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Supplementary Information

**Optimal tagging strategies for illuminating expression profiles of genes
with different abundance in zebrafish**

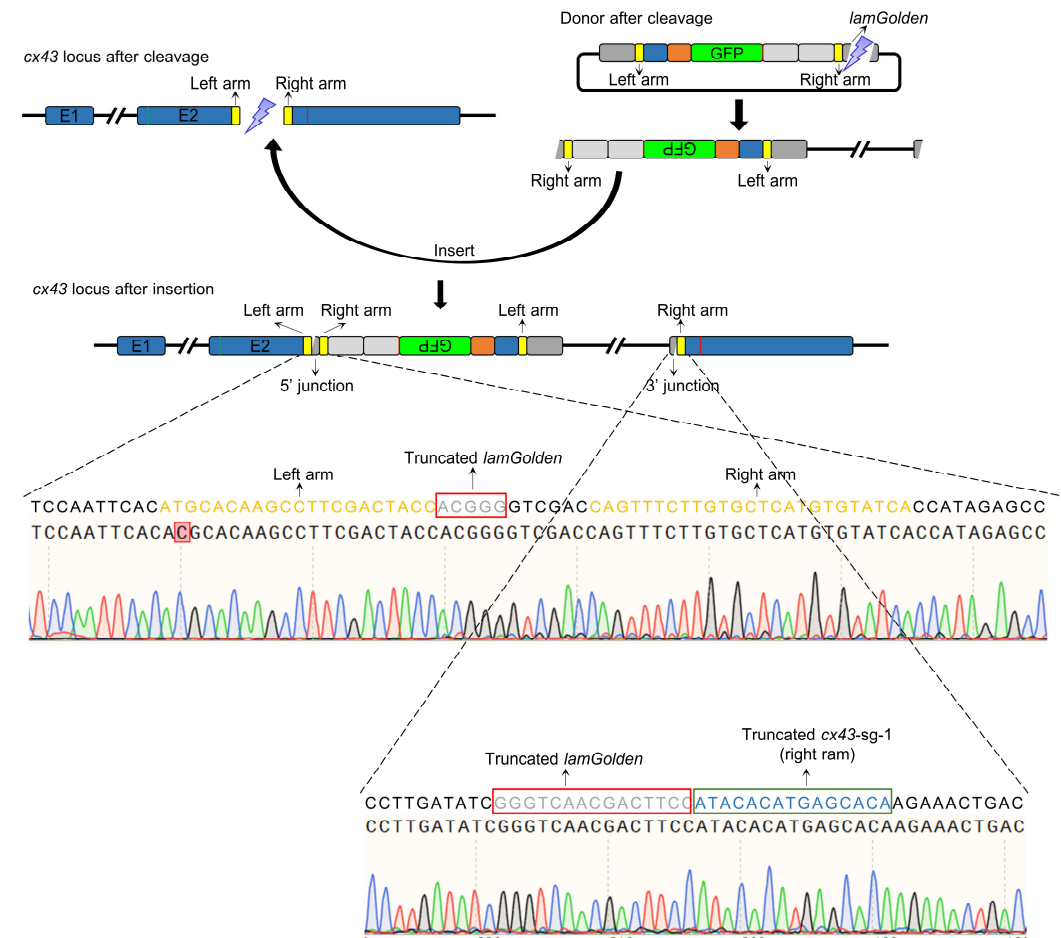
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Zhang^{1,*}

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This PDF file includes: Supplementary Figure 1-29

17

Supplementary Figure 1



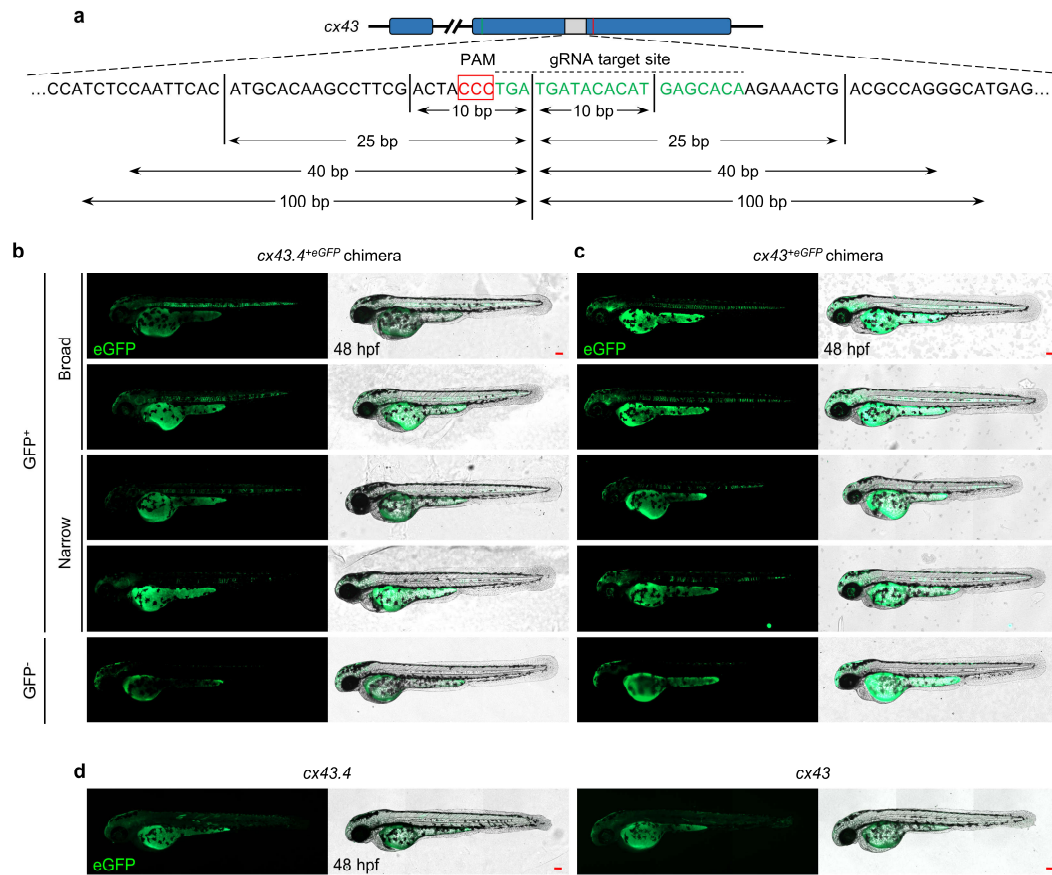
18

Supplementary Fig. 1. Two artificial Cas9/sgRNA sites in KI donors increase errors in MMEJ-mediated repair.

Incorrect MMEJ-repair caused by two artificial Cas9/sgRNA sites in the donor as exemplified by KI at the *cx43* locus. Cas9 cleaved only the *lamGolden* site adjacent to the right homologous arm and the entire donor was reversely inserted into the genome by NHEJ-mediated repair as revealed by junction PCR and sequencing analysis.

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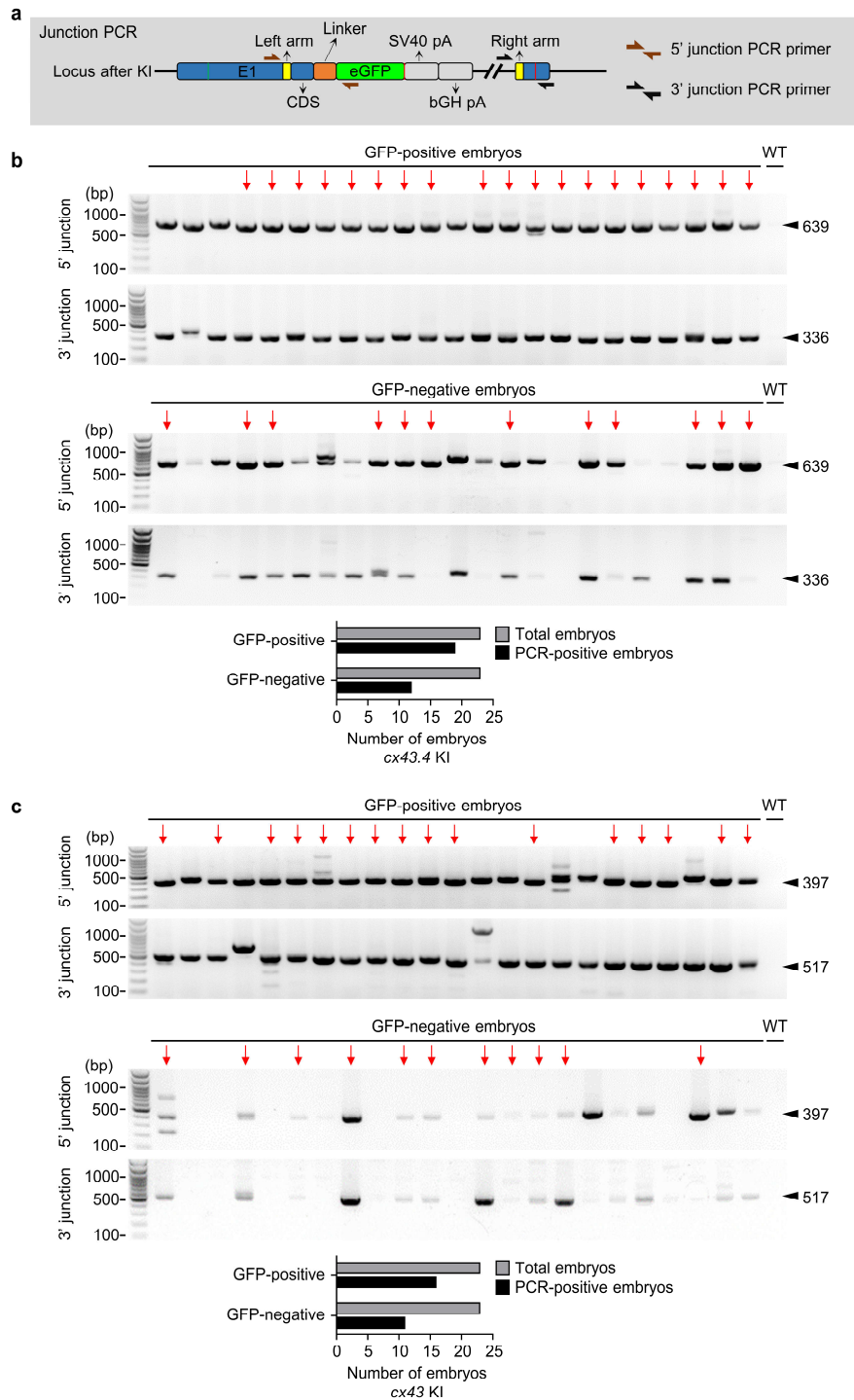
Supplementary Figure 2



Supplementary Fig. 2. S-25 is an optimized donor for MMEJ-mediated KI.

(a) Taking *cx43* as an example, mircohomologous right and left arms are sequences downstream and upstream of the double-strand break (DSB), respectively. 10 bp, 25 bp, 40 bp, and 100 bp mircohomologous arms were tested. (b and c) Images of microinjected zebrafish embryos using the MMEJ-single strategy at the *cx43.4* or *cx43* locus. GFP signals were observed in the notochord of *cx43.4* or *cx43* F₀ at 48 hpf. Embryos were separated based on the range of GFP expression, broad or narrow. Scale bars, 100 μm. Images are representatives of at least 100 F₀. (d) Images of microinjected zebrafish embryos using the MMEJ-double strategy at the *cx43.4* or *cx43* locus. GFP signals were randomly detected in the muscle in some F₀ embryos at 48 hpf. Scale bars, 100 μm. Images are representatives of at least 30 F₀.

42 **Supplementary Figure 3**



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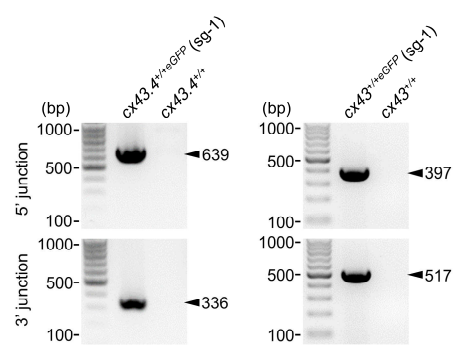
44 **Supplementary Fig. 3. Junction PCR of GFP-positive or GFP-negative**
45 **embryos after microinjection.**

46 **(a)** A schematic diagram of the 5'-junction PCR and 3'-junction PCR analysis
47 for the S-25 KI strategy. For the 5'-junction PCR, the forward primer was

designed in the genome upstream of the left arm, and the reverse primer was located in the GFP sequence of the donor. For the 3'-junction PCR, the forward primer was designed in the donor, and the reverse primer was located downstream of the right arm. **(b and c)** Junction PCR analysis of GFP-positive or GFP-negative embryos for *cx43.4* (b) or *cx43* (c) KI. Embryos after microinjection were classified into two groups, GFP-positive and GFP-negative groups at 48 hpf. Twenty-three microinjected embryos in each group were randomly selected and genotyped. PCR-positive F₀ embryos were indicated by red arrowheads.

58

Supplementary Figure 4



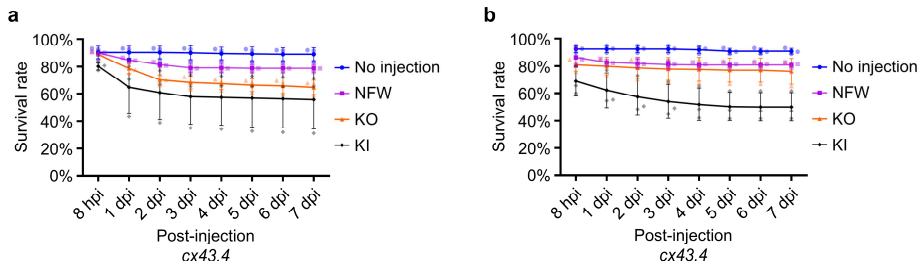
59

60 **Supplementary Fig. 4. Junction PCR analysis of *cx43.4^{+/+eGFP}* and**
61 ***cx43^{+/+eGFP}* F₁.**

62 F₁ embryos carrying GFP-labeled *cx43.4* or *cx43* alleles were identified by 5'-
63 and 3'-junction PCR. Data are representatives of at least 10 F₁.

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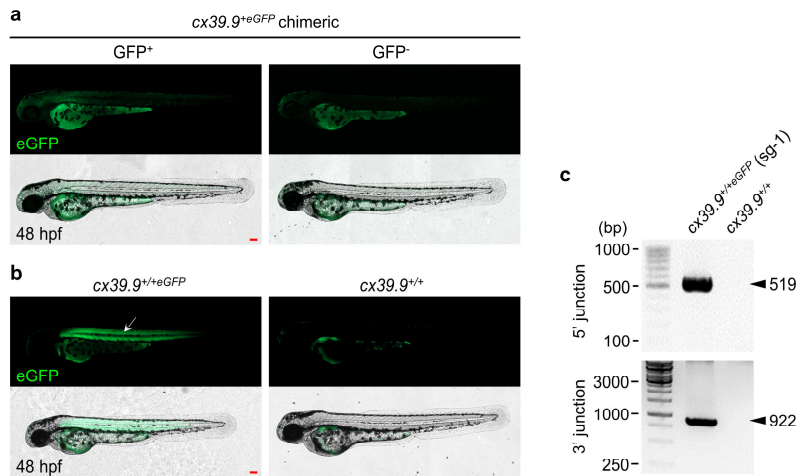
Supplementary Figure 5



Supplementary Fig. 5. Effects of the S-25-based KI on survival rates of the microinjected embryos.

Survival rates of embryos microinjected with different gene-editing materials for *cx43.4* (a) or *cx43* (b). Different materials, including NFW (nuclease-free water), KO mix (Cas9 mRNA and sgRNA), and KI mix (Cas9 mRNA, two sgRNAs, and the corresponding S-25 donor), were injected into at least 100 one-cell-stage WT embryos. Survival rates were analyzed at different time points. Non-injected embryos were included as the control. Data represent mean $\pm$ SD of 3 independent experiments. hpi, hours post-injection; dpi, days post-injection.

77 **Supplementary Figure 6**



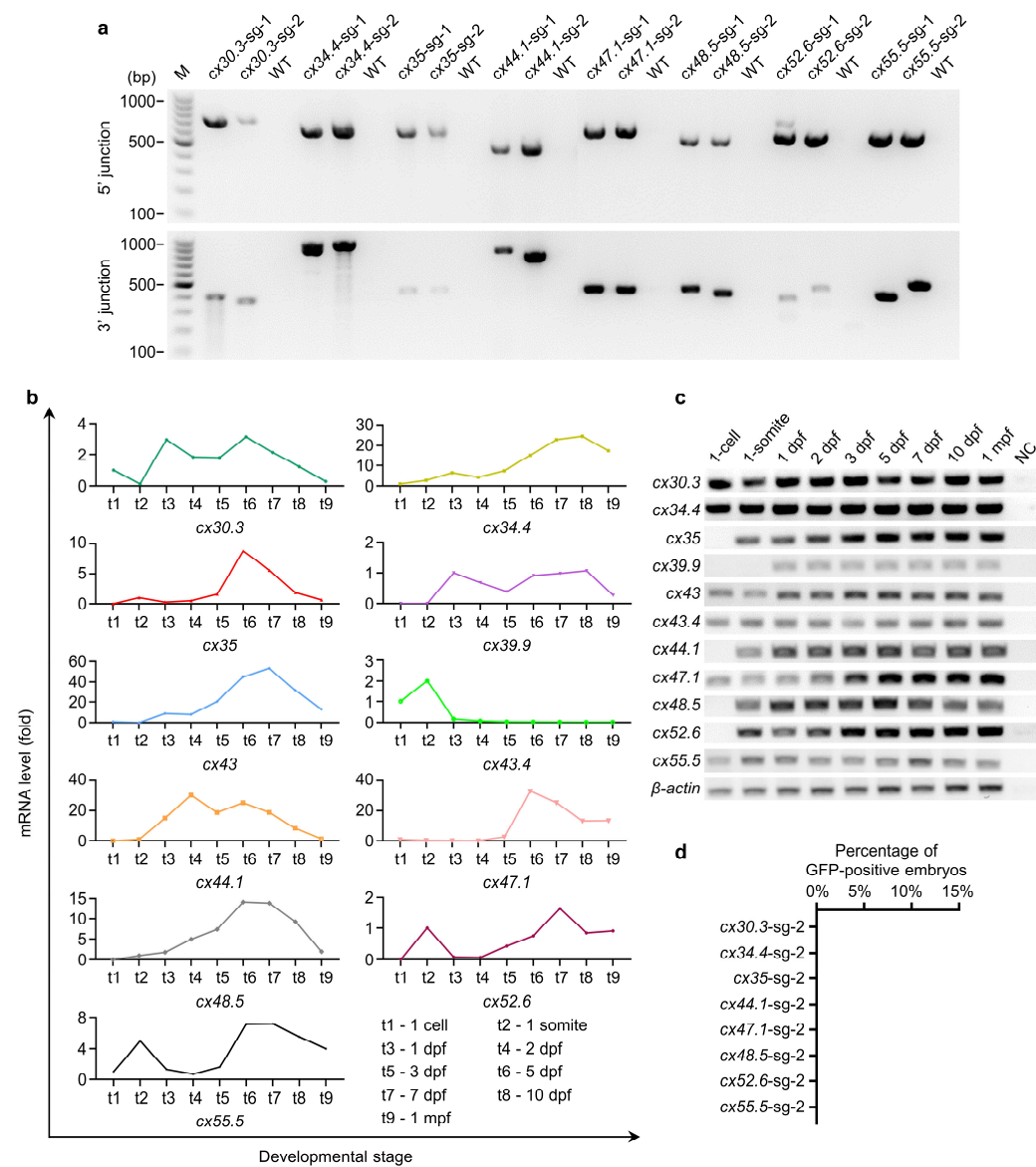
78

79 **Supplementary Fig. 6. Generation of the GFP-labeled *cx39.9* KI zebrafish.**

80 **(a and b)** Images of microinjected F₀ (a) and F₁ (b) embryos for *cx39.9* KI by
 81 using the S-25 strategy at 48 hpf. Scale bars, 100 μm. Images are
 82 representatives of at least 7 embryos. **(c)** Junction PCR analysis of the
 83 *cx39.9^{+/+}eGFP* F₁ zebrafish. Data are representatives of at least 10 F₁.

84

Supplementary Figure 7

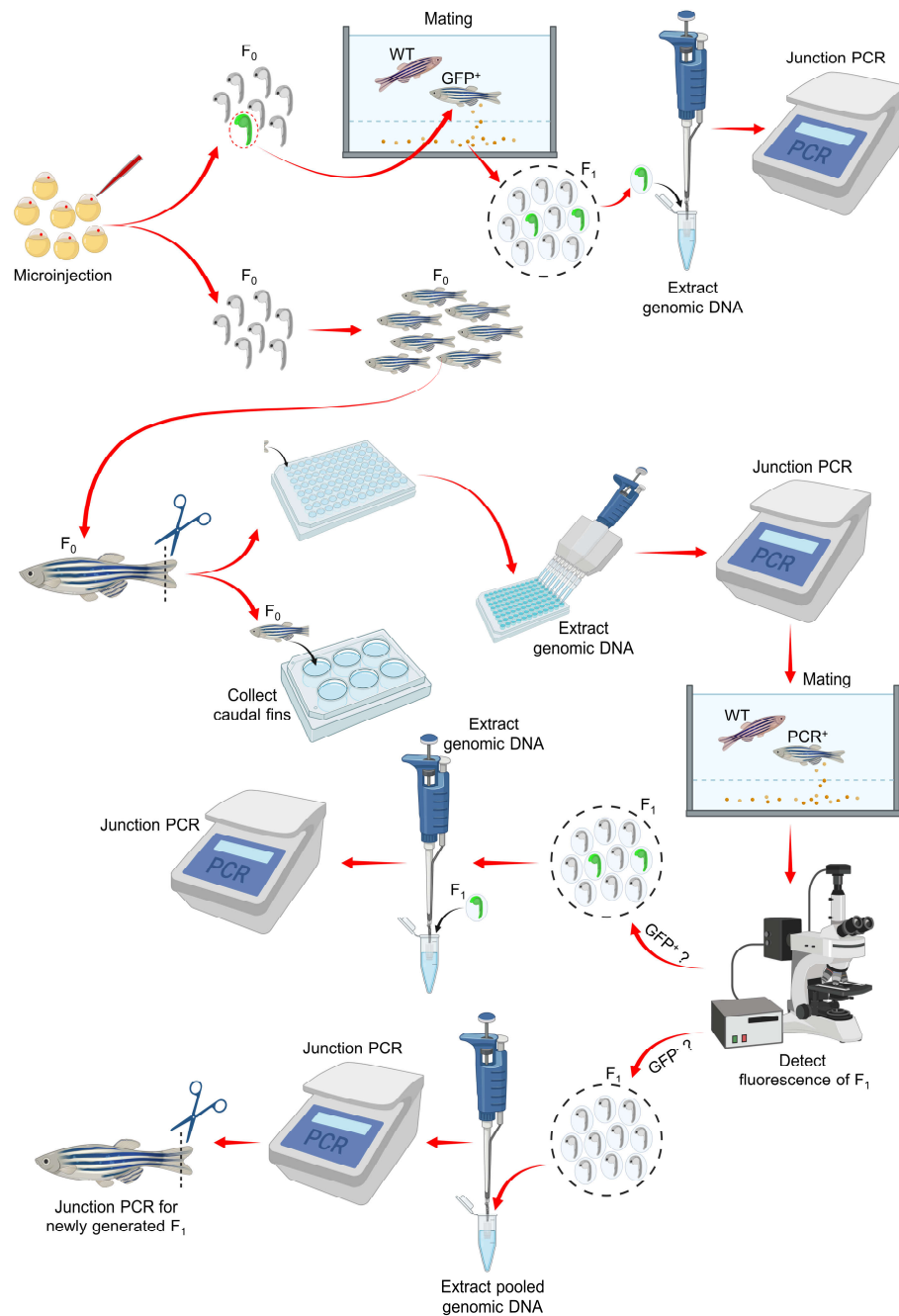


Supplementary Fig. 7. Transcriptional analysis of connexins.

(a) Junction PCR analysis of microinjected F₀ embryos. Two high-efficiency sgRNAs were chosen for each gene. For each sgRNA, 5 microinjected embryos were randomly selected and pooled for genomic DNA extraction and junction PCR analysis at 24 hpf. WT embryos were used as negative controls. (b and c) Real-time quantitative PCR (RT-qPCR) analysis of connexins at different developmental stages of zebrafish. Thirty WT zebrafish embryos were pooled to extract RNA for time points before 1-month post fertilization (mpf). At 1 mpf, a whole WT zebrafish was frozen to death and treated with 1 mL of RNAiso

96 Plus reagent for total RNA extraction and the subsequent RT-qPCR. For a
97 specific gene in each sample, expression of the target gene was normalized to
98 that of *β-actin*, and then the threshold cycle ($2^{-\Delta\Delta C_t}$) method was used and the
99 fold change of the target gene in each sample relative to the time point the
100 expression was first detected was plotted (b). qPCR products were verified by
101 electrophoresis (c). NC, negative control. **(d)** Percentages of GFP-positive F₀
102 embryos after tagging *cx30.3*, *cx34.4*, *cx35*, *cx44.1*, *cx47.1*, *cx48.5*, *cx52.6*, or
103 *cx55.5*. S-25 strategy was performed on at least 100 WT embryos using an
104 alternative high-efficiency sgRNA for each gene and GFP-positive F₀ embryos
105 were counted at 48 hpf.

Supplementary Figure 8

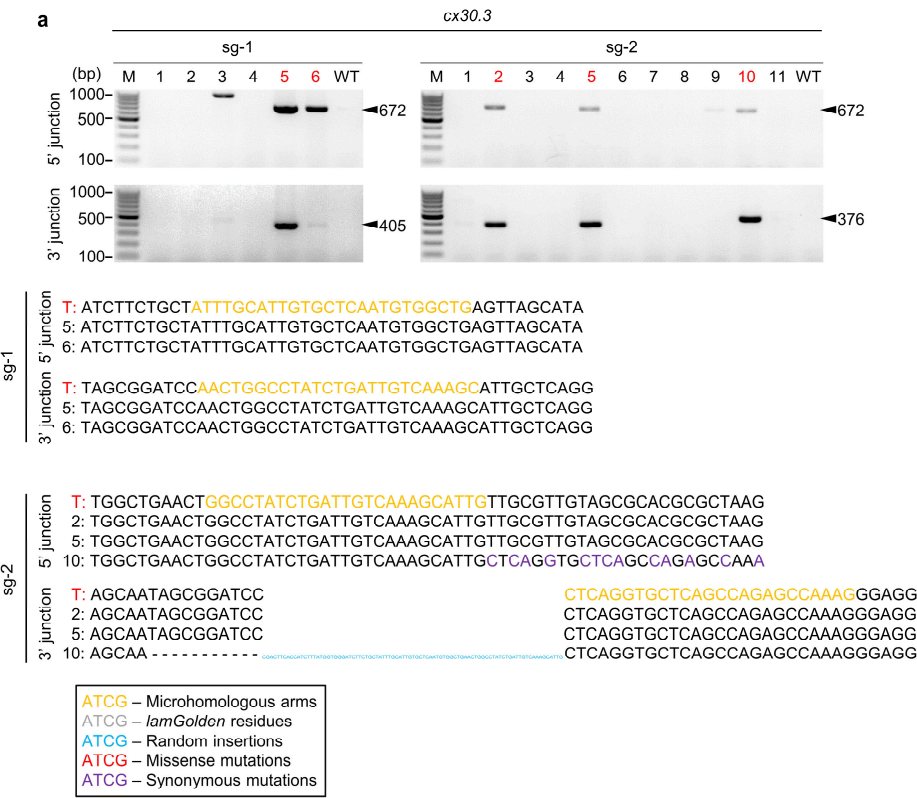


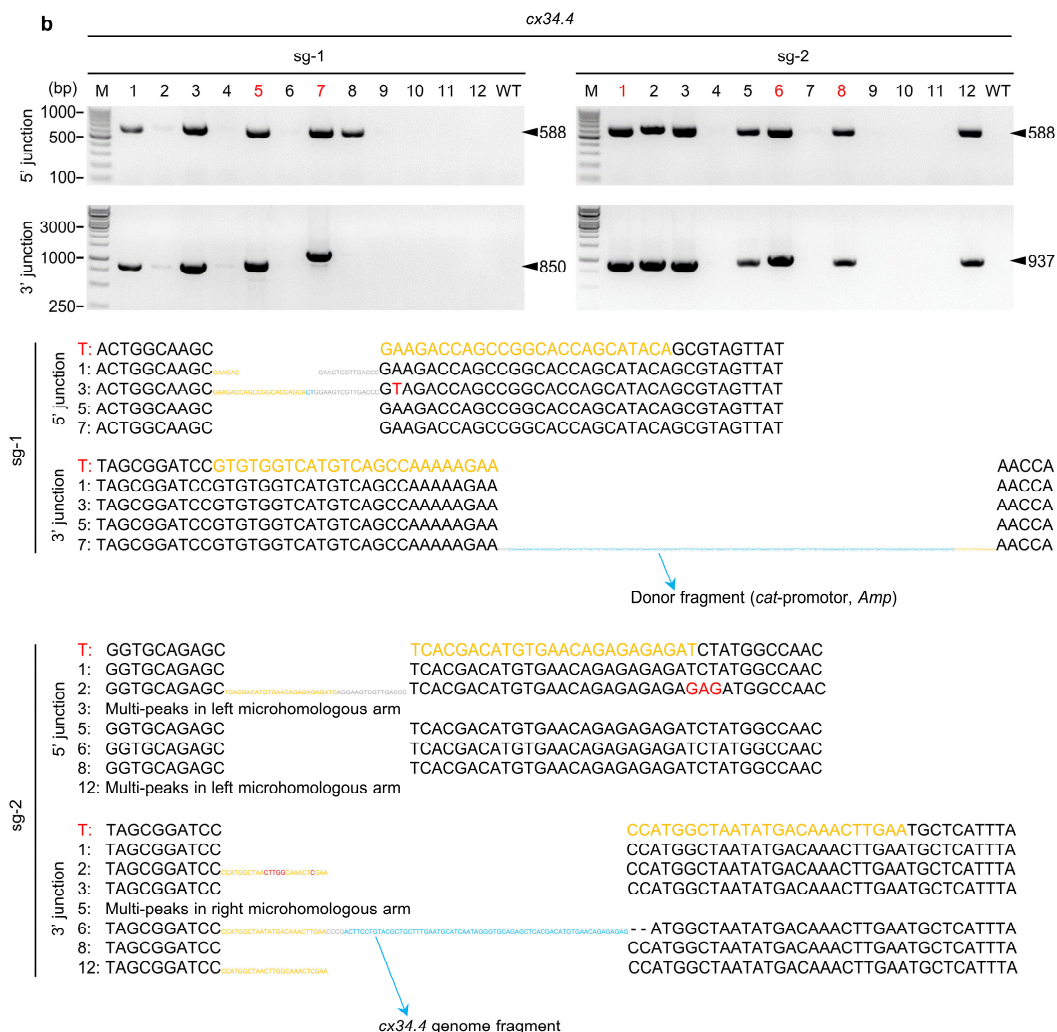
Supplementary Fig. 8. A schematic diagram of a germline transmission screen procedure combining fluorescence examination and junction PCR.

If GFP-positive embryos are observed in microinjected F_0 zebrafish, all of the GFP-positive F_0 embryos are raised to adulthood and outbred with WT. The F_1 embryos are identified by GFP expression patterns and genotyping. However,

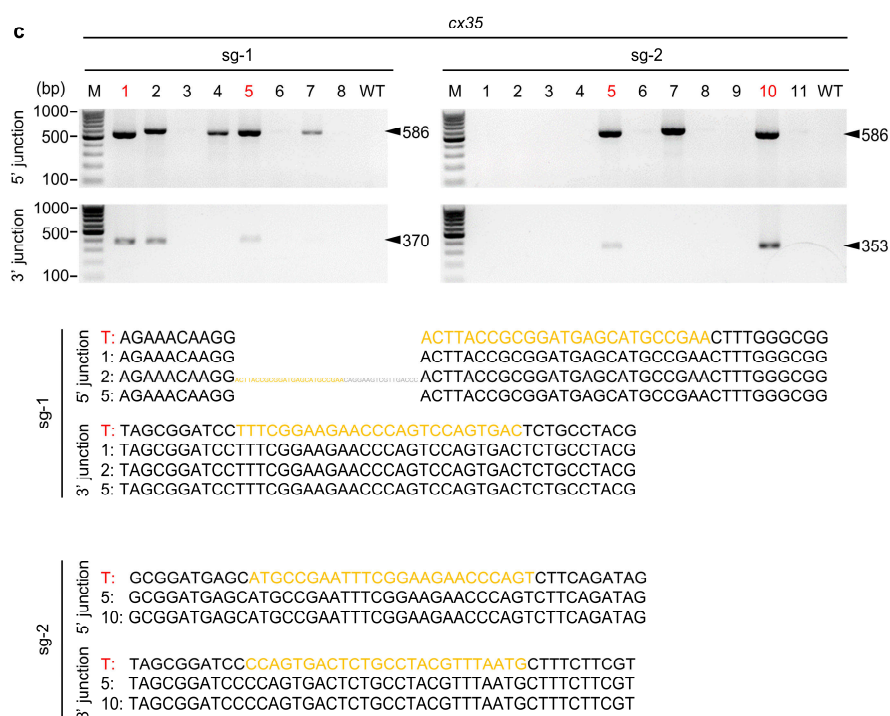
if GFP signals cannot be detected in F_0 embryos after microinjection, all of the F_0 embryos are raised to 1 month old. Genomic DNA is then isolated from the caudal fins of these 1-month-old F_0 zebrafish and used for junction PCR to identify PCR-positive F_0 . Next, fluorescence is examined in F_1 embryos obtained from outbreeding PCR-positive F_0 with WT zebrafish. If fluorescence is detected in F_1 embryos, junction PCR would be performed to determine the genotype of the F_1 . Otherwise, all of the GFP-negative F_1 embryos are pooled for genomic DNA extraction and junction PCR analysis. If KI is detected in the pooled DNA, the corresponding F_0 adults would be outbred with WT again to generate new F_1 . These F_1 are genotyped using caudal fins at 1-month-old to confirm KI.

Supplementary Figure 9



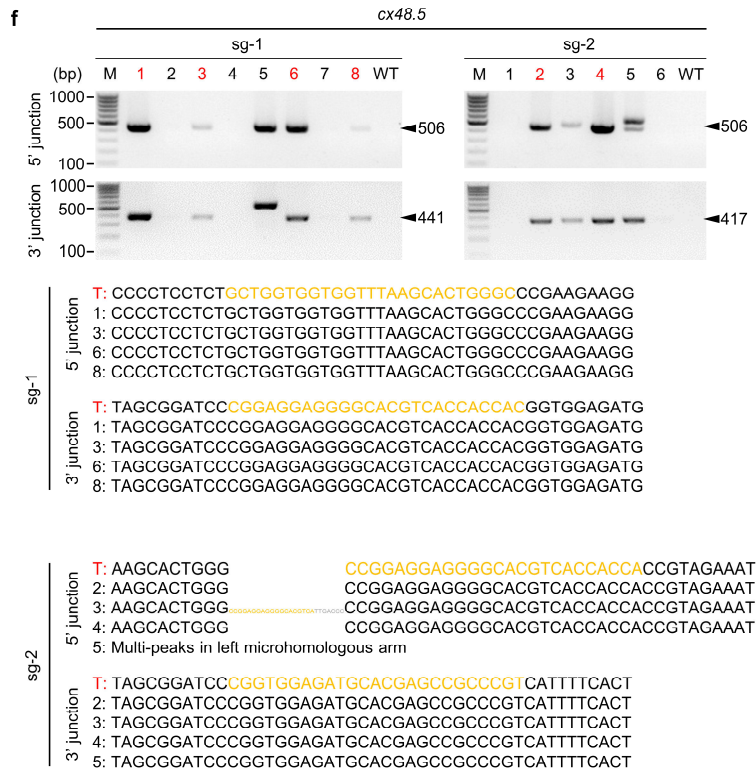


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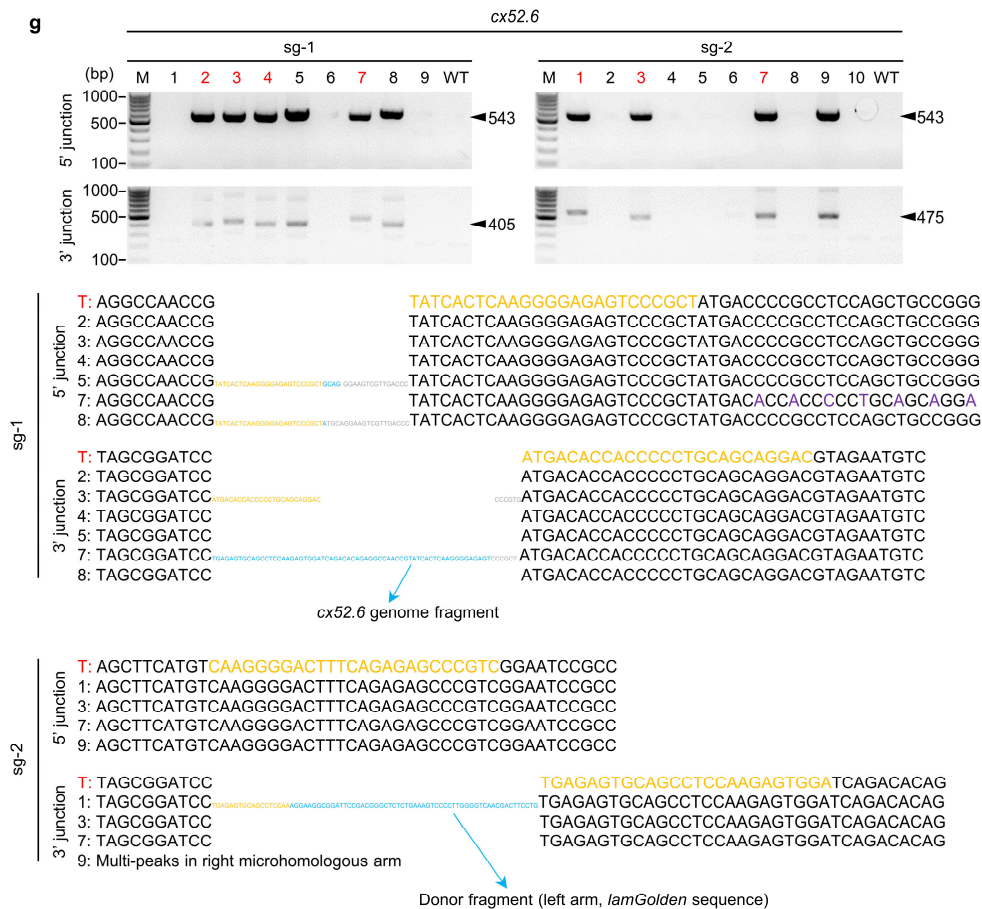


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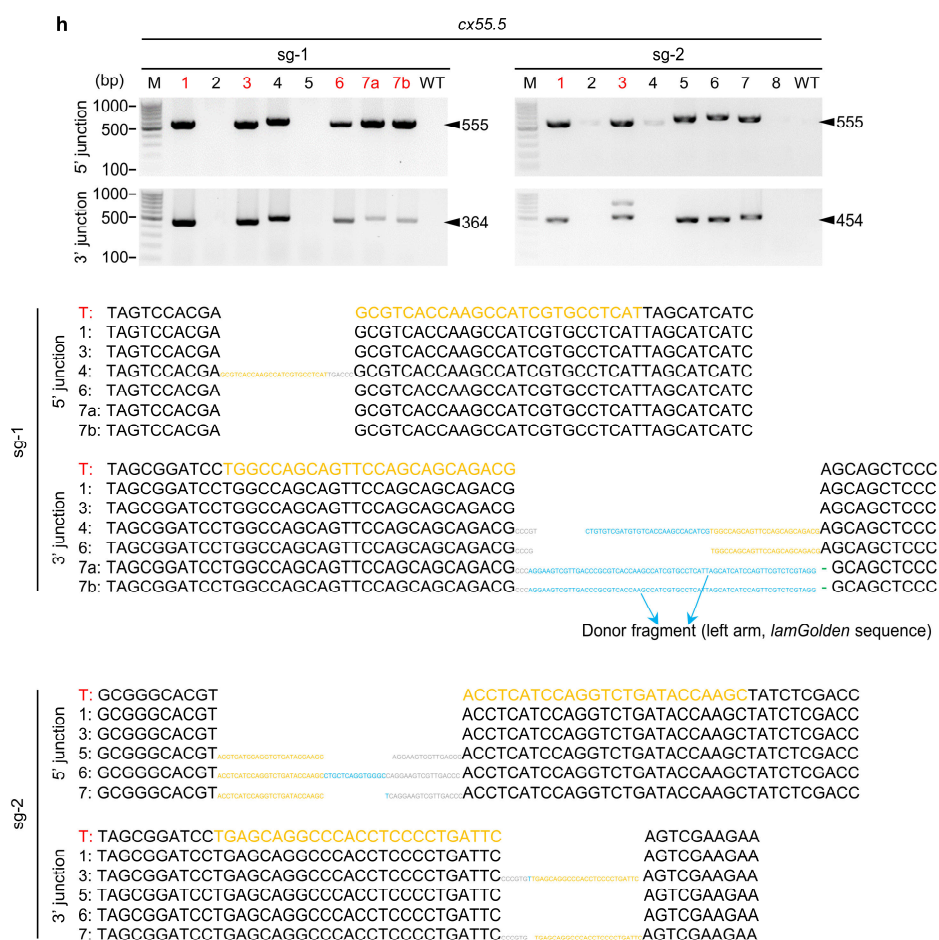




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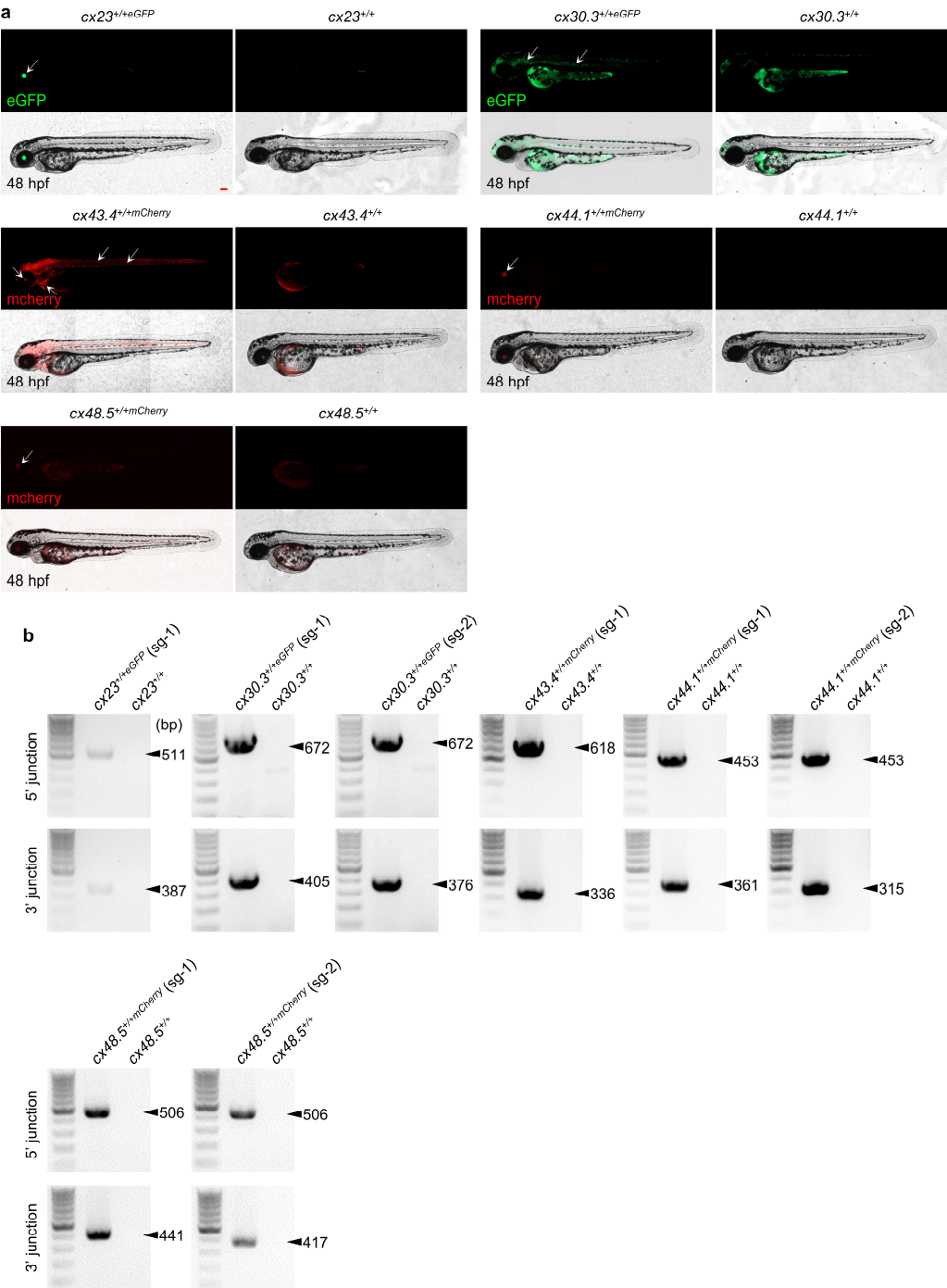


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Supplementary Fig. 9. Germline transmission screen results.

Germline transmission screens for GFP-tagged *connexins* including *cx30.3* (a), *cx34.4* (b), *cx35* (c), *cx44.1* (d), *cx47.1* (e), *cx48.5* (f), *cx52.6* (g), and *cx55.5* (h) following procedures introduced in Supplemental Figure 8. GFP was detected in F₁ embryos for *cx30.3*, *cx44.1*, and *cx48.5* KI but not in F₁ embryos for *cx34.4*, *cx35*, *cx47.1*, *cx52.6*, or *cx55.5* KI. Germline transmission was confirmed by junction PCR and sequencing for all tested genes. Numbers on the top of the gels represent F₁ zebrafish pools generated by different F₀ zebrafish. Desired F₁ were indicated by red numbers. T, the desired sequence used as template for the alignment.

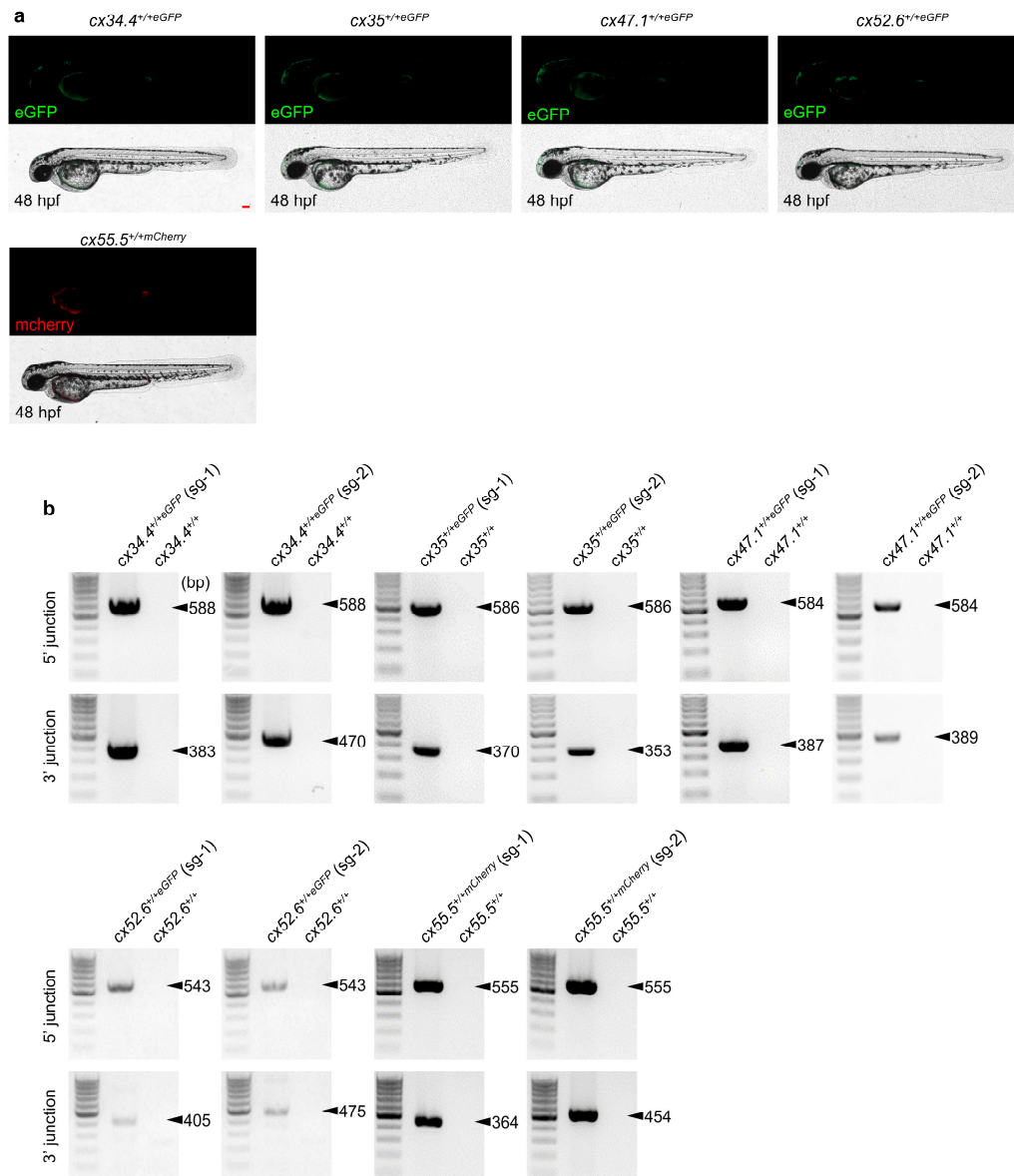


149 **Supplementary Fig. 10. Images and junction PCR analysis of F₁ zebrafish**
150 **carrying fluorescence-labeled *connexins*.**

151 **(a)** *Cx23*, *Cx30.3*, *Cx43.4*, *Cx44.1*, or *Cx48.5* KI F₁ zebrafish with detectable
152 fluorescence at 48 hpf. *Cx23*^{+/+}eGFP, *Cx44.1*^{+/+}mCherry, and *Cx48.5*^{+/+}mCherry larvae
153 showed fluorescence in the lens. *Cx30.3*^{+/+}eGFP larvae showed GFP expression

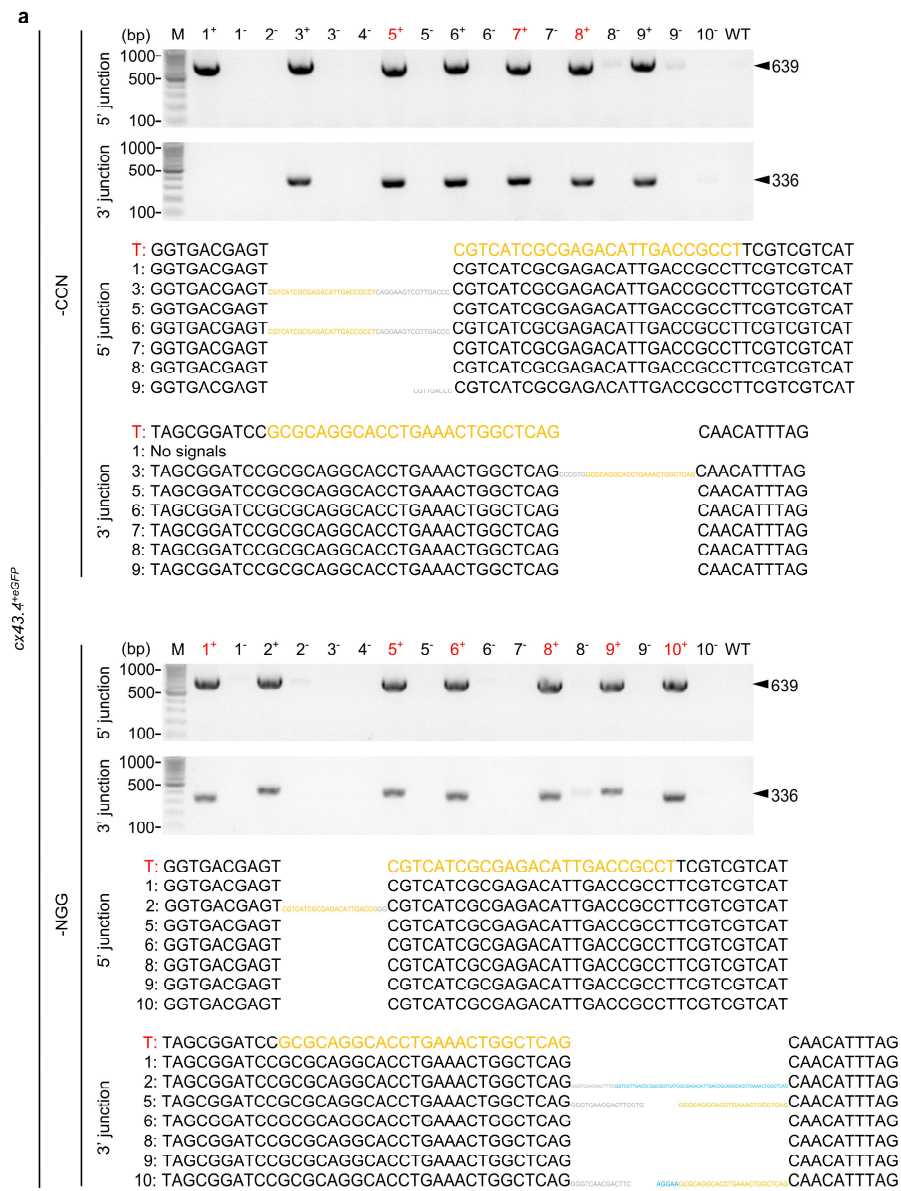
154 in the anterior notochord and skin. *cx43.4^{+/+mCherry}* larvae showed the same
155 expression patterns as that of the *cx43.4^{+/+eGFP}* larvae in Fig. 1D, 4D, and 5E.
156 Scale bars, 100 μ m. Images are representatives of at least 10 F₁. **(b)**
157 *cx23^{+/+eGFP}*, *cx30.3^{+/+eGFP}*, *cx43.4^{+/+mCherry}*, *cx44.1^{+/+mCherry}*, and *cx48.5^{+/+mCherry}*
158 F₁ embryos carrying fluorescence-labeled *connexin* alleles were confirmed by
159 5'- and 3'-junction PCR. Littermates were used as negative controls. Data are
160 representatives of at least 10 F₁.

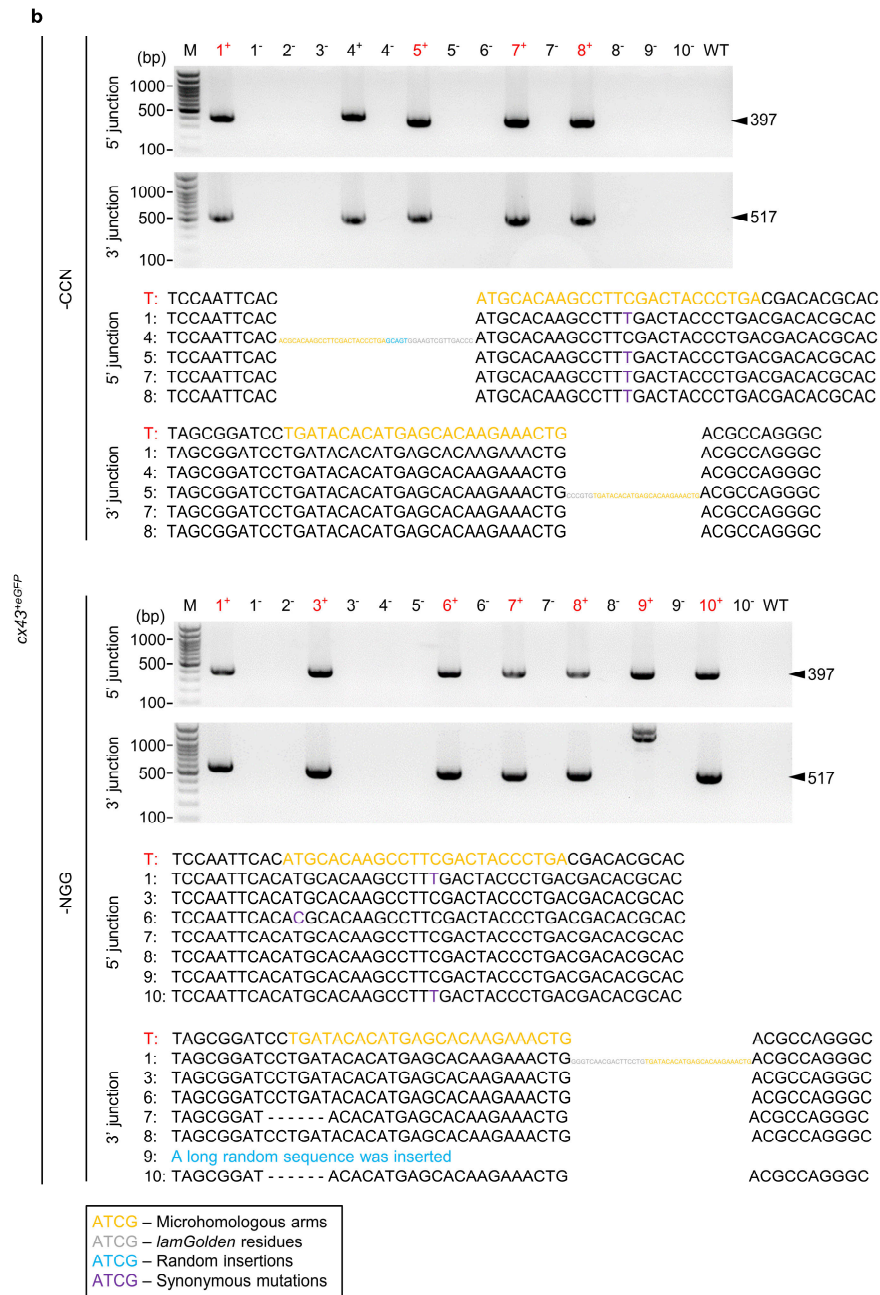
Supplementary Figure 11



Supplementary Fig. 11. Images and Junction PCR analysis of *cx34.4*, *cx35*, *cx47.1*, *cx52.6*, or *cx55.5* KI F₁ zebrafish.

(a) *cx34.4^{+/+}eGFP*, *cx35^{+/+}eGFP*, *cx47.1^{+/+}eGFP*, *cx52.6^{+/+}eGFP*, and *cx55.5^{+/+}mCherry* larvae showed no fluorescence at 48 hpf. Scale bars, 100 μ m. Images are representatives of at least 10 F₁. **(b)** *cx34.4^{+/+}eGFP*, *cx35^{+/+}eGFP*, *cx47.1^{+/+}eGFP*, *cx52.6^{+/+}eGFP*, and *cx55.5^{+/+}mCherry* F₁ embryos were confirmed by 5'- and 3'-junction PCR. Littermates were included as negative controls. Data are representatives of at least 10 F₁.



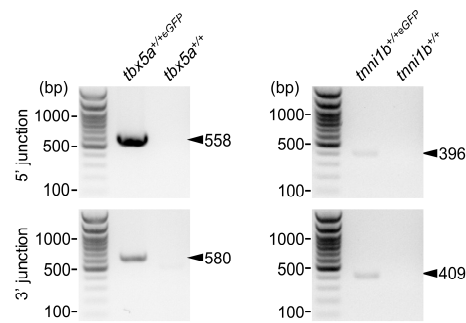


Supplementary Fig. 12. A comparison of the S-NGG-25 and S-CCN-25 strategies in generating desired F₀.

Junction PCR analysis of *cx43.4^{+/+eGFP}* (a) and *cx43^{+/+eGFP}* (b) F₁ generated by the S-CCN-25 or S-NGG-25 strategy. Ten randomly selected GFP-positive F₀ were screened for germline transmission. F₁ derived from outbreeding each F₀ with WT were examined for GFP expression patterns and genotypes. Numbers on the top of the gels represent F₁ generated by different F₀ zebrafish. “+” at

183 the upper right of each number means “GFP-positive” and “-” means “GFP-
184 negative”. Desired F_1 were indicated in red. T, the desired sequence used as
185 template for the alignment.
186

Supplementary Figure 13



Supplementary Fig. 13. Junction PCR analysis of *tbx5a*^{+/+eGFP} and *tn timer*^{+/+eGFP} F₁ zebrafish.

F₁ embryos carrying GFP-labeled *tbx5a* or *tn timer* alleles were identified by 5'- and 3'-junction PCR analysis. Data are representatives of at least 10 F₁.

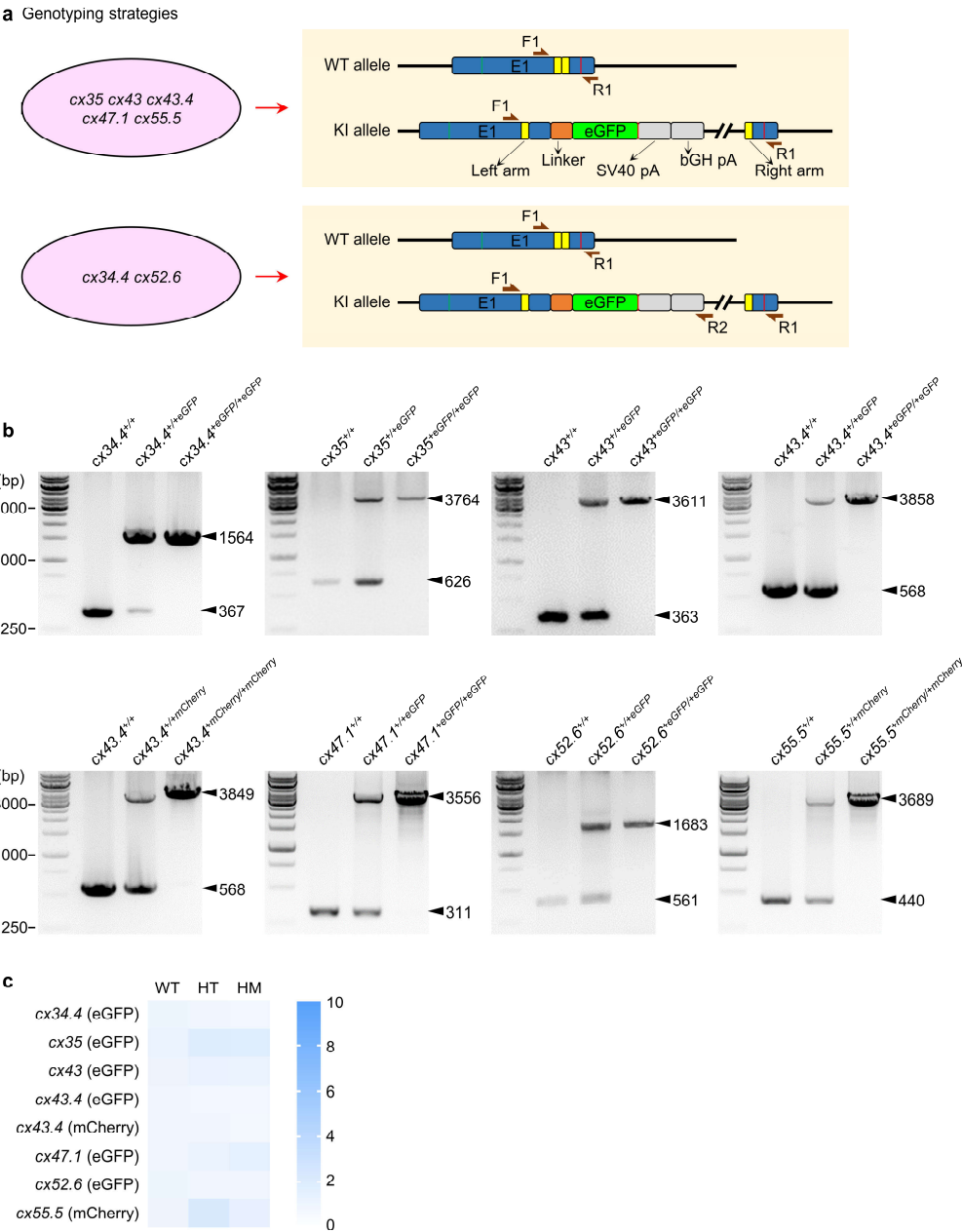
Supplementary Figure 14

F ₁ progeny carrying fluorescence-labeled <i>connexin</i> allele					
<i>Cx23</i> ^{+/+eGFP}	✓	<i>Cx34.1</i> ^{+/+eGFP}		<i>Cx43.4</i> ^{+/+eGFP} (<i>Cx43.4</i> ^{+/+mCherry})	✓
<i>Cx27.5</i> ^{+/+eGFP}		<i>Cx34.4</i> ^{+/+eGFP}		<i>Cx44.1</i> ^{+/+mCherry}	✓
<i>Cx28.1</i> ^{+/+eGFP}		<i>Cx34.5</i> ^{+/+eGFP}		<i>Cx44.2</i> ^{+/+eGFP}	
<i>Cx28.6</i> ^{+/+eGFP}		<i>Cx35</i> ^{+/+eGFP}		<i>Cx45.6</i> ^{+/+eGFP}	
<i>Cx28.8</i> ^{+/+eGFP}		<i>Cx35.4</i> ^{+/+eGFP}		<i>Cx47.1</i> ^{+/+eGFP}	
<i>Cx28.9</i> ^{+/+eGFP}		<i>Cx36.7</i> ^{+/+eGFP}		<i>Cx48.5</i> ^{+/+mCherry}	✓
<i>Cx30.3</i> ^{+/+eGFP}	✓	<i>Cx39.4</i> ^{+/+eGFP}		<i>Cx52.6</i> ^{+/+eGFP}	
<i>Cx30.9</i> ^{+/+eGFP}		<i>Cx39.9</i> ^{+/+eGFP}	✓	<i>Cx52.7</i> ^{+/+eGFP}	
<i>Cx31.7</i> ^{+/+eGFP}		<i>Cx40.8</i> ^{+/+eGFP}		<i>Cx52.9</i> ^{+/+eGFP}	
<i>Cx32.2</i> ^{+/+eGFP}		<i>Cx41.8</i> ^{+/+mCherry}		<i>Cx55.5</i> ^{+/+mCherry}	
<i>Cx32.3</i> ^{+/+eGFP}		<i>Cx43</i> ^{+/+eGFP}	✓	<i>Cx79.8</i> ^{+/+eGFP}	

Supplementary Fig. 14. A summary of successfully tagged *connexins* and whether or not fluorescence was observed in the KI F₁.

“✓” means that fluorescence-positive F₁ were obtained.

Supplementary Figure 15



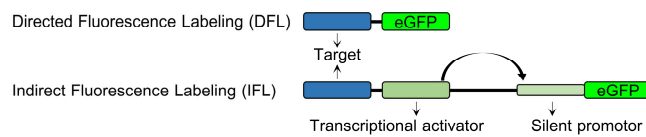
201

202 **Supplementary Fig. 15. A strategy for genotyping *connexin* KI zebrafish.**

203 **(a)** A schematic diagram of a genotyping method to identify WT, heterozygous
204 (HT), or homozygous (HM) KI zebrafish. For *cx35*, *cx43*, *cx43.4*, *cx47.1*, and
205 *cx55.5*, primers (F1 and R1) were designed to flank the homologous arms. For
206 *cx34.4* and *cx52.6*, the reverse primer R2 targets the bGH polyA sequence and
207 genotyping was done using primers F1, R1, and R2. **(b)** Genotyping results for
208 *cx34.4*, *cx35*, *cx43*, *cx43.4*, *cx47.1*, *cx52.6*, and *cx55.5* KI. Data are
209 representatives of at least 10 F₂. **(c)** Expression levels of *connexins* in

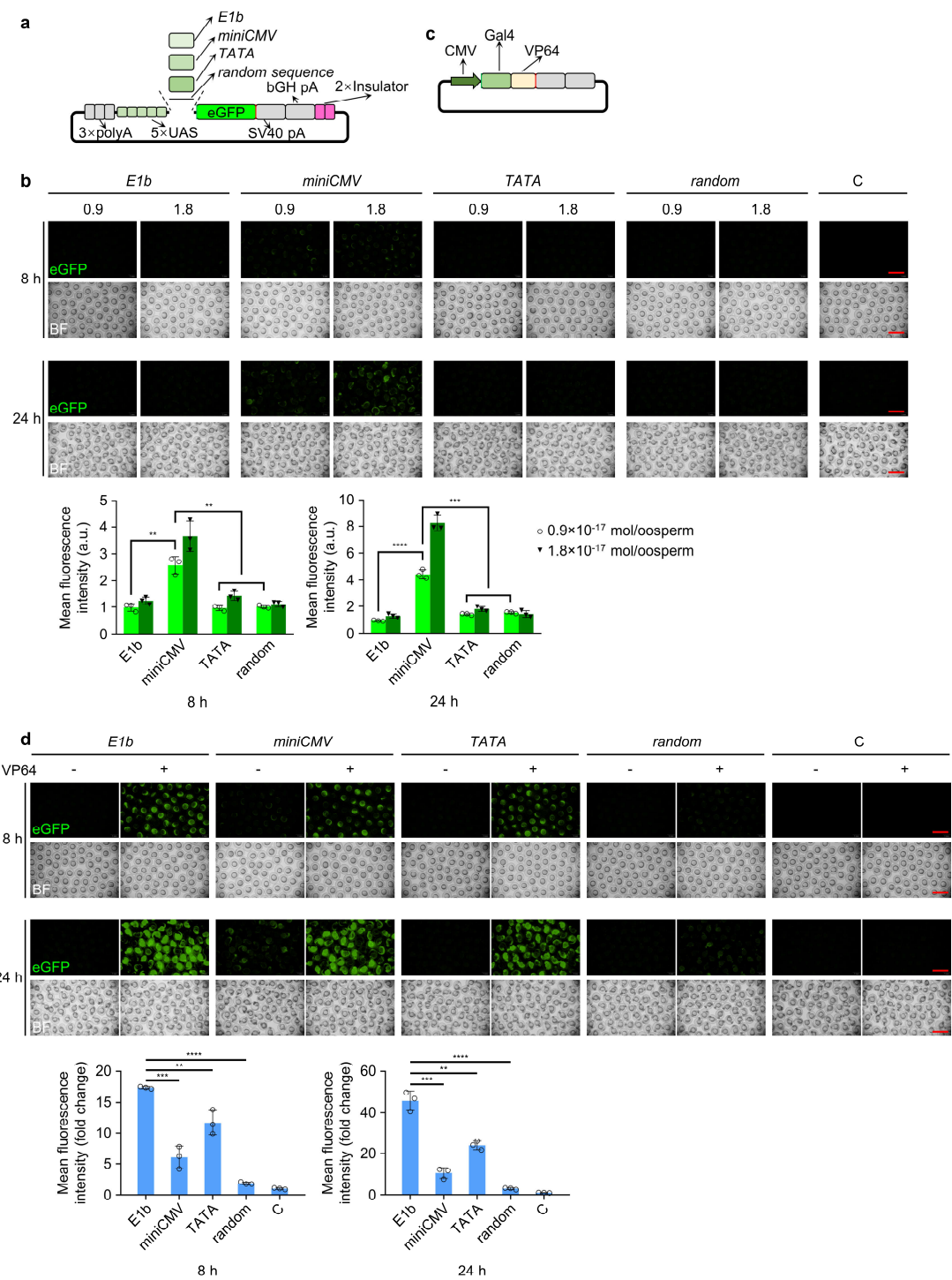
fluorescence-labeled HT or HM F₃ relative to WT after tagging. The total mRNA of genotype-confirmed WT, HT, or HM F₃ zebrafish for each *connexin* was extracted and used for RT-qPCR analysis. Expression of the tagged *connexin* in each of its F₃ progeny was normalized to that of *β-actin*, and then the threshold cycle ($2^{-\Delta\Delta C_t}$) method was used and the fold change of the *connexin* in each sample relative to the WT sample was plotted. At least 30 F₃ embryos per genotype were analyzed and 3 repeats were included for each sample in an RT-qPCR.

Supplementary Figure 16



Supplementary Fig. 16. A schematic diagram of how the indirect fluorescence labeling (IFL) works.

Direct fluorescence labeling (DFL) generates fluorescent proteins (e.g. GFP) directly fused with the target protein by a linker. Indirect fluorescence labeling (IFL) indirectly produces GFP mediated by a transcriptional activator which is fused and co-expressed with the target protein, initiating GFP expression by binding a specific promoter ahead of the GFP CDS.

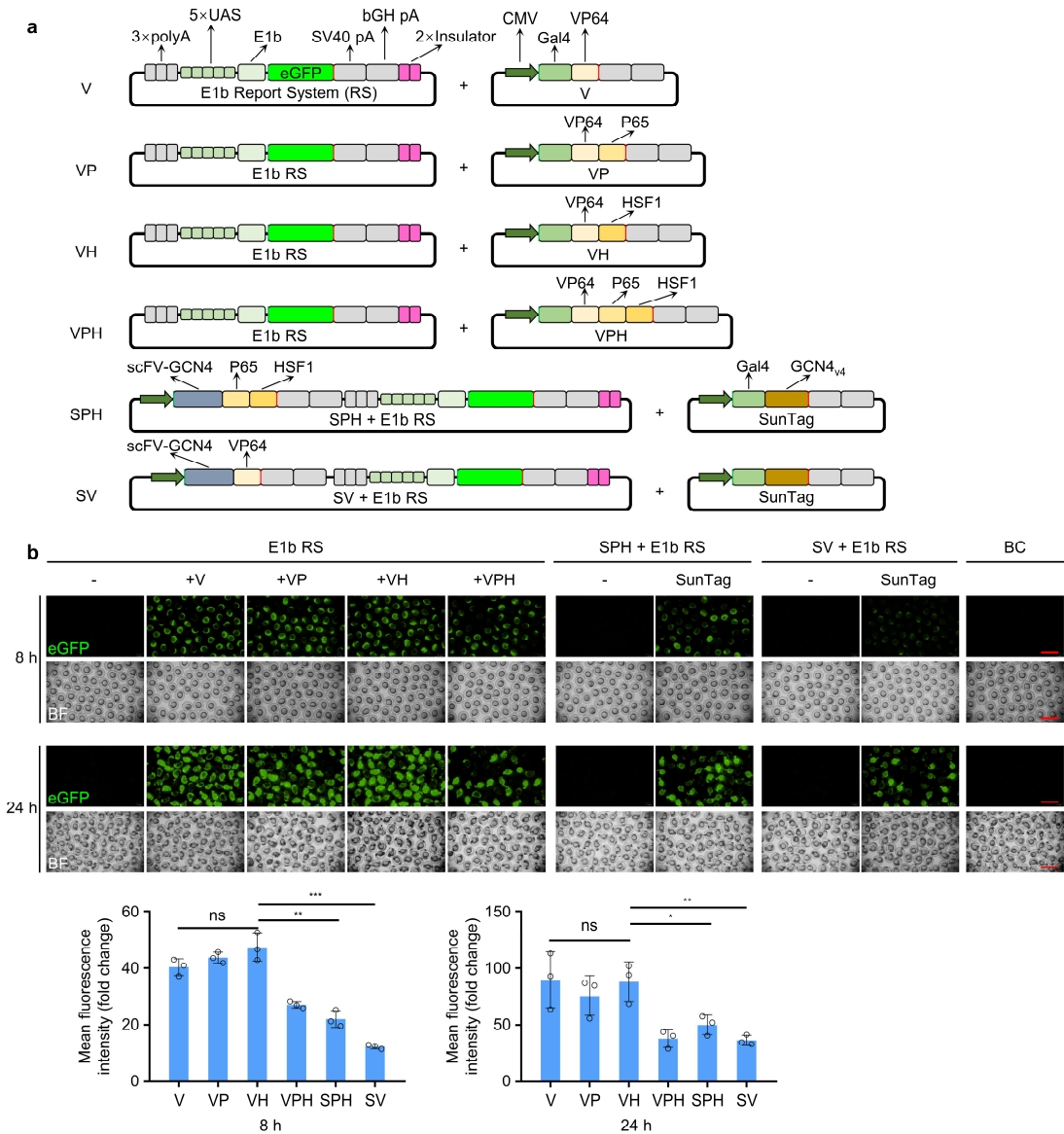


230

231 **Supplementary Fig. 17. Promotor optimization for the fluorescence**
232 **amplification system.**

233 **(a)** A schematic diagram of the fluorescence reporter plasmid. Different
234 promoters, including *E1b*, *miniCMV*, *TATA* (random sequence containing *TATA*

box), and random sequence (without *TATA* box), are located downstream of 5×nrUAS and upstream of eGFP CDS. **(b)** Comparison of leaky expression driven by different promoters. Different plasmids were microinjected into WT zebrafish embryos at the one-cell stage respectively. Images of embryos were taken at 8 hpf and 24 hpf to calculate MFI. C, vector control without promoters. Data are presented as mean ± SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001. Scale bars, 2 mm. **(c)** A schematic diagram of the Gal4-VP64 transcriptional activator plasmid. Gal4-VP64 is driven by a CMV promoter. **(d)** Comparison of transcriptional activation of different promoters by Gal4-VP64. Gal4-VP64 and fluorescence reporter plasmids (0.9×10^{-17} mol per plasmid per embryo) were co-injected into 1-cell-stage WT zebrafish embryos. Images of embryos were taken at 8 hpf and 24 hpf to calculate MFI. C, vector control without promoters. Data are presented as mean ± SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001. Scale bars, 2 mm.

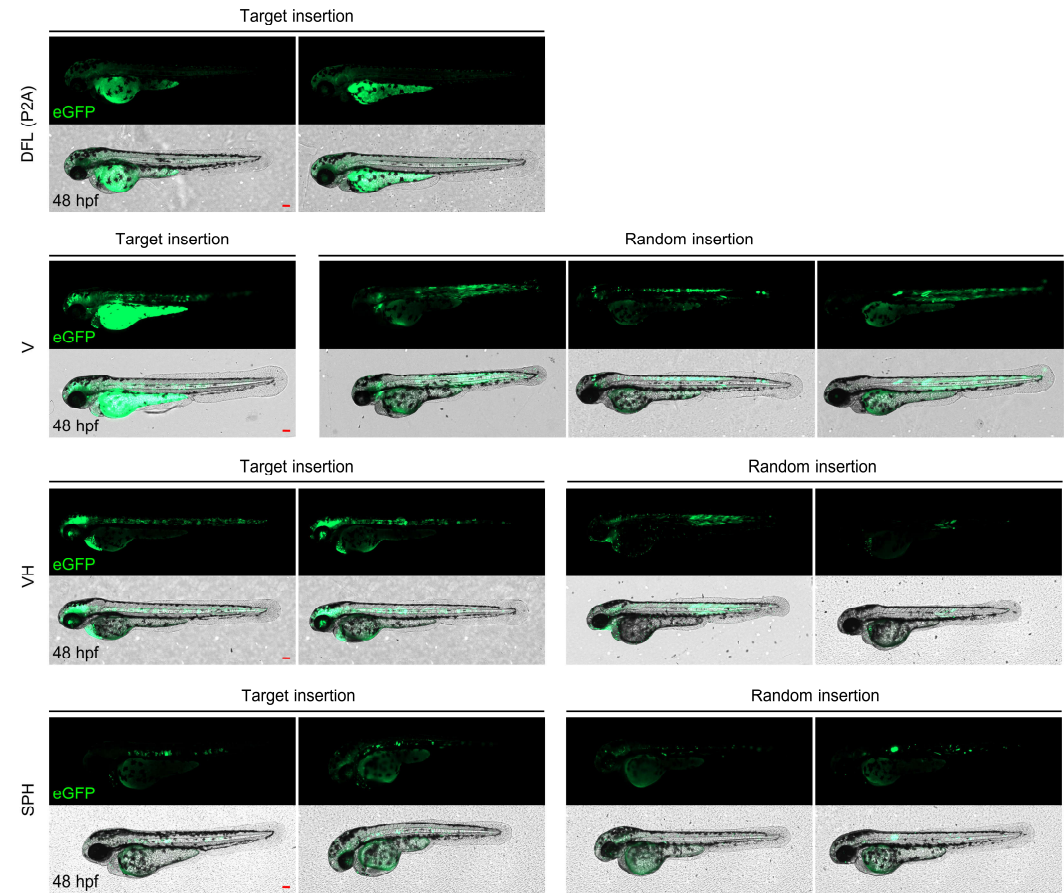


Supplementary Fig. 18. Optimization of the transcriptional activator for the fluorescence amplification system.

(a) Schematic diagrams of different transcriptional activation systems. Gal4-VP64 (V), Gal4-VP64-P65 (VP), Gal4-VP64-HSF1 (VH), or Gal4-VP64-P65-HSF1 (VPH) was co-injected with the *5×nrUAS-E1b-eGFP* plasmid into 1-cell-stage WT embryos (0.9×10^{-17} mol per plasmid per embryo). SunTag-P65-HSF1 (SPH) or SunTag-VP64 (SV) was cloned into the *5×nrUAS-E1b-eGFP* plasmid and was co-injected with the Gal4-GCN₄ plasmid into WT embryos (0.9×10^{-17}

mol per plasmid per embryo). **(b)** Comparison of fluorescence generated by different transcriptional activation systems. Images of embryos were taken at 8 hpf and 24 hpf to calculate MFI. BC, blank control without plasmids. Data are presented as mean $\pm$ SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. ns, not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Scale bars, 2 mm.

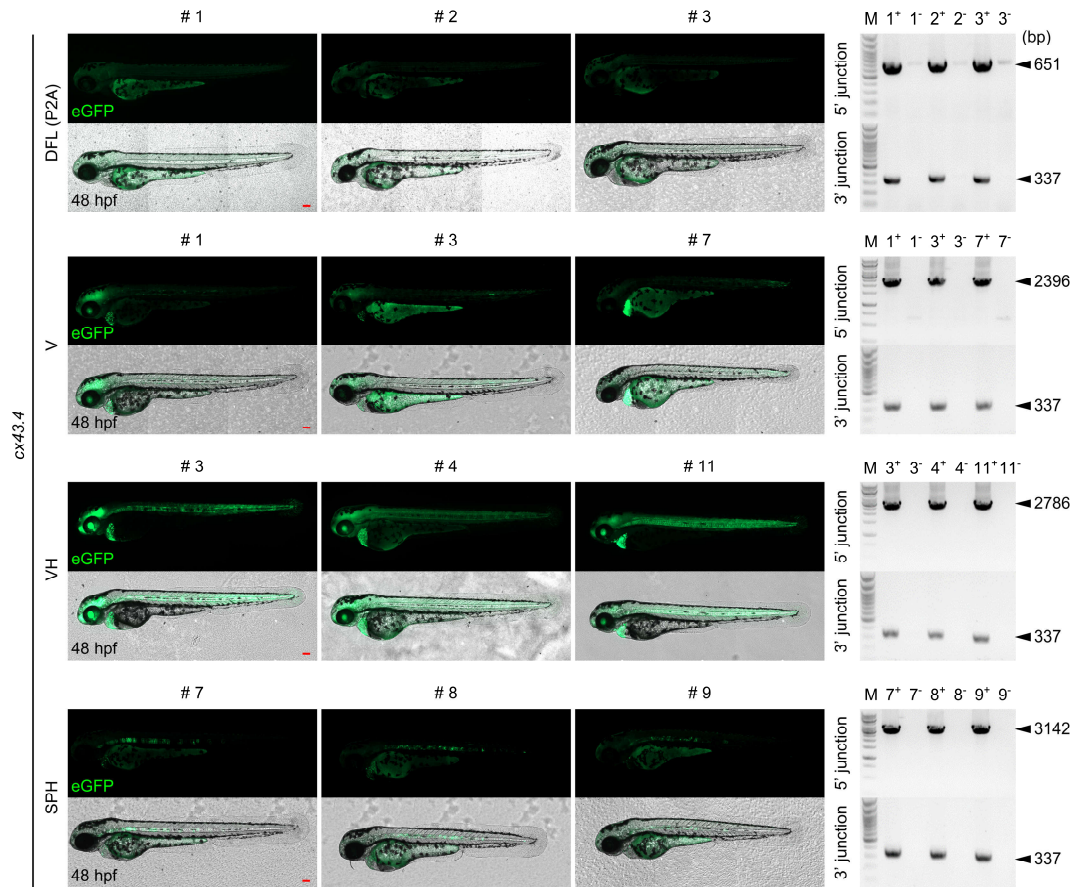
Supplementary Figure 19



Supplementary Fig. 19. Images of *cx43.4* KI embryos using DFL, V, VH, or SPH strategy.

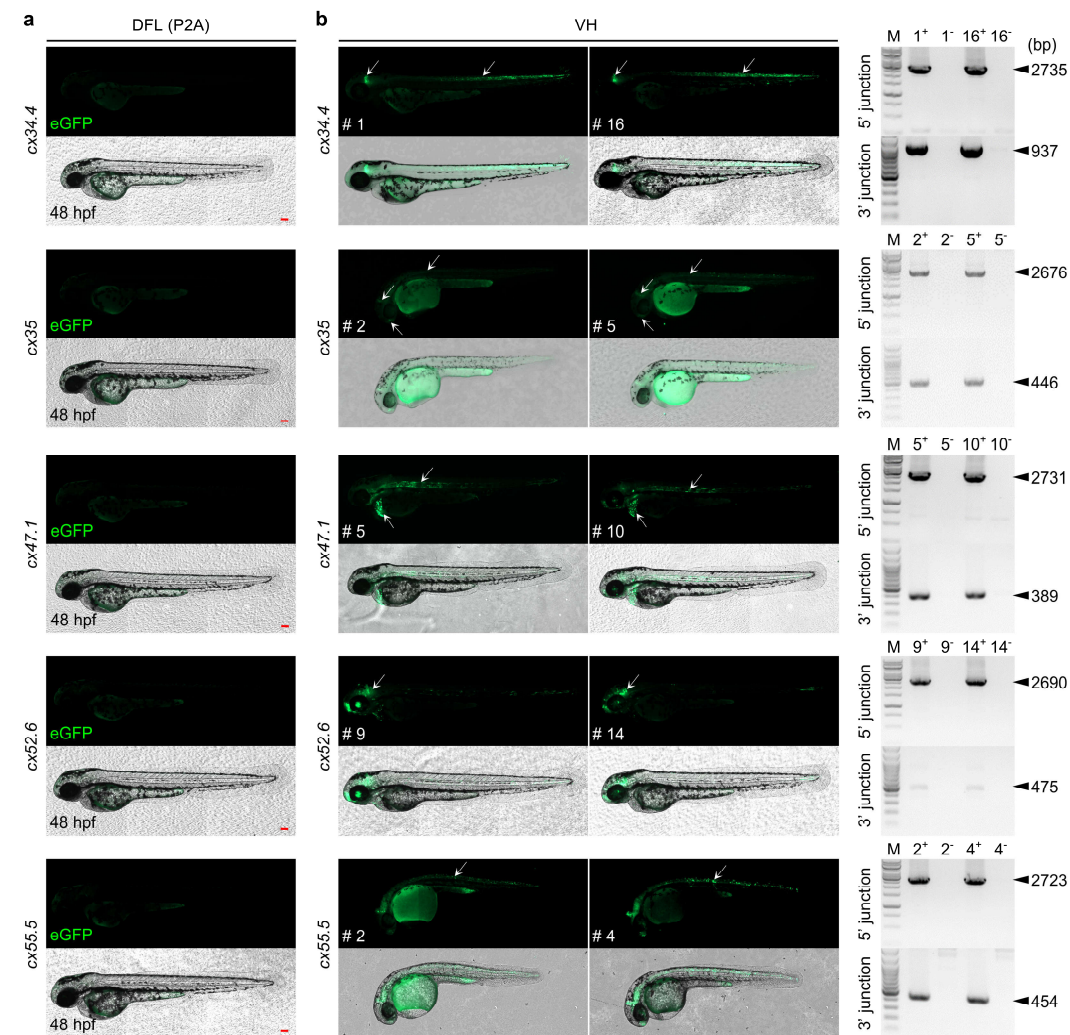
Different KI strategies were performed to generate *cx43.4* KI. After co-injection of the Cas9/sgRNA system and the IFL or DFL donor into 1-cell-stage WT embryos, images of microinjected GFP-positive embryos were collected at 48 hpf. IFL-labeled GFP-positive F_0 embryos were classified into target-insertion and random-insertion groups based on the expression patterns of *cx43.4* in the DFL group (see also Fig. 1d). F_0 embryos of the target-insertion groups were raised to adulthood for germline transmission screen. Scale bars, 100 μ m. Images are representatives of at least 30 F_0 for each strategy.

Supplementary Figure 20



Supplementary Fig. 20. Images and junction PCR analysis of F₁ *cx43.4*-KI zebrafish generated by DFL, V, VH, or SPH strategy.

Different KI strategies were performed to fluorescence-label *cx43.4*. (Left panel) Images of GFP-expressing desired *cx43.4*-KI F₁ embryos derived from three different F₀ adults generated by using DFL, V, VH, or SPH strategy. Images were taken at 48 hpf. The number of the corresponding F₀ zebrafish was shown. Scale bars, 100 μ m. (Right panel) Junction PCR tests were performed to confirm genotypes of the desired F₁. GFP-negative littermates were used as negative controls. Numbers on the top of the gels represent F₁ zebrafish generated by F₀ with different numbers shown in (Left panel). "+" at the upper right of each number means GFP-positive F₁ embryos, and "-" means GFP-negative F₁ embryos.



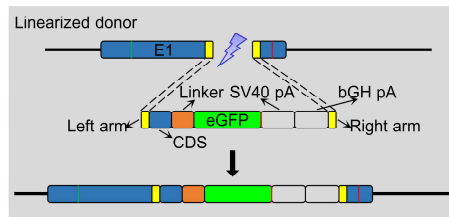
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299 **Supplementary Fig. 21. Images of F₁ embryos generated by the DFL or VH**
300 **strategy for *cx34.4*, *cx35*, *cx47.1*, *cx52.6*, or *cx55.5* KI.**

301 **(a)** F₁ embryos generated by the 2A-linker-mediated DFL strategy were
302 included as controls and no fluorescence was detected in these F₁. Images are
303 representatives of at least 10 F₁ at 48 hpf. **(b)** Left panel, for each VH-
304 engineered *connexin*, F₁ carriers were derived from outbreeding at least two F₀
305 founders with WT. Images are representatives of at least 10 at 48 hpf. The
306 number of the corresponding F₀ was labeled at the bottom left. Scale bars, 100
307 μm. Right panel, junction PCR was performed to confirm genotypes of the VH
308 strategy-generated F₁. GFP-negative littermates were used as negative
309 controls. The numbers on the top of the gels are the numbers of the

310 corresponding F_0 zebrafish, the same as those shown in the left panel. “+”
311 means GFP-positive F_1 , and “-“ means GFP-negative F_1 .
312

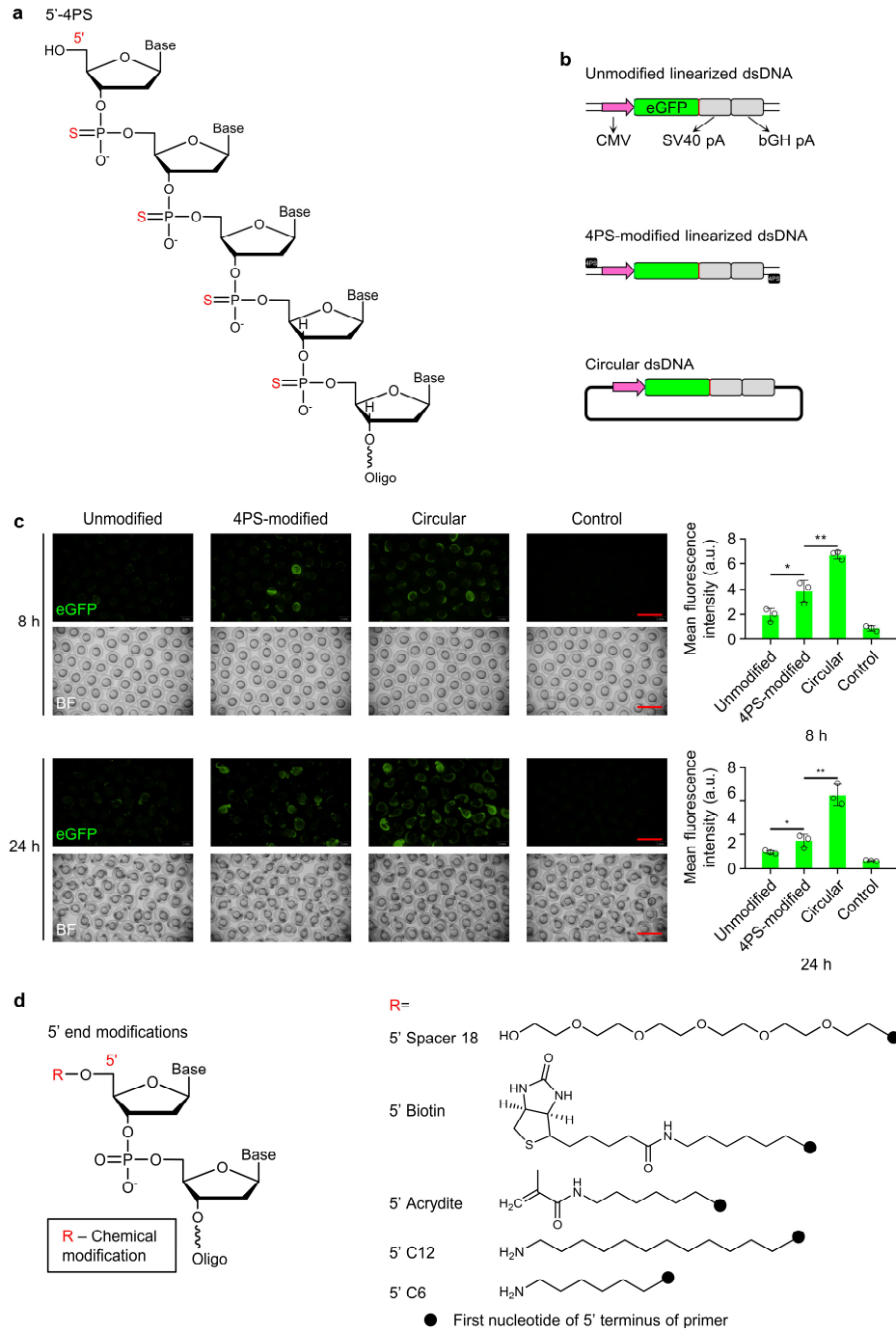
Supplementary Figure 22



Supplementary Fig. 22. A schematic diagram of the linearized dsDNA-mediated KI strategy.

Linearized dsDNA containing functional cassettes was amplified by PCR and was used as the donor. When MMEJ-repair happens, the linearized donor would be integrated into the target gene without a redundant sequence.

Supplementary Figure 23

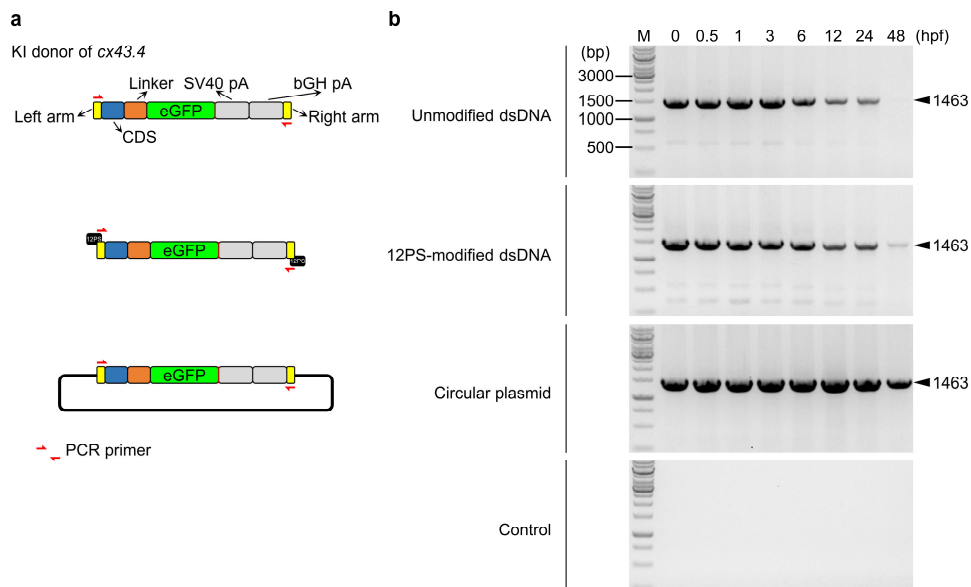


Supplementary Fig. 23. 5'-end modifications improved the stability of dsDNAs.

(a) Structure of the 5'-4PS modification. Replacing 1 P=O in 1 phosphate group between 2 deoxyribonucleotides generates 1 P=S and replacing 4 P=O in 4 phosphate groups between 5 deoxyribonucleotides generates 4 P=S, and the like. **(b)** A schematic diagram of the unmodified linearized dsDNA, 4PS-

modified linearized dsDNA, and circular dsDNA. All of the dsDNAs have the same GFP expression cassette, including a *CMV* promotor, a GFP coding sequence, and polyA signals. 5'-4PS modifications were added to the 5' terminus of the linearized dsDNAs by using modified PCR primers. **(c)** dsDNAs in **(b)** were microinjected into one-cell-stage WT zebrafish embryos at 0.9×10^{-17} mol/embryo. Images of embryos were taken at 8 hpf and 24 hpf to calculate MFI. Control, the vector plasmid control without functional cassettes. Data are presented as mean $\pm$ SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. *, *P* < 0.05; **, *P* < 0.01. Scale bars, 2 mm. **(d)** Structures of other 5'-chemical modifications (R), including spacer18, biotin, acrydite, C12, and C6. These 5'-chemical modifications were added to the first deoxyribonucleotide of the 5' terminus of each primer.

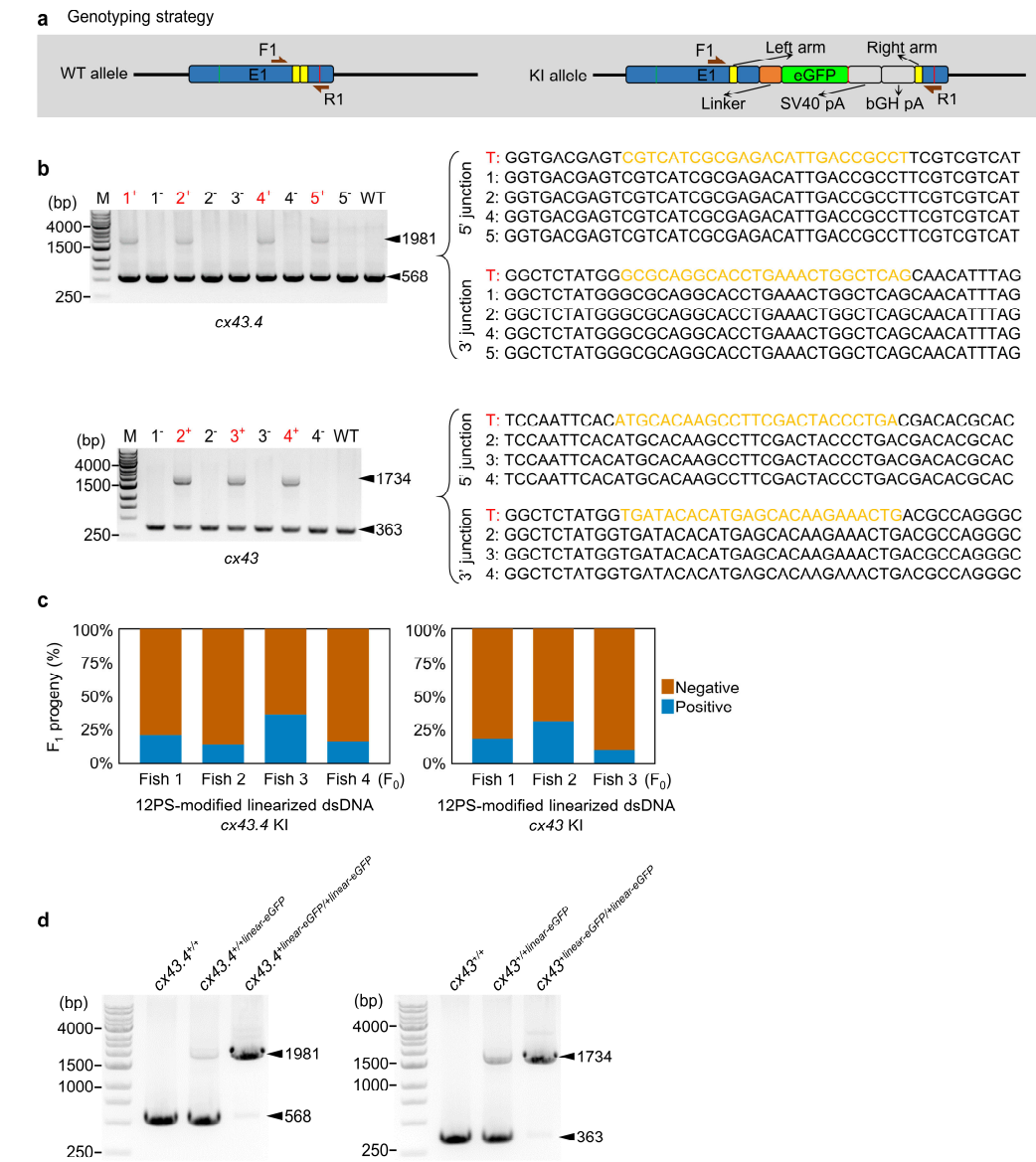
Supplementary Figure 24



Supplementary Fig. 24. Comparison of the degradation of different forms of dsDNAs in embryos.

(a) A schematic diagram of three types of KI donors for *cx43.4*, the unmodified linearized dsDNA, the 12PS-modified linearized dsDNA, and the circular plasmid. PCR primers were designed in the left and right arms. **(b)** Time-course analysis of the degradation of different forms of dsDNA donors shown in (A). Equal amount (0.9×10^{-17} mol/embryo) of each donor was injected into one-cell-stage WT embryos. Five embryos per donor type were randomly selected and mixed to extract total DNA at different time points. DNA samples were then subjected to PCR analysis. Data are representatives of 3 independent experiments.

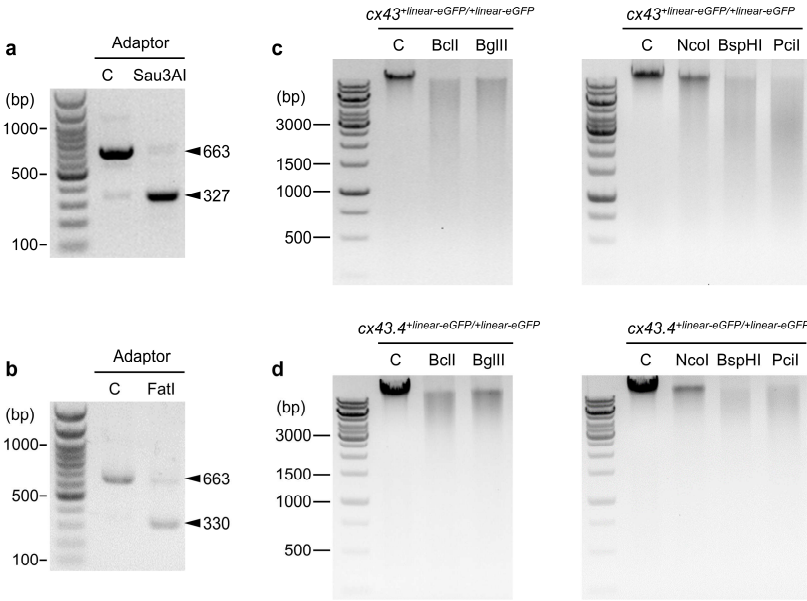
Supplementary Figure 25



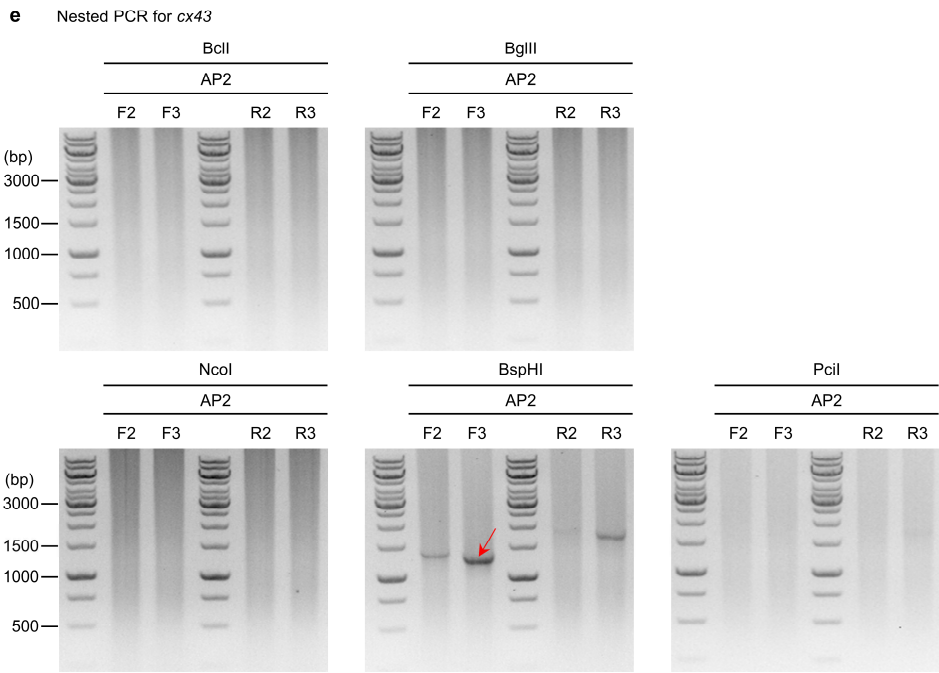
Supplementary Fig. 25. GFP-labeled *cx43* or *cx43.4* alleles were successfully generated by the 12PS-modified linearized dsDNA mediated KI.

(a) A schematic diagram of a genotyping method for the linearized dsDNA mediated KI. Primers were designed to flank the homologous arms. **(b)** Genotyping and sequencing analysis of GFP-positive *cx43*- or *cx43.4*-KI F₁ progeny from outbreeding F₀ adults generated by using the 5'-12PS donors. Numbers on the top of the gels represent F₁ zebrafish generated by F₀ labeled with different numbers. "+" at the upper right of each number means "GFP-

positive” and “-” means “GFP-negative”. Desired F₁ were indicated in red. T, the
desired sequence used as the template for the alignment. **(c)** Mosaicism of the
germline of F₀ founders for *cx43.4* and *cx43* KI generated by using the 12-PS
modified linearized dsDNA was determined by the percentage of F₁ carrying
the KI cassette. GFP-positive *cx43.4*^{+/+eGFP} or *cx43*^{+/+eGFP} F₁ were shown in blue
and GFP-negative *cx43.4*^{+/+} or *cx43*^{+/+} F₁ were in orange. At least 60 F₁ were
examined for each PCR-positive F₀ founder. **(d)** Genotyping of F₂ progeny
obtained from inbreeding GFP-positive *cx43*- or *cx43.4*-KI F₁ zebrafish. Data
are representatives of at least 10 F₂.



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Sequence blast of BspHI-F3

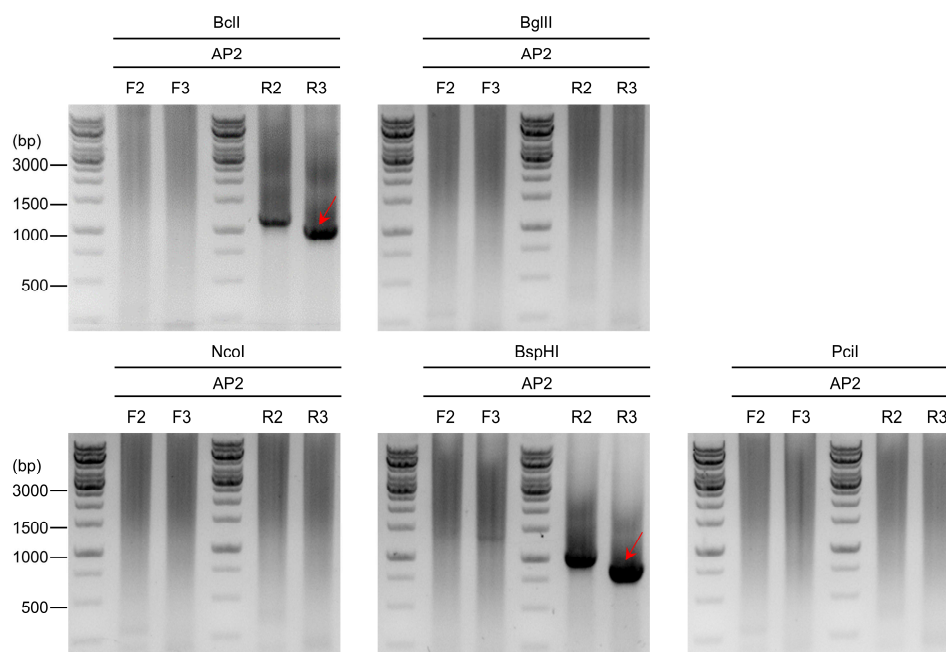
Danio rerio connexin 43, mRNA (cDNA clone MGC:55225 IMAGE:5915503), complete cds
Sequence ID: [BC049297.1](#) Length: 2821 Number of Matches: 1

Range 1: 1395 to 1675 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
520 bits(281)	1e-142	281/281(100%)	0/281(0%)	Plus/Plus
Query 1	GCAGAGCTAACATGCCCTAACTACGACAGTCAAGGGGCTGTCCGGGACGAACGCA	60		
Sbjct 1395	GCAGAGCTAACATGCCCTAACTACGACAGTCAAGGGGCTGTCCGGGACGAACGCA	1454		
Query 61	CTGAACCTGGCAGCTCTCTCAACTCAGCCACCAAGACAGACCTTGGGATGTGATTGAT	120		
Sbjct 1455	CTGAACCTGGCAGCTCTCTCAACTCAGCCACCAAGACAGACCTTGGGATGTGATTGAT	1514		
Query 121	TTTGTGTGTGCTTGTGAAATGCCACACAAATGATTCACATTAAACACTTGCACCTCTAC	180		
Sbjct 1515	TTTGTGTGTGCTTGTGAAATGCCACACAAATGATTCACATTAAACACTTGCACCTCTAC	1574		
Query 181	AGTTGTGTAGATTGTGTCTAAccccccTACAGTCGATCCGGTTATAACGTCAATC	240		
Sbjct 1575	AGTTGTGTAGATTGTGTCTAAccccccTACAGTCGATCCGGTTATAACGTCAATC	1634		
Query 241	GTTCGATGATCTCAATCCACAGGGGTGTTCAAGTCATG	281		
Sbjct 1635	GTTCGATGATCTCAATCCACAGGGGTGTTCAAGTCATG	1675		

379

f Nested PCR for cx43.4



Sequence blast of BclI-R3

Danio rerio connexin 43.4, mRNA (cDNA clone MGC:191652 IMAGE:100059961), complete cds
Sequence ID: [BC164477.1](#) Length: 1210 Number of Matches: 1

Range 1: 385 to 684 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
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Sbjct 445	TATGATTATGGAAGAGATGCTGCTGAGAAGAAAGGCTCCAGAGAGTCTGCTGTAA	504		
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Sbjct 505	ACATGACGGCCGGGAGAAATAAGCGAGATGGCTCATGAGGTGTACATCTGCAGCT	564		
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Query 241	CGAGGTGCCCCGTCATACGTGTGCACTGCGAGTCCCTGCCCGACACCGTAGAGTCT	300		
Sbjct 625	CGAGGTGCCCCGTCATACGTGTGCACTGCGAGTCCCTGCCCGACACCGTAGAGTCT	684		

Sequence blast of BspHI-R3

Danio rerio connexin 43.4, mRNA (cDNA clone MGC:191652 IMAGE:100059961), complete cds
Sequence ID: [BC164477.1](#) Length: 1210 Number of Matches: 1

Range 1: 541 to 840 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

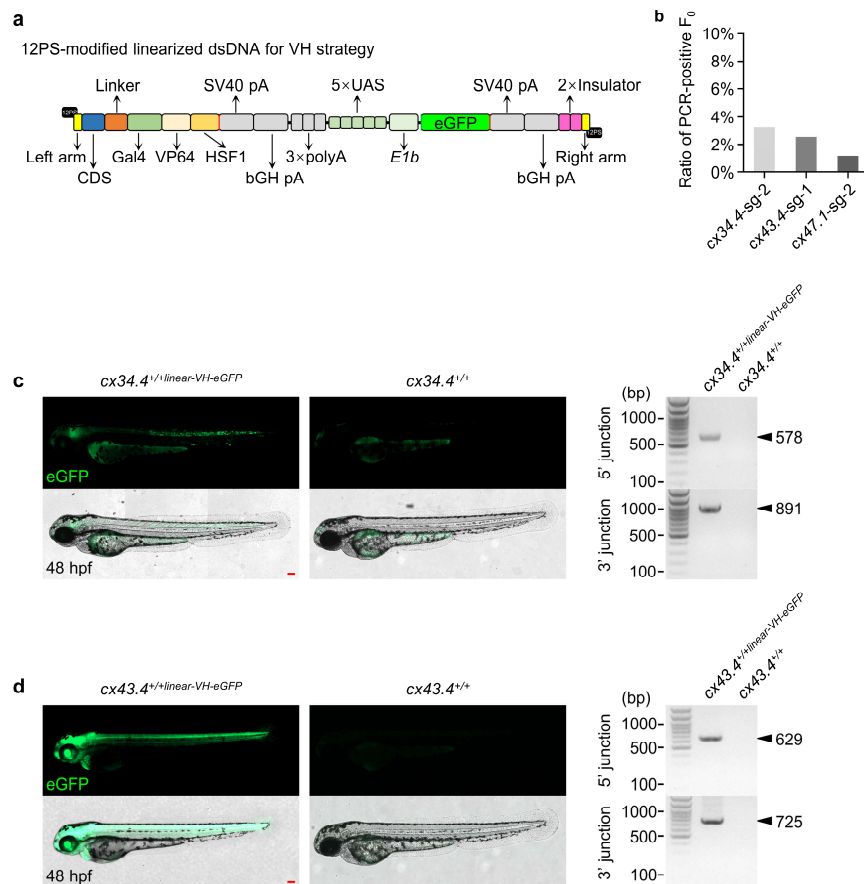
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Sbjct 601	TGGCCAGTATATCTCTGTATGTTTTCAGGTGCCCCGTCATACGTGTGCACTGCGAGTCC	660		
Query 121	CTGCCCGCACACGAGACTGTTTGTGTGAGTGTGAGAGAAACCATCTTTCTGCT	180		
Sbjct 661	CTGCCCGCACACGAGACTGTTTGTGTGAGTGTGAGAGAAACCATCTTTCTGCT	720		
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Sbjct 721	GATTATGTATGCGGTGAGCTGTCTGCTTGTCTTACGGTGTGAGATTCTTCATT	780		
Query 241	GGGCTCAGCGGAATTCTGTATGCTTTTCAGCAGCTGCACGCCATCAAGTGTTCAGCG	300		
Sbjct 781	GGGCTCAGCGGAATTCTGTATGCTTTTCAGCAGCTGCACGCCATCAAGTGTTCAGCG	840		

Supplementary Fig. 26. Examination of random integration by the Cyclic Digestion and Ligation-Mediated PCR (CDL-PCR) method.

(a and b) Preparation of the adaptors. Pre-adaptors were amplified by PCR from the pMD18T-APS plasmid. Purified pre-adaptors were enzymatically digested by *Sau3A*I and *Fat*I, respectively. "C", the undigested pre-adaptor

control. **(c and d)** Enzymatic digestion of the genomic DNA. Genomic DNA of *cx43.4^{+linear-eGFP/+linear-eGFP}* or *cx43^{+linear-eGFP/+linear-eGFP}* zebrafish was extracted from the caudal fins using the Genomic DNA Clean & Concentrator-10 Kit, and then was enzymatically digested by BclI, BglII, NcoI, BspHI, and PciI, respectively. “C”, the undigested genomic DNA control. **(e and f)** Integration was detected by nested PCR and confirmed by sequencing. BclI- or BglII-digested genomic DNA was ligated with the Sau3AI-digested adaptor by T4 DNA ligase. NcoI-, BspHI-, or PciI-digested genomic DNA was ligated with the FatI-digested adaptor by T4 DNA ligase. Nested PCR was performed to detect integration in the ligated genomic DNA. “AP2” was the primer of the adaptor, “F2”, “F3”, “R2”, and “R3” were primers of the genomic DNA. Amplified PCR products were then subjected to sequencing analysis.

Supplementary Figure 27

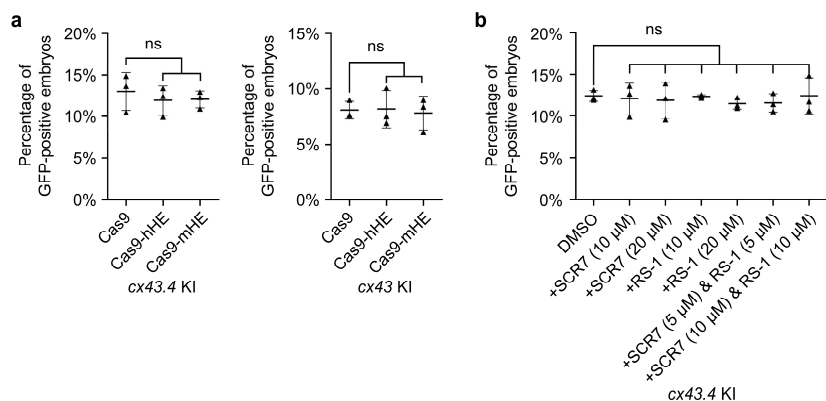


Supplementary Fig. 27. A combination of the 5'-modified linearized dsDNA with the VH-mediated KI strategy.

(a) A schematic diagram of a 12PS-modified linearized dsDNA for the VH strategy. The 12PS-modification was added to the 5'-terminus of the microhomologous arms. **(b)** Ratios of the PCR-positive F_0 after KI using the 12PS-modified linearized dsDNA-mediated VH strategy for some *connexins*. One high-efficiency sgRNA was used for each *connexin*. Genomic DNA was isolated from the caudal fins of one-month-old F_0 and used for 5'-junction PCR to identify PCR-positive F_0 . At least 800 F_0 were tested for each gene. The ratio of PCR-positive F_0 in all tested F_0 zebrafish was then calculated. **(c and d)** Images of F_1 larvae for *cx34.4* or *cx43.4* KI generated by the 12PS-modified linearized dsDNA-mediated VH strategy. F_1 embryos were identified by 5'- and 3'-junction PCR analysis and were representatives of 10 PCR-positive F_1 .

414 Images were taken at 48 hpf. Scale bars, 100 μ m.
415

Supplementary Figure 28



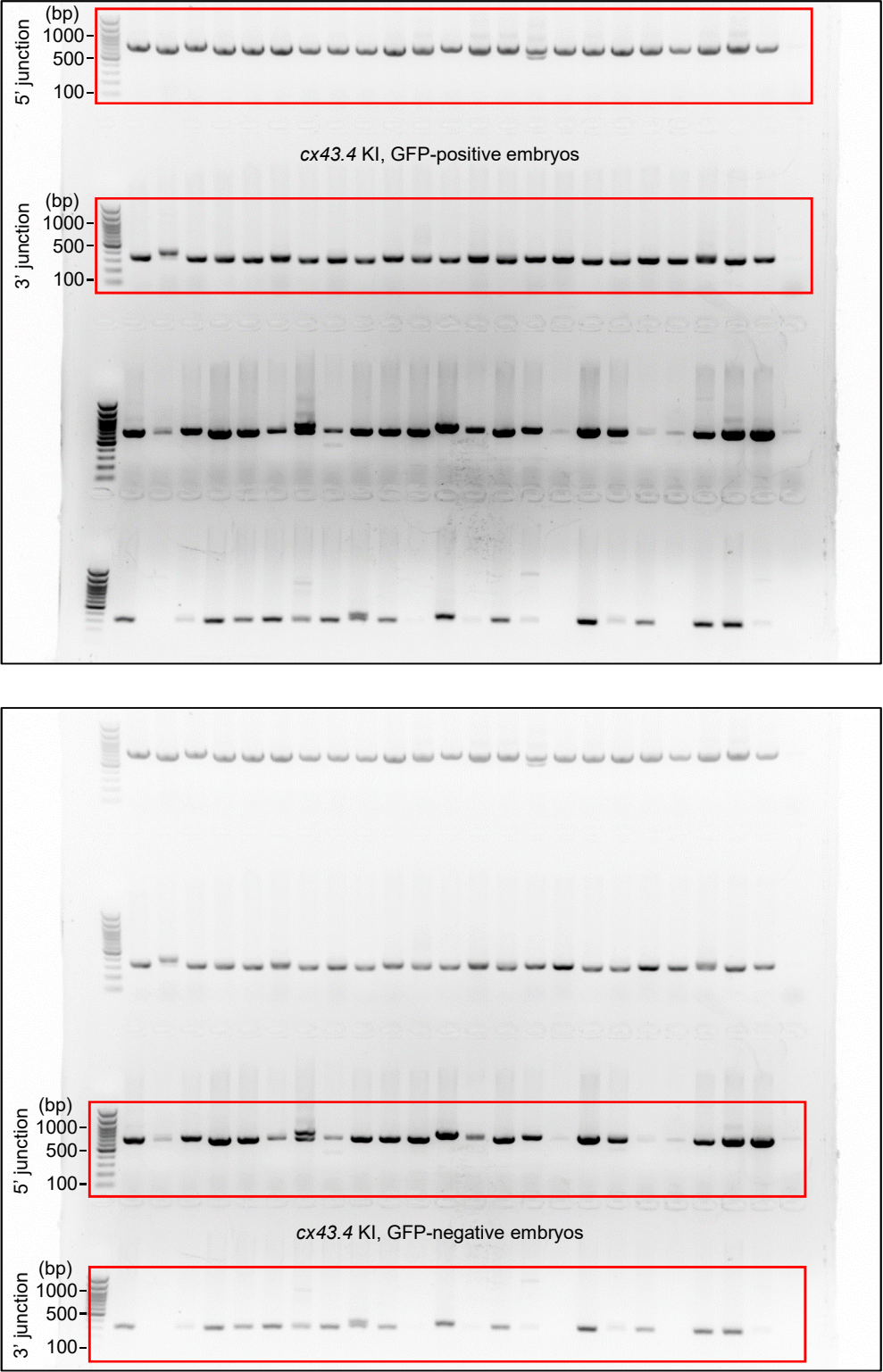
Supplementary Fig. 28. Effects of CtIP, SCR7, and RS-1 on KI efficiency.

(a) Percentages of GFP-positive embryos obtained by using Cas9, Cas9-hHE, or Cas9-mHE to perform KI for *cx43.4* or *cx43*. Cas9, Cas9-hHE, or Cas9-mHE mRNA was co-injected with the donor plasmid, sgRNA for *cx43* or *cx43.4*, and *lamGolden* sgRNA into at least 200 WT embryos at the one-cell stage. GFP-positive embryos were counted at 48 hpf. Data represent mean $\pm$ SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. ns, not significant. **(b)** Percentages of GFP-positive embryos obtained by treatment of SCR7 or RS-1 after microinjection of Cas9 mRNA, donor plasmid, sgRNA for *cx43.4*, and *lamGolden* sgRNA into at least 200 WT embryos at the one-cell stage. SCR7, RS-1, or SCR7 & RS-1 were used at different concentrations for 6 hours. DMSO was used as the vehicle control. GFP-positive embryos were counted at 48 hpf. Data represent mean $\pm$ SD of 3 independent experiments. *P* values were calculated using an unpaired Student *t* test. ns, not significant.

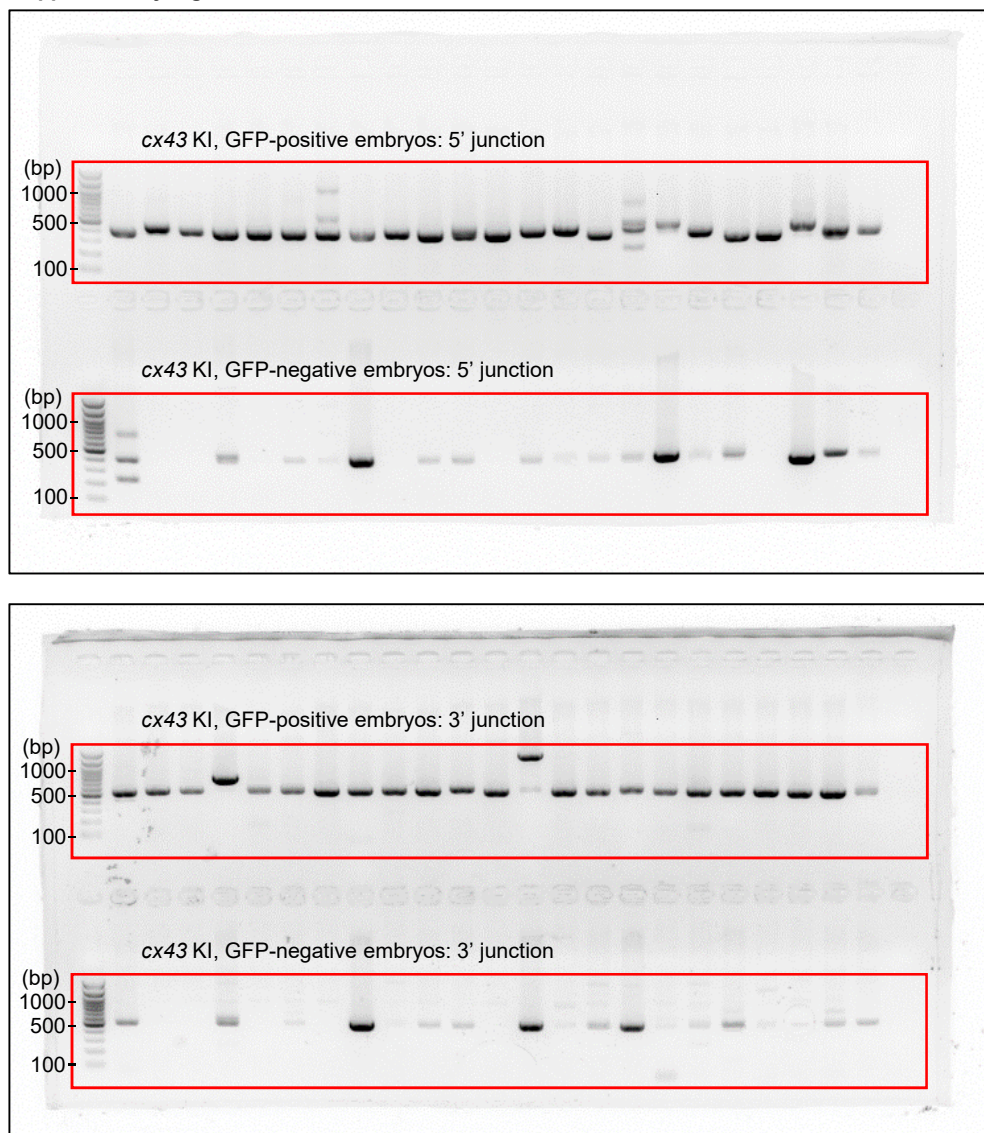
Supplementary Fig. 29. Uncropped agarose gels are shown below.

Including Supplementary Fig. 3b-c, 4, 6c, 7a, 7c, 9a-h, 10b, 11b, 12a-b, 13, 15b, 20, 21, 24, 25b, 25d, 26a-f, 27c.

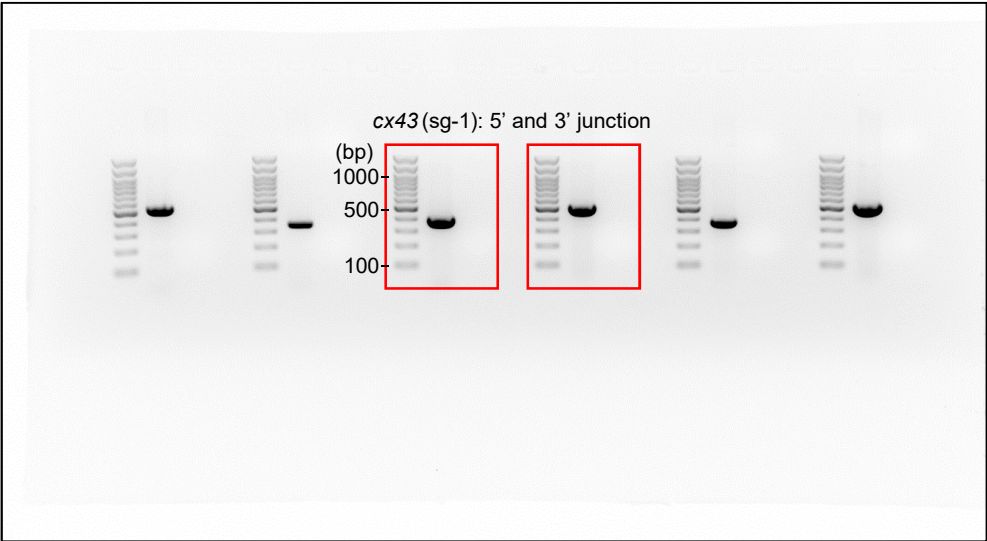
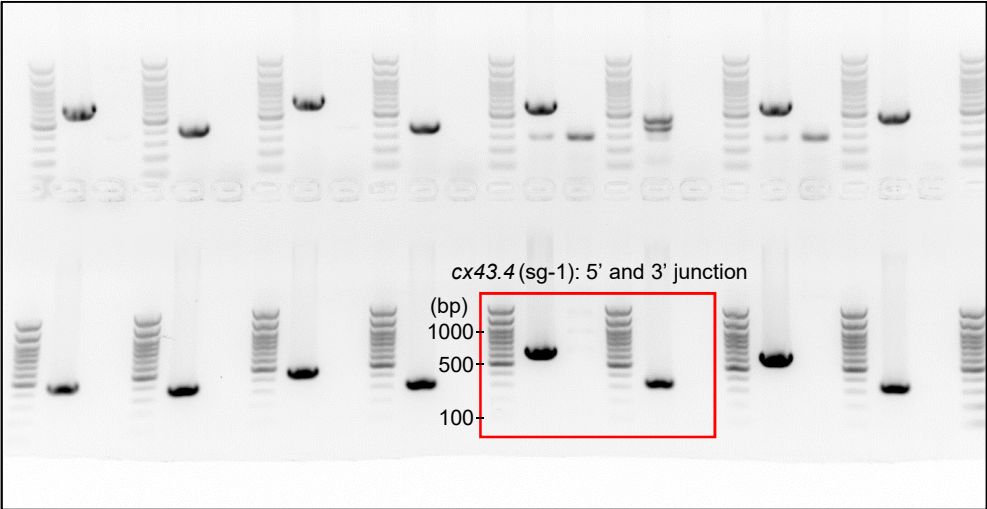
Supplementary Fig. 3b



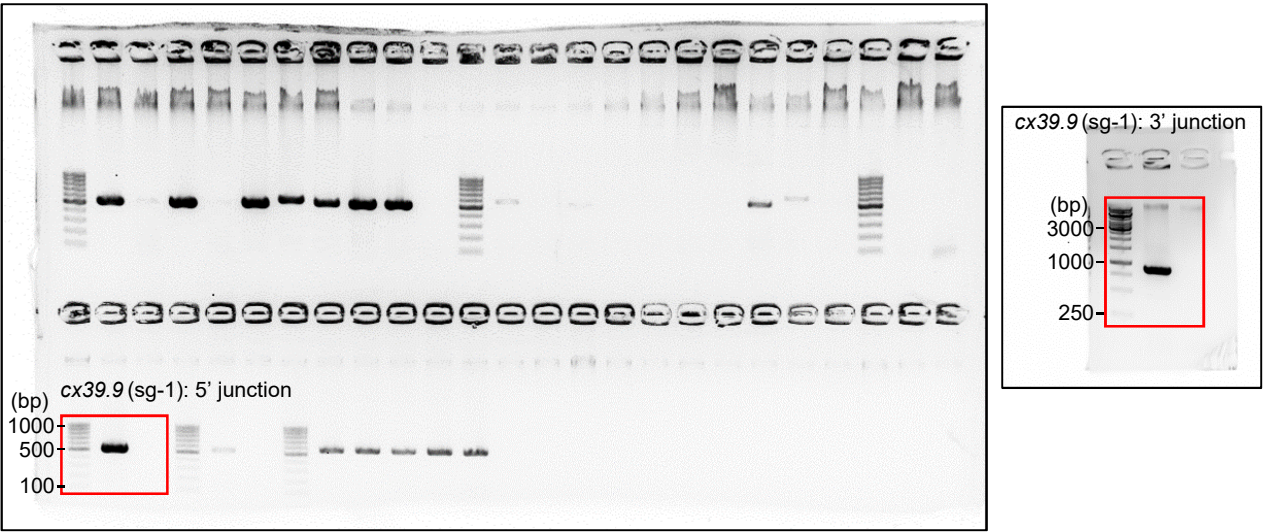
Supplementary Fig. 3c



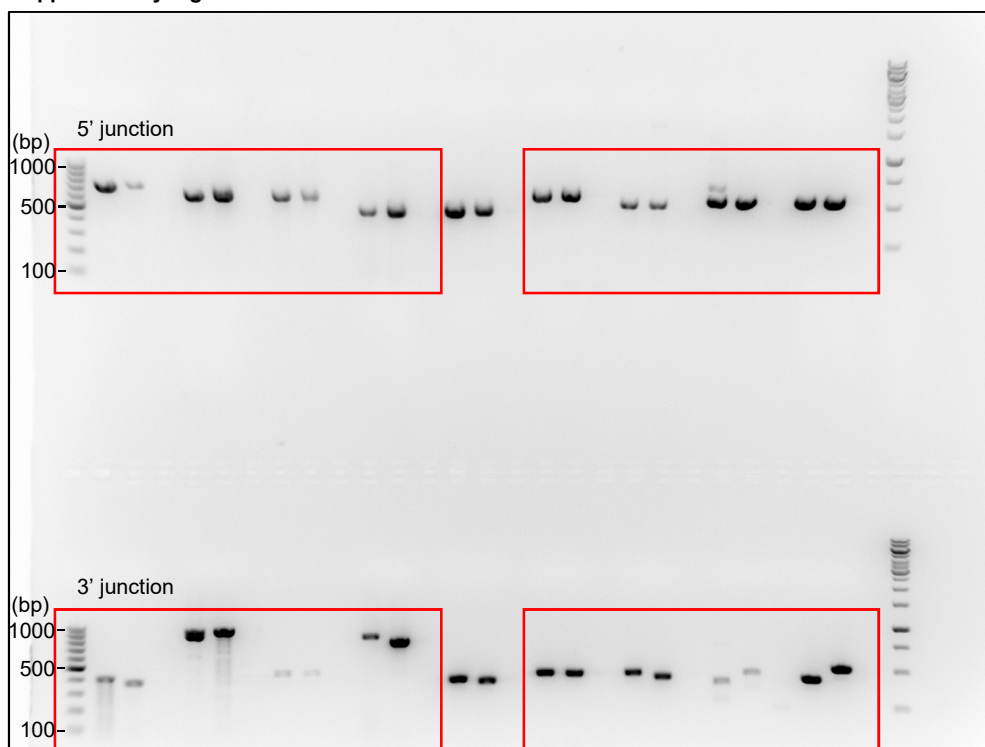
Supplementary Fig. 4



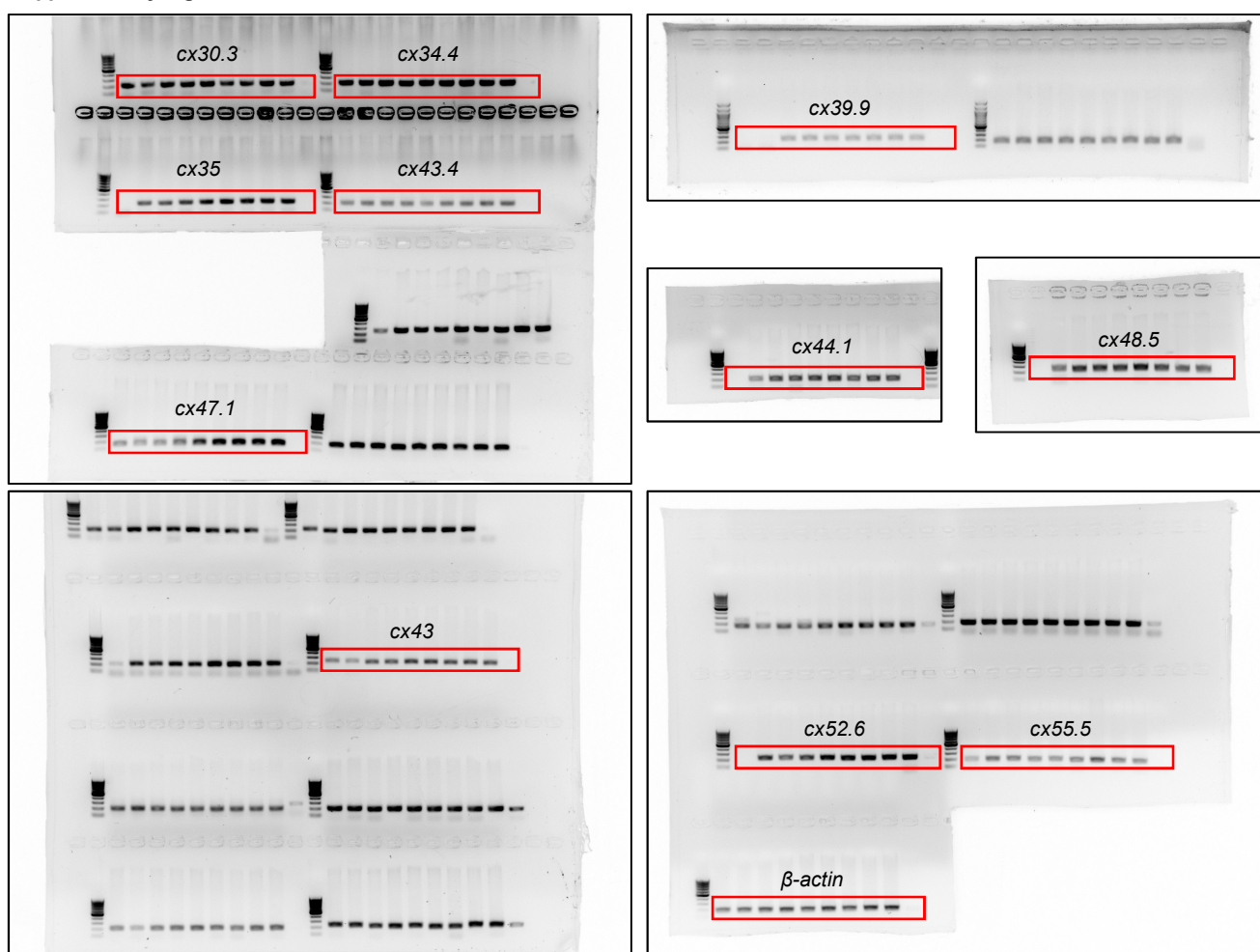
Supplementary Fig. 6c



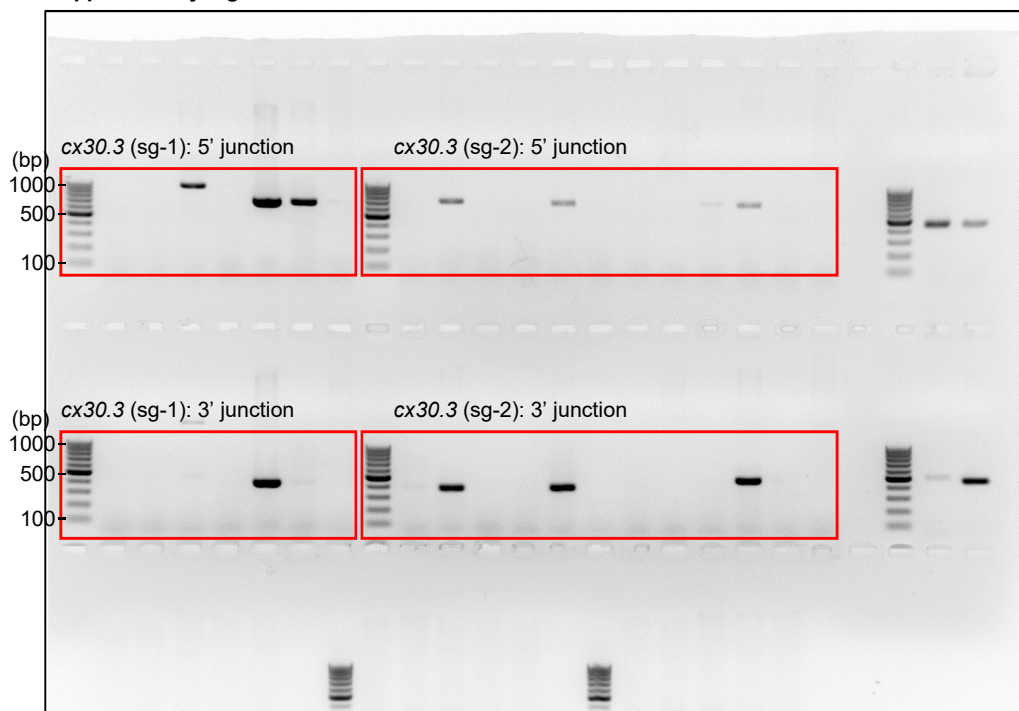
Supplementary Fig. 7a



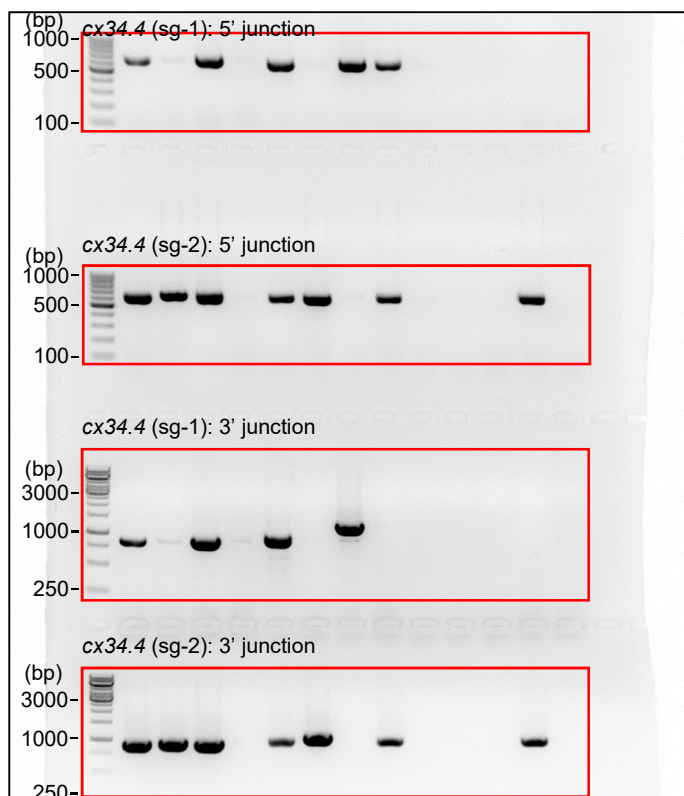
Supplementary Fig. 7c



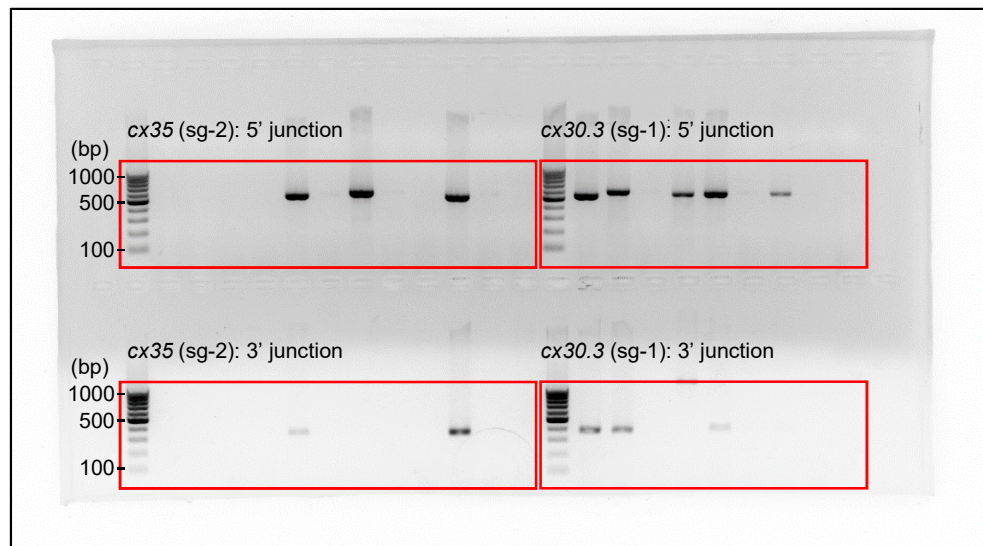
Supplementary Fig. 9a



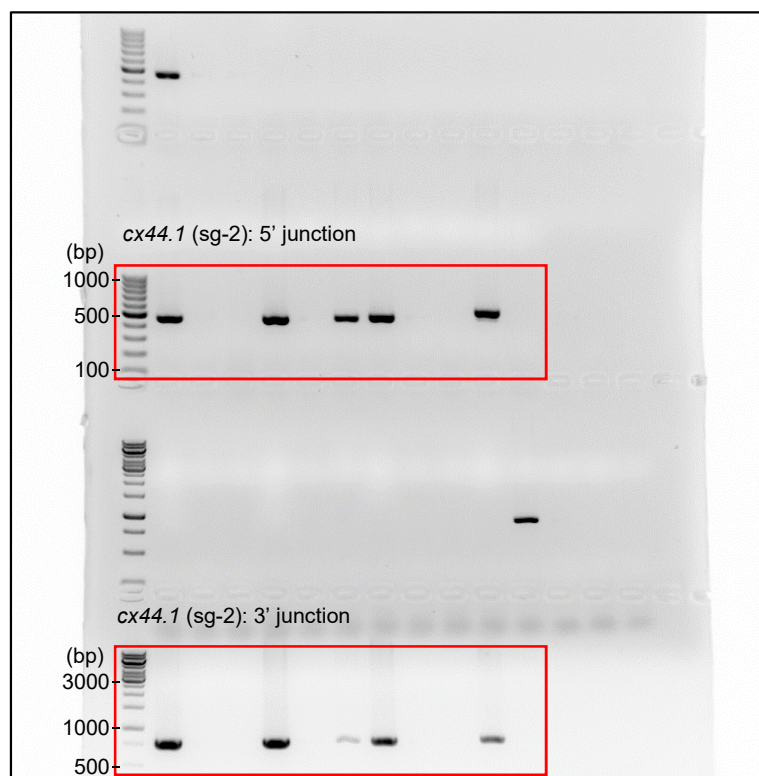
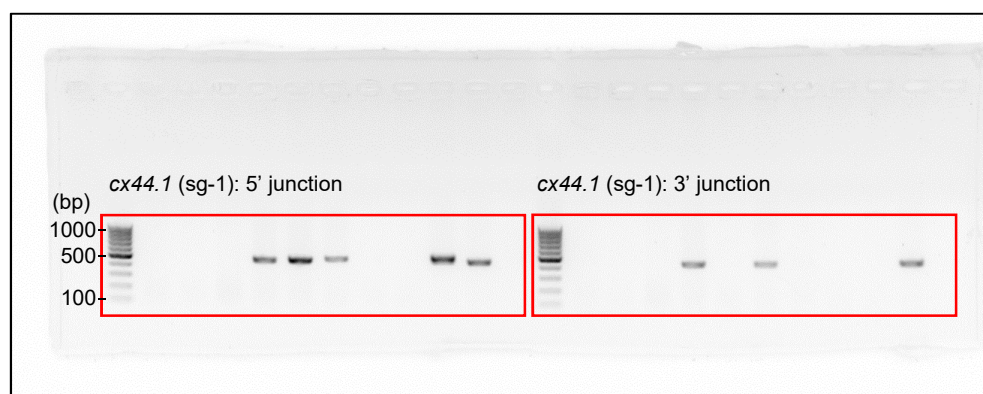
Supplementary Fig. 9b



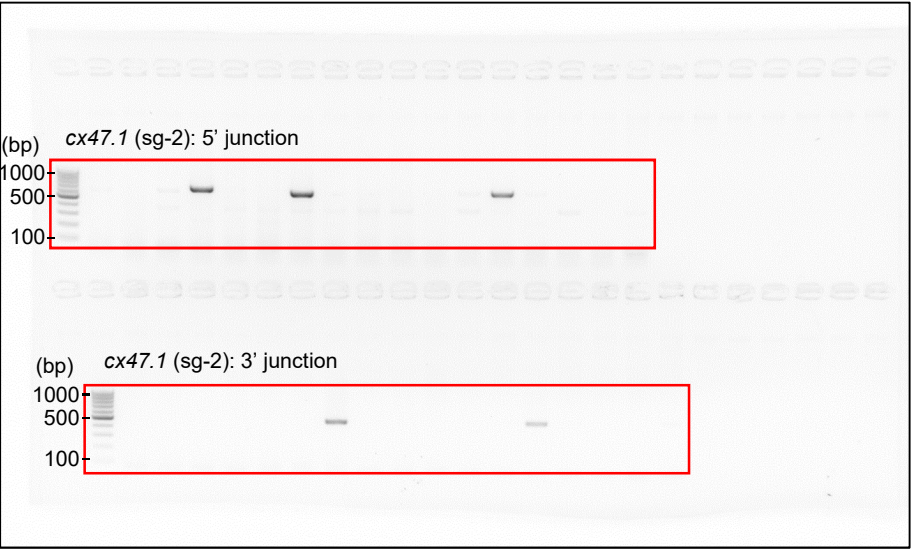
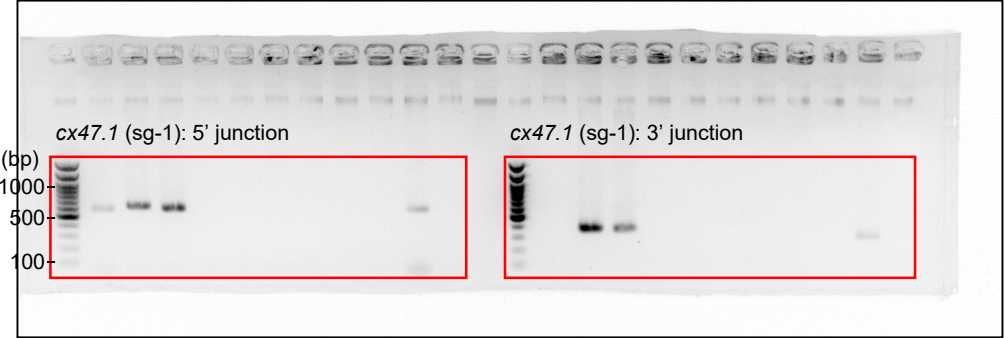
Supplementary Fig. 9c



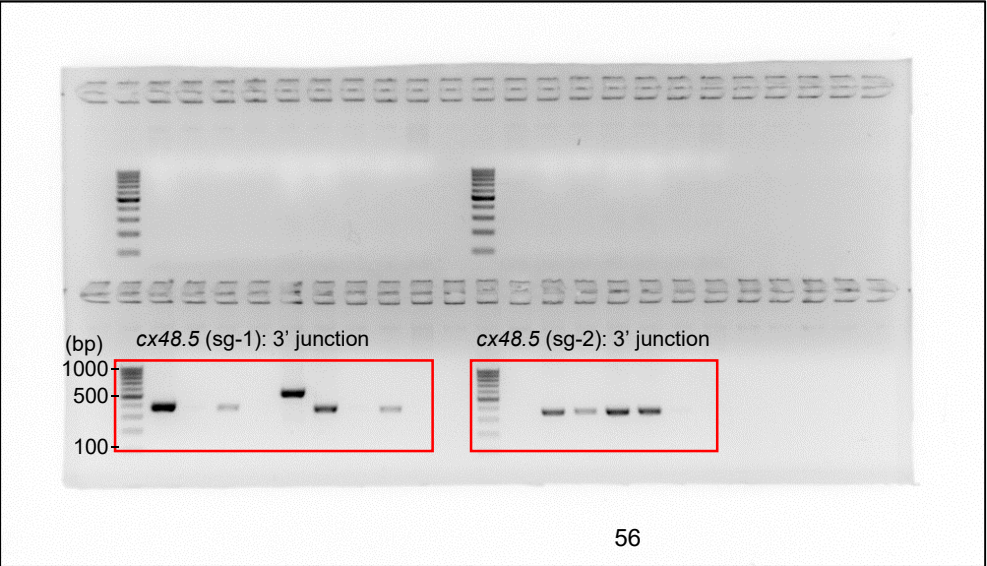
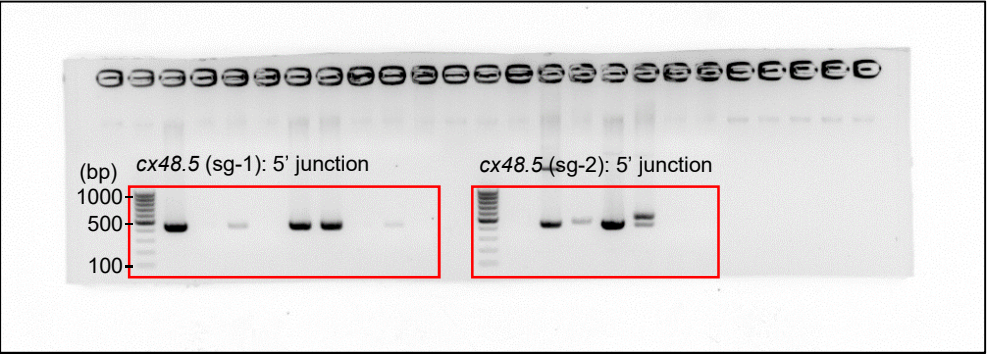
Supplementary Fig. 9d



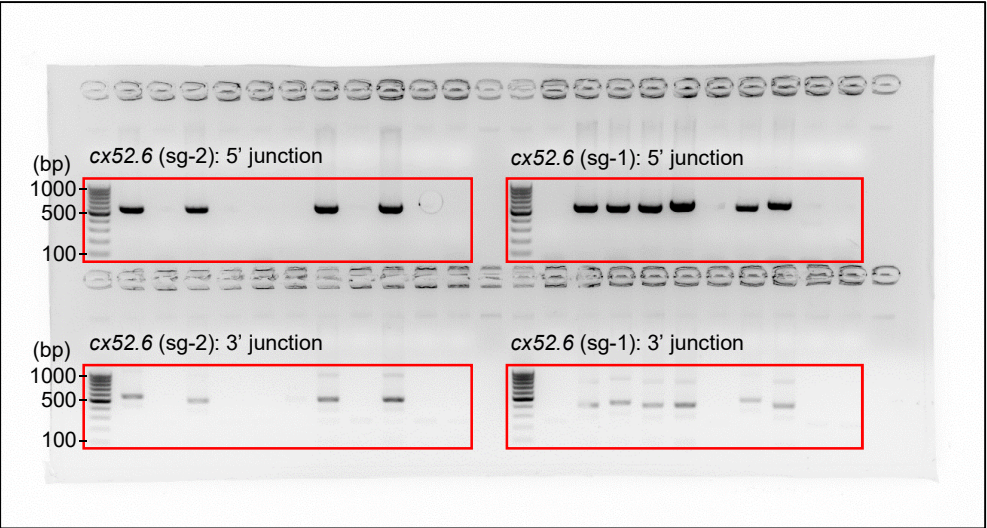
Supplementary Fig. 9e



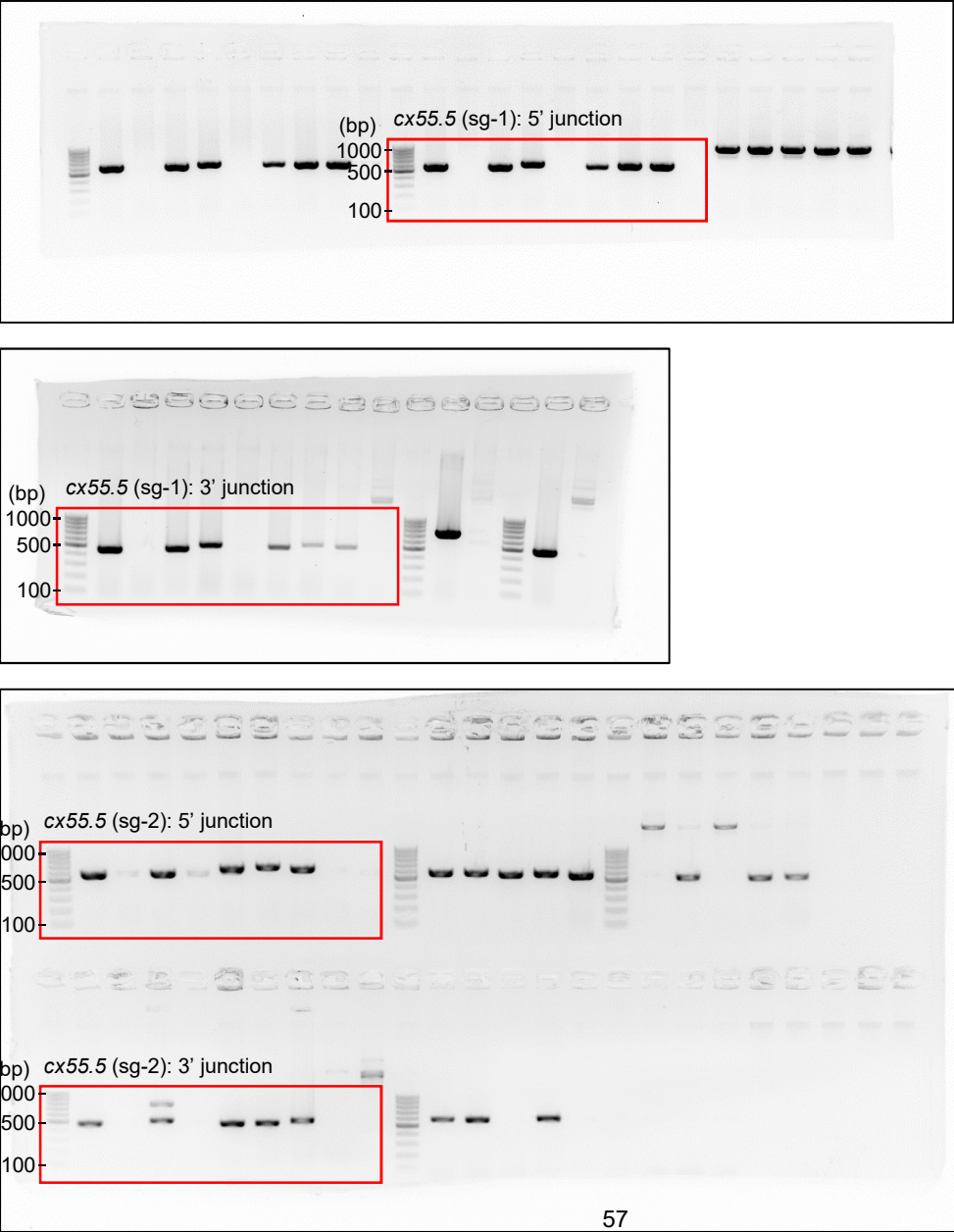
Supplementary Fig. 9f



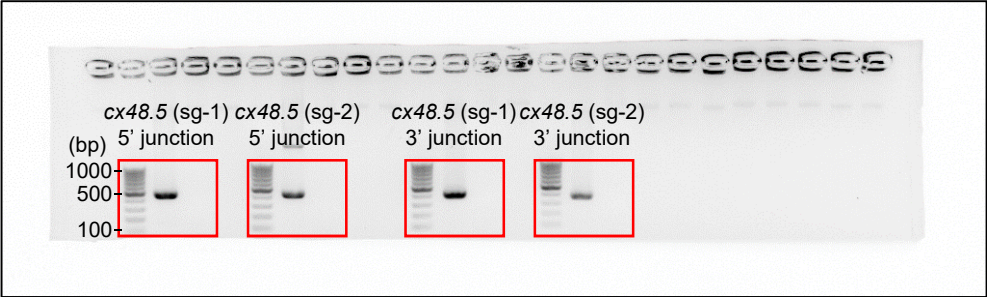
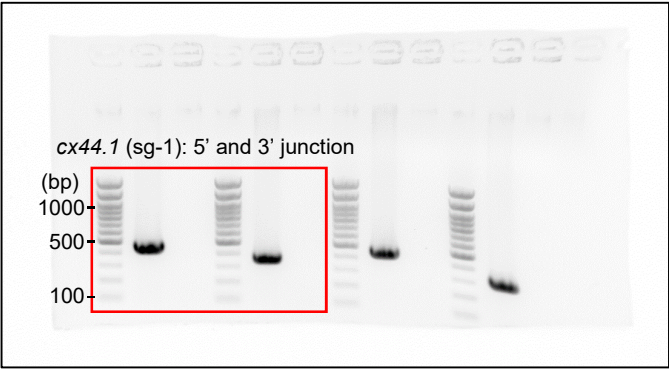
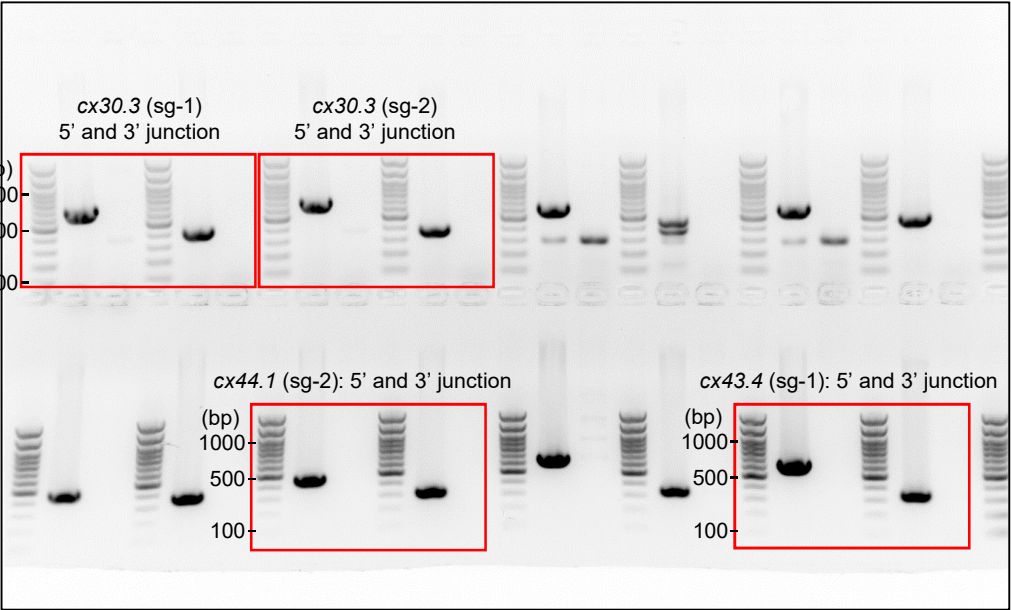
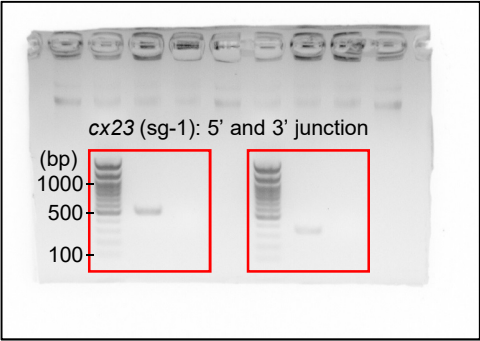
Supplementary Fig. 9g



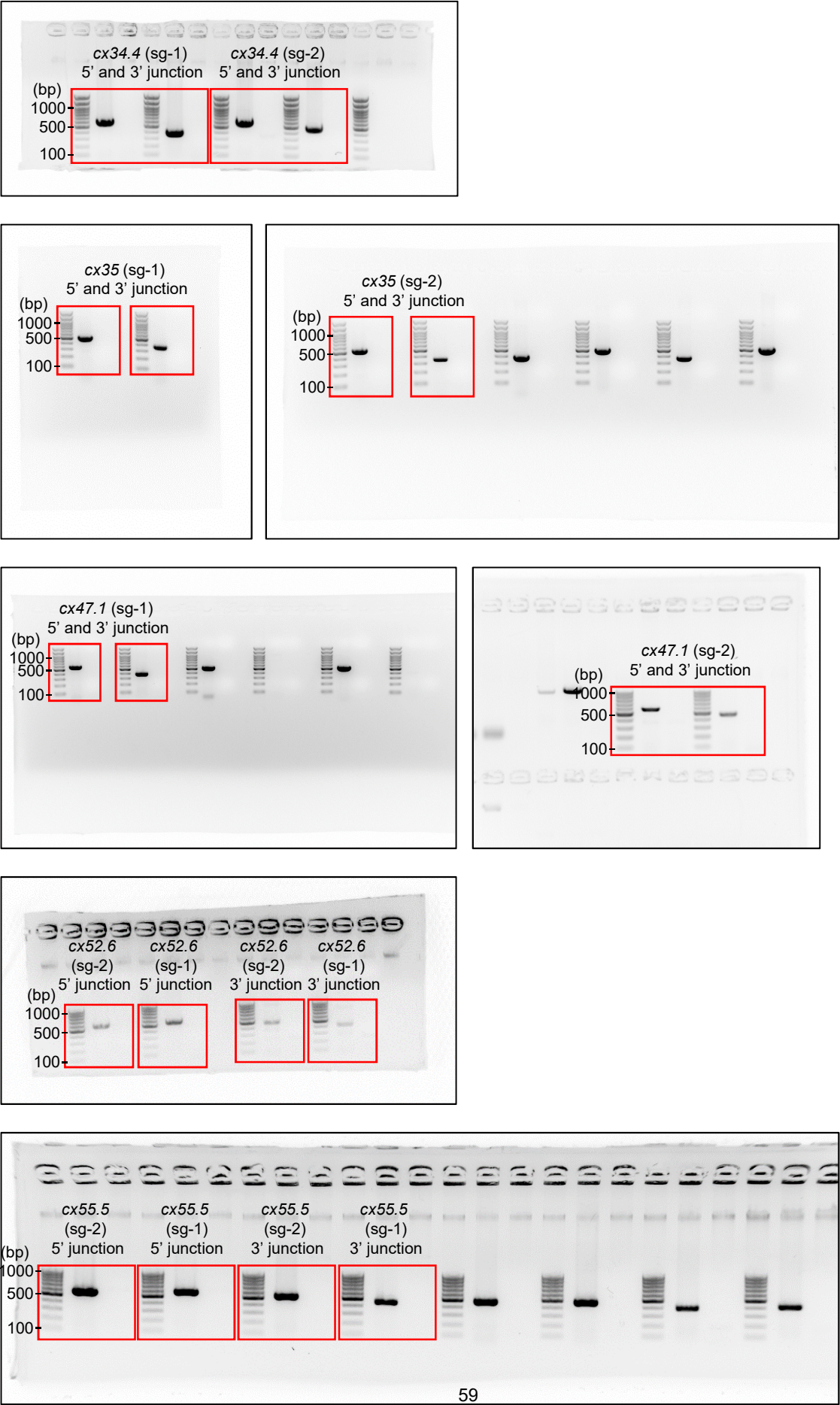
Supplementary Fig. 9h



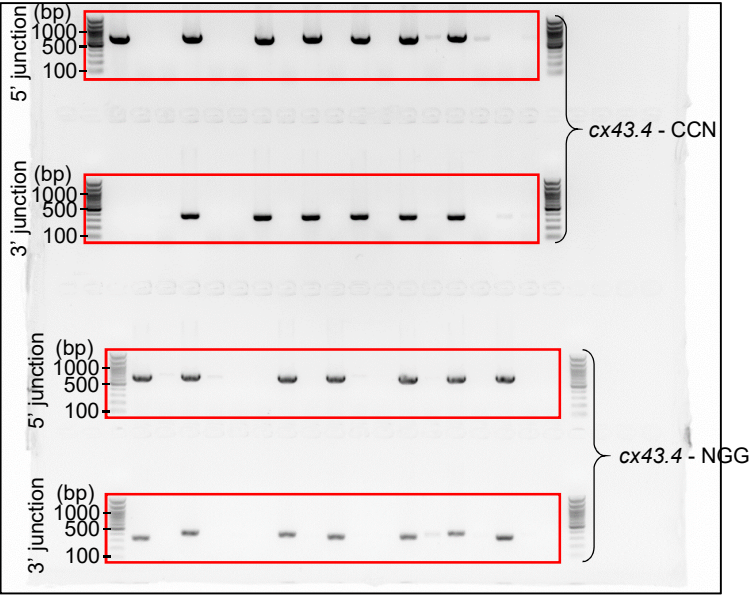
Supplementary Fig. 10b



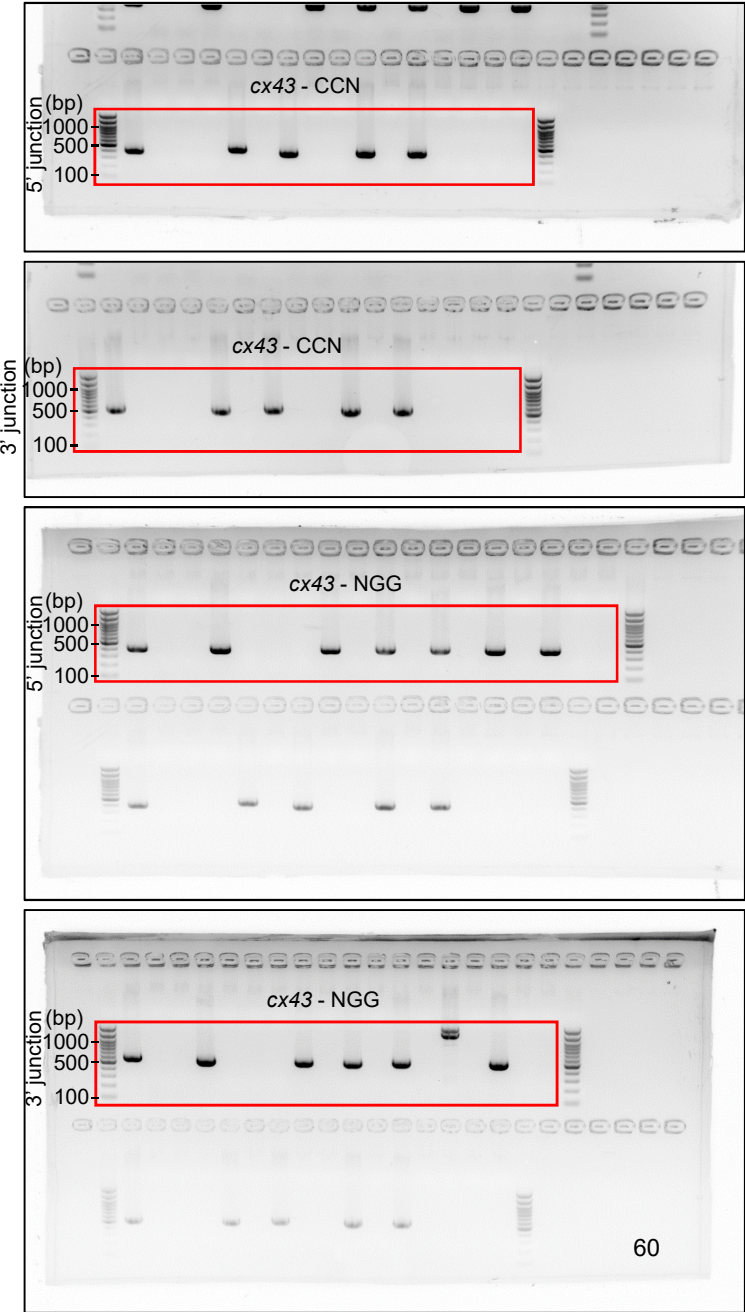
Supplementary Fig. 11b



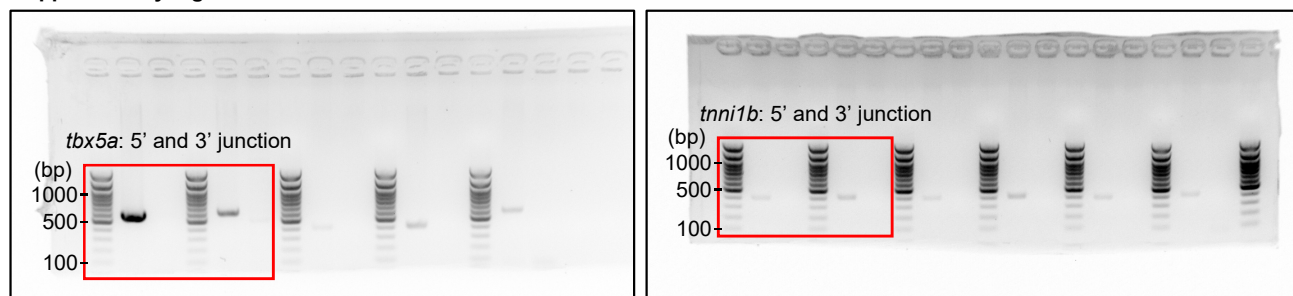
Supplementary Fig. 12a



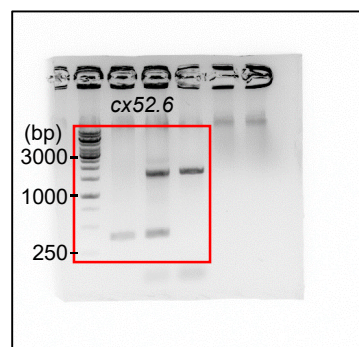
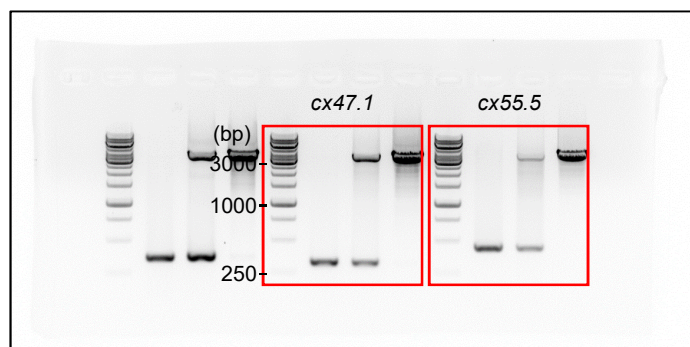
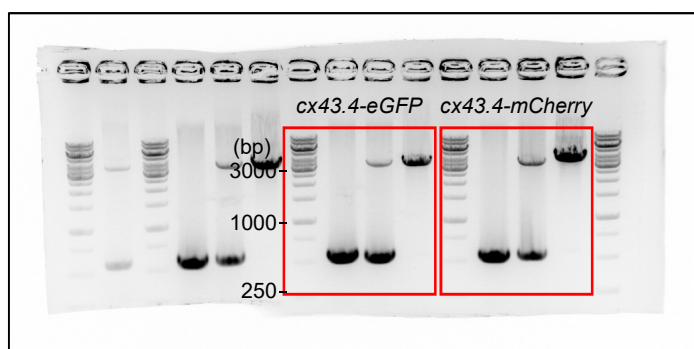
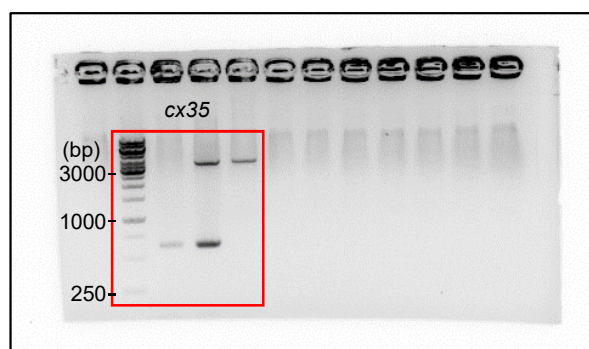
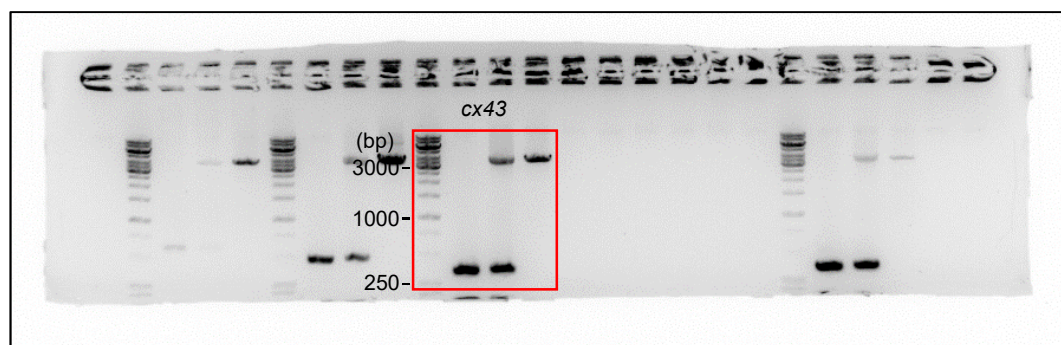
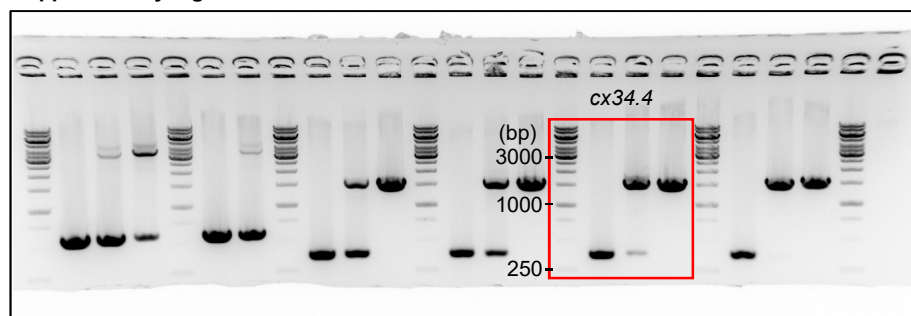
Supplementary Fig. 12b



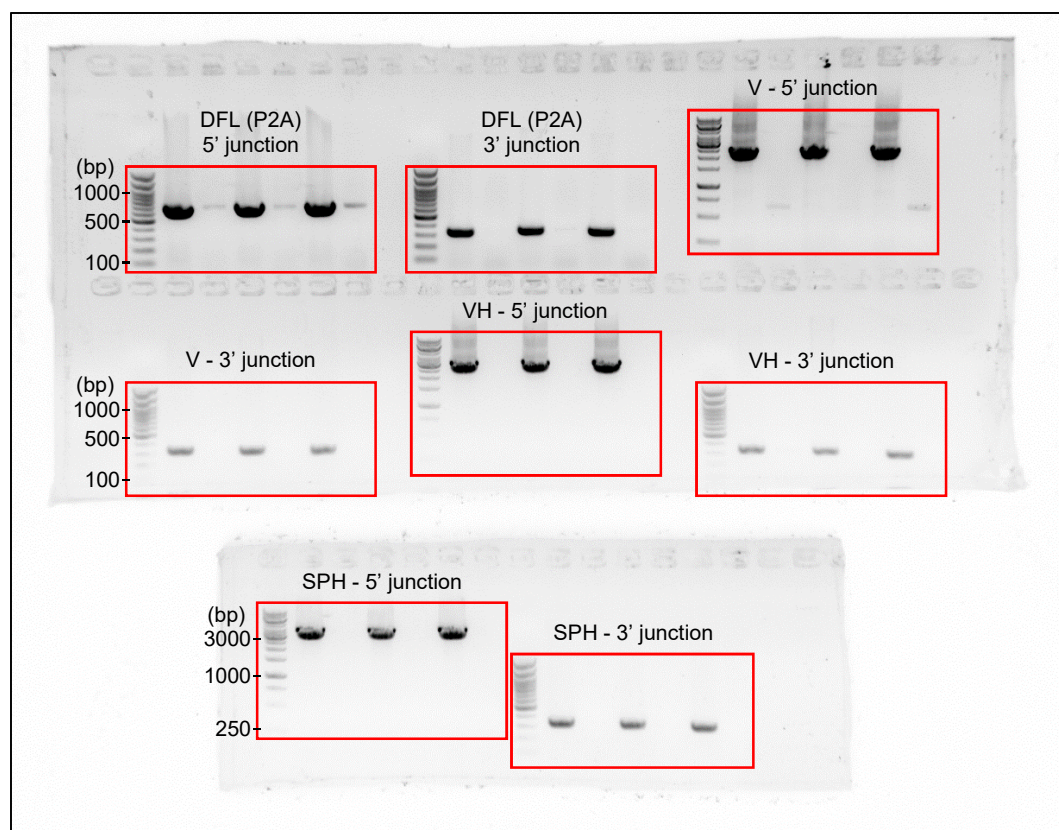
Supplementary Fig. 13



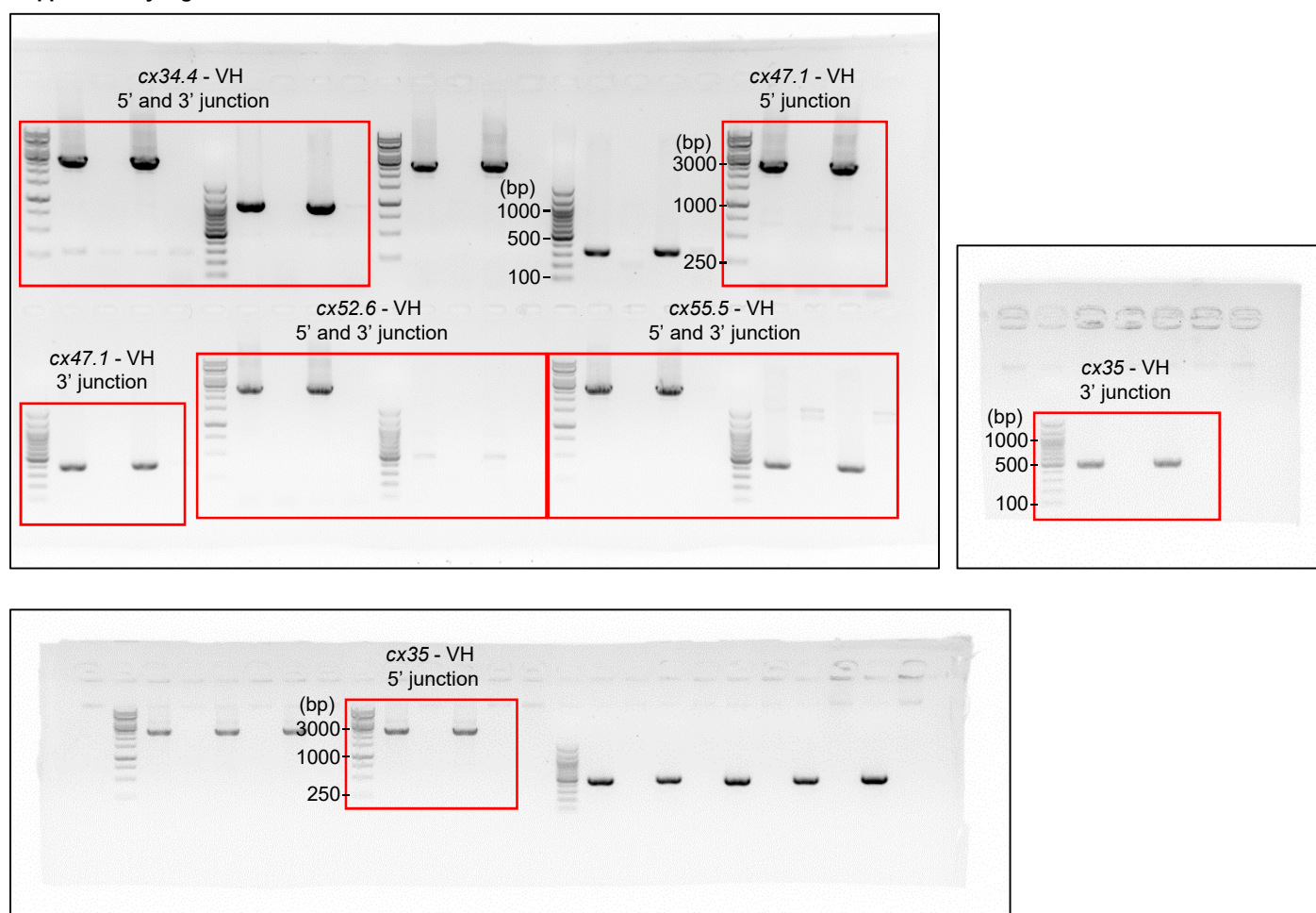
Supplementary Fig. 15b



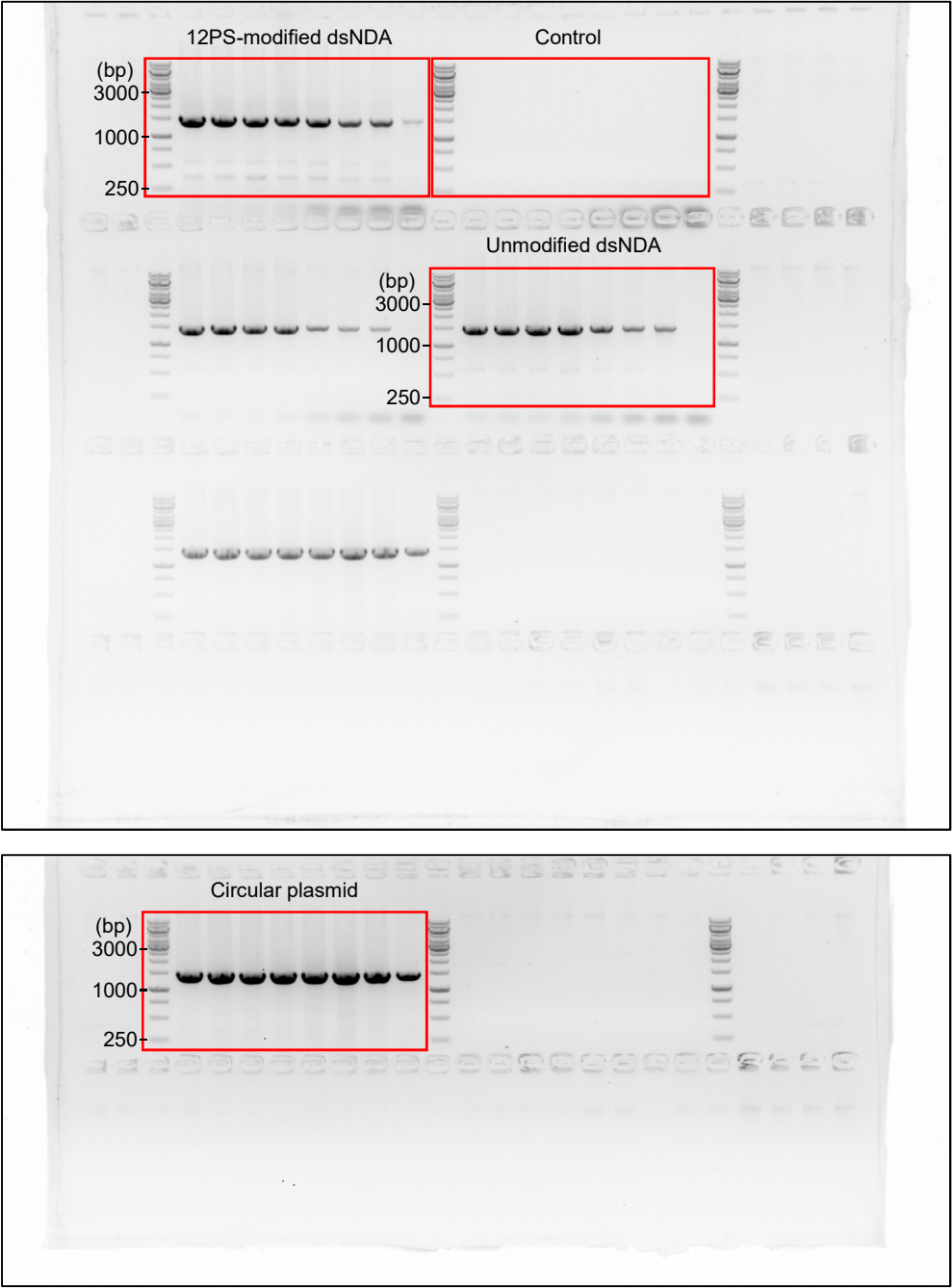
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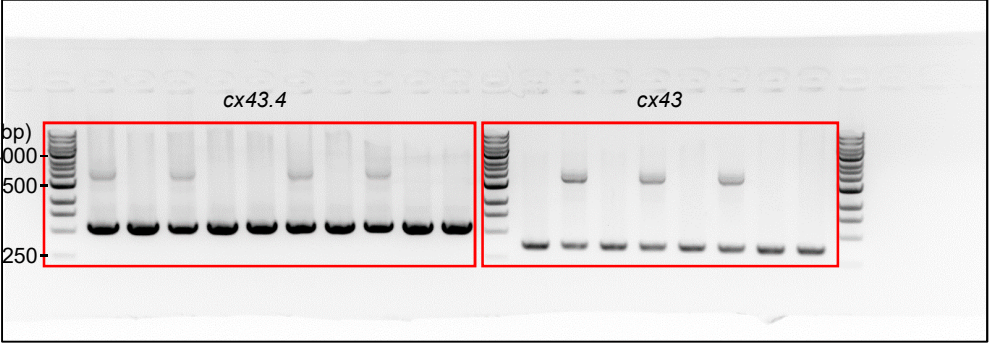
Supplementary Fig. 21



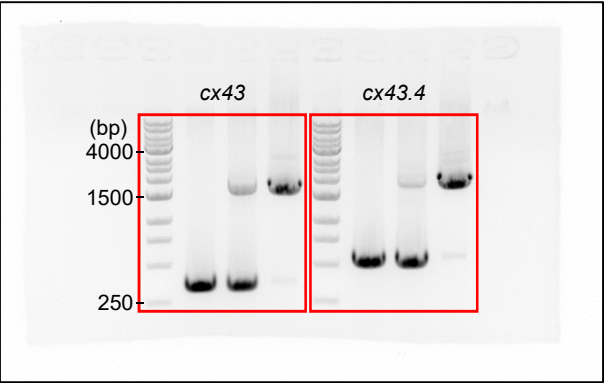
Supplementary Fig. 24



Supplementary Fig. 25b



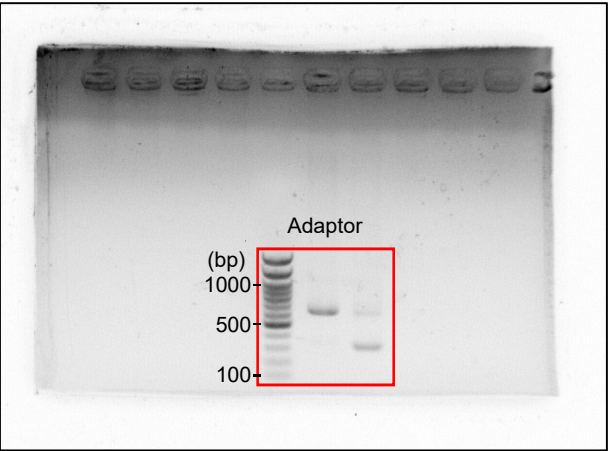
Supplementary Fig. 25d



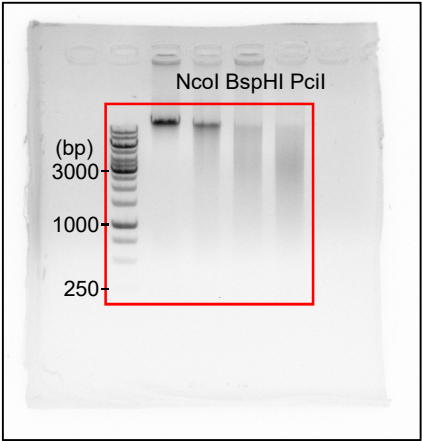
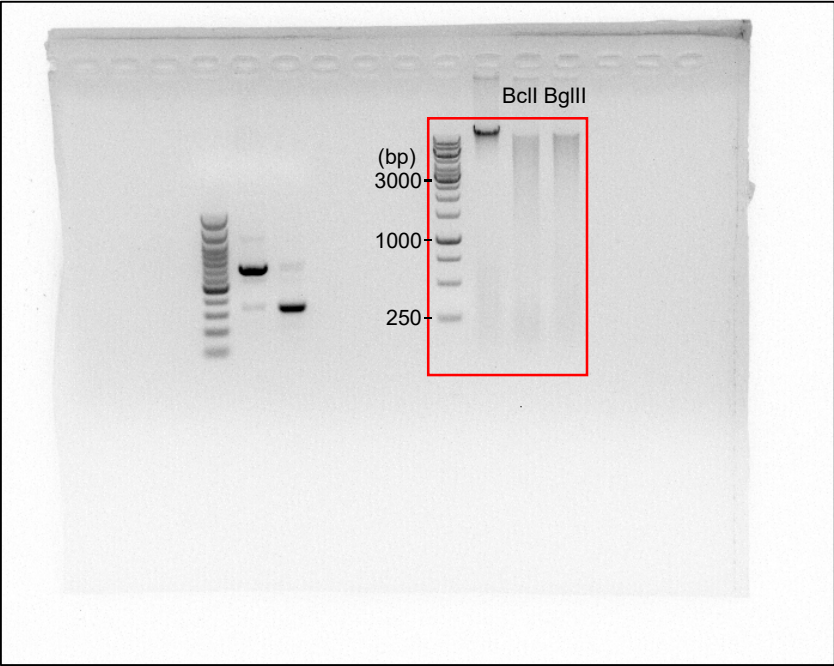
Supplementary Fig. 26a



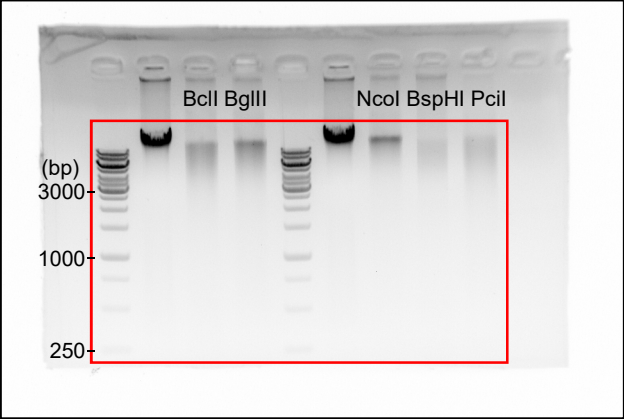
Supplementary Fig. 26b



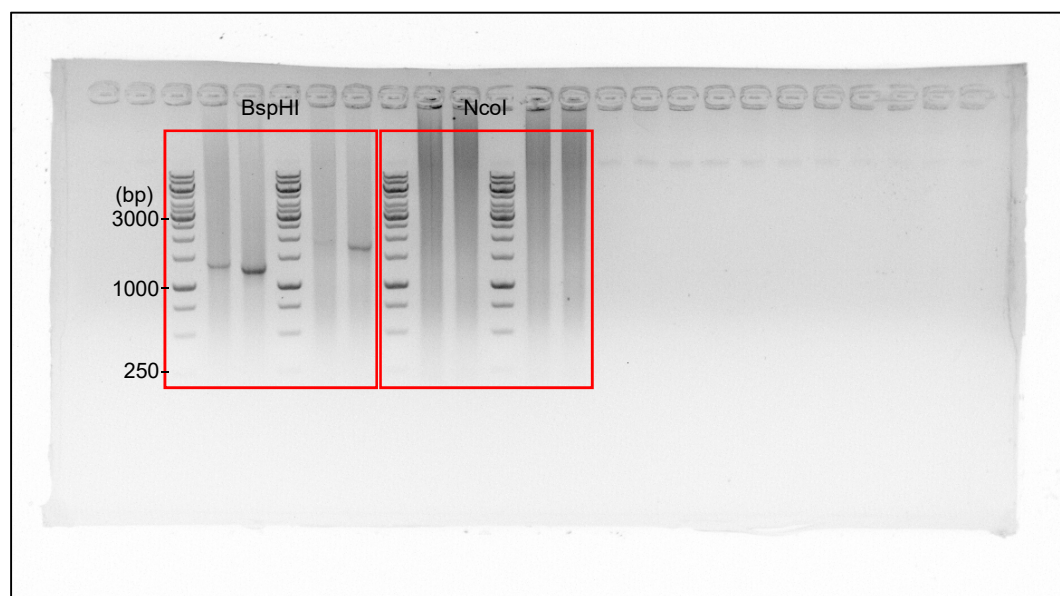
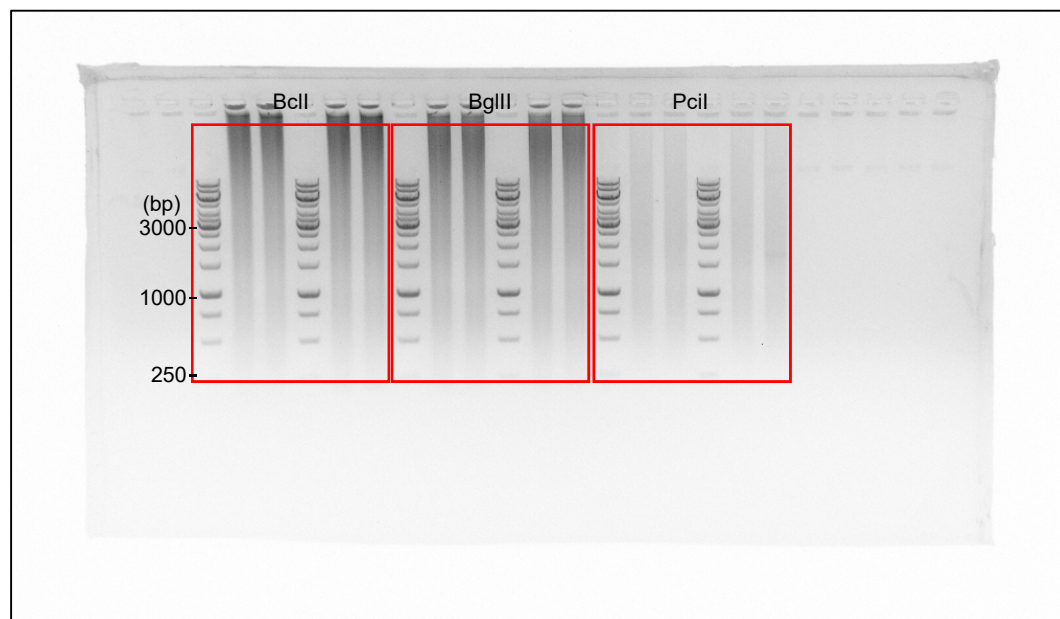
Supplementary Fig. 26c



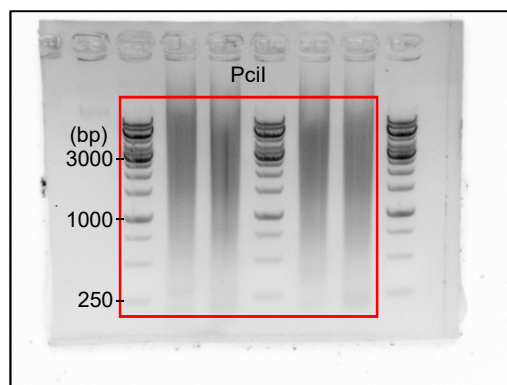
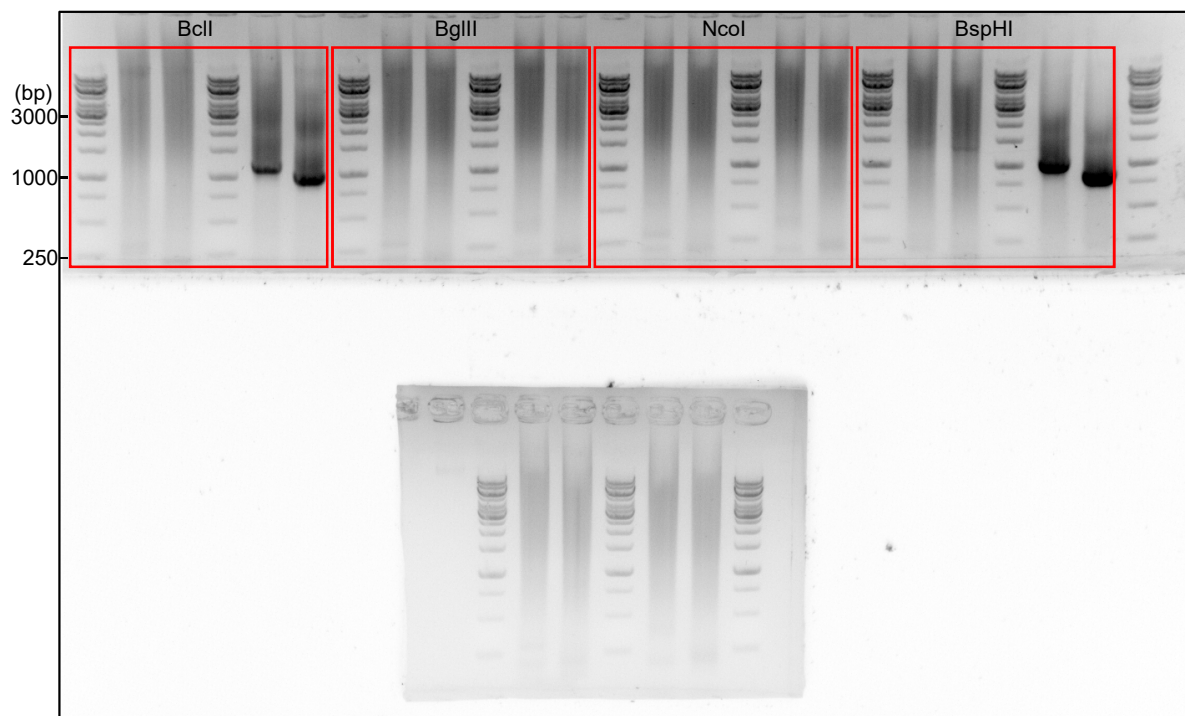
Supplementary Fig. 26d



Supplementary Fig. 26e



Supplementary Fig. 26f



Supplementary Fig. 27c

