

## RESEARCH ARTICLE

## TECHNIQUES AND RESOURCES

# Efficient RNA/Cas9-mediated genome editing in *Xenopus tropicalis*

Xiaogang Guo<sup>1,2,\*</sup>, Tiejun Zhang<sup>3,\*</sup>, Zheng Hu<sup>1,2,4,\*</sup>, Yanqi Zhang<sup>5</sup>, Zhaoying Shi<sup>1,2</sup>, Qinhu Wang<sup>6</sup>, Yan Cui<sup>1,2,4</sup>, Fengqin Wang<sup>1,2,7</sup>, Hui Zhao<sup>8</sup> and Yonglong Chen<sup>1,2,‡</sup>

**ABSTRACT**

For the emerging amphibian genetic model *Xenopus tropicalis* targeted gene disruption is dependent on zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), which require either complex design and selection or laborious construction. Thus, easy and efficient genome editing tools are still highly desirable for this species. Here, we report that RNA-guided Cas9 nuclease resulted in precise targeted gene disruption in all ten *X. tropicalis* genes that we analyzed, with efficiencies above 45% and readily up to 100%. Systematic point mutation analyses in two loci revealed that perfect matches between the spacer and the protospacer sequences proximal to the protospacer adjacent motif (PAM) were essential for Cas9 to cleave the target sites in the *X. tropicalis* genome. Further study showed that the Cas9 system could serve as an efficient tool for multiplexed genome engineering in *Xenopus* embryos. Analysis of the disruption of two genes, *ptf1a/p48* and *tyrosinase*, indicated that Cas9-mediated gene targeting can facilitate direct phenotypic assessment in *X. tropicalis* embryos. Finally, five founder frogs from targeting of either *elastase-T1*, *elastase-T2* or *tyrosinase* showed highly efficient transmission of targeted mutations into F1 embryos. Together, our data demonstrate that the Cas9 system is an easy, efficient and reliable tool for multiplex genome editing in *X. tropicalis*.

**KEY WORDS:** CRISPR, Cas9, *Xenopus tropicalis*, Genome editing

**INTRODUCTION**

Bacterial and archaeal clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) adaptive immune systems rely on small RNAs in complex with Cas proteins to silence foreign nucleic acids, including viruses and plasmids. There are three major types of CRISPR/Cas systems (Makarova et al., 2011; Wiedenheft et al., 2012). In the type II CRISPR system, the Cas9 protein forms a complex with two short non-coding RNAs, namely the spacer-containing RNA (crRNA) and the trans-activating

CRISPR RNA (tracrRNA), to selectively cleave the invading DNA. With the recapitulation of this DNA cleavage activity *in vitro* with purified Cas9 and an engineered single guide RNA (gRNA) molecule containing the minimal features of both spacer and tracrRNAs (Jinek et al., 2012), it was anticipated that the system could potentially be used in place of zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) for targeted genomic cleavage in higher organisms (Carroll, 2012; Jinek et al., 2012). Indeed, it has been shown that, via generation of site-specific DNA double-strand breaks in the target loci, RNA-guided Cas9 nuclease facilitates genome editing in yeast, nematode, fly, zebrafish and mice, in mouse and human cell lines, as well as in plants (Bassett et al., 2013; Chang et al., 2013; Cho et al., 2013; Cong et al., 2013; DiCarlo et al., 2013; Dickinson et al., 2013; Ding et al., 2013; Fujii et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Mali et al., 2013; Shen et al., 2013; Wang et al., 2013; Yang et al., 2013).

In the past decade, the diploid frog *Xenopus tropicalis* has emerged as an excellent amphibian genetic model (Harland and Grainger, 2011). We and others have established ZFN- or TALEN-mediated gene targeting protocols in this species (Ishibashi et al., 2012; Lei et al., 2012; Young et al., 2011). In comparison to ZFNs, TALENs are more effective in frogs. Despite the ease of designing TALE modules, it is by no means trivial to generate TALENs in the laboratory for use in large-scale reverse genetics. Thus, efficient genome engineering tools that can be easily and cost-effectively generated are still highly desirable. Here, we report that gRNA/Cas9 can serve as an easy, economic, efficient and reliable tool for targeted gene disruption in *X. tropicalis*.

**RESULTS****Optimization of gRNA and Cas9 doses in *X. tropicalis* embryos**

First, we injected Cas9 mRNA at a dose of 500 pg per embryo together with gRNAs (50 pg/embryo) targeting *ptf1a/p48*, *hhx* or *pat* into one-cell stage *X. tropicalis* embryos. All three injection groups showed high levels of dead and deformed embryos (Fig. 1A,B), indicating non-specific toxicity. We then chose *hhx* and *pat* gRNAs to optimize the doses of Cas9 mRNA and gRNA for *X. tropicalis* embryos based purely on the morphological phenotype. The data obtained indicate that the optimal Cas9 mRNA dose is 300 pg/embryo (Fig. 1C) and the quantity of gRNA should not exceed 500 pg/embryo (Fig. 1D,E). In all subsequent experiments, we set the Cas9 mRNA dose at 300 pg/embryo; for a given locus, the gRNA dose was further optimized in the range 1–500 pg per embryo.

**gRNA/Cas9 is an efficient and reliable tool for genome editing in *X. tropicalis***

We initially designed gRNAs targeting 12 loci in ten different genes (Table 1; supplementary material Table S1). Those targeting

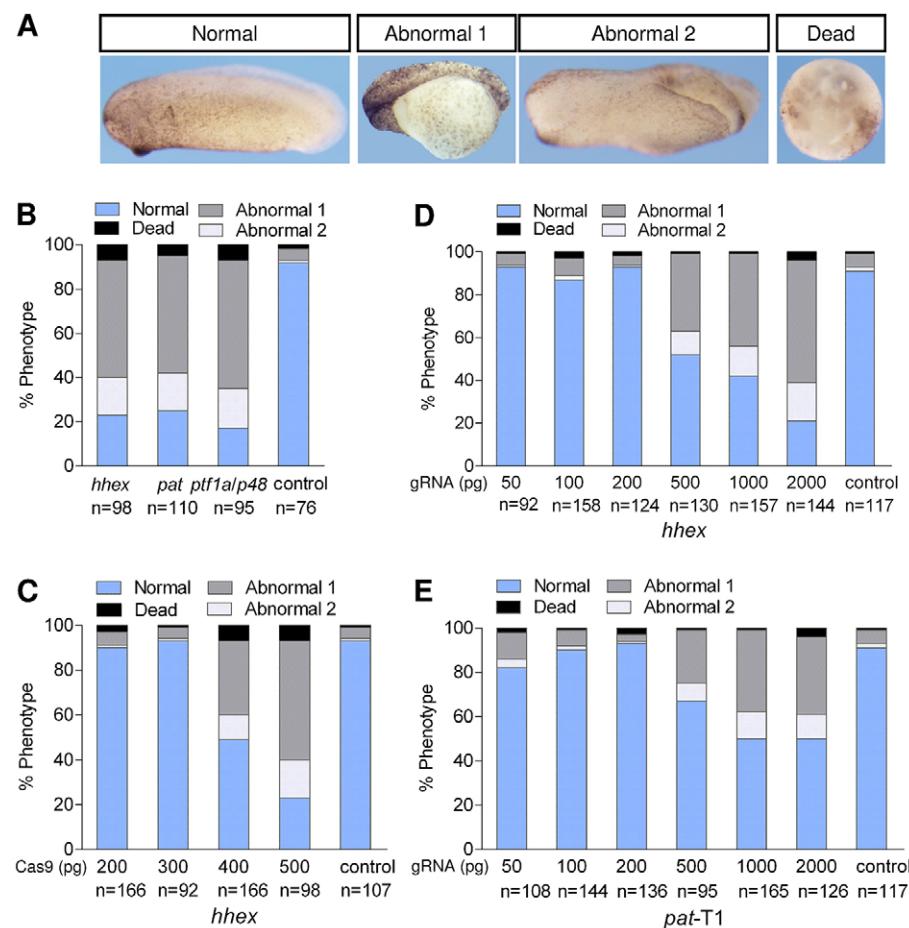
<sup>1</sup>Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, Guangdong 510530, China.

<sup>2</sup>Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Guangzhou, Guangdong 510530, China. <sup>3</sup>Department of Biotechnology, Guangzhou Medical University, Guangzhou 510182, China. <sup>4</sup>University of Chinese Academy of Sciences, Beijing 100049, China. <sup>5</sup>School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, Guangdong 510006, China. <sup>6</sup>State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China. <sup>7</sup>School of Life Sciences, Anhui University, Hefei, Anhui 230039, China. <sup>8</sup>School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China.

\*These authors contributed equally to this work

<sup>†</sup>Author for correspondence (chen\_yonglong@gibh.ac.cn)

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**Fig. 1.** The optimal dose of Cas9 mRNA for *X. tropicalis* embryos is 300 pg/embryo and the quantity of gRNA should not exceed 500 pg/embryo. (A) Representative morphology of dead and abnormal embryos evaluated when control siblings reached stage 30. The deformation was mainly caused by gastrulation defects. (B) Cas9 mRNA at 500 pg/embryo appeared toxic to *X. tropicalis* embryos. Dead and abnormal embryos were scored upon injection of Cas9 mRNA (500 pg/embryo) and gRNA (50 pg/embryo) targeting the indicated genes. (C) Constant amount (50 pg/embryo) of gRNA targeting *hhx* and graded doses of Cas9 mRNA (in pg/embryo) were injected into one-cell stage *X. tropicalis* embryos and the resulting dead and abnormal embryos were scored. (D,E) Constant amount of Cas9 mRNA (300 pg/embryo) and various doses (in pg/embryo) of gRNAs targeting either *hhx* (D) or *pat* (E) were injected into one-cell stage *X. tropicalis* embryos and the resulting dead and abnormal embryos were scored. (B-E) The total embryos for each injection (*n*) is given under each column.

*elastase-T1*, *ets2*, *tm4sf4-T2*, *grp78*, *elastase-T2* and *ptf1a/p48* readily exhibited targeting efficiencies above 72% at a dose of 50 pg/embryo and the first three even achieved 100% efficiency (Fig. 2A; supplementary material Fig. S1). The mutagenesis rates induced by gRNAs targeting *hhx*, *tm4sf4-T1* and *tyrosinase* were raised from 31.3%, 60% and 60% to 100%, 86.7% and 82.4% when the gRNA doses were increased from an initial 50 pg/embryo to 500,

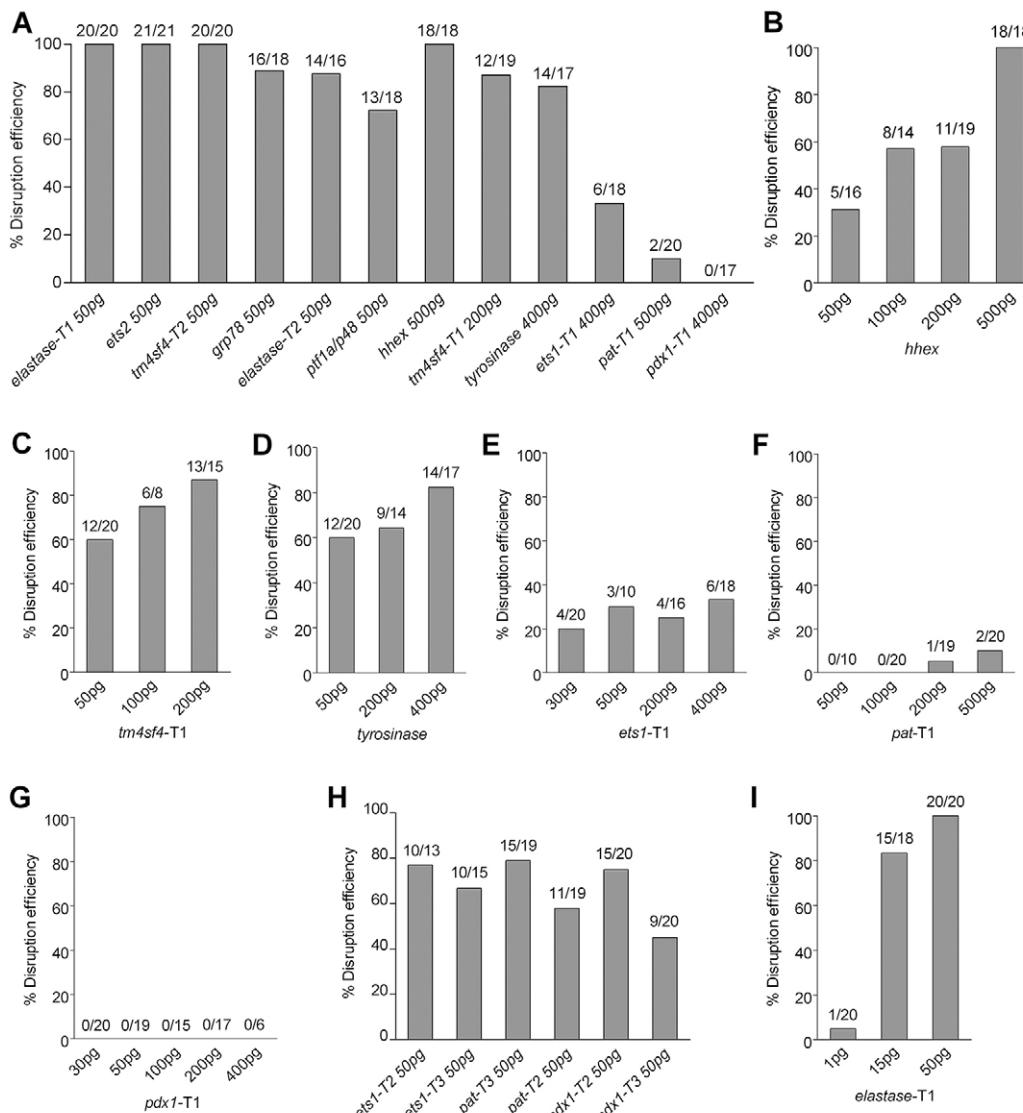
200 and 400 pg/embryo, respectively (Fig. 2A-D; supplementary material Fig. S1). The highest efficiency obtained for *ets1* gRNA was 33.3%, and gRNAs targeting *pat* and *pdx1* showed either very low efficiency or no effect at the various gRNA doses tested (Fig. 2A,E-G; supplementary material Fig. S1). We then designed two additional gRNAs for each of *ets1*, *pat* and *pdx1*. The data obtained indicate that all caused mutations with high efficiencies

**Table 1.** The 18 targeting loci in ten *X. tropicalis* genes and the oligonucleotides used to construct the corresponding gRNA constructs

Target gene	Target site	PAM	Oligonucleotide 1	Oligonucleotide 2
<i>elastase-T1</i>	GGCAGTTGGTACCATACCTG	TGG	TAGGCAGTTGGTACCATACCTG	AAACAGGTATGGTACCAACTG
<i>elastase-T2</i>	GGTGGTATGGAGTCGGTTC	AGG	TAGGTGGTATGGAGTCGGTTC	AAACGAACGGACTCCATCACCA
<i>ets1-T1</i>	GGAGCAGCATTATGTGGCCT	GGG	TAGGAGCAGCATTATGTGGCCT	AAACAGGCCACATAATGCTGCT
<i>ets1-T2</i>	GGTTCAGAGAATTCTAGAGGG	CGG	TAGGTTCAAGAATTCTAGAGGG	AAACCCCTCTGAATTCTCTGAA
<i>ets1-T3</i>	GGGTCACGCAGCAATGCTAA	AGG	TAGGGTCACGCAGCAATGCTAA	AAACTTAGCATTGCTGCGTGAC
<i>ets2</i>	GGTCTGGACTCTTACTCTCA	TGG	TAGGTCTGGACTCTTACTCTCA	AAACTGAGAGTAAGAGTCCAGA
<i>grp78</i>	GGCAGACACCAGAACACCA	AGG	TAGGCAGACACCAGAACACCA	AAACTGGTGTGCTGGTGTCTG
<i>hhx</i>	GGGCTGAGGAGCTGGGGTGC	TGG	TAGGGCTGAGGAGCTGGGGTGC	AAACGCACCCCCAGCTCCCTCAGC
<i>pat-T1</i>	GGCCTGTAAGCAAACATT	TGG	TAGGCCTGTAAGCAAACATT	AAACAAATGTTTGCTTACAGG
<i>pat-T2</i>	GGCACATTCTGATGCAGCAG	TGG	TAGGCACATTCTGATGCAGCAG	AAACCTGCTGCTGATCAGAATGTG
<i>pat-T3</i>	GGCAGCTTGGAAAGAACAG	TGG	TAGGCAGCTTGGAAAGAACAG	AAACCTGTTCTCCAAGAGCTG
<i>pdx1-T1</i>	GGGCCAGCATTCTATTCT	TGG	TAGGGCCAGCATTCTATTCT	AAACAGAAAATGAAATGCTGGC
<i>pdx1-T2</i>	GGTGAGGGATCCCTGGGTGA	TGG	TAGGTGAGGGATCCCTGGGTGA	AAACTCACCCAGGGATCCCTCA
<i>pdx1-T3</i>	GGGAATGGCATTGCTGGTG	GGG	TAGGGAATGGCATTGCTGGTG	AAACCACCAGCAAATGCCATT
<i>ptf1a/p48</i>	GGAAGACGATGTGGACTTCT	TGG	TAGGAAGACGATGTGGACTTCT	AAACAGAAAGTCCACATCGTCTT
<i>tm4sf4-T1</i>	GGTGGTTGCGCAAAGTGCTT	GGG	TAGGTGGTTGCGCAAAGTGCTT	AAACAAGCACTTGTGCGAACCA
<i>tm4sf4-T2</i>	GGACTCATAGGAACAATCTG	TGG	TAGGACTCATAGGAACAATCTG	AAACAGATTGTTCTATGAGT
<i>tyrosinase</i>	GGCCCTCAGTTCCATTAC	TGG	TAGGCCTCAGTTCCATTAC	AAACGTGAATGAAACTGAGGG

Sequences are shown 5'-3'.

PAM, protospacer adjacent motif.



**Fig. 2. gRNA/Cas9 is an efficient and robust tool for gene targeting in *X. tropicalis*.** (A) gRNA/Cas9 induced efficient targeted gene disruption in *X. tropicalis* embryos. The genes targeted and the doses of gRNAs (in pg/embryo) used are indicated. The dose of Cas9 mRNA was set at 300 pg/embryo for all injections in this figure. (B-G) Targeting efficiencies can be improved by increasing the amount of gRNA (pg/embryo shown), as evaluated with a constant amount of Cas9 mRNA (300 pg/embryo). (H) The targeting efficiencies of further gRNAs (50 pg/embryo). (I) At 15 pg/embryo, *elastase-T1* gRNA was still 83.3% efficient. The numbers above each bar indicate mutations detected among total samples sequenced.

(45–79%) at 50 pg/embryo (Fig. 2H; supplementary material Fig. S2). Thus, all of the genes tested were readily targeted by gRNA/Cas9 with efficiencies above 45%. Finally, we chose one of the most effective gRNAs to scale down the gRNA dose, and found that 15 pg/embryo of *elastase-T1* gRNA still exhibited 83.3% efficiency (Fig. 2I). Together, our results strongly suggest that gRNA/Cas9 can efficiently target most loci in the *X. tropicalis* genome.

#### A perfect match between the spacer and protospacer sequences proximal to the PAM is essential for Cas9 to cleave target DNA in the *X. tropicalis* genome

To investigate the specificity of the gRNA/Cas9 system for genome editing in whole organisms, we chose two loci (*ets2* and *tm4sf4-T2*) that displayed 100% targeting efficiency and systematically analyzed the consequence of single-nucleotide mismatches between the spacer and the protospacer sequences for targeting efficiency in *X. tropicalis* embryos. For both genes, point mutations up to the eleventh base pair upstream of the protospacer adjacent motif (PAM) completely abolished the targeting activity of gRNA/Cas9. By contrast, gRNA/Cas9-mediated target cleavage is partially tolerant to point mutations 12, 13, 15, 17, 18, 19 and 20 bp 5' of the PAM (Fig. 3; supplementary material Figs S3 and S4).

To further assess whether gRNA/Cas9 creates any off-target mutations in frog embryos, we first computationally identified all the potential off-target sites with up to five mismatches to all the loci targeted in this study (supplementary material Table S2). Since no sites with one mismatch were identified, we selected 119 sites in total, including all four sites with two mismatches, seven sites with three mismatches distal to the PAM sequence, and all sites with up to four mismatches for the *ets2*, *ptfla/p48* and *tyrosinase* target loci, and performed a T7EI assay to identify any off-target disruptions (supplementary material Table S3). In contrast to the on-target sites, no potential off-target sites analyzed showed reliable gRNA/Cas9-dependent T7EI assay positive signals (data not shown). Our study suggests that the frequency of cleavage within potential off-target sites with two to four mismatches is too low to be detected by our T7EI assay.

#### Multiplexed gene targeting in *X. tropicalis*

To test whether this approach is suitable for multiplexed editing of genomic loci in *Xenopus* embryos, we co-injected Cas9 mRNA together with two gRNAs targeting *grp78* and *elastase-T1*. The data indicate that the targeting efficiencies for each gene from the co-injection are almost identical to those obtained from the individual injections (Fig. 4A; supplementary material Fig. S5). Single-cell

<b>A</b>		<b>B</b>			
	PAM		PAM		
<i>ets2</i> locus	5' GGTCTGGACTCTTACTCTCAT <b>TGG</b> 3'	disruption efficiency	<i>tm4sf4-T2</i> locus	5' GGACTCATAGGAACAAATCTG <b>TGG</b> 3'	disruption efficiency
wt crRNA	GGUCUGGACUCUUACUCUCA	100% (21/21)	wt crRNA	GGACUCAUAGGAACAAUCUG	100% (20/20)
m1	GGUCUGGACUCUUACUCU <b>C</b>	0% (0/19)	m1	GGACUCAUAGGAACAAUCUG	0% (0/19)
m3	GGUCUGGACUCUUACUC <b>A</b>	0% (0/20)	m3	GGACUCAUAGGAACAAUGUG	0% (0/16)
m5	GGUCUGGACUCUUAC <b>U</b> CUCA	0% (0/20)	m5	GGACUCAUAGGAACATUCUG	0% (0/20)
m7	GGUCUGGACUCUU <b>U</b> CUCUCA	0% (0/20)	m7	GGACUCAUAGGAAGAACUG	0% (0/20)
m9	GGUCUGGAC <b>U</b> CUACUCUCA	0% (0/19)	m9	GGACUCAUAGGTACAAUCUG	0% (0/18)
m11	GGUCUGGAC <b>A</b> CUACUCUCA	0% (0/18)	m11	GGACUCAUA <b>C</b> GAACAAUCUG	0% (0/20)
m12	GGUCUGGAC <b>U</b> CUACUCUCA	10% (2/20)	m12	GGACUCAU <b>U</b> GGAAACAAUCUG	95% (19/20)
m13	GGUCUGGU <b>C</b> CUACUCUCA	53% (10/19)	m13	GGACUCA <b>T</b> AGGAACAAUCUG	35% (7/20)
m15	GGUCUG <b>G</b> ACUUACUCUCA	18% (3/17)	m15	GGACU <b>G</b> AGGAACAAUCUG	63% (12/19)
m17	GGUGUGGACUCUUACUCUCA	18% (3/17)	m17	GGAG <b>U</b> CAUAGGAACAAUCUG	42% (8/19)
m18	GGACUGGACUCUUACUCUCA	80% (16/20)	m18	GG <b>T</b> CACUAA <b>U</b> GGAAACAAUCUG	71% (12/17)
m19	GGCUCUGGACUCUUACUCUCA	68% (13/19)	m19	GG <b>C</b> ACUCAUAGGAACAAUCUG	61% (11/18)
m20	GG <b>C</b> UCUGGACUCUUACUCUCA	6% (1/16)	m20	GG <b>G</b> ACUCAUAGGAACAAUCUG	63% (12/19)

**Fig. 3. A perfect match between the spacer and the protospacer sequences proximal to the PAM is essential for Cas9 to cleave target sites in the *X. tropicalis* genome.** (A,B) *ets2*- or *tm4sf4-T2*-targeting crRNAs containing single-point mutations (red) were generated to investigate the consequences of single-nucleotide mismatches between the spacer and the protospacer sequences for Cas9-mediated gene targeting efficiency in *X. tropicalis* embryos. The targeting efficiency is indicated on the right of each mutant. The PAM sequence is indicated (blue). wt, wild type.

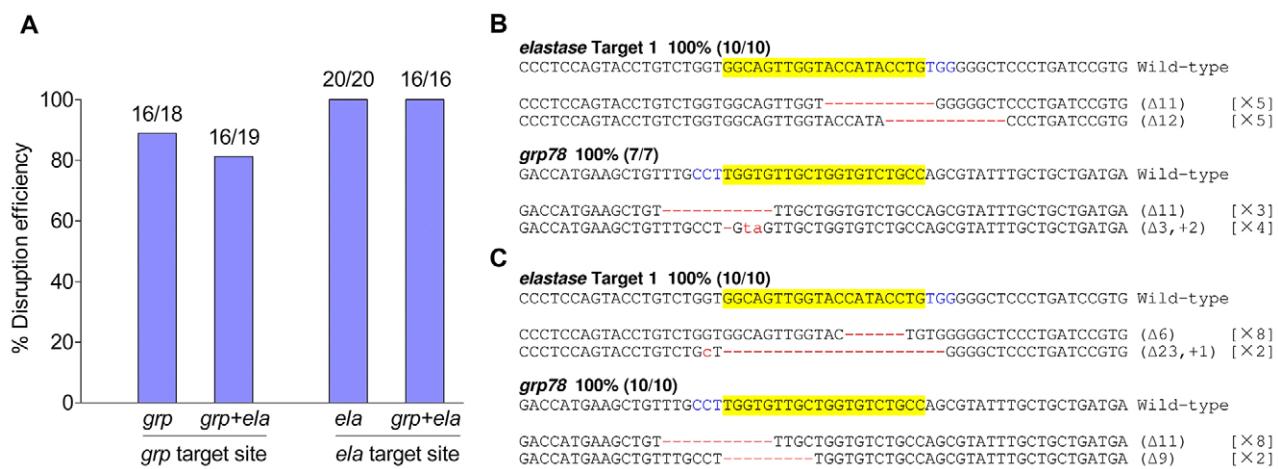
analysis for stage 9 embryos (blastulae) indicates that both alleles of the two loci targeted were mutated in the same cell (Fig. 4B,C). Given the high targeting efficiency in the founder embryos and high germ line transmission rates observed in this study with other genes, these data suggest that double or triple knockout lines of genes of interest in *X. tropicalis* could be established from a single injection of Cas9/gRNAs, which also appears to be achievable in mice (Wang et al., 2013).

#### Phenotyping of gRNA/Cas9-targeted G0 embryos, froglets and frogs

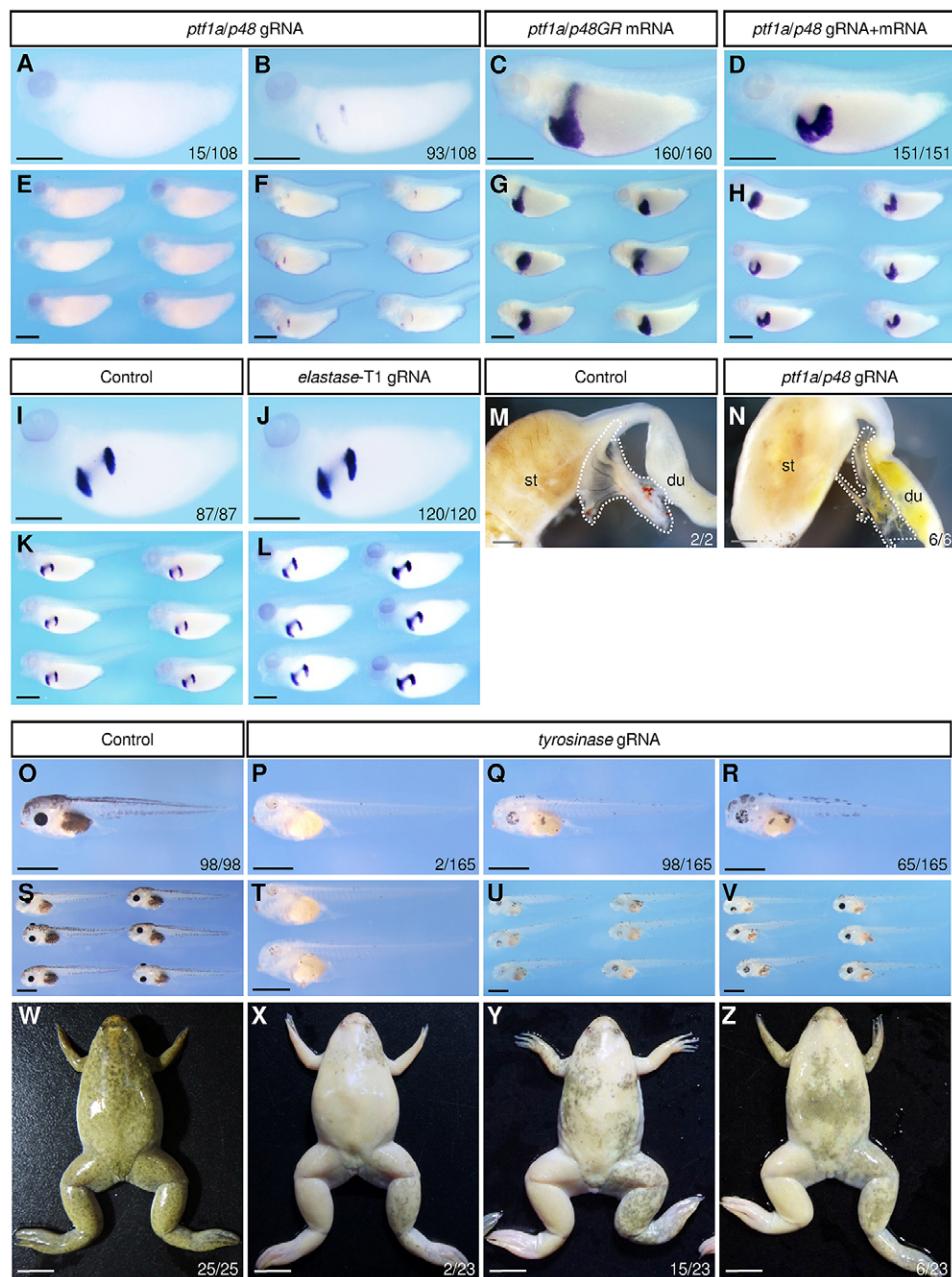
In principle, the high efficiency of gene disruption induced by Cas9 nuclease could allow for direct phenotype assessment in gRNA/Cas9-injected *Xenopus* embryos. Our data indicated that the expression of the pancreas-specific marker gene *pdip* is indeed completely inhibited in a portion of *ptfla/p48* gRNA-injected embryos (Fig. 5A,E). The rest of the targeted embryos showed severe inhibition of *pdip* expression (Fig. 5B,F), with hardly any showing the strong signals seen in wild-type or *elastase*-targeted embryos (Fig. 5I-L). Co-

injection of a dexamethasone-inducible variant of *Ptfla/p48* (*Ptfla/p48GR*), which was activated after gastrulation, resulted in 100% rescue of the *ptfla/p48* gRNA-induced phenotype (Fig. 5D,H). Together, these findings are reminiscent of those obtained upon application of *ptfla/p48* morpholinos to *Xenopus laevis* embryos (Afelik et al., 2006; Jarikji et al., 2007). We also dissected six *ptfla/p48*-targeted froglets that all showed severe pancreatic hypoplasia (Fig. 5N), consistent with our previous findings with *ptfla/p48* TALENs (Lei et al., 2012).

As a second example, we chose to phenotype the disruption of *tyrosinase*, which causes the ablation of pigmentation (Beermann et al., 2004; Damé et al., 2012; Ishibashi et al., 2012; Koga et al., 1995; Oetting et al., 2003). Upon *tyrosinase* gRNA/Cas9 injection, the majority of tadpoles (100/165, ~61%) showed severe perturbation of pigmentation, with two showing almost full albinism, whereas the remainder displayed partial albinism with none showing the pigmentation pattern seen in wild-type siblings (Fig. 5O-V). The various levels of albinism were maintained to adulthood (Fig. 5X-Z).



**Fig. 4. gRNA/Cas9 is suitable for multiplexed genome editing in *X. tropicalis*.** (A) Co-injection of Cas9 mRNA (300 pg/embryo) together with two gRNAs targeting *grp78* (*grp*) and *elastase-T1* (*ela*) did not affect the targeting efficiencies obtained from individual injections. The gRNA dose for each gene was set at 50 pg/embryo. (B,C) DNA sequencing data obtained from the progenies of two different blastomeres (shown separately in B and C) of a stage 9 embryo demonstrate that both loci were disrupted in the same cell. The wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue. Red dashes indicate deletions and lowercase letters in red indicate insertions. The number of deleted (Δ) or inserted (+) base pairs is indicated in parentheses; numbers in square brackets show the frequencies of the mutation among the sequenced samples. The data indicate that both alleles of both loci were mutated in progenies of each blastomere analyzed.



**Fig. 5. gRNA/Cas9-mediated gene targeting is suitable for G0 phenotyping.** (A-L) Whole-mount *in situ* hybridization analysis of expression of the pancreas-specific marker *pdip* in *X. tropicalis* normal control tadpoles (stage 40), tadpoles injected with Cas9 mRNA (300 pg/embryo) and gRNAs (50 pg/embryo), and tadpoles injected with either *ptf1a/p48GR* mRNA (20 pg/embryo) alone or in combination with Cas9 and *ptf1a/p48* gRNA. Dexamethasone (working concentration of 10 µM) was added at stage 14 to activate Ptf1a/p48. (A,E) Complete inhibition of *pdip* expression upon targeting *ptf1a/p48*. (B,F) Partial inhibition of *pdip* expression upon targeting *ptf1a/p48*. (C,G) Overexpression of *ptf1a/p48* expands *pdip* expression in the territory of the stomach and duodenum. (D,H) The inhibition of *pdip* expression upon gRNA/Cas9-mediated targeting of *ptf1a/p48* was completely rescued by co-injection of *ptf1a/p48GR* mRNA. (I,K) Uninjected control embryos. (J,L) As a negative control, *pdip* expression was unaffected upon targeting *elastase*. All the images are lateral views with the head to the left. E-H,K,L show further examples of the types represented in A-D,I,J, respectively. The number of embryos showing the illustrated phenotype is given in the representative image. (M,N) Dissection of 1-week-old froglets revealed severe pancreatic hypoplasia in *ptf1a/p48* gRNA/Cas9-injected G0 froglets, with stomach and duodenum unaffected. The pancreas is outlined (dashed line). du, duodenum; st, stomach. (O-V) Albinism phenotype caused by *tyrosinase* gRNA/Cas9. (O,S) Uninjected control tadpoles. (P,T) Almost full albinism. (Q,U) Tadpoles showing severe perturbation of pigmentation. (R,V) Partial albinism. S-V show further examples of the types represented in O-R, respectively. The number of embryos showing the illustrated phenotype is given in the representative image. (W-Z) Dorsal view of adult frogs. (W) Wild type. (X) Almost full albinism caused by *tyrosinase* gRNA/Cas9. (Y,Z) Partial albinism. The numbers of frogs showing the illustrated phenotypes are listed. Scale bars: 400 µm in A-L; 2 mm in M,N; 1 mm on O-V; 1 cm in W-Z.

### gRNA/Cas9-injected founder frogs show high germ line transmission rates

The high efficiency of somatic targeting in gRNA/Cas9-injected embryos would suggest a similarly high targeting efficiency in

germ cells of G0 frogs. Just as expected, all five founder male frogs from the targeting of either *elastase-T1*, *elastase-T2* or *tyrosinase* transmitted their targeted mutations through the germ line with high efficiencies ranging from 40-100% (Fig. 6). The

**A elastase-T1 gRNA/Cas9 targeted founder 1 100% (10/10)**

CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCAT---GTGGGGGCTCCCTGATCCGTG Wild-type  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCAT---TGTTGGGGCTCCCTGATCCGTG (Δ4)  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGT---GGGGGCTCCCTGATCCGTG (Δ11) [× 3]  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGT---TGTTGGGGCTCCCTGATCCGTG (Δ12) [× 3]  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCATcAgggagccccccacaggggggCCTGT (+19)

**elastase-T1 gRNA/Cas9 targeted founder 2 80% (8/10)**

CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCATACCTGTTGGGGCTCCCTGATCCGTG Wild-type  
 CCCTC---CTGTGGGGGCTCCCTGATCCGTG (Δ31) [× 5]  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCATACCAGTGTGGGGGCTCCCTGATCCGTG (Δ1)  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCAT---CTGATCCGTG (Δ17)  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGGGCTCCCTGATCCGTG (Δ13,+9) [× 2]  
 CCCTCCAGTACCTGTCGGTGGCAGTTGGTACCATACCTGTTGGGGGCTCCCTGATCCGTG

**B elastase-T2 gRNA/Cas9 targeted founder 70% (7/10)**

AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT Wild-type  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (+1)  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (+3)  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (Δ4)  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (Δ5)  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (Δ16) [× 2]  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT (Δ16) [× 3]  
 AGTCCACCATGGTGTGTCGTCGGTGGGATGGAGTCGTCAGGATGCCAGGTACAATGAT

**C tyrosinase gRNA/Cas9 targeted founder 1 90% (9/10)**

TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---TGGGGTTGACGATAGAGAGA Wild-type  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---CTGGGGTTGACGATAGAGAGA (Δ3) [× 2]  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---GGGGTTGACGATAGAGAGA (Δ4) [× 2]  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATTGGGAAACCCACTGGGGTTGACG (+9) [× 4]  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATTACTGGGGTTGACGATAGAGAGA

**tyrosinase gRNA/Cas9 targeted founder 2 40% (4/10)**

TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---GGGGTTGACGATAGAGAGA Wild-type  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---GGGGTTGACGATAGAGAGA (Δ2) [× 2]  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---GGGGTTGACGATAGAGAGA (Δ4) [× 2]  
 TCC TGACCA GAGCTCTGCTACTGGCCCTCAGTTCCATT---CTGGGGTTGACGATAGAGAGA (Δ4) [× 6]

data indicate that gRNA/Cas9-induced mutagenesis in *X. tropicalis* is highly heritable.

**DISCUSSION**

We have shown that gRNA/Cas9 is an efficient, simple and robust tool for *X. tropicalis* genome editing with high precision and specificity. Specificity in genome editing is crucial to both basic research and therapeutic application. Our data from the systematic analysis of the effects of single-nucleotide mismatches between the spacer and the protospacer sequences on Cas9-mediated gene targeting efficiencies in *X. tropicalis* embryos are consistent with findings obtained *in vitro* and in bacteria and mammalian cell lines (Cong et al., 2013; Fu et al., 2013; Hsu et al., 2013; Jinek et al., 2012; Sapranauskas et al., 2011), further highlighting the importance of the 3' protospacer sequence close to the PAM in designing gRNAs to eliminate off-target effects. In contrast to the high level of off-target cleavage reported in human cell lines using the CRISPR/Cas system (Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013), our data suggest that the gRNA/Cas9-induced off-target mutation rate is very low in *X. tropicalis* embryos, consistent with data obtained with mouse embryos (Yang et al., 2013). Future studies using whole-genome sequencing would generate more comprehensive information. Meanwhile, the use of paired gRNA/Cas9 nickases significantly improves the specificity (Ran et al., 2013).

Sequencing data indicate that both Cas9 and TALEN induce diverse indels (supplementary material Fig. S1) (Lei et al., 2012), suggesting that different embryonic cells are likely to harbor different genotypes of the targeted gene. If some of the mutations do not result in a loss of function, the injected embryos would display a mosaic phenotype. It is also possible that a few cells carry a disruption in only one allele, or even in neither allele. Thus, the

**Fig. 6. gRNA/Cas9-induced targeted mutations are highly heritable.** (A-C) DNA sequencing data showing the genotypes of each F1 embryo obtained from founder frogs treated as indicated. The wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue. Red dashes indicate deletions and lowercase letters in red indicate insertions. The number of deleted (Δ) or inserted (+) base pairs is indicated in parentheses; numbers in square brackets show the frequencies of the genotype among the ten sequenced samples.

which was purified with the RNeasy Mini Kit (Qiagen) according to the RNA clean protocol.

To create a gRNA expression vector, we placed a T7 promoter followed by two *BbsI* sites upstream of the recently described gRNA scaffold (Mali et al., 2013), which was synthesized by GenScript and cloned into the pUC57-Simple vector (GenScript) (supplementary material Fig. S6). The gRNAs were designed to target protospacer sequences in genes of interest with the form 5'-GG-(N)<sub>18</sub>-NGG-3' (Table 1). NGG is the PAM. The locus-specific 20 bp protospacer containing the cloning cohesive sites was obtained by annealing two synthesized partially complementary oligonucleotides (Table 1), and was then cloned into *BbsI*-digested gRNA expression vector. The resulting construct was digested with *Dra*I and transcribed using the MAXIscript T7 Kit (Ambion). The gRNA was purified by miRNeasy Mini Kit (Qiagen).

Capped *ptf1a/p48GR* mRNA was generated as described (Afelik et al., 2006).

### Manipulation of *X. tropicalis* embryos and evaluation of gRNA/Cas9-associated toxicity

*X. tropicalis* frogs were purchased from Nasco. Ovulation and *in vitro* fertilization were carried out according to the protocol described previously (Khokha et al., 2002; Young et al., 2011). The desired amount of Cas9 mRNA and gRNA in 2 nl was co-injected into one-cell stage embryos. During subsequent development, dead and abnormal embryos (mainly due to gastrulation defects) were sorted out and counted for the purposes of morphological phenotyping.

### Evaluation of gene targeting efficiency in gRNA/Cas9-injected embryos

Forty-eight hours after microinjection (about stage 40), we randomly pooled five healthy embryos from each injection, extracted genomic DNA, amplified the targeted region by PCR (for primers see supplementary material Table S4), and then cloned the purified PCR products into the pMD18-T vector (Takara) by TA cloning. Twenty single colonies were randomly picked for DNA sequencing analysis to detect any insertion or deletion (indel) mutations resulting from error-prone non-homologous end joining (NHEJ)-based repair of Cas9-created double-strand breaks. The targeting efficiency was determined by the ratio of mutant to total colonies.

For single-cell analysis, stage 9 embryos co-injected with Cas9 mRNA together with two gRNAs targeting *grp78* and *elastase-T1* were freed from the vitelline membrane and dissociated in calcium- and magnesium-free medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 7.5 mM Tris pH 7.6). Single blastomeres from the dissociated embryos were separately cultured with 1× MBS solution (88 mM NaCl, 2.4 mM NaHCO<sub>3</sub>, 1 mM KCl, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 10 mM HEPES pH 7.4) in 24-well plates lined with 0.8% agar overnight and then subjected to proteinase K digestion, PCR amplification and TA cloning. Ten single colonies from each blastomere progeny were sequenced to determine the genotype of individual blastomeres.

### Measurement of germ line transmission

gRNA/Cas9-injected *X. tropicalis* embryos were raised to sexual maturity. Male founder frogs were crossed with wild-type females and individual F1 embryos were collected 48 hours postfertilization for genomic DNA extraction. Evaluation of mutations was carried out by PCR amplification, TA cloning and DNA sequencing of single colonies. Ten embryos from each founder frog were analyzed, and for each F1 embryo ten colonies were sequenced.

### Identification of potential off-target sites in the *X. tropicalis* genome

All genomic loci containing up to five mismatches compared with the coding sequence for a given 20 nt gRNA followed by the NGG PAM sequence were identified by mapping the targeted site to *X. tropicalis* genome V4.1 using a PERL script developed according to the SeqMap method (Jiang and Wong, 2008).

### T7 endonuclease I (T7EI) assay for detecting off-target mutagenesis

The T7EI assay was performed essentially as described (Guschin et al., 2010). For each injection, gRNA/Cas9-injected embryos or uninjected control embryos at stage 40 were pooled in groups of five for genomic DNA extraction. The regions of interest containing the off-target sites were amplified by PCR with gene-specific primers (supplementary material Table S3). PCR products were denatured and annealed under the following conditions: 95°C for 5 minutes, 95–85°C at –2°C/s, 85–25°C at –0.1°C/s, hold at 4°C. The annealed samples were digested with T7EI (NEB M0302L), separated and measured on an ethidium bromide-stained 10% polyacrylamide TBE gel and quantified using ImageJ software (NIH).

### Whole-mount *in situ* hybridization

The digoxigenin-labeled antisense *X. tropicalis pdip* probe was transcribed with T7 RNA polymerase using an RT-PCR-amplified template containing the T7 promoter (forward, 5'-GAGGAGGAGACATCAGACGA-3'; reverse, 5'-CAGTAATACGACTCACTATAAGGAATACTCAAGGACCGAAGAAA-3'). Whole-mount *in situ* hybridization was performed as described (Harland, 1991).

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

X.G., T.Z., Z.H., H.Z. and Y. Chen designed the work and analyzed experiments. X.G., T.Z., Z.H., Y.Z., Z.S., Y. Cui, and F.W. carried out the experiments. Q.W. designed the PERL script. Y. Chen, T.Z. and X.G. wrote the manuscript.

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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099853/-DC1>

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**Table S1.** The GenBank accession numbers of genes targeted in this study.

Target gene	GenBank accession number
<i>elastase</i>	NM_001011493.1
<i>ets1</i>	NM_001130368.1
<i>ets2</i>	NM_001127061.1
<i>grp78</i>	XM_002941644.1
<i>hhex</i>	NM_204089.1
<i>pat</i>	XM_002940115.1
<i>pdx1</i>	XM_002934019.1
<i>ptf1a/p48</i>	XM_002933135.1
<i>tm4sf4</i>	NM_203627.1
<i>tyrosinase</i>	NM_001103048.1

**Table S2.** Computationally identified total number of potential off-target sites in *Xenopus tropicalis* genome with up to 5 mismatches to all the loci targeted in this study.

Target ID	Number of mismatch				
	1	2	3	4	5
<i>elastase</i> -T1	0	0	4	45	427
<i>elastase</i> -T2	0	0	2	22	278
<i>ets1</i> -T1	0	0	5	37	578
<i>ets1</i> -T2	0	0	5	59	652
<i>ets1</i> -T3	0	0	0	34	329
<i>ets2</i>	0	0	0	26	354
<i>grp78</i>	0	0	13	78	716
<i>hhex</i>	0	1	24	220	1941
<i>pat</i> -T1	0	0	8	93	822
<i>pat</i> -T2	0	1	9	84	866
<i>pat</i> -T3	0	0	5	89	653
<i>pdx1</i> -T1	0	1	9	76	763
<i>pdx1</i> -T2	0	0	40	278	2459
<i>pdx1</i> -T3	0	1	11	131	936
<i>ptf1a/p48</i>	0	0	4	36	368
<i>tm4sf4</i> -T1	0	0	5	27	307
<i>tm4sf4</i> -T2	0	0	3	48	402
<i>tyrosinase</i>	0	0	2	40	345

**Table S3.** 119 potential off-target sites examined by T7EI assay and corresponding PCR primers used for T7EI assay.

Site ID	Potential Off Target Site	Coordinate (Start)	Forward PCR Primer	Reverse PCR Primer
hhex-2	GGGCTGGGAGCTGGGGGCCGG	scaffold_5011:(6508)	TGGTGCCCCACATCCTGTGGCAC	GGCCCCCCCCAGCTCTCCAG
pat-T2-2	GGCACATTCTGATGCAC <b>T</b> AGAGG	scaffold_471:(239289)	CTATGAACTCGAGAGCCGCTCT	CCGCATTCTCCATGATGTT
pdx1-T1-2	GGGCCTGCATTTCGTTTCTGGG	scaffold_163:(1506895)	CCCTCTGGTATGTGCTACACC	ATAGGGCTTATAGCTGTGTGTC
pdx1-T3-2	GGGAATGCATTTCGAGAGG	scaffold_82:(2396453)	CAGAAGAGGAAGACAAGTATCAGG	ACTCTGTTGTCACTTTCCAC
pat-T2-3	G <b>T</b> TCATTCTGATGCAGCTGG	scaffold_567:(560009)	TTTACATGGCTGTATGCTACACC	TTTCCCATGAGCATCTAAAGG
pdx1-T1-3	GGGCCAC <b>A</b> TTTCATTTCCTAGG	scaffold_75:(1531776)	TCCATCTTGCTACAGAAAGCC	TTTAGGCCCCCTACATAGGAG
pdx1-T3-3	GGGG <b>G</b> ATTGGATTGCTGGTGGG	scaffold_618:(156398)	CAGCCAGATTGTTCTCCAG	GTACCTGAAGCACCCTG
ets1-T1-3	GGAT <b>C</b> TGCTTATGTCGGCTGGG	scaffold_19:(386769)	AGGGTGCATTGAGACACTTTG	GGGTAGCTGGCCTATAAATAGG
pat-T1-3	GGGCTTAGGCAAACATTGGG	scaffold_90:(1242588)	TACCATTTCATTTAGCAAAGCG	TTAATTCTTAGAGCCCCCTTGAG
elastase-T1-3-1	TGAAGATGGTACCATACCTGTGG	scaffold_384:(6780)	ATAATGGATTTGCTGAATTGC	ATGATACATTGCTTCAGCTG
elastase-T1-3-2	GG <b>T</b> TATTGGTACCATACCTGTGG	scaffold_67:(1803421)	TGGGTATCGCTTCAGACTATTG	GGTCAACAAGCACCTGATAGC
ets2-4-1	GAGGTGGACTATTACTCTCATGG	scaffold_104:(2007422)	CAAGATGCCATAGATACTGCTGTG	TGGACCATTAAGTGAGCCCCATAC
ets2-4-2	GGTCAGGC <b>T</b> TTACTGTCAGGG	scaffold_12:(2621242)	CCTCTTCTACACTCTCCATCTGG	ACAGTCAGGGAAATGATGC
ets2-4-3	AGACTGGACTCTTC <b>T</b> CCCATGG	scaffold_12:(2854544)	AACTAATTGATTGCCATATCGG	TGGTGCCTCTAAAGAATTAC
ets2-4-4	AGTCTGCACTCTTA <b>A</b> TCTTAAGG	scaffold_13:(3467302)	GGCATAACTTAGACAGTCCACCAG	TTTCATACTTGCAATGTGTCTG
ets2-4-5	GGTCAG <b>T</b> TACTTACTCTCAGGG	scaffold_132:(2363382)	ATAGAATTTCCTTGAGCCTGCC	GTATGGGTTTGCTATTGTTGAT
ets2-4-6	TGTCTGGTTCTTC <b>C</b> CTCTCAAGG	scaffold_144:(139498)	TATATTGTAGGGATGACCAGATT	CTATGGGGTGACAGGATT
ets2-4-7	GGACTGGATTCT <b>G</b> ACTCTAACGG	scaffold_163:(1321382)	TCAGCCAGTGCAGAAACTTC	GGCTGGCCTTATCAATTATTCTAC
ets2-4-8	TGTCTGGTTCTTC <b>C</b> CTCTCAAGG	scaffold_171:(2122408)	CCTAGGATAGGCCATAGGAG	AGGTGCAATACTGCTTAATGGC
ets2-4-9	GGGCTGGCTCTTA <b>T</b> TAATGG	scaffold_175:(2078354)	CAAGTGGTGCCTGTGTTGC	TGACAGTGCTGTGCTCTTTG
ets2-4-10	GGTCTAGT <b>C</b> TATTCTCTCATGG	scaffold_214:(1626645)	GTAAAGTGTGACTGAAACACC	CAGCCTTCTGGGTTAGAG
ets2-4-11	GGCCTGTACTTTACTCA <b>A</b> GG	scaffold_313:(732450)	TATTATACTTATGTTGCAGTGC	ATTATTACACCCATTGTTGCTTG
ets2-4-12	AGTCTGGC <b>C</b> TTACTGTAA <b>CG</b> GG	scaffold_374:(908485)	TGACACTGTAGGCACAAATATGG	CGTTGCATTGTTCTTATTCC
ets2-4-13	GGTCTGTCTCTTAC <b>C</b> CTCCAGGG	scaffold_435:(862492)	TAAAGGTTGTTGGTGTCCG	CTCCATTGACTCCATTGCTTG
ets2-4-14	GGTCTGGATTCT <b>G</b> ACACTCTAGG	scaffold_463:(421903)	GTTTACATTCTATTACAGGGCTTG	CTTCCTTCACTGCATAACCTC
ets2-4-15	GGTCTGG <b>T</b> ACTTACCCAGGG	scaffold_482:(377203)	GGGAGACCATCCTAGAACAGG	CGGTCTATCTCTGCCTAGTG
ets2-4-16	GGTT <b>T</b> GCATTCTTACTCACAGGG	scaffold_510:(337303)	GGTACAAACTGGCTCCCTTTC	GACAGATGCAGCAGAGCTTATG
ets2-4-17	GGT <b>C</b> GG <b>A</b> T <b>C</b> CTACTCTCAGGG	scaffold_518:(816043)	TTCACGTGCATTATAATCCAAC	GGTCAGTTGAAGTGTATCTGGAG
ets2-4-18	TGTCTGG <b>C</b> CTCT <b>C</b> TCT <b>G</b> ATGG	scaffold_578:(441317)	GGCAGGAAACCCCTACAAAG	TTAGCTTATGCAAAGGCTGG
ets2-4-19	GGTCAGGG <b>T</b> CTTA <b>G</b> TCTCAGGG	scaffold_58:(274580)	GTTCACAAAACAATTGTCAGTG	GCTAAGTGGAAATCAAAGGTTCTC
ets2-4-20	ATT <b>C</b> TTGACTCTTC <b>T</b> CTCTCAGGG	scaffold_59:(1960990)	CATGTGCTATTCTCAGGGAAAGG	CCCCCTTTAATAGTGTCTCTG
ets2-4-21	GGTCTGGACT <b>G</b> TAA <b>G</b> C <b>T</b> CATGG	scaffold_68:(1440099)	ATTACAGGCCACTGGGCTCTAC	AGACACGCAATGTTCTTGG
ets2-4-22	GGTT <b>T</b> GGACTTTACT <b>G</b> GCAGG	scaffold_7:(5245156)	GTGTTGATGTCGTGGTGA <b>G</b> TTCTTG	GACCAGCAAA <b>A</b> CTAACAGAGG
ets2-4-23	GGG <b>C</b> TGG <b>A</b> CTATTACT <b>C</b> AGATGG	scaffold_753:(150)	CGGAAATGAATAGTGTCA <b>G</b> CT	CATAGTTGA <b>A</b> CTTCATGGCAGAG
ets2-4-24	GGG <b>C</b> TGG <b>A</b> CTATTACT <b>C</b> AGATGG	scaffold_753:(7514)	CAGTTTATAGGCG <b>A</b> ATGAATAG	CCCTGTAAGTTGC <b>C</b> ATTGTC
ets2-4-25	GTTC <b>T</b> GG <b>C</b> TCTTACT <b>T</b> TGAAGG	scaffold_8529:(5756)	GCACAGAAAGAGGGTGC <b>A</b> TTAC	CTCCTGGATTCT <b>C</b> AAATGGAC
ets2-4-26	GGACTGG <b>C</b> AC <b>T</b> ACTCTCATGG	scaffold_917:(200870)	GGGGCATTACACACAGGGGA	CAAGGGCCACTAAC <b>G</b> TATGC
ptf1a/p48-3-1	AGAT <b>G</b> CA <b>A</b> TGTGGACT <b>T</b> CTAGG	scaffold_381:(76541)	TTGAACATAATGTC <b>T</b> CT <b>G</b> CTG	TTCTTCTTTGTT <b>C</b> ATGGTCC
ptf1a/p48-3-2	GGAA <b>G</b> AGGATT <b>T</b> GGACT <b>T</b> CT <b>G</b>	scaffold_47:(3047525)	CTCAGGGTTGC <b>A</b> ACTATTG	TCTTACCC <b>C</b> TATGCAGGTAAGC
ptf1a/p48-3-3	GGAA <b>G</b> AC <b>C</b> CT <b>T</b> GTGC <b>A</b> CT <b>T</b> CT <b>G</b>	scaffold_81:(1528612)	ATTGTTATGG <b>A</b> CAATGGCT	AAATAGTAG <b>C</b> GCACAGCACAA
ptf1a/p48-3-4	GGAG <b>G</b> AC <b>T</b> GTGC <b>A</b> CT <b>T</b> CT <b>G</b>	scaffold_9095:(7960)	CTTC <b>G</b> TCT <b>T</b> GGAG <b>C</b> CTAGA <b>A</b> TC	GCATATTG <b>C</b> TAT <b>CG</b> GTGTAG
ptf1a/p48-4-1	GGAA <b>G</b> GGATT <b>T</b> TGACT <b>T</b> CT <b>G</b>	scaffold_11680:(787)	CAACCAAGAT <b>GG</b> CATTGGAG <b>T</b> AC	CCTGTTGGAGCTTGGACTGC
ptf1a/p48-4-2	GGAA <b>A</b> AC <b>G</b> CT <b>T</b> GT <b>G</b> ACT <b>T</b> TT <b>G</b>	scaffold_125:(2489873)	GGAT <b>T</b> AAAGTGTGCC <b>A</b> TAGGAC	TATCG <b>G</b> TATT <b>C</b> ATCACCTGG
ptf1a/p48-4-3	GGAA <b>G</b> AC <b>A</b> GG <b>T</b> GT <b>G</b> ACT <b>T</b> AA <b>AG</b> GG	scaffold_128:(556723)	TACAA <b>G</b> TGC <b>A</b> TGTGTGC <b>A</b> GG	TTAGGTAAACAGATTAGGCCATG
ptf1a/p48-4-4	TGAAG <b>A</b> AT <b>G</b> AT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_139:(1729414)	ATCCC <b>A</b> CA <b>A</b> ATTAGGTT <b>T</b> TC	TCTG <b>C</b> AAA <b>A</b> CATCAG <b>C</b> AAATC
ptf1a/p48-4-5	GGAA <b>A</b> GC <b>T</b> GT <b>G</b> AC <b>C</b> CT <b>C</b> T <b>G</b>	scaffold_144:(1611751)	CAGTT <b>C</b> TT <b>A</b> TAGGGGG <b>T</b> GG <b>T</b> TC	CGGAA <b>A</b> TT <b>A</b> CACTACC <b>C</b> CCAC
ptf1a/p48-4-6	GGAT <b>G</b> AG <b>G</b> TGT <b>G</b> ACT <b>T</b> CC <b>A</b> GG	scaffold_150:(1771007)	TGACGG <b>T</b> GT <b>C</b> TATTACAAA <b>A</b> T	ACTAGTGGAAAG <b>C</b> ATA <b>T</b> GTAGC
ptf1a/p48-4-7	GGAG <b>G</b> AG <b>G</b> TCT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_155:(58578)	AATTATGC <b>C</b> TT <b>G</b> TA <b>A</b> AT <b>G</b> TT <b>C</b> AGC	AGTTGC <b>C</b> TA <b>G</b> T <b>A</b> T <b>C</b> CT <b>C</b> CAG <b>A</b> TG
ptf1a/p48-4-8	GGAG <b>G</b> AG <b>G</b> TCT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_155:(58617)	TTC <b>C</b> AC <b>A</b> GT <b>T</b> CT <b>A</b> AAA <b>A</b> GT <b>C</b> A <b>G</b>	TACATTGG <b>A</b> T <b>C</b> A <b>T</b> CT <b>G</b> T <b>G</b> CTA
ptf1a/p48-4-9	GGAA <b>G</b> T <b>C</b> GT <b>T</b> GT <b>G</b> AC <b>G</b> T <b>C</b> T <b>T</b> <b>G</b>	scaffold_1804:(27886)	AAAT <b>C</b> CT <b>A</b> AG <b>C</b> TT <b>T</b> TC <b>G</b> C <b>A</b> G	GAG <b>T</b> G <b>C</b> AG <b>C</b> AG <b>T</b> ATT <b>C</b> AAAG <b>A</b> GG
ptf1a/p48-4-10	GGAA <b>G</b> AC <b>T</b> GT <b>G</b> AG <b>G</b> AC <b>C</b> T <b>T</b> <b>G</b>	scaffold_2:(2433980)	AAAC <b>G</b> CT <b>T</b> GG <b>T</b> CT <b>C</b> T <b>C</b> T <b>G</b> C	AGACATT <b>T</b> G <b>C</b> TT <b>T</b> TG <b>C</b> TT <b>T</b> G
ptf1a/p48-4-11	AGGAG <b>A</b> CG <b>T</b> GT <b>G</b> GG <b>C</b> CT <b>T</b> <b>G</b>	scaffold_2:(4338728)	TGTT <b>T</b> GT <b>A</b> AC <b>C</b> CC <b>G</b> CC <b>A</b> G <b>A</b> CC	AAC <b>C</b> CT <b>C</b> ACT <b>T</b> CC <b>A</b> C <b>A</b> T <b>G</b> G <b>C</b> TA
ptf1a/p48-4-12	GCAG <b>G</b> AC <b>T</b> GT <b>G</b> AA <b>T</b> TT <b>C</b> T <b>G</b>	scaffold_212:(1294306)	AGAAG <b>C</b> AT <b>A</b> GT <b>T</b> GT <b>G</b> AT <b>C</b> TT <b>T</b> <b>G</b> C	CGT <b>G</b> T <b>C</b> T <b>T</b> AG <b>C</b> CC <b>T</b> TA <b>A</b> AGC
ptf1a/p48-4-13	GTAAG <b>T</b> AG <b>T</b> GT <b>G</b> ACT <b>T</b> ATT <b>G</b>	scaffold_24:(1548124)	ATTAA <b>A</b> AG <b>C</b> AA <b>A</b> AT <b>G</b> AC <b>C</b> AT <b>T</b> GT <b>G</b>	AAT <b>GG</b> C <b>A</b> CT <b>T</b> CT <b>C</b> AC <b>C</b> CT <b>A</b> AG
ptf1a/p48-4-14	TGTAG <b>A</b> CC <b>T</b> GT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_242:(750479)	CTAT <b>C</b> AC <b>T</b> GG <b>G</b> GT <b>A</b> AT <b>G</b> AA <b>A</b> AC	GCAG <b>C</b> AA <b>C</b> AC <b>T</b> TT <b>C</b> TA <b>A</b> AC <b>A</b> G
ptf1a/p48-4-15	GGAAG <b>G</b> GT <b>T</b> TT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_267:(152383)	TTGC <b>A</b> TT <b>C</b> T <b>A</b> TT <b>G</b> AG <b>T</b> GA <b>A</b> AA <b>A</b> AG	GT <b>C</b> TT <b>A</b> CC <b>C</b> T <b>C</b> T <b>A</b> T <b>G</b> CAG <b>T</b> AA <b>A</b> C
ptf1a/p48-4-16	GGAAG <b>G</b> GT <b>T</b> TT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_268:(337535)	GAAG <b>C</b> AT <b>T</b> GT <b>G</b> AA <b>C</b> CC <b>T</b> C <b>A</b> GG	GT <b>C</b> TT <b>A</b> CC <b>C</b> T <b>C</b> T <b>A</b> T <b>G</b> CAG <b>T</b> AA <b>A</b> C
ptf1a/p48-4-17	GGAAG <b>G</b> GT <b>T</b> TT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_268:(342264)	AGT <b>G</b> ACT <b>G</b> GT <b>T</b> CC <b>A</b> TT <b>T</b> CC <b>A</b> TT <b>T</b> CC	CAG <b>C</b> AT <b>C</b> AC <b>C</b> T <b>G</b> GT <b>A</b> AC <b>A</b> C
ptf1a/p48-4-18	GGAAG <b>G</b> GT <b>T</b> TT <b>G</b> ACT <b>T</b> CT <b>G</b>	scaffold_268:(348088)	TTT <b>A</b> CT <b>T</b> GT <b>G</b> CT <b>T</b> GG <b>C</b> GG <b>A</b> GG	GGGT <b>C</b> TT <b>A</b> CC <b>C</b> T <b>C</b> T <b>A</b> T <b>G</b> CAG <b>G</b>
ptf1a/p48-4-19	GGAG <b>G</b> GG <b>A</b> AT <b>G</b> AA <b>T</b> CT <b>G</b>	scaffold_274:(172841)	GAAG <b>G</b> ATT <b>A</b> GC <b>A</b> GG <b>G</b> GT <b>A</b> TT <b>G</b>	AT <b>C</b> AA <b>C</b> TT <b>T</b> T <b>C</b> TT <b>GG</b> GT <b>C</b> AT

Site ID	Potential Off Target Site	Coordinate (Start)	Forward PCR Primer	Reverse PCR Primer
ptf1a/p48-4-20	GGAAGAGGATTGACTTCCTGG	scaffold_291:(113367)	TTGCAGACTAGCATGTCCACATC	ACACTGCCTGAGAATGGGAATC
ptf1a/p48-4-21	GGAAGCCAAGTGCACTTCTGGG	scaffold_387:(351500)	GATTATTCAAGGTGCTGCTGAGG	ACAGGGGCTCCCTTATAACC
ptf1a/p48-4-22	GGAAGATTATGTCATTTCTGGG	scaffold_408:(152759)	CTAAGGGCAGGTAAAGGCTCTCT	CCACAAGGAGGTGATACAGGAGT
ptf1a/p48-4-23	GGAGGACAATATGGACTTCAGG	scaffold_441:(983968)	GCAGACTCTCGTCGATTATGC	AACATCTGCTGCAAGATAAAACC
ptf1a/p48-4-24	GGAAGAGGATTGACTTCCTGG	scaffold_569:(721734)	TTCTCCATAGTTGTTGAAATGAGTG	CTCCTGTTGGAGCTTGGACT
ptf1a/p48-4-25	GGAAGAGGATTGTTAGTTCTGG	scaffold_625:(443803)	GTGCATTCTGGATAATGGGTC	CTCATTATATATCAGTCCTGC
ptf1a/p48-4-26	GAAAGAGGATGTGCCACTTGG	scaffold_63:(205522)	CATCTTCCGATCAGCACGTTTC	GTTCTCTTGTGCTGATATTTC
ptf1a/p48-4-27	GGAAGAGGATTGACTTCCTGG	scaffold_6344:(1703)	GTTTGAGAGACCAACATCCCC	ACTGCTCAGCTACCCCTGACTG
ptf1a/p48-4-28	GGATGACGTGAGACTTGTGG	scaffold_649:(10287)	CTTTGCCTATGGAGGCTATTG	GTGGTGGCTACTGAAGCAGAC
ptf1a/p48-4-29	GGAA TACCTGTGGAATTGGG	scaffold_653:(376103)	ATTTAGTCGCCATAATATGCACTAC	GTACCCCTGACTACTTCATCCT
ptf1a/p48-4-30	GGAACACTATTGGAACCTCTGG	scaffold_709:(400270)	CCTGCCCTAAGCTAGTGTGAC	GGCGGATACAACTCTGTATTG
ptf1a/p48-4-31	GGAGGAAGATGTGGATTTCCTGG	scaffold_742:(350425)	GTAGCAGATTGCATCTACTTCAGTC	GGGATTGCCTATGTCATT
ptf1a/p48-4-32	AGAAGACGATGGGGCTACTGGG	scaffold_782:(110628)	GCAGCAATGATTCAAGGACTTC	TGATGTTGGGCTCTATACG
ptf1a/p48-4-33	GGAAGAGGATTGACTTCCTGG	scaffold_791:(6875)	ACAGTTGAGAGAACACATCCC	ACTGCTCAGCTACCCCTGACTG
ptf1a/p48-4-34	GGAAGAAGATGGGGCTACTGGG	scaffold_8389:(8767)	GCAAATAATCGAGCAGAAAATAAG	GGAGAACTAAAGGTTAGGAC
ptf1a/p48-4-35	AGAAGACAATATGGATTCTGG	scaffold_93:(1041592)	GCAGATCCAAAGAGATATAGTTG	TGAATCTATTGCCAGTAAATGC
ptf1a/p48-4-36	GAAAGAGGATGTGCCACTTGG	scaffold_9648:(349)	GCATAGGGAGCTGAGATGAAG	GTGTTACTTTGATTTGGCCGC
tyrosinase-3-1	GACCCCCAGTTCCCTTCACTGG	scaffold_440:(120683)	CACACTGCAGAACCTCATTCAAC	CGTGTGAACTGACCACAAAC
tyrosinase-3-2	GGCCTTGTAGTTCCAGTCACTGG	scaffold_99:(2814102)	GTGGGATTCAGGGTGTGATG	GAATGGAATGCTTCCACCTGC
tyrosinase-4-1	GGCAATCAGTTCCAATGACAGG	scaffold_10:(1775587)	CTCCTAGATCCCTGAAATTGAAG	TTTGTGGACATATGAATGTTATTG
tyrosinase-4-2	GGCTCCCAGTTACAATCACTGG	scaffold_10:(3396052)	CAAATACAGTCCACCAGAAAGGC	TAACTCCTCAACCGACACAGATT
tyrosinase-4-3	GACCTCAGTTCTGTTCAAGG	scaffold_101:(2091511)	TTAGAATTGCCAGTGATTAGGGA	CTGGTGAGAAAGCATTCTGTA
tyrosinase-4-4	TGGCCTCAGTTACTTCACTGG	scaffold_102:(757505)	AATGATTGATTAATGGCACCA	CTTCTTATAACACTGGCATGCTG
tyrosinase-4-5	CGCCCTCAGTTCTAGTCATGG	scaffold_105:(172804)	GCTTCTTGTTCAGAAATAACCCAT	TATCTAATTCTGGCTTAGGAG
tyrosinase-4-6	GGCCCTTAGTGCCCATACACGGG	scaffold_106:(171699)	GGTGGGGATATCAGGCTAAT	AACAAATGCACATGCAATTGATT
tyrosinase-4-7	TGCGTCAGTCCATTACACGGG	scaffold_123:(654169)	GTGGACATGGCCATTATTATTG	CCCACTTAAAGAGACAGTCAGAGAC
tyrosinase-4-8	GTCTCTCAGTTCCATGCACTGG	scaffold_12888:(1272)	GTCAGGTCTGTGTTTGAGGTTG	GAAATTGATAAAGCATATAAAGGGA
tyrosinase-4-9	GGCCCTCGGTTCCCGCACAGG	scaffold_137:(2005057)	CGAAAGGCAATGCTCTGTCA	TGTGGGTTCTGAGCAAGCGTA
tyrosinase-4-10	GCCCTCAGTGTTCATTCAACGGG	scaffold_14:(5389042)	GCAGCTCAGCAGTAAAGTGAGA	AGTTTGCCTAATGTAAGCAGAT
tyrosinase-4-11	GTGCCTCAGTTGCATTCCCTGG	scaffold_143:(518441)	TTTGGTAAAACCTCCATGTTAGTAT	ATGTAGCGTTGCACATAGTAGCA
tyrosinase-4-12	GGCCCTTCAGCTCCATTGCCCTGG	scaffold_149:(202527)	TGCTCCTCTGGAACAGATGT	CTACAGGATGTGGCTATGACTAT
tyrosinase-4-13	GCCCCTCATGTTCCCTTCACAGG	scaffold_180:(662866)	GGCTGCAAATAAAACAAATATGTC	TATAAAATCCGTTTTGTTCC
tyrosinase-4-14	GGCATA CAGTTACATTCACTGG	scaffold_210:(341070)	GACAGTTCCTGACTCTCTGATTA	ACAGCCCTATCTCCTTCATTGG
tyrosinase-4-15	GGCAGTCAGATTCCATTCAACGGG	scaffold_228:(627447)	AAACAGAAATAAACATGGAGTTG	TGAGACTTCCTCAAGGAAGAG
tyrosinase-4-16	GCCCCTAAGTGTCCACTCACTGG	scaffold_27:(1013047)	GTAGCCGTTACGACTTGCAG	TTAAATCTGTTTCATTCTGGACTT
tyrosinase-4-17	GGGCATCAGTTACCATACACAGG	scaffold_274:(1373068)	AGTTTCCCTAGACGCATGGCAA	GCCCAACGAAAGTGGCTGAT
tyrosinase-4-18	GGCCGTCAGGTTCCATCAACTGG	scaffold_277:(630877)	AGGTAATCAGGGCAATATAGAATAA	CAGTTGTACAAAGCTGTTCAAATA
tyrosinase-4-19	GAGCCTCAGTTCTATTCCAGG	scaffold_305:(90979)	GCTGCAATTGTTAGTAAACG	GTTTGTGATTTCTTAAACATTGGC
tyrosinase-4-20	GTCCCCTCAGTTCACTTACAGG	scaffold_33:(2154089)	CAGGGACTGGTTGCCATCTT	TGTAGCACACAACACATCAGTAAT
tyrosinase-4-21	GGCCCTCATTTCTATTACAGG	scaffold_359:(468247)	AGCCAAACAGATTATTCTCTCA	TAAATTGAATAGTTGAGAACAC
tyrosinase-4-22	TGTCCTCAGTTCACTTACAGG	scaffold_409:(662043)	GTGAAATGCACCTGCACTCTAT	GTGGGTGAATAATCAGAGTGGTT
tyrosinase-4-23	GTCCCACAGTTCCATTATTGG	scaffold_414:(811399)	AGAGTGTGTTGTAGATGTATGTA	GCTTGTGTCGCTTCCCTTCTT
tyrosinase-4-24	GCCCCTTAATGTCATTACAGG	scaffold_441:(601770)	AAACGTTGCGCCTTTAAGTTA	GACAGCAGACTATGGCTACTTACC
tyrosinase-4-25	GGCTCCCATTTCCATTCCCTGG	scaffold_451:(852715)	ACAGAGAGATTCTGATATTCTGCTT	GCGACAATCTGCAGTTACAATA
tyrosinase-4-26	GGTGCACAGTTCCATGCACTGG	scaffold_459:(263553)	CGTCAAGAACATTGCGGTGAA	AGGATGTCTCAAACCATAAATCTG
tyrosinase-4-27	GCCTCTCTGTTCCATTACAGG	scaffold_479:(495121)	ATGGACAGAGAGATCAAAGATAGA	GTCCAAGGAAAGGGGACACA
tyrosinase-4-28	GTCTCTCAGTTCCATGCACTGG	scaffold_491:(181703)	GTCATGGCTATGGTTCTAGATTC	GCTATGCTAATATGAACCATGACCA
tyrosinase-4-29	GTCTCTCAGTTCCATGCACTGG	scaffold_491:(194802)	TTTGAGGTTGCACACTTCTCATT	TTCTTGTGACAGACCCATAATCCTT
tyrosinase-4-30	TGCCCTCAGTTACATGCACTGG	scaffold_5:(5861390)	ATATAGGGAGATGCTCTGAGTC	TTAGTTAAAATCTCTGATGTGCG
tyrosinase-4-31	CGCAATCAGTTGCCATTCACTGG	scaffold_537:(315212)	CCGAGGCAGAAGGACTAGCTAT	CACAGGAAAGAACAGAGGCAT
tyrosinase-4-32	GGCCATCAGTTCTTACACAGG	scaffold_57:(2987708)	AGACATCAGGCTCAGTTACTTGC	ATCACACTAGAATATGGATCACCAC
tyrosinase-4-33	GACAGGAGTTCCATTCACTGG	scaffold_570:(345550)	GCCATGAAAGCTTGGAGATAATTTC	CGTAGTGTGCTATTTCAGCAGGT
tyrosinase-4-34	GGCCCTGTGTTCCAGGCCACAGG	scaffold_577:(227920)	CTGTCAGTTCACTGCCCTGCA	CGCTGTCACGGTAGCCTTATC
tyrosinase-4-35	GTCCCCTCGTTCCATTAGAGG	scaffold_64:(3047845)	CTGCTTCTGTAGTATAACTGGTAC	TGTAACACCCCTGTAACAGAGAAATGG
tyrosinase-4-36	GGCTTCACTTACATTCTCAGG	scaffold_937:(132022)	CAAATGTCATCTACAATAATCAG	ATATGACTGAAACAGCCTGGTGA
tyrosinase-4-37	GGCCCTCAGTCTGCACACTGG	scaffold_99:(530571)	TTTCACTCCAACCTGGCCTTCA	GCAGCTCAATAAGGTCATTGTAT
tyrosinase-4-38	GGCCTTCAGTCTGCACACTGG	scaffold_99:(551360)	TTTCACTCCAACCTGGCCTTCA	GCAGCTCAATAAGGTCATTGTAT
tyrosinase-4-39	GGCCAGCAGCTCCATTCACTGG	scaffold_992:(42836)	TTTGTGACCTTGGGAATGAATTCTGTT	CCTCATTGTGGTGTGGCGT
tyrosinase-4-40	GGCCAGCAGCTCCATTCACTGG	scaffold_992:(62574)	TTGTGACCTTGGGAATGAATTCTGTT	CCTCATTGTGGTGTGGCGT

In a given potential off-target sequence, the red letters indicate the nucleotides that are different from those in the corresponding on-target site illustrated in Table 1.

**Table S4.** List of PCR primers used to amplify the targeted loci.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>elastase</i> -T1	GGAGTGGCATCATACTGACTGTC	AAGGGAAGGGATGTAAGAGC
<i>elastase</i> -T2	ATGGTCATCCAATCTGGAAGC	CTGTCCCTTCCGTAATGATGC
<i>ets1</i> -T1	GGTTCGTGTGATACAAGTACC	AAAAGTATGTTCAACCCAAGCC
<i>ets1</i> -T2	GAATCTGCTTGTTCTTCAGGAAC	GCAGTAATGAGATGGTCACGG
<i>ets1</i> -T3	GAATCTGCTTGTTCTTCAGGAAC	GCAGTAATGAGATGGTCACGG
<i>ets2</i>	CCAAATTAGAAGGCTTCCATGTAG	CAATATTAACAGAGTTGGCACCG
<i>grp78</i>	AAAC CCTATTGAATTAGTTGGAGGC	TCCCTTAACATGTGACTCAAACC
<i>hhex</i>	ATGTTGATTCCGATCTCTCATTT	CCCATAACAATGGCAGTTAGTTG
<i>pat</i> -T1	AAATCCATGCTGCATTTACAAG	AACAAGCTGCTCTTATATCTAATGC
<i>pat</i> -T2	CCATATAATTGCATGTGGCATAC	AAATAACCACATCTGCTCTGAGGG
<i>pat</i> -T3	CCATATAATTGCATGTGGCATAC	AAATAACCACATCTGCTCTGAGGG
<i>pdx1</i> -T1	CGTGCAACCACAGCTAAATAGTG	CGGAGTCTGAATATTGCACC
<i>pdx1</i> -T2	AACAGGTGTCCACTGCCAAG	CCCAGCCTGAGTCTCCTACTC
<i>pdx1</i> -T3	AACAGGTGTCCACTGCCAAG	CCCAGCCTGAGTCTCCTACTC
<i>ptf1a/p48</i>	GCAGAACGCAATGCTATG	GGATGAGAAGGAGAAGTTGCC
<i>tm4sf4</i> -T1	GCATGATAACATTAAGGGCACA	CAAATTCCAAGGCCATCTC
<i>tm4sf4</i> -T2	GGAGGTTAGTGGCTAAGGTAAT	CTGACAAACAGCAGGATAAAGAT
<i>tyrosinase</i>	GAGATCCTGCGTGTCAATTGG	CATAGGGTTGGAGCCATTATTC

**grp78 88.9% (16/18)**

GACCATGAAGCTGTTGCCTCCTGGTGTCTGCCAGCGTATTGCTGATGA Wild-type

GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ2) [×2]  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ3,+1)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ6)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ9) [×3]  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ10)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ11)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ14)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ48)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (+1)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ4,+14)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (+5) [×2]  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ9,+24)  
 GACCATGAAGCTGTTGCCTGCTGGTGTCTGCCAGCGTATTGCTGATGA [×2]

**elastase Target 1 100% (20/20)**

CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA Wild-type

CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ1)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ3)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ3)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ3)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ5) [×3]  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ5,+4)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ6,+1)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ6)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ6)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ9) [×2]  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ11)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ14)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ15)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ17)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ7,+11)  
 CCCTCAGTACCTGTCCTGGTGGAGCTTACCTCTGGTGTCTGCCAGCGTATTGCTGATGA (Δ18,+1)

**elastase Target 2 87.5% (14/16)**

AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT Wild-type

AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ1)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ1,+1)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ1)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ5)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ5)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ5)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ16,+9)  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ11) [×2]  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ13) [×2]  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ13) [×2]  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT (Δ19) [×2]  
 AGTCACCATGGTGTGGCTGGAGTCCGTTCAAGATGCCAGGTACAATGAT [×2]

**ets1 Target 1 33.3% (6/18)**

TTCAAGAGTTCTGTATGAGCGGAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG Wild-type

TTCAAGAGTTCTGTATGAGCGGAGCAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG (Δ5) [×2]  
 TTCAAGAGTTCTGTATGAGCGGAGCAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG (Δ5)  
 TTCAAGAGTTCTGTATGAGCGGAGCAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG (Δ8,+6)  
 TTCAAGAGTTCTGTATGAGCGGAGCAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG (Δ17)  
 TTCAAGAGTTCTGTATGAGCGGAGCAGCATTATGTCCTGGTGTCTGGCCTGGGAAGGAATGTTTCTAG (Δ20) [×12]

**ets2 100% (21/21)**

AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT Wild-type

AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ1,+3) [×2]  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ2) [×2]  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ2,+5)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ3,+7)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ3,+1)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ4)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ5)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ6)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ6) [×2]  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ8) [×2]  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ9)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ13,+5)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ13,+5)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ16)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ38)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ+2)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ+4)  
 AGAACAGCGGTACCAACGGCTGGACTCTTACTCTCATGGTATGTCCTGCTTACCT (Δ+6)

**hhx 100% (18/18)**

AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG Wild-type

AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ1,+3)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (+7)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ8) [×9]  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ14)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ15)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ16)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ16)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ17)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ23)  
 AGAATAGCCATAGCAGTAACGACCCCCAGCTCCAGGCCCTGGGCTCAGTGTCCCCCTG (Δ40)

**ptf1a/p48 72.2% (13/18)**

TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT Wild-type

TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ8)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ9)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ9) [×4]  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ11)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ14,+10)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ20)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ28)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ52) [×2]  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT (Δ55)  
 TGGACCCAGACGACTTTTCTGGAGAGCTTCTGGCCGGTCAAGTCAAGACT [×5]

**pat Target 1 10% (2/20)**

GTAGCATTGGGGATATTGGCTGTAAGCAAAACATTCTGGTTAGATAAGGTTCT Wild-type

GTAGCATTGGGGATATTGGCTGTAAGCTTCTGGTTAGATAAGGTTCT (Δ24)  
 GTAGCATTGGGGATATTGGCTCTGGCT (Δ26)  
 GTAGCATTGGGGATATTGGCTGTAAGCAAAACATTGGTTAGATAAGGTTCT [×18]

**tm4sf4 Target 1 86.7% (12/14)**

TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC Wild-type

TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ2,+5)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ3)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ4) [×2]  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ4)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ4,+4)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ10,+10)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ11,+10)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ13)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ14,+2)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ16)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC (Δ27)  
 TAACCTGAAAATGTGTCCTGGTGTGGCAAGTGCCTGGCATTACCTTATACAC [×7]

**tm4sf4 Target 2 100% (20/20)**

GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG Wild-type

GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ2)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ2)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ2,+1)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ3)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ4)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ5,+6)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ6)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ6,+3)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ6,+13)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ6,+13)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ7,+9)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ9,+6)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ10)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ12) [×2]  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ12) [×2]  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ13)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ14)  
 GGCCATTCAAGCTATCAACGGACTCATAGGAACATCTGGGACTGCAATGCTGTG (Δ24)

**tyrosinase 82.4% (14/17)**

TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA Wild-type

TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ3)  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ4) [×3]  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ4) [×4]  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ5)  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ5) [×2]  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ6)  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ6,+15)  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA (Δ17)  
 TCCCTGACCAGCTCTGCTACTGGCCCTCAGTTCCATTCTGGGACTGACGATAGAGAGA [×3]

**Fig. S1.** DNA sequencing data show the targeted mutations induced by gRNA/Cas9 targeting the loci illustrated in Fig. 2A. For all the panels, the wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue text. Mutated regions are shaded in gray with red dashes indicating deletions and lowercase letters in red indicating insertions. The numbers in parentheses show the number of deleted (Δ) or inserted (+) base pairs, whereas numbers in square brackets show the frequencies of the mutation in all the sequenced samples.

***etsI* Target 2 76.9% (10/13)**

```

AACATGCACAGTGTGTTCCGCCCTCTGAATTCTCTGAACCAGCTTCATCACAGAGTCAT Wild-type
AACATGCACAGTGTGTTCCGCT-GAATTCTCTGAACCAGCTTCATCACAGAGTCAT (Δ2) [×2]
AACATGCACAGTGTGTTCCG-TGAAATTCTCTGAACCAGCTTCATCACAGAGTCAT (Δ4)
AACATGCACAGTGTGTTCC-TGAAATTCTCTGAACCAGCTTCATCACAGAGTCAT (Δ6)
AACATGCACAGTGTGTT-TGAAATTCTCTGAACCAGCTTCATCACAGAGTCAT (Δ7) [×2]
AACATGCACAGTGTGTT-GTAACCCAGCTTCATCACAGAGTCAT (Δ9)
AACATGCACAGTGTGTTCCG-GAACCCAGCTTCATCACAGAGTCAT (Δ12)
AACATGCACAGTGAATT-TCTGAACCCAGCTTCATCACAGAGTCAT (Δ14)
AACATGCACAGTGTGacagtgtgtTCTGAATTCTCTGAACCCAGCTTCATCACAGAGTCAT (Δ9,+11)
AACATGCACAGTGTGTTCCGCCCTGAATTCTCTGAACCAGCTTCATCACAGAGTCAT [×3]

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***pat* Target 2 57.9% (11/19)**

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CACAGACTCTGAATCCAAAGGCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAAC Wild-type
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCGAA-TGGCAGCTTGGAAAGAAC (Δ2,+1)
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCG-TGGCAGCTTGGAAAGAAC (Δ3) [×2]
CACAGACTCTGAATCCAAAAGGCACATTCTGATGC-AGTGGCAGCTTGGAAAGAAC (Δ3)
CACAGACTCTGAATCCAAAAGGCACATTCTGAT-GCAGTGGCAGCTTGGAAAGAAC (Δ3) [×3]
CACAGACTCTGAATCCAAAAGGCACATTCTGAT-AGTGGCAGCTTGGAAAGAAC (Δ9)
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCG-TCTGGAAAGAAC (Δ9)
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCG-AGTGGCAGCTTGGAAAGAAC (Δ9)
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCG-TGGCAGCTTGGAAAGAAC (Δ11)
CACAGACTCTGAATCCAAAAGGCACATTCTGATGCGAGCTTGGCAGCTTGGAAAGAAC [×8]

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***pdx1* Target 2 75% (15/20)**

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CACACCACATCATCACACCATCACCCCAGGGATCCCTCACCCCACAGCAATGCCATTC Wild-type
CACACCACATCACACCACATCACACCACCAGGGATCCCTCACCCCACAGCAATGCCATT (Δ2)
CACACCACATCACACCACATCACACCACCAGGGATCCCTCACCCCACAGCAAATGCCATT (Δ3)
CACACCACATCACACCACATCACACCACCGGATCCCTCACCCCACAGCAAATGCCATT (Δ4)
CACACCACATCACACCACATCACACCACCGGATCCCTCACCCCACAGCAAATGCCATT (Δ4) [×2]
CACACCACATCACACCACATCACACCACCGGATCCCTCACCCCACAGCAAATGCCATT (Δ6)
CACACCACATCACACCACATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ6,+5)
CACACCACATCACACCACATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ6,+6)
CACACCACATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ7,+10)
CATACCATACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ14,+5)
CACACCATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ27) [×2]
CACACCATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ30,+7)
CACACCATCACACCACCGGGGATCCCTCACCCCACAGCAAATGCCATT (Δ50) [×5]

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***etsI* Target 3 66.7% (10/15)**

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AATATGAGAATGACTACCCTTTAGCATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG Wild-type
AATATGAGAATGACGACCC-CATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ6)
AATATGAGAATGACTAAC-TTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ6) [×2]
AATATGAGAATGACTAC-CATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ6) [×2]
AATATGAGAATGACT-ATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ7)
AATATGAGAATGACT-CATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ7)
AATATGAGAATGA-CATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ11)
AATATGAGAATGA-CATTGCTGCGTGACCCCTGCAGCCTGAATCTCAGG (Δ47)
AATATGAGAATGACgACcaCCgTGCTGCGgACCCCCgGCaCCaGATTCTG (Δ11,+11)
AATATGAGAATGACTACCCCCTTAGTTGCTGACCCCCTGACCCTGATTCAG [×5]

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***pat* Target 3 78.9% (15/19)**

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GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAACAGTGGAGTACATTCACCATCT Wild-type
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAACAGtACAGTGGAGTACATTCACCATCT (Δ2)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtACAGTGGAGTACATTCACCATCT (Δ2,+2)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGC-TGGAGTACATTCACCATCT (Δ3,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGC-ACATtTGGAGTACATTCACCATCT (Δ2,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGC-ACATtTGGAGTACATTCACCATCT (Δ5,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGC-ACATtTGGAGTACATTCACCATCT (Δ5,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGC-ACATtTGGAGTACATTCACCATCT (Δ6,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (+7)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ3,+6)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ9,+1)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ11)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ11) [×2]
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ11)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ11) [×2]
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT (Δ2,+11)
GCACATTCTGATGCAGCAGTGGCAGCTTGGAAAGAGtTGGAGTACATTCACCATCT [×4]

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***pdx1* Target 3 45% (9/20)**

```

ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA Wild-type
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ4)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ4)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ4)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ12)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ5,+7)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ7,+9)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ9,+5)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ19)
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA (Δ36) [×11]
ACCCAGGGATCCCTACCCACCCAGCCATCCTCAGATGCATCTGCAA [×11]

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Fig. S2. DNA sequencing data show the targeted mutations induced by gRNA/Cas9 targeting the genes showed in Fig. 2H. For all the panels, the wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue text. Mutated regions are shaded in gray with red dashes indicating deletions and lowercase letters in red indicating insertions. The numbers in parentheses show the number of deleted (Δ) or inserted (+) base pairs, whereas numbers in square brackets show the frequencies of the mutation in all the sequenced samples.

<b>ets2 m1 0% (0/19)</b>	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		<b>ets2 m15 18% (3/17)</b>	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	[×19]	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	(Δ5,+12)
<b>ets2 m3 0% (0/20)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	(Δ2)
	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC	[×14]	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	(Δ31)
<b>ets2 m5 0% (0/20)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		<b>ets2 m17 18% (3/17)</b>	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	[×20]	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ8) [×2]
<b>ets2 m7 0% (0/20)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ8)
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	[×20]	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ8)
<b>ets2 m9 0% (0/19)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ2) [×5]
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	[×19]	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ5)
<b>ets2 m11 0% (0/18)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ24)
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC	[×18]	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cACT</b> <b>catggactca</b> TGGTATGTGTCTTGCCTTCCC	(Δ8)
<b>ets2 m12 10% (2/20)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		<b>ets2 m19 68% (13/19)</b>	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCAT <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ2)		AAGAACACGGCGGTACCAACGGGCTGGACTCTTACT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ2)	[×5]
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ4)		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ8)	
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b>	[×18]	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ8)	
<b>ets2 m13 53% (10/19)</b>	AAGAACACGGCGGTACCAACGGC <b>GGTCTGGACTCTTACTCTCAT</b> <b>TGG</b> TATGTGTCTTGCTTCCC Wild-type		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>GGACTCTTAC</b> <b>GGGTCTGGA</b> CATGGTATGTGTCTTGCCTTCCC (Δ9,+1)	
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ2) [×3]		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>GGACTCTTAC</b> <b>GGGTCTGGA</b> CATGGTATGTGTCTTGCCTTCCC (Δ11,+9)	
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ4,+2)		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>GGACTCTTAC</b> <b>GGGTCTGGA</b> CATGGTATGTGTCTTGCCTTCCC (Δ17)	
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ8) [×2]		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>GGACTCTTAC</b> <b>GGGTCTGGA</b> CATGGTATGTGTCTTGCCTTCCC (Δ19)	
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ9)		AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>GGACTCTTAC</b> <b>GGGTCTGGA</b> CATGGTATGTGTCTTGCCTTCCC	[×6]
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ5,+7)		<b>ets2 m20 6% (1/16)</b>	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCATGGTATGTGTCTTGCTTCCC Wild-type
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ18)		AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCATGGTATGTGTCTTGCTTCCC	(Δ7,+1)
	AAGAACACGGCGGTACCAACGGGCTGGACTCT <b>cT</b> <b>ATGGTATGTGTCTTGCTTCCC</b> (Δ27)		AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCATGGTATGTGTCTTGCTTCCC	[×15]
	AAGAACACGGCGGTACCAACGGGCTGGACTCTTACTCTCATGGTATGTGTCTTGCTTCCC	[×10]		

Fig. S3. DNA sequencing data show the consequence of single-nucleotide mismatches between the spacer and the protospacer sequences on the Cas9 mediated gene targeting efficiencies as illustrated in Fig. 3A for *ets2* loci. For all the panels with mutations, the wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue text. Mutated regions are shaded in gray with red dashes indicating deletions and lowercase letters in red indicating insertions. For all the panels without mutations, only the wild-type sequence is shown. The numbers in parentheses show the number of deleted (Δ) or inserted (+) base pairs, whereas numbers in square brackets show the frequencies of the mutation in all the sequenced samples.

Fig. S4. DNA sequencing data show the consequence of single-nucleotide mismatches between the spacer and the protospacer sequences on the Cas9 mediated gene targeting efficiencies as illustrated in Fig. 3B for *tm4sf4*-T2 loci. For all the panels with mutations, the wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue text. Mutated regions are shaded in gray with red dashes indicating deletions and lowercase letters in red indicating insertions. For all the panels without mutations, only the wild-type sequence is shown. The numbers in parentheses show the number of deleted ( $\Delta$ ) or inserted (+) base pairs, whereas numbers in square brackets show the frequencies of the mutation in all the sequenced samples.

**elastase T1 + grp78 gRNA****elastase Target 1 100% (16/16)**

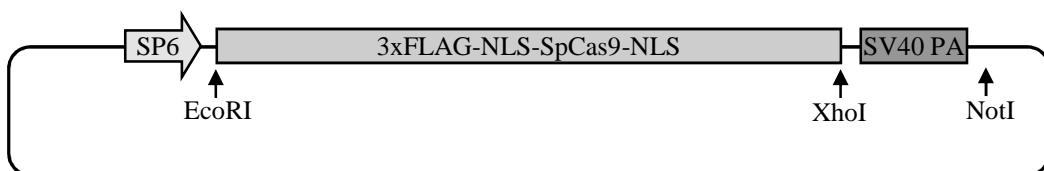
CCCTCCAGTACCTGTCTGGT **GGCAGTTGGTACCATACCTG** **TGG** GGGCTCCCTGATCCGTG Wild-type  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCATAC **TGT** GGGGGGCTCCCTGATCCGTG (Δ1)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCATAC **Tag** GGGGGGCTCCCTGATCCGTG (Δ3, +2)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCA **gacaggagat** GGGGGGCTCCCTGATCCGTG (Δ4)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCA **gacaggagat** GGGGGGCTCCCTGATCCGTG (Δ4, +10)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCA **atac** GGGGGGCTCCCTGATCCGTG (Δ5)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCA **atac** GGGGGGCTCCCTGATCCGTG (Δ5, +4)  
 CCCTCCAGTACCTGTCTGGCAGTTGGTACCA **gacaggtaactgagggagatc** GGGGG (Δ6, +21)  
 CCCTCCAGTACCTGTCTGGCAGTTGG **aACCATct-gt** GGGGGGCTCCCTGATCCGTG (Δ6, +5)  
 CCCTCCAGTACCTGTCTGGCAGTTGG **ggggg** GGGGGGCTCCCTGATCCGTG (Δ8, +5)  
 CCCTCCAGTACCTGTCTGGCAGTTGGT **-----** GGGGGGCTCCCTGATCCGTG (Δ9) [×2]  
 CCCTCCAGTACCTGTCTGGCAGTTGGT **-----** GGGGGGCTCCCTGATCCGTG (Δ14) [×2]  
 CCCTCCAGTACCTGTCTGGCAGTTGGT **-----** GGGGGCTCCCTGATCCGTG (Δ15)  
 CCCTCCAGTACCTGTCTGGCAGTTGGT **-----** GGGGGCTCCCTGATCCGTG (Δ16)  
 CCCTCCAGTACCTGTCTGGCAGTTGGT **-----** GGCTCC **gtgatggctc** GGGGG (Δ26, +11)

**grp78 84% (16/19)**

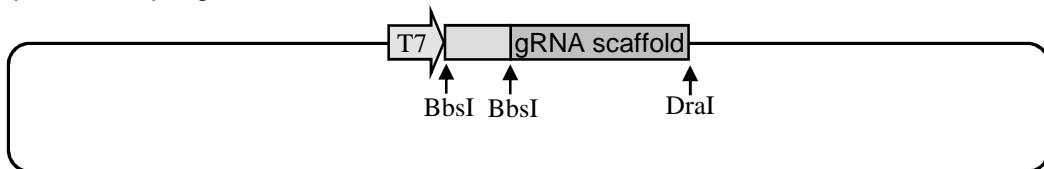
GACCATGAAGCTGTTGCCCTGGT **CCT** TGGTGTGCTGGTGTCTGCC **AGCGT** ATTTGCTGCTGATGA Wild-type  
 GACCATGAAGCTGTTGCCCTGGT **-----** CTGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ2)  
 GACCATGAAGCTGTTGCCCTGGC **cc** TGGTGTGCTGCCAGCGTATTTGCTGCTGATGA (Δ3, +2)  
 GACCATGAAGCTGTTGCCCTT **-----** GCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ6)  
 GACCATGAAGCTGTTGCCCTG **c** TGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ7, +1)  
 GACCATGAAGCTGTTGCCCTTG **c** TGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ7, +1)  
 GACCATGAAGCTGTTGCCCTGGT **-----** TGCCAGCGTATTTGCTGCTGATGA (Δ9)  
 GACCATGAAGCTGTTGCCCTGGT **-----** TGCCAGCGTATTTGCTGCTGATGA (Δ10)  
 GACCATGAACCTGT **tg** GCCCTGG **c** GCTGCCAGCGTCTTGT **tgctgatga** GACCATGAACCTGT **tg** GCCCTGG **c** GCTGCCAGCGTCTTGT **tgctgatga** (Δ14, +11)  
 GACCATGCAGC **gttg** GCC **-----** TG **tg** T **-----** CTGCCAGCGC **at** TTGCTGCTGATGA GACCATGCAGC **gttg** GCC **-----** TG **tg** T **-----** CTGCCAGCGC **at** TTGCTGCTGATGA (Δ15, +5)  
 GACCATGAAG **-----** GCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA GACCATGAAG **-----** GCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ21)  
 GACCATGAAGCTGTTGCCCTGGT **g** TTGCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA GACCATGAAGCTGTTGCCCTGGT **g** TTGCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ25, +1)  
 GACCATGAAGCTGTTGCCCTGGT **c** TTGCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA GACCATGAAGCTGTTGCCCTGGT **c** TTGCTGGTGTCTGCCAGCGTATTTGCTGCTGATGA (Δ+1) [×2]  
 GACCATGAAGCTGTTGCCCTGGT **accatgaagctg** TTGCTGGTGTCTGCCAGCGTATT GACCATGAAGCTGTTGCCCTGGT **accatgaagctg** TTGCTGGTGTCTGCCAGCGTATT (Δ+12)  
 GACCATGAAGCTGTTGCCCTGGT **tttctcaactgctaagctt** TTGCTGGTGTCTGCCAGCGTATT GACCATGAAGCTGTTGCCCTGGT **tttctcaactgctaagctt** TTGCTGGTGTCTGCCAGCGTATT (Δ+19) [×3]

Fig. S5. DNA sequencing data show the targeted mutations in *elastase* (the upper panel) and *grp78* (the lower panel) induced by co-injection of Cas9 mRNA together with two gRNAs targeting *elastase-T1* and *grp78*. For both panels, the wild-type sequence is shown at the top with the target site highlighted in yellow and the PAM sequence in blue text. Mutated regions are shaded in gray with red dashes indicating deletions and lowercase letters in red indicating insertions. The numbers in parentheses show the number of deleted (Δ) or inserted (+) base pairs, whereas numbers in square brackets show the frequencies of the mutation in all the sequenced samples.

pCS2- 3xFLAG-NLS-SpCas9-NLS



pUC57-Simple-gRNA backbone



gRNA backbone sequence

TAATACGACTCACTATA**G**GTTCGTCTTCGAGAAGACCTGTTTAGAGCTAGAAATAGCAA  
 T7 promoter                    BbsI                    BbsI  
 GTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTAAA  
 DraI

Fig. S6. Cas9 and gRNA expression vectors used in this study. The upper and middle diagrams illustrate the pCS2-3xFLAG-NLS-SpCas9-NLS vector and the pUC57-Simple-gRNA backbone vector, respectively. The bottom panel shows the DNA sequence of T7 promoter, BbsI cloning sites, and the gRNA backbone. T7 promoter is underlined and the transcription start site is highlighted in yellow. The BbsI and DraI restriction sites are shaded in grey. NLS, nuclear localization signal; SpCas9, *Streptococcus pyogenes* Cas9.