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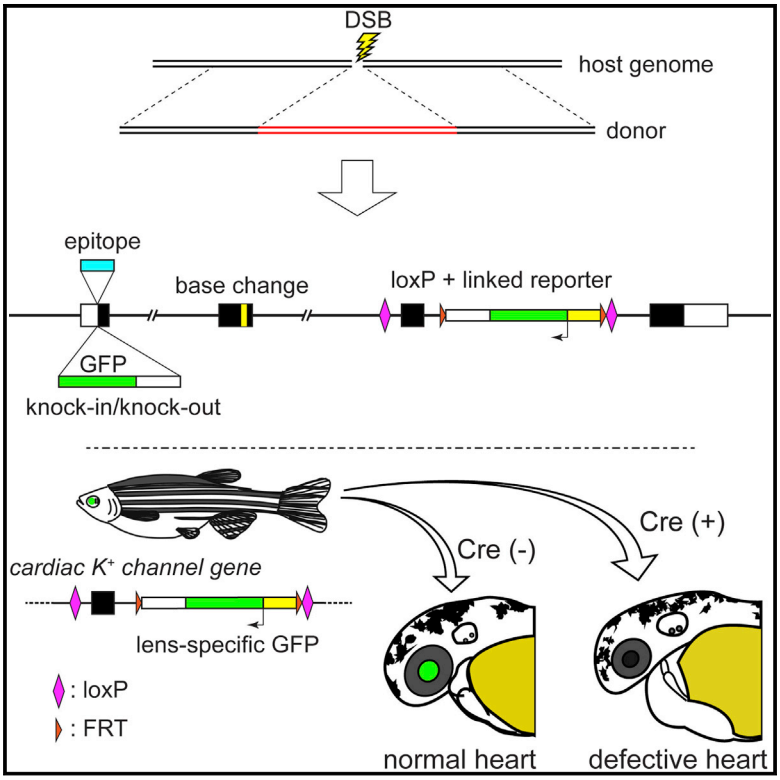
# Precise Editing of the Zebrafish Genome Made Simple and Efficient

## Graphical Abstract

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## In Brief

In this Technology article, Hoshijima et al. use homologous recombination to generate precise genome modifications in zebrafish, allowing for a range of gene editing events. Marking donor sequences with reporter genes facilitates efficient recovery of phenotypically silent changes, including acquisition of *loxP* sites and creation of functional conditional alleles.

## Highlights

- Efficient methods for precise modification of the zebrafish genome are introduced
- New vectors allow facile recovery of phenotypically silent changes
- Editing events ranging from single codon changes to knockin alleles are generated
- Functional conditional alleles using *loxP* sites and Cre recombinase can be created

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# Precise Editing of the Zebrafish Genome Made Simple and Efficient

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## SUMMARY

**We present simple and efficient methods for creating heritable modifications of the zebrafish genome. Precisely modified alleles are generated by homologous recombination between the host genome and dsDNA donor molecules, stimulated by the induction of chromosomally targeted double-strand breaks. Several kilobase-long tracts of genome sequence can be replaced. Tagging donor sequences with reporter genes that can be subsequently excised improves recovery of edited alleles by an order of magnitude and facilitates recovery of recessive and phenotypically silent conditional mutations. We generate and demonstrate functionality of (1) alleles with a single codon change, (2) an allele encoding an epitope-tagged version of an endogenous protein, (3) alleles expressing reporter proteins, and (4) a conditional allele in which an exon is flanked by recombinogenic *loxP* sites. Our methods make recovery of a broad range of genome editing events very practicable, significantly advancing applicability of the zebrafish for studying normal biological processes and modeling diseases.**

## INTRODUCTION

The zebrafish has been established as a formidable experimental system for the discovery and analysis of gene functions regulating a wide spectrum of developmental and physiological processes as they occur in the intact organism (Hammerschmidt and Mullins, 2002; North et al., 2009; Ceol et al., 2011; Kikuchi and Poss, 2012; Wolman and Granato, 2012; Barbosa et al., 2015; Ruprecht et al., 2015). The near transparency of the embryo has even permitted visualization and analysis of the dynamics of signaling molecules in intact tissues (Yu et al., 2009; Muller et al., 2012). Furthermore, the extent of functional conservation among vertebrates makes the zebrafish an outstanding system for examining the mechanistic basis of disease (Jurynec et al., 2008), assaying human gene activity (Bamford et al., 2000; Jou et al., 2013), or discovering compounds that augment or inhibit developmental processes or disease-like conditions (Peterson et al., 2004; North et al., 2007; Hagedorn et al., 2014). The sophistication with which biological processes can be probed in the zebrafish make it poised to exploit tools of genome manipulation, which could be used to generate models of human disease, study gene function in specific tissue or temporal contexts, or generate alleles that allow study of tagged protein activities under physiological conditions. Whereas early efforts have demonstrated the feasibility of genome editing in the zebrafish (Bedell et al., 2012; Hruscha et al., 2013; Zu et al., 2013; Auer et al., 2014; Irion et al., 2014; Kimura et al., 2014; Shin et al., 2014; Hisano et al., 2015), available methods are inefficient and limited in their ability to allow recovery of genome modifications, such as conditional alleles or novel recessive alleles, which do not confer an immediate phenotype (Auer and Del Bene, 2014). As a result, there is not yet an established procedure for the routine recovery of edited genomes. To more fully utilize the potential of the zebrafish, we have developed an easy-to-apply approach for creating and recovering genome modifications that facilitates the study of normal and disease gene function and analyses of biological processes in vivo.

The development of synthetic sequence-specific DNA nucleases (also called programmable nucleases) that are easy to generate and implement opened a new realm of possibilities for genome modification (Hsu et al., 2014; Kim and Kim, 2014). Double-strand breaks (DSBs) trigger host-cell repair responses that can be utilized to produce altered alleles: in the absence of a template guiding repair, the lesion is likely to be healed via the error-prone non-homologous end-joining pathway; in the presence of single- or double-stranded DNA sharing homology with the targeted locus, the template can either guide repair or participate in an exchange of sequences via homologous recombination (HR). The induction of chromosomal DSBs greatly stimulates recombination between the targeted locus and available homologous DNA sequences (Jasin, 1996; Paques and Haber, 1999). Cleavage of chromosomal loci by synthetic nucleases can be highly efficient, with $\geq$50% of the genomes in a developing zebrafish embryo experiencing a targeted DSB (Dahlem et al., 2012). Thus targeted DSBs can be harnessed to stimulate genome modification through homology-directed repair of induced lesions or true HR exchange events with exogenously supplied templates.

Here we describe a comprehensive approach to accurate genome editing in the zebrafish that involves targeted HR, allowing tracts of genomic sequence to be precisely replaced with donor sequences. We also introduce methods that greatly increase the efficiency of inducing and recovering precisely edited alleles. Typically, 5%–15% of the treated animals transmit edited alleles through their germlines. The identification and recovery of edited alleles is made extremely simple and efficient by temporarily tagging donor sequences with reporter genes whose acquisition initially marks the edited allele and which can be

subsequently excised. The tools and approaches we report make it possible to generate and rapidly recover most types of desired gene modifications. We illustrate application of these methods by generating different types of edited alleles at four distinct genome sites, including designed coding changes, alleles encoding antigenically tagged versions of endogenous proteins expressed under physiological conditions, alleles that drive expression of introduced proteins under the control of endogenous promoters, and conditional floxed alleles.

### Design

In our approach to genome editing in the zebrafish, DSBs were generated continually at a locus of interest during early developmental stages of a zebrafish embryo while exogenous double-stranded DNA (dsDNA) donor sequences were provided as a template to guide repair of the breaks (Zu et al., 2013; Irion et al., 2014; Shin et al., 2014). Targeted cleavage of the genome was accomplished by injecting just-fertilized zygotes with a pair of mRNAs encoding a heterodimeric TALEN (Dahlem et al., 2012) or with CRISPR/Cas9 components (Charpentier and Doudna, 2013; Hwang et al., 2013; Jao et al., 2013) designed to cleave a unique sequence within the genome. Nuclease activity in vivo was assessed by detection of targeted mutations in the genomes of 1 day post-fertilization (dpf) injected embryos (Dahlem et al., 2012); only TALENs or sgRNAs that induced targeted DSBs in every embryo were selected to trigger HR (Table S1 lists target sites and the mutagenesis efficiency of each TALEN). A donor template was provided by simultaneous injection of plasmid or plasmid-derived dsDNA. Donor templates consisted of novel sequences flanked by homology arms of approximately 1 kb identical to the host sequences bordering the nuclease target site. Animals with minimal sequence heterogeneity at the targeted locus were selected as hosts (Experimental Procedures). Donor sequences were always designed so their incorporation would destroy the nuclease recognition site in the genome and introduce novel diagnostic restriction sites or primer-binding sequences. Hence, successfully targeted loci could not be re-cleaved by the nuclease and could be distinguished unambiguously by PCR anchored at unique donor sequences and host genome-specific sequences distal to the homologies. Following injection of zygotes, genomic DNAs of individual embryos were always analyzed by diagnostic PCR (Table S2 lists primers). These analyses indicated virtually all founder embryos harbored correctly edited alleles. The relative abundance of edited alleles in treated F0 embryos was used as an assay to optimize editing conditions. In all cases reported here, the configuration of germline-transmitted edited alleles was verified by complete sequence analysis of single amplicons that bridged the host sequences that lay distal to the homologies and thus extended across all recombination junctions and newly introduced sequences.

## RESULTS

### Effecting Precise Coding Changes by Targeted Homologous Recombination

As previous studies (Dahlem et al., 2012) indicated DSB cleavage activity commences about 2 hr after injection of TALEN mRNA into zebrafish zygotes, we anticipated DSB-stimulated HR would occur in a mosaic fashion during multicellular stages of embryonic development. To detect such events, we developed a simple assay in which individual cells with edited alleles could be readily recognized in the somatic tissue of developing embryos. Embryos homozygous for the null *golden*$^{b1}$ mutation have severely reduced pigmentation at 2–3 dpf (Streisinger et al., 1981). As *golden* (*gol*) functions cell autonomously to promote pigmentation, we measured the ability to convert the *gol*$^{b1}$ allele, which encodes a premature termination product (Y151X) due a C→A mutation at the 3′ end of exon 5 (Lamason et al., 2005), to a wild-type (WT) form (Figure 1A). Recombination/repair was stimulated by injecting *gol*$^{b1}$ mutant zygotes with *gol-int5* TALEN mRNA to generate a DSB within intron 5, approximately 30 bp 3′ of the *b1* mutation. Donor sequences were provided by co-injection of 50 pg of *gol*(b1→WT) donor plasmid DNA harboring modified *gol* locus sequences that would restore WT coding in exon 5 and introduce nearby intronic changes destroying the TALEN target site and creating a diagnostic *Not*I restriction site. The modified donor sequences were flanked by perfect homology arms (1 kb left arm and 1.1 kb right arm) derived by PCR from the *gol* locus of the targeted genome. Whereas injection of TALEN mRNA or donor plasmid alone did not yield embryos with pigmentation at 2 dpf, nearly every embryo (95%, n = 368) with both TALEN and donor plasmid had normally pigmented cells (Figure 1B and Table 1). PCR analysis indicated virtually all targeted embryos had acquired donor sequences at the *gol* locus (Figure 1C).

To determine if founders transmitted WT alleles to offspring, injected F0 founder embryos were raised to adulthood and mated with *gol*$^{b1/b1}$ partners. Eight of 51 (16%) F0 founders produced wholly pigmented offspring, indicating transmission of *gol*$^{+}$ gametes (WT gametes represented 0.6%–23.1% of germlines, $\mu$ = 6.6%, Table S3). Sequence analysis of the eight transmitted *gol*$^{b1\rightarrow WT}$ alleles (Figure S1) indicated seven had precisely replaced genomic sequences with donor sequences, whereas the eighth also suffered a 7-bp deletion at the TALEN cut site within intron 5. It appears all WT alleles arose through precise HR events, but one of these also experienced the kind of mutagenic event typical of non-homologous end-joining repair. In summary, gene editing in which a small number of nucleotides replace the endogenous sequence via HR can be triggered to occur efficiently in zebrafish.

CRISPR/Cas9 components can also be used to initiate DSBs that significantly stimulate HR. Mutant embryos with normally pigmented melanophores were readily produced following co-injection of *gol* mutant fertilized eggs with *gol*(b1→WT) donor plasmid DNA, Cas9 protein, and sgRNA targeted to the *gol-int5* TALEN recognition site (Figures 1A and 1B). Although it is difficult to use the two approaches to induce genomic DSBs at precisely the same site and with precisely the same efficiency, it appears DSBs created by CRISPR/Cas9 components or TALENs are similarly capable of stimulating HR events (Figures 1B and 1D).

### Precise Coding Sequence Additions

The replacement of short stretches of sequence at the *gol* locus indicated it should also be possible to introduce defined coding sequences at a precise location. Given the relative dearth of proven functional antibodies against zebrafish proteins, one

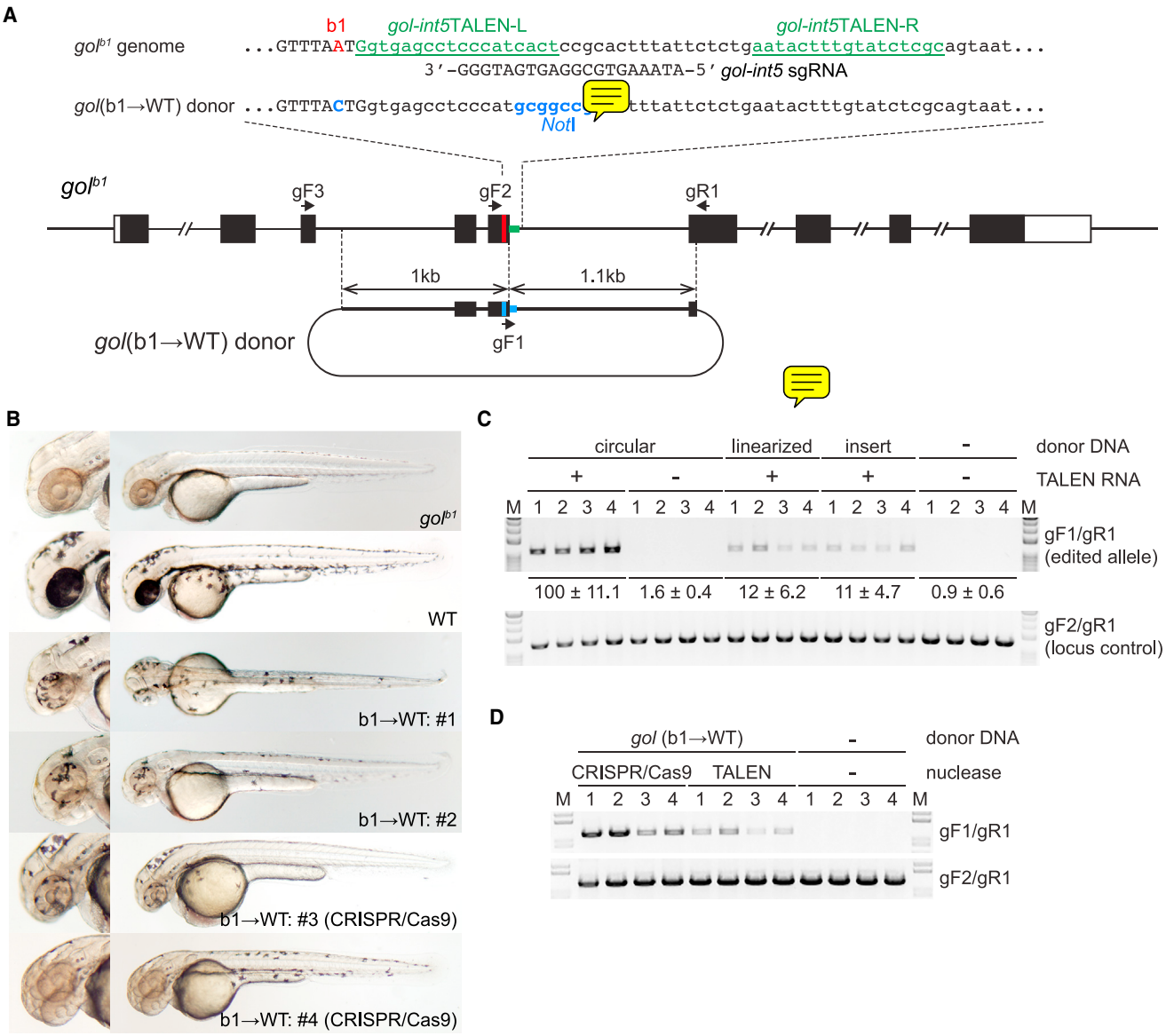


**Figure 1. Nuclease-Stimulated Gene Editing at the *Golden* Locus**

(A) Schematic representation of the genomic structure of the *golden* (*gol*) gene (exons are boxes, 5′ and 3′ UTRs in white), indicating the positions and sequences of the *gol*$^{b1}$ null mutation (red) in exon 5, the target of the *gol-int5* TALEN (green and underlined), the sequence and target of the *gol-int5* sgRNA, as well as the structure of the *gol*(b1→WT) donor DNA, highlighting the modified donor sequences (blue), which restore WT coding in exon 5, destroy the TALEN and sgRNA recognition sequences, and introduce a *Not*I site to intron 5. Modified sequences are flanked by 1 kb (upstream) and 1.1 kb (downstream) homology arms generated by amplification of genomic sequences. Positions of diagnostic primers are depicted with short arrows: gF1 is specific to donor sequences; gF2 recognizes exon 5 whether derived from the host or donor; gF3 and gR1 recognize host sequences outside the region of homology with the donor.

(B) Genome editing produces darkly pigmented cells in the soma of *gol*$^{b1}$ homozygous mutants. 2-dpf uninjected *gol*$^{b1}$ homozygous mutants lack pigment in the retinal pigmented epithelium (RPE, highlighted on left) and on the body (right), which is normally seen in comparable WT embryos. Injection of *gol*$^{b1}$ mutant embryos with circular plasmid *gol*(b1→WT) donor DNA with either *gol-int5* TALEN RNA or a mixture of *gol-int5* sgRNA and Cas9 protein yields 2-dpf mutant embryos with darkly pigmented cells scattered throughout the RPE and body.

(C) Diagnostic PCR of genomic DNA from 2-dpf injected embryos demonstrates targeted integration of donor sequences is dependent on TALEN activity and the configuration of donor DNA. *gol*$^{b1}$ zygotes were injected with 50 pg of intact circular plasmid DNA, linearized plasmid DNA, or gel-purified *gol*(b1→WT) donor sequences. Four embryos were individually analyzed per injection category by amplification of genomic DNA with the gF2/gR1 primer pair, which detects the original *b1* as well as edited *gol* alleles (locus control), and with gF1/gR1 primer pair, specific for the edited allele. The intensity of each edited allele amplicon was normalized with respect to the pan-*gol* amplicon; the average intensities (±SE) of edited allele amplicons are presented as a percentage of the amplicons produced from embryos injected with circular plasmid DNA and TALEN RNA.

(D) Genome modification by HR can be stimulated by DSBs produced by the CRISPR/Cas9 system. *gol*$^{b1}$ zygotes were injected with circular donor DNA along with either *gol-int5*TALEN RNA or *gol-int5*sgRNA and Cas9 protein. The genomes of individual injected embryos were analyzed by diagnostic PCR to detect edited alleles that acquired *gol*(b1→WT) donor sequences. Either method for introducing targeted DSBs facilitated generation of edited *gol* alleles.

See also Figures S1 and S3 and Table S3.

**Table 1. DSB-Dependent Induction of Pigmented Cells in *gol*$^{b1}$ Mutant Embryos**

| Donor DNA | | | | Injected *gol*$^{b1}$ Embryos: Morphologically Normal at 2 dpf | |
|---|---|---|---|---|---|
| | Structure | Amount (pg) | TALEN RNA | Total | With Pigment |
| *gol(b1→WT)* | circular plasmid | 50 | + | 368 | 351 (95%) |
| | | | – | 72 | 0 |
| | | 100 | + | 22 | 17 (77%) |
| | | | – | 23 | 0 |
| | linear plasmid | 50 | + | 16 | 13 (81%) |
| | linear insert | 50 | + | 56 | 46 (82%) |
| | | | – | 21 | 0 |
| *gol(b1→WT; CV)* | circular plasmid | 50 | + | 188 | 157 (84%) |
| | | | – | 26 | 0 |
| | linearized plasmid | 50 | + | 28 | 9 (32%) |
| None | | 0 | + | 35 | 0 |
| | | | – | 292 | 0 |

*gol*$^{b1}$ mutant zygotes were injected with donor DNA and TALEN mRNA (50 pg *gol-int5* TALEN-L + 50 pg *gol-int5* TALEN-R mRNAs) as indicated. Circular plasmid, super-coiled dsDNA; linear plasmid, linear dsDNA generated by restriction enzyme cleavage of the vector backbone; linear insert, gel-purified linear dsDNA donor sequences devoid of vector backbone sequences. Data indicate the total number of morphologically normal 2-dpf embryos scored and the number (%) of those embryos with darkly pigmented *gol*$^{+}$. On average, 72% of zygotes injected with 50 pg of DNA appeared normal at 2 dpf, whereas only 48% of zygotes injected with 100 pg of DNA developed normally.

application of sequence insertion would be to generate a modified allele encoding an antigen-tagged version of a protein in order to track an endogenous protein expressed under native conditions and/or recover interacting DNA or protein partners. We used DSB-stimulated HR to introduce sequences encoding the V5 epitope in frame immediately downstream of the AUG translation initiation codon of the *no tail* (*ntl*) gene. *ntl* is an essential gene required for development of the notochord and posterior mesoderm (Halpern et al., 1993). It encodes a T-box transcription factor expressed in the primitive mesoderm and the nascent notochord (Schulte-Merker et al., 1994). Previous work indicated additional proteins likely interact with No Tail to modify its transcription function (Goering et al., 2003).

The strategy for editing the *ntl* locus is illustrated in Figure 2A. To stimulate HR, the *ntl-ex1* TALEN was engineered to target about 45 bp downstream of the *ntl* translation start site. Donor sequences encoded the V5 epitope fused to the normal N terminus of No Tail; additional silent codon changes were engineered so that incorporation of the donor would render the edited *ntl* allele resistant to further TALEN activity. The novel sequences in the donor plasmid were flanked by perfect homology arms of 1 and 1.2 kb.

All embryos co-injected with TALEN mRNA and circular donor plasmid harbored HR products as detected by PCR using primer pairs specific to donor and host genome sequences (Figures 2A and S2A). A V5-epitope-modified *ntl* allele was recovered from the germlines of founders and established in a true-breeding line. F2 *ntl*$^{V5/+}$ embryos expressed nuclear V5-tagged protein specifically in the *ntl* expression domain (Figures 2B–2E). To test if the V5-tagged No Tail protein provided WT activity, *ntl*$^{V5/+}$ heterozygotes were intercrossed and their progeny analyzed. All progeny, including *ntl*$^{V5/V5}$ homozygotes, appeared morphologically normal (Figures 2F and 2G) and were viable to adulthood (not shown). Thus genome editing by HR allows for production of modified alleles in which epitope-tagged proteins, detectable by common commercially available antibodies, are expressed under conditions that truly mimic native proteins.

## DSBs Stimulate Genome Editing Events in Zebrafish

We investigated factors that affect HR. We found genome editing is quite sensitive to the degree to which DSBs are induced at the target locus (Jasin, 1996; Paques and Haber, 1999). In the absence of induced DSBs, we failed to detect recombination between genomic sequences and homologous donor DNAs (Figures 1C and S2). To test the sensitivity to DSB induction, we generated pairs of TALENs with identical DNA binding motifs but different DNA cleavage domains: one harboring the first-generation heteromeric DD/RR version of the Fok I nuclease domain (Miller et al., 2007), the other harboring the more active derived heteromeric DDD/RRR version of the nuclease domain (Doyon et al., 2011). As illustrated by analysis of mutagenesis at the *gol* locus (Figure S3A), the DDD/RRR form reproducibly induced higher levels of mutations. The ability of DD/RR or DDD/RRR variants of the *gol-int5* TALEN to stimulate gene editing of the *gol*$^{b1}$ mutation was assayed as described in Figure 1. Precise editing events were more frequent among the genomes of embryos injected with the DDD/RRR variant (Figure S3B), demonstrating the efficiency with which DSBs are induced to stimulate HR notably affects the frequency of gene editing events. Using the induction of targeted mutations as a proxy measurement for the efficiency with which DSBs were induced, the TALENs used in the experiments reported here induced DSBs in 23%–86% of the genomes of injected F0 embryos (Table S1).

## Conformation of Donor DNA Affects Gene Editing

The ability of intact plasmid circles, linearized plasmids, or purified dsDNA fragments containing only donor sequences and flanking homology arms to function as donor molecules for HR was analyzed. Under our experimental conditions, plasmid circles always proved most efficient as donor molecules (Figure 1C

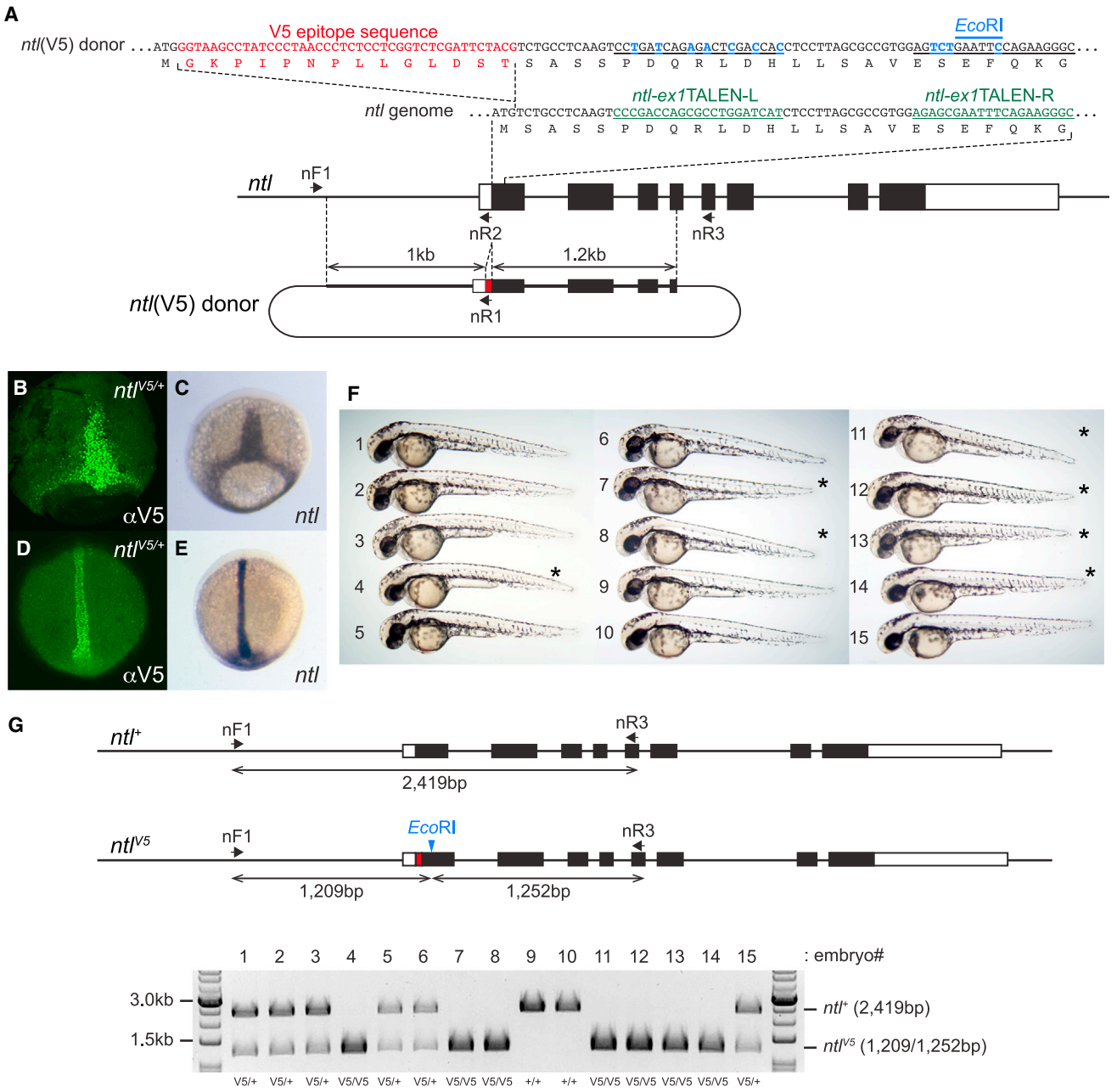


**Figure 2. An Edited Allele that Encodes an Epitope-Tagged Version of No Tail**

(A) Schematic representation of the genomic structure of the *no tail* (*ntl*) gene, the *ntl-ex1* TALEN target (green and underlined), and the *ntl(V5)* donor DNA, highlighting novel V5 epitope sequences (red) and modified *ntl* sequences (blue). Modified sequences are flanked by 1 kb (upstream) and 1.2 kb (downstream) homology arms. Diagnostic primers are depicted.

(B–E) The edited $ntl^{V5}$ allele is expressed faithfully. The expression pattern of the V5 antigen in (B) mid- and (D) late-gastrula $ntl^{V5/+}$ embryos revealed by immunohistochemistry mimics the expression of *ntl* transcripts detected by in situ hybridization in comparably staged embryos (C and E).

(F) The $ntl^{V5}$ allele has WT function. All progeny of $ntl^{V5/+}$ intercross matings appear morphologically WT at 2 dpf. Homozygous $ntl^{V5/V5}$ embryos, which are indistinguishable from their WT siblings, are indicated with *.

(G) The 15 embryos depicted in (F) were genotyped by amplifying the *ntl* locus with primers (nF1 and nR3) distal to the homologies present in donor DNA, and cleaving the amplicon with *Eco*RI, which only recognizes the edited *V5* allele.

See also Figure S2.

and Table 1, and data not shown). Although the reason for these differences is not clear, similar findings have been made in *Drosophila* (Carroll and Beumer, 2014) and in some zebrafish studies (Irion et al., 2014). We hypothesize that linear molecules may be more prone than intact circles to degradation or concatenation, either of which might affect the availability of donor

sequences for HR. Noting that linear molecules generated by cleavage with the *I-Sce*I meganuclease are excellent substrates for random insertion and tend to produce single copy transgenes in zebrafish (Grabher et al., 2004), we tested whether linear donor molecules generated by *I-Sce*I cleavage might function well for gene editing purposes. As *I-Sce*I enzyme cleaves its target site asymmetrically and associates in a relatively stable manner with the longer digestion product (Perrin et al., 1993), we generated a series of pKHR donor vectors (Figure S4) containing a central domain that accepts donor sequences flanked by a pair of head-to-head oriented *I-Sce*I sites. The effect of *I-Sce*I-digestion on the efficiency of gene editing was tested at the zebrafish *kcnh6a* locus.

The *kcnh6a* gene encodes a potassium channel expressed exclusively in the heart that is critical for normal cardiac excitability (Arnaout et al., 2007). Mutations in the human homolog *KCNH2*/*HERG* cause inherited arrhythmia disorders associated with sudden cardiac death (Splawski et al., 2000). Cardiac activity in zebrafish embryos lacking *kcnh6a* function can be rescued by expression of WT human *KCNH2* RNA but not RNA-encoding pathogenic *KCNH2* variants associated with long QT syndrome (Jou et al., 2013). To further develop this bioassay, we chose to produce zebrafish lines in which fluorescent reporter proteins would be expressed from the *kcnh6a* locus in lieu of its normal protein product. The strategy for editing the *kcnh6a* locus is shown in Figure 3A. Two donor constructs were generated in pKHR4 with the purpose of introducing a fluorescent reporter protein into the *kcnh6a* locus. In each, either eGFP or mCherry coding sequences followed by translation and transcription termination motifs were flanked by perfect homology arms of about 1 kb in such a manner that HR between each arm and the host genome would introduce the reporter sequence in frame just downstream of the *kcnh6a* AUG translation initiation codon. Recombination was stimulated with the *kcnh6a-int1* TALEN designed to induce a DSB in intron 1, about 150 bp 3′ of the initiation codon. Donor DNA was injected into one-cell zygotes with or without *kcnh6a-int1* TALEN mRNA or *I-Sce*I enzyme. Integration of reporter sequences at the *kcnh6a* locus of injected embryos was detected by PCR amplification with diagnostic primers (Figures 3A and 3B).

Without TALEN activity to cleave the target locus and stimulate HR, neither *gfp* nor *mcherry* sequences were detected at the *kcnh6a* locus. Following induction of DSBs, although intact plasmid DNA served as an adequate substrate for HR events, in experiments with two different donor plasmids, co-injection of donor plasmid DNA with *I-Sce*I enzyme further stimulated the generation of *kcnh6a* locus-specific integration events (Figure 3B). As demonstrated by quantitative assessment of edited alleles in injected F0 embryos, the enhancement of genome editing events was dependent on the amount of *I-Sce*I enzyme digestion and could be accomplished by digestion of donor DNA with *I-Sce*I enzyme in vitro, prior to injection of zygotes (Figure 3C).

## Production of Knockin/Knockout Alleles

Embryos injected with both *kcnh6a*(eGFP) donor plasmid and *I-Sce*I enzyme were grown to adulthood, and germline-transmitted alleles were recovered from 2 of 14 (14%) F0 founders. The resulting *kcnh6a*$^{GFP}$ alleles were amplified from the genomes of F1 embryos and sequence analysis confirmed that donor sequences had been perfectly integrated as expected from HR.

*kcnh6a*$^{GFP/+}$ heterozygous embryos were fully viable and expressed the GFP reporter protein exclusively in the heart (Figures 3D and 3E). F2 embryos homozygous for the knockin allele were phenotypically indistinguishable from previously described *kcnh6a* null mutants (Arnaout et al., 2007), exhibiting cardiac edema (Figures 3F and 3G) and hearts with contractile defects (Movies S1 and S2).

Reporter sequences can be readily introduced at additional loci for uses such as the in vivo lineage analysis of cells that fail to express a gene regulating developmental fate. The TALEN and homology arm sequences used to mediate introduction of V5 epitope sequences just downstream of the *ntl* AUG initiation codon (Figure 2A) were also used to introduce *eGFP* coding sequences followed by translation and transcription termination motifs (Figure 4A). Virtually all injected embryos had evidence of HR at the *ntl* locus (Figure S2B). Embryos carrying the *ntl*$^{GFP}$ allele expressed GFP appropriately in the notochord of developing embryos (Figures 4B and 4C). As expected, the knockin allele destroyed *ntl* function as the *ntl*$^{GFP}$ allele failed to complement the null *ntl*$^{b195}$ mutation (Figures 4D and 4E).

## Tagging Editing Events with a Linked Reporter Gene

One goal of genome editing in the zebrafish is to recapitulate and analyze the functions of human disease alleles. Many of these editing events, such as those that produce recessive alleles, will be difficult to recognize phenotypically in the F1 generation. With current genome editing methods in the zebrafish, it would be difficult to routinely recover silent sequence modifications, as detection of these would require labor-intensive DNA screening of the genomes of thousands of F1 individuals. Given that non-homologous stretches of sequences encoding reporter proteins can be integrated efficiently into the genome via HR, we reasoned that donor-sequence-linked reporter genes could be used to tag the acquisition of donor sequences. To test this concept, we determined whether acquisition of a reporter gene could be used to identify genomes that had acquired linked sequence modifications at the *gol* locus.

The donor plasmid used to correct the *gol*$^{b1}$ null mutation was modified so it harbored a reporter gene within the intron of donor sequences, 3′ of the TALEN recognition site (Figure 5A). The 1.8-kb $\alpha$-*crystallin::Venus* (*CV*) reporter gene expressing a GFP variant under the control of the $\alpha$-crystallin lens promoter (Hesselson et al., 2009) was bordered by FRT recombination sites that could mediate excision of the reporter gene upon expression of FLP recombinase.

Homozygous *gol*$^{b1}$ zygotes were injected with a combination of *gol-int5* TALEN RNA and *gol*(b1→WT; CV) donor plasmid, resulting in normal-appearing embryos, 84% of which exhibited pigmented tissue (Table 1). Analysis of genomic DNA indicated each F0 embryo had some genomes in which reporter sequences were integrated into the *gol* locus as expected (Figure S2C). F0 embryos were raised to adulthood and mated with *gol*$^{b1}$ mutant partners to examine their germlines for the ability to transmit an expressed *CV* transgene and a WT *gol* allele. Of 39 F0 adults screened, 13 (33%) produced progeny expressing the fluorescent reporter in the lens. Among the 13 F0s that

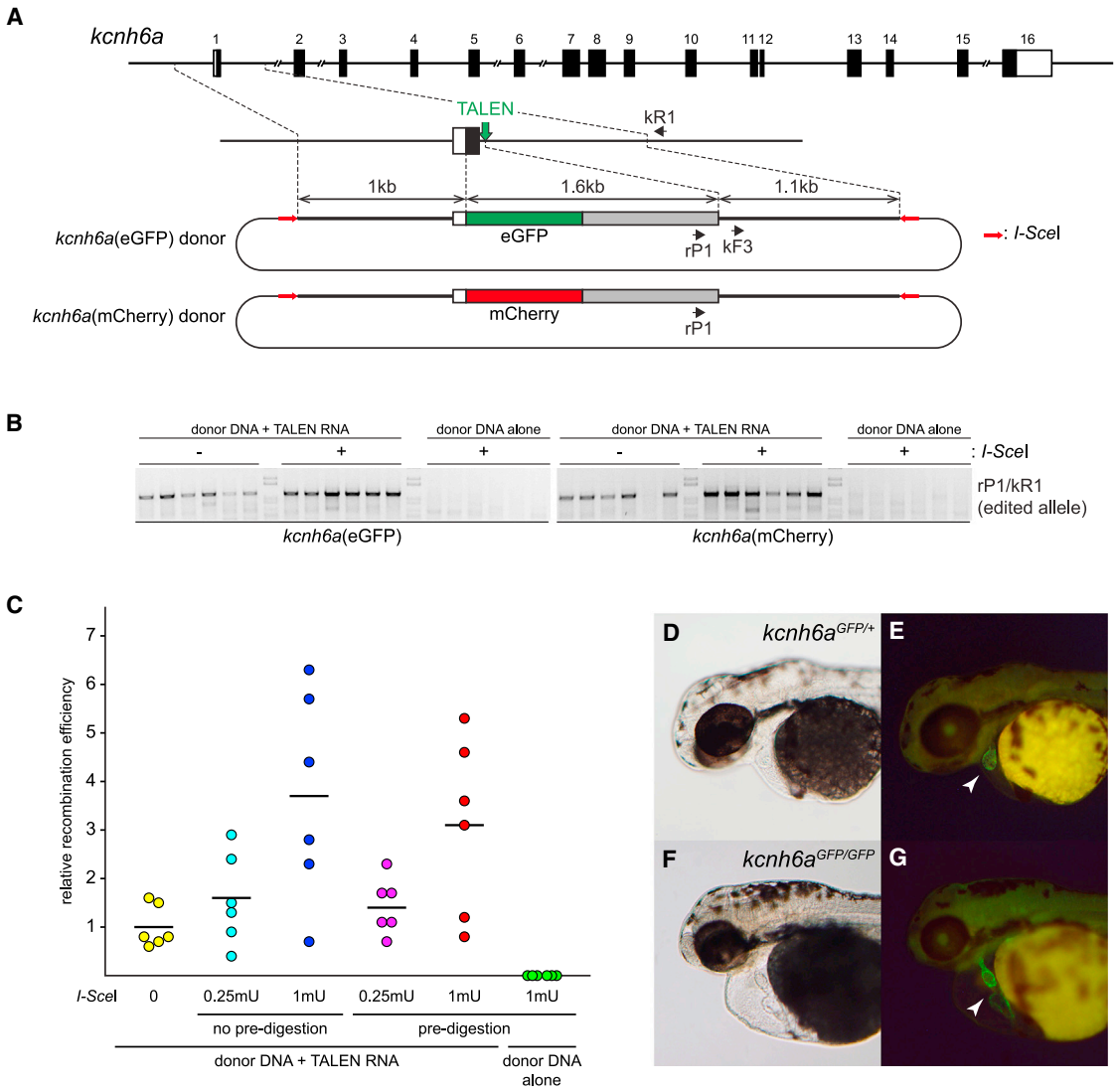


**Figure 3. Reporter Knockin/Knockout Alleles at the *kcnh6a* Locus**

(A) Schematic representation of the genomic structure of the *kcnh6a* gene, indicating the *kcnh6a-int1* TALEN target, and the structures of the donor DNAs (composed in pKH4 vector), highlighting the reporter coding sequences (colored) and translation/transcription termination signal sequences (gray) that are introduced by the donor. Left and right homology arms are bordered by *I-Sce*I recognition sites in head-to-head orientation (red arrows). Diagnostic primers are depicted. The rP1/kR1 pair specifically amplifies edited alleles, whereas the kF3/kR1 pair amplifies edited and unedited forms of the *kcnh6a* gene.

(B) In vivo *I-Sce*I-digestion of donor plasmids stimulates genome editing. Individual embryos were injected with *kcnh6a*(eGFP) or *kcnh6a*(mCherry) donor DNAs with or without the *I-Sce*I meganuclease. Edited alleles were detected by PCR with diagnostic primers.

(C) Genome editing is enhanced following *I-Sce*I digestion of donor plasmids, performed either in vivo or in vitro, prior to injection. Zygotes were injected with TALEN RNA and donor plasmid DNA mixed with differing amounts of *I-Sce*I enzyme on ice until injection (no pre-digestion) or digested in vitro prior to injection (pre-digestion). As a control, in vitro-digested donor plasmid was injected alone. The fraction of edited alleles (detected with the rP1/kR1 primer pair) relative to total *kcnh6a* alleles (detected with the kF3/kR1 primer pair) present in injected 2-dpf embryos was determined by qPCR. The relative recombination efficiency was determined by normalizing to 1.0 the mean fraction of edited alleles following injection of TALEN RNA and undigested donor plasmid DNA. Six individual embryos were analyzed (circles) for each condition, and the mean relative recombination efficiency is indicated (horizontal dash). Unpaired t test analysis indicated that in vivo or in vitro digestion of donor DNA with 1 mU enzyme significantly stimulated the production of edited alleles compared with untreated donor DNA ($p < 0.01$). Digestion with 0.25 mU enzyme did not yield a significant increase in recombination efficiency compared with untreated DNA.

(D–G) The *kcnh6a*$^{GFP}$ allele lacks WT function but drives GFP reporter expression, as detected by immunohistochemistry. (D and E) *kcnh6a*$^{GFP/+}$ heterozygotes are viable and express GFP in the heart; white arrowhead in (E) and (G). (F and G) In contrast, *kcnh6a*$^{GFP/GFP}$ homozygotes have cardiac deficiencies and are inviable. See also Movies S1 and S2.

transmitted the *CV* transgene, four (31%) produced offspring that both expressed the reporter and were fully pigmented (Figures 5B and 5C). Analysis of genomic DNA sequences indicated the embryos that expressed both the reporter and pigment had precisely replaced mutant *gol* sequences with a single copy of the donor sequences.

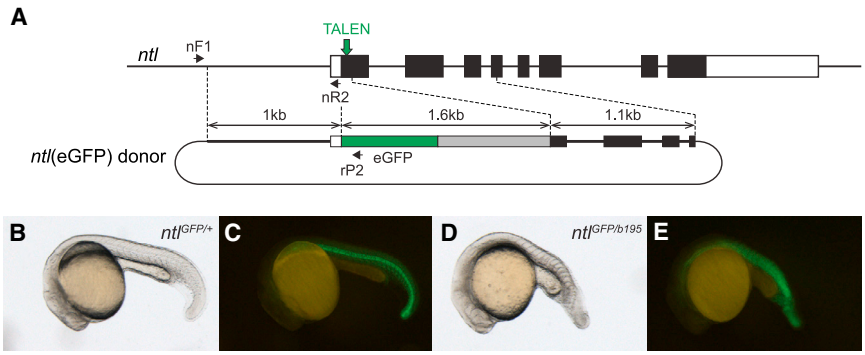


**Figure 4. Reporter Knockin/Knockout Allele of *No Tail***

(A) Schematic representation of the genomic structure of the *ntl* gene, the position of the *ntl-ex1* TALEN target, and the structure of the donor DNA. Left and right homology arms flank the novel donor sequences. Diagnostic primers are depicted.

(B–D) The $ntl^{GFP}$ allele is a functional null that drives GFP fluorescence expression in the *ntl* expression domain. (B and C) $ntl^{GFP/+}$ embryos (20 hpf) are morphologically normal with GFP expression in the notochord. (D and E) $ntl^{GFP}$ fails to complement the null $ntl^{195}$ allele. $ntl^{GFP/b195}$ embryos display abnormal somites and loss of posterior mesoderm seen in *ntl* mutants.

See also Figure S2.

Following recovery of the reporter-tagged genome editing event, the FRT-bordered reporter gene can be readily excised (Boniface et al., 2009). Zygotes carrying the edited $gol^{b1\rightarrow WT;CV}$ allele were injected with FLP recombinase mRNA. Resulting 2-dpf embryos lost GFP expression but still exhibited normal pigmentation (Figures 5D and 5E). Consistent with the phenotypic analyses, genomic DNA from each injected embryo experienced precise loss of reporter sequences from edited alleles (Figure 5F).

In summary, the reporter gene can be used to simplify and streamline the recovery of genomes that have been modified by HR and enrich for those that have acquired linked donor sequences of interest. Once an edited allele is recovered, the reporter gene can be removed efficiently using sequence-specific recombination tools.

## Efficient Recovery of Floxed Alleles using a Linked Reporter Gene

The experiments at *gol* indicated acquisition of a linked reporter gene could be used to identify accompanying silent editing events. We tested the utility of the linked reporter for recovering one type of phenotypically silent editing event, generation of a conditional floxed allele, which would enable tissue-specific and temporal analyses of gene function that are not available in the zebrafish. The strategy for introducing *loxP* sites flanking the 203-bp exon 6 of *kcnh6a* is presented in Figure 6A. HR was stimulated with DSBs induced in intron 6 by the *kcnh6a-int6* TALEN. Donor sequences were assembled in the pKHR5 backbone (Figure S4), which provides a *loxP* site immediately 3′ to the FRT-flanked *CV* reporter gene. Right arm homology was derived from genomic sequences extending about 1 kb 3′ of the TALEN site. The left arm extended about 1.5 kb upstream from the TALEN site, spanning the entirety of exon 6. The left arm was constructed by overlapping PCR to introduce a *loxP* site into intron 5, 0.4 kb 5′ of the TALEN site, with additional 1.1-kb host sequences extending distal to the *loxP* site. Incorporation of all novel donor sequences would yield a floxed allele of the configuration 5′-loxP-exon6-FRT-*CV* reporter-FRT-loxP-3′.

Zygotes were injected with TALEN mRNA, *kcnh6a*(*loxP*) donor plasmid, and *I-Sce*I enzyme. PCR analysis diagnostic of locus-specific integration events indicated every injected embryo had evidence of HR (not shown), and thus F0 embryos were raised to adulthood. Six independently segregating $CV^{+}$ alleles were recovered among the offspring of five of the 43 (12%) F0 adults that were screened. Individual F1 progeny that expressed the reporter were analyzed for the presence of left and right donor-host genome junctions and for the retention of backbone vector sequences. These preliminary analyses revealed two patterns of donor sequence integration (Figure S5): (1) *kcnh6a* integrants with expected donor/target locus junctions; and (2) random integrants with vector backbone and donor sequences but lacking target locus junctions. Sequence analysis of *CV* reporter-expressing F1 individuals indicated edited *kcnh6a* alleles arose from precise exchange of sequences, incorporating both flanking *loxP* sites and the *CV* reporter gene.

In summary, to recover conditional alleles of *kcnh6a*, the offspring of 43 F0 individuals were initially screened for GFP expression in the lens, and five founders that transmitted the *CV* reporter were identified. To identify precisely edited conditional *kcnh6a* alleles, only the genomes of reporter-expressing F1 offspring were screened. Three independently induced conditional *kcnh6a* alleles were recovered, representing one-half of all the $CV^{+}$ alleles transmitted by F0 founders. To propagate the conditional allele, only $CV^{+}$ progeny carrying $kcnh6a^{loxP}$ were raised to adulthood. Hence recovery of the conditional *kcnh6a* allele was streamlined dramatically by virtue of expression of the linked reporter gene.

To test the functionality of the conditional $kcnh6a^{loxP}$ allele, $kcnh6a^{loxP/+}$ heterozygotes were intercrossed, and the development of their progeny was examined in the absence or presence of Cre recombinase. As the *loxP* sites resided in introns flanking both the $\alpha$-*crys::Venus* reporter gene and exon 6, we anticipated intercross progeny carrying the conditional allele to be viable and express GFP in the lens. Approximately three-fourths (78/112 = 0.70) of the offspring expressed the reporter, and all of these appeared morphogically normal at 2 dpf (Figures 6B, 6C, and 6F). Genotyping of intercross progeny revealed that the conditional $kcnh6a^{loxP}$ allele segregated as a fully viable allele (Figure 6G). In contrast, following injection of intercross zygotes with 50 pg of *cre* mRNA, none (0/49) of the embryos expressed the lens reporter gene and approximately one-quarter (12/49 = 0.24) displayed cardiac edema and a non-beating ventricle (Figures 6D–6F), characteristics typical of loss of *kcnh6a* function. Analysis of the genomic DNA of mutants indicated efficient excision of all sequences between the *loxP* sites such that we could not detect intact conditional alleles in the genomes of injected embryos (Figure 6G). These results indicate the conditional allele is fully functional: the floxed allele has WT activity and the

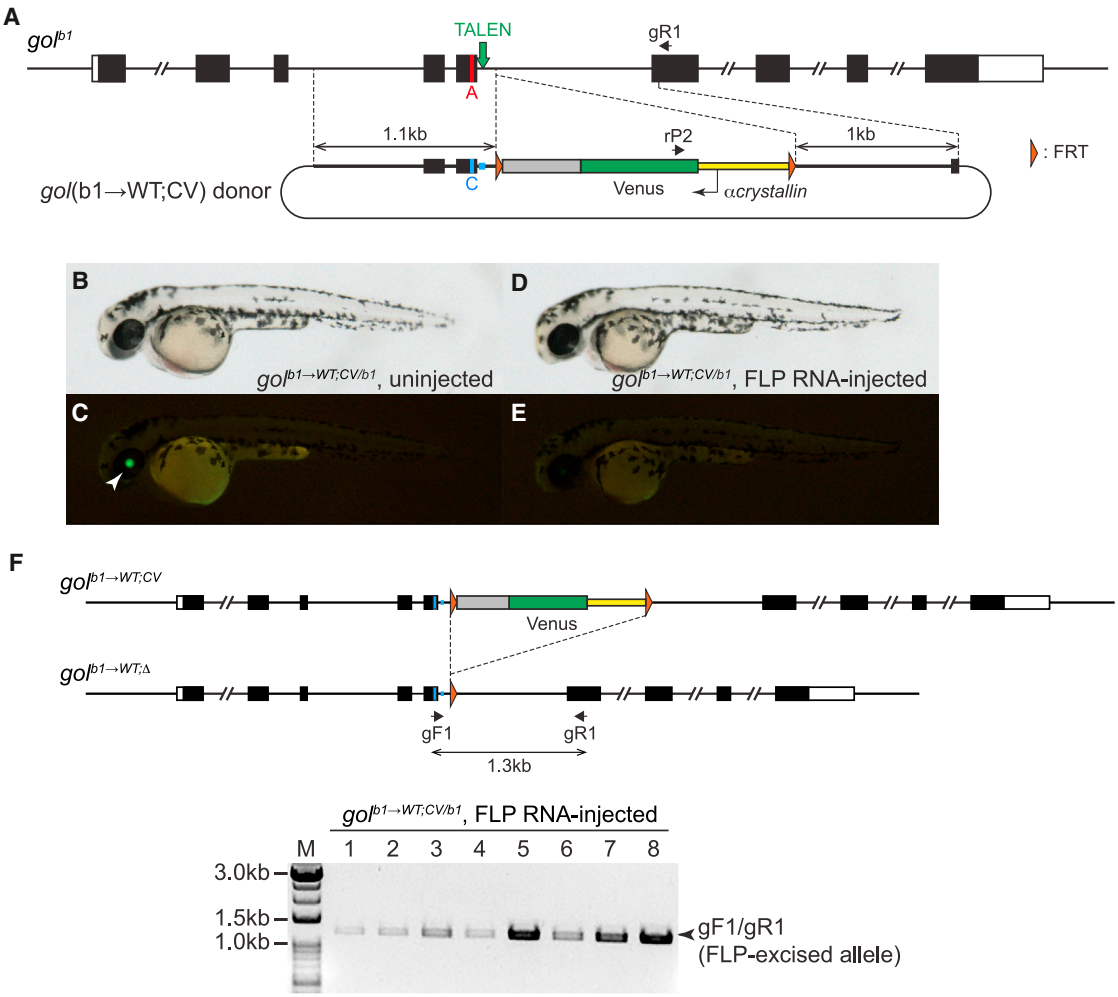


**Figure 5. Recovery of an Edited Genome by Virtue of a Linked Reporter Gene that Can Be Subsequently Excised**

(A) Schematic representation of the *gol* gene and *gol*(b1→WT) donor DNA modified to include within intron 5 an FRT-*CV*-FRT-loxP cassette with tandemly repeated FRT sites (orange arrowheads) bordering the 1.8-kb α-*crystallin::Venus* (*CV*) reporter gene, which is in inverted orientation relative to *gol* transcription. Diagnostic primers are depicted.

(B–E) The donor-linked *CV* reporter can be used to recover gene editing events and can be subsequently excised from the genome. (B and C) A 2-dpf $gol^{b1\rightarrow WT;CV/b1}$ embryo is normally pigmented with strong GFP fluorescence expression in the lens (white arrowhead). (D and E) Following injection of one-cell embryos with FLP mRNA, a 2-dpf $gol^{b1\rightarrow WT;CV/b1}$ embryo is fully pigmented but lacks lens expression of the GFP reporter.

(F) The FRT-*CV*-FRT-loxP cassette is effectively removed from the genome following injection of $gol^{b1\rightarrow WT;CV/b1}$ zygotes with FLP mRNA. Each of eight injected embryos analyzed by diagnostic PCR at 2 dpf had $gol^{b1\rightarrow WT;\Delta}$ edited alleles from which the *CV* reporter had been excised successfully.

See also Figure S2.

*loxP*-flanked sequences are excised efficiently in the presence of Cre recombinase, producing a non-functional allele.

## DISCUSSION

Here we demonstrate that precise genome editing stimulated by targeted induction of HR can be accomplished with high efficiency in the zebrafish. Genome modification by HR allows for the generation of a large spectrum of designed changes to the genome. We have created modified alleles that (1) harbor single codon alterations, (2) express an antigen-tagged version of an endogenous product, or (3) express GFP from a targeted locus under control of the endogenous promoter. In addition, we used HR to introduce *loxP* sites flanking an essential exon and thus produced a fully functional conditional *kcnh6a* allele, which can be readily converted from WT to mutant function in the presence of the Cre recombinase. These types of genome modifications will allow analysis in the zebrafish of disease-associated variants, candidate enhancer sequences, trafficking and interactions of proteins, or tissue-specific utilization of genes. It appears most loci can be modified by the methods presented here; in this work four different sequences were targeted to produce seven different alleles.

Almost all genome editing events produced via HR, with dsDNA used as donor molecules, result in precisely altered alleles in the zebrafish. This is in stark contrast to the use of ssDNA oligonucleotides as donor templates for genome editing in the zebrafish, where a large fraction of the modified alleles are

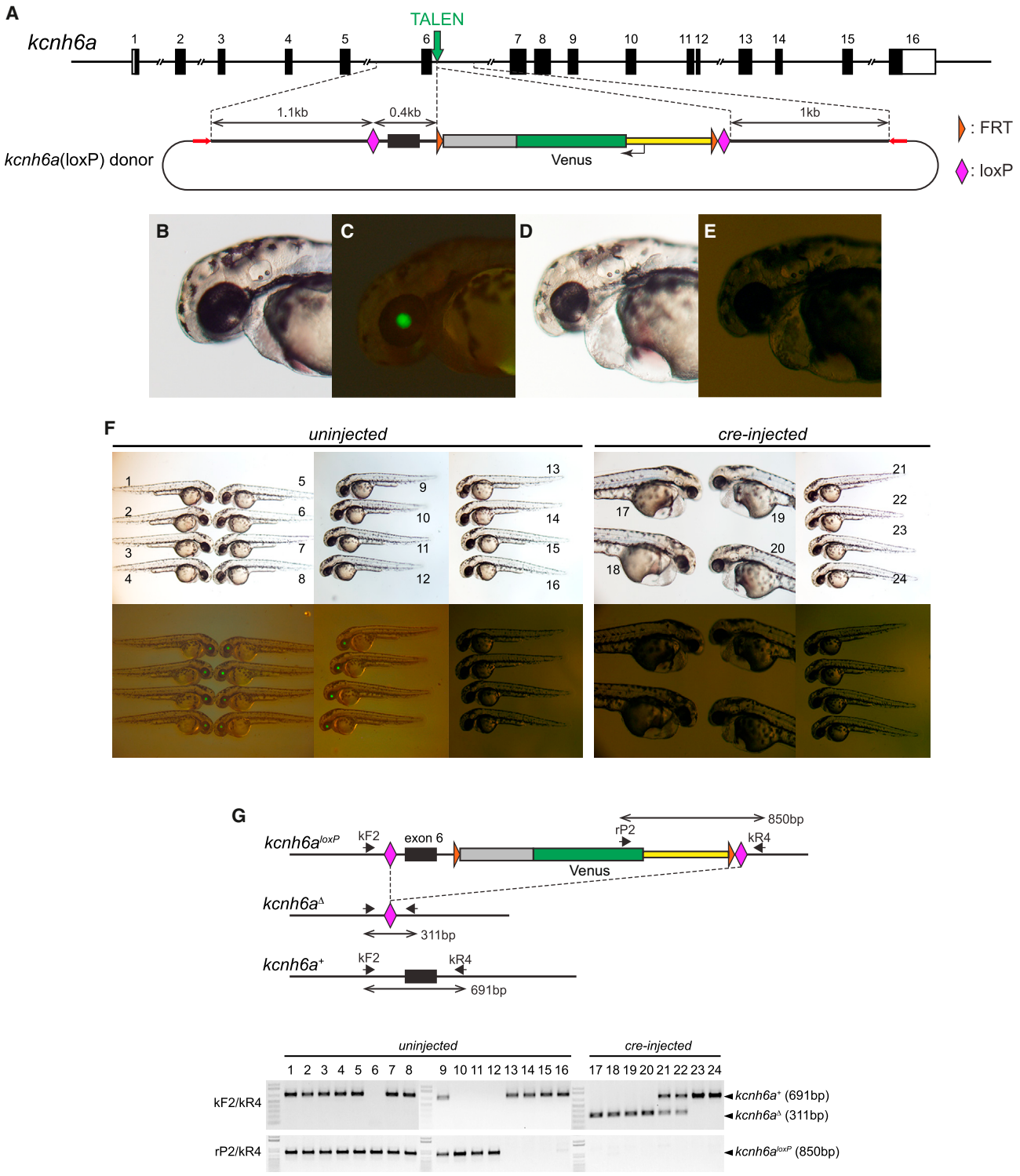


**Figure 6. A Conditional *kcnh6a* Allele**

(A) Strategy for generating a floxed *kcnh6a* allele. The *kcnh6a-int6* TALEN target site lies 125 bp 3′ of exon 6. The donor plasmid was constructed in pKHR5, which provides the FRT-*CV*-FRT-loxP cassette. Overlapping PCR was used to introduce a *loxP* site (pink diamond) into intron 5 of the donor sequences. Left and right homology arms are bordered by *I-Sce*I recognition sites (red arrows). Two kinds of GFP$^{+}$ F1 offspring were recovered: those with precise and those with imprecise integration events (see Figure S5).

(B–E) The *kcnh6a*$^{loxP}$ allele is conditionally functional. (B and C) A 2-dpf *kcnh6a*$^{loxP/loxP}$ embryo appears WT and expresses GFP fluorescence in the lens. (D and E) Following injection of a *kcnh6a*$^{loxP/loxP}$ zygote with Cre mRNA, at 2 dpf the embryo has cardiac defects typical of a *kcnh6a* null mutant and lacks GFP expression.

*(legend continued on next page)*

accompanied by unintended mutations (Bedell et al., 2012; Hruscha et al., 2013; Auer and Del Bene, 2014).

## Factors Affecting the Efficiency of Genome Editing via HR

Work in both yeast and mammalian cells established the significant impact of DSBs on the stimulation of HR between a targeted host locus and exogenously supplied homologous sequences (Jasin, 1996; Paques and Haber, 1999). Our work with TALENs carrying FokI domains with differing activities indicating the efficiency with which targeted DSBs are induced has a pronounced effect on the efficiency of HR. In addition, the structure of the donor template DNA affects the efficiency of HR in the zebrafish embryo. Notably, we find linear dsDNA bordered by a pair of head-to-head oriented *I-SceI* recognition sites recently cleaved by the *I-SceI* meganuclease functions effectively as a source of donor template for HR events that lead to genome editing. We have developed a series of vectors that should be useful for generating *I-SceI*-cleaved templates for genome modification. In our hands, digestion of donor molecules generated in these vectors with *I-SceI* stimulated the production of edited alleles at multiple loci (data not shown), but we cannot assert this will be true for all editing events. In all, induction of DSBs at the target locus to stimulate HR, combined with the use of *I-SceI*-digested donor DNA molecules, leads to the routine and efficient production of precisely edited alleles.

## Use of a Linked Marker to Efficiently Recover Precisely Edited Alleles

Because our strategy of using a reporter gene nested within donor sequences allows for the extremely efficient identification and recovery of genomes with modifications, it dramatically changes the numbers and types of genome alterations that can be practically isolated on a routine basis. Using any of the previously reported approaches to genome editing in zebrafish (Auer and Del Bene, 2014), it is necessary to screen the genomes of approximately 500–5,000 F1 individuals to identify carriers of sequence modifications that do not produce a phenotype in the F1 generation. This is because current approaches to genome editing produce founders whose germlines are genetically mosaic. Pooling the data from all the heritable edited alleles recovered in this study, we find that an edited allele is transmitted by 6.3% of the F0 gametes on average (median = 5.4%) (Table S3). Thus only a few percent of the gametes of a small subset of potential founders carry a modified allele of interest. To identify an altered allele, it is often necessary to screen by DNA analysis up to 100 offspring from each of 20–100 founders. Furthermore, to recover the edited allele from a transmitting founder once identified, it has been necessary to raise large numbers of F1 progeny and then genotype these as adults.

The approach presented here improves both the production of edited alleles in the germlines of founders and the efficiency with which the alleles are recognized. By providing the *α-crys::Venus* gene within donor sequences, animals with genomes that have acquired donor sequences can be unambiguously recognized by virtue of expression of GFP in the lens. In our experience, the presence of the *CV* reporter gene, within an intron and in opposite transcriptional orientation to the host gene, does not have a detectable effect on expression of the endogenous gene. Moreover, if needed, the reporter can be subsequently excised. As the unintegrated donor plasmid bearing the reporter gene is capable of driving GFP expression in the injected embryo, this expression cannot be used to identify F0 embryos with integration events. However, use of the linked reporter significantly enhances the recovery of desired genome editing events: In the two examples described in this study, 30%–50% of the F1 individuals expressing the reporter gene had precisely edited alleles that arose via HR. This finding is wholly consistent with expectations, given our finding that about 10% of F0s transmit a bona fide edited allele and about 10% of F0s transmit donor sequences that have integrated elsewhere in the genome (as determined by PCR analysis, data not shown). Thus one need only raise a handful of F1 offspring that express the donor-linked *CV* reporter gene, anticipating that a large fraction of these will be heterozygous for the modified allele. In rare cases where non-targeted integration of donor sequences may occur with high frequency, we have found use of the donor vectors that additionally carry the *cmlc2::mCherry* reporter outside of the homology regions (Figure S4) further streamlines recovery of correct editing events. When these vectors are used, the simultaneous expression of both reporter genes indicates germlines that must have acquired donor sequences by means other than precise HR. Germlines that only harbor the *α-crys::Venus* reporter will be enriched for edited alleles that arose via HR.

## One Approach to Generate a Spectrum of Precisely Modified Alleles

We demonstrate how HR can be used to generate several highly useful types of alleles in the zebrafish. First, we show that, at the *gol* locus, it is simple to recover and propagate coding sequence changes by virtue of the expression of a linked reporter that was acquired along with the sequence change of interest. Second, at *ntl* we demonstrate that in-frame introduction of epitope-coding sequences can be used to tag endogenous proteins that are produced under physiological conditions and maintain WT function. Third, at *kcnh6a* we show single HR-mediated gene editing events can produce fully functional, conditional floxed alleles, whose *loxP* sites can be induced to recombine efficiently in the presence of Cre recombinase. We envision zebrafish with engineered conditional alleles could be used routinely as bioassays

(F) Whereas the conditional $kcnh6a^{loxP}$ allele has WT function, Cre-mediated excision efficiently generates a derivative null $kcnh6a^{\Delta}$ allele. Uninjected: 16 2-dpf $kcnh6a^{loxP/+}$ in-cross progeny, all of which appeared morphologically WT and viable, were examined for GFP lens expression and genotyped (G) as illustrated. All 12 carriers of the $kcnh6a^{loxP}$ conditional allele expressed the *CV* lens reporter. Genotyping indicated the conditional allele segregated as a normal viable allele. *cre*-injected: zygotes produced from a $kcnh6a^{loxP/+}$ in-cross were injected with *cre* mRNA; four mutant and four WT progeny were analyzed at 2 dpf. None of the embryos had GFP expression, consistent with the finding (G) that none had an intact $kcnh6a^{loxP}$ allele.

(G) Genotyping revealed each mutant had only $kcnh6a^{\Delta}$ alleles, indicating each was originally homozygous for the conditional allele and excision is efficient. Each of the WT progeny carried at least one copy of a WT $kcnh6a^{+}$. Each separate gel panel represents a distinct portion of a gel.

See also Figure S5.

to distinguish functional from pathogenic variants of human disease-associated genes. Conditional mutations also allow study of the tissue- and temporal-specific utilization of genes (Ni et al., 2012). Finally, the ability to manipulate conditional alleles will have a great impact on studies of regeneration or cancer in the zebrafish, in which it is important to preserve normal gene function during early stages of animal development and growth.

## EXPERIMENTAL PROCEDURES

### Zebrafish Handling and Maintenance

Zebrafish *Danio rerio* were maintained in accordance with approved institutional protocols at the University of Utah. WT zebrafish were from the Tübingen (Tü) or AB strains. Embryos and adults were maintained under standard conditions (Westerfield, 2000).

### Nuclease Design and Generation

Potential TALEN target sites were identified using the TALEN Targeter program at https://tale-nt.cac.cornell.edu/node/add/talen-old. TALEN targets are listed in Table S1. The RVD repeat array of each TALEN monomer was assembled using Golden Gate assembly (Dahlem et al., 2012). The RVD repeat arrays for left and right TALEN monomers were cloned into the pCS2TAL3DDD (Addgene 48637) and pCS2TAL3RRR (Addgene 48636) vectors, respectively, which contain FokI domain sequences (Doyon et al., 2011). TALEN mRNA was generated by transcription in vitro of pCS2-TALEN plasmids linearized with *Not*I (mMESSAGE mMACHINE SP6 kit; Life Technologies). The *gol-int5* sgRNA target site (Figure 1A) was identified using the CRISPR design program at http://crispr.mit.edu. *gol-int5* sgRNA was prepared by transcription in vitro essentially as described (Gagnon et al., 2014).

### Founder Selection and Donor Plasmid Construction

As individuals from WT strains carry polymorphisms, a small breeding population of WT F0 animals with minimal sequence heterogeneity at the targeted locus was used for each experiment. Briefly, once the presence of the predicted nuclease target site was confirmed in founder genomes, high-resolution melting analysis (HRMA) (Dahlem et al., 2012) and/or sequence analysis was used to identify founders with little sequence heterogeneity surrounding the target site. To make homology arms, amplicons were generated from selected genomes and cloned into donor vectors. Modified sequences were introduced using oligonucleotides and overlapping PCR.

### Microinjection to Produce Targeted DSBs and HR

To generate DSBs, 50 pg left and 50 pg right TALEN mRNA or 250 pg *gol-int5* sgRNA and 600 pg Cas9 protein (PNA BIO) were injected into the cytoplasm of zygotes. Following injection of nuclease components with 50 pg donor DNA, on average 68% of embryos (range, 59%–78%) appeared normal at 1–2 dpf.

### Preparation of Donor DNA for Microinjection

For circular plasmid injection, plasmids were purified by phenol/chloroform extraction followed by ethanol precipitation. For linearized plasmid injection, plasmids were digested with *Sca*I and purified by phenol/chloroform extraction and ethanol precipitation. For injection of purified donor fragments without vector backbone, plasmids were digested with *Xba*I and *Xho*I and the insert fragments were isolated following agarose gel electrophoresis and purified by phenol/chloroform extraction and ethanol precipitation. Aliquots of *I-Sce*I enzyme (NEB) were stored at −80°C. For co-injection with *I-Sce*I, 0.25 mU or 1 mU *I-Sce*I enzyme was mixed with 50 pg donor DNA and 100 pg total TALEN mRNA in 0.5× *I-Sce*I buffer (NEB). To pre-digest donor DNA with *I-Sce*I enzyme, 500 ng of donor DNA was incubated in 5 μl of 1× *I-Sce*I buffer with 2.5 U or 1 U *I-Sce*I enzyme at 37°C for 1 hr, and subsequently mixed with 5 μl of 100 ng/μl each of left and right TALEN mRNA. All injection samples were maintained on ice until injection of fertilized eggs with approximately 1 nl.

### Genomic DNA Extraction and Analyses

To prepare genomic DNA, individual 2- or 7-dpf embryos were incubated in 30 μl or 70 μl, respectively, of 50 mM NaOH at 95°C, 20 min. After cooling to 4°C, 1/10 volume of 1 M Tris-HCl (pH 8.0) was added to neutralize. PCR primers are listed in Table S2. PCR amplification and subsequent imaging were performed under non-saturating conditions. qPCR was performed to measure the effect of digestion of donor DNA with *I-Sce*I on the efficiency of gene editing. A 10-μl qPCR reaction contained 1 μl of embryonic genomic DNA, 0.5× KAPA SYBR FAST qPCR Kit Master Mix (KAPA Biosystem), 0.5× KAPA2G Fast HotStart PCR Kit (KAPA Biosystem), and 500 nM each forward and reverse primers (see Table S2 and Figure 3). The amplification reaction consisted of initial denaturation at 95°C, 3 min followed by 40–45 cycles (95°C, 10 s; 66°C, 15 s; 72°C, 45 s). The reactions were monitored in an Eco Real-Time PCR System (Illumina) and data were analyzed using EcoStudy Software (Illumina).

### Immunohistochemistry and In Situ Hybridization

Embryos were fixed (0.4% Triton X-100, 4% paraformaldehyde in PBS) for 2 hr at room temperature, washed, blocked, and incubated overnight at 4°C in 1:250 V5 Epitope Tag Antibody (catalog no. MA515253; InVitrogen) or 1:250 anti-GFP antibody (Millipore). Signal was developed following tyramide amplification using the TSA Kit #2 (Molecular Probes/Life Technologies). Whole-mount in situ hybridization was performed according to standard procedures (Westerfield, 2000).

## ACCESSION NUMBERS

Sequences for the donor vectors pKHR 4, 5, 7, and 8 (see Figure S4) can be found at GenBank: KU144822, KU144823, KU144824, and KU144825, respectively.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, three tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.02.015.

## AUTHORS CONTRIBUTIONS

K.H., M.J.J., and D.J.G. each contributed to the design and conduct of experiments and collectively wrote the manuscript.

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## Supplemental Information

# Precise Editing of the Zebrafish Genome Made Simple and Efficient

**Kazuyuki Hoshijima, Michael J. Jurynec, and David Jonah Grunwald**

# *Supplemental Inventory*

## Supplemental Figures



## Supplemental Tables



## Supplemental Movies



## Supplemental References

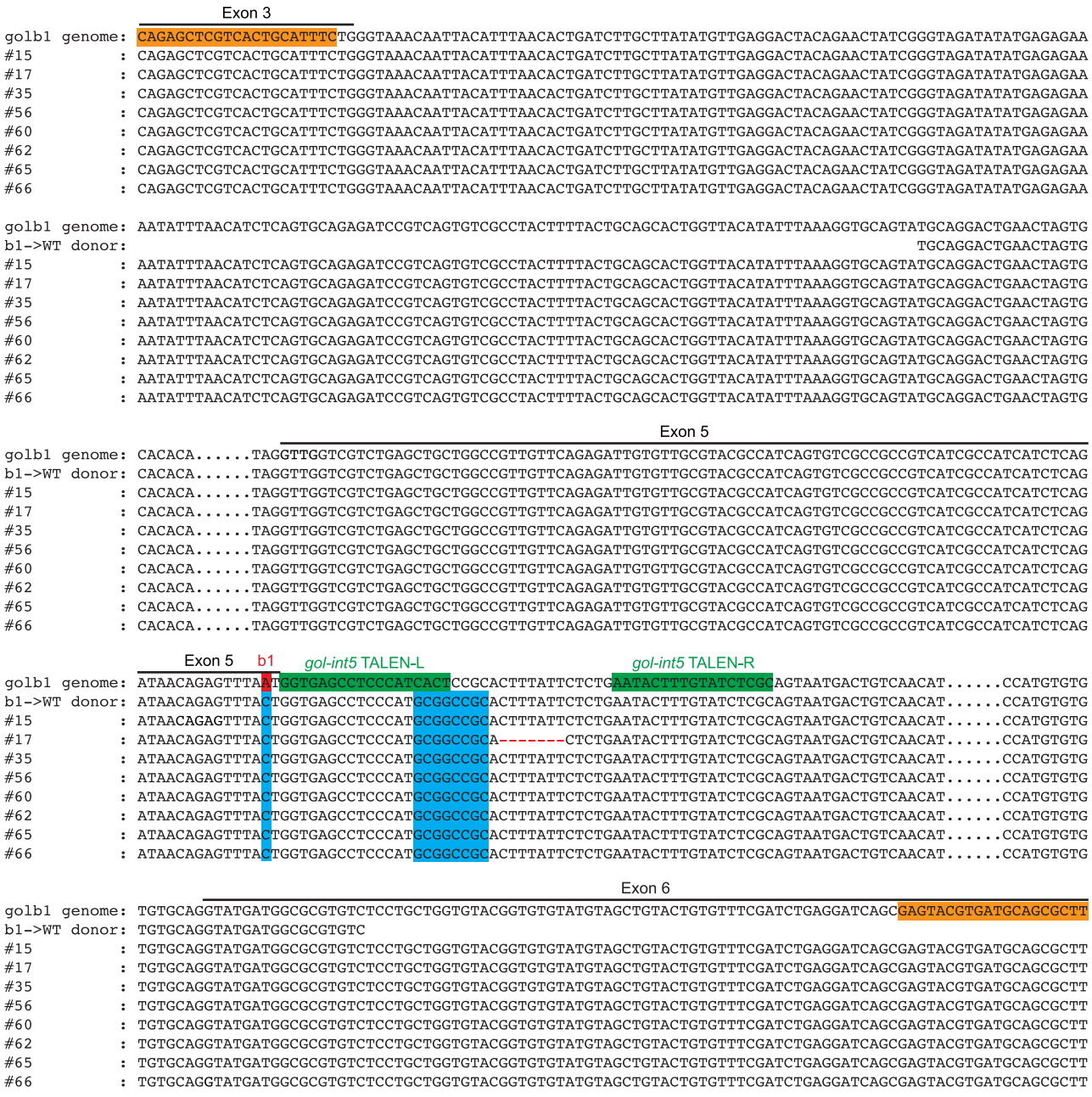


**Figure S1. Genome sequences of eight *gol*$^{b1\rightarrow WT}$ edited at the *golden* locus, Related to Figure 1**

Edited *gol*$^{b1\rightarrow WT}$ alleles were amplified from genomic DNA using the primer pair gF3/gR1 (position of primers is indicated in orange here and as arrows in Figure 1), which generated a single amplicon spanning host sequences that lay distal to the homology arms. The complete sequence of each recovered allele was determined, and relevant sections are shown here (some of the sequence is omitted here, indicated as ....), along with corresponding sequences of the original *gol*$^{b1}$ locus and the donor DNA. Some sequence features are highlighted: the *gol*$^{b1}$ mutation and the induced 7 bp deletion are in red; the *gol-int5*TALEN target site is green; and donor-specific sequences are blue. Except as noted here, the sequence of each edited allele was identical to the original *gol*$^{b1}$ allele and none of the edited alleles had additional modifications.

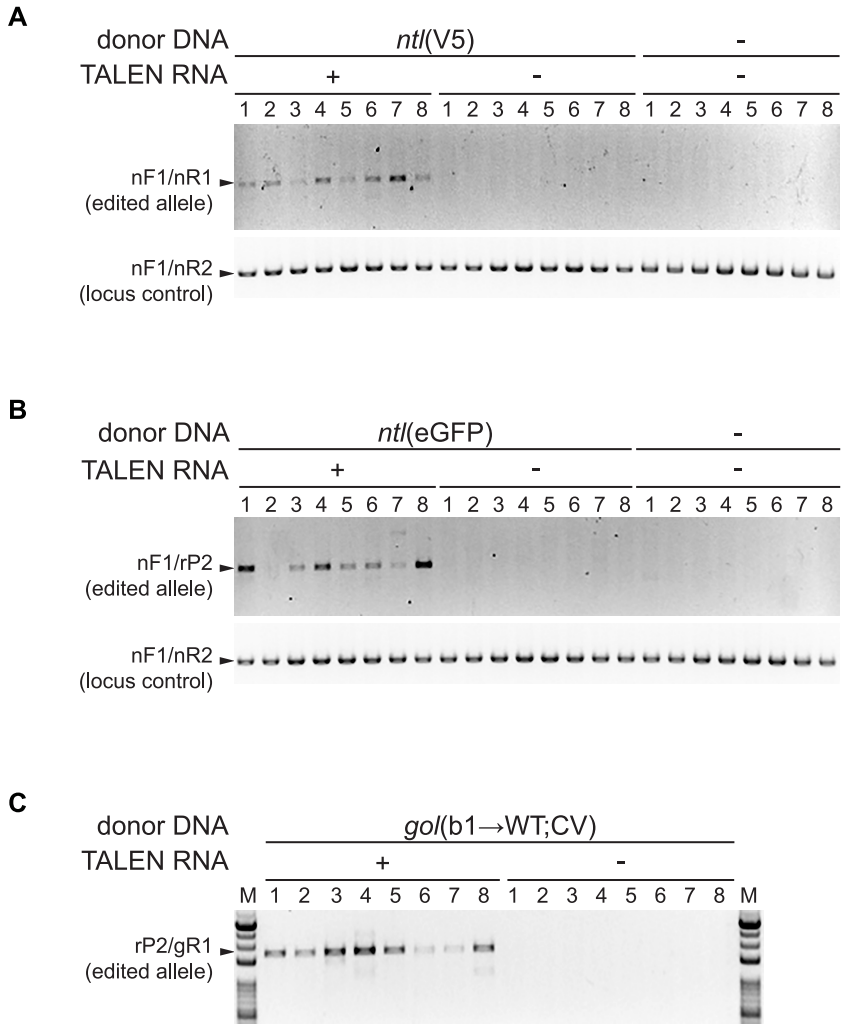


**Figure S2. Detection of edited alleles in F0 embryos, Related to Figures 2, 4, and 5**
To detect generation of edited alleles in F0 embryos, zygotes were injected with or without donor DNA and with or without TALEN RNA, and the genomic DNAs of individual 7 dpf embryos were analyzed. Edited alleles were detected with allele-specific primer pairs. As a control for the ability to detect the locus, primers that recognized both original host and edited alleles were also utilized. (A) Detection of edited $ntl^{V5}$ alleles using the $ntl^{V5}$-specific primer pair nF1/nR1 in combination with the locus control primer pair nF1/nR2 (see Figure 2). (B) Detection of edited $ntl^{GFP}$ alleles using the $ntl^{GFP}$-specific primer pair nF1/rP2 in combination with the locus control primer pair nF1/nR2 (see Figure 4). (C) Detection of edited $gol^{b1\rightarrow WT;CV}$ alleles using the $gol^{b1\rightarrow WT;CV}$-specific primer pair rP2/gR1 (see Figure 5).

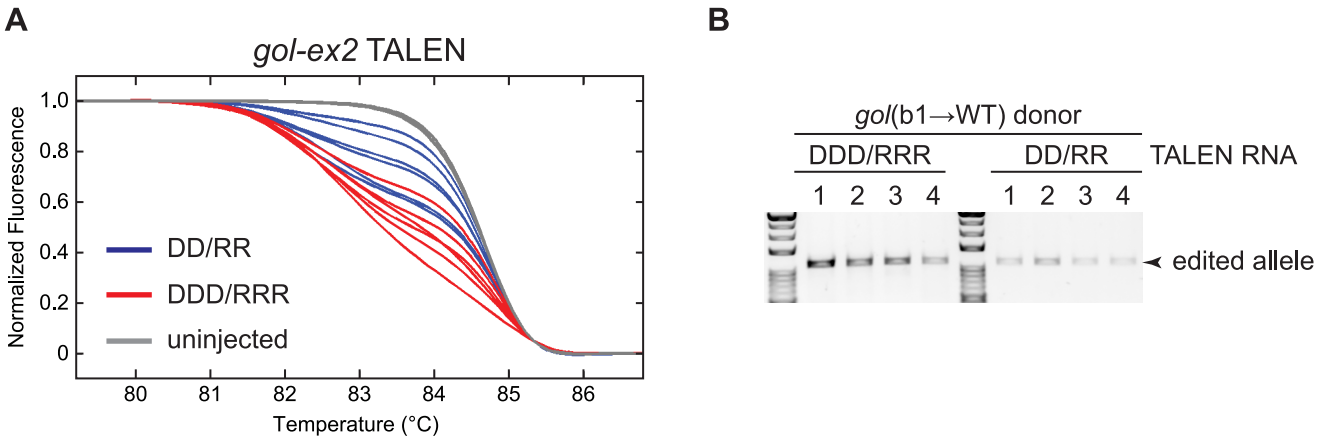


**Figure S3. TALEN activity at the target site affects gene editing, Related to Figure 1**
(A) HRMA analysis indicates the DDD/RRR version of the *gol-ex2* TALEN is more active than the DD/RR version at inducing mutations in the *gol* locus. The *gol-ex2* TALEN, which targets exon 2 of the *gol* locus, has been described previously (Dahlem et al. 2012). WT zygotes were either not injected or injected with 50pg TALEN-L and 50pg TALEN-R mRNAs, and the induction of target-specific *gol* mutations 24 hpf embryos was analyzed by HRMA as described (Dahlem et al. 2012). Deflection away from the WT melt curve results from the presence of newly induced polymorphisms (mutations) at the *gol-ex2* TALEN target site. (B) Detection of $gol^{b1\rightarrow WT}$ edited alleles following initiation of DSBs with either the DDD/RRR or DD/RR forms of the *gol-int5* TALEN. Genome editing of the $gol^{b1}$ mutation was performed as in Figure 1. DSBs were initiated following injection of 100pg total RNA encoding the DDD/RRR or DD/RR forms of the *gol-int5* TALEN. Donor sequences were provided by co-injection with 50pg plasmid *gol*(b1→WT). Edited alleles in 2 dpf F0 injected embryos were detected with the $gol^{b1\rightarrow WT}$ allele-specific primers gF1/gR1 (see Figure 1).

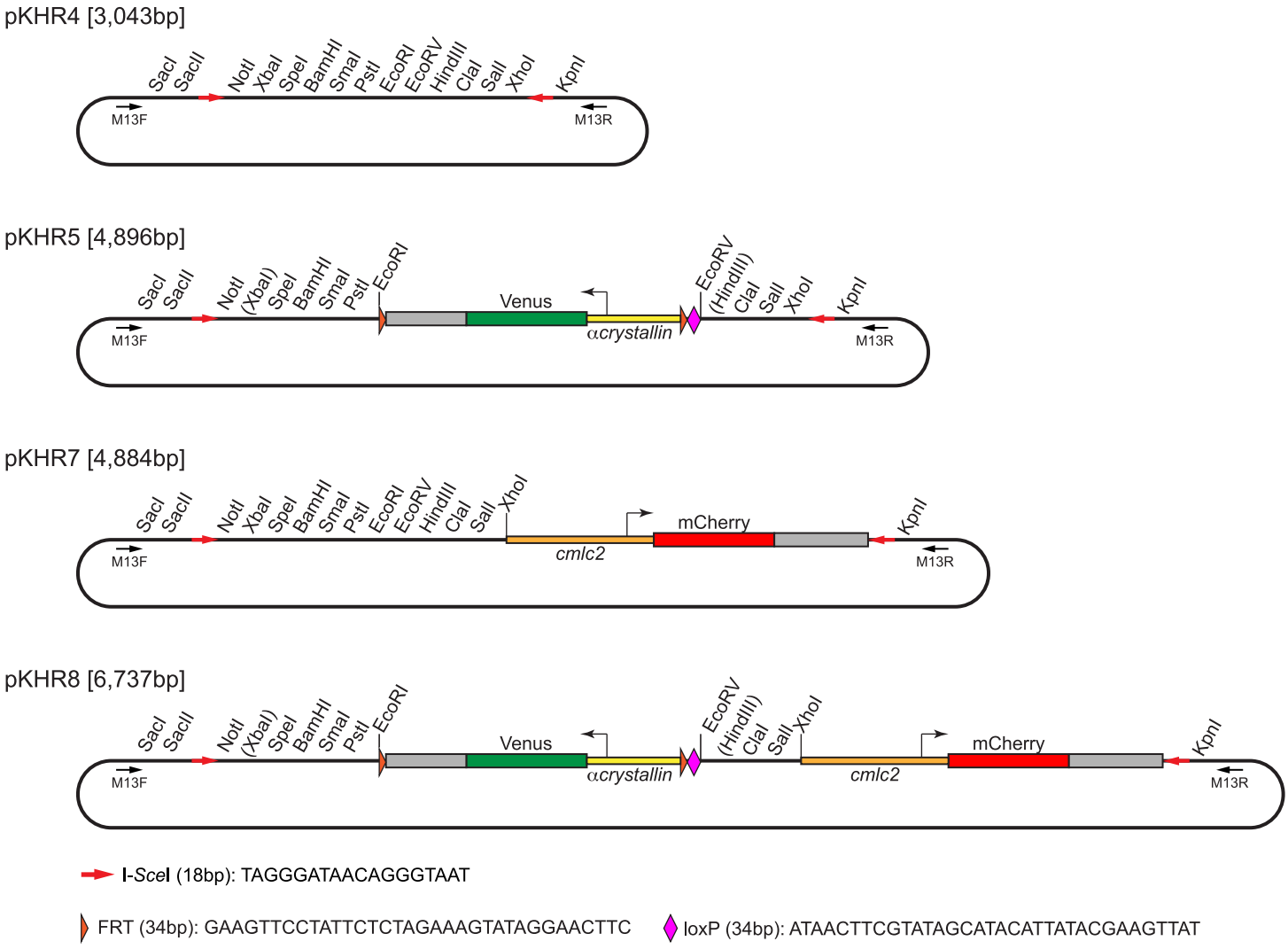


**Figure S4. Vectors useful for gene editing, Related to Experimental Procedures**
*pKHR4:* pKHR4 provides a simple multiple cloning site (mcs) flanked by inverted *I-Sce*I sites. Unique restriction sites are indicated. The backbone is an *amp*$^{R}$ plasmid built from Bluescript SK(+).

*pKHR5:* pKHR5 provides the *CV* reporter gene cassette, FRT-*CV*-FRT-loxP, inserted between the *Eco*R1 and *Eco*RV sites of pKHR4. The cassette consists of the α-crystallin promoter driving expression of the Venus protein (Hesselson et al. 2009) flanked by FRT sites and bordered on the 3' with a single loxP site. pKHR5 contains two multiple cloning sites (mcs) for introducing Left Arm and Right Arm homologous sequences. As indicated by (), the *Hind*III and *Xba*I sites are still present but they are no longer unique. Successful integration of left *and* right arm sequences leads to integration of the reporter gene. Reporter gene transcription is terminated with polyA termination sites provided from the Bovine Growth Hormone gene and SV40 late polyA site (the α-crystallin::venus-BGH-pA-SV40pA sequence was subcloned from pCM326, kindly provided by Christian Mosimann).

*pKHR7:* pKHR7 contains a simple modification of pKHR4 that allows detection of random insertion events. pKHR7 contains a *cmlc2::mCherry* (red heart) reporter gene that resides *within* the donor fragment that is produced by *I-Sce*I digestion, but *outside* any homology region. Random integration of the donor fragment leads to incorporation of the reporter gene.

*pKHR8:* pKHR8 contains a simple modification of pKHR5 that allows detection of random insertion events. pKHR8 carries the *CV* reporter gene cassette, FRT-*CV*-FRT-loxP, flanked by mcs to receive Right Arm and Left Arm homologies. pKHR8 also contains the *cmlc2::mCherry* (red heart) reporter gene that resides *within* the donor fragment produced by *I-Sce*I digestion, but *outside* any homology region. Hence Homologous Recombination events of interest should acquire the green lens reporter but *not* the red heart reporter.

Sequences for the donor vectors pKHR4, 5, 7, 8 have been deposited in Genbank (KU144822-KU144825).

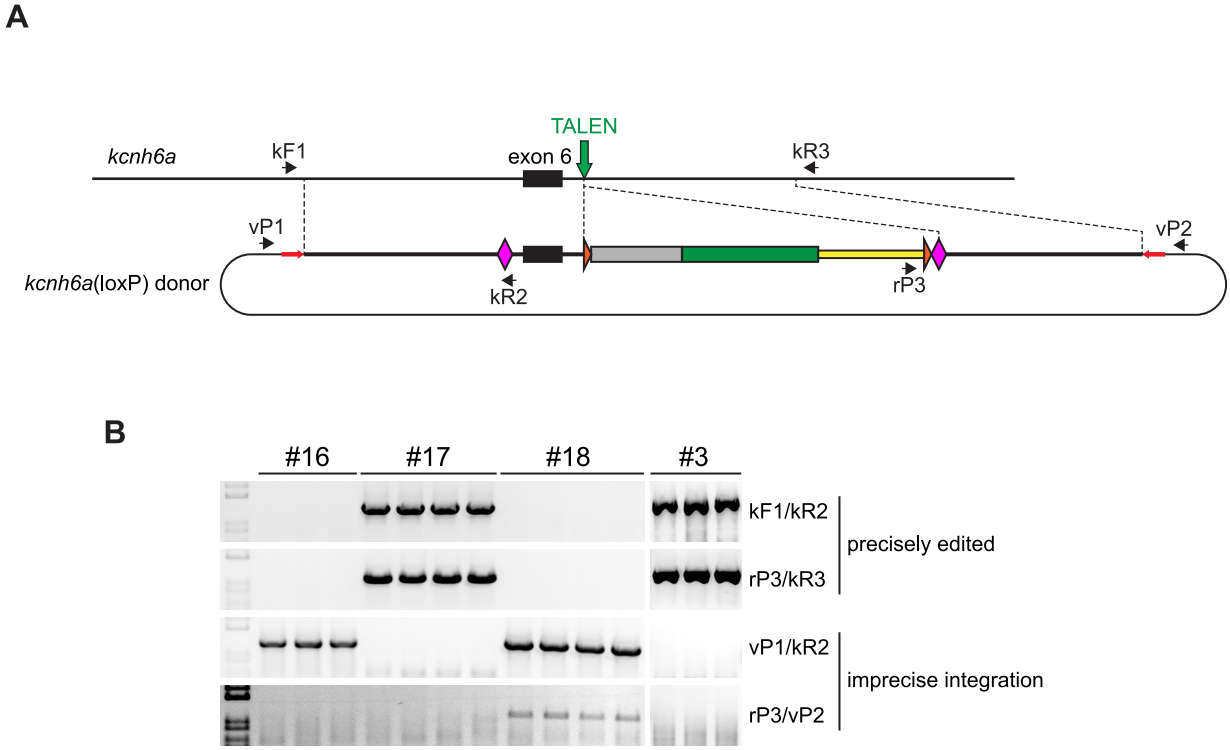


**Figure S5. Recovery of a *kcnh6a*$^{loxP}$ allele, Related for Figure 6**
(A) Strategy for detecting *kcnh6a*-specific or random location integration events involving the *kcnh6a*(loxP) donor plasmid. (B) F1 embryos expressing lens GFP from 4 different F0 founders (#3, #16, #17, #18) that transmitted a single integrant were analyzed for the presence of *kcnh6a*(loxP) donor plasmid sequences. Each lane represents a different F1 genome. The primer pairs kF1/kR2 and rP3/kR3 amplify kcnh6a host - donor junction fragments. The primer pairs vP1/kR2 and rP3/vP2 amplify donor - vector backbone junction fragments. Only two types of patterns were detected: F1 embryos from founders #17 and #3 had junction sequences indicating that donor sequences integrated precisely into the *kcnh6a* locus (precisely edited), whereas offspring of founders #16 and #18 lacked junction sequences and appeared to have donor sequences along with vector backbone sequences integrated at other locations in the genome (imprecise integration). Each separate gel panel represents a distinct portion of a gel.

**Table S1. TALEN Target Sequences and Mutagenesis Activities, Related to Experimental Procedures**

| TALEN | Target sequence (5' → 3') | Fraction targeted loci with induced mutations |
|---|---|---|
| *gol-int5*TALEN | TGGTGAGCCTCCCATCACTCCGCACTTTATTCTCTGAATACTTTGTATCTCGCA | 0.45 |
| *gol-ex2*TALEN | TACATGCTGCTGTCCGTCTCCATCGTGTGTGATGAATATTTTCTGCCA | 0.58 |
| *ntl-ex1*TALEN | TCCCGACCAGCGCCTGGATCATCTCCTTAGCGCCGTGGAGAGCGAATTTCAGAAGGGCA | 0.34 |
| *kcnh6a-int1*TALEN | TATATCTCAGAAATGCTGTAGGCTAGTCTAATATAGTAGATGGATAGCGA | 0.23 |
| *kcnh6a-int6*TALEN | TATTTCCTACAGAGCTTTCGCAGCCTATTATTTATTTATGGGGTTAATGATA | 0.86 |

Binding sequences of left and right TALEN monomers are underlined. However, note that the right TALEN monomers actually bind to the complementary strand. As a measure of the relative ability of each TALEN to induce DSBs, 50pg left and 50pg right TALEN RNAs were together injected into zygotes under standard conditions and the induction of targeted indel mutations at 2 dpf was calculated: PCR-generated amplicons covering the target site were generated from control and TALEN RNA-injected embryos and analyzed by capillary electrophoresis-based fragment analysis on the Applied Biosystems 3730 platform. AppliedBiosystems GeneMapper v3.7 software was used to determine peak size and height. The change in the fraction of amplicon product that migrated at the WT peak was used to calculate the portion of template genomes that had acquired indel mutations, essentially as in (Carrington et al. 2015).

**Table S2. Primer Sequences, Related to Experimental Procedures**

| Target | Label | Sequence (5' → 3') |
|---|---|---|
| *gol* | gF1 | GTGAGCCTCCCATGCGG |
| | gF2 | GCCGTTGTTCAGAGATTGTGTT |
| | gF3 | CAGAGCTCGTCACTGCATTTC |
| | gR1 | AAGCGCTGCATCACGTACTC |
| *ntl* | nF1 | TGCAACCAGGATGCTTTTACAG |
| | nR1 | TAGAATCGAGACCGAGGAGAGG |
| | nR2 | CATATTTCCGATCAAATAAAGCTTGAG |
| | nR3 | GGTCTGGGACTTCCTTGTGGT |
| *kcnh6a* | kF1 | CATCTCTGCTGCTATTAAACTCCTCT |
| | kF2 | GCTGCAGTCCTCTAGGAACTGATT |
| | kF3 | AATGGAAGCGTGGAGGTCAG |
| | kR1 | TTGAAACAGGGCACTCAGGTT |
| | kR2 | GGCAAATAACTTCGTATAATGTATGCT |
| | kR3 | AGGCTGTGCTTTTTGTTTTCAC |
| | kR4 | GAAGCCTTTATATTTGCCTTACTAATGT |
| reporter | rP1 | TGCATTCTAGTTGTGGTTTGTCC |
| | rP2 | GAACTTGTGGCCGTTTACGTC |
| | rP3 | CATCCTGCACTGAATGCACA |
| vector | vP1 | GTAAAACGACGGCCAGTGA |
| | vP2 | GGAAACAGCTATGACCATGAT |

**Table S3. Germ line transmission of edited alleles, Related to Figure 1**

| Editing event: Donor DNA | Founder | % F0 germ line with an edited allele |
|---|---|---|
| gol(b1→WT) | F0#15 | 2.0% (7/358) |
| | F0#17 | 3.6% (7/192) |
| | F0#35 | 1.6% (7/442) |
| | F0#56 | 23.1% (70/303) |
| | F0#60 | 9.6% (20/209) |
| | F0#62 | 10.5% (26/247) |
| | F0#65 | 1.9% (5/265) |
| | F0#66 | 0.6% (2/362) |
| gol(b1→WT; CV) | F0#07 | 1.0% (3/288) |
| | F0#12 | 8.9% (13/146) |
| | F0#23 | 2.6% (13/506) |
| ntl(V5) | F0#24 | 5.4% (8/147) |
| ntl(eGFP) | F0#26 | 2.4% (6/255) |
| kcnh6a(eGFP) | F0#12 | 11.0% (6/56) |
| | F0#20 | 6.9% (5/72) |
| kcnh6a(loxP) | F0#03 | 10% (93/929) |
| | F0#17 | 4% (9/224) |
| *average germ line clone size:* | | 6.3% |

The fraction of F1 offspring that inherited an edited allele was determined for each transmitting F0 founder reported in this study. To identify transmission of the edited $gol^{b1\_WT}$ allele, founders were mated with $gol^{b1}$ homozygotes and pigmentation of F1 embryos was scored at 2 dpf. Transmission of the reporter-expressing alleles was scored by virtue of tissue-specific green fluorescence in F1 embryos. Transmission of other alleles was detected by PCR analysis of genomic DNA prepared from fin biopsies of adult F1 animals. For each edited allele recovered, extensive genomic DNA analysis was performed to confirm that the edited allele was intact at the targeted locus and devoid of unintended alterations. All edited alleles were captured as a single amplicon that bridged host-specific sequences flanking the acquired donor DNA, and then completely sequenced. Only the edited *gol*(b1→WT) allele recovered from F0#17 contained unintended sequence rearrangements (Figure S1).

**SUPPLEMENTAL MOVIES**

**Movie S1: Normal cardiac contractions of a 2 dpf WT embryo, Related to Figure 3**

**Movie S2: Aberrant cardiac contractions of a 2 dpf *kcnh6a*$^{GFP/GFP}$, which lack ventricle contraction, Related to Figure 3**

**SUPPLEMENTAL REFERENCES**

Carrington B, Varshney GK, Burgess SM, Sood R. 2015. CRISPR-STAT: an easy and reliable PCR-based method to evaluate target-specific sgRNA activity. *Nucleic acids research* **43**: e157.