

A tRNA-based multiplex sgRNA expression system in zebrafish and its application to generation of transgenic *albino* fish

Tomoya Shiraki and Koichi Kawakami

Supplementary information

Sequences of tRNA genes and ribozymes used in this study

tRNA 5' leader sequences are shown in lower-case

> Dr-tRNA^{Gly}(GCC)

gtgaGCATTGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTT
CGATTCCCGGCCAATGCA

> Dr-tRNA^{Lys}(CTT)

ggtctcatcaGCCCCGGCTAGCTCAGTCGGTAGAGCATGAGACTCTTAATCTCAGGGTCGTG
GGTTCGAGCCCCACGTCGGGCG

> Dr-tRNA^{Asn}(GTT)

gctatctGTCTCTGTGGCGCAATCGGTTAGCGCGTTCGGCTGTTAACCGAAAGGTTGGT
GGTTCGAGCCCCACCCAGGGACG

> Dr-tRNA^{Met}(CAT)

gcctgaagGTTTCCGTAGTGTAGTGGTTATCACGTTGCCTCATACGCGAAAGGTCCCCA
GTTTCGAAACTGGGCGGAAACA

> Dr-tRNA^{Gln}(CTG)

gacttgaGGTTCCATGGTGTAAATGGTTAGCACTCTGGACTCTGAATCCAGCGATCCGAGT
TCAAATCTCGGTGGGACCA

> Dr-tRNA^{Ser}(GCT)

ggaaaatGACGAGGTGGCCGAGTGGTTAAGGCGATGGACTGCTAATCCATTGTGCTTTG
CACGCATGGGTTCGAATCCCATCCTCGTCG

> Dr-tRNA^{Thr}(AGT)

gcagcGGCGCCGTGGCTTAGTTGGTTAAAGCGCCTGTCTAGTAAACAGGAGATCCTGG
GTTTCGAATCCCAGCGGTGCCT

> Dr-tRNA^{His}(GTG)

gctcGCCGTGATCGTACAGTGGTTAGTACTCTGCGTTGTGGCCGCAGCAACCCCGGTT
CGAATCCGGGTCACGGCA

> Dr-tRNA^{Leu}(CAG)

gcatGTCAGGATGGCCGAGTGGTCTAAGGCGCTGCGTTCAGGTCGCAGTCTCCCCTG
GAGGCGTGGGTTTCAATCCCACTTCTGACA

>Os-tRNA^{Gly}(GCC)

gaacaaaGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGG
TTCGATTCCCGGCTGGTGCA

>Os-tRNA^{Gly}(GCC)-scrambled

GAACCTCTTACACGCGCAGATCAACTAAATGTACACTGCGACGGTCCGTGGCTCCGA
GAGGGGTTACAGGGTACGCTG

>Dr-tRNA^{Gly}(GCC)-scrambled

GCGCTGTGGCGTACCGGGTACGTACTCGCTTGACTGGGTTGGTACTAGGCGAAACC
AGCTCCGTGGGATTGCACC

>Hammerhead ribozyme (HH)

gttccccCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTC

>Hepatitis delta virus ribozyme (HDV)

GGCCGGCATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCAT
GGCGAATGGGAC

Supplementary Table S1.

Summary of zebrafish tRNA genes used in this study

tRNA Isotype	Anticodon	Gene number	tRNA # used in this study	tRNA-Score
Asn	GTT	1138	Chr15.tna38	82.89
Lys	CTT	1018	Chr5.tna677	81.83
Gly	GCC	826	Chr22.tna999	81.62
Met	CAT	612	Zv9-scaffold3503.tna115	84.46
Ser	GCT	436	Chr4.tna543	86.48
His	GTG	405	Chr4.tna5002	67.62
Thr	AGT	384	Chr7.tna183	84.06
Gln	CTG	297	Chr4.tna512	75.59
Leu	CAG	314	Chr22.tna605	78.35

Supplementary Table S2.

DNA oligos used for sgRNA synthesis

sgRNA Name	Sequence
drslc45a2_sg1	taatacgactcactataGGGGAAGGTTGATTATGCACgtttagagctagaa
drslc45a2_sg2	taatacgactcactataGGGGTCGCCATGTTTGGAAGtttagagctagaa
drslc45a2_sg3	taatacgactcactataGGAGGTCGTCATGGGGCCGAgtttagagctagaa
drslc45a2_sg4	taatacgactcactataGGGGGTTGAAGTGGGCTGTTgtttagagctagaa
drslc45a2_sg5	taatacgactcactataGGGAGATGGAGTGGCTCCTGgtttagagctagaa
drmpv17_sg1	taatacgactcactataGGGGTCCAGTGGTCGGTGGAGtttagagctagaa
drmpv17_sg2	taatacgactcactataGGTGGTCGGTGGATGGTACAgtttagagctagaa
drmpv17_sg3	taatacgactcactataGGTTGTGATTGGCCAGTCCAgtttagagctagaa
drmpv17_sg4	taatacgactcactataGGAATAACTGGAACCCCTCAAgtttagagctagaa
drmpv17_sg5	taatacgactcactataGGGAGGGATGAAATAAAAAATgtttagagctagaa
sgTail primer1	aaaagcaccgactcgggtgccacttttcaagttgataacggactagcctattttaacttgctatttctagctctaaac
drslc45a2_sg1-2 (for sgTail primer2)	taatacgactcactataGGGGAAGGTTGATTATGCACgtttaagagctatgc
sgTail primer2	aaaagcaccgactcgggtgccacttttcaagttgataacggactagcctattttaacttgctatgctgttccagcatagctcttaaac

Targeting sequences of sgRNAs are shown in upper-case

Supplementary Table S3.

DNA oligos used for the synthesis of 5'-tRNA-sgRNA, sgRNA-tRNA-3', 5'-HH-sgRNA, and sgRNA-HDV-3'

sgRNA Name	Primer Name	Sequence
5'-Dr-tRNA ^{Gly} (GCC)-sgRNA	T7_Gly-GCC Fw slc45a2-sg1_Gly-GCC Rv	taatacgactcactataGGTGAGCATTGGTGGTTCAGTG GTGCATAATCAACCTTCCCCTGCATTGGCCGGAATCGAAC
5'-Dr-tRNA ^{Gly} (GCC)scr-sgRNA	T7-DrGly-GCC-sh Fw slc45a2-sg1-DrGly-GCC-scr Rv	taatacgactcactataGGCGCTGTGGCGTACCGGG GTGCATAATCAACCTTCCCCGGTGCAATCCCACGGAGCTG
5'-Os-tRNA ^{Gly} (GCC)-sgRNA	T7-OsGly-GCC Fw slc45a2-sg1-OsGly-GCC Rv	taatacgactcactataGGAACAAAGCACCAGTGGTCT GTGCATAATCAACCTTCCCCTGCACCAGCCGGAATCGAA
5'-Os-tRNA ^{Gly} (GCC)scr-sgRNA	T7-OsGly-GCC-sh Fw slc45a2-sg1-OsGly-GCC-scr Rv	taatacgactcactataGGAACCTCTTACACGCGCAGAT GTGCATAATCAACCTTCCCCCAGCGTACCCTGTAACCCCTCT
5'-HH-sgRNA	T7-HH Fw slc45a2-sg1-HH Rv	taatacgactcactataGGTTCCCCCTGATGAGTCCGT GTGCATAATCAACCTTCCCCGACGAGCTTACTCGTTTC
5'-Dr-tRNA ^{Asn} (GTT)-sgRNA	T7_Asn-GTT Fw slc45a2-sg1_Asn-GTT Rv	taatacgactcactataGGCTATCTGTCTCTGTGGC GTGCATAATCAACCTTCCCCCGTCCCTGGGTGGGCTCGAA
5'-Dr-tRNA ^{Lys} (CTT)-sgRNA	T7_Lys-CTT Fw slc45a2-sg1_Lys-CTT Rv	taatacgactcactataGGTTCTCATCAGCCCGGCTA GTGCATAATCAACCTTCCCCCGCCGACGTGGGGCTCGAA
5'-Dr-tRNA ^{Met} (CAT)-sgRNA	T7_Met-CAT Fw slc45a2-sg1_Met-CAT Rv	taatacgactcactataGGCCTGAAGGTTTCCGTAGTG GTGCATAATCAACCTTCCCCTGTTTCCGCCAGTTTCG
5'-Dr-tRNA ^{Ser} (GCT)-sgRNA	T7_Ser-GCT Fw slc45a2-sg1_Ser-GCT Rv	taatacgactcactataGGAAAATGACGAGGTGGCCGA GTGCATAATCAACCTTCCCCGACGAGGATGGGATTCTGAAC
5'-Dr-tRNA ^{His} (GTG)-sgRNA	T7_His-GTG Fw slc45a2-sg1_His-GTG Rv	taatacgactcactataGGCTCGCCGTGATCGTACAGT GTGCATAATCAACCTTCCCCTGCCGTGACCCGGATTCTGAAC
5'-Dr-tRNA ^{Thr} (AGT)-sgRNA	T7_Thr-AGT Fw slc45a2-sg1_Thr-AGT Rv	taatacgactcactataGGCAGCGGCGCCGTGGCTTA GTGCATAATCAACCTTCCCCAGGCACCGCTGGGATTCTGAAC
5'-Dr-tRNA ^{Gln} (CTG)-sgRNA	T7_Gln-CTG Fw slc45a2-sg1_Gln-CTG Rv	taatacgactcactataGGACTTGAGGTTCCATGGTGT GTGCATAATCAACCTTCCCCTGGTCCCACCGAGATTGAA
5'-Dr-tRNA ^{Leu} (CAG)-sgRNA	T7_Leu-CAG Fw slc45a2-sg1_Leu-CAG Rv	taatacgactcactataGGCATGTCAGGATGGCCGAGT GTGCATAATCAACCTTCCCCTGTCAGAAGTGGGATTCTGAAC
universal sgRNA scaffold for 5'-tRNA-sgRNA	slc45a2-sg1_gRNA Fw AAAA-gRNA Rv	GGGGAAGGTTGATTATGCACGTTTAAGAGCTATGCTGGAAACA AAAAGCACCGACTCGGTGCCACTTT
universal Fw primer for sgRNA-tRNA(ribozyme)-3'	T7-slc45a2-sg1-gRNA Fw	aatacgactcactataGGGGAAGGTTGATTATGCACGTTTAAGAGCTA TGCTGGAAACA
sgRNA-Dr-tRNA ^{Gly} (GCC)-3'	DrGly-GCC Rv	AAAATGCATTGGCCGGAATCGAAC
sgRNA-Dr-tRNA ^{Gly} (GCC)-scr-3'	DrGly-GCC-scr Rv	AAAAGGTGCAATCCCACGGAGCTG
sgRNA-Os-tRNA ^{Gly} (GCC)-3'	OsGly-GCC Rv	AAAATGCACCAGCCGGAATCGAA
sgRNA-Os-tRNA ^{Gly} (GCC)-scr-3'	OsGly-GCC-scr Rv	AAAACAGCGTACCCTGTAACCCCTCT
sgRNA-HDV-3'	HDV Rv	AAAAGTCCCATTGCGCATGCCGAAG
3xalb-sgRNAs 3xalb-3xmpv-sgRNA	T7_Gly-GCC Fw multi_sgRNA_IVT Rv	taatacgactcactataGGTGAGCATTGGTGGTTCAGTG GAGCTAGTTGGTACCATCGATA

T7 promoter sequence is shown in lower-case.

Each 5'-tRNA (ribozyme)-sgRNA fragment was amplified from a respective plasmid vector by PCR, and then combined with the universal sgRNA scaffold for 5'-tRNA-sgRNA by overlap extension PCR.

Supplementary Table S4.**Primers used for genotyping PCR**

Target	Primer Name (in Fig.2)	Forward (5' to 3')	Reverse (5' to 3')	Amplicon Length (bp)
drslc45a2_sg1&sg2	F1 & R1	TGGACTCGGAGCTTAAAGAGG	AGCAACACTGGCGTGACAAAC	169
drslc45a2_sg3_#1 (Fig.2)	F2 & R2	AGTGTGCTGAGCGTTGGAC	TGCCCACTAACATCAGAATCCC	176
drslc45a2_sg3_#2 (Fig.4-6)	F2b & R2b	ACAGTCTGGTGTGGCTCATAAG	CTGTTGTGACTGCATCTC	177
drslc45a2_sg4	F3 & R3	AAGGGGAATCCGTATGCTGAAC	AGCCAGAAAGAATGAGCAGTTG	160
drslc45a2_sg5	F4 & R4	CTTTCTGTACAGGAGCAAAGG	TGAGCCAACTGAACCATACAGG	108
drmpv17_sg1&sg2	F1 & R1	TGGGCTTGTGTTCTGTGATTG	TTGTGGCCACCTGGTCAAC	196
drmpv17_sg3	F2 & R2	CAAACAGATGTCTGTCTCGGTC	TGCAAATGGAGCAGAATCAC	197
drmpv17_sg4_#1 (Fig.2)	F3 & R3	CTGCCGTTTATATCTCCACAGG	TCAGGCGTGTGATAGATTACCC	137
drmpv17_sg4_#2 (Fig.4)	F3 & R3b	CTGCCGTTTATATCTCCACAGG	AGTACATTTGTGCCATCACAGTC	172
drmpv17_sg5	F4 & R4	GTTTCATTTCTCCAGCTTTGGC	GCTGCAGTGGCATTAGCATTAG	138
SpCas9	–	TGCTGAACGCCAAGCTGATTAC	ACGAACTCGCTTTCAGCTTAG	370

Supplementary Table S5.**Primers used for plasmid construction**

Fragment Name	Primer Name	Sequence
3xalb-A-sgRNA_Fragment1 (template: Dr-tRNA ^{Asn} (GTT))	slc45a2 sg1-Primer F1 BseRI	GATTCCCGGCCAATGCAGGGGAAGGTTGATTATGCACGTTT AAGAGCTATGCTGGAA
	slc45a2 sg3-Asn-Primer R1	TCGGCCCCATGACGACCTCCCGTCCCTGGGTGGGCTCGAA
3xalb-A-sgRNA_Fragment2 (template: Dr-tRNA ^{Lys} (CTT))	slc45a2 sg3-Primer F2	GGAGGTCGTCATGGGGCCGAGTTTAAGAGCTATGCTGGAA
	slc45a2 sg4-Lys-Primer RL BseRI	CAGCATAGCTCTTAAACAACAGCCCACTTCAACCCCCCGCC CGACGTGGGGCTCGAA
3xalb-B-sgRNA_Fragment1 (template: Dr-tRNA ^{Asn} (GTT))	slc45a2 sg1-Primer F1 BseRI	GATTCCCGGCCAATGCAGGGGAAGGTTGATTATGCACGTTT AAGAGCTATGCTGGAA
	slc45a2 sg4-Asn-Primer R1	AACAGCCCACTTCAACCCCCGTCCCTGGGTGGGCTCGAA
3xalb-B-sgRNA_Fragment2 (template: Dr-tRNA ^{Lys} (CTT))	slc45a2 sg4-Primer F2	GGGGGTTGAAGTGGGCTGTTGTTTAAGAGCTATGCTGGAA
	slc45a2 sg3-Lys-Primer RL BseRI	CAGCATAGCTCTTAAACTCGGCCCATGACGACCTCCCGCC CGACGTGGGGCTCGAA
3xalb-C-sgRNA_Fragment1 (template: Dr-tRNA ^{Lys} (CTT))	slc45a2 sg1-Primer F1 BseRI	GATTCCCGGCCAATGCAGGGGAAGGTTGATTATGCACGTTT AAGAGCTATGCTGGAA
	slc45a2 sg3-Lys-Primer R1	TCGGCCCCATGACGACCTCCCGCCGACGTGGGGCTCGAA
3xalb-C-sgRNA_Fragment2 (template: Dr-tRNA ^{Asn} (GTT))	slc45a2 sg3-Primer F2	GGAGGTCGTCATGGGGCCGAGTTTAAGAGCTATGCTGGAA
	slc45a2 sg4-Asn-Primer RL BseRI	CAGCATAGCTCTTAAACAACAGCCCACTTCAACCCCCGTCC CTGGGTGGGCTCGAA
3xalb-3xmpv-sgRNA_Fragment1 (template: Dr-tRNA ^{Asn} (GTT))	slc45a2 sg1-Primer F1 BseRI	GATTCCCGGCCAATGCAGGGGAAGGTTGATTATGCACGTTT AAGAGCTATGCTGGAA
	slc45a2 sg3-Primer R1	TCGGCCCCATGACGACCTCCCGTCCCTGGGTGGGCTCGAA
3xalb-3xmpv-sgRNA_Fragment2 (template: Dr-tRNA ^{Met} (CAT))	slc45a2 sg3-Primer F2	GGAGGTCGTCATGGGGCCGAGTTTAAGAGCTATGCTGGAA
	slc45a2 sg4-Primer R2	AACAGCCCACTTCAACCCCCGTGTTCCGCCAGTTTCG
3xalb-3xmpv-sgRNA_Fragment3 (template: Dr-tRNA ^{Ser} (GCT))	slc45a2 sg4-Primer F3	GGGGGTTGAAGTGGGCTGTTGTTTAAGAGCTATGCTGGAA
	mpv17 sg1-Primer R3	CACCGACCACTGGACCCCCGACGAGGATGGGATTCTGAAC
3xalb-3xmpv-sgRNA_Fragment4 (template: Dr-tRNA ^{His} (GTG))	mpv17 sg1-Primer F4	GTCCAGTGGTCGGTGGAGTTTAAGAGCTATGCTGGAA
	mpv17 sg3-Primer R4	GACTGGCCAATCACAACCTGCCGTGACCCGGATTCTGAAC
3xalb-3xmpv-sgRNA_Fragment5 (template: Dr-tRNA ^{Lys} (CTT))	mpv17 sg3-Primer F5	TGTGATTGGCCAGTCCAGTTTAAGAGCTATGCTGGAA
	mpv17 sg4-Primer RL BseRI	CAGCATAGCTCTTAAACTTGAGGGTCCAGTTATTCCCGCCC GACGTGGGGCTCGAA

Supplementary figure legends

Fig. S1 Schematic diagram of cRT-PCR (Related to Figure 3D). The 5'-Dr-tRNA^{Gly}(GCC)-sg1 transcript or processed mature sgRNA was circularized by self-ligation with T4 RNA ligase, and then reverse-transcribed with a primer designed near the 5'-end of the sgRNA. Subsequently, the fragments originated from the unprocessed or processed sgRNA were amplified by a pair of specific primers to investigate the processing efficiency of the tRNA-sgRNA transcript.

Fig. S2 Related to Figure 3E. Sequence chromatographs of the mature sgRNA processed from the 5'-Dr-tRNA^{Gly}(GCC)-sg1 transcript. The white arrowhead indicates the presumptive processing site, and the black arrowheads indicate the actual cleavage sites.

Fig. S3 Related to Figure 6. Generation of transgenic albino (Tg-albino) zebrafish. (A) Representative images of the lateral views of 3-mpf F0 fish. (lower) Some fish show mosaic albino phenotype. (B) Representative images of the dorsal views (left) and lateral views (right) of 3-dpf F1 larvae derived from F0#11 (founder) fish. F1 larvae with normal pigmentation (top), mild *albino* phenotype (middle), and severe *albino* phenotype (bottom). (C) Representative images of the lateral views of adult wild-type (WT; upper) and transgenic (Tg; lower) fish. (D) Magnified images of the trunk region of the fish shown in D. Scale bars: 5 mm (A), 1 mm (B), 1 cm (C), 2 mm (D).

Fig. S4 Related to Figure 6. Presence of transgenes in F1 larvae with *albino* phenotypes from F0#11 (A) and F0#14 (B) fish. The transgenes are detected with SpCas9 primers (top), whereas the primer pair against the exon4 of *mpv17* (control; mpv17-F1 and mpv17-

R1) is used as an internal positive control primer pair (bottom). Full gels are shown in Supplementary Fig. S12.

Fig. S5 Related to Figure 7. Eye and body pigmentation in Tg-albino at Day 5. Representative bright field images of dorsal views of wild-type (WT), PTU-treated WT (WT+PTU), *albino*, and Tg-albino backgrounds. Scale bars: 1 mm.

Fig. S6 A detailed method for the one-step cloning of tRNA-sgRNAs into pT2TS-ubb:Cas9;u6c:Dr-tRNA^{Gly}(GCC)-sgRNA-scaffold.

Fig. S7 Full scanned gels for heteroduplex mobility assay shown in Figure 2. (A) Full scanned gels of Fig. 2B. (B) Full scanned gels of Fig. 2D.

Fig. S8 Full scanned gels shown in Figure 3. (A) Full scanned gels used for quantification in Fig. 3A. The two gel were processed in parallel. The left gel is the full scanned image of Fig. 3A. (B) Full scanned gels used for quantification in Fig. 3B. The two gel were processed in parallel. The left gel is the full scanned image of Fig. 3B. Lanes unrelated to this study on the right gel were removed. (C) Full scanned gels of Fig. 3C. (D) Full scanned image shown in Fig. 3D.

Fig. S9 Full scanned gels for heteroduplex mobility assay shown in Figure 4. (A) Full scanned gels shown in Fig. 4B and Fig. 4E. (B) Full scanned gels shown in Fig. 4C and Fig. 4G. (C) Full scanned gel shown in Fig. 4D. (D) Full scanned gel shown in Fig. 4F.

Fig. S10 Full scanned gels for heteroduplex mobility assay shown in Figure 5. (A) Full scanned gel shown in Fig. 5B. (B) Full scanned gel shown in Fig. 5C. (C) Full scanned gel shown in Fig. 5D.

Fig. S11 Full scanned gels shown in Figure 6E.

Fig. S12 Full scanned gels shown in Supplementary Figure S4. (A) Full scanned gel shown in Supplementary Fig. S4A. (B) Full scanned gels shown in Supplementary Fig. S4B.

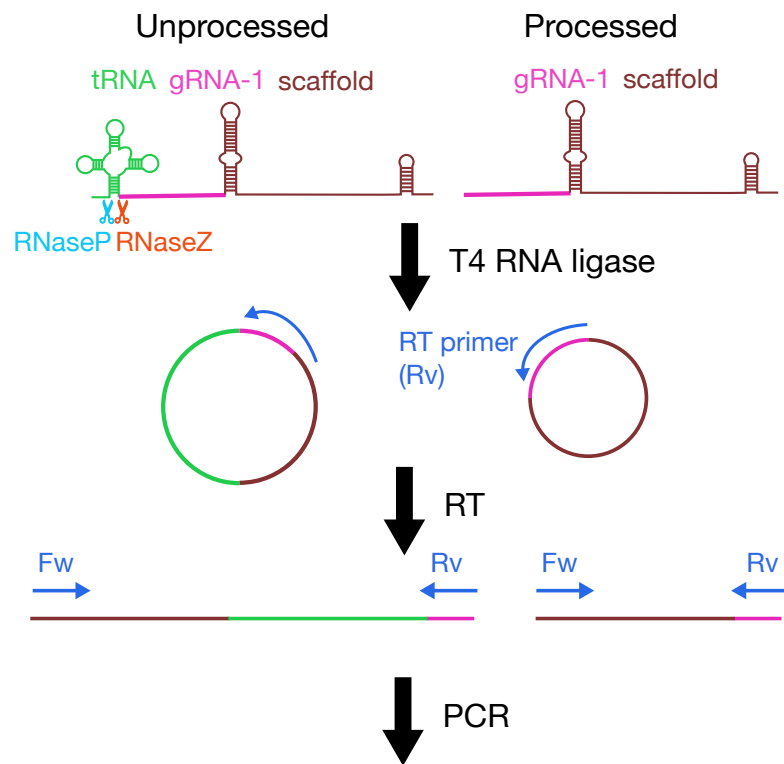


Figure-S1

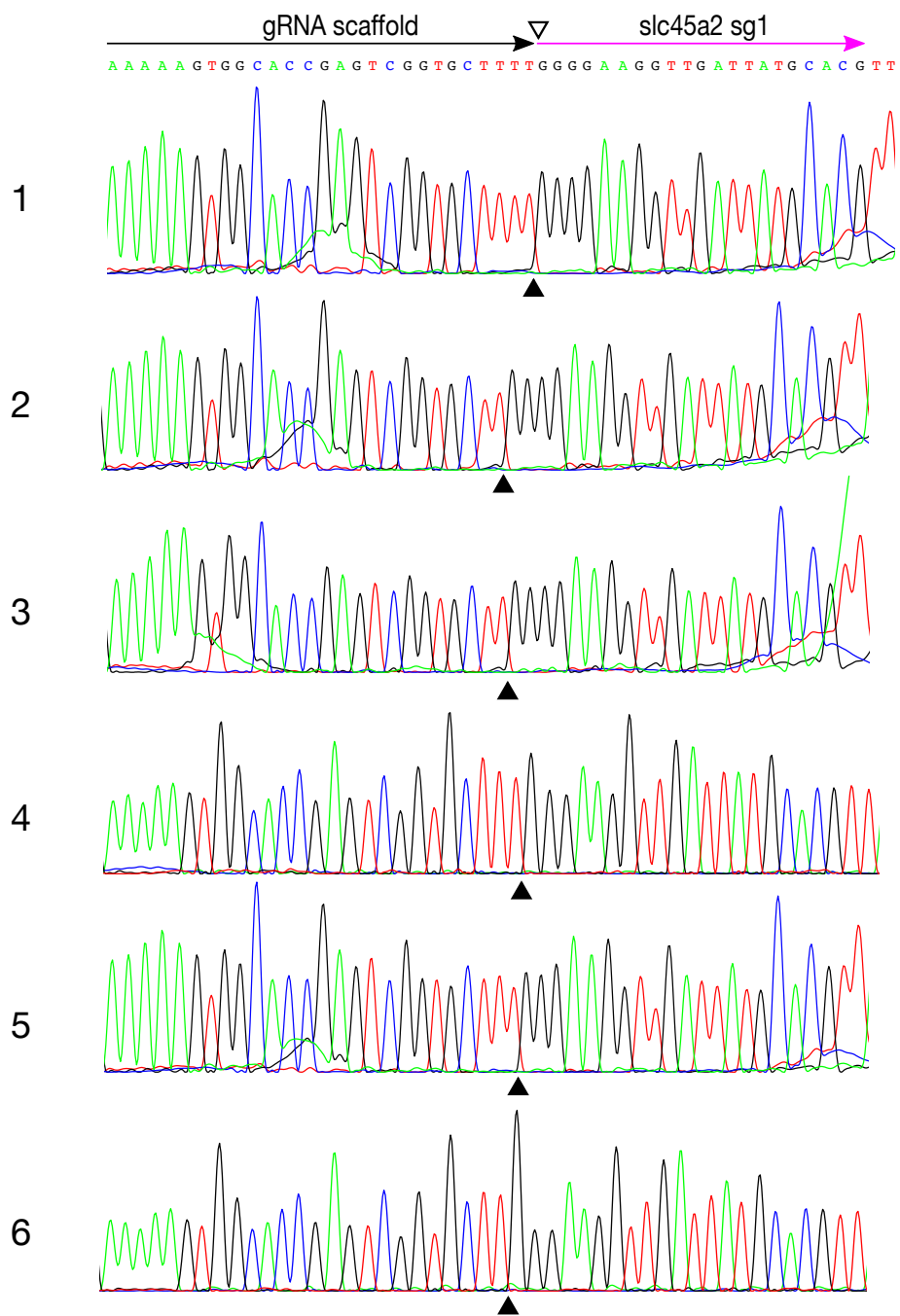


Figure-S2

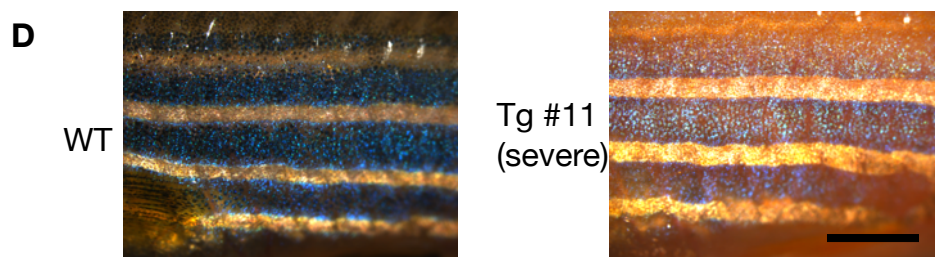
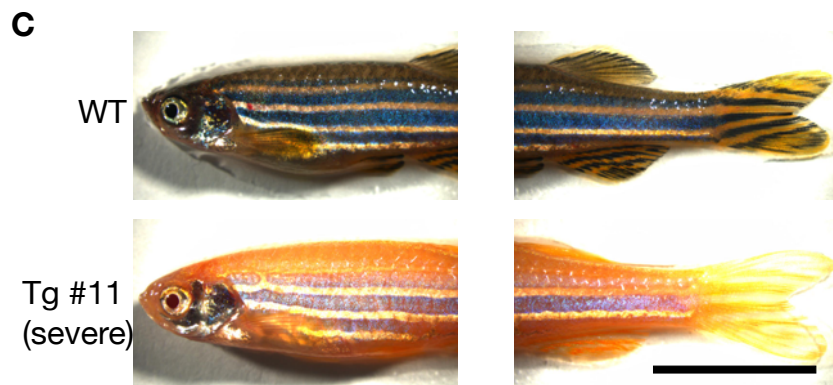
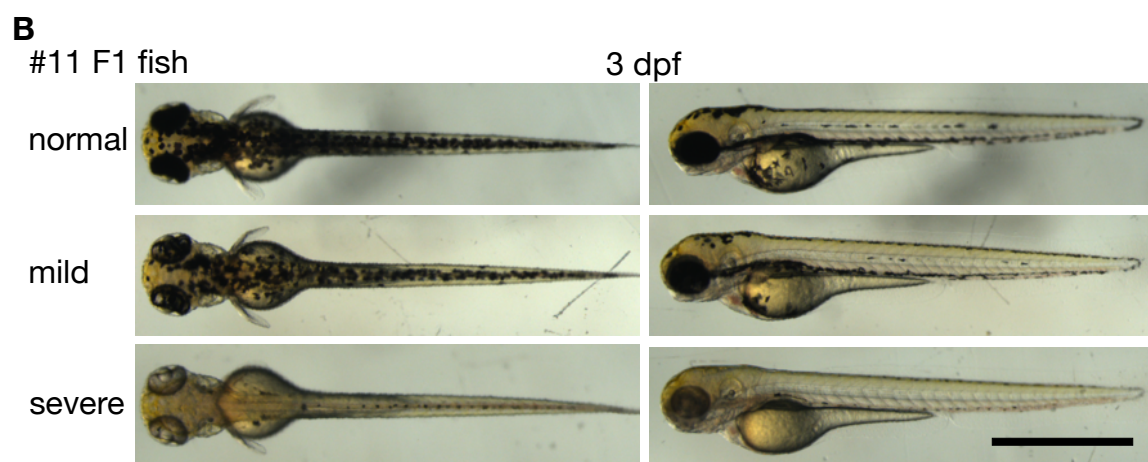
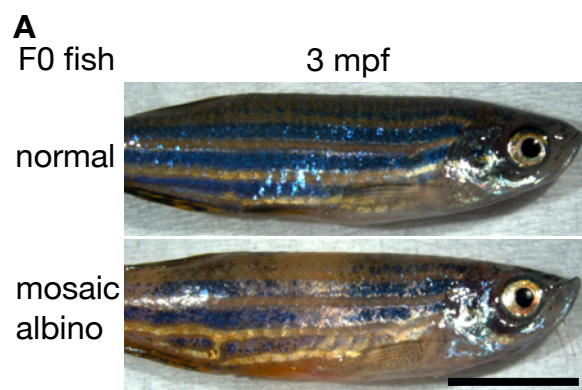
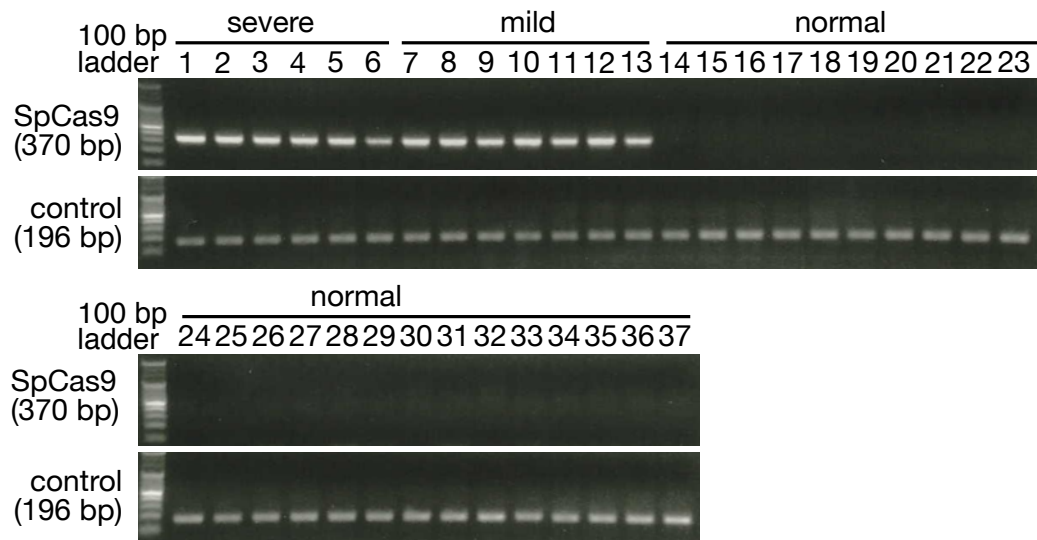


Figure-S3

A Tg-albino #11 F1



B Tg-albino #14 F1

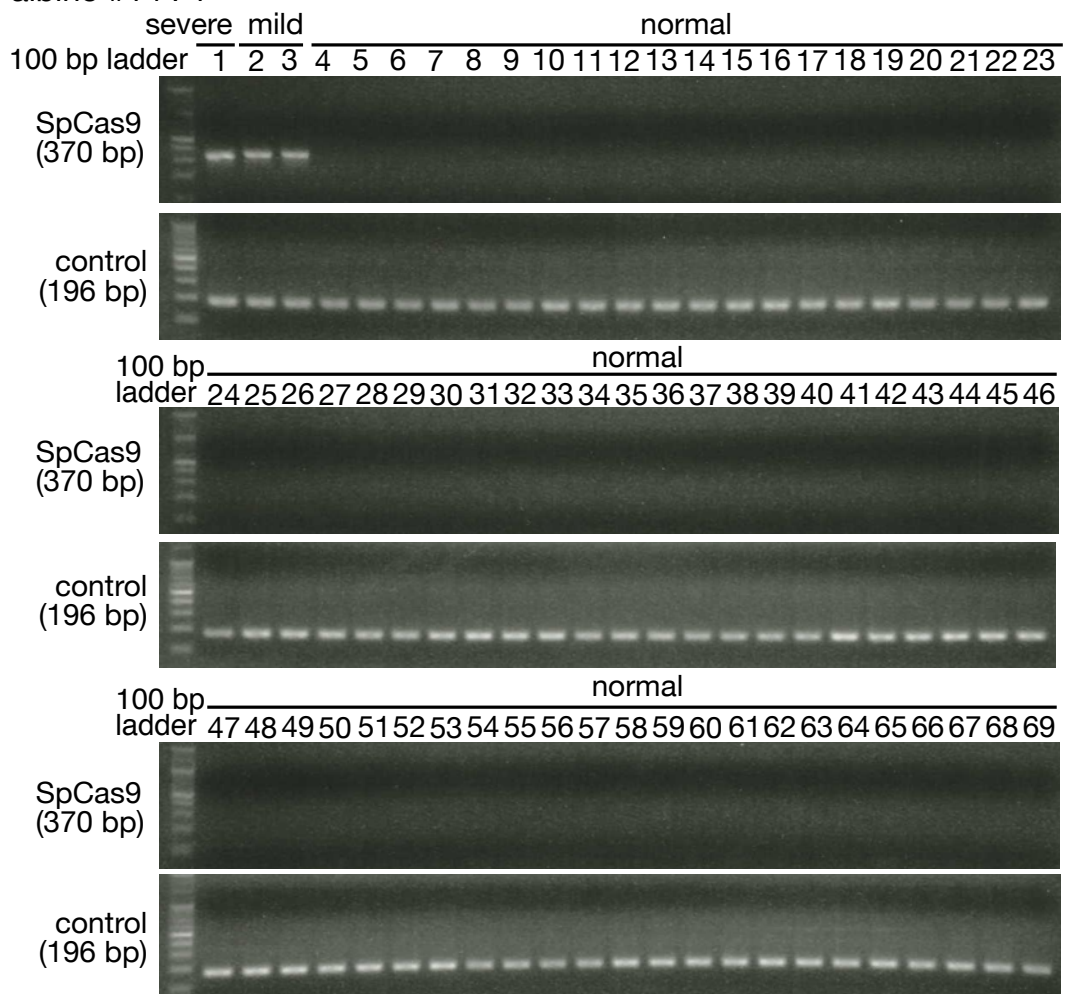


Figure-S4

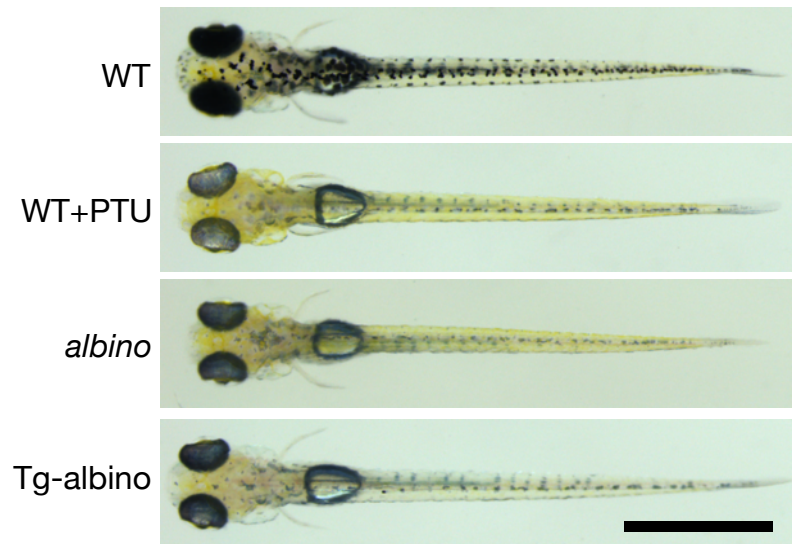
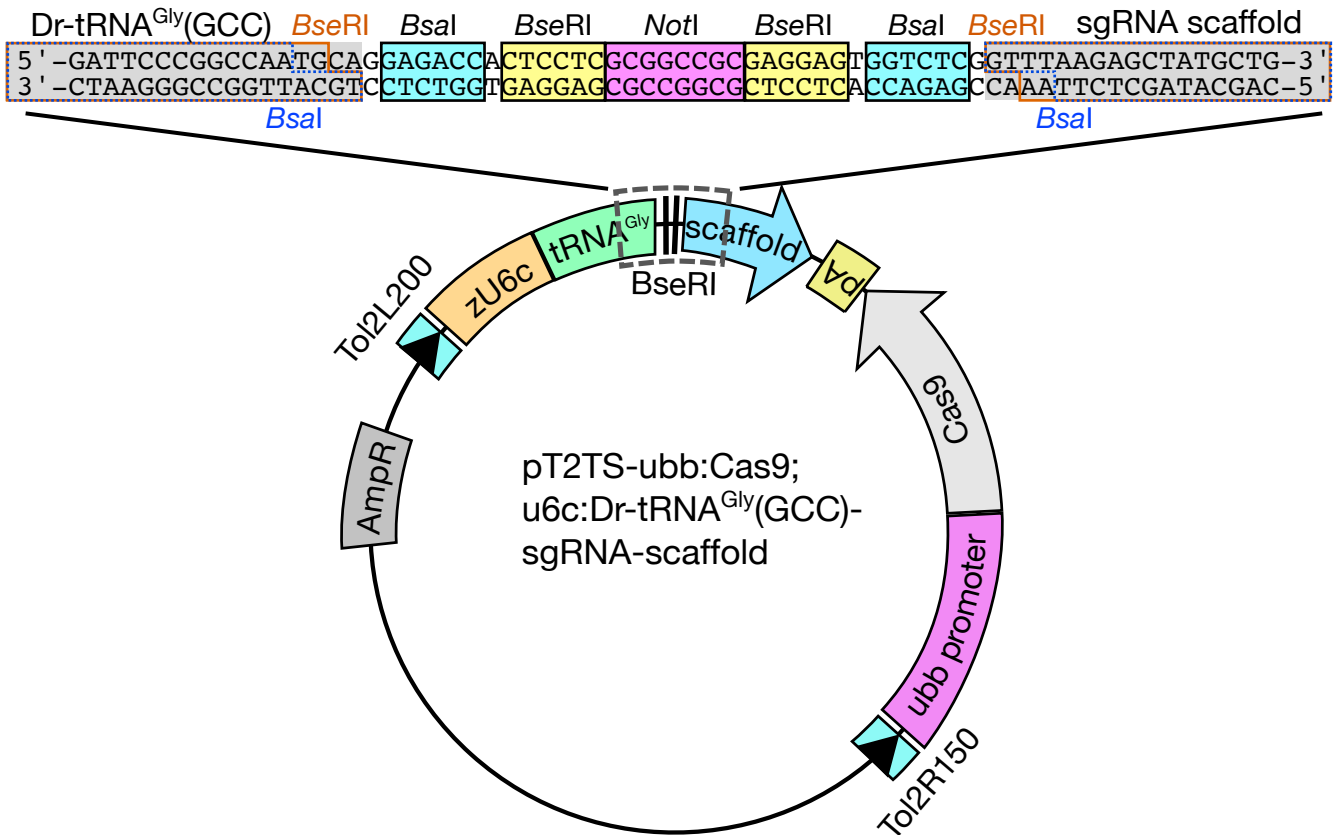


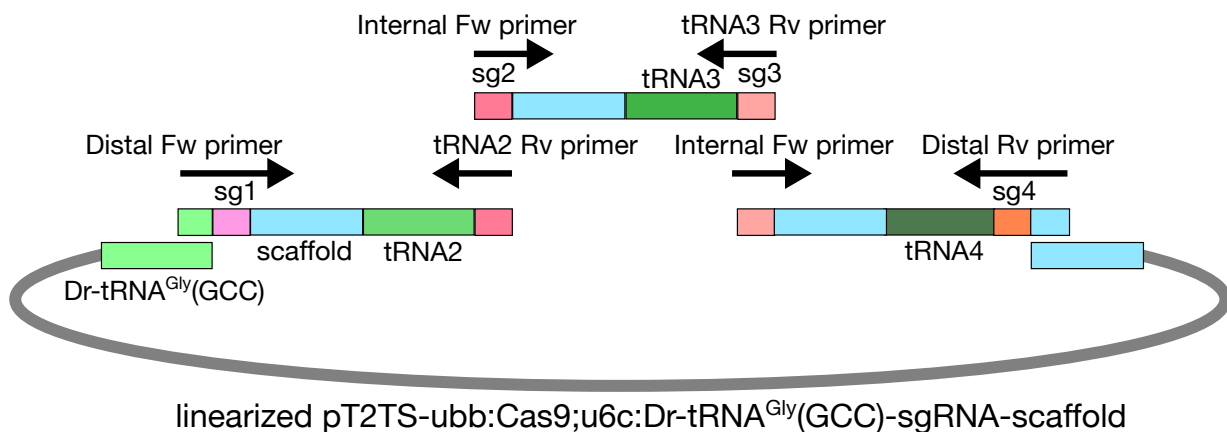
Figure-S5

1. Digest the pT2TS-ubb:Cas9;u6c:Dr-tRNA^{Gly}(GCC)-sgRNA-scaffold with *Bse*RI
(Only *Bse*RI is available due to the existence of *Bsa*I sites within the *ubb* promoter)
*Bse*RI produces cohesive ends that allow seamless cloning of sgRNA sequences.
(*Bse*RI cleavage sites are shown in orange, while *Bse*RI recognition sites are highlighted in yellow.)



2. Prepare sgRNA fragments by PCR amplification

sgRNA fragments are amplified from pgRNA-drRNA vectors by PCR, and then cloned into the linearized pT2TS-ubb:Cas9;u6c:Dr-tRNA^{Gly}(GCC)-sgRNA-scaffold by seamless cloning methods such as Gibson Assembly and In-Fusion as illustrated in the schematic below. The following schematic shows an example of the case of four sgRNAs.



Internal primers contain ~20-nt sgRNA sequences used as homology arm for seamless ligation, while distal primers include 15-bp overlap with *Bse*RI-treated pT2TS-ubb:Cas9;u6c:Dr-tRNA^{Gly}(GCC)-sgRNA-scaffold.

Figure-S6

2-1. Design primers as shown in the table below

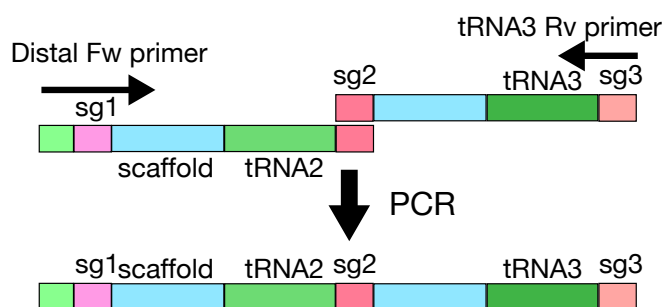
[illegible]

2-2. Run PCRs

Run the individual PCRs using a high-fidelity polymerase according to the manufacturer's instructions. The protocol shown below is an example of the use of PrimeSTAR GXL (TaKaRa).

Set up the 1st PCR mixture		Run PCR			
	final conc.				
PrimeSTAR GXL Buf.	1x	98°C	1 min	} 20 cycles	➡ Run the PCR products on 2% agarose gel. The expected sizes are ~200 bp. Purify the PCR products using a commercial kit.
dNTPs	0.2 mM each	98°C	10 sec		
Primer	0.3 µM each	55°C	30 sec		
Template Plasmid	1 ng/nL	68°C	20 sec		
PrimeSTAR GXL	0.5 U/20 µL				

For cloning more than four sgRNAs (three fragments), we fuse fragments by overlap extension PCR.



Set up the 2nd PCR mixture without primers (~100 ng each fragment in 20 μ L reaction)

Run PCR without primers

98°C	1 min	] 15 cycles
98°C	10 sec	
55°C	30 sec	
68°C	20 sec	
Add primer pair (final conc., 0.3		
98°C	10 sec	] 20 cycles
55°C	30 sec	
68°C	30 sec	

2-3. Digest the PCR products with *DpnI* to remove contaminating template plasmid DNA

Figure-S6
(continued)

3. Cloning sgRNA fragments

3-1. Seamless cloning using In-Fusion (or Gibson assembly)

A) Prepare the In-Fusion mixture

5x In-Fusion	0.5 µL
Vector backbone	~50 ng
Inserts (~200 bp)	~2 ng each
Inserts (~400 bp)	~4 ng each
Water	up to 2.5 µL

B) Incubate 50°C for 15 min

C) Transform the mixture into chemically competent cells, and plate cells on Ampicillin plates.

3-2. Select clones with correct inserts by colony PCR (This step can be skipped)

Perform colony PCR using primers below.

zU6c_seq Fw: 5' –TAAGCGTTTGCAGGTTTGCC–3'
h-globin-pA_seq Fw: 5' –AAAGGGAATGTGGGAGGTCAGTG–3'

3-3. Verify the recombinant colonies by Sanger sequencing with primers below

zU6c_seq Fw: 5' –TAAGCGTTTGCAGGTTTGCC–3'
h-globin-pA_seq Fw: 5' –AAAGGGAATGTGGGAGGTCAGTG–3'

Figure-S6
(continued)

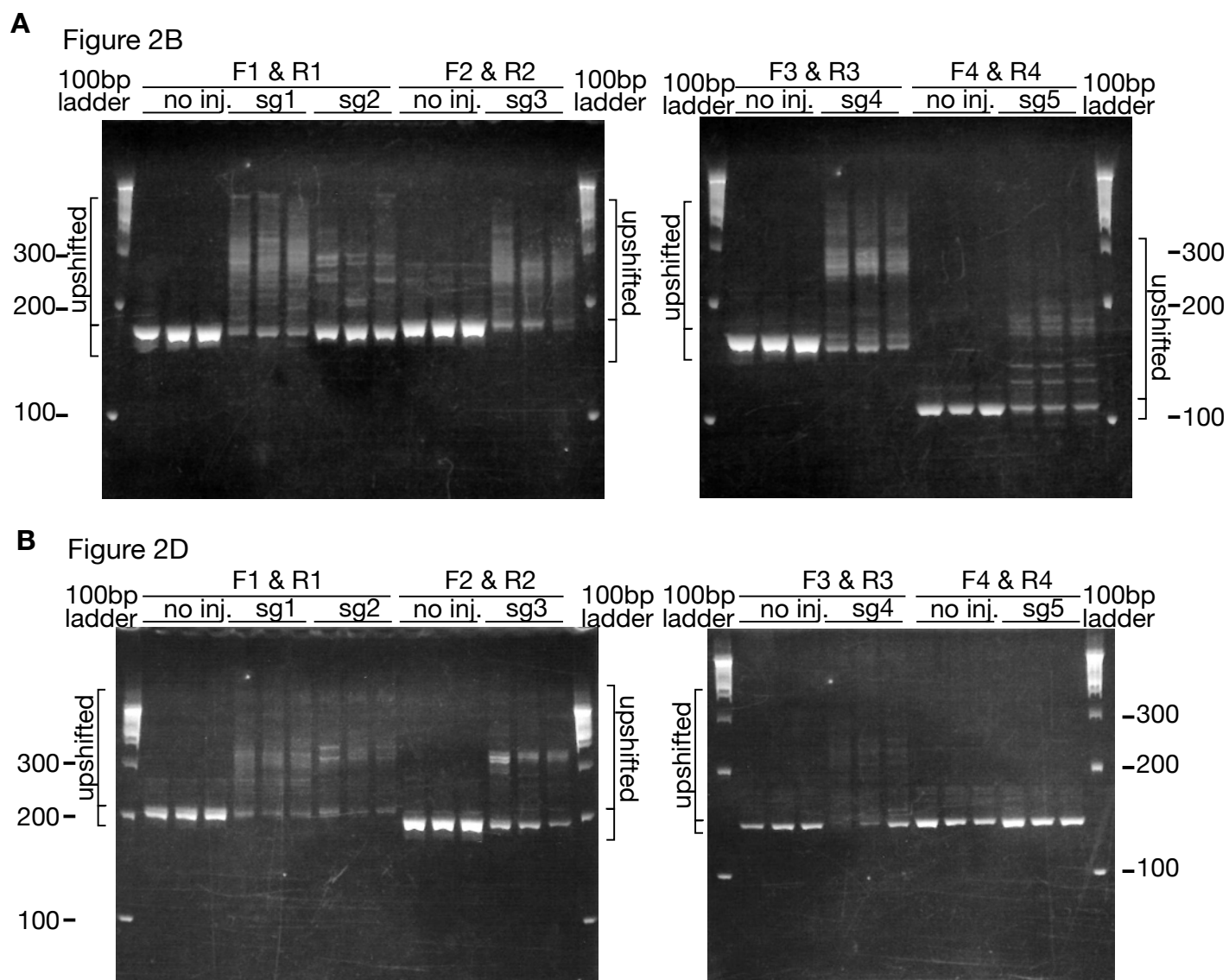


Figure-S7

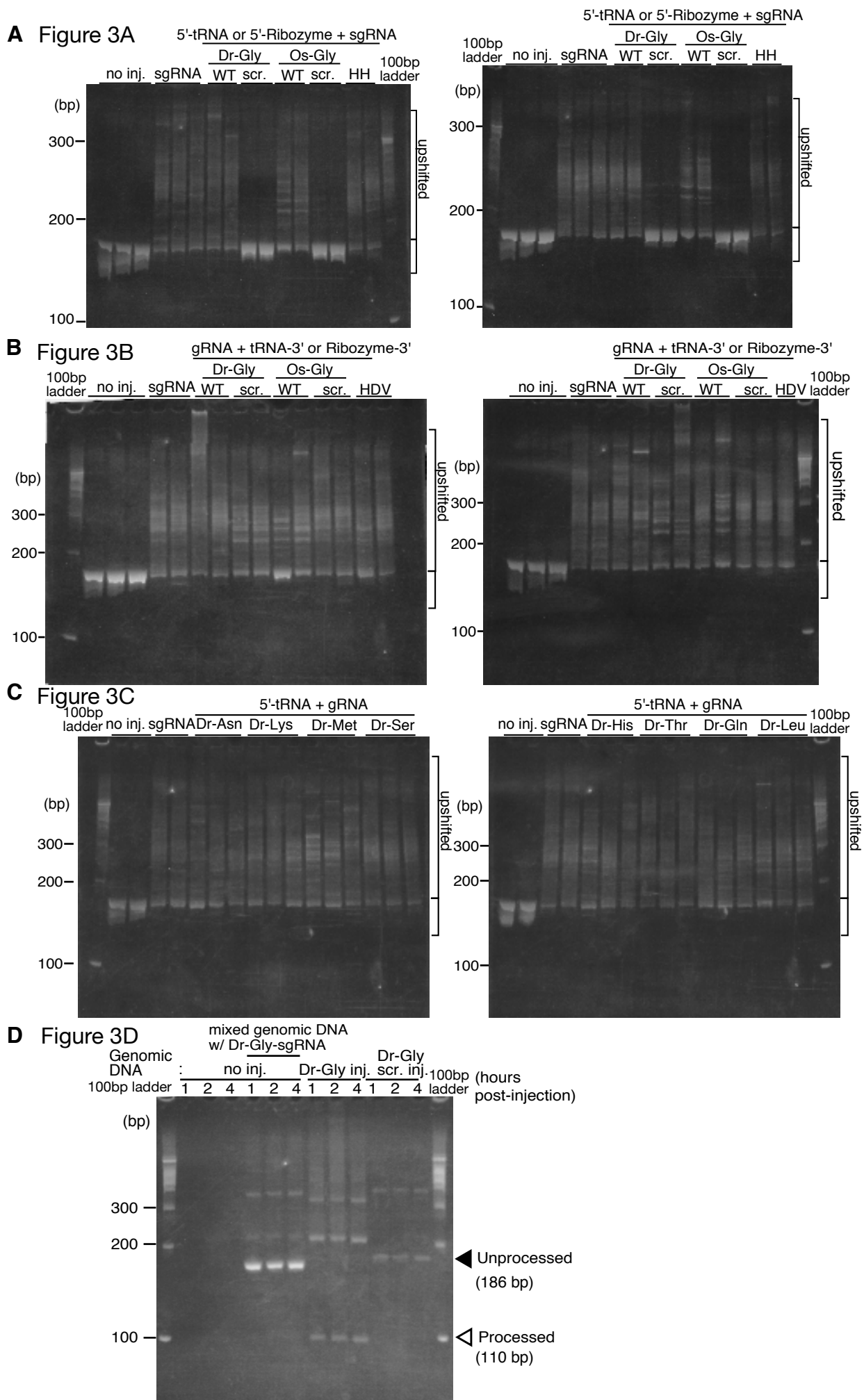


Figure-S8

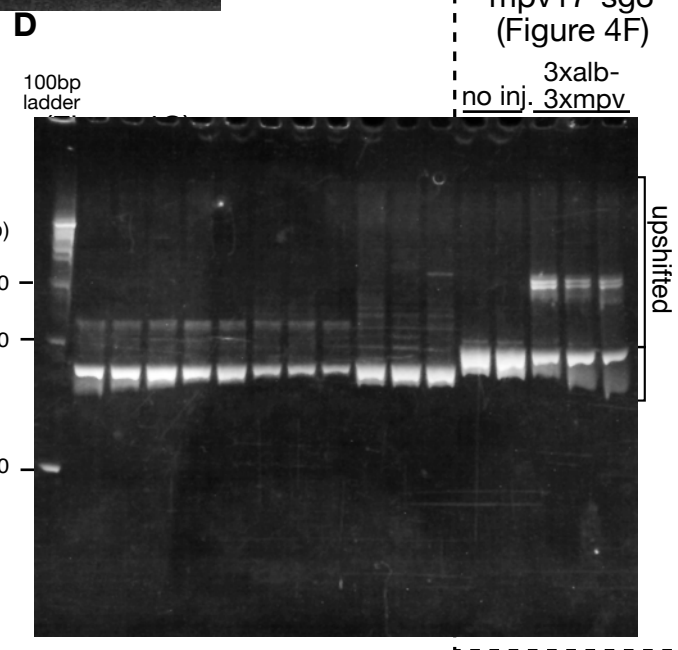
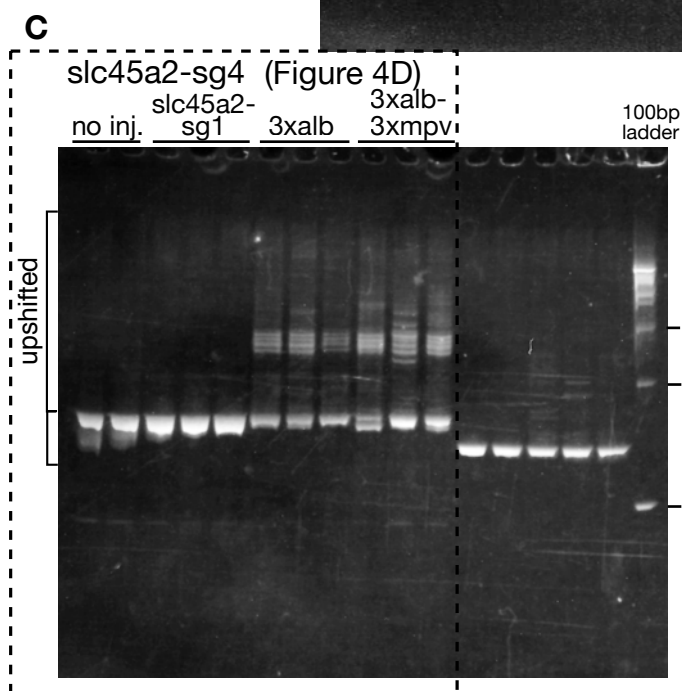
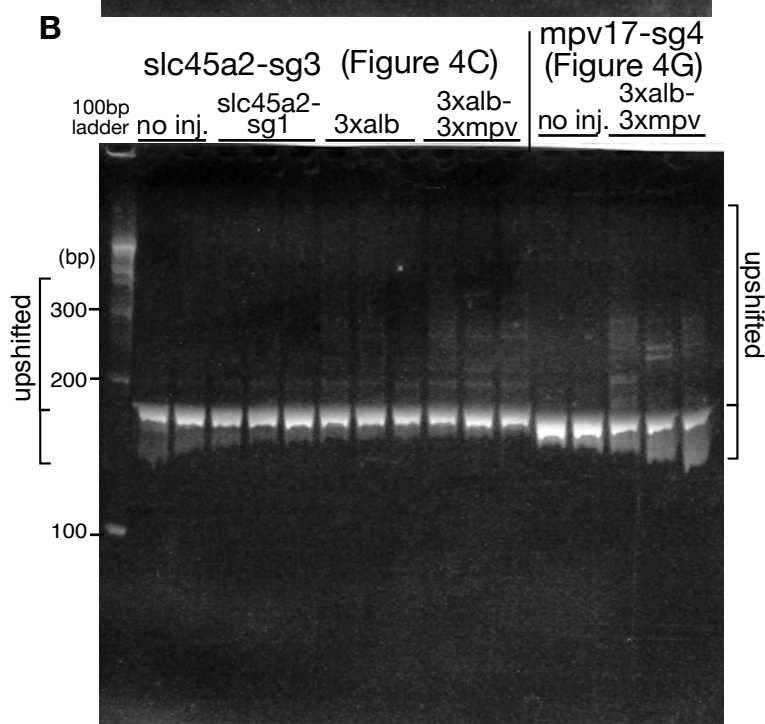
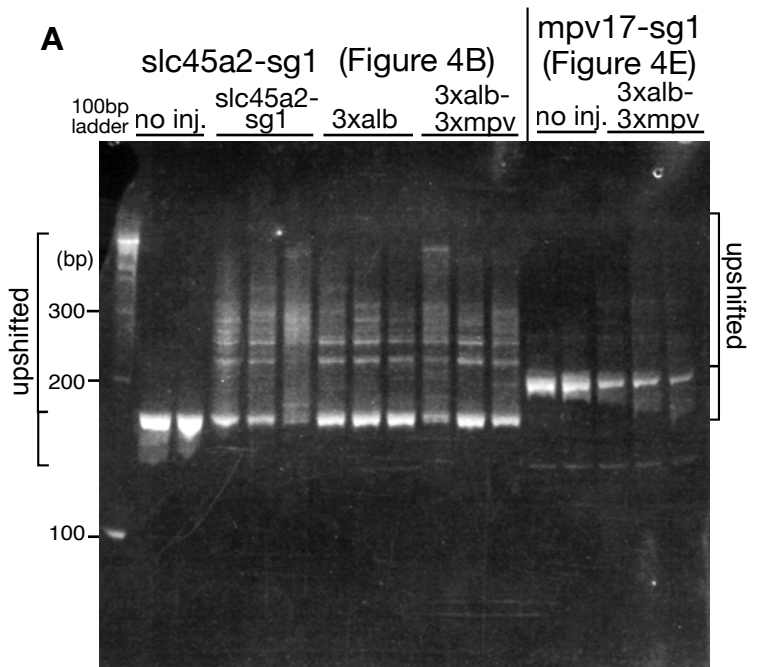


Figure-S9

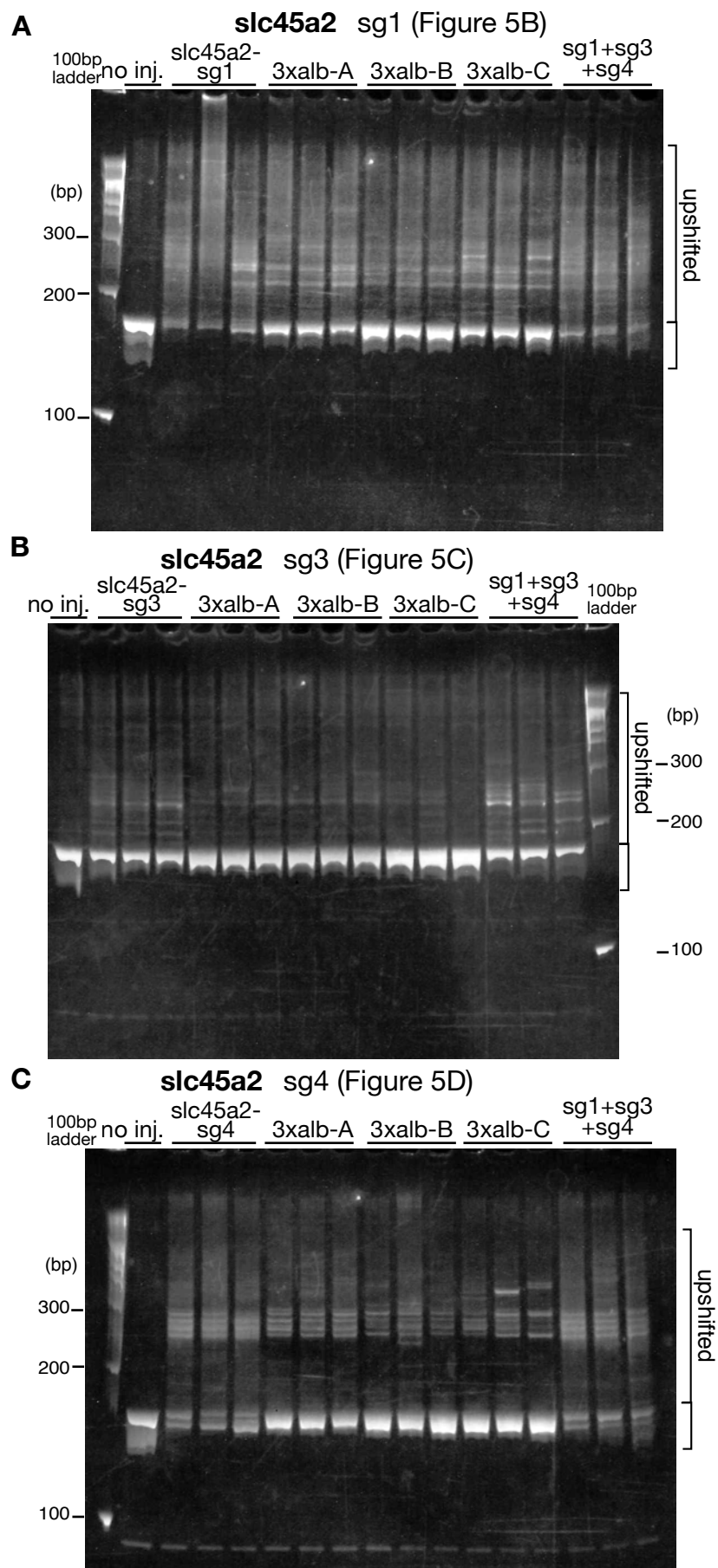


Figure-S10

Figure 6E

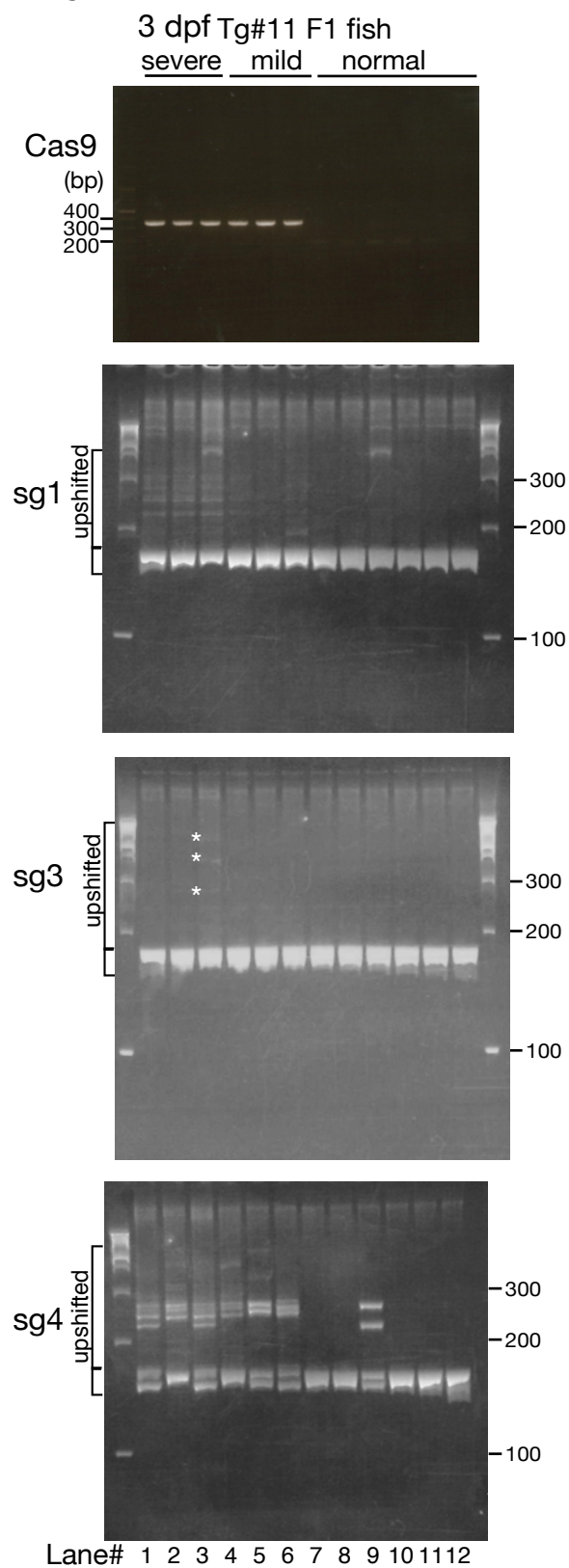
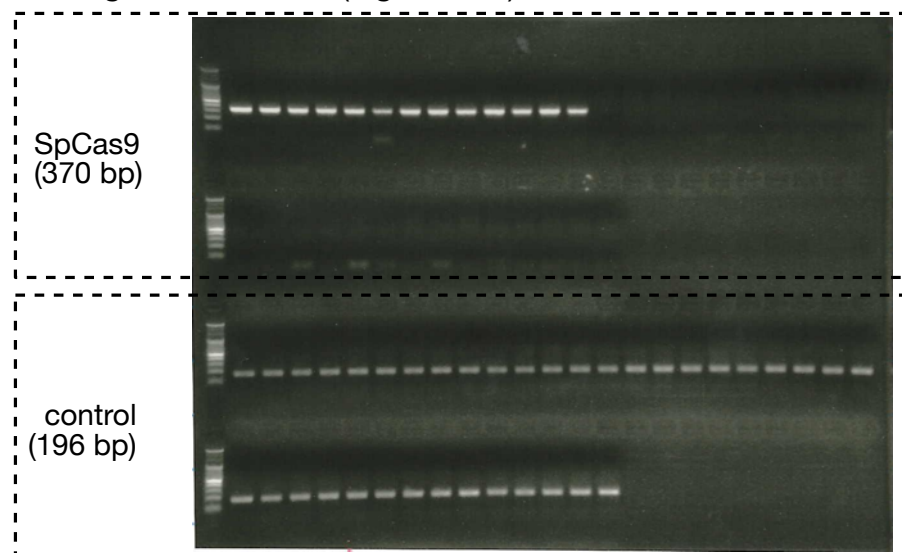


Figure-S11

A Tg-albino #11 F1 (Figure S4A)



B Tg-albino #14 F1 (Figure S4B)

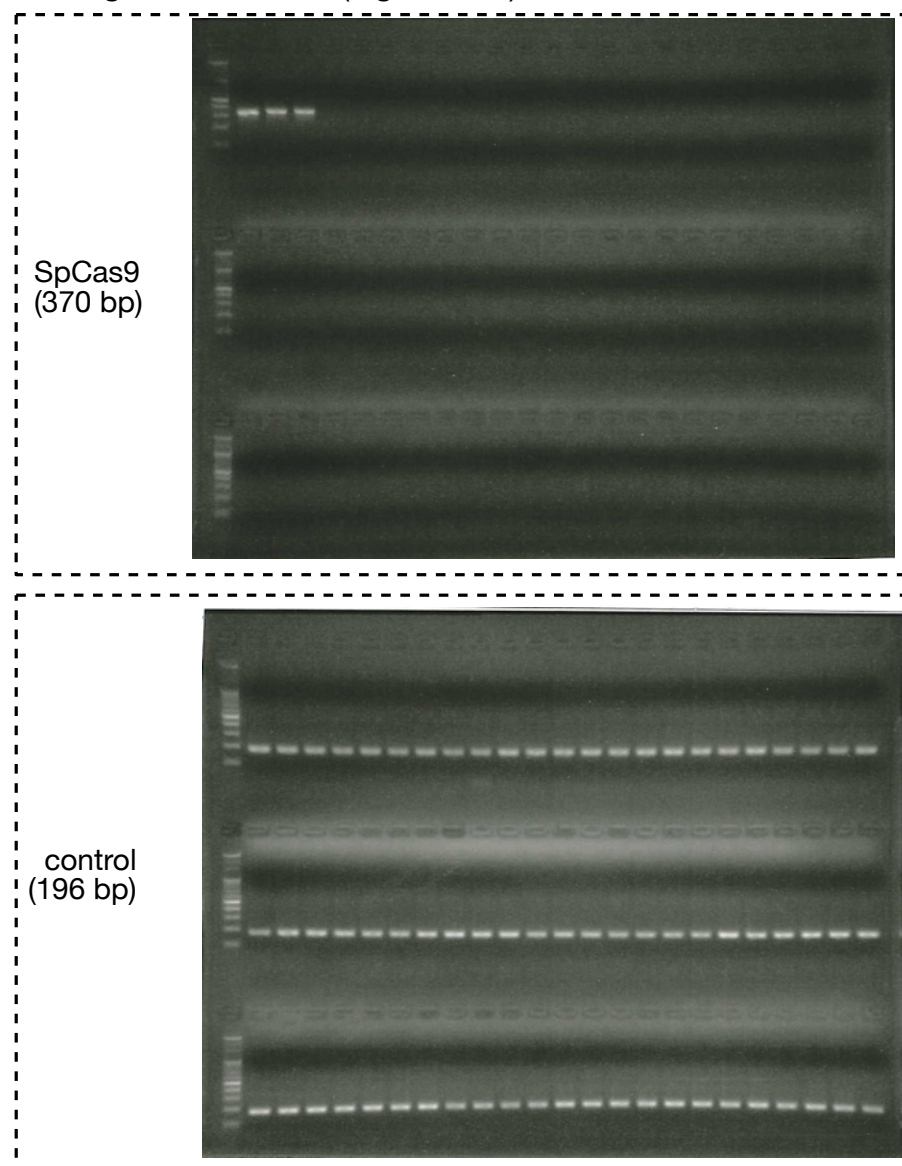


Figure-S12