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Application and optimization of CRISPR–Cas9-mediated genome engineering in axolotl (*Ambystoma mexicanum*)

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Genomic manipulation is essential to the use of model organisms to understand development, regeneration and adult physiology. The axolotl (*Ambystoma mexicanum*), a type of salamander, exhibits an unparalleled regenerative capability in a spectrum of complex tissues and organs, and therefore serves as a powerful animal model for dissecting mechanisms of regeneration. We describe here an optimized stepwise protocol to create genetically modified axolotls using the CRISPR–Cas9 system. The protocol, which takes 7–8 weeks to complete, describes generation of targeted gene knockouts and knock-ins and includes site-specific integration of large targeting constructs. The direct use of purified CAS9-NLS (CAS9 containing a C-terminal nuclear localization signal) protein allows the prompt formation of guide RNA (gRNA)–CAS9-NLS ribonucleoprotein (RNP) complexes, which accelerates the creation of double-strand breaks (DSBs) at targeted genomic loci in single-cell-stage axolotl eggs. With this protocol, a substantial number of F_0 individuals harboring a homozygous-type frameshift mutation can be obtained, allowing phenotype analysis in this generation. In the presence of targeting constructs, insertions of exogenous genes into targeted axolotl genomic loci can be achieved at efficiencies of up to 15% in a non-homologous end joining (NHEJ) manner. Our protocol bypasses the long generation time of axolotls and allows direct functional analysis in F_0 genetically manipulated axolotls. This protocol can be potentially applied to other animal models, especially to organisms with a well-characterized transcriptome but lacking a well-characterized genome.

Introduction

Salamanders have become a key tetrapod model organism for studying regeneration, owing to their ability to regenerate complete organs such as limbs and spinal cord, abilities that are notably absent in mammals. Among salamanders, axolotls (*A. mexicanum*) have been widely used because they are historically the most readily bred species in the laboratory[1,2] and they offer the most comprehensive toolkit for molecular genetic manipulation[2–10]. Studies using transcriptomics, functional genomics, viral and transgenesis approaches have uncovered a number of genes required for regeneration[2–10]. These methods provide a solid basis for further exploration of the crucial gene regulatory networks controlling regeneration in axolotl. Transgenesis (introduction of an exogenous gene—called a transgene—into a living organism) has been an important aspect of recent progress in the system and has until now relied on I-Sce1 and transposon-based methods for introducing transgenes[2,3]. These methods were used to map the genetic fate of muscle satellite cells to study their lineage during regeneration[11], to block the cell cycle in the spinal cord[3] and to rescue the pigment cell defects in white (*d/d*) mutant axolotls[12]. In addition, Whited et al.[13] have established a LacI-inducible system and used it to test candidate gene function during axolotl limb regeneration. However, with these transgenesis methods, the insertion locations of the integrated sequences were not predictable, which necessitated screening of promoters for accurate tissue-specific expression[2,3]. Creating targeted gene knockout and knock-in animals would bypass these limitations, expand the study of different cell types and allow inducible deletion of gene sequences to evaluate gene functions during regeneration.

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Targeted gene knockouts and knock-ins were recently achieved in axolotls using CRISPR–Cas9-mediated gene editing[14,15]. The CRISPR–Cas9 system was first identified in bacteria as a viral defense mechanism and was adapted in other model organisms for targeted genome modification[16–19]. The CRISPR–Cas9 editing system consists of two components: the endonuclease CAS9 and a gRNA that associates with CAS9 and directs its activity toward cDNA sequences to create DSBs. The DSBs are repaired either by the NHEJ pathway, which often introduces insertions or deletions (indels) at the DSB site and thus may result in frameshift mutations at the targeted genomic loci, or by homology-directed repair (HDR), when an exogenous repair template is available[18,19].

The CRISPR–Cas9 system introduced into single-cell stage axolotl eggs can efficiently modify targeted genomic DNA in axolotls and has been used to generate knockouts, thus demonstrating the essential roles of *Brachyury* and *Pax7* during development, and *Sox2* during spinal cord regeneration[9,14,15,20]. In addition, the CRISPR–Cas9 system was used to insert exogenous gene sequences into the axolotl *Sox2* and *Pax7* genomic loci to faithfully label neural stem cells and satellite cells[21], two cell types that had been difficult to mark genetically via classical transgenesis[3,11,21]. In this protocol, we summarize our approach for efficient generation of gene mutations and site-specific insertion of exogenous sequences such as those encoding tamoxifen-inducible *Cre*-recombinase, which can be placed under the control of endogenous regulatory elements. With the recent release of the full axolotl genome and the availability of comprehensive transcriptome data[4,6,8,9,22], it is now possible to target any axolotl genomic locus, and to design better gRNAs and assess their off-target effects more accurately. In addition, for any animal model with a well-characterized transcriptome but a poorly characterized genome, the exon knock-in approach described here allows for gene insertion at a desired exonic location. These protocols will substantially expand the application of molecular genetics in axolotl and other salamanders.

Advantages of CAS9 protein-based gene knockout and NHEJ-mediated knock-in

To efficiently introduce DSBs at defined genomic loci, we use purified CAS9-NLS protein instead of *Cas9* mRNA. Several studies, including ours, have shown that CAS9-NLS proteins are more efficient than *Cas9* mRNA in creating modifications at targeted genomic loci in zebrafish, mouse, axolotl and cultured cells[23–29]. It has also been reported that CAS9-NLS protein, as compared with CAS9 protein lacking the NLS, is generally more efficient in generating indels at targeted genomic loci in zebrafish[30]. Use of purified recombinant CAS9 protein bypasses the *Cas9* mRNA translation process and leads to the rapid formation of gRNA–CAS9 RNP complexes[21,26–28]. This probably allows for the immediate interaction of gRNA–CAS9 complexes with the targeted genomic locus, which may in turn lead to the prompt formation of DSBs in injected single-cell-stage axolotl embryos. The imprecise NHEJ repair of DSBs located in the coding sequence may lead to frameshift mutations and therefore loss of function of the targeted gene, producing gene-knockout embryos. Using the current protocol, we have successfully created axolotl knockouts with pre-characterized gRNAs (i.e., gRNA that mediates ≥90% of target DNA modifications as described in this protocol; see Step **22**) at several genomic loci, such as *Tyrosinase*, *Pax7*, *Sox2* (ref. [9,15,29]), *BMP* and *Egr1* (J.-F.F. and E.M.T., data not shown). Importantly, a substantial proportion (~45% for *Tyrosinase* knockouts[29]) of the F_0-injected axolotl embryos harbor homozygous-type frameshift mutations (only one or two types of modifications are detectable in a single CRISPR animal, probably indicating that either a single modification has occurred on both the paternal and maternal alleles or that two different types of modifications have occurred independently on the paternal and maternal alleles in injected single-cell-stage axolotl embryos), according to genotyping and/or immunohistochemistry[9,29].

To generate gene-knock-in axolotls, we used a NHEJ-mediated knock-in approach. A circular plasmid, which contains a gRNA recognition site (bait sequence) in front of the gene of interest (GOI), is provided as a targeting construct. Linearization of the targeting plasmid can be achieved by the same gRNA that mediates the production of genomic DSB, as identical gRNA recognition sites are presented in the genome and the targeting construct, which leads to the integration of the donor DNA including the GOI into the genomic DSB site[21,31,32]. We compared the performance of CAS9-NLS protein versus *Cas9* mRNA in our knock-in experiments and found CAS9-NLS protein to be considerably better than *Cas9* mRNA[21]. We found that when CAS9-NLS protein is used (i) knock-in efficiency is higher and (ii) expression of the GOI from the knock-in allele is more ubiquitous as compared to that of F_0 knock-in axolotls derived from *Cas9* mRNA injection[21]. This is consistent

with the observation that CAS9-NLS protein is more efficient in generating indels at targeted genomic loci in other animal models (e.g., zebrafish)[24–29]. With this protocol, we have achieved efficient knock-in of exogenous genes (e.g., *Cherry* or *ER^{T2}-Cre-ER^{T2}*) into axolotl *Sox2* or *Pax7* genomic loci in injected F_0 embryos. For *Cherry* integration, ~15% of F_0 individuals in all injected embryos express CHERRY in the majority, if not all of the expected targeting tissue/domain. The knock-in efficiency declines with increasing size of the targeting construct[21]. Furthermore, we have demonstrated the broad applicability of the NHEJ-mediated knock-in approach in axolotl and successfully inserted *Cherry* or *ER^{T2}-Cre-ER^{T2}* donor constructs into several other loci, such as *Sox9* and *HoxA13* axolotl genomic loci (D.K. and E.M.T., data not shown).

Overall, the protocols presented here are simple and efficient approaches for generation of F_0 knockout and knock-in axolotls with high penetrance, i.e., in which a higher percentage of F_0 CRISPR animals are edited at the targeted genomic loci and a substantial number of F_0 individuals are non-mosaic (showing homogeneous transgene expression) or nearly non-mosaic genetically modified animals. These protocols bypass the long generation time of axolotls needed to produce F_1 animals and allow for rapid phenotype analysis in F_0 CRISPR animals. The exon-knock-in approach (Experimental design) can potentially be applied to other salamanders, even non-salamander species with poorly characterized genomes, and thus provides a practical genome manipulation method. Taking advantage of these protocols, we have studied the essential roles of *Pax7* during development and *Sox2* during spinal cord regeneration in F_0 CRISPR axolotls[9,15]. Moreover, we have also used F_0 knock-in axolotls to demonstrate that satellite cells are the direct cell source of myogenesis during limb regeneration[21].

Applications of CAS9 protein-based gene knockout and NHEJ-mediated knock-in

The techniques described here have a broad range of applications. We have used gRNA-CAS9-NLS RNP complexes to generate gene knockout axolotls to study targeted gene functions, and NHEJ-mediated knock-in axolotls to label or manipulate specific cell populations under the control of endogenous genomic regulatory sequences. It has been reported that NHEJ-mediated knock-in is much more efficient than HDR-mediated knock-in in zebrafish and mammalian cell culture[31,32]. gRNA-CAS9-NLS complexes also work very well in a wide range of species, ranging from plants, worms, insects and fish, to mammals[23,24,26,33–37]. Therefore, a gRNA-CAS9-NLS complex–based, NHEJ-mediated knock-in approach may potentially be applied to many other species, including other salamanders. It may be particularly useful for generating knock-in animals of species that lack well-characterized genomes.

Depending on the system of study, the gRNA-CAS9-NLS RNP complexes can also be introduced into somatic tissues by electroporation to mutate the target gene in specific cell types. We have successfully used this method to target axolotl neural stem cells in the spinal cord and blastema cells of regenerating limbs[20]. In the spinal cord, we obtained a high percentage of genetic modifications in the targeted neural stem cells. We were able to achieve knockout of GFP transgene or endogenous *Sox2* gene in >90% and >75% of luminal neural stem cells, respectively. Moreover, simultaneous delivery of gRNA-CAS9-NLS complexes targeted to multiple genes results in multiple gene knockouts with efficiency comparable to those of individual gene knockouts[20].

Comparison to other methods

TALEN

Several modern genomic engineering methods, such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and CRISPR–Cas9, were developed in the recent decades[38]. It was demonstrated that TALEN can be applied to many species to manipulate the genome at targeted loci, for generating gene knockout and knock-in animals[15,39]. We and others have shown that injection of TALEN mRNA into the single-cell stage of axolotl eggs leads to target modification in F_0 animals[9,15,40]. In comparison with that of the CRISPR–Cas9 system, which creates highly efficient genetic editing in individual F_0 axolotls, allowing for direct experimental analysis, the efficiency of TALEN-mediated genomic modification is lower (e.g., upon *Tyrosinase* targeting, 99.3% and 6.7% of F_0 CRISPR and TALEN animals show a complete loss of melanophore pigments, respectively)[15]. In most cases, owing to the low penetrance of TALEN-mediated target modification in F_0 animals, one has to wait for germline transmission to F_1 or F_2 generations for phenotype analysis (e.g., we can observe typical *Pax7* mutant phenotypes in F_0 CRISPR, but not F_0 TALEN axolotls)[9,15].

Furthermore, unlike gRNA, which can be prepared easily by in vitro transcription, assembly of repeat-variable di-residues into TALEN repeats is time- and labor-consuming.

I-Sce1 and transposon-based transgenic techniques

I-Sce1 and transposon-mediated transgenesis methods have been established in axolotl and are relatively simple and straightforward approaches to creating transgenic animals, provided that well-characterized promoter sequences are available to drive gene expression in the target tissue, organ, or cell type of interest[2,3,41]. Compared to CRISPR–Cas9, which allows precise targeting of specific genomic loci, I-Sce1 and transposon (e.g., Tol2)-based transgenic methods randomly integrate a gene cassette into the axolotl genome. This random insertion has several drawbacks: (i) random integration of the transgene may trigger inactivation (silencing) of the transgene or disrupt an endogenous gene; (ii) the copy number of the transgene is not controlled; and (iii) depending on the integration locus, the transgene may be expressed mosaically only in a subpopulation of desired cells[2,3]. Furthermore, if lacking a well-characterized promoter sequence, it is necessary to screen and characterize the promoter to drive faithful expression of transgene in the desired cell types. CRISPR–Cas9-mediated knock-in inserts the GOI precisely into the desired genomic locus, thus allowing homogeneous expression of the GOI under the control of an endogenous promoter for faithful cell-type labeling. It also prevents variations in copy number of the transgene. We were able to produce transgenic axolotls with faithful labeling of $SOX2^+$ cells, including neural stem cells, in the central nervous system by the NHEJ-mediated knock-in method using CRISPR–Cas9 (ref. [21]), but not by I-Sce1-mediated random integration of a transgene plasmid in which an 11.68-kb sequence upstream of the *Sox2* coding sequence was placed in front of a fluorescence reporter. In the latter case, only neural stem cells in the brain were labeled, probably due to the lack of proper expression regulatory elements[3,42].

Limitations of CAS9 protein–based gene knockout and NHEJ-mediated knock-in

We use the most commonly used *Cas9* variant isolated from the bacterium *Streptococcus pyogenes* (*spCas9*) for targeted gene editing in axolotls. The *spCAS9*-mediated genomic target cleavage requires the presence of a '5′-NGG' protospacer adjacent motif (PAM) sequence, which must directly follow the 3′ end of the 20-bp gRNA recognition site on the genomic DNA[16–19]. The selection of gRNA is restricted to the presence of the PAM 'NGG'. To increase CRISPR genomic accessibility, scientists have identified and characterized new CRISPR nucleases that recognize different PAM sequences, such as *Staphylococcus aureus Cas9* (ref. [43]), *Campylobacter jejuni Cas9* (ref. [44]), *Streptococcus thermophilus Cas9* (ref. [45]), *Neisseria meningitidis Cas9* (ref. [46]), *Acidaminococcus* sp. and *Lachnospiraceae bacterium Cpf1* (ref. [47]), or evolved CRISPR nucleases to recognize alternative PAM sequences[48–50]. To further expand the CRISPR genomic accessibility in axolotl, it may be worth trying other CRISPR systems, such as *Cpf1* or the engineered *spCas9* that recognizes a different PAM.

When inserting a GOI into the targeted axolotl genomic locus via a NHEJ-mediated knock-in method, a 'scar' is formed at the integration junction between the genomic DSB and the donor-targeting construct. A scar is an unintended indel resulting from error-prone NHEJ repair[21,31]. If the gRNA targeting site is located in an intron, the scar is removed during mRNA processing and leads to the production of a transcript identical to the wild type (for details, see Experimental design)[51]. However, for a gene lacking introns, the scar remains in the mature mRNA (e.g., *Sox2*). In this case, proper translation of the entire knock-in cassette (including the endogenous gene) requires an in-frame insertion (e.g., 0, +3, +6, … nt) or deletion (e.g., −3, −6, … nt) in the scar region. Furthermore, the donor plasmid backbone is integrated into the targeted genomic locus at the 3′ of the GOI. To date, we have not observed any negative effects when a vector backbone is present in our knock-in axolotl lines. Establishment of an efficient CRISPR–Cas9 HDR-mediated knock-in approach will overcome these potential issues. CRISPR–Cas9 has been used to achieve HDR-mediated knock-in in a broad range of organisms and cells[23,24,26,36,52]. We have compared NHEJ- with HDR-mediated knock-in methods and found that NHEJ-mediated knock-in is much more efficient than the latter approach (~15% versus 3–5% efficiency, respectively) for inserting the *T2A-Cherry* cassette into the axolotl *Sox2* genomic locus (J.-F.F. and E.M.T., data not shown), consistent with former observations[21,31,32]. Considering that NHEJ-mediated knock-in is very efficient and widely applicable to both genes containing introns (which compose the majority of protein-coding genes) and genes without introns, we included only the NHEJ-mediated knock-in approach in this protocol.

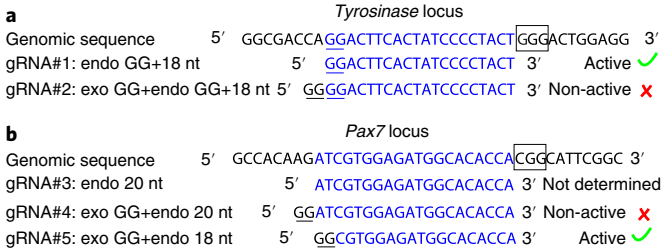

Fig. 1 | gRNA design. Examples of gRNAs we designed and tested for two different axolotl genomic loci: *Tyrosinase* (**a**) and *Pax7* (**b**). gRNAs were designed to contain a 5′ GG (underlined), either as part of the sequence complementary to the target genomic region or added exogenously (exo). This sequence is followed by 18 or 20 nt complementary to the target genomic region (shown in blue for both the gRNA and the genomic sequences) and directly preceding a PAM (NGG sequence; black boxes). **a**, Two gRNAs targeting the *Tyrosinase* locus with 20 nt complementary to the target genomic region: 'gRNA#1: endo GG+18 nt' contains an endogenous GG, and 'gRNA#2: exo GG+endo GG+18 nt' contains both an endogenous and an exogenous GG. **b**, Two gRNAs with 20 nt complementary to the target region: 'gRNA#3: endo 20 nt' has no GG and 'gRNA#4: exo GG+endo 20 nt' has an exogenous GG. 'gRNA#5: exo GG+endo 18 nt' has only 18 nt homologous to the target region and an exogenous GG. Green check marks indicate the gRNA design works; red crosses indicate the gRNA design did not work.

Experimental design

gRNA design

The functioning of the CRISPR–Cas9 system naturally requires CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) to form an RNA complex that recognizes the DNA-binding site and recruits CAS9 to the targeting site[18,19,53]. Jinek et al.[16] engineered crRNA and tracrRNA as a single gRNA molecule that carries the functions of those two RNAs. Both gRNA and crRNA/tracrRNA are used broadly in a wide range of organisms, and it was shown that they efficiently mediate targeted gene knockout and knock-in in these species[16,23,24,54–58]. Compared to crRNA/tracrRNA, gRNA is a single RNA molecule that can be easily synthesized by in vitro transcription. We chose the engineered gRNA instead of the native crRNA/tracrRNA, and injected gRNA-CAS9-NLS RNP complexes into one-cell-stage eggs for axolotl genomic manipulation. It is reported that the first 20 nucleotides at the 5′ end of the gRNA, which is in total 100 nt long, determine the binding specificity to its target[16,17,54].

We recommend using the online program sgRNA Designer from the Broad Institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) to design gRNAs targeting the desired axolotl genomic loci (Steps **7–9**). sgRNA Designer lists all 20 nt followed by an 'NGG' PAM sequence as potential gRNA-targeting sites. However, efficient gRNA preparation via in vitro transcription generally requires the presence of the 5′ 'GG. To satisfy this requirement, we tried the online software Zifit (http://zifit.partners.org/ZiFiT/), which selects gRNA sequences with the constraint that the 5′ end must start with GG (5′–GGnnnnnnnnnnnnnnnnnn+NGG–3′; 'GG+18n' stands for 20 nt homologous to the gRNA target; 'n' and 'N' reprent any nucleotide presented in gRNA and PAM sequences, respectively) in order to obtain efficient in vitro transcription with the T7 promoter (Fig. 1a)[15]. However, this limited the number of valid sites available for targeting. To increase the flexibility to choose gRNAs, we asked if we could add GG to the 5′ end of any gRNA (Fig. 1), even if the GG did not exist in the genomic locus (Fig. 1b). We found that addition of the GG to a 20-nt homologous region did not yield effective gRNAs (Fig. 1). Recent studies have shown that it is not necessary to have all 20 nt perfectly matched to the target for efficient cleavage[59]. To test whether this principle holds true for axolotl, we first used sgRNA Designer to design gRNAs. We then replaced two nucleotides at the 5′ end of the 20-nt homology region with GG. In this way, we obtained effective gRNAs (Fig. 1b) and were able to expand the possible locations to be targeted by gRNAs. By using this strategy, we obtained very satisfactory gRNA efficiencies for gRNAs designed against *Pax7*[9]. In conclusion, we chose 18 nt of target homology sequence followed by the PAM sequence 'NGG' for gRNA design and 'off-target' prediction[60].

It is important to screen the gRNAs for their cleavage efficiency by injection of the gRNA-CAS9-NLS complexes into axolotl eggs (as described in Steps **15–17**). We generally design several (at least three) gRNAs and select the best one or two, on the basis of in vivo evaluation (Steps **15–22**), for knockout and knock-in experiments. On average, one of those designed gRNAs leads to highly efficient (≥90%) modification of the targeted locus. If none of the designed gRNAs fulfills this

criterion in the in vivo test, it is necessary to design new gRNAs, and it may be worthwhile considering designing new gRNAs targeting a different genomic sequence of the same locus. We found that gRNAs targeting different exons (regions) of the same gene sometimes show marked differences in modification efficiencies, which may reflect differences in chromatin organization between adjacent exons (regions) within the same locus. Therefore, in the initial screening for efficient gRNAs, we recommend considering several exons as targets for gRNAs. The efficiency of the gRNA affects not only the ability to produce knockout mutant axolotls, but also the efficiency of targeting genomic loci for integration of exogenous sequences.

Selection of gRNAs for targeted gene knockout

In cases in which we aim to make a loss-of-function allele of a given gene, we normally use at least one of the following two criteria to design the gRNAs. (i) The first option is to design the gRNAs close to the translation start codon 'ATG'. In this strategy, only the production of frameshifting indels will lead to the lack of protein product. In general, we try to avoid the 5′ coding region of a given gene (the first 30 amino acids from the start codon) for gRNA design to avoid the production of a truncated protein from a potential downstream in-frame 'ATG', that may occur in eukaryotes[61]. We observed such an effect in *Pax7* mutants when the mutation was introduced very close to the start codon[9]. Therefore, it is necessary to examine the sequences and to be wary of the presence of downstream instances of in-frame ATGs that may allow the translation of a truncated protein lacking the N terminus. (ii) The second option is to design the gRNA to target a key functional domain of the gene. The advantage of this strategy is that most of the indels will cause loss of function of the targeted gene. Alternatively, it is also possible to use two gRNAs in parallel to remove the sequence in between[62]. To date, we have mainly focused on single-gRNA-mediated gene knockout in axolotl, as we are aiming to obtain homozygous-type mutants whereby the gene modification occurs in single-cell-stage axolotl eggs. The application of two gRNAs together may cause more mosaicism, if the DNA cleavage efficiencies from these two gRNAs are very different[62]. However, if the aim is to breed the mutant into the F_1 generation to perform phenotype analysis in the F_2 generation, then use of two gRNAs is a viable strategy.

Selection of gRNAs for targeted gene knock-in

Selection of gRNAs for targeted gene insertion involves several considerations. At the genomic level, some genes harbor multiple exons and introns, whereas others contain a single exon. Insertion of the exogenous DNA via NHEJ-mediated knock-in is more efficient than HDR but generally creates a scar at the integration junctions[21,31]. According to the regions selected for exogenous sequence insertion, we categorized the integration events as exon and intron knock-in as described below.

(i) Exon knock-in: the aim is to insert the GOI into the open reading frame region of a given genomic locus. In this case, the gRNA-mediated DSB occurs in the coding exons (Fig. 2a–d). The entire or the 3′ portion of the coding cDNA (in general, without a stop codon) is used as the bait sequence to reconstitute the endogenous gene function, and is followed by the genes of interest (Fig. 2a–d, middle panels). For genes containing a single exon, this is the only available NHEJ-mediated knock-in method of inserting exogenous DNA into the targeted genomic locus (Fig. 2a,b). For exon knock-in, gRNAs are normally designed at the most 5′ or 3′ region of the coding sequence for exogenous gene integration, concomitant with the formation of a scar at the integration junctions (Fig. 2a–d). Only the formation of an in-frame scar allows for the expression of an integrated GOI. The in-frame scar normally leads to a few amino acid modifications at either the most 5′ or 3′ ends that will probably not affect the protein function encoded by most endogenous genes. It is recommended to design the targeting event at the 3′ end of a given gene, unless the C terminus of the encoded protein has important biological functions.

(ii) Intron knock-in: the aim is to insert the GOI into the intron region of a given genomic locus. In this case, the gRNA-mediated DSB is designed to occur in the intron (Fig. 2e,f). The bait-targeting vector contains the 3′ portion of an intron, followed by the rest of the cDNA coding sequence, starting from the immediately 3′ adjacent exon, then followed by the GOI (Fig. 2e,f). For the intron knock-in, the integration junction (scar) is located in the intron, which is removed during mRNA splicing to produce a 'scar-free' mRNA transcript. Because introns contain sequences essential to the splicing process, it is critical to ensure that the scar resulting from gRNA-mediated DSBs does not occur in the intron region essential for splicing. In principle, any intron can be targeted for an intron knock-in. However, the intron in front of the last coding exon is generally an ideal position at which

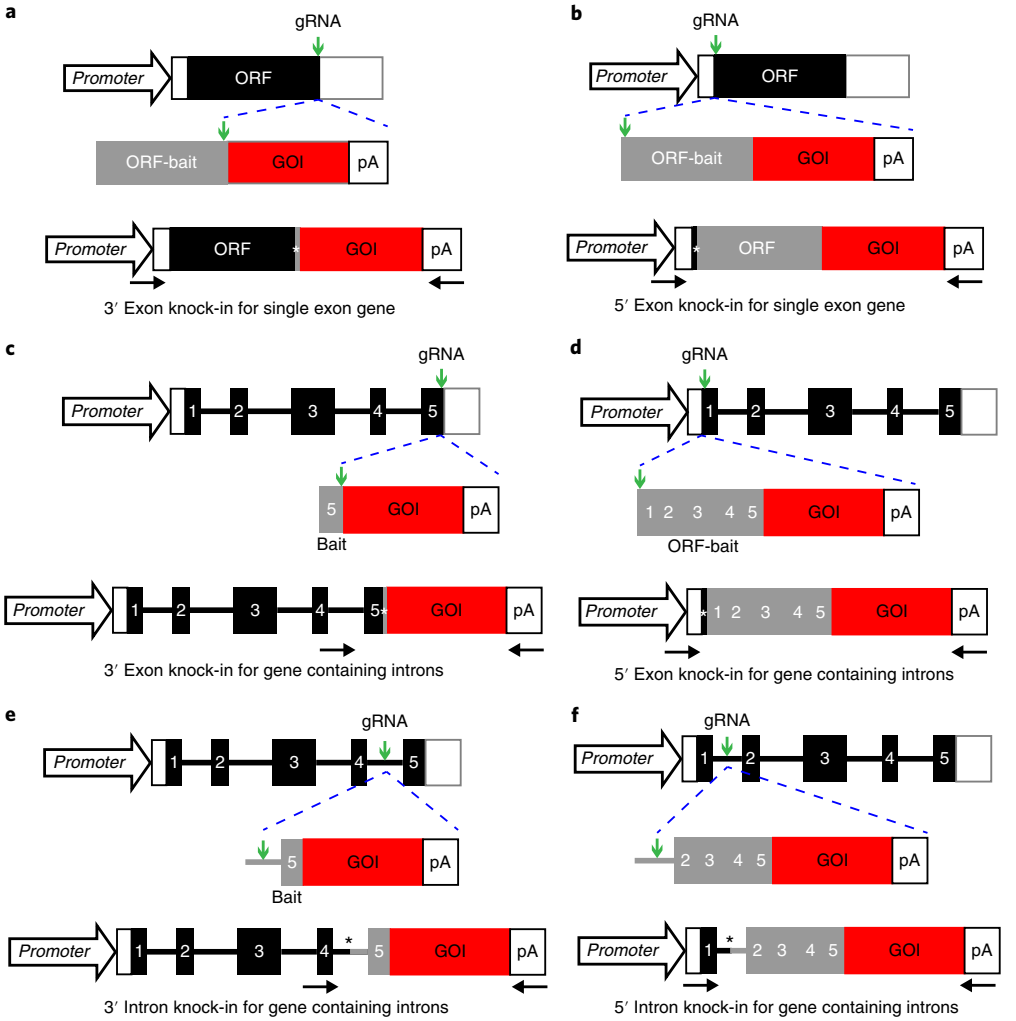

Fig. 2 | Gene knock-in approaches. a,**b**, The 3′ (**a**) and the 5′ (**b**) exon knock-in strategies for a single-exon coding gene. (Top) Wild-type allele contains a single-exon coding gene. (Middle) Targeting construct design. (Bottom) Knock-in allele after targeting-construct integration. **c**–**f**, The 3′ exon (**c**), 5′ exon (**d**), 3′ intron (**e**) and 5′ intron (**f**) knock-in strategies for an intron-containing gene. (Top) The wild-type allele contains five exons and four introns. (Middle) Targeting construct design. (Bottom) Knock-in allele after targeting-construct integration. 1–5 indicate the exons; black bars indicate introns. In **b**, **d** and **f**, the presence of a large portion of bait sequence between the gRNA-binding site and the GOI allows for bringing the desired modifications (e.g., single amino acid substitution) into the targeted genomic locus. Block arrow, promoter; solid rectangles, coding exons (black, endogenous exons; gray, exons from donor construct); empty rectangles, untranslated regions; lines, introns (black, endogenous introns; gray, introns from donor construct); green arrows, gRNA-targeting sites; asterisks, the integration junctions ('scars') formed between the genomic DNA and the targeting constructs; black arrows, the positions of the forward (fw) and reverse (rev) genotyping primers; ORF, open reading frame; pA, exogenous polyadenylation sequence.

to insert the exogenous DNA sequences (Fig. 2e). In this case, the bait sequence can be easily amplified from a single piece genomic DNA and is relatively short as compared with the bait sequence used in other strategies (Fig. 2f).

For systematically characterized salamander genomes, such as axolotl and Iberian newt (*Pleurodeles waltl*)[63], there are multiple possible knock-in options as described above. However, if planning to knock in the GOI into a defined locus in a salamander species with a poorly characterized genome, it is ideal to choose the exon knock-in approach based on cDNA sequence acquired by a transcriptome assembly or PCR amplification of cDNA using degenerate primers. The exon–intron genomic structure is generally highly conserved in vertebrates and therefore the cDNA sequence is sufficient for gRNA design for exon knock-in.

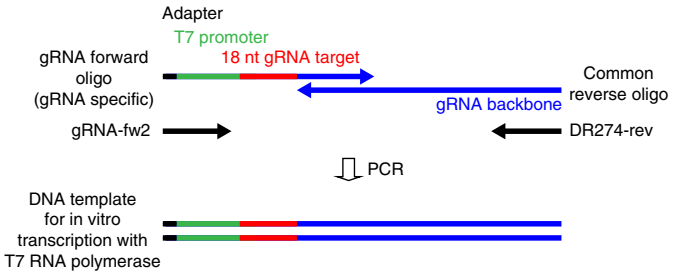

Fig. 3 | Scheme of preparation of DNA template for gRNA synthesis using a one-step PCR method. gRNA-forward oligo and common gRNA-reverse oligo anneal to each other at the gRNA backbone region (blue, overlapping region) and extend to full-length double-strand DNA during PCR (Step **10B(i,ii)**). They are further amplified using two short primers, gRNA-fw2 and DR274-rev. The common reverse oligo is a universal primer, which binds to the gRNA backbone and can be used for any designed gRNA. The final PCR products, from 5′ to 3′, will contain an adapter sequence (black, critical for improving T7 promoter activity), T7 promoter (green), gRNA targeting sequence (red) and gRNA backbone (blue).

Table 1 | gRNA oligo design principle for cloning into the DR274 vector (Step 10A)

	gRNA target (5′ to 3′)	Sense oligo (5′ to 3′)	Antisense oligo (5′ to 3′)
General template	nnnnnnnnnnnnnnnnnnNGG (n18 followed by PAM NGG)	TAGGnnnnnnnnnnnnnnnnnn	AAACnnnnnnnnnnnnnnnnnn (reverse and complement of n18)
Specific example (*Tyrosinase*)	acttcactatcccctactGGG (PAM GGG)	TAGGacttcactatcccctact	AAACagtaggggatagtgaagt

'n' indicates any nucleotide in gRNA, excluding the two 5′ nucleotides.

gRNA synthesis

We describe two options for preparing DNA templates for gRNA synthesis: PCR amplification from cloned plasmids (Step **10A**) and PCR amplification from synthesized oligonucleotides (Step **10B**). In Step **10A**, we clone the gRNA sequences into the DR274 vector, which contains a T7 promoter for in vitro transcription. We then carry out a PCR amplification step using the DR274-gRNA vector (verified by Sanger sequencing) as template to enrich the DNA template for gRNA synthesis. In option B, we describe a one-step PCR method in which the T7 promoter and gRNA sequences are introduced into PCR products via designed primers (Fig. 3). We provide the DNA oligo design principles, as well as an example (*Tyrosinase*-gRNA[15], used as a positive control in Steps **16** and **17**) for Step **10A** and Step **10B** in Table 1 and Table 2, respectively.

Assessment of gRNA efficiency by determination of F_0 axolotl genotype

The evaluation of gRNA efficiency in vivo (Steps **15–22**) is an essential aspect of identifying appropriate gRNAs for generating knockout or knock-in animals. A good gRNA is critical for the production of high-penetrance F_0 CRISPR axolotls (animals developed from CRISPR-injected axolotl eggs), in which the vast majority, even all the CRISPR targets, are modified in individuals[9,15,21]. It allows for mutant phenotypic analysis in a cleaner background (Steps **26** and **27**). In addition, gRNAs that lead to efficient target cleavage are more competent in producing high-penetrance gene-knock-in animals[21,31].

Several methods, such as traditional Sanger sequencing, next-generation sequencing (NGS)[64–66], surveyor/T7E1 nuclease assay[67–69], heteroduplex mobility assay[70–72] and high-resolution melting analysis[73,74], have been reported to identify modifications (indels) at gRNA-targeted genomic loci. We list the main genotyping methods for detection of mutations in CRISPR–Cas9 samples and compare their advantages and drawbacks (Table 3). Most of the methods, such as the T7E1 (Surveyor) assay[68,75], although very sensitive for detecting the presence of indels, are only qualitative assays. To obtain a quantitative readout of gRNA efficiency, we inject the selected gRNA-CAS9-NLS complexes into single-cell stage eggs, grow them up to hatchlings, and then evaluate the gRNA activity by using either Sanger sequencing (Fig. 4a) or NGS (Fig. 4b). Cloning and Sanger sequencing are routine procedures that can be carried out in any research laboratory. Furthermore, relatively

Table 2 | Primer design principle for DNA template preparation for gRNA synthesis using the one-step PCR method (Step 10B)

	gRNA Target (5′ to 3′)	gRNA forward oligo (5′ to 3′)[a]	gRNA reverse oligo (common, 5′ to 3′)[b]
General template	nnnnnnnnnnnnnnnnnnGG (n18 followed by PAM NGG)	TTGAAATTAATACGACTCACTATAGG nnnnnnnnnnnnnnnnnn GTTTTAGAGCTAGAAATAGCAAGT	AAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTAT TTTAACTTGCTATTTCTAGCTCTAAAAC
Specific example (*Tyrosinase*)	acttcactatcccctactGGG (PAM GGG), *Tyrosinase*	TTGAAATTAATACGACTCACTATAGG acttcactatcccctactGTTTTAGAGCTAGAAA TAGCAAGT	

[a]The gRNA forward oligo contains the T7 promoter sequence and a few adapter nucleotides at the 5′ end of the oligo (Fig. 3).
[b]The common gRNA reverse oligo includes solely the gRNA backbone for CAS9 recruitment that is common among all gRNAs. The 3′ ends of the sense and antisense oligos are complementary, therefore serving as the primers for the extension of the annealed oligo pair (Fig. 3).
'n' indicates any nucleotide in gRNA, excluding the two 5' nucleotides.

Table 3 | Comparison of main methods to genotype F_0 CRISPR animals

Technique	Indel size detected[a]	Quantitative data	Sequence information	Cost[b]	Labor[c]	Throughput[d]	Advantages
SNA[67–69]	Small	No	No	+	++	Low–moderate	Inexpensive, simple
HMA[70–72]	Small	No	No	+	+	Low–moderate	Inexpensive, simple
HRM[73,74]	Small	No	No	+	+	Moderate	Inexpensive, simple
Sanger sequencing	Small–moderate	Yes[e]	Yes	+++	+++	Low–moderate	Sequence data obtained, quantitative
NGS[64–66]	Small–moderate	Yes	Yes	+++ ++	+++	High	High throughput, sequence data obtained, quantitative

[a]SNA, HMA and HRM are normally suitable for detecting small indels (e.g., <30–50 bp), but the T7E1 assay (a type of SNA) cannot be used to detect single-nucleotide mismatches[68]. Sanger sequencing and NGS can be used to detect a broad range of indels, ranging from a single nucleotide to hundreds of nucleotides.
[b]Estimated cost per assay: +, €1–2; +++, €50–100; +++++, €500–1,000.
[c]Estimated timing: + and ++, <1 d; +++, within 1 week.
[d]Low–moderate, <100; moderate, hundreds; high, thousands.
[e]To be quantitative, a sufficient number (e.g., ≥20) of clones need to be sequenced.

large deletions of >100 bp can occur in CRISPR axolotls, which can be detected by Sanger sequencing but are likely to be overlooked by the other techniques described in Table 3. NGS is the leading-edge high-throughput method for quantitative characterization of mutations in CRISPR animals. Through barcoding and pooling of the PCR products generated from multiple gRNA targets and samples, millions of reads can be obtained in a single sequencing event. Both Sanger sequencing and NGS can directly identify frameshift mutations that allow us to bypass the long generation time of axolotls, characterize phenotypes and correlate the phenotypes with genotypes in F_0 CRISPR animals. We select gRNAs with a ≥90% mutagenesis rate for knock-in and knockout experiments (Steps **15–22**).

NGS library preparation

The NGS library preparation involves a two-step PCR. In the first PCR (PCR1), a genomic region around the gRNA target site in CRISPR axolotls or controls is amplified using a gene-specific primer pair, NGS-PCR1-forward and NGS-PCR1-reverse (Step **21B**(v)). In addition to the gene-specific sequences, these primers also contain the following adapters at their 5′–ends: forward primer adapter, 5′–ACACTCTTTCCCTACACGACGCTCTTCCGATCT; and reverse primer adapter, 5′–GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT (Fig. 4b, Table 4). In the second PCR (PCR2), a unique barcode combination (double indexing) is added to each sample by using primer pair P5-indexes-fw and P7-indexes-rev during amplification, such that many samples can be multiplexed[66] (Step **21B(vii, viii)**). The 3′ portion of the barcoded primers anneals to the adapter sequences that were integrated into the PCR product from PCR1 (Fig. 4b). Therefore, the same indexing primers can be used with any amplicon that was created during PCR1. A list of indexing primers can be found in Gansauge and Meyer[76], and Supplementary Tables 1 and 2.

It is practical to have 12× P5-indexes-forward primers (Supplementary Table 1) and 8× P7-indexes-reverse primers (Supplementary Table 2), allowing for 96 (12×8) unique combinations for

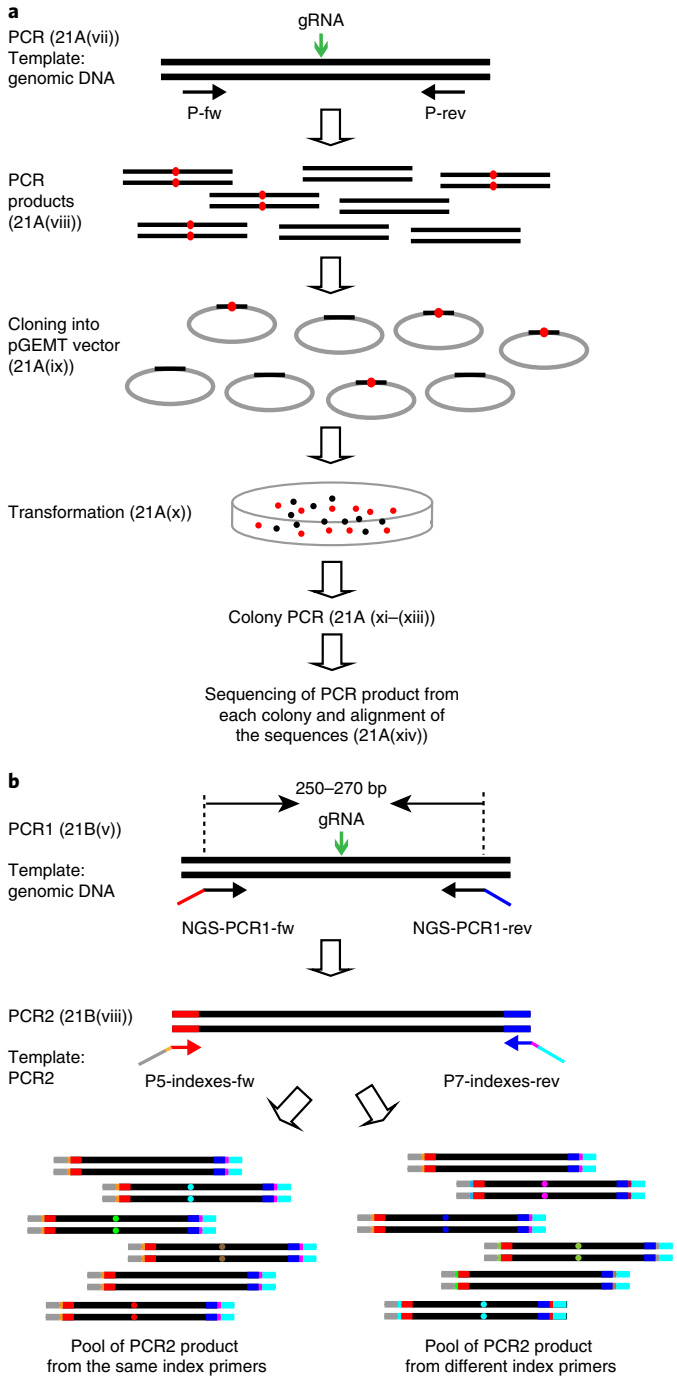

Fig. 4 | Scheme of gRNA evaluation using Sanger sequencing or NGS. a, PCR amplification, cloning and Sanger sequencing of the gRNA targeted genomic region by PCR from CRISPR F_0 axolotls (Step **21A**). The PCR product contains the amplicons from the wild-type and the modified (red dots) alleles. Vertical green arrows, gRNA-targeting site; horizontal arrows, forward (P-fw) and reverse (P-rev) primer binding sites. **b**, Scheme of the NGS procedure (Step **21B**), which can be used as an alternative to Sanger sequencing. Red and blue bars in NGS-PCR1-forward (fw) and reverse (rev) primers indicate the adapters of the two primers. In P5-indexes-forward (fw) and P7-indexes-reverse (rev) primers, gray and cyan bars indicate P5 and P7 sequences for Illumina sequencing, respectively, and orange and pink bars indicate indexing barcodes. The bottom panel illustrates two pools obtained from a single (left) or multiple (right) pairs of P5-indexes-fw and P7-indexes-rev primers. In the bottom panel, dots in varied colors represent mutations in PCR2 products derived from CRISPR-edited alleles, and colored bars adjacent to the P5 and P7 sequences represent barcodes from the indexing primers.

Table 4 | Primer sequences

Step	Primer	Sequence (5′–3′)	Purpose
10A(i)	Sense gRNA oligo for DR274	TAGGnnnnnnnnnnnnnnnnn	Anneal with antisense gRNA oligo for cloning of gRNA target into DR274 vector to synthesize gRNA
10A(i)	Antisense gRNA oligo for DR274	AAACnnnnnnnnnnnnnnnnn	Anneal with sense gRNA oligo for cloning of gRNA target into DR274 vector to synthesize gRNA
10B(i)	gRNA-fw	TTGAAATTAATACGACTCACTATAGGnnnnnnnn nnnnnnnnnnGTTTTAGAGCTAGAAATAGCAAGT	Oligo harbors T7 promoter and gRNA target to prepare the PCR template for gRNA synthesis
10B(i)	Common-gRNA-rev	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Oligo harbors the common 3′ portion of the gRNA for preparation of the PCR template to synthesize gRNA
10A(xiv), (xv)	DR274-fw	AAATGGTCAGTATTGAGCCTCAG	For sequencing and amplification of the template for gRNA synthesis
10A(xv), 10B(i)	DR274-rev	AAAAGCACCGACTCGGTGCCAC	For amplification of the template for gRNA synthesis
10B(i)	gRNA-fw2	TTGAAATTAATACGACTCACTATAGG	For amplification of the template for gRNA synthesis
21A(i)	Tyr-fw	GCTCTTCCTGCTGCTCTGGGAG	For genotyping of tyrosinase CRISPR animals
21A(i)	Tyr-rev	CTGCCAAGAAGCGAAGAAGGAG	For genotyping of tyrosinase CRISPR animals
21A(i)	Pax7-fw	CCAACTCCTCCCAAGAACTCTG	For genotyping of Pax7 CRISPR animals
21A(i)	Pax7-rev	GTCTGGAGCTCGAAACGCTCAAC	For genotyping of Pax7 CRISPR animals
21A(i)	Oct4-fw	GAGGCTGCAGCTGGAATTAGTGAG	For genotyping of Oct4 CRISPR animals
21A(i)	Oct4-rev	TGCTGCTGTGCGGGCTGTCATCG	For genotyping of Oct4 CRISPR animals
21A(xi)	T7	TAATACGACTCACTATAGGG	For colony PCR
21A(xi)	SP6	GATTTAGGTGACACTATAG	For colony PCR
21B(v)	NGS-PCR1-fw	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN NNNNNNNNNNNNNNNNNN	For NGS, PCR1
21B(v)	NGS-PCR1-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNN NNNNNNNNNNNNNNNNNNNN	For NGS, PCR1
21B(viii)	P5-indexes-fw	AATGATACGGCGACCACCGAGATCTACACcctgcga **ACACTCTTTCCCTACACGACGCTCTT**	For NGS, PCR2
21B(viii)	P7-indexes-rev	CAAGCAGAAGACGGCATACGAGATgcctctc **GTGACTGGAGTTCAGACGTGT**	For NGS, PCR2
36	pA-rev	GATCTTCATAAGAGAAGAGGGACAG	For genotyping of knock-in axolotls

Instances of 'n' in sense, antisense gRNA oligos for DR274 and gRNA-fw indicate 18-nt gRNA-binding sequence. Instances of 'N' in NGS-PCR1-fw and NGS-PCR1-rev indicate gene-specific primer sequence. The lowercase letters in P5-indexes-fw and P7-indexes-rev indicate indexes. The bold characters in P5-indexes-fw and P7-indexes-rev indicate the sequence annealing to the 5′ region in NGS-PCR1-fw and NGS-PCR1-rev, respectively.

a 96-well plate format (Supplementary Fig. 1). The number of indexes and possible combinations can be increased as needed when combining >96 samples of the same amplicon. It is convenient to distribute 12 P5-primers in a 12-well PCR-tube strip and eight P7-primers in an eight-well PCR-tube strip. P5-primers can then be added to each row of a 96-well plate by using a 12-channel pipette, and P7-primers can be added to each column of the 96-well plate by using an 8-channel pipette (Supplementary Fig. 1). In the final pool of PCR2 products for NGS, the same PCR amplicons (same genomic locus amplified from the same gene-specific primers) of individual CRISPR axolotls can be distinguished bioinformatically on the basis of the combination of indexes. Furthermore, different PCR amplicons (different genomic loci amplified from different gene-specific primers) that share the same index combination can also be distinguished bioinformatically on the basis of the sequence of the amplified region.

Targeting construct design

For NHEJ-mediated knock-in, the targeting construct (donor DNA) normally consists of the following components: the bait sequence, the GOI and the polyadenylation (pA) sequence. The bait sequence serves two purposes: it provides a gRNA cutting site in the targeting construct to linearize it, and it provides the cDNA for modifying or reconstituting the locus of interest. For exon knock-in, the

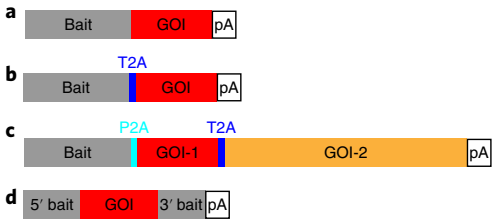

Fig. 5 | Several targeting construct designs. The basic structure of the targeting constructs contains the bait, followed by the GOI. The baits are either entirely coding cDNA or include a portion of an intron, followed by the coding cDNA (for details, see Fig. 2). The GOIs (e.g., a fluorescence reporter) can be designed immediately after the bait and translated as a fusion protein from the mRNA arising from the knock-in allele (**a**), separated from the bait by a 2A peptide (**b**), or with multiple 2A peptides separating the coding sequences (**c**); in particular cases, the GOI can also be placed in the middle of the bait (**d**; for details, see Experimental design). pA, polyadenylation signal; P2A, porcine teschovirus-1 2A; T2A, *Thosea asigna* virus 2A.

Box 1 | Targeting construct preparation using Gibson assembly ● Timing 3 d

We describe here a stepwise cloning protocol to create the knock-in targeting constructs by assembling the bait sequence, GOI and other necessary elements into a common vector backbone.

Procedure

1 Design PCR primers for each element in the targeting construct, including a vector backbone (e.g., pGEMT vector), the bait sequence, the GOI and a pA sequence (e.g., β-globin poly(A) signal). Use the NEB online tool (http://nebuilder.neb.com/) to design the PCR primers that harbor overlapping sequences between the adjacent DNA fragments. The exemplary primer design for preparing the pGEMT-Pax7bait-T2A-Cherry-pA targeting construct (Reagents) can be found in Fei et al.[21].

2 Set up a PCR reaction, using a high-fidelity DNA polymerase to amplify each DNA fragment. Each amplicon should harbor ~20 nt at the 5′ and 3′ends, which overlap with its adjacent DNA fragments. Depending on the region that is targeted, use either axolotl genomic DNA or cDNA as template for the bait amplification.

3 Set up the Gibson assembly reaction[79] with the PCR-amplified fragments, following the NEB Gibson Assembly Cloning Kit manufacturer's instructions, and incubate the mixture at 50 °C for 1 h in a heat block or water bath.

4 Transform the assembled product into *E. coli* TOP10 competent cells, pick six to ten clones, and culture each in 3–5 ml of LB liquid medium with appropriate antibiotics, following the instructions in the Gibson assembly manual. Prepare plasmid minipreps according to the QIAprep Spin Miniprep Kit manufacturer's instructions and validate the sequence by Sanger sequencing.

bait sequence can be the entire cDNA coding sequence (normally including the 'ATG' translational start codon, but without the stop codon) or the 3′ portion of the cDNA coding sequence (including the gRNA cleavage site, without the stop codon) (Fig. 2a–d, middle panels); for the intron knock-in, the bait sequence includes the 3′ part of an intron, followed by the rest of the cDNA coding sequence, starting from the immediate 3′ exon (Fig. 2e,f, middle panels). In most cases, the bait sequence does not contain the stop codon from the original cDNA, and it is placed in front (5′) of the GOI. Note that desired modifications, such as single amino acid substitutions, can be brought into the targeted genomic locus by placing those modifications after the gRNA-binding site in the bait sequence. The GOIs are often exogenous genes, such as the fluorescent-protein-coding genes *GFP* and *Cherry*, or the *Cre* recombinase containing a stop codon. Usually, they are placed after the bait sequence and translated as a fusion protein (Fig. 5a), or separated from the bait sequence by a 2A cleaving peptide sequence (e.g., T2A, P2A). Cleavage of a 2A peptide yields two separate molecules, the peptides encoded by the bait sequence and the GOI[77] (Fig. 5b). (Note: In our experience, internal ribosomal entry site sequences[78], which allow for the binding of ribosomes to an internal sequence and translation initiation from this sequence, have not worked in axolotl[3]). When several genes of interest are placed after the bait sequence, they can be further separated by other variations of the 2A cleavage peptide sequence, if necessary (Fig. 5c). In some particular cases, the exogenous sequence of interest can be inserted into the middle of the bait sequence (coding sequence) (Fig. 5d). For example, to modify a secretory molecule, it may be best to insert the GOI between the sequence coding for the signaling peptide and that coding for a peptide carrying out other key functions, as interruption at either the N or the C terminus of such a molecule may abolish its proper function. Because many endogenous promoters are relatively weak, we normally choose to include a strong pA sequence (e.g., rabbit beta-globin pA signal) after the stop codon in order to stabilize the mRNA transcript against disruption by the knock-in allele (Figs. 2 and 5).

There are several methods for preparing the targeting construct, such as restriction enzyme–based conventional cloning and Gibson assembly[79]. We recommend preparing the targeting construct via Gibson assembly as described in Box 1, because conventional cloning offers lower versatility with regard to cloning multiple DNA fragments[79]. In brief, we first perform PCR by using a high-fidelity DNA polymerase to amplify each individual element. For example, to prepare a targeting construct, pGEMT-Pax7bait-T2A-Cherry-pA, for insertion of the *Cherry* reporter gene into the axolotl *Pax7* locus, we carry out PCR to amplify the pGEMT vector, Pax7bait and T2A-Cherry-PA, in total three fragments[21]. If a proper template for amplification of a particular fragment, such as T2A-Cherry-PA, is lacking, it is necessary to perform PCR separately to obtain T2A, Cherry and PA fragments. Gibson enzyme mix is then used to assemble all PCR fragments together to form a circular plasmid (Box 1). Using this method, we have successfully assembled up to eight fragments into a single plasmid. We have deposited two basic targeting vectors with Addgene (http://www.addgene.org) that allow for the expression of a membrane-tagged CHERRY and ER^{T2}-Cre-ER^{T2} or solo CHERRY reporter gene in the desired cells after integration[21] (Reagents). The Pax7bait sequence in both plasmids can be easily replaced with other baits, using MluI and SphI double-restriction enzyme digestion.

Control design

It is critical to include positive and negative controls, particularly a positive control, when setting up the CRISPR–Cas9 system to knock out a target gene in axolotls for the first time. We recommend choosing a previously published gRNA targeting the axolotl *Tyrosinase* gene (genome recognition site, 5′–GGACTTCACTATCCCCTACT) as a positive control[15] because (i) it is very efficient: injection of *Tyr*-gRNA-CAS9-NLS RNP complexes into single-cell-stage eggs results in nearly 100% knockout in the derived F_0 CRISPR axolotls; (ii) knockout of *Tyrosinase* gene leads to loss of melanophore pigmentation, an easily detectable phenotype; (iii) knockout of the *Tyrosinase* gene does not cause any other obvious phenotype[15,29]. If necessary, one can use a previously published gRNA targeting EGFP (genome recognition site, 5′–GGCCACAAGTTCAGCGTGTC) as a negative control[15]. The DNA templates for gRNA synthesis can be obtained by cloning of the indicated gRNA sequences into the DR274 vector or by PCR (Step **10A** or **10B**, respectively).

It is worthwhile to include a positive control when designing and carrying out an axolotl knock-in experiment. We have shown that NHEJ-mediated insertion of the *Cherry* reporter gene into the endogenous *Pax7* locus yields ~15% positive transgenic F_0 axolotls[21]. In *Pax7:Cherry* knock-in axolotl embryos, (i) CHERRY is expressed in a clearly distinguished domain, in the central nervous system; (ii) CHERRY signal can be easily detected in ~2-week-old transgenic embryos under a normal fluorescence stereomicroscope, which makes it an ideal positive control. The targeting construct pGEMT-Pax7bait-T2A-Cherry-pA is available from Addgene (cat. no. 11154). The *Pax7*-gRNA used for the knock-in control binds to exon1 of the *Pax7* coding sequence (5′–GGGC AGAACTACCCACGGAC)[21].

Materials

Biological materials

- Axolotls (white axolotl, *d/d*) are available from the Ambystoma Genetic Stock Center (http://www.ambystoma.org/genetic-stock-center/: white embryos, RRID:AGSC_107J; white larvae, RRID: AGSC_101L; white adults, RRID:AGSC_101A) or from research laboratories that breed axolotls. **! CAUTION** All animal experiments must be carried out in accordance with local and national regulations and with the approval of the relevant institutional review board. We advise adherence to the ARRIVE guidelines[80]. All axolotl experiments discussed here were carried out in accordance with the applicable animal welfare legislation and were approved by the State Authorities of Saxony in Germany and the Magistrate of Vienna in Austria (license no. 9418/2017/12). **▲ CRITICAL** We breed all of our own animals and obtained axolotl eggs (white axolotl, *d/d*) from the axolotl colony at the DFG Center for Regenerative Therapies, Dresden (CRTD) or from the Research Institute of Molecular Pathology (IMP) in Vienna for injection. Woodcock et al.[12] have reported that the axolotls from the Ambystoma Genetic Stock Center, from which we imported axolotls and set up our colony many years ago, may carry tiger salamander DNA for historical reasons.

Reagents

- Artemia cysts (http://www.aquariumline.com)
- *Escherichia coli* strain DH5α (Thermo Fisher Scientific, cat. no. 18265017)
- DR274 (Addgene, cat. no. 42250)
- pGEMT (Promega, cat. no. A3600)
- Targeting constructs, pGEMT vector harboring the bait sequence and the genes of interest, obtained by Gibson assembly and verified by sequencing; e.g., pGEMT-Pax7bait-P2A-GAP43-Cherry-T2A-ER^{T2}-Cre-ER^{T2} (Addgene, cat. no. 111153), pGEMT-Pax7bait-T2A-Cherry-pA (Addgene, cat. no. 111154)
- PCR primers and DNA oligos (Sigma; Table 4)
- CAS9-NLS protein (PNA Bio, cat. no. CP03; or MPI-CBG protein expression facility[20,29], 5 mg/ml)
- Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, cat. no. F530L)
- Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, cat. no. F548L)
- Pfu DNA polymerase (Promega, cat. no. M7741)
- REDExtract-N-Amp PCR ReadyMix, 2× Taq master mix (Sigma, cat. no. R4775)
- BsaI-HF (NEB, cat. no. R3535L)
- T4 DNA ligase (NEB, cat. no. M0202)
- Gibson Assembly Master Mix (NEB, cat. no. E2611L)
- Gibson Assembly Cloning Kit (NEB, cat. no. E5510S)
- Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, cat. no. 28-9034-71)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28106)
- QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27106)
- Qiagen Plasmid Maxi Kit (Qiagen, cat. no. 12163)
- MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific, cat. no. AM1354)
- 1 kb plus DNA ladder (Invitrogen, cat. no. 10787-026)
- dNTPs (NEB, cat. no. N0447L)
- RedSafe Nucleic Acid Staining Solution (JH Science, cat. no. 21141)
- Ampicillin (Amresco, cat. no. 0339-25)
- Kanamycin (Sigma, cat. no. K4000)
- Penicillin–streptomycin (pen–strep; Thermo Fisher Scientific, cat. no. 15070063)
- Ficoll PM 400 (Sigma-Aldrich, cat. no. 46327-500G-F)
- Benzocaine (to anesthetize the axolotls; Sigma-Aldrich, cat. no. E1501) **! CAUTION** Benzocaine may cause skin irritation; handle it with gloves.
- UltraPure agarose (Thermo Fisher Scientific, cat. no. 16500500)
- Phenol/chloroform (Sigma, cat. no. P2069) **! CAUTION** Phenol is toxic; wear gloves and a mask, and handle it in a fume hood. Dispose of waste according to local institutional rules.
- Agar (Serva, cat. no. 11393.04)
- LB medium powder (Sigma, cat. no. L7658)
- SOC medium (Sigma, cat. no. S1797)
- IPTG (Sigma, cat. no. I6758)
- X-gal (Sigma, cat. no. B4252)
- NaCl (Merck, cat. no. 106404)
- NaAc (Sigma, cat. no. S7670)
- $MgCl_2$ (Merck, cat. no. 105833)
- KOH (Merck, cat. no. 814353)
- NaOH (Sigma, cat. no. 71687)
- EDTA (Sigma, cat. no. E5134)
- HEPES (Merck, cat. no. 391338)
- KCl (Merck, cat. no. 104936)
- $CaCl_2$ (Merck, cat. no. 102382)
- $MgSO_4$ (Merck, cat. no. 105886)
- Acetic acid, glacial (Sigma, cat. no. ARK2183)
- 37% (vol/vol) HCl (fuming; Merck, cat. no. 100317)
- TE (pH 8; Sigma, cat. no. 93283-100ml) **! CAUTION** HCL is corrosive and can damage skin and eyes; handle it in a fume hood while wearing gloves and a mask.
- Ethanol, absolute (VWR, cat. no. 20821-330)
- Tris base (Sigma, cat. no. T6066)
- Boric acid (Sigma, cat. no. 31146)

- RNaseZap (Thermo Fisher Scientific, cat. no. AM9780) **! CAUTION** RNaseZap may cause irritation to lungs and mucous membranes; wear gloves and avoid breathing the fumes directly.
- PAX7 mouse monoclonal primary antibody (DSHB, cat. no. PAX7)
- RFP rabbit polyclonal primary antibody (Rockland, cat. no. 600-401-379)
- RFP rat monoclonal primary antibody (Chromotek, cat. no. 5f8-100)
- GFAP goat polyclonal primary antibody (Abcam, cat. no. ab53554)
- SOX2 rabbit polyclonal primary antibody (homemade)[15]
- Alexa Fluor 488–conjugated donkey anti-mouse secondary antibody (Invitrogen, cat. no. A21202)
- Alexa Fluor 488–conjugated donkey anti-rabbit secondary antibody (Invitrogen, cat. no. A21206)
- Alexa Fluor 555–conjugated donkey anti-rabbit secondary antibody (Invitrogen, cat. no. A31572)
- Alexa Fluor 647–conjugated donkey anti-goat secondary antibody (Invitrogen, cat. no. A21447)
- Cy3-conjugated donkey anti-rat IgG (H+L) secondary antibody (Jackson ImmunoResearch, cat. no. 712-165-153)
- UltraPure DNase/RNase-free dH_2O (Thermo Fisher Scientific, cat. no. 10977015)
- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63880)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q32851)
- MiSeq Reagent Nano Kit v2 (300 cycles)

Equipment

- Disposable scalpel for tail sample collection (B. Braun, cat. no. 5518075)
- Containers for collecting eggs and for keeping axolotl larvae (VWR, cat. no. 216-3416)
- Cups for keeping axolotls (250 ml; Sarstedt, cat. no. 75 560)
- Sieve for rinsing eggs (Carl Roth, cat. no. 8098.1)
- Nets for washing and transferring axolotls (Zoo Zajac: http://www.zajac.de/): 20 cm (cat. no. AQ161); 10 cm (cat. no. AQ164); 8 cm (cat. no. AX100165)
- Forceps for dejellying of axolotl eggs and trimming the injection needles (Fine Science Tools, cat. no. 11295-51)
- Ring forceps for transferring axolotls (Fine Science Tools, cat. no. 11106-09)
- Petri dishes for keeping the axolotl eggs (145, 94, 60 and 35 mm; Greiner Bio-One, cat. nos. 639 102, 633 180, 628 102 and 627 102)
- RNase-free pipette filter tips (0.1–10, 2–20, 10–100 and 20–200 µl; Fisher Scientific, cat. nos. 02-707-439, 02-707-432, 02-707-431 and 02-707-430)
- 24-Well plates for keeping the injected axolotl embryos during the first 2 weeks (Nunc, cat. no. 142475)
- Sterile plastic pipettes (2 ml) for transferring the embryos (Sarstedt, cat. no. 86.1171.001)
- PCR tubes (0.2 ml; Bio-Rad, cat. no. TWI-0201)
- RNase-free Microfuge tubes (1.5 ml; Thermo Fisher Scientific, cat. no. AM12400)
- Stage micrometers (0.1 mm) to determine the volume of the injection droplet (Fine Science Tools, cat. no. 29025-01)
- Plastic mold for making injection plates (manufactured at the MPI-CBG workshop)[2]
- Gradient PCR machine (Bio-Rad, model no. C1000 Touch Thermal Cycler)
- Gel imaging system (ChemiDoc Imaging System; Bio-Rad)
- Centrifuge (Eppendorf, model no. 5424 R)
- Centrifuge (Eppendorf, model no. 5920 R)
- Pneumatic Pico pump (WPI, model no. PV830)
- Spectrophotometer (Thermo Fisher Scientific, model no. Nanodrop 2000)
- ThermoMixer (Eppendorf, ThermoMixer R model)
- Micromanipulator (Narishige, model no. MN-153)
- Magnetic stand (Narishige, model no. GJ-1)
- Iron plate to fix the magnet stand (Narishige, model no. IP)
- Injection needles: borosilicate glass capillaries (1.00 mm outer diameter × 0.58 mm inner diameter; Harvard Apparatus, cat. no. 30-0020)
- Flaming/brown micropipette puller (Sutter Instrument, model no. P-97)
- Stereomicroscope for axolotl egg injection (Olympus, model no. SZX10)
- Light source (Olympus, model no. KL 1500 compact)
- Stereomicroscope for examining the CRISPR animal phenotypes and the fluorescent protein expression in knock-in axolotls (Olympus, model no. SZX16)
- VWR mini centrifuge (VWR, cat. no. 75993-410)

- Tube rotator (Stuart, model no. SB3)
- Magnetic separator (MagRack 6; GE Healthcare, cat. no. 28-9489-64)
- Qubit 4 fluorometer (Thermo Fisher Scientific, cat. no. Q33226)
- Qubit assay tubes (Thermo Fisher Scientific, cat. no. Q32856)
- Illumina MiSeq System (Illumina; alternatively, the samples can be sent to a company for sequencing)

Software

- Primer3Plus (https://primer3plus.com)
- MacVector (http://www.macvector.com)
- Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)
- sgRNA Designer from the Broad Institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design)
- Lasergene (https://www.dnastar.com)
- CRISPResso (http://crispresso.rocks)

Biological material setup

Axolotl husbandry

In our colony, axolotls are kept at 18–20 °C in water with a 12-h light/dark cycle. We feed daily freshly hatched *Artemia* to axolotl larvae (snout to tail tip = <5 cm) and fish pellets to juvenile and adult axolotls. For detailed axolotl husbandry conditions, please see the protocol previously published by our lab[2].

Reagent setup

DNA oligo preparation

For oligos >40 nt, we order HPLC-purified oligos. Otherwise, desalted oligos are sufficient. We reconstitute oligos in deionized water at 100 µM and store them at −20 °C for up to 24 months. If necessary, dilute the 100-µM oligo stocks to 5–10 µM with deionized water.

Kanamycin stock solution (50 mg/ml)

Prepare 10 ml of kanamycin stock solution by dissolving 0.5 g of kanamycin in deionized water. Filter the solution to sterilize it. Divide the solution into 1-ml aliquots, and store the aliquots at −20 °C for up to 12 months.

1 M Tris buffer (pH 8)

Prepare 1 liter of Tris buffer by dissolving 121.1 g of Tris base in deionized water. Adjust the pH to 8.0 with HCl and autoclave the solution. The solution can be stored at room temperature (21–25 °C) for up to 6 months.

LB liquid medium

Prepare 1 liter of LB liquid medium by dissolving 20.6 g of LB medium powder in deionized water. Bring the volume to 1 liter and autoclave the solution. The solution can be stored at 4 °C for up to 6 months.

LB–kanamycin liquid medium (50 mg/L)

Prepare 500 ml of LB–kanamycin liquid medium by mixing 500 µl of kanamycin stock solution with 500 ml of LB liquid medium. The solution can be stored at 4 °C for up to 1 month.

LB–agar plates

Prepare 1 liter of LB–agar medium by mixing 20.6 g of LB medium powder and 15 g of agar and bringing the volume to 1 liter with deionized water. Autoclave the medium, let it cool to 50 °C, and add the appropriate antibiotics (and also 0.1 mM IPTG and 40 µg/ml X-gal when necessary). Pour ~25 ml per plate and allow the agar to solidify at room temperature. The plates can be stored at 4 °C for up to 3 months.
▲ **CRITICAL** In the presence of X-gal, cover the plates with aluminum foil to protect the plates from light.

10× CAS9 buffer (200 mM HEPES, 1.5 M KCl, pH 7.5)

Prepare 500 ml of 3M sodium acetate solution by dissolving 204.1 g of sodium acetate in deionized water. Adjust the pH to 5.2 with glacial acetic acid, fill up the volume to 500 ml, and filter-sterilize the solution. The solution can be stored at room temperature for up to 24 months.

Sodium acetate solution (3M, pH 5.2)
Prepare 50 ml of 10× CAS9 buffer by dissolving 2.38 g of HEPES and 5.59 g of KCl in RNase-free water. Adjust the pH to 7.5 with NaOH, fill up the volume to 50 ml and filter-sterilize the solution. Divide the solution into 1-ml aliquots, and store the aliquots at −20 °C for up to 24 months.

10× and 1×TBE
Prepare 1 liter of 10× TBE by mixing 121.1 g of Tris base, 61.8 g of boric acid and 7.4 g of EDTA in deionized water and bringing the volume to 1 liter. The solution can be stored at room temperature for up to 6 months. Dilute 100 ml to 1 liter in deionized water to make the gel-running buffer (1× TBE).

70% (vol/vol) Ethanol
Prepare 100 ml of 70% (vol/vol) ethanol by measuring 70 ml of absolute ethanol and bringing the final volume to 100 ml with deionized water. The solution can be stored at room temperature for up to 6 months.

10× Marc's modified Ringer's solution
Prepare 2 liters of 10× Marc's modified Ringer's (MMR) solution by mixing 400 ml of 5 M NaCl, 40 ml of 1 M KCl, 20 ml of 1 M $MgCl_2$, 40 ml of 1 M $CaCl_2$, 4 ml of 0.5 M EDTA and 100 ml of 1 M HEPES (pH 7.2). Add 1.5 liters of deionized water, adjust the pH to 7.8 with 10 M KOH, and fill up the volume to 2 liters with deionized water. Autoclave the solution. The solution can be stored at room temperature for up to 6 months.

1× MMR solution with pen–strep
Prepare 1 liter of 1× MMR solution with pen–strep by mixing 100 ml of 10× MMR solution with 13 ml of 100× pen–strep and bringing the volume to 1 liter with deionized water. Filter the solution to sterilize it. The solution can be stored at room temperature for up to 2 months.

1× MMR solution with 20% (wt/vol) Ficoll and pen–strep
Prepare 250 ml of this solution by mixing 25 ml of 10× MMR solution, 50 g of Ficoll and 7 ml of 100× pen–strep (5,000 U/ml penicillin and 5,000 μg/ml streptomycin). Bring the volume to 250 ml with deionized water and filter the solution. The solution can be stored at 4 °C for up to 2 months.
▲ **CRITICAL** Stir the solution and make sure the Ficoll is completely dissolved.

0.1× MMR solution with 5% (wt/vol) Ficoll and pen–strep
Prepare 1 liter of this buffer by mixing 10 ml of 10× MMR solution, 50 g of Ficoll and 13 ml of 100× pen–strep. Bring the volume to 250 ml with deionized water and filter the solution. The solution can be stored at 4 °C for up to 2 months.

0.1× MMR solution with pen–strep
Prepare 1 liter of this solution by mixing 10 ml of 10× MMR solution and 13 ml of 100× pen–strep. Bring the volume to 1 liter with deionized water and filter the solution. The solution can be stored at 4 °C for up to 2 months.

400% (wt/vol) Holtfreter's solution
Prepare 10 liters of Holtfreter's solution by dissolving 11.125 g of $MgSO_4{\cdot}7H_2O$, 5.36 g of $CaCl_2{\cdot}2H_2O$, 158.4 g of NaCl and 2.875 g of KCl in 10 liters of dH_2O. The solution can be stored at room temperature for up to 6 months.

10× Tris-buffered saline
Prepare 1 liter of 10× tris-buffered saline (TBS) by dissolving 24.2 g of Tris base and 90 g of NaCl in 990 ml of dH_2O and adjusting the pH to 8.0 by adding 10 ml of HCl (37% (vol/vol)). The solution can be stored at room temperature for up to 6 months.

10% (wt/vol) Benzocaine stock solution
Prepare 500 ml of 10% (wt/vol) benzocaine by mixing 50 g of benzocaine in 500 ml of 100% (vol/vol) ethanol. The solution can be stored at room temperature for up to 12 months.

0.03–0.006% (vol/vol) Benzocaine solution

Prepare 10 liters of axolotl anesthetic solution by mixing 500 ml of 10× TBS, 500 ml of 400% (wt/vol) Holtfreter's solution and 6–30 ml of 10% (wt/vol) benzocaine stock solution. Bring the volume to 10 liters with dH_2O. The solution can be stored at room temperature for up to 6 months.

Procedure

Validation of the axolotl genomic sequence targeted by CRISPR-Cas9 ● Timing 3 d

▲ **CRITICAL** The steps in this section serve two purposes: (i) to identify appropriate primers for amplifying the genomic locus of interest for downstream analysis of mutants and (ii) to unambiguously define the genomic sequence of the locus, as sequence polymorphisms or sequence inaccuracies in genome or transcriptome assemblies would confound the gRNA design process.

1 Identify the cDNA and the genomic sequences of the GOIs at the Axolotl-Omics.org website (https://www.axolotl-omics.org/). For a detailed stepwise procedure, see Supplementary Method 1.

2 Design PCR primers using the software Primer3Plus (https://primer3plus.com) for amplification of genomic DNA sequences of appropriate sizes (in general ~1 kb), and order two to three pairs of forward and reverse primers. We normally design the melting temperature (T_m) of both the forward and reverse primers for genomic PCR to be 60–65 °C. The difference between forward and reverse primer T_m values should be <2–3 °C.

3 Assemble the following PCR reaction on ice, using genomic DNA prepared from laboratory axolotls[9] as template. Perform PCR for all possible combinations (nine combinations, if three primer pairs are used) of the forward and reverse primers. For each primer combination, prepare enough PCR master mix to be sufficient for a gradient PCR with annealing temperatures from 50 to 70 °C, as listed in Step **4**.

▲ **CRITICAL STEP** The axolotl genome was assembled from a single male axolotl (https://www.axolotl-omics.org/assemblies) or (http://genome.axolotl-omics.org/cgi-bin/hgTracks?db=ambMex3). To identify possible single-nucleotide polymorphisms (SNPs) or other sequence variations between individuals, it may be necessary to use genomic DNA from several laboratory axolotls (e.g., five to ten axolotls obtained from the breedings of different parents) as PCR templates. It may be worthwhile to characterize the genomic sequence of the parental axolotls, which will be used for breeding to produce eggs for knockout and knock-in experiments.

Component	Amount	Final concentration
Axolotl genomic DNA	200 ng	10 ng/µl
Taq reaction buffer, 10×	2 µl	1×
dNTPs (10 mM each)	0.3 µl	150 µM
Forward primer (10 µM; Step **2**)	1 µl	0.5 µM
Reverse primer (10 µM; Step **2**)	1 µl	0.5 µM
Taq DNA polymerase (5 U/µl)	0.1 µl	
Nuclease-free H_2O	To 20 µl	

4 Perform a gradient PCR using the following cycler program.

Cycle number	Denature	Anneal	Extend
1	94 °C, 2 min		
2-36	94 °C, 30 s	50-70 °C, 30 s	72 °C, 1 kb/min
37			72 °C, 5 min

5 Run 5 µl of each PCR reaction and DNA ladder on a 1–1.5% (wt/vol) agarose gel prepared in 1× TBE buffer with 1× RedSafe DNA dye. Visualize the gel (emission/detection at 537 nm) using a gel imaging system such as a ChemiDoc Gel Imaging System.

▲ **CRITICAL STEP** PCR using the optimal primer combination under optimal conditions should yield a single product at the expected size. For an exemplary image, see Fig. 6a.

6 Sequence the single-band PCR products[81] and align the sequencing result to the original sequences from the database (Step **1**), using MacVector or the online free software Clustal Omega[82]. Usually, we expect the sequencing results to match the sequences in the database. If SNPs or other sequence

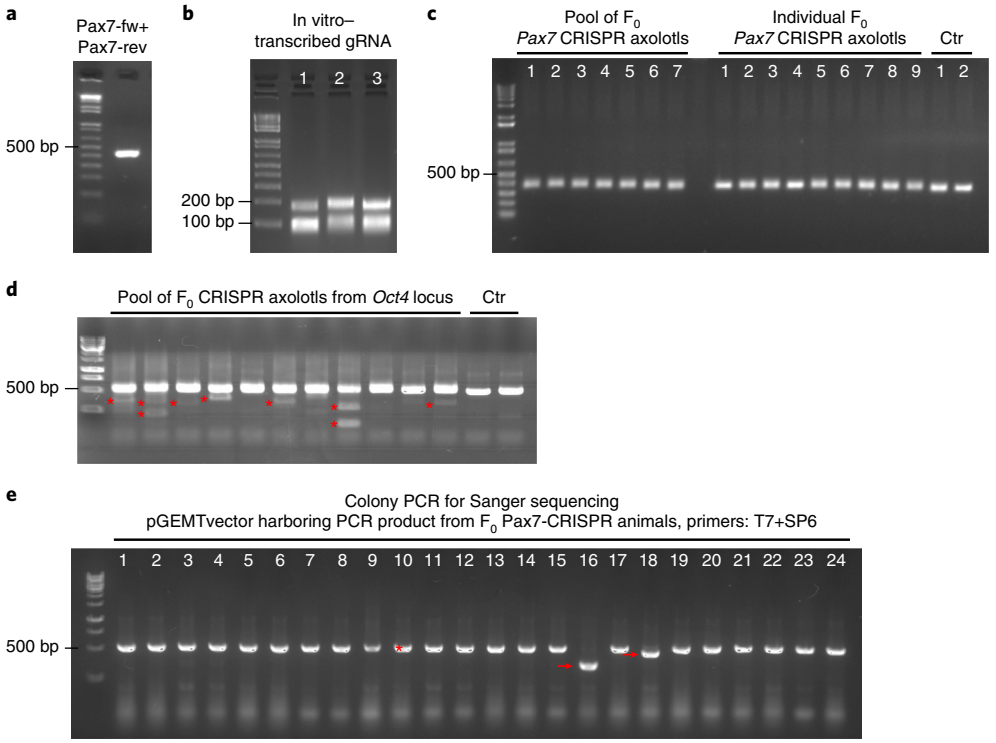

Fig. 6 | gRNA synthesis and genotyping of F_0 CRISPR axolotls. a, Representative image of an optimized genotyping PCR (Step **5**) using *d/d* axolotl genomic DNA as template. A single PCR product is amplified from the *Pax7* locus, using Pax7-fw and Pax7-rev primers. **b**, Representative image of purified gRNAs (Step **13**). They appear on a non-denaturing gel as two bands, one of which represents the native single-strand RNA; the other one may represent a secondary structure formed by the gRNA. **c**,**d**, Representative images of genotyping PCR using genomic DNA templates from pooled (**c**, left panel, **d**) or individual (**c**, right panel) F_0 CRISPR axolotls injected with gRNA-CAS9-NLS RNP complexes, or negative controls (Ctr, uninjected siblings of the F_0 CRISPR axolotls). Each pool contains genomic DNA from approximately ten tail tips of F_0 CRISPR axolotls. Genomic PCR products are amplified from the *Pax7* locus, using Pax7-fw and Pax7-rev primers (**c**), or the *Oct4* locus, using Oct4-fw and Oct4-rev primers (**d**). The size of the PCR products from the CRISPR axolotls in **c** is comparable to that of the control (**c**). Many PCR products in **d** contain smaller bands (red stars), in addition to the bands at the expected size, as compared to the negative control (Ctr). It is impossible to determine the presence of indels solely from the gel picture, as PCR products that harbor small indels are indistinguishable from the wild-type PCR product. Therefore, in order to evaluate the gRNA efficiency in an unbiased manner, all PCR products should be cloned and sequenced. **e**, Colony PCR for Sanger sequencing. Colony PCR is carried out as described in Step **21A(xiii)**. pGEMT vector containing PCR product from F_0 Pax7-CRISPR animals (as illustrated in **c**) is used as template for colony PCR. Red arrows indicate the colony PCR products harboring large indels, which can be distinguished. Most of the colony PCR products contain small indels that are indistinguishable from each other. All PCR products should be sequenced to determine the presence of indels.

variations are present at the gRNA-targeting sites, use the PCR product sequences as the final reference sequence for gRNA and targeting vector design. In very rare cases, if the CRISPR targeting loci of multiple animals (e.g., five to ten axolotls) are sequenced and the results differ from each other and are different from the sequences in the database, use the sequences from particular axolotls for gRNA design (Steps **7–9**), and then use these axolotls for breeding to produce eggs for injections (Steps **16** and **17**).

▲ **CRITICAL STEP** The procedure is more relevant when targeting introns, which generally harbor more sequence variations.

gRNA design and 'off-target' prediction ● Timing 2 h

7 Upload the genomic reference sequence from Step **6** for targeting to an online gRNA design tool, such as the sgRNA Designer from the Broad Institute . CAS9 (*S. pyogenes*) requires a PAM sequence 'NGG' downstream of the target sequence. The output from sgRNA Designer includes the list of all potential targeting sites from the input sequence with the feature 5′–nnnnnnnnnnnnnnnnnnnnNGG–3′ (n20+NGG).

8 Blast the full list of potential gRNA targets 5′–nnnnnnnnnnnnnnnnnnNGG–3′ (n18+NGG, removing the first two nucleotides at 5′) against the axolotl genome to predict potential off-targets at the Axolotl-Omics.org website (https://axolotl-omics.org/blast). For details, see Supplementary Method 2.

9 Choose preferentially gRNAs within these 18 nucleotides ('n18', Step **8**) that meet the following criteria: (i) having ≥3 nucleotide mismatches with other axolotl genomic loci; (ii) GC content between 40 and 80%; (iii) few or no regions of repetitive sequences and (iv) no presence of long (≥6 bp) stretches of single-nucleotide repeats, such as GGGGGG or TTTTTT. Order the sense and antisense oligonucleotides for the selected and control gRNAs as needed, according to the gRNA synthesis strategy that will be followed in Step **10** (option A or B; see Experimental design for further information on oligo design principles and control design).
▲ **CRITICAL STEP** It is strongly recommended to design more than three gRNAs, preferentially targeting several exons for the initial screening (see Steps **15–22**).

Template preparation for gRNA synthesis ● Timing 2–5 d

10 Synthesize gRNAs from DNA templates produced by one of the following two methods. We have provided a classic cloning method (option A) and a simple one-step PCR method (option B) for preparing gRNA synthesis templates. Using option B to prepare DNA template for gRNA synthesis is faster. However, one of the potential risks is mutations created during the synthesis of long oligonucleotides, which are difficult to detect in the PCR product without a cloning step.

(A) **Generation of DNA templates for gRNA in vitro transcription by PCR amplification from cloned plasmids**
▲ **CRITICAL** Steps **10A(i, ii)**, and **10A(iii, vi)** can be carried out in parallel.

(i) Mix the paired sense and antisense oligonucleotides in PCR tubes as detailed below:

Component	Amount
Sense oligo (100 μM; Table 1)	2 μl
Antisense oligo (100 μM; Table 1)	2 μl
10 mM Tris (pH 8.0)	46 μl
Total	50 μl

(ii) Anneal the oligonucleotides, using the following program in a PCR machine.

Step	Temperature	Time
1	95 °C	2 min
2	95–80 °C	Lower the temperature at 1 °C/s
3	80–25 °C	Lower the temperature at 0.1 °C/s
4	25 °C	Hold

(iii) Digest the cloning vector DR274 with the restriction enzyme BsaI-HF. Set up the reaction as outlined below:

Component	Amount
10× CutSmart buffer	2 μl
DR274	2–3 μg
BsaI-HF (20 U/μl)	0.5 μl
Nuclease-free H_2O	To 20 μl

(iv) Incubate the reaction at 37 °C for 4–16 h.

(v) Run all the digested vector and the DNA ladder on a 1.2% (wt/vol) agarose gel prepared in 1× TBE buffer supplied with the 1× RedSafe DNA dye. Check the gel using a gel imaging system. BsaI cuts twice on the DR274 plasmid and will lead to the release of a 25-bp DNA fragment that is too small to be visible on the gel. Excise only the gel piece containing the fully linearized vector (~2.1 kb) and place the gel piece in a 1.5-ml Eppendorf tube.
▲ **CRITICAL STEP** Because the single (partially) and double (fully) BsaI-linearized DR274 vectors are undistinguishable on the gel, it is critical to digest the vector completely with

BsaI in order to minimize the cloning background in the following steps. BsaI is a type IIS restriction enzyme that recognizes an asymmetric DNA sequence and cleaves outside of its recognition site. Therefore, the fully linearized vector does not self-ligate.

(vi) Recover the linearized vector from the agarose gel slice, using an Illustra GFX PCR DNA and Gel Band Purification Kit or a similar kit and quantify the DNA concentration using a NanoDrop or UV-visible spectrophotometer.

(vii) Ligate the annealed gRNA oligonucleotides (Step **10A(ii)**) to the linearized DR274 vector. Set up the reaction as detailed below and incubate at 4 °C overnight. It is important to include a control ligation in which the annealed oligonucleotides are replaced by water.

Component	Amount
Linearized DR274 vector (Step **10A(vi)**)	50–100 ng
Annealed oligos (Step **10A(ii)**)	3 µl
10× T4 ligase buffer	1 µl
T4 DNA ligase (400 U/ µl)	0.5 µl
Nuclease-free H_2O	To 10 µl

(viii) *Bacterial transformation and selection*. Add 5 µl of the ligation product or negative control (no gRNA oligos) to 20–30 µl of ice-cold competent cells of an *E. coli* strain, such as DH5α. Incubate the mixture on ice for 20 min, heat-shock at 42 °C for 1 min in a water bath or heat block, and then incubate again on ice for 3–5 min.

(ix) Add 600 µl of SOC medium to the transformed bacteria mixture and culture at 37 °C for 1 h with vigorous shaking (~700 r.p.m.).

(x) Centrifuge the bacteria at 8,000*g* for 1 min at room temperature. Re-suspend the bacterial pellet in 50–100 µl of LB liquid medium and then spread the bacteria on a pre-warmed LB–agar plate containing 50 µg/ml kanamycin.

(xi) Incubate the LB–agar plate at 37 °C overnight for selection of transformants.
▲ **CRITICAL STEP** It is expected that hundreds of colonies will be obtained on the gRNA-ligation plates, at least three times more those on the negative control (no gRNA oligos) plate.
? TROUBLESHOOTING

(xii) Pick three colonies from each gRNA-ligation plate, inoculate each colony into 3 ml of LB–kanamycin medium (50 mg/L) and culture at 37 °C overnight with shaking at 300 r.p.m.

(xiii) Isolate plasmid DNA from the bacterial cultures, using the Qiagen Plasmid Miniprep Kit or a similar kit and following manufacturer's instructions.

(xiv) Sequence the plasmids with primer DR274-fw as listed in Table 4. The majority of sequenced plasmids should harbor the correct insert.

(xv) Set up the PCR reaction as below, using the primers DR274-fw and DR274-rev, listed in Table 4, with the sequence-verified plasmids from Step **10A(xiv)** as templates.
▲ **CRITICAL STEP** This step amplifies and enriches the DNA fragments harboring the T7 promoter and gRNA sequences from the sequence-verified plasmids to obtain high amounts of synthesized gRNA in the coming steps. It is important to use a high-fidelity DNA polymerase (e.g., Phusion, Pfu) to perform the PCR reaction in order to minimize mutations in the derived PCR products.

Component	Amount	Final concentration
DR274-gRNA plasmid (Step **10A(xiv)**)	3–5 ng	60–100 pg/µl
Phusion HF Buffer, 5×	10 µl	1×
dNTPs (10 mM each)	0.5 µl	100 µM
DR274-fw (100 µM; Table 4)	0.35 µl	0.7 µM
DR274-rev (100 µM; Table 4)	0.35 µl	0.7 µM
Phusion DNA polymerase (2 U/µl)	0.5 µl	
Nuclease-free H_2O	To 50 µl	

(xvi) Perform the PCR, using the following cycler program.

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–36	98 °C, 20 s	60 °C, 20 s	72 °C, 20 s
37			72 °C, 5 min

(xvii) Run 2 µl of the PCR product on a 1.5% (wt/vol) agarose gel to check the amplification. Expect a single and strong 282-bp PCR product. Purify the rest of the PCR product, using a QIAquick PCR Purification Kit according to the manufacturer's instructions and elute in 20 µl of DNase- and RNase-free water. Measure the DNA concentration using a NanoDrop spectrophotometer. The DNA concentration generally ranges between 100 and 200 ng/µl.
▲ **CRITICAL STEP** Completely remove the extra wash buffer on the edges of the O-ring in the QIAquick column, using vacuum suction or a piece of autoclaved filter paper. Any leftover wash buffer may inhibit the subsequent RNA in vitro transcription. For elution, directly add DNase/RNase-free water to the center of the QIAquick column membrane.
■ **PAUSE POINT** The purified PCR product can be stored at −20 °C for at least 1 month.

(B) **Generation of DNA templates for gRNA in vitro transcription by PCR amplification from synthesized oligonucleotides**

(i) Set up the PCR reaction as detailed below. We use a very low concentration of the primer pair (gRNA forward oligo and gRNA reverse oligo) to produce the initial full-length single guide RNA (sgRNA) template that will be further amplified by gRNA-fw2 and DR274-rev primer pair (Fig. 3) to yield a high-quality PCR product.

Component	Amount	Final concentration
Phusion GC buffer, 5×	10 µl	1×
dNTPs (10 mM each)	0.5 µl	100 µM
gRNA-fw2 (100 µM; Table 4)	0.35 µl	0.7 µM
DR274-rev (100 µM; Table 4)	0.35 µl	0.7 µM
gRNA forward oligo (100 µM; Table 2)	0.02 µl	0.04 µM
gRNA reverse oligo (100 µM; Table 2)	0.02 µl	0.04 µM
Phusion DNA polymerase (2 U/µl)	0.5 µl	
Nuclease-free H_2O	To 50 µl	

(ii) Perform the PCR using the program as listed in Step **10A(xvi)**.
▲ **CRITICAL STEP** This step amplifies and enriches the DNA fragments harboring a T7 promoter and gRNA sequences for gRNA synthesis. Perform the PCR reaction using a high-fidelity DNA polymerase (e.g., Phusion, Pfu) to minimize mutations in the derived PCR product.

(iii) Check and purify the PCR product as previously described in Step **10A(xvii)**.

In vitro transcription of gRNA ● Timing 2 d

11 Carry out the gRNA preparation procedure according to the MEGAshortscript T7 Transcription Kit manufacturer's instructions, as briefly described in Steps **11–14**. Assemble the in vitro transcription reaction for gRNA synthesis, using 500–750 ng of purified PCR products (from Step **10A(xvii)** or Step **10B(iii)**) as template.
▲ **CRITICAL STEP** Prepare in parallel *Tyrosinase*-gRNA and *GFP*-gRNA as controls for gene knockout, and *Pax7*-gRNA as control for knock-in experiments[9,15,21] (for details, see Experimental design).
▲ **CRITICAL STEP** Wear gloves to protect the samples from RNase contamination during gRNA synthesis and purification.

12 Incubate the reaction at 37 °C overnight in a PCR machine, followed by DNase treatment at 37 °C for 15 min.

13 Run 0.5 µl of the RNA sample on a 2% (wt/vol) agarose gel (prepared in TBE buffer) to estimate the yield and the quality of the synthesized gRNA.

▲ CRITICAL STEP On a 2% (wt/vol) agarose gel, high-quality gRNAs run as a single, often double, sharp and clear band(s). For an exemplary image, see Fig. 6b.

▲ CRITICAL STEP Clean the electrophoresis tank with RNaseZap before running the gel and wear gloves to protect the samples from RNase contamination.

? TROUBLESHOOTING

14 Use lithium chloride solution (7 M lithium chloride, 50 mM EDTA, from the MEGAshortscript T7 Transcription Kit) to precipitate the gRNA, and then pellet the gRNA by centrifugation at 16,200*g* for 10–15 min at 4 °C. Wash the RNA pellet carefully three times with 70% (vol/vol) ethanol, spinning at at 16,200*g* for 5 min at 4 °C each time and discarding the supernatant. Air-dry the pellet for 5–10 min and dissolve it in 30–50 µl of RNase-free water. Measure the concentration using a NanoDrop spectrophotometer. The gRNA concentration normally ranges between 2 and 4 µg/µl.

▲ CRITICAL STEP It is critical to completely remove the lithium chloride during the 70% (vol/vol) ethanol washing steps. The presence of lithium chloride may cause developmental defects in the injected embryos.

▲ CRITICAL STEP RNA pellets should be clearly visible. Perform 70% (vol/vol) ethanol washing steps carefully to avoid the loss of the RNA pellets.

? TROUBLESHOOTING

■ PAUSE POINT Purified gRNAs can be divided into aliquots (5–10 µl each) and stored at −80 °C for up to 6 months.

gRNA activity evaluation in vivo ● Timing 2 weeks

▲ CRITICAL We have tested several methods to determine gRNA activity and found that evaluation of the gRNA activity in vivo in axolotls is the most reliable method so far. We evaluate gRNAs by injection of gRNA-CAS9-NLS RNP complexes into single-cell-stage eggs, growing up the eggs to hatchlings, and then genotyping injected animals. We include two genotyping protocols to detect indels: Sanger sequencing (Step **21A**) and deep sequencing (NGS) of a PCR-amplified genomic region (Step **21B**).

15 Prepare an injection needle and injection plate as previously described[2].

16 Collect freshly laid (ideally within 2–4 h) single-cell-stage axolotl eggs, and then transfer and dejelly the eggs in 1× MMR solution with pen–strep as previously described[2].

▲ CRITICAL STEP During the dejellying step, avoid poking eggs with the forceps and carefully remove the jelly. Improper dejellying may cause defects in axolotl development.

17 Mix CAS9-NLS protein and gRNA as detailed in the table below, and then incubate the mixture at room temperature for 5 min, allowing the formation of gRNA-CAS9-NLS RNP complexes. Fill the injection needle with 2–5 µl of gRNA-CAS9-NLS solution. We determined the amount of gRNA for axolotl egg injection according to previously published literature[24,28,31,83-86], calculated based on the volume of eggs compared to that from other species.

▲ CRITICAL STEP It is recommended to prepare *Tyrosinase*-gRNA-CAS9-NLS (and, optionally, *Pax7*-gRNA-CAS9-NLS), and *GFP*-gRNA-CAS9-NLS) RNP complexes as the positive and negative controls[9,15,21], respectively (for details, see Experimental design).

Component	Amount	Final concentration
CAS9-NLS protein (5 µg/µl), prepared in 1× CAS9 buffer	1 µl	0.5 µg/µl
gRNA (from Step **14**)	4 µg	0.4 µg/µl
CAS9 buffer, 10×	0.9 µl	1× (CAS9 protein is reconstituted in 1× CAS9 buffer)
Nuclease-free H_2O	To 10 µl	

▲ CRITICAL STEP Wear gloves to protect the samples from RNase contamination during gRNA-CAS9-NLS complex preparation.

18 Carefully transfer the axolotl eggs (from Step **16**) to the injection plate pre-filled with 1× MMR solution with 20% (wt/vol) Ficoll and pen–strep, inject 5 nl of gRNA-CAS9-NLS complex solution into each egg, and incubate the injected eggs in 1× MMR solution with 20% (wt/vol) Ficoll and pen–strep for 2–3 h to allow the closure of the holes from the injections as previously described[2]. For each gRNA, inject ~30 eggs for gRNA activity evaluation. In parallel, inject ~30 eggs with

Tyrosinase-gRNA-CAS9-NLS complexes (Step **17**) as the positive control[15] (for details, see Experimental design). Keep around ten dejellied, uninjected eggs as a developmental/quality control.
▲ **CRITICAL STEP** The first cell division in axolotls occurs ~6 h after the egg is laid. It is critical to carry out the injections in freshly laid eggs; the earlier (normally within 3 h), the better. If the eggs are laid overnight, screen all eggs under a stereomicroscope and keep only single-cell-stage eggs for injection.
▲ **CRITICAL STEP** It is possible to reduce the Ficoll concentration. 5–10% (wt/vol) Ficoll is sufficient to prevent leakiness of the injected eggs. However, using the lower range of Ficoll concentrations can be challenging for new experimenters, because if the hole created by the injection is too big, then the embryo's content can leak when using a lower concentration of Ficoll. Therefore, it is safer to use higher Ficoll concentrations (e.g., 20% (wt/vol) Ficoll) in the injection buffer.

19 Transfer the injected axolotl eggs in 0.1× MMR solution with 5% (wt/vol) Ficoll and pen–strep, and incubate the eggs overnight as previously described[2].

20 Transfer the injected axolotl eggs to 24-well plates (one egg per well) prefilled with 0.1× MMR solution with pen–strep and raise the eggs in 0.1× MMR solution with pen–strep until the hatching stage (~2 weeks at room temperature). In general, it is expected that 20–30 larvae will be obtained.
▲ **CRITICAL STEP** Make sure to transfer the injected eggs to 0.1× MMR solution with pen–strep before reaching the gastrula stage. The presence of Ficoll may cause the malformation of the developing axolotl embryos after the gastrula stage.
▲ **CRITICAL STEP** Injection of certain gRNA-CAS9-NLS complexes may cause an early embryonic phenotype, perhaps even early embryonic lethality. To determine whether the phenotypes in CRISPR-injected axolotls are derived from particular gRNA-CAS9-NLS complexes rather than from an artifact (variations in egg quality between individual matings and improper handling of axolotl eggs may cause unspecific developmental defects), it is critical to compare the CRISPR–Cas9-injected axolotls with the control *Tyr*-gRNA, PBS injected or uninjected eggs. If early embryonic lethality occurs specifically in axolotls injected with the newly designed gRNA but not in the controls before the larval (hatching) stage, carry out sample collection and genotyping as soon as possible (Step **21A** or **21B**).
? TROUBLESHOOTING

21 Perform genotyping on F_0 CRISPR axolotl larvae, using either option A (Sanger sequencing) or option B (NGS). Both methods are suitable for evaluation of gRNA activity to identify efficient gRNAs (a critical first step in the generation of knockout and knock-in axolotls) and for subsequent genotyping of F_0 CRISPR knockout axolotls. Option A provides a detailed Sanger sequencing–based protocol that is ideal for genotyping a small number (e.g., <30) of animals. It is a routine molecular biology procedure and is easy to establish, particularly in a lab carrying out CRISPR experiments for the first time. Option B provides a detailed indel detection protocol based on NGS. NGS is advantageous for genotyping of F_0 animals because it is much more cost effective than Sanger sequencing when analyzing a large number (hundreds to a thousand) of animals; an Illumina Nano flowcell delivers ~2 million paired-end reads, which is sufficient to analyze 500 animals.

(A) **Genotyping of F_0 CRISPR axolotl larvae by Sanger sequencing**

(i) While waiting for development of axolotl embryos, use Primer3Plus to design the genotyping primers based on the verified genomic sequence (Step **6**), and using axolotl genomic DNA (Step **2**) as template to determine the optimized genotyping primer pair and PCR reaction conditions following Steps **3–5**.
▲ **CRITICAL STEP** Design the primer pairs to amplify ideally 500- to 600-bp PCR products, in which the gRNA cutting site is situated roughly in the middle. Choose the primer pair and the PCR conditions that produce a strong, single PCR product at the expected size for further genotyping. Alternatively, primers identified in Steps **2–5**, which yield a genomic PCR product at ~1 kb, are also acceptable, although not optimal for the following genotyping PCR. Note: genotyping primers for *Tyrosinase, Pax7* and *Oct4* CRISPR animals are listed in Table 4.

(ii) Anesthetize axolotl larvae in 0.006% (vol/vol) benzocaine solution. Transfer each individual carefully with ring forceps and place it on the surface of a Petri dish. Alternatively, cut the tip of a plastic Pasteur pipette and use it for transferring larvae.

(iii) Use a sharp scalpel to cut a tiny piece of tail tip (~0.5–1 mm in length) from each individual animal, pick up the tissue with forceps, and transfer it to the bottom of a 1.5-ml Eppendorf tube. Collect all the tail tips from axolotls injected with the same gRNA in the same Eppendorf tube. Keep the tissues on ice during sample collection.

▲ **CRITICAL STEP** Axolotl larvae dry quickly after being placed in a Petri dish. Do not handle too many larvae in the same time. It is better to place the second axolotl larva in the Petri dish after finishing the tail sample collection from the first one.

▲ **CRITICAL STEP** Collecting too much tissue from each individual may result in excessive impurities in the crude DNA extract, which may inhibit the subsequent PCR reaction. Tail tips from a maximum of 20 axolotls can be collected in one Eppendorf tube. If you have >20 axolotls, collect approximately equal numbers of tail tips in two tubes.

▲ **CRITICAL STEP** Include all surviving axolotls, even the ones showing obviously unfavorable developmental phenotypes for sample collection. The pooling of animals injected with the same gRNA can help reduce potential experimental variation to yield a more accurate assessment of gRNA cutting efficiency.

(iv) After collecting tissue samples from animals injected with one gRNA, use an alcohol burner to flame the scalpel and forceps. For the next gRNA, repeat Step **21A(ii, iii)**.

(v) Prepare crude DNA extracts by adding 200 µl of 50 mM NaOH to the Eppendorf tubes containing the collected tail tips (Step **21A(iii)**) and incubate the tubes at 95 °C for 10–15 min.

▲ **CRITICAL STEP** Make sure all tail tissues collected are fully submerged in the NaOH buffer. Mix the samples at ~500 r.p.m. on a shaking heat block or flick the tubes one to two times by hand during the incubation. In general, the tissue completely disappears within 5–10 min.

(vi) Add a 1/10 volume (20 µl) of 1 M Tris (pH 8) to the crude DNA extract, mix well by flicking, and centrifuge briefly for 5–10 s at 16,200*g* at room temperature.

■ **PAUSE POINT** The crude DNA preparation can be stored at 4 °C for at least 1 month.

(vii) Assemble one PCR reaction per crude DNA sample on ice and perform the PCR for ≤30 amplification cycles, using the optimal cycler program identified in Step **21A(i)**. If tail tips for one gRNA are split between multiple tubes, keep the crude DNA extracts separate and assemble multiple PCRs for this gRNA. Prepare the PCR mix for all samples (including the control) by mixing the components listed below.

Component	Amount	Final concentration
Crude DNA extract (Step **21A(vi)**)	10 µl	
Sigma Red Taq master mix, 2×	25 µl	1×
Primer-fw (10 µM; Step **21A(i)**)	2.5 µl	0.5 µM
Primer-rev (10 µM; **Step 21A(i)**)	2.5 µl	0.5 µM
Nuclease-free H_2O	10 µl	
Total	50 µl	

▲ **CRITICAL STEP** Set up the PCR reaction using Sigma 2× Taq Master mix (REDExtract-N-Amp PCR Ready Mix), which efficiently amplifies the target sequences from crude DNA extract.

(viii) Check the PCR results by running 2–5 µl of each PCR product and the DNA ladder on a 1.5% (wt/vol) agarose gel in 1× TBE buffer. Because of the genomic modifications, you may observe PCR products other than the PCR product of the expected size on the gel. For exemplary images, see Fig. 6c,d.

? TROUBLESHOOTING

(ix) Ligate 1 µl of each unpurified PCR product to the pGEMT vector by following the pGEMT Vector Systems manufacturer's instructions. Set up the ligation reaction and keep it at 4 °C overnight.

■ **PAUSE POINT** The ligation products can be stored at −20 °C for at least 1 week.

(x) Transform each ligation product into a competent bacteria strain (e.g., DH5α). In brief, add 5 µl of ligation product to 20–30 µl of competent cells, incubate the bacteria on ice for 20 min, heat-shock at 42 °C for 1 min in a water bath or heat block, and then incubate again on ice for 3–5 min. Spread all bacteria on an LB–agar plate containing 0.1 mM IPTG, 40 µg/ml X-gal and 100 µg/ml ampicillin. Keep the LB–agar plate at 37 °C overnight for the selection of transformants.

▲ **CRITICAL STEP** It is expected that a few hundred white colonies (the colonies harboring PCR products cloned into pGEMT vectors) will be obtained.

■ **PAUSE POINT** The bacterial LB plates can be stored at 4 °C for at least 2 weeks.

(xi) Pick 30–40 white colonies from each plate and set up colony PCR according to the table below:

Component	Amount	Final concentration
Single colony of bacteria	—	—
Sigma Red Taq master mix, 2×	5 µl	1×
T7 primer (10 µM; Table 4)	0.5 µl	0.5 µM
Sp6 primer (10 µM; Table 4)	0.5 µl	0.5 µM
Nuclease-free H_2O	4 µl	
Total	10 µl	

(xii) Perform the PCR using the following program.

Cycle number	Denature	Anneal	Extend
1	94 °C, 3 min		
2–36	94 °C, 45 s	52 °C, 45 s	72 °C, 1 kb/min
37			72 °C, 5 min

(xiii) Run 2–5 µl of each PCR product and a DNA ladder on a 1.5% (wt/vol) agarose gel to examine the PCR results. The size of PCR products from individual colonies may vary, owing to the presence of the indels. For an exemplary image, see Fig. 6e.

(xiv) Send the unpurified PCR products directly for Sanger sequencing, with a T7 or Sp6 primer. Analyze the sequencing results using software (e.g., MacVector or Lasergene, https://www.dnastar.com), and calculate the mutagenesis rate (the percentage of clones carrying indels out of the total number of colonies sequenced) in each DNA pool.

(B) **Genotyping of F_0 CRISPR axolotl larvae by NGS**

(i) Design, synthesize and test gene-specific primers for NGS genotyping.

▲ **CRITICAL STEP** While waiting for the development of axolotl embryos, use Primer3Plus to design genotyping primers based on the verified axolotl genomic sequence (Step **6**), and determine the optimized genotyping primer pair and PCR reaction conditions following Steps **3–5**. Note: gene-specific primers for *Tyrosinase, Pax7* and *Oct4* CRISPR animals are listed in Table 4.

▲ **CRITICAL STEP** Design the primer pairs (desalted primers are sufficient for this purpose) to amplify a 250- to 270-bp region of genomic DNA. This PCR product length is important in order to later allow separation of primer–dimers from the amplicon and also to allow the paired-end Illumina reads to overlap. This length is recommended for use in conjunction with a MiSeq Reagent Nano Kit v2 (300 cycles). Note that newer versions of the Illumina chemistry (MiSeq Reagent Kit v3 for 300-bp paired-end reads) support sequencing of longer fragments and can be useful when three different gRNAs are designed in nearby regions.

▲ **CRITICAL STEP** Design the primers so that the gRNA cutting site is situated roughly in the middle (ideally, not closer than 50 nt from the cut site). Deletions are usually <20 nt long, but if the primers are placed too close to the cutting site, a long deletion could remove the primer-binding site and thus conceal the mutation.

(ii) Add the forward primer adapter and reverse primer adapter sequences (see Experimental design and Table 4) to the verified gene-specific primers from Step **21B(i)**. Combining with the gene-specific primer (the forward gene-specific primer sequence will replace the 'N' letters in NGS-PCR1-fw, and the reverse gene-specific primer sequence will replace the 'N' letters in NGS-PCR1-rev) yields a complete primer pair (see NGS-PCR1-fw and NGS-PCR1-rev in Table 4). Desalted primers are sufficient for this purpose.

▲ **CRITICAL STEP** The 33 or 34 bases added as adapters to the PCR primers are in addition to the gene-specific primer at each end. As such, they are not homologous to the 250–270 bases of genomic sequence to be amplified.

(iii) *Collect tissue and prepare genomic DNA of F_0 CRISPR axolotls.* We designed the DNA preparation procedure for large-scale (from dozens to a few hundreds, even a thousand) samples. In Step **21B**, each individual tail tip is placed into a separate

PCR tube. This is in contrast to Step **21A(iii)**, in which multiple tail tips from the same gRNA are pooled in 1.5-ml Eppendorf tubes to reduce the number of samples for Sanger sequencing. In Step **21B**, however, the barcoding system allows assignment of individual sequence reads to individual samples; therefore, multiple samples can be sequenced in a single NGS event. Cut the tail tip (an ~1.0-mm-long piece) from each anesthetized individual as previously described (Step **21A(ii)**), and place each tissue sample into a separate well of eight-well strips or 96-well format 0.2-ml PCR tubes, depending on the number of samples. Flame the scalpel between tail collections to avoid DNA cross-contamination. Collect tail tissue of all F_0 CRISPR axolotls (generally 20–30 animals from each designed gRNA).

PAUSE POINT For convenience, collected tissue biopsies can be kept at −80 °C for up to 1–2 months, until samples from all animals to be processed together are collected.

(iv) Carry out DNA preparation according to Step **21A(v, vi)**. Incubate the PCR tubes in a PCR thermocycler.

CRITICAL STEP Make sure that all tail pieces are submerged in the digestion buffer. Mix the samples and briefly (~10–30 s) spin down at ~3800*g* at room temperature, using a VWR mini centrifuge (for eight-well strips) or the Eppendorf 5920 R centrifuge (for 96-well plates) before placing them in the PCR machine. In general, tissue pieces completely disappear within 10 min of incubation.

PAUSE POINT The crude DNA preparation can be stored at 4 °C for at least 1 month.

(v) Set up the first PCR (PCR1) reaction using 2× Sigma Red Taq master mix, which efficiently amplifies the target sequences from crude DNA extract. Assemble one PCR reaction per sample (Step **21B(iii)**, tail tip of an individual axolotl) on ice as follows, and perform the PCR for 38 amplification cycles, using the optimal cycler program identified in Step **21B(i)**. Prepare the PCR master mix for all samples (including the control injected with *Tyr*-gRNA; Steps **16** and **17**) by combining all listed components except the genomic DNA.

Component	Amount	Final concentration
Axolotl genomic DNA (Step **21B(iii)**)	1 µl	
Sigma Red Taq master mix, 2×	10 µl	1×
NGS-PCR1 forward primer (10 µM; Step **21B(ii)**)	0.5 µl	0.25 µM
NGS-PCR1 reverse primer (10 µM; Step **21B(ii)**)	0.5 µl	0.25 µM
Nuclease-free H_2O	To 20 µl	

CRITICAL STEP Use a limiting concentration of primers in the PCR reaction so that they will be consumed during the amplification cycles. This is to avoid carryover of leftover primers into PCR2 (Step **21B(viii)**) and also to ensure that all samples will be amplified to a similar level.

(vi) Check the PCR results by running 2–5 µl of each PCR product (or a few randomly selected samples, if handling a large number of samples) alongside a DNA ladder on a 2% (wt/vol) agarose gel. For an exemplary image, see Supplementary Fig. 2a.

CRITICAL STEP Normally, a single PCR product at the expected size will be observed on the gel. The PCR amplicons harboring indels are normally indistinguishable from the PCR amplicons from the unmodified allele, because the indels are typically short. Alternatively, there may be multiple bands other than the PCR product with the expected size on the gel, owing to the presence of large indels and the mosaicism in F_0 CRISPR axolotls.

(vii) Design and order barcoded forward-P5 and reverse-P7 primers for the second, indexing PCR (see Table 4, Supplementary Tables 1 and 2, and Experimental design for further information on the design of primers)[66].

(viii) Assemble the second PCR (indexing PCR, NGS PCR2) using P5-indexes-forward and P7-indexes-reverse primers to create double-indexed sequencing library molecules for Illumina sequencing[66]. To do so, dilute the PCR product from PCR1 by adding 60 µl of water to the remaining 15 µl of PCR product from PCR1 (Step **21B(v, vi)**). Use 1 µl of this diluted PCR1 as template for PCR2 and amplify the template with a high-fidelity DNA polymerase (e.g., 2× Phusion High-Fidelity PCR Master Mix) as follows. We recommend

first pipetting the 2× PCR Master Mix into each well, then adding the template, and, finally, adding 2 µl of P5-indexes-fw primer (6.25 µM) to each column and 2 µl of P7-indexes-rev (6.25 µM) to each row, using a multichannel pipette (final PCR volume, 10 µl; final concentration of each primer, 1.25 µM), to give each sample a unique barcoded primer combination. For details, see Experimental design.

Component	Amount	Final concentration
Phusion High-Fidelity PCR Master Mix, 2×	5 µl	1×
Diluted template from PCR1 (Step **21B(v)** and **(vi)**)	1 µl	
Nuclease-free H_2O	To 6 µl	

▲ **CRITICAL STEP** Be very careful to avoid cross-contamination when pipetting. To minimize cross-contamination, always spin PCR-tube strips before opening the lids. Keep multiple aliquots of primer sets.

(ix) Carry out PCR using the thermocycler program below, which should result in good amplification.

Cycle number	Denature	Anneal	Extend
1	94 °C, 5 m		
2–39	94 °C, 20 s	64 °C, 30 s	72 °C, 20 s, 1 kb/min
40			72 °C, 5 min

▲ **CRITICAL STEP** Perform the primer annealing step at 64 °C. Adjust the other PCR conditions according to the manufacturer's recommendations accompanying the high-fidelity polymerase used.

▲ **CRITICAL STEP** Amplify, using enough cycles to reach the plateau stage (38 cycles), to adjust for different starting amounts of the template.

(x) Run 2 µl of several random samples from PCR2 (Step **21B(viii)**) on a 1.5% (wt/vol) agarose gel. Because of the high number of amplification cycles, all samples should have similar band intensity. For an exemplary image, see Supplementary Fig. 2b.

▲ **CRITICAL STEP** Each sample is now uniquely barcoded, so they can be pooled to purify them all together.

(xi) Pool a defined volume (3 µl) of all PCR2 fragments from Step **21B(viii)** and purify with Agencourt AMPure XP beads, following the Agencourt protocol, with a bead/sample (vol/vol) ratio = 0.9:1. Elute the PCR amplicon with 30 µl of H_2O.

▲ **CRITICAL STEP** This step is important for removing all short PCR artifacts. As the beads are expensive, it is sufficient to purify 30–50 µl of the pooled PCR mix.

(xii) Measure the DNA concentration using a NanoDrop spectrophotometer or a Qubit fluorometer. The DNA concentration of purified PCR products generally ranges between 400 and 1,000 ng/µl.

(xiii) Perform sequencing using the Illumina MiSeq system. Up to 500 samples can easily be pooled and sequenced in a single event, which reduces the cost per sample. Perform paired-end sequencing according to the protocol provided by Illumina (https://support.illumina.com/downloads/miseq_system_user_guide_15027617.html). We use a MiSeq Reagent Nano Kit v2, which supports up to 1 million 150-nt-long paired-end reads.

(xiv) Analyze the sequencing results, using a free online analysis tool such as CRISPResso[87]. Provide the forward and reverse reads obtained from the Illumina MiSeq system in FASTQ format, along with the unmodified amplicon sequence (from Step **6**), the open reading frame of the targeted gene (https://www.axolotl-omics.org/; see also Supplementary Method 1), and the gRNA sequence. Run the program with the embedded parameters in the online software to obtain information about the frequency and distribution of the generated indels and the proportion of created frameshift mutations. As an example, we included the NGS analysis of three F_0 *Tyrosinase* CRISPR axolotls in Supplementary Fig. 3.

22 Pool the Sanger sequencing or NGS results to determine gRNA efficiency. Choose gRNAs yielding a ≥90% mutagenesis rate for subsequent knockout and knock-in experiments.

▲ CRITICAL STEP gRNAs producing a high percentage of indels are also more efficient in generating knock-in axolotls.
? TROUBLESHOOTING

Characterization of gene knockout axolotls ● Timing 2 weeks

23 The axolotls obtained at Steps **15–20** can already be used for phenotyping analysis. If only the pools of animal tails were genotyped by Sanger sequencing (Step **21A**), then it is necessary to carry out the second round of genotyping analysis on individual CRISPR-axolotls. If necessary, inject more single-cell-stage axolotl eggs with the selected gRNA to produce more mutants.

24 If the F_0 CRISPR-axolotls show obviously unfavorable early developmental phenotypes, categorize them into three or four groups according to the severity of the phenotypes for further analysis: (i) mild or no phenotype, (ii) medium phenotype, (iii) strong phenotype and (iv) lethal phenotype. If there is no obviously unfavorable early developmental phenotype in F_0 CRISPR-axolotls, go directly to Step **25**. Also check the phenotypes in the positive controls, *Tyrosinase* and *Pax7* CRISPR axolotls. *Tyrosinase* knockout axolotls lose melanophore pigmentation that can be observed immediately after hatching (within ~2 weeks)[15], and *Pax7* knockout animals develop strong limb and trunk muscle defects that become obvious in ~3 months[9].
▲ CRITICAL STEP The criteria for determination of the severity of phenotypes vary from case to case. As an example, see the classification of F_0 *Pax7* CRISPR animals[9].

25 Carry out genotyping PCR and sequencing on individual F_0 CRISPR axolotls as described in Step **21A** or **B**, with the following modifications: Collect a tiny piece of tail tip (~1 mm) from each individual axolotl into single tubes for DNA extraction (at Step **21A(iii)**); add 50–100 µl of 50 mM NaOH for tissue lysis (at Step **21A(v)**); pick ~20–25 clones per each individual axolotl genotyped when using the Sanger sequencing method (at Step **21A(xi)**).

26 Analyze the sequencing results. Identify the homozygous types of F_0 frameshift CRISPR-axolotl mutants. In Sanger sequencing (Step **21A**) or NGS (Step **21B**) results, the homozygous-type CRISPR mutants should harbor only a single or two types of frameshift mutations at the gRNA-targeted genomic loci, and should lack the unmodified wild-type sequence (Supplementary Fig. 3a). The presence of two different modifications in individuals very likely represents two independent gene modification events on the paternal and maternal alleles that occurred in injected single-cell stage axolotl embryos. A single-type frameshift mutation very likely represents an identical modification on the paternal and maternal alleles, or else one of the two modifications (e.g., a large deletion) on either allele could not be detected by PCR and sequencing.
▲ CRITICAL STEP To exclude the possibility that the phenotypes observed are from unexpected off-target modifications, it is necessary to characterize the phenotypes from a second gRNA targeting the same genomic locus. Two gRNAs targeting the same genomic locus should produce the same spectrum of phenotypes.
▲ CRITICAL STEP It is important to correlate the phenotype with genotypes to prove that the phenotype is caused by the targeted gene mutation. For gRNAs yielding early developmental phenotypes, the homozygous types of F_0 CRISPR mutants should fall into the categories of 'strong phenotype' or 'lethal phenotype' (Step **24**). The categories of F_0 CRISPR mutants showing 'mild' or 'no phenotype' and 'medium phenotype' (Step **24**) are in general mosaics, in which there is a proportion of unmodified wild-type or in-frame mutant (functional) alleles.
▲ CRITICAL STEP In axolotls, it is often observed that the mutant phenotypes occur in only half (left or right) of the body axis[9,15], when mutagenesis has occurred in only one cell within two-cell stage embryos.
? TROUBLESHOOTING

27 Examine the expression of the targeted gene upon frameshift mutation in F_0 CRISPR-axolotls by western blot or immunohistochemistry analysis[9,15], to determine whether the F_0 CRISPR mutants are true knockouts.
▲ CRITICAL STEP The frameshift mutations might not always result in a complete loss of the gene product[9]. We have observed the production of a truncated PAX7 protein lacking the N-terminal portion of the protein when the frameshift mutation is close to the original start codon 'ATG'[9]. To completely knock out the GOI, it is necessary to identify an alternative downstream 'ATG' and design new gRNAs 3′ of the alternative start codon.

28 Raise selected F_0 CRISPR axolotls to adulthood for germline transmission.
▲ CRITICAL STEP Choose F_0 CRISPR axolotls showing mild or no phenotype, but containing a considerable proportion of frameshift mutations, as founders. CRISPR axolotls showing severe phenotypes may fail to develop to adults for germline transmission.

Generation and characterization of gene knock-in axolotls ● Timing 2 weeks

29 Design targeting constructs (Experimental design) based on the characterized gRNA (Step **22**) that target the intron or coding exon of the GOI.

30 Prepare the targeting construct, using Gibson assembly[79] according to the manufacturer's instructions. For details, see Experimental design and Box 1.

31 Carry out plasmid maxipreps of the correct targeting constructs, using a Qiagen Plasmid Maxi Kit according to the manufacturer's instructions.

■ PAUSE POINT The plasmid maxipreps can be stored at −20 °C for at least 6 months.

32 Purify the maxipreps with phenol/chloroform. In brief, bring up the volume of the maxiprep sample to 500 µl with TE buffer and add an equal volume of phenol/chloroform, then place the tube on a tube rotator for 15–30 min at 25 r.p.m. at 4 °C. Centrifuge the tube at 16,200*g* for 10 min at 4 °C, and carefully transfer the upper aqueous phase to a new tube. Repeat the extraction step with an equal volume of chloroform only, again transfer the upper aqueous phase to a new tube, and add a 1/10 volume of sodium acetate (pH 5.2) and 2.5× volumes of ice-cold 100% (vol/vol) ethanol. Mix the solution and keep the tube at −20 °C for 30 min, pellet the plasmid DNA by centrifuging for 10 min at 16,200*g* at 4 °C, wash the DNA pellet in 70% (vol/vol) ethanol for 5 min at room temperature, and centrifuge the tube for 5 min at 16,200*g* at 4 °C. Remove the 70% (vol/vol) ethanol and repeat the 70% (vol/vol) ethanol washing step once. Air-dry the DNA pellet for 5–10 min at room temperature, and dissolve the DNA pellet in 100 µl of RNase-free TE buffer.

33 Prepare the CAS9-NLS protein, gRNA (Step **22**) and circular targeting construct (Step **32**) mixture for injection according to the table below. We prepare 10 µl of mixture, which is sufficient for ~200 eggs. Incubate the mixture at room temperature for 5 min before injection.

Component	Amount	Final concentration
CAS9-NLS protein (5 µg/µl)	1 µl	0.5 µg/µl
gRNA (Step **22**)	4 µg	0.4 µg/µl
Targeting construct (Step **31**)	200–500 ng	20–50 ng/µl
CAS9 buffer, 10×	0.9 µl	1×
Nuclease-free H_2O	To 10 µl	

! CAUTION Wear gloves to protect the samples from RNase contamination during gRNA-CAS9-NLS complex preparation.

▲ CRITICAL STEP It is recommended to inject the mixture of *Pax7*-gRNA-CAS9-NLS complexes and the targeting construct pGEMT-Pax7bait-T2A-Cherry-pA to generate Pax7-Cherry knock-in axolotls[21] as a positive control for knock-in experiments.

34 Inject ~200 freshly laid single-cell-stage axolotl eggs (~5 nl of mixture/egg) and allow the eggs to develop until the hatching stage (~2 weeks) as previously described[2].

▲ CRITICAL STEP We use the eggs obtained from breedings of either *d/d* or particular transgenic axolotls for injections. Mostly, we inject the *d/d* eggs to produce F_0 knock-in axolotls. However, we also carry out injections into the eggs of given transgenic axolotls (e.g., a floxed reporter line *CAGGS:loxP-EGFP-loxP-Cherry*) to produce double-transgenic F_0 knock-in axolotls, which allows for direct phenotype analysis in F_0 founders[21].

35 (Optional) Anesthetize the F_0 developing axolotl larvae from Step **34** (including the positive control) using 0.006% (vol/vol) benzocaine, and examine the fluorescent protein (if present in the targeting construct) expression from the knock-in allele under a fluorescence stereomicroscope as previously described[2]. Note: It is expected that clear CHERRY expression will be observed in the central nervous system of positive controls derived from *Pax7-Cherry* axolotls (Fig. 7; ref. [21]).

▲ CRITICAL STEP Knock-in efficiency decreases with increasing size of the targeting construct.

? TROUBLESHOOTING

36 Design primer pairs for genomic PCR, perform genotyping PCR and sequence the product to check or confirm the proper integration of the targeting construct (as described in Step **21**; for details, see Experimental design). One of the genomic primers should bind to the 5′ genomic flanking sequence (not present in the targeting construct), and the other primer (such as pA-rev in Table 4) should bind to the end of the pA sequence (see Experimental design, Fig. 2 and Supplementary Fig. 4).

▲ CRITICAL STEP This step is essential for checking for targeting construct integration if no desired fluorescent signal is observed in the F_0 founder or there is no fluorescent marker in the targeting construct.

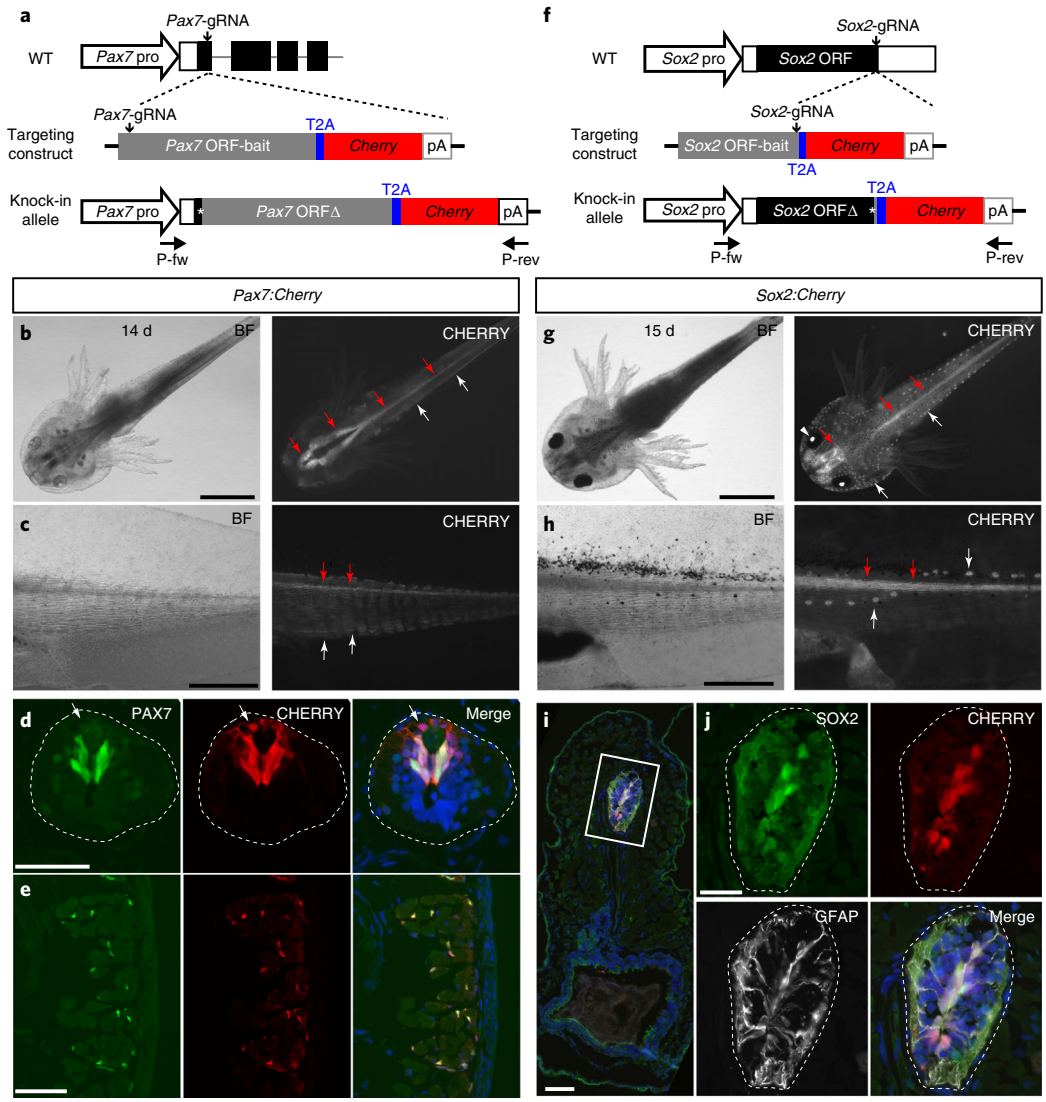

Fig. 7 | Generation and characterization of *Pax7:Cherry* and *Sox2:Cherry* knock-in axolotls. a,**f**, Targeting strategies for insertion the *Cherry* reporter gene into the *Pax7* (**a**) and *Sox2* (**f**) genomic loci. (Top) The wild-type (WT) axolotl *Pax7* (**a**) and *Sox2* (**f**) alleles. Empty arrows, *Pax7* promoter (**a**, *Pax7* pro) and *Sox2* promoter (**f**, *Sox2* pro) regions; solid rectangles, coding exons (black, endogenous exons; gray, exons from donor construct); empty rectangles, untranslated regions; gray solid lines, introns; vertical arrows, gRNA-targeting sites. (Middle) The targeting constructs contain the complete axolotl *Pax7* (**a**) or *Sox2* (**f**) ORF (without stop codon) as the bait, followed by T2A, the *Cherry* coding sequence (with a stop codon) and the polyadenylation signal (pA). Vertical arrows, the gRNA-targeting sites. (Bottom) The *Pax7* (**a**) and *Sox2* (**f**) knock-in alleles after *Cherry* reporter gene integration. The newly formed *Pax7* (**a**, *Pax7* ORFΔ) and *Sox2* (**h**, *Sox2* ORFΔ) ORFs contain in-frame scars at the targeting construct integration junctions (asterisks). Horizontal black arrows indicate the binding sites of the forward (fw) and reverse (rev) genotyping primers (P). **b**,**c**, The dorsal (**b**) and lateral (**c**) view bright-field (BF, left panels) and CHERRY fluorescence (right panels) images of 14-day-old *Pax7:Cherry* knock-in F_0 axolotls highlight the CHERRY expression in the central nervous system (red arrows) and trunk muscle compartment (white arrows). Scale bars, 2 mm (**b**); 1 mm (**c**). **d**,**e**, Immunofluorescence for PAX7 (green), CHERRY fluorescence (red) combined with DAPI staining (blue) on 10-µm tail cross-cryosections of 83-day-old *Pax7:Cherry* knock-in F_0 axolotls. The CHERRY expression matches the PAX7 expression in dorsal spinal cord (**d**) and trunk satellite cells (**e**). Primary antibodies: PAX7 mouse monoclonal antibody, RFP rabbit polyclonal primary antibody. Secondary antibodies: Alexa Fluor 488–conjugated donkey anti-mouse antibody, Alexa Fluor 555–conjugated donkey anti-rabbit antibody. Dashed lines, spinal cord; arrows, inherited CHERRY in a PAX7-negative new-born neuron. Scale bars, 100 µm. **g**,**h**, The dorsal (**g**) and lateral (**h**) view bright-field (BF, left panels) and CHERRY fluorescence (right panels) images of 15-day-old *Sox2:Cherry* knock-in F_0 axolotls highlight the CHERRY expression in the central nervous system (red arrows), lens (arrowhead) and lateral-line neuromasts (white arrows). Scale bars, 2 mm (**g**); 1 mm (**h**). **i**,**j**, Immunofluorescence for SOX2, GFAP (glial fibrillary acidic protein, an intermediate filament marker to highlight the neural stem cell morphology), and CHERRY fluorescence combined with DAPI staining on 10-µm tail cross-cryosections of 2-month-old *Sox2:Cherry* knock-in F_0 axolotls. The CHERRY expression matches the SOX2 expression in the spinal cord. The rectangular region outlined in the merged image in **i** is shown at a higher magnification in **j** as separate channels. Primary antibodies: SOX2 rabbit polyclonal antibody[15,21], GFAP goat polyclonal antibody, RFP rat monoclonal antibody. Secondary antibodies: Alexa Fluor 488–conjugated donkey anti-rabbit antibody, Cy3-conjugated donkey anti-rat IgG (H+L) antibody, and Alexa Fluor 647–conjugated donkey anti-goat antibody. Dashed lines, spinal cord. Scale bars, 100 µm (**i**); 50 µm (**j**). Adapted with permission from Fei, J.F. et al. Efficient gene knock-in in axolotl and its use to test the role of satellite cells in limb regeneration. *Proc. Natl. Acad. Sci. USA* 114, 12501–12506 (2017). All animal experiments were carried out according to the relevant institutional and national regulations.

▲ **CRITICAL STEP** If one plans to use the same targeting construct design for different genomic loci, design appropriate restriction enzyme cutting sites flanking the bait sequence to allow easy replacement of the bait sequence.

37 Raise the F_0 founders showing correct fluorescent signal expression (as observed by microscopy) and targeting construct integration (as tested by genotyping PCR) to adults for germline transmission, or use them for experimental analysis (e.g., western blot[9], immunohistochemistry[9,15] or lineage tracing[21]) directly at the desired developmental stages. The immunohistochemical characterizations of *Sox2:Cherry* and *Pax7:Cherry* F_0 knock-in axolotls are illustrated in (Fig. 7)[21].
▲ **CRITICAL STEP** It is fine to collect tissues (mostly appendage tissues such as tail and limbs) of F_0 knock-in founders for experimental analysis. The founders will regenerate the lost tissues and continue to mature for germline transmission tests.
▲ **CRITICAL STEP** The F_0 knock-in founders can be directly bred to either *d/d* axolotls or another transgenic line to test for germline transmission.

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting table

Step	Problem	Possible reason	Solution
10A(xi)	Too many colonies growing on the control plate	Incomplete digestion of the DR274 vector	Check Bsa1-HF activity; reduce the amount of DR274 vector used for digestion; incubate the reaction overnight at 37 °C
13	Low gRNA yield from in vitro transcription	Low-quality template because of wash buffer contamination during PCR purification, or sgRNA secondary structure prevents T7 RNA polymerase binding	Repeat the gRNA preparation or reduce the amount (use half the amount) of PCR template used for in vitro transcription; design new gRNA
14	gRNA concentration is too low after purification	Loss of RNA pellet during the 70% (vol/vol)ethanol washing step	Carefully remove the 70% (vol/vol) ethanol at each washing step
20	Unspecific developmental defects in embryos	Bad batch of eggs or improper dejellying of the eggs	Repeat injection or improve dejellying skills
21A(viii)	Failure of the genomic PCR	Too much animal tissue in the crude DNA extract	Collect less tissue for DNA preparation or dilute the DNA to minimize the influence of impurities on the genomic PCR
22	Low-efficiency modification at the gRNA-targeted genomic locus	Improper gRNA	Re-design the gRNA, targeting the same genomic locus
26	Fraction of animals showing phenotypes do not correlate to the frequency of DNA modifications	Off-target effect from the gRNA, or lithium chloride contamination in the gRNA solution	Design and test new gRNAs targeting another exon or region of the locus, or prepare a new batch of gRNA by in vitro transcription
26	No F_0 homozygous types of mutants	gRNA-CAS9-NLS complexes were injected into late single-cell- or two-cell-stage axolotl eggs	Collect and inject freshly laid single-cell-stage axolotl eggs for injection
35	No detectable fluorescence in F_0 knock-in axolotls	Fluorescence is too weak or there was no integration of targeting construct	Carry out genotyping to determine whether the targeting construct has been integrated

Timing

Steps **1–6**, validation of the axolotl genomic sequence targeted by CRISPR–Cas9: 3 d
Steps **7–9**, gRNA design and 'off-target' prediction: 2 h
Step **10**, template preparation for gRNA synthesis: 2–5 d
Steps **11–14**, in vitro transcription of gRNA: 2 d
Steps **15–22**, gRNA activity evaluation in vivo: 2 weeks
Steps **23–28**, characterization of gene knockout axolotls: 2 weeks
Steps **29–37**, generation and characterization of gene knock-in axolotls: 2 weeks
Box 1, targeting construct preparation using Gibson assembly: 3 d

Anticipated results

By combining the application of the CAS9-NLS protein and the NHEJ-mediated knock-in approach, this protocol permits the generation of genetically modified knockout and knock-in axolotl lines in the lab. Evaluation of gRNA activity for selection of highly efficient gRNAs that normally mediate ≥90% of modifications at the targeted genomic loci (Step **22**) is critical for creation of high-penetrance F_0 knockout and knock-in CRISPR axolotls. gRNAs mediating low cleavage activity are inefficient in generating genetically modified axolotls.

Using this protocol, we have targeted several genomic loci and created axolotl mutants, even homozygous-type F_0 knockouts for phenotype analysis[9,15,29]. When targeting the *Tyrosinase* locus, we designed and tested three gRNAs. Nearly all F_0 *Tyrosinase* CRISPR axolotls injected with *Tyr*-gRNA#1-CAS9-NLS complexes exhibited a complete loss of melanophore pigmentation[15,29]. Among the F_0 CRISPR axolotls edited with *Tyr*-gRNA#1, we screened 23 individuals (20 by Sanger sequencing and 3 by NGS) and obtained 47.8% (11 out of 23) homozygous knockout axolotls (Supplementary Fig. 3). When targeting the *Sox2* and *Pax7* loci, by immunohistochemistry analysis, SOX2 or PAX7 expression was massively or completely lost in the desired cell types in F_0 *Sox2* and *Pax7* CRISPR axolotls, respectively, compared to the controls. Using F_0 *Sox2* mutant axolotls, we showed that *Sox2* is essential for spinal cord regeneration but not development[15]. Using F_0 *Pax7* knockout axolotls, we observed that animals demonstrate phenotypes of both *Pax7* and *Pax3* mutations in other species, suggesting that axolotl *Pax7* also performs the functions of *Pax3*, which is absent in this species[9].

For knock-in, we used the protocol to insert a *Cherry* fluorescence reporter into *Pax7* and *Sox2* loci to label specific cell populations[21]. We designed the targeting construct to contain the complete *Pax7* or *Sox2* open reading frame (but without the stop codon), followed by the T2A self-cleaving peptide sequence, the *Cherry* coding sequence and the pA signal (Fig. 7a,f). We designed three gRNAs, each targeting *Pax7* or *Sox2* loci, and used Sanger sequencing to determine the most efficient gRNAs for NHEJ-mediated knock-in (Supplementary Fig. 4). When injecting the selected *Pax7*-gRNA-CAS9-NLS or *Sox2*-gRNA-CAS9-NLS RNP complexes together with the relevant targeting construct, we screened 157 *Pax7:Cherry* and 310 *Sox2:Cherry* F_0 knock-in axolotls, respectively[21]. 12.7% (20 out of 157) of resulting F_0 *Pax7:Cherry* animals showed the expected CHERRY expression in PAX7-positive muscle satellite cells and the dorsal domain of the spinal cord in *Pax7:Cherry* knock-in axolotls (Fig. 7b–e). Likewise, CHERRY was expressed correctly in SOX2-positive spinal cord neural stem cells and in the lens and neuromast of the lateral line in 14.5% (45 out of 310) of F_0 *Sox2:Cherry* knock-in axolotls (Fig. 7g–j). In addition, knock-in of an ER^{T2}-*Cre*-ER^{T2} donor construct into the *Pax7* locus in *CAGSS: loxp-GFP-loxP Cherry* reporter axolotls[3] allowed for the direct tracing of the progeny of PAX7-positive satellite cells in the derived F_0 double-transgenic axolotls and demonstrated that satellite cells are the source of myogenesis during axolotl limb regeneration[21].

Reporting Summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

References

1. Voss, S. R., Epperlein, H. H. & Tanaka, E. M. *Ambystoma* mexicanum, the axolotl: a versatile amphibian model for regeneration, development, and evolution studies. *Cold Spring. Harb. Protoc.* **2009**, pdbemo128 (2009).
2. Khattak, S. et al. Optimized axolotl (*Ambystoma mexicanum*) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nat. Protoc.* **9**, 529–540 (2014).
3. Khattak, S. et al. Germline transgenic methods for tracking cells and testing gene function during regeneration in the axolotl. *Stem Cell Rep.* **1**, 90–103 (2013).
4. Campbell, L. J. et al. Gene expression profile of the regeneration epithelium during axolotl limb regeneration. *Dev. Dyn.* **240**, 1826–1840 (2011).
5. Putta, S. et al. From biomedicine to natural history research: EST resources for ambystomatid salamanders. *BMC Genomics* **5**, 54 (2004).
6. Stewart, R. et al. Comparative RNA-seq analysis in the unsequenced axolotl: the oncogene burst highlights early gene expression in the blastema. *PLoS Comput. Biol.* **9**, e1002936 (2013).
7. Habermann, B. et al. An *Ambystoma mexicanum* EST sequencing project: analysis of 17,352 expressed sequence tags from embryonic and regenerating blastema cDNA libraries. *Genome Biol.* **5**, R67 (2004).

8. Bryant, D. M. et al. A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell Rep.* **18**, 762–776 (2017).
9. Nowoshilow, S. et al. The axolotl genome and the evolution of key tissue formation regulators. *Nature* **554**, 50–55 (2018).
10. Voss, S. R. et al. Gene expression during the first 28 days of axolotl limb regeneration I: Experimental design and global analysis of gene expression. *Regeneration* **2**, 120–136 (2015).
11. Sandoval-Guzman, T. et al. Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell* **14**, 174–187 (2014).
12. Woodcock, M. R. et al. Identification of mutant genes and introgressed tiger salamander DNA in the laboratory axolotl, *Ambystoma mexicanum*. *Sci Rep.* **7**, 6 (2017).
13. Whited, J. L., Lehoczky, J. A. & Tabin, C. J. Inducible genetic system for the axolotl. *Proc. Natl. Acad. Sci. USA* **109**, 13662–7 (2012).
14. Flowers, G. P., Timberlake, A. T., Mclean, K. C., Monaghan, J. R. & Crews, C. M. Highly efficient targeted mutagenesis in axolotl using Cas9 RNA-guided nuclease. *Development* **141**, 2165–71 (2014).
15. Fei, J. F. et al. CRISPR-mediated genomic deletion of Sox2 in the axolotl shows a requirement in spinal cord neural stem cell amplification during tail regeneration. *Stem Cell Rep.* **3**, 444–459 (2014).
16. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
17. Mali, P., Esvelt, K. M. & Church, G. M. Cas9 as a versatile tool for engineering biology. *Nat. Methods* **10**, 957–963 (2013).
18. Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262–1278 (2014).
19. Doudna, J. A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096 (2014).
20. Fei, J. F. et al. Tissue- and time-directed electroporation of CAS9 protein–gRNA complexes in vivo yields efficient multigene knockout for studying gene function in regeneration. *npj Regen. Med.* **1**, 16002 (2016).
21. Fei, J. F. et al. Efficient gene knockin in axolotl and its use to test the role of satellite cells in limb regeneration. *Proc. Natl. Acad. Sci. USA* **114**, 12501–12506 (2017).
22. Smith, J. J. et al. Sal-Site: integrating new and existing ambystomatid salamander research and informational resources. *BMC Genomics* **6**, 181 (2005).
23. Wang, W. et al. Delivery of Cas9 protein into mouse zygotes through a series of electroporation dramatically increases the efficiency of model creation. *J Genet. Genomics* **43**, 319–327 (2016).
24. Albadri, S., Del Bene, F. & Revenu, C. Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. *Methods* **121–122**, 77–85 (2017).
25. Burger, A. et al. Maximizing mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes. *Development* **143**, 2025–2037 (2016).
26. Liang, X., Potter, J., Kumar, S., Ravinder, N. & Chesnut, J. D. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *J. Biotechnol.* **241**, 136–146 (2017).
27. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24**, 1012–1019 (2014).
28. Gagnon, J. A. et al. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS ONE* **9**, e98186 (2014).
29. Mircetic, J. et al. Purified Cas9 fusion proteins for advanced genome manipulation. *Small Methods* **1**, 1600052 (2017).
30. Hu, P., Zhao, X., Zhang, Q., Li, W. & Zu, Y. Comparison of various nuclear localization signal-fused Cas9 proteins and Cas9 mRNA for genome editing in zebrafish. *G3* **8**, 823–831 (2018).
31. Auer, T. O., Duroure, K., De Cian, A., Concordet, J. P. & Del Bene, F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res.* **24**, 142–153 (2014).
32. He, X. et al. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. *Nucleic Acids Res.* **44**, e85 (2016).
33. Liang, Z. et al. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* **8**, 14261 (2017).
34. Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K. & Mark Cigan, A. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat. Commun.* **7**, 13274 (2016).
35. Cho, S. W., Lee, J., Carroll, D., Kim, J. S. & Lee, J. Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9-sgRNA ribonucleoproteins. *Genetics* **195**, 1177–1180 (2013).
36. Paix, A., Folkmann, A., Rasoloson, D. & Seydoux, G. High efficiency, homology-directed genome editing in *Caenorhabditis elegans* using CRISPR-Cas9 ribonucleoprotein complexes. *Genetics* **201**, 47–54 (2015).
37. Lee, J. S. et al. RNA-guided genome editing in *Drosophila* with the purified Cas9 protein. *G3* **4**, 1291–5 (2014).
38. Gaj, T., Gersbach, C. A. & Barbas, C. F. 3rd ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405 (2013).
39. Joung, J. K. & Sander, J. D. TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* **14**, 49–55 (2013).

40. Kuo, T. H. et al. TALEN-mediated gene editing of the thrombospondin-1 locus in axolotl. *Regeneration* **2**, 37–43 (2015).
41. Grabher, C. & Wittbrodt, J. Meganuclease and transposon mediated transgenesis in medaka. *Genome Biol.* **8**, S10 (2007).
42. Saigou, Y., Kamimura, Y., Inoue, M., Kondoh, H. & Uchikawa, M. Regulation of Sox2 in the pre-placodal cephalic ectoderm and central nervous system by enhancer N-4. *Dev. Growth Differ.* **52**, 397–408 (2010).
43. Ran, F. A. et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* **520**, 186–191 (2015).
44. Kim, E. et al. In vivo genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*. *Nat. Commun.* **8**, 14500 (2017).
45. Muller, M. et al. *Streptococcus thermophilus* CRISPR-Cas9 systems enable specific editing of the human genome. *Mol. Ther.* **24**, 636–644 (2016).
46. Lee, C. M., Cradick, T. J. & Bao, G. The *Neisseria meningitidis* CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Mol. Ther.* **24**, 645–654 (2016).
47. Zetsche, B. et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771 (2015).
48. Kleinstiver, B. P. et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481–485 (2015).
49. Kleinstiver, B. P. et al. Broadening the targeting range of *Staphylococcus aureus* CRISPR-Cas9 by modifying PAM recognition. *Nat. Biotechnol.* **33**, 1293–1298 (2015).
50. Hu, J. H. et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**, 57–63 (2018).
51. Li, J. et al. Intron targeting-mediated and endogenous gene integrity-maintaining knockin in zebrafish using the CRISPR/Cas9 system. *Cell Res.* **25**, 634–637 (2015).
52. Hisano, Y. et al. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. *Sci. Rep.* **5**, 8841 (2015).
53. Sander, J. D. & Joung, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* **32**, 347–355 (2014).
54. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
55. Mali, P. et al. RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).
56. Wang, H. et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918 (2013).
57. Kotani, H., Taimatsu, K., Ohga, R., Ota, S. & Kawahara, A. Efficient multiple genome modifications induced by the crRNAs, tracrRNA and Cas9 protein complex in zebrafish. *PLoS ONE* **10**, e0128319 (2015).
58. Sung, Y. H. et al. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. *Genome Res.* **24**, 125–131 (2014).
59. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* **32**, 279–284 (2014).
60. Fu, Y. et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–866 (2013).
61. Kochetov, A. V. Alternative translation start sites and hidden coding potential of eukaryotic mRNAs. *BioEssays* **30**, 683–691 (2008).
62. Harrison, M. M., Jenkins, B. V., O'Connor-Giles, K. M. & Wildonger, J. A CRISPR view of development. *Genes Dev.* **28**, 1859–1872 (2014).
63. Elewa, A. et al. Reading and editing the *Pleurodeles waltl* genome reveals novel features of tetrapod regeneration. *Nat. Commun.* **8**, 2286 (2017).
64. Bell, C. C., Magor, G. W., Gillinder, K. R. & Perkins, A. C. A high-throughput screening strategy for detecting CRISPR-Cas9 induced mutations using next-generation sequencing. *BMC Genomics* **15**, 1002 (2014).
65. Sentmanat, M. F., Peters, S. T., Florian, C. P., Connelly, J. P. & Pruett-Miller, S. M. A survey of validation strategies for CRISPR-Cas9 editing. *Sci Rep.* **8**, 888 (2018).
66. Kircher, M., Sawyer, S. & Meyer, M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic Acids Res.* **40**, e3 (2012).
67. Kim, J. M., Kim, D., Kim, S. & Kim, J. S. Genotyping with CRISPR-Cas-derived RNA-guided endonucleases. *Nat. Commun.* **5**, 3157 (2014).
68. Vouillot, L., Thelie, A. & Pollet, N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3* **5**, 407–415 (2015).
69. Qiu, P. et al. Mutation detection using surveyor nuclease. *Biotechniques* **36**, 702–707 (2004).
70. Ota, S. et al. Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. *Genes Cells* **18**, 450–458 (2013).
71. Shigeta, M. et al. Rapid and efficient analysis of gene function using CRISPR-Cas9 in *Xenopus tropicalis* founders. *Genes Cells* **21**, 755–771 (2016).
72. Zhu, X. et al. An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Sci. Rep.* **4**, 6420 (2014).
73. Dahlem, T. J. et al. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet.* **8**, e1002861 (2012).

74. Samarut, E., Lissouba, A. & Drapeau, P. A simplified method for identifying early CRISPR-induced indels in zebrafish embryos using high resolution melting analysis. *BMC Genomics* **17**, 547 (2016).
75. Guschin, D. Y. et al. A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247–256 (2010).
76. Gansauge, M. T. & Meyer, M. Single-stranded DNA library preparation for the sequencing of ancient or damaged DNA. *Nat. Protoc.* **8**, 737–748 (2013).
77. Kim, J. H. et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS ONE* **6**, e18556 (2011).
78. Pelletier, J. & Sonenberg, N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320–355 (1988).
79. Gibson, D. G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345 (2009).
80. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412 (2010).
81. Wilton, S. Direct sequencing of PCR products. *eLS* https://doi.org/10.1038/npg.els.0003769 (2002).
82. Sievers, F. et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
83. Brocal, I. et al. Efficient identification of CRISPR/Cas9-induced insertions/deletions by direct germline screening in zebrafish. *BMC Genomics* **17**, 259 (2016).
84. Irion, U., Krauss, J. & Nusslein-Volhard, C. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* **141**, 4827–4330 (2014).
85. Armstrong, G. A. et al. Homology directed knockin of point mutations in the zebrafish *tardbp* and *fus* genes in ALS using the CRISPR/Cas9 system. *PLoS ONE* **11**, e0150188 (2016).
86. Nakayama, T. et al. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in *Xenopus tropicalis*. *Genesis* **51**, 835–843 (2013).
87. Pinello, L. et al. Analyzing CRISPR genome-editing experiments with CRISPResso. *Nat. Biotechnol.* **34**, 695–697 (2016).

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Author contributions

J.-F.F., D.K. and E.M.T. conceived and designed the experiments. J.-F.F., D.K., P.M., T.G., Y.T. and S.N. performed the experiments. W.P.-K.L. and S.K. contributed to the data analysis. J.-F.F., W.P.-K.L. and E.M.T. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Related links
Key references using this protocol
Nowoshilow, S. et al. *Nature*. **554**, 50–55 (2018): https://doi.org/10.1038/nature25458
Fei, J.-F. et al. *Proc. Natl. Acad. Sci. USA* **114**, 12501–12506 (2017): https://doi.org/10.1073/pnas.1706855114
Fei, J.-F. et al. *Stem Cell Rep*. **3**, P444–P459 (2014): https://doi.org/10.1016/j.stemcr.2014.06.018

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Life Sciences Reporting Summary

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Experimental design

1. Sample size

   Describe how sample size was determined.

   We inject 30 eggs for gRNA evaluation, that is a good number to determine gRNA-mediated target DNA cleavage efficiency and to exclude the injection variation. For the knock-in experiment, we inject around 200 eggs. It produce sufficient number of F0 axolotls showing the correct integration of the exogenous sequence, since the knock-in efficiency ranges from around 5-15%.

2. Data exclusions

   Describe any data exclusions.

   We did not exclude data from the manuscript.

3. Replication

   Describe the measures taken to verify the reproducibility of the experimental findings.

   For Gene knockout, we have achieved high efficient knockout of GFP, Tyrosinase, Sox2, Pax7 and several other unpublished genomic loci in axolotl. For CRISPR/Cas9 mediated gene knock-in, we have successfully inserted varied exogenous gene (e.g. Cherry, ER-Cre-ER) into two independent axolotl genoimc loci (Pax7 and Sox2) (Fei et. al., 2017, PNAS, 114(47):12501-12506). In our lab, others have successfully inserted exogenous gene cassette into several other genomic loci (e.g. Sox9, HoxA9) in axolotl.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   The category of the F0 axolotl CRISPR-mutants can be determined by the severity of the given phenotypes. For example, in F0 Pax7 mutant analysis, all progeny were initially categorized into different groups based on the presence (severity) of the limb and body muscle mass phenotypes, then confirmed by the genotyping analysis (Fei et. al., 2017, PNAS, 114(47):12501-12506). The presence of the phenotype must correlate to the genotype analysis. And two separate gRNAs targeting the same genomic locus must produce the same spectrum of phenotypes.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   The investigators were not blinded. We select one or two most efficient gRNA for subsequent knockout and knockin experiments.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☒	☐ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☒	☐ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ A statement indicating how many times each experiment was replicated
☒	☐ The statistical test(s) used and whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☒	☐ Test values indicating whether an effect is present *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
☒	☐ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☒	☐ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Adobe Photoshop (CS6)
Adobe Illustrator (CS6)
MacVector (10.6)
Primer3 (Plus)
Zeiss ZEN 2 (blue edition)
Olympus CellSens Standard (1.5)
sgRNA Designer (Broad Institute, link: https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design)
CRISPResso (For NGS analysis, link: http://crispresso.rocks)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All unique materials are openly available.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

monoclonal anti-PAX7 (catalog number: PAX7, DSHB), validation on axolotl tissue was performed by Schnapp et al (2005) Development, 132:3243-53.
RFP (catalog number: 600-401-379, rabbit polyclonal antibody, Rockland), RFP (catalog number: 5f8-100, rat monoclonal antibody, Chromotek), validation on axolotl tissue was performed by Fei et al (2017) PNAS, 114(47):12501-12506.
GFAP (catalog number: ab53554, goat polyclonal antibody, Abcam), validation on axolotl tissue was performed by Fei et al (2017) PNAS, 114(47):12501-12506.
SOX2 (rabbit polyclonal antibody, homemade), validation on axolotl tissue was performed by Fei et al (2014) Stem Cell Reports, 3(3):444-59. Loss of SOX2 immuno-activity in Sox2 CRISPR mutant axolotls.

Alexafluor 488-conjugated donkey anti-mouse (catalog number: A21202, Invitrogen), Alexafluor 488-conjugated donkey anti-rabbit (catalog number: A21206, Invitrogen), Alexafluor 555-conjugated donkey anti-rabbit (catalog number: A31572, Invitrogen), Alexafluor 647-conjugated donkey anti-goat (catalog number: A21447, Invitrogen) and Cy3-conjugated donkey anti-rat IgG (H+L)secondary antibody (712-165-153, Jackson ImmunoResearch) secondary antibodies, were validated by staining sections with secondary antibody only to determine that there was no signal in the cell types to be analyzed.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.
No eukaryotic cell lines were used

b. Describe the method of cell line authentication used.
No eukaryotic cell lines were used

c. Report whether the cell lines were tested for mycoplasma contamination.
No eukaryotic cell lines were used

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
No eukaryotic cell lines were used

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

Ambystoma mexicanum, strain: white (d/d).
We used single cell stage eggs (d/d) for injection.
For CRISPR-axolotls, phenotypes characterization can be carried out at diverse stages.
Up to juvenile stages, it is not possible to determine the sex of the axolotls by morphology and no cytochemical or molecular assay is available. For Pax7-CRISPR axolotls, the phenotypes obtained are very likely independent of the gender (Nowoshilow et. al., 2018, Nature, 554(7690):50-55).
For Pax7:Cherry and Sox2:Cherry knockin axololtls, characterization were performed at diverse stages, 14-day, 15-day, 2-month and 83-day old axolotls (Fei et. al., 2017, PNAS, 114(47):12501-12506).

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not include human research participants