Efficient RNA/Cas9-mediated genome editing in
Xenopus tropicalis

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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 tracr RNAs (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; Gratzi 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, hhex 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 hhex 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

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 hhex, 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>
<thead>
<tr>
<th>Target gene</th>
<th>Target site</th>
<th>PAM</th>
<th>Oligonucleotide 1</th>
<th>Oligonucleotide 2</th>
</tr>
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<tr>
<td>elastase-T1</td>
<td>GGCAGTTTGTACCATACCTG</td>
<td>TGG</td>
<td>TAGGCGATTGTGACCATACCTG</td>
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<td>GGAGCAGCATTATGTGGCCT</td>
<td>GGG</td>
<td>TAGGGTCAGCATTATGTGGCCT</td>
<td>AACACGCACTACATGCCTCA</td>
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<tr>
<td>ets1-T2</td>
<td>GGTTCAGAGAATTCAGAGGG</td>
<td>CAG</td>
<td>TAGGGTCAGAATTCAGAGGG</td>
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<tr>
<td>ets1-T3</td>
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<td>TAGGGTCACGCAGCAATTCTCA</td>
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<tr>
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<td>CAG</td>
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<td>TGG</td>
<td>TAGGGGCATTGAACAAACATT</td>
<td>AACAACTGAACTTGGCAATGCCTCA</td>
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</tbody>
</table>

Sequences are shown 5'-3'.
PAM, protospacer adjacent motif.
(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, ptf1a/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
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 *ptf1a/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 Ptf1a/p48 (Ptf1a/p48GR), which was activated after gastrulation, resulted in 100% rescue of the *ptf1a/p48* gRNA-induced phenotype (Fig. 5D,H).

Together, these findings are reminiscent of those obtained upon application of *ptf1a/p48* morpholinos to Xenopus laevis embryos (Afelik et al., 2006; Jarikji et al., 2007). We also dissected six *ptf1a/p48*-targeted froglets that all showed severe pancreatic hypoplasia (Fig. 5N), consistent with our previous findings with *ptf1a/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. 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.**

**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.**
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).
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).

In conclusion, our results demonstrate that gRNA/Cas9 is a superb tool for multiplex genome editing in *X. tropicalis*. Given the simplicity and low cost of gRNA construction, the high targeting efficiency of the gRNA/Cas9 system, the high efficiency of germ line transmission, the relatively short generation time of frogs (4-6 months), and the availability of the frog genome sequence (Hellsten et al., 2010), there is no doubt that the diploid frog *X. tropicalis* is jumping into the future of developmental genetics.

**MATERIALS AND METHODS**

**Production of Cas9 mRNA, gRNAs and ptf1a/p48 mRNA**

The recently reported (Cong et al., 2013) codon-optimized *Streptococcus pyogenes* Cas9 cDNA together with the two attached nuclear localization signals (3xFLAG-NLS-SpCas9-NLS) was synthesized by GenScript and cloned into the pCS2+ vector (supplementary material Fig. S6). The construct was linearized with NotI and transcribed with the mMessage mMachine SP6 Kit (Ambion) to produce capped Cas9 RNA.
which was purified with the RNasey Mini Kit (Qiagen) according to the RNA clean protocol.

To create a gRNA expression vector, we placed a T7 promoter followed by two Bsal 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)₃NGG-3' (Table 1). NGG is the PAM. The locus-specific 2 bp protospacer containing the cloning cohesive sites was obtained by annealing two synthesized partially complementary oligonucleotides (Table 1), and was then cloned into Bsal-digested gRNA expression vector. The resulting construct was digested with DraI and transcribed using the MAXIscript T7 Kit (Ambion). The gRNA was purified by miRNeasy Mini Kit (Qiagen).

Capped pT1a/p88GR 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) 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 NaHCO₃, 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 NaHCO₃, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mM HEPES pH 7.4) in 24-well plates lined with 0.8% agar over night and then subjected to proteinase K digestion, PCR amplification and TA cloning. Ten single colonies from each blastomere progeny were sequenced to determine the genome 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'-GAGGAGGAGAATCATCAGCGA-3'; reverse, 5'-CAGTATACGACTCTATAGGGAATATCTCAAGGACCGAAGAAA-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., H.Z. and Y. Chen designed the work and analyzed experiments. X.G., T.Z., H.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

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