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Supplemental Data

**Analysis of U8 snoRNA Variants in Zebrafish Reveals
How Bi-allelic Variants Cause Leukoencephalopathy
with Calcifications and Cysts**

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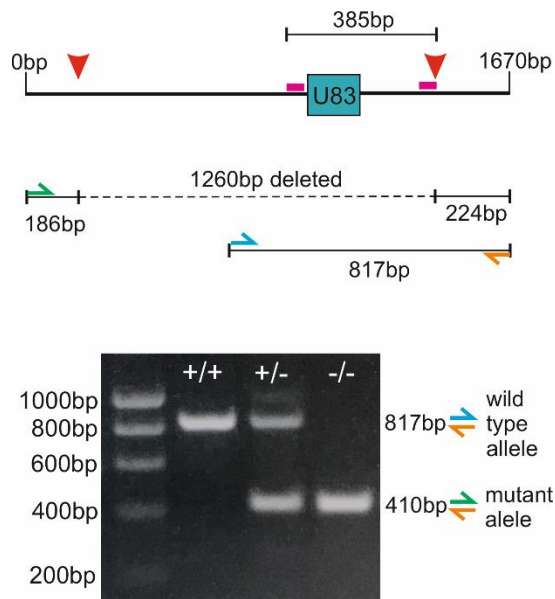


Figure S2. The $\Delta U8-3$ mutant is homozygous for a 1.26kb deletion encompassing the *U8-3* gene locus

Schematic depicting the genotyping strategy for the zebrafish $\Delta U8-3$ mutant. Two sgRNAs predicted to excise 385bp encompassing the *U8-3* gene are represented by pink bars. Red arrowheads depict actual excision sites that delete 1260bp (dashed line) encompassing the *U8-3* gene locus. A three primer PCR was performed to genotype whereby 817bp (blue, orange primer) is amplified in the presence of a wildtype copy of *U8-3* and 410bp (green, orange primer) amplified in the presence of a deleted $\Delta U8-3$ allele. bp, base pairs.

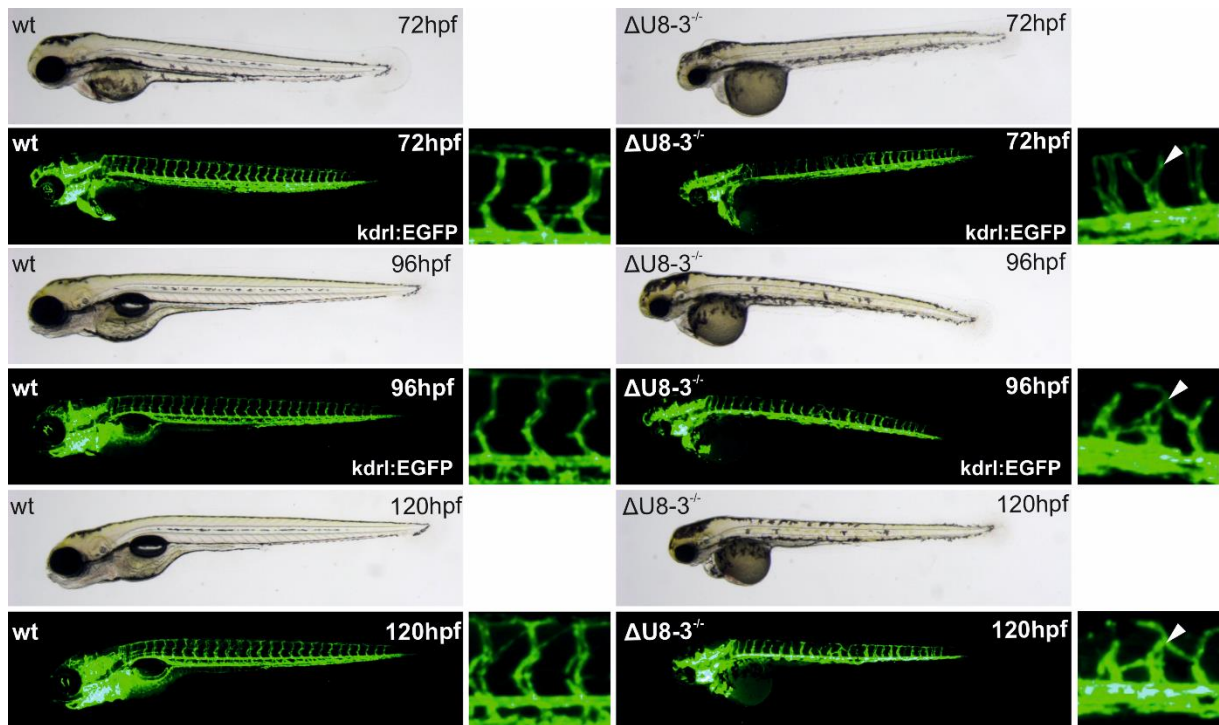


Figure S3. The $\Delta U8-3$ mutant exhibits a range of gross morphological abnormalities and comes to a developmental standstill

From 72hpf $\Delta U8-3$ mutants display impaired yolk resorption, reduced craniofacial structures and hindbrain swelling. By 96hpf $\Delta U8-3$ mutants fail to inflate their swim bladders, display an underdeveloped intestine, and by 120hpf display cardiac oedema. At all stages disorganized trunk vasculature is observed (white arrowheads) when compared to wildtype (inserts).

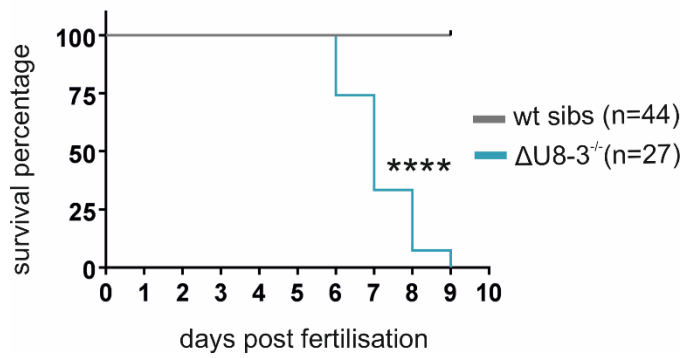


Figure S4. The $\Delta U8-3$ mutant phenotype is lethal

Kaplain-Meier plot demonstrating that $\Delta U8-3$ mutants begin to die from 6dpf and exhibit 100% mortality by 9dpf. dpf - days post fertilization.

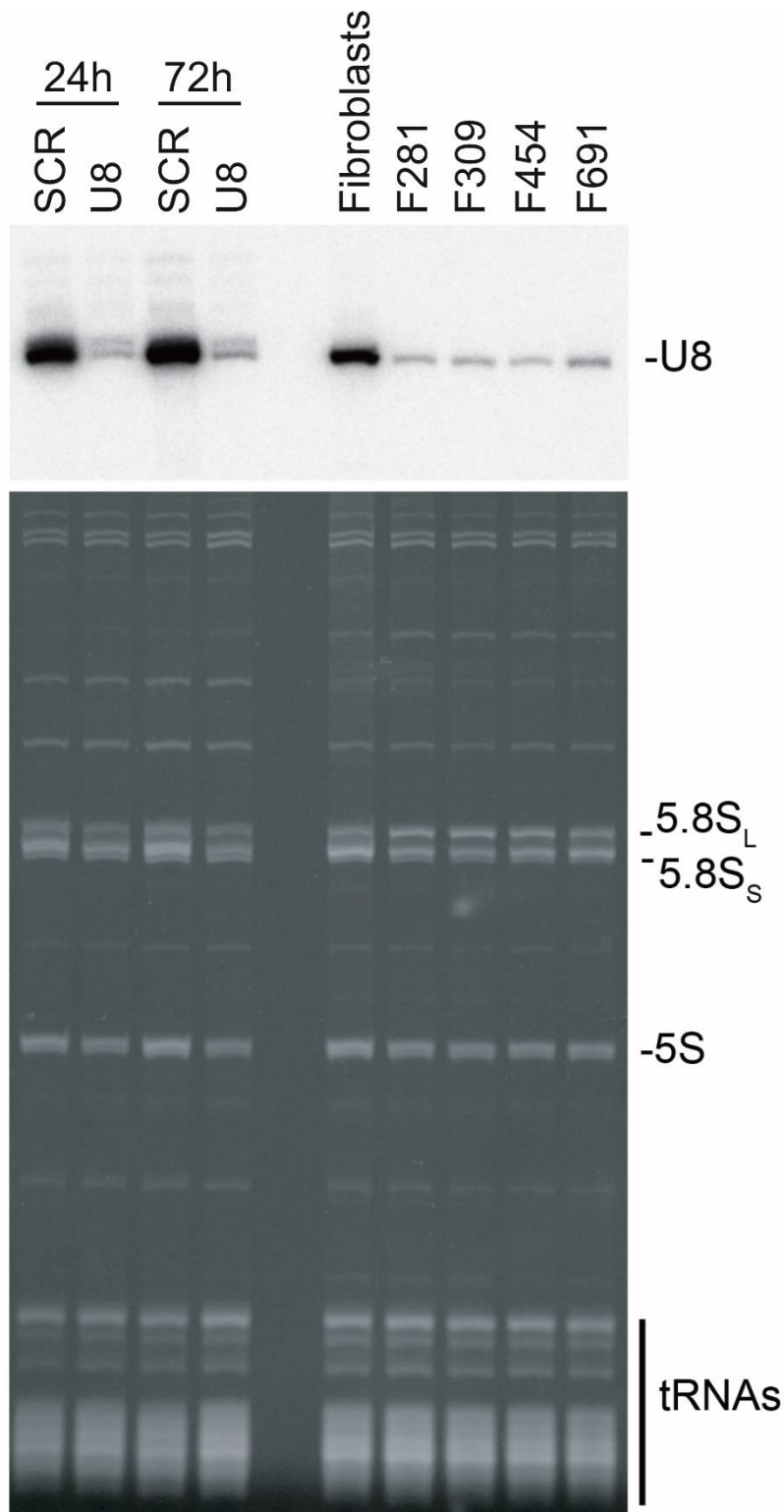


Figure S5. LCC fibroblasts express less total U8 compared to wildtype fibroblasts

Northern blot analysis with a probe specific to U8 snoRNA. U8 expression is shown after ASO-mediated depletion of human U8 snoRNA from HCT116 cells in which rRNA processing was analyzed (see Figure 2E), or in LCC fibroblast cell lines F281, F309, F454 and F691. ASO – antisense oligonucleotide. The same amount of total RNA (OD₂₆₀) was loaded in each lane of the gel. Ethidium bromide staining of the acrylamide gel demonstrates equal loading, as determined by tRNAs which are produced independently.

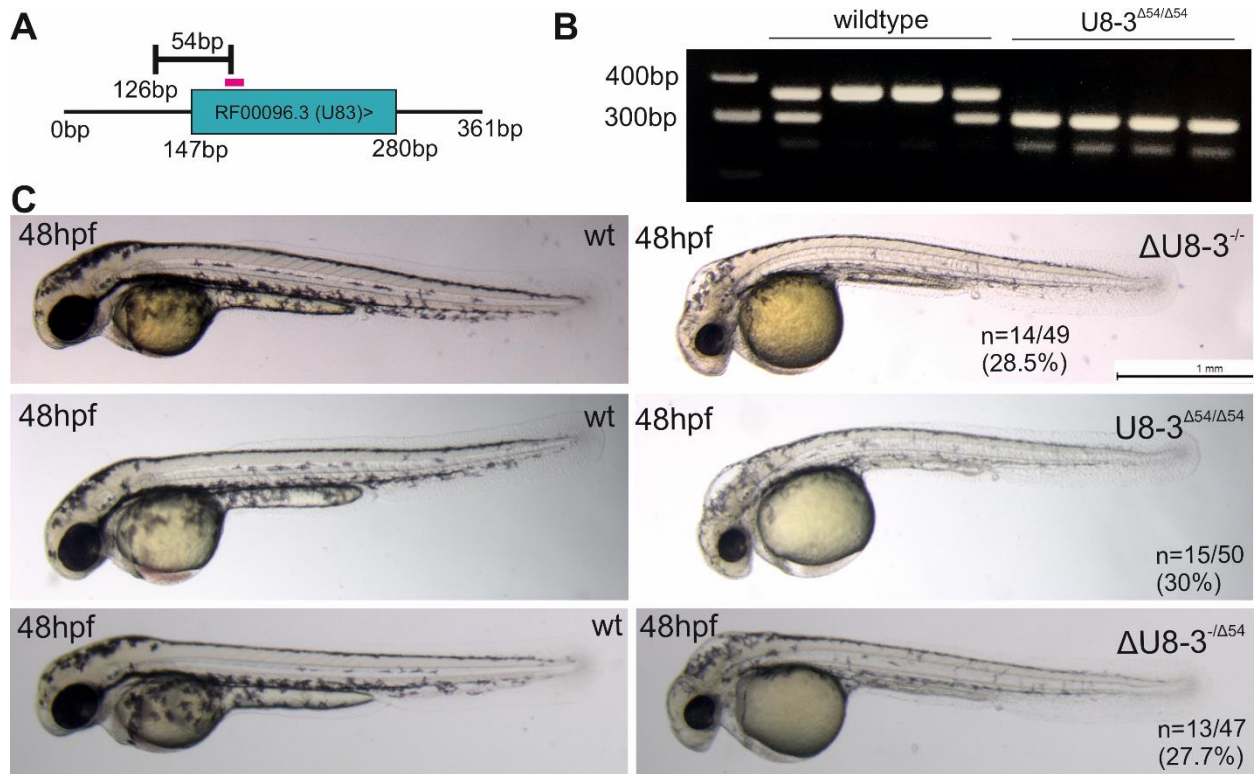


Figure S6. Non-complementation with independent mutant alleles of *U8-3* demonstrates that the $\Delta U8-3$ mutant phenotype is specific

A) Schematic depicting the 54bp deletion allele ($\Delta 54U8-3$) generated with an independent sgRNA (pink bar) from the sgRNAs used to excise *U8-3* shown in Figure 2A.

B) Genotyping demonstrates the 54bp deletion (307bp band) segregates with the *U8-3* mutant phenotype.

C) Both $\Delta U8-3$ and $\Delta 54U8-3$ alleles display mendelian recessive inheritance i.e. approximately 25% of mutant progeny are produced from incrossing heterozygous carriers. When heterozygous carriers of each allele were crossed to each other non-complementation (failure to produce 100% wildtype progeny) was observed, demonstrating the alleles result from loss-of-function in the same gene.

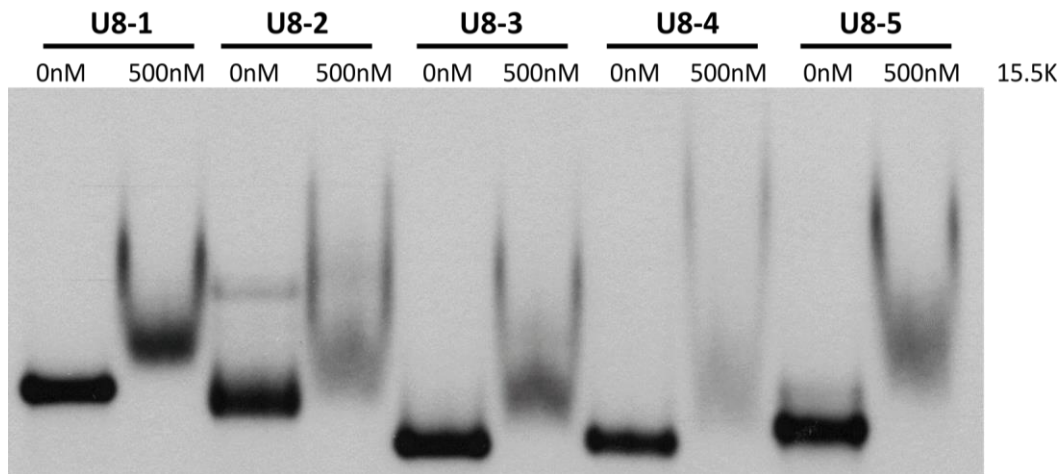


Figure S7. EMSAs demonstrates all five zebrafish U8s bind 15.5K

Protein binding of zebrafish U8 variants. Electrophoretic mobility shift assays (EMSAs) using 5'-end-radiolabeled *in vitro* transcribed mature zebrafish *U8-1*, *U8-2*, *U8-3*, *U8-4* and *U8-5* with 500nM of His₆-tagged 15.5K human protein (15.5K). Binding of 15.5K to zebrafish U8 is indicated by a mobility shift.

		3' processed region			3'BOX
				GTYY (N2-5)	AAARRYAGA
human U8	CTTTCTGACGATCACTTACATTTGT			GTTATGCT	GATTAGCAGA
zebrafish U8-3	ACAT TTT AATAACC			GTTT	CAAATCAGA

Figure S8. Alignment of human U8 and zebrafish U8-3 3' extension sequences

Human and putative zebrafish 3' extension sequences (blue) are divergent. A 3' BOX is predicted for zebrafish U8-3 (pink) according to the 3' BOX consensus sequence (black).

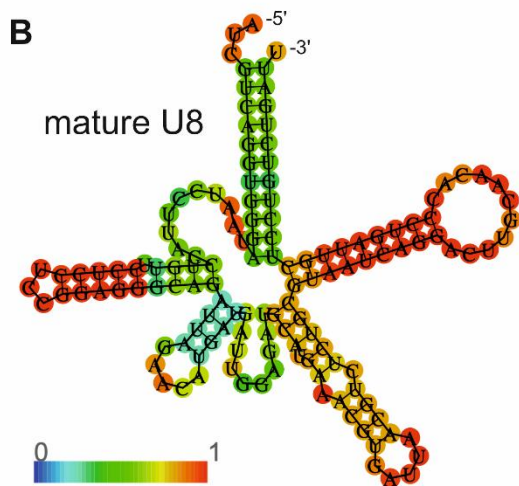
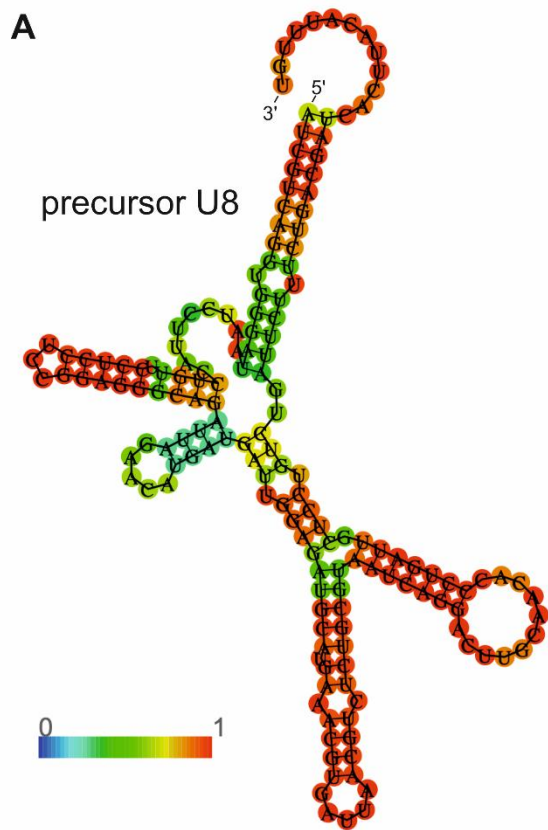


Figure S9. Minimum free energy secondary structure predictions of precursor and mature human U8

A) Minimum free energy secondary structure prediction by RNA fold of the precursor human U8. Duplex formation between the 5' end and 3' extension is predicted with high probability. The probability of each base-pairing interaction is represented by a colorimetric scale ranging from 0 (blue) to 1 (red).

B) Minimum free energy secondary structure prediction by RNA fold of the mature human U8. Duplex formation between the 5' end and 3' end is predicted with low probability. The probability of each base-pairing interaction is represented by a colorimetric scale ranging from 0 (blue) to 1 (red).

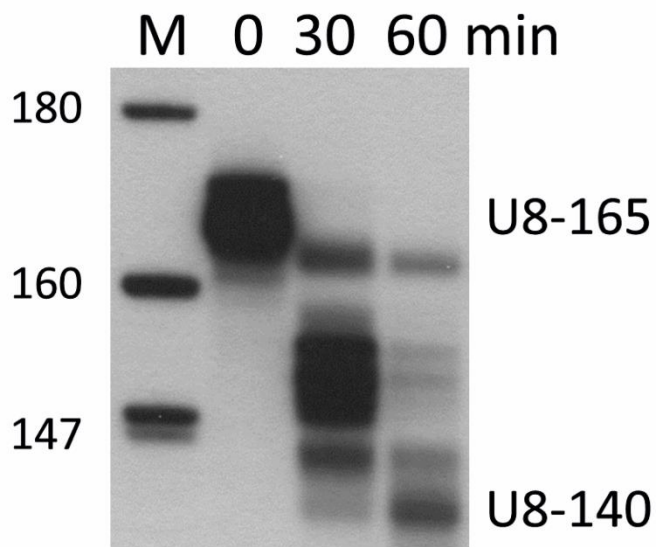


Figure S10. Time course of wild type human pre-U8 snoRNA processing.

Processing of 5' end-radiolabelled *in vitro* transcribed precursor U8 wildtype snoRNA (U8-165) was assessed in HeLa nuclear extracts at 0, 30 and 60 minutes (min) to monitor production of mature U8 (U8-140). Mature U8 is clearly present after 60 min of processing.

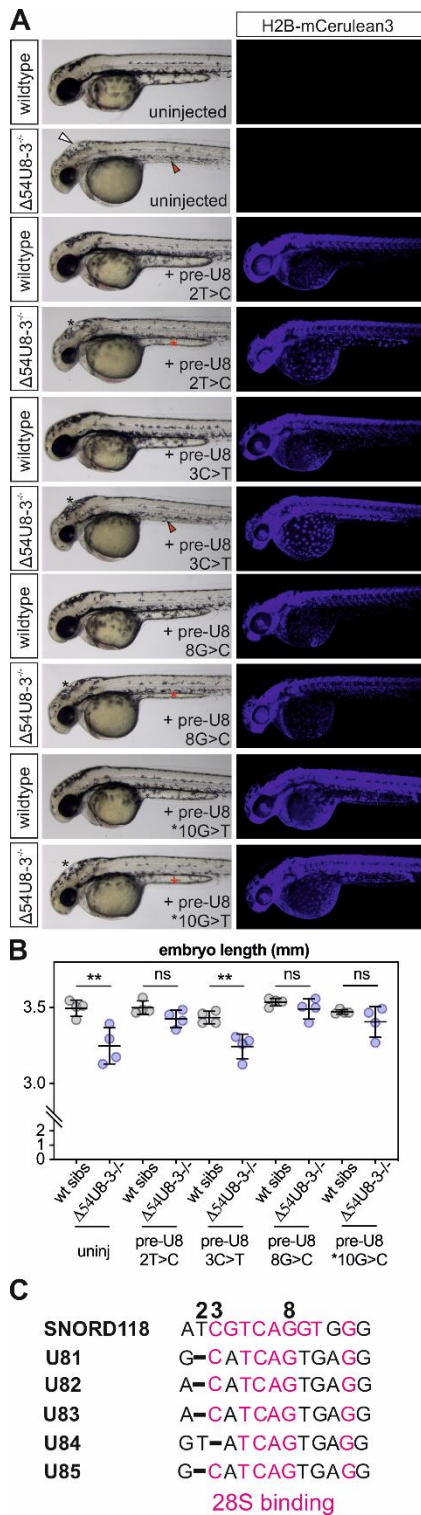


Figure S11. Functional testing of four LCC mutant U8 snoRNAs in zebrafish

A) Representative images of rescue experiments conducted with four putatively hypomorphic U8 mutations detected in LCC individuals. White arrowhead indicates hindbrain swelling and red arrowheads indicate abnormal yolk extension. Black and red asterisks indicate rescued hindbrain swelling and yolk extension respectively.

B) pre-U8^{3C>T} does not rescue the reduced embryo length of $\Delta U8-3$ mutants, whereas pre-U8^{2T>C}, pre-U8^{8G>C} and pre-U8^{*10G>T} restores $\Delta U8-3$ mutant embryo length to that of wildtype siblings. n=4 embryos per genotype. Error bars indicate S.D. from the mean.

C) alignment of human U8 and zebrafish U8 28S interaction region. Shared identity between zebrafish and human 28S-binding nucleotides is shown in pink.

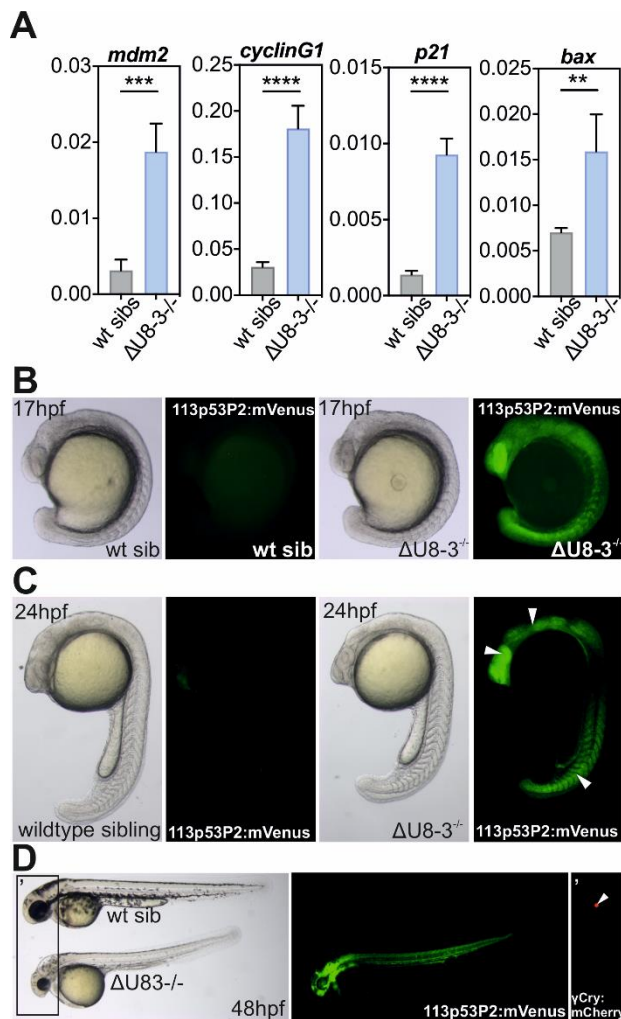


Figure S12. tp53 is trans-activated in response to loss of *U8-3* in zebrafish

A) quantitative RT-PCR demonstrates significant upregulation of the tp53 target genes *mdm2*, *cyclinG1*, *p21* and *bax* in $\Delta U8-3$ mutants when compared to wildtype siblings at 24hpf. Error bars indicate S.D. from the mean.

B) tp53 activity is observed throughout the $\Delta U8-3$ mutant at 17hpf, prior to the onset of observable gross morphological abnormalities, particularly in the eye.

C) tp53 activity is observed in numerous tissues that develop abnormally in $\Delta U8-3$ mutants, including the eye, hind-brain and somites (arrowheads) at 24hpf.

D) tp53 activity is observed throughout the $\Delta U8-3$ mutant at 48hpf. Integrated 113p53P2:mVenus transgene is reported by γ -crystallin:mCherry in the backbone of the plasmid which drives lens specific mCherry expression from approximately 36hpf. The developmental delay in $\Delta U8-3$ mutants delays expression of this reporter.

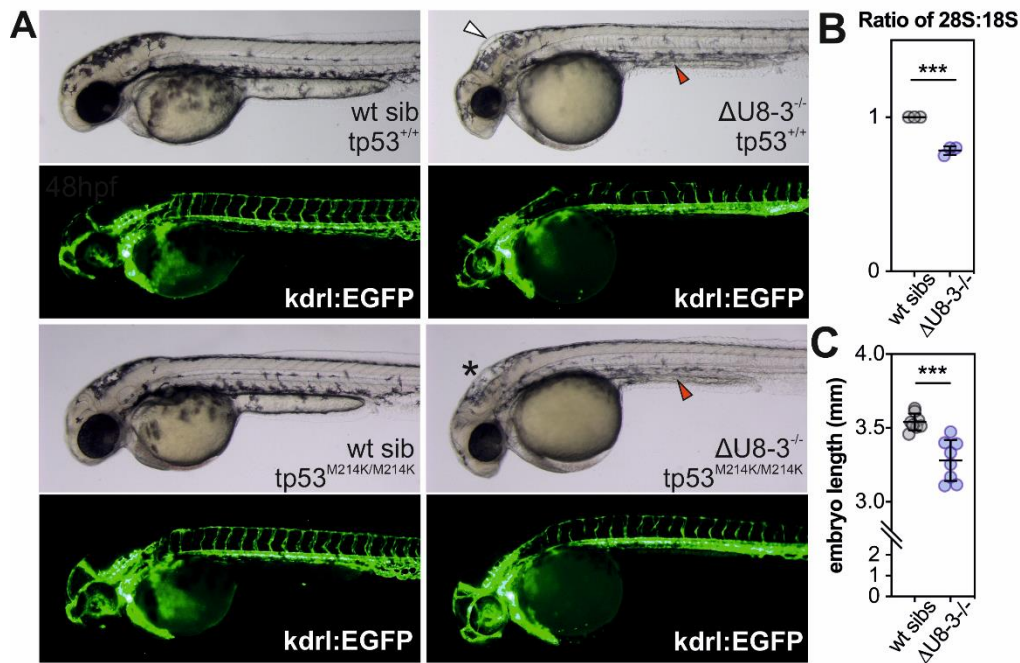


Figure S13. Inactivation of tp53 partially rescues the $\Delta U8-3$ mutant phenotype although fails to rescue the embryo length or rRNA biogenesis defect

A) Representative images showing inactivation of tp53 signaling rescues the hindbrain swelling of $\Delta U8-3$ mutants (white arrowhead compared to black asterisk) and the trunk vasculature, but not the yolk extension (red arrowhead) abnormality.

B) Tapestation assay demonstrates tp53 mutant $\Delta U8-3$ mutant embryos display a preferential reduction in 28S biogenesis at 48hpf. $n=3$ biological replicates per genotype. *Error bars* indicate S.D. from the mean.

C) tp53 mutant $\Delta U83$ mutant embryos are significantly shorter than tp53 mutant $\Delta U8-3$ wildtype sibling embryos at 48hpf. $n=8$ biological replicates per genotype. *Error bars* indicate S.D. from the mean.

Table S1. LCC cohort published in Jenkinson 2016

Family	Number of affected individuals	Chromosomal position (Hg19)	Variants detected	Zygoty	gnomAD frequency
F172	1	g.8076761C>A	n.10*G>T	Het	0.002495
		g.8076851dup	n.56dup	Het	0.000008617
F278 #	1	g.8076832T>C	n.75A>G	Het	0.00001723
		g.8076899C>G	n.8G>C	Het	0.00001523
F281	1	g.8076826C>G	n.81G>C	Het	Novel
		g.8076904G>A	n.3C>T	Het	0.001482
F285	1	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076850C>T	n.57G>A	Het	0.00002657
F309	1	g.8076776G>C	n.131C>G	Het	0.00001520
		g.8076904G>A	n.3C>T	Het	0.001482
F330	1	g.8076825T>C	n.82A>G	Het	0.00006832
		g.8076899C>T	n.8G>A	Het	0.002756
F331	2	g.8076761C>A	n.*10G>T	Het	0.002495
		g.8076835T>C	n.72A>G	Het	0.00006894
F334	1	g.8076825T>C	n.82A>G	Het	0.00006832
		g.8076761C>A	n.*10G>T	Het	0.002495
F337	1	g.8076905A>G	n.2T>C	Het	0.00002167
		g.8076849dup	n.58dup	Het	Novel
F343	1	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076885_8076913dup	n.-7_22dup	Het	Novel
F344	1	g.8076794G>A	n.113C>T	Hom	0.00004177
		g.8076899C>G	n.8G>C	Hom	0.00001523
F362	2	g.8076766G>C	n.*5C>G	Het	0.0006056

		g.8076887G>A	n.20C>T	Het	0.00003021
F414	1	g.8076770G>A	n.*1C>T	Het	0.005054
		g.8076846T>C	n.61A>G	Het	0.00006073
F426	2	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076826C>T	n.81G>A	Het	0.00004740
F433	1	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076887G>A	n.20C>T	Het	0.00003021
F445	1	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076780G>C	n.127C>G	Het	0.00004316
F446	1	g.8076766G>C	n.*5C>G	Hom	0.0006056
F454	2	g.8076766G>C	n.*5C>G	Het	0.0006056
		g.8076955_8076960del	n.-54_-49del	Het	Novel
F465	1	g.8076825T>C	n.82A>G	Het	0.00006832
		g.8076846_8076847insA	n.60_61insT	Het	0.000007591
F521	2	g.8076803C>T	n.104G>A	Het	0.0004176
		g.8076776G>C	n.131C>G	Het	0.00001520
F551	1	g.8076762G>A	n.*9C>T	Het	0.001923
		g.8076780G>C	n.127C>G	Het	0.00004316
F564	1	g.8076770G>A	n.*1C>T	Het	0.005054
		g.8076781G>A	n.126C>T	Het	0.0001178
F691	1	g.8076762G>A	n.*9C>T	Het	0.001923
		g.8076849T>C	n.58A>G	Het	0.00002277
F730	1	g.8076761C>A	n.*10G>T	Het	0.002495
		g.8076826C>T	n.81G>A	Het	0.00004740
F766	1	g.8076865C>T	n.42G>A	Het	0.001070
		g.8076904G>T	n.3C>A	Het	0.000004327
F780	2	g.8076770G>A	n.*1C>T	Het	0.005054
		g.8076776G>C	n.131C>G	Het	0.00001520

F819	2	g.8076762G>A	n.*9C>T	Het	0.001923
		g.8076770G>A	n.*1C>T	Het	0.005054
		g.8076696_8076977del§		Het	Novel
F906	1	g.8076804C>T	n.103G>A (<i>de novo</i>)	Het	0.00001724
		g.8076868C>G	n.39G>C	Het	0.0001253
F1127	1	g.8076762G>A	n.*9C>T	Het	0.001923
		g.8076807A>C	n.100T>G	Het	Novel
F1172	1	g.8076762G>A	n.*9C>T	Het	0.001923
		g.8076776G>C	n.131C>G	Het	0.00001520
F1288	1	g.8076848A>C	n.59T>G	Het	Novel
		g.8076762G>A	n.*9C>T	Het	0.001923
F1424	1	g.8076777A>G	n.130T>C	Het	0.000008628
		g.8076912C>T	n.-6G>A	Het	0.0008539
F1445	1	g.8076904G>A	n.3C>T	Het	0.001482
		g.8076826C>T	n.81G>A	Het	0.00004740

#Also reported in McNeill N et al. *Neurol Genet* 14: e162 (2017)

Bolded mutations proposed to be hypomorphic – individuals highlighted in green lack mutations in any of the affected seven nucleotides located in the novel base-pairing region between the 5' end and 3' extension.

Nomenclature in gnomAD does not correspond to nomenclature in Alamut

Het = heterozygous; Hom = homozygous. gnomAD = Genome Aggregation Database

§ Deletion extends beyond these boundaries, but boundaries have not been fully defined. One sib carries g.8076762G>A

plus g.8076696_8076977del. The other sib carries g.8076770G>A plus g.8076696_8076977del. Mum carries g.8076762G>A plus g.8076770G>A.

Table S2. Oligonucleotides used in this study

PURPOSE	OLIGONUCLEOTIDE SEQUENCE 5' TO 3'
Sequence zebrafish U81 from genomic DNA	S ACGATACCAATGCTGTAC AS GTAAGGATACACAGAATACC
Sequence zebrafish U82 from genomic DNA	S CTCCACCAATAGTATCGCAG AS CTGTGGTGAGAAGTAACTTTC
Sequence zebrafish U83 from genomic DNA	See Genotype Δ54U8-3 allele
Sequence zebrafish U84 from genomic DNA	S CCAATAGGATCACAGGAGAC AS GCATAGTAAGCTATAGTGCTC
Sequence zebrafish U85 from genomic DNA	S GGAGCCATTAGCATGCTAAC AS TTTCTAGCACACCCTGCTG
Quantitative RT-PCR to U8-1	S GGTTTATCCTTACCTGTTGTC AS CAGACAGGATTCATAAAGGTC
Quantitative RT-PCR to U8-2	S ACATCAGTGAGGTTTGTC AS CACATAGGATCCAGAGTTGTG
Quantitative RT-PCR to U8-3	S GAGGTACATCCTTACCTGTTAC AS GATTTCGTAAGGGGTTGCAG
Quantitative RT-PCR to U8-4	S GGTTTATCCTTACCTGTTGTC AS TAAAGAGGTTGCAAGTCATG
Quantitative RT-PCR to U8-5	S GCATCAGTGAGGTTTATCCTTG AS CAAGCCCTATATGAACACAGAG
DNA template for production of guide RNA to cleave 5' of U8-3	S TAATACGACTCACTATAGGCGTCGTTTAAAAAGCATGTGGTTTTAGAGC AS AAAAGCACCGACTCGGTGCCACTTTTTCAAG
DNA template for production of guide RNA to cleave 3' of U8-3	S TAATACGACTCACTATAGGCGCCGGCGTAGAGACGAGGGTTTTAGAGC AS AAAAGCACCGACTCGGTGCCACTTTTTCAAG
DNA template for production of guide RNA specific to U8-3 Genotype ΔU8-3 allele	S TAATACGACTCACTATAGATCTGTAACCTTATTGCTAGTTTTAGAGC AS AAAAGCACCGACTCGGTGCCACTTTTTCAAG
Genotype Δ54U8-3 allele	S1 ACCCTTGACAGAGGAGTTTG S2 TCCAATCCAACCAATGAGAC AS CATGCAGAAATGTCTCACTATC
Genotype Δ54U8-3 allele	S TAAACGATCGTCTCGTCCAC AS GGAATGGAGTCACAGACTTAC
DNA template for production of zebrafish U8-3 snoRNA	S TAATACGACTCACTATAGGGGACATCAGTGAGGTACATCC AS GTCAGACAGGATTTCGTAAAG
DNA template for production of zebrafish pre-U8-3 snoRNA	S TAATACGACTCACTATAGGGGACATCAGTGAGGTACATCC AS GGTTATTTAAAATGTGTCAGACAGGATTC
DNA template for production of human U8 snoRNA	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS AATCAGACAGGAGCAATCAGGGGTGTTGCAAG
DNA template for production of human pre-U8 snoRNA	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 57G>A	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCCTTACCTGTTCCCTCCTCCG GAGGGCAGATTAGAACATAATGATTGGAGATGCATG AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 58A>G	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCCTTACCTGTTCCCTCCTCCG GAGGGCAGATTAGAACATGGTATTGGAGATGCATG AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 61A>G	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCCTTACCTGTTCCCTCCTCCG GAGGGCAGATTAGAACATGATGGTTGGAGATGCATGAAAC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA *1C>T	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCAGAAAAAATCAGACAGGAG
DNA template for production of human pre-U8 snoRNA *5C>G	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCACAAAGAATCAGACAGGAG
DNA template for production of human pre-U8 snoRNA *9C>T	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 2T>C	S TAATACGACTCACTATAGGGGACCGTCAGGTGGGATAATCCTTACC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 3C>T	S TAATACGACTCACTATAGGGGATGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA 8G>C	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATCGTCAG
DNA template for production of human pre-U8 snoRNA *10G>T	S TAATACGACTCACTATAGGGGATCGTCAGGTGGGATAATCC AS ACAAATGTAAGTGATAGTCAGAAAGAATCAGACAGGAG
Amplification of 113p53 promoter	S GACAGACTCGAGTAGCTCGTTGGTCTGACATC AS GACAGAAGTACTAGTCTGCAGTTTGTGCTGTG
Quantitative RT-PCR to zebrafish Δ113p53	S ATATCCTGGCGAACATTTGG AS ACGTCCACCACCATTTGAAC
Quantitative RT-PCR to zebrafish mdm2	S TGACAACGAGAACTGGTAAGA AS AAACATAACCTCCTTCATGGT
Quantitative RT-PCR to zebrafish p21	S AGCTGCATTCTCTCGTAGC AS CGGTTGAAATAAAAACGGAATA

Quantitative RT-PCR to zebrafish cyclinG1	S GTGCGGAGACGTTTTCTT AS AAGACAGATGCTTGGGCTGA
Quantitative RT-PCR to zebrafish bax	S GGAGGCGATACGGGCAGTG AS TCGGCTGAAGATTAGAGTTGTTT
Quantitative RT-PCR to zebrafish elf1a	S CTGGAGGCCAGCTCAAAC AS ATCAAGAAGAGTAGTACCGCTAGCATTAC
Genotyping of the M214K substitution in p53 mutant zebrafish. The p53 locus is amplified with S1-AS1 for 35 cycles. 1ul of the PCR product is used as a DNA template for amplification with S2-AS2. Sequencing is performed with M13	S1 CCATGTAGTGAAGTATAGTTGC AS1 GTCGGGTCTTCAGTTTTATGC S2 TGTAACGACGCGCCAGTGAATTATCAA AS2 AGGAAACAGCTATGACCATCCAATGGCATG M13 CAGGAAACAGCTATGAC
Probe sequence for human 3'-ETS - LD2612	GAGGAGGCGGGAACCGAAGAAGCGG