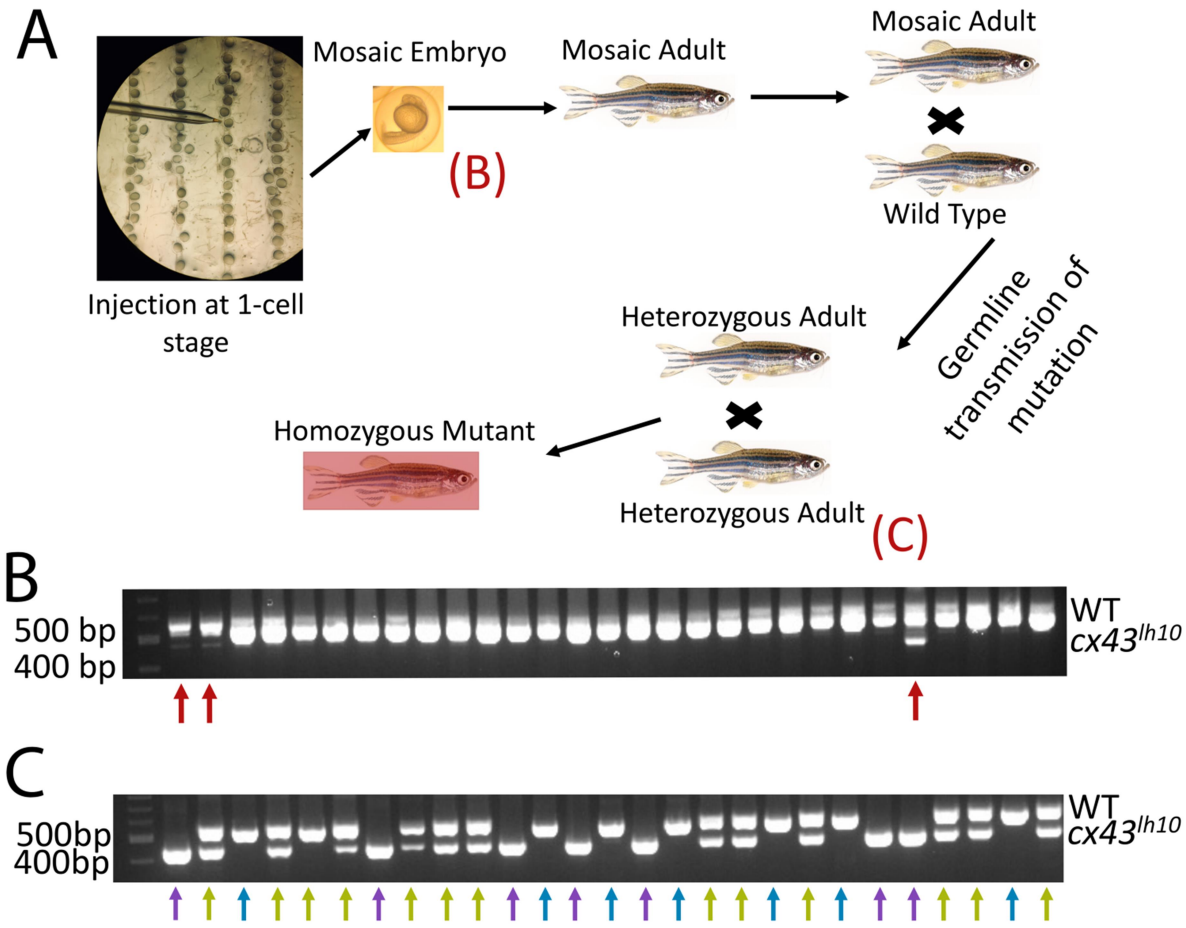
381

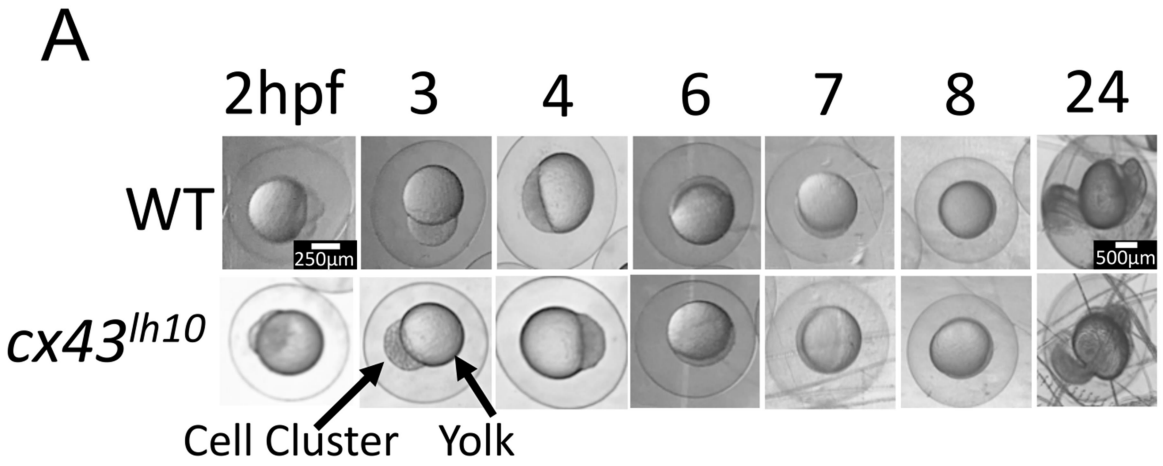
B

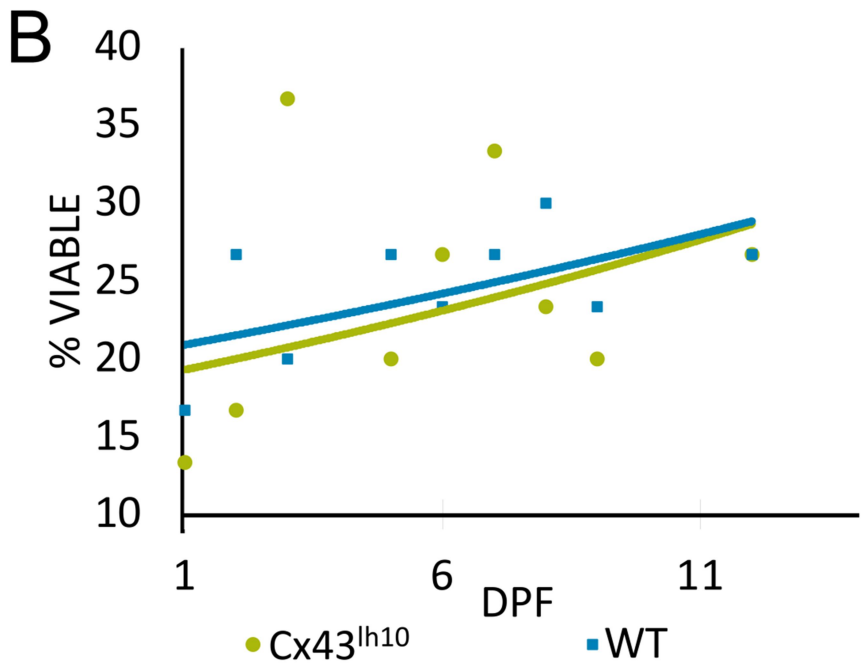
gRNA Sequence (including downstream *PAM*)	Strand	GC Content (%)	MM0	MM1	MM2	MM3	Efficiency
TAGACAGTTCCTTCGGCGTG*GGG*	-	55	1	0	0	0	59.55
CCAGGCTACAAACTGGCCAC*CGG*	+	60	1	0	0	0	60.32

C




A
Mosaic Embryo
Mosaic Adult
Mosaic Adult
(B)
Wild Type
Injection at 1-cell stage
Heterozygous Adult
Germline transmission of mutation
Homozygous Mutant
Heterozygous Adult
(C)
B
500 bp
400 bp
WT
cx43lh10
C
500bp
400bp
WT
cx43lh10


A
2hpf
3
4
6
7
8
24
WT
cx43lh10
250µm
500µm
Cell Cluster
Yolk


B
40
35
30
25
20
15
10
% VIABLE
1
6
11
DPF
Cx43lh10
WT

1. Quantitative RT-PCR Results

Gene	*Average C_T*	*ΔC_T (gene of interest-keratin)*	*$\Delta\Delta C_T$ ($\Delta C_{T(exp)}$ - $\Delta C_{T(control)}$)*	*Fold Difference*
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 $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T$ -s, where s= standard deviation of the ΔΔCT value. Three biological replicates (Rep 1-3) were performed for all comparisons.