LETTER

doi:10.1038/nature11537

In vivo genome editing using a high-efficiency TALEN system

Victoria M. Bedell[1]*, Ying Wang[2]*, Jarryd M. Campbell[1]*, Tanya L. Poshusta[1], Colby G. Starker[3], Randall G. Krug II[1], Wenfang Tan[3], Sumedha G. Penheiter[1], Alvin C. Ma[1,4], Anskar Y. H. Leung[4], Scott C. Fahrenkrug[3,5], Daniel F. Carlson[3,5], Daniel F. Voytas[3], Karl J. Clark[1], Jeffrey J. Essner[2] & Stephen C. Ekker[1]

The zebrafish (*Danio rerio*) is increasingly being used to study basic vertebrate biology and human disease with a rich array of *in vivo* genetic and molecular tools. However, the inability to readily modify the genome in a targeted fashion has been a bottleneck in the field. Here we show that improvements in artificial transcription activator-like effector nucleases (TALENs) provide a powerful new approach for targeted zebrafish genome editing and functional genomic applications[1–5]. Using the GoldyTALEN modified scaffold and zebrafish delivery system, we show that this enhanced TALEN toolkit has a high efficiency in inducing locus-specific DNA breaks in somatic and germline tissues. At some loci, this efficacy approaches 100%, including biallelic conversion in somatic tissues that mimics phenotypes seen using morpholino-based targeted gene knockdowns[6]. With this updated TALEN system, we successfully used single-stranded DNA oligonucleotides to precisely modify sequences at predefined locations in the zebrafish genome through homology-directed repair, including the introduction of a custom-designed *EcoRV* site and a modified *loxP* (*mloxP*) sequence into somatic tissue *in vivo*. We further show successful germline transmission of both *EcoRV* and *mloxP* engineered chromosomes. This combined approach offers the potential to model genetic variation as well as to generate targeted conditional alleles.

Custom zinc finger nucleases (ZFNs)[7–9] and TALENs[1–5] have been used to introduce locus-specific double-stranded breaks in the zebrafish genome, generating dozens of mutant alleles[10]. Recent work has been facilitated by the relatively straightforward DNA base recognition cipher underlying TALEN technology[11,12]. However, the efficacy of previously described custom sequence-specific nucleases was limiting in some applications[1–5,7–9]. For example, standard TALENs using the pTAL scaffold[13] (Supplementary Fig. 1) targeting exon 2 of the zebrafish *ponzr1* locus[14] resulted in a measurable level of locus modification in somatic tissue (median value of 5%; Fig. 1b, c). This pTAL-*ponzr1* pair yielded 4 germline-transmitting founder animals carrying a mutation in *ponzr1* out of the 24 tested (Supplementary Fig. 3d). TALENs against a second locus (*crhr1*) using the pTAL scaffold yielded a modest rate of locus modification (<1%; Fig. 1b, c). These results are characteristic of the standard TALEN efficacy range, demonstrating room for improvement.

Multiple TALEN scaffold designs have been described[13,15,16], including those with different amino- and carboxy-terminal truncations, diverse FokI nuclease linkers, and various nuclear localization sequences. To improve *in vivo* efficacy, we tested the GoldyTALEN scaffold (Supplementary Fig. 1 and Supplementary Fig. 2) in a messenger RNA expression vector backbone (pT3TS[17]) using DNA analysis that measures the loss of a restriction enzyme recognition sequence at the TALEN cut site (Fig. 1a). Using the same recognition domains in the GoldyTALEN scaffold, there is a sixfold increase in somatic gene modification at the *ponzr1* locus (Fig. 1b, c and Supplementary Fig. 3b) over the pTAL scaffold. The germline modification rate was similarly increased when switching scaffolds, from 17% (4/24; pTAL-*ponzr1*; Supplementary Fig. 3d) to 71% (10/14; GoldyTALEN-*ponzr1*; Supplementary Fig. 3e). We also detected improved efficacy using a cell-free assay system with *in vitro*-translated TALEN protein and purified *ponzr1* PCR DNA (Fig. 1d). The GoldyTALENs against *crhr1* showed an increase in the genome modification rate, improving from <1% to 7% median cutting efficacy (Fig. 1b, c and Supplementary Fig. 3c). Sequence comparisons of pTAL and GoldyTALEN scaffolds in both loci demonstrate similar insertions or deletions (indels) at the cut site, which is diagnostic of non-homologous end joining (NHEJ) repair (Supplementary Fig. 3).

To further test the efficacy of the GoldyTALEN scaffold, we generated TALENs against three additional loci (*moesina*, also known as *msna*, *ppp1cab* and *cdh5*; Supplementary Fig. 4a). We observed efficient gene modification at each locus (5 out of 5 loci in total; Fig. 1 and Fig. 2a). In three instances, the mutagenesis efficiency ranged from 70 to 100% as demonstrated by loss of the restriction enzyme recognition sequence at the TALEN cut sites (Fig. 2a) and DNA sequence analyses (Supplementary Fig. 4b–d) of amplicons from pooled injected embryos. To determine the time course of the GoldyTALEN-induced changes, we examined restriction enzyme nuclease activity at 256-cell, 28 h post-fertilization (hpf) and 50 hpf stages. A majority of the DNA was modified by the 256-cell stage (Supplementary Fig. 5). Together, these results indicate early, efficient gene targeting in somatic tissues, including biallelic conversion in some animals. Somatic targeting efficacy using the GoldyTALEN scaffold compares favourably with previous TALEN scaffolds in zebrafish, with three out of five GoldyTALENs demonstrating as high or higher mutation frequency as any of the previously reported loci using the first generation TALEN systems[1–5].

In response to the increased efficacy of the GoldyTALENs, we investigated whether injection of TALENs could recapitulate a known morpholino[6] loss of function phenotype. We conducted a dose–response curve of the *moesina*, *ppp1cab* and *cdh5* GoldyTALEN pairs, optimizing GoldyTALEN concentration to the number of embryos with biallelic changes, and per cent dead or malformed embryos (Supplementary Fig. 6). Embryos injected with either *cdh5* GoldyTALENs (Fig. 2d) or morpholinos[18] (Fig. 2c) showed similar vascular phenotypes: pronounced cardiac oedema (Fig. 2b, top panels), loss of patent lumens in the Tg(*fli1-egfp*)^{y1} vasculature[19] (Fig. 2c, d, bottom panels), and loss of circulating Tg(*gata1:dsred*)^{sd2} red blood cells[20] (Fig. 2c, d, bottom panels, and Supplementary Movies 1–3). A similar pericardial oedema phenotype was observed in F1 offspring from F0 *cdh5* founder incrosses (data not shown), suggesting specificity of the phenotype described in F0 fish to *cdh5* loss of function. Furthermore, *cdh5* GoldyTALEN-injected embryos have little or no Cdh5 protein

[1]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905, USA. [2]Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa 50011, USA. [3]Department of Genetics, Cell Biology and Development, the Center for Genome Engineering, and the Department of Animal Science, University of Minnesota, Minneapolis, Minnesota 55455, USA. [4]Department of Medicine, LKS Faculty of Medicine, the University of Hong Kong, Pok Fu Lam Road, Hong Kong. [5]Recombinetics, Inc., 2575 University Ave. West, Suite 100, Saint Paul, Minnesota 55114, USA.
*These authors contributed equally to this work.

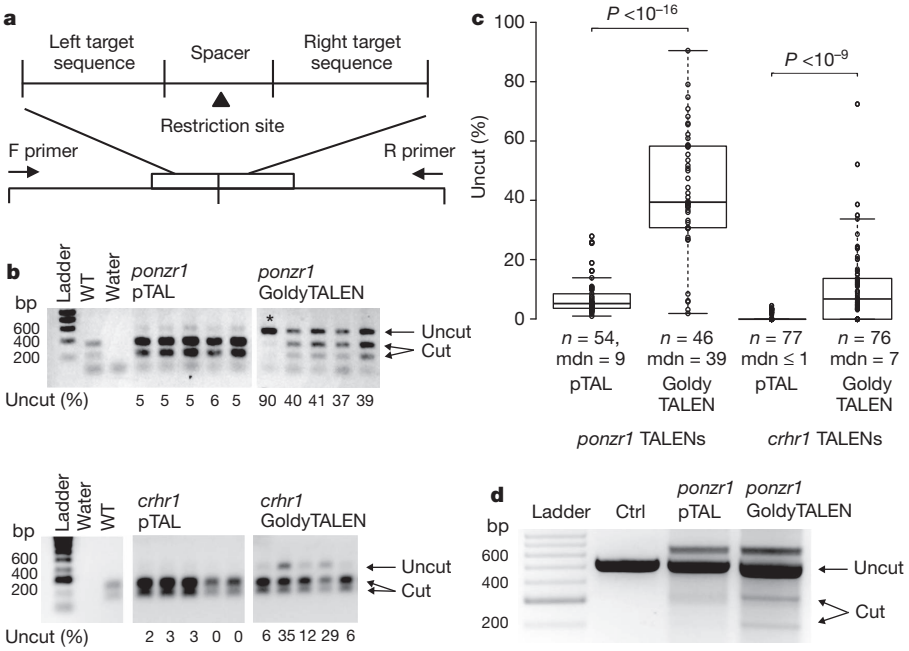

Figure 1 | Second-generation GoldyTALEN scaffold improves genome-editing efficacy. **a**, Schematic showing the layout of TALEN target sites. TALENs were targeted to flanking sequences surrounding a restriction enzyme site for easy screening through introduction of a restriction fragment length polymorphism. **b**, Relative activity of the GoldyTALEN and pTAL scaffolds at two loci, *ponzr1* and *crhr1*. Under each lane is the percent uncut DNA of a single larva, illustrating the increased activity of GoldyTALEN. WT, wild type. **c**, Whisker plots of the percent uncut DNA demonstrates TALEN cutting efficiency at two loci. *ponzr1* TALENs demonstrate a significant ($P < 10^{-16}$), sixfold increase in activity using GoldyTALEN. *crhr1* TALENs also demonstrate a significant ($P < 10^{-9}$), 15-fold increase in activity. *n*, number of embryos screened; mdn, the median percent cut. **d**, The *ponzr1* GoldyTALENs were more active in a cell-free restriction enzyme digestion assay. *ponzr1* DNA is labelled in both uncut and cut forms. Ctrl, negative control.

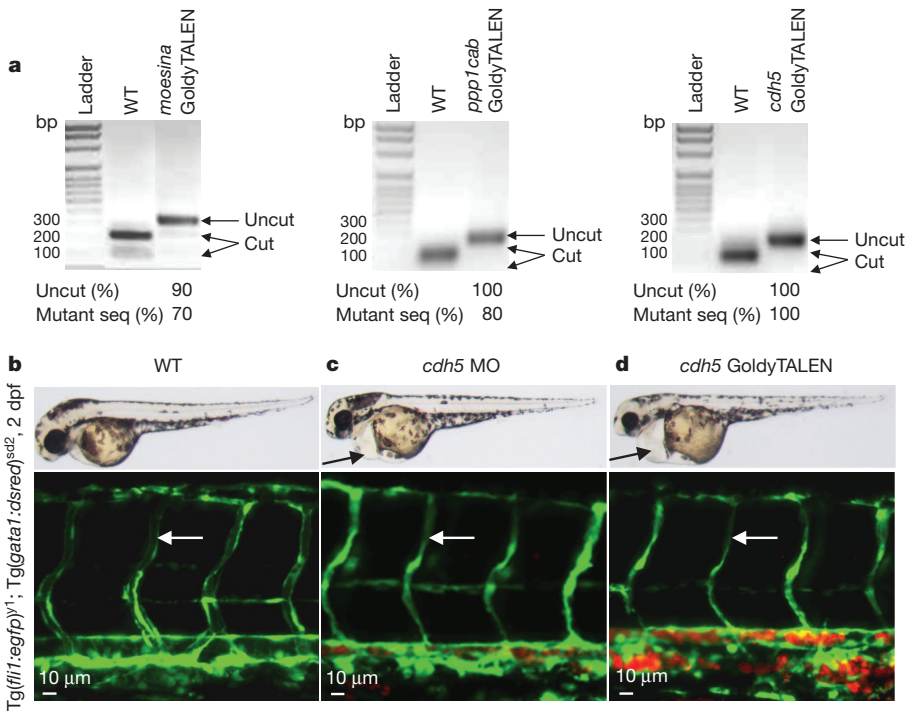

Figure 2 | Increased TALEN efficiency results in biallelic gene targeting. **a**, GoldyTALENs were designed against the *moesina*, *ppp1cab* and *cdh5* genes. All three gene targets contained a restriction enzyme site within the spacer region between the TALEN binding sites. Injection of GoldyTALEN mRNAs demonstrated a nearly complete loss of the restriction enzyme site in the amplicons of somatic tissue. Each lane is the amplification product from a group of 10 embryos. Mutant seq (%), percentage of amplicons that carry mutant sequences as determined by sequencing 10 clones (Supplementary Fig. 4). **b–d**, Injection of *cdh5* GoldyTALENs (**d**) phenocopies the morpholino-based loss-of-function phenotype (**c**). Bright field images (top panels) show pronounced cardiac oedema (arrows) in both GoldyTALEN-injected (**d**) and morpholino-injected (**c**) larvae at 2 days post fertilization (dpf). Using the Tg(*fli1-egfp*)^{y1} line, the intersomitic vessels were visualized (bottom panels) and show a loss of lumen formation (white arrow) in both the morpholino-injected (**c**) and GoldyTALEN-injected larvae (**d**). The Tg(*gata1:dsred*)^{sd2} line revealed reduced circulation in GoldyTALEN- and morpholino-injected larvae, demonstrated by the increase in red fluorescence in the confocal images (see Supplementary Movies 1–3).

(Supplementary Fig. 7). Together, these results indicate that the GoldyTALEN platform can achieve efficient biallelic targeting recapitulating known loss-of-function phenotypes. Furthermore, these data demonstrate that GoldyTALENs have the potential to be a complementary, but distinct, approach to morpholino-based somatic phenotype assessment.

The biallelic GoldyTALEN-injected fish were raised to assess germline mutation transmission. The *moesina*, *ppp1cab* or *cdh5* F0 founders were outcrossed. Ten pooled F1 embryos were screened and showed a 9 to 55% locus mutation frequency (Supplementary Fig. 8a–c). From two founder F0 outcrosses per locus, 10 individual F1 embryos were sequenced with mutant alleles identified in 20% to 100% of the F1 offspring (Supplementary Fig. 8). Furthermore, in two out of three of these loci we detected germline mosaicism, indicating several independent repair events. These data indicate that the efficient somatic TALEN targeting is effectively passed through the germline.

Recent *in vitro* work demonstrates that single-stranded DNA (ssDNA) can be an effective donor for homology-directed repair (HDR)-based genome editing at a ZFN-induced double-stranded break[21,22]. With the highly efficient genome modification success of GoldyTALENs, we hypothesized that synthetic oligonucleotides designed to span the predicted TALEN cut site could serve as a template for HDR *in vivo* (Fig. 3). Using *ponzr1* as a test locus, we introduced an EcoRV restriction site by co-injection of *ponzr1* GoldyTALENs and a ssDNA oligonucleotide (Fig. 3a). In these experiments, 42 out of 74 injected embryos had a detectable level of chromosomes containing the introduced *EcoRV* sequence with an estimated 9% ratio of converted chromosomes in these animals (Supplementary Fig. 9a). Sequence analysis indicated two precisely modified chromosome events from different larvae (Supplementary Fig. 9b) demonstrating successful somatic HDR at the *ponzr1* locus. Other events show precise addition at the 3′ end while small indels were noted at the 5′ side of the modification site (Supplementary Fig. 9b). Several homology arm lengths were tested for the highest HDR signal. In this experimental approach, an increase in homology arm length that spanned the TALEN binding site decreased the frequency of HDR events (Supplementary Table 1).

To test whether the HDR sequence modification was stably maintained in zebrafish somatic tissue, fin biopsies from 2-month-old fish were assayed for addition of the *EcoRV* sequence at the *ponzr1* locus. Out of 186 fish, 8 showed a visible incorporation of *EcoRV* (Supplementary Fig. 9c). To determine whether a lack of somatic *EcoRV* incorporation also indicated a lack of germline incorporation, 13 randomly selected fish with *EcoRV*-negative fin biopsies were outcrossed. The offspring from all 13 adults were negative for *EcoRV* incorporation at the *ponzr1* locus (clutch sizes ranged from 16 to 96 embryos). Therefore, fin-biopsy-positive fish were prioritized for determining germline transmission. Outcross embryos from three out of four fin-tissue-positive fish yielded clutches with introduction of the *EcoRV* site at the *ponzr1* locus (Fig. 3b). Two out of three of these germline fish demonstrated precise *EcoRV* addition (Fig. 3c).

We next asked whether TALEN/oligonucleotide co-injection could introduce larger sequences such as a *loxP* site, an essential step in making Cre-dependent conditional genetic alleles. We used TALENs against an intron in the *crhr2* gene and a ssDNA oligonucleotide were used to add a modified $loxP^{JTZ17}$ (*mloxP*)[23] site at this location (Fig. 4a). PCR analysis demonstrates somatic introduction of the *mloxP* sequence at the *crhr2* TALEN cut site (Supplementary Fig. 10a). Sequence characterization confirmed integration of the *mloxP* site in three assayed somatic chromosomes (Supplementary Fig. 10b). A similar method was used to introduce an *mloxP* sequence at the *ponzr1* locus (Supplementary Fig. 11a). Sequencing confirmed precise somatic addition at this locus (Supplementary Fig. 11b, c).

Maintenance of somatic *mloxP*-modified *crhr2* chromosomes by fin biopsy was used to identify germline transmission of the *mloxP* sequence. Positive chromosomes were detected by quantitative PCR in 20 out of 53 animals (Supplementary Fig. 10a). Embryos were

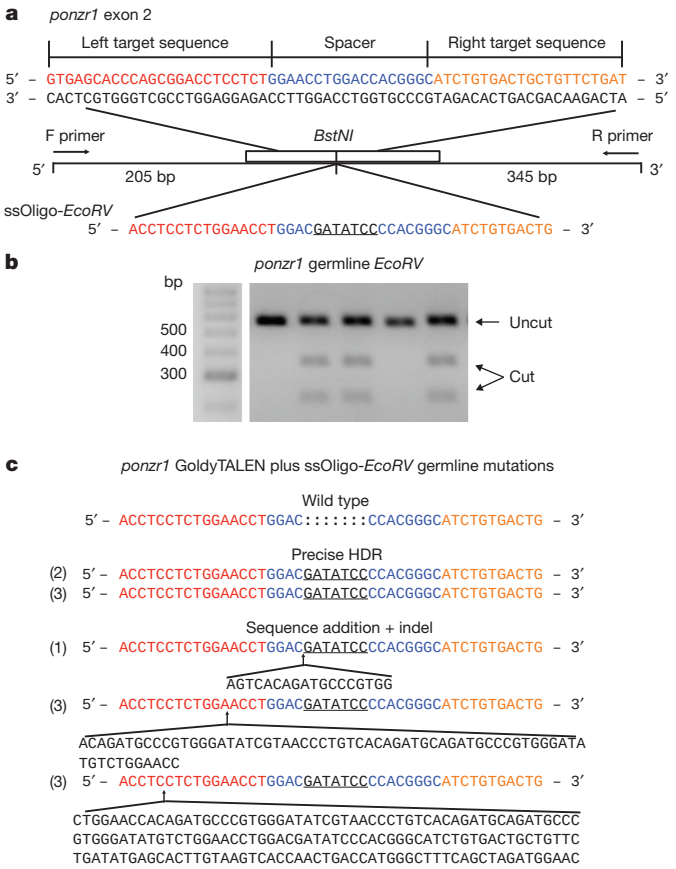

Figure 3 | Targeted genome editing using GoldyTALENs. a, A schematic of the *ponzr1* locus with the ssDNA sequence used to introduce a targeted exogenous *EcoRV* sequence (underlined, note the extra C added to make the sequence mutagenic) into the genome *in vivo*. The left and right TALEN binding sites are shown in red and orange, respectively, and the spacer region is in blue. **b**, A representative gel from founder fish no. 2 demonstrating germline transmission of the HDR-based *EcoRV* sequence. Three out of four fin-tissue-positive fish demonstrated germline transmission of the *EcoRV* sequence. **c**, Sequence analysis of the three germline-transmitting lines. The first fish transmitting HDR-based genome changes through the germline (1) yielded 7 out of 96 embryos with an incorporated *EcoRV* site. The genomes of all 7 embryos showed the same modified sequence. The second founder fish (2) yielded 7 out of 46 embryos with *EcoRV* incorporation. All 7 embryos showed precise HDR-based addition of the *EcoRV* sequence. The third fish with germline transmission (3) yielded 5 out of 18 embryos with an incorporated *EcoRV* site, and showed a mosaic germline as demonstrated by offspring with three different modified sequences. One embryo included precise HDR-based *EcoRV* addition. The other 4 embryos contained sequence insertions on the 5′ end with two embryos each harbouring the specific sequences changes.

obtained from 16 of the somatic-positive fish as well as 42 fish that had not been pre-screened by PCR. Both groups transmitted HDR events through the germline (Fig. 4b). However, no significant enrichment for probable germline transmitting animals was noted, perhaps owing to the less stringent PCR assay than that used for *ponzr1*. In total, 6 out of 58 injected animals transmitted *mloxP*-modified chromosomes through the germline at the *crhr2* locus (Fig. 4b). Sequence confirmation of three of these fish demonstrated a precise HDR event as well as other, non-precise events (Fig. 4c).

Here, we focused on local genome-editing changes induced by TALENs, especially those induced by HDR. However, more complete analyses will be required to assess any off-target effects of TALENs or ssDNA-based HDR. Whole-genome sequencing on germline-transmitting fish from different parental lines would be particularly instructive. Should this analysis demonstrate off-target mutations, TALENs using obligate heterodimer-based nuclease fusions have

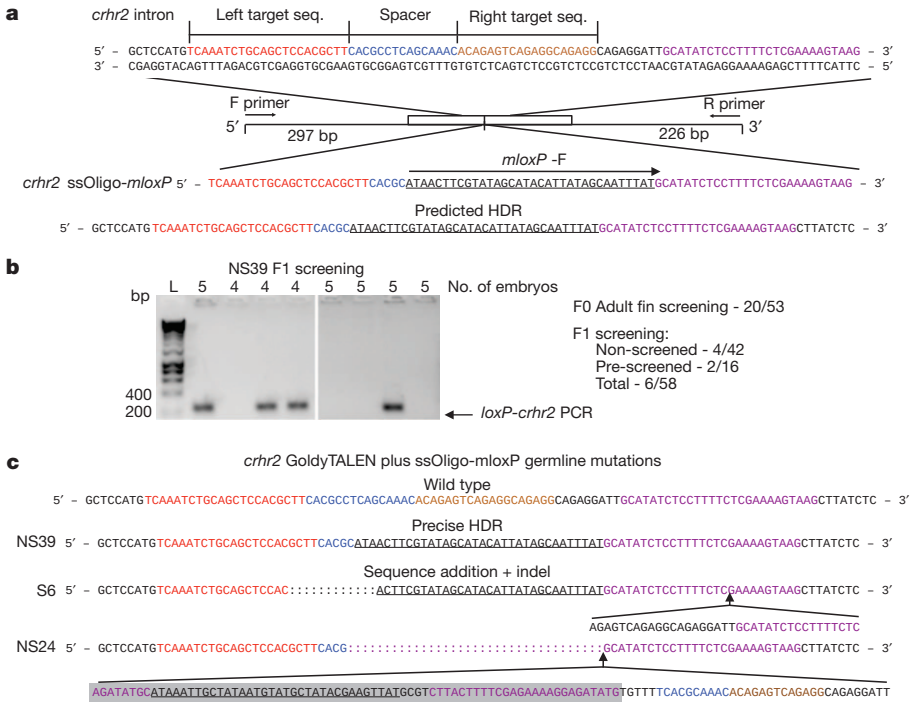

Figure 4 | Germline *mloxP* integration into the *crhr2* locus. a, A diagram of the TALEN target sites with the *mloxP* ssDNA oligonucleotide. The left and right TALEN target sequences are red and orange, respectively, the spacer region is blue, and the right homology arm of the oligonucleotide is in purple. The *mloxP* sequence is underlined. **b**, Germline screening of the *crhr2* locus. 53 adult fish were pre-screened via fin biopsy. Of those pre-screened, 20 demonstrated mloxP maintenance. A total of 16 F0 fish were outcrossed with 2 showing germline transmission. A total of 42 unscreened F0 fish were outcrossed and 4 demonstrated germline transmission. **c**, Sequence confirmation of three *mloxP* germline fish. One fish demonstrated precise germline HDR whereas two showed indels. In NS24, the reverse complement of the *mloxP* was noted (shaded in grey).

recently been reported as an alternative approach[3,5,24]. Using obligate heterodimers in the GoldyTALEN scaffold is one future method for potentially optimizing HDR-directed gene-editing specificity.

To our knowledge, these results represent the first description of successful HDR in zebrafish and the first demonstration of HDR using ssDNA as a donor template *in vivo*. This approach complements the error-prone NHEJ toolkit for model organisms (Fig. 5). The use of ssDNA facilitates an array of genome changes, including the introduction of single-nucleotide polymorphisms for vertebrate genetic applications. The asymmetry in precise editing suggests an additional mechanism for genome editing that incorporates both HDR and NHEJ (Fig. 5). For example, the donor ssDNA may serve as a primer for new strand synthesis at the TALEN break. Extension from the 3′ end of the oligonucleotide would create long regions of homology for recombination. However, the 5′ end of the oligonucleotide limits the extent of strand invasion and a limited opportunity for HDR. This leads to 5′ end resolution by either HDR or NHEJ. For applications where new sequences are introduced into non-coding genomic regions, such as the introduction of *loxP* sites into intronic sequences, either event will probably be of high utility.

Using the zebrafish, we report an updated TALEN system for use in genome modification and functional genomic applications. The high efficacy enables new approaches, including somatic gene targeting for reverse genetics applications. Furthermore, we show that synthetic ssDNA oligonucleotides can be used with this TALEN system for genome editing, including the precise introduction of exogenous DNA sequence at a specific locus. Although deployed here in zebrafish, this approach has the potential to be effective for *in vivo* applications in a wide array of model organisms.

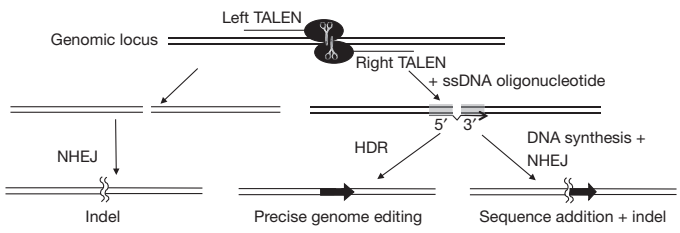

Figure 5 | *In vivo* TALEN-induced genome editing outcomes. TALENs efficiently create double-stranded breaks in chromosomal DNA and catalyse three major outcome classes. First, error-prone NHEJ produces an indel in and near the spacer region of the TALEN binding site. If a complementary ssDNA oligonucleotide is also added, two different outcomes are noted. First, HDR precisely uses the exogenous sequence information in the ssDNA to add sequence at the cut site. Alternatively, ssDNA acts as a primer for 3′ integration of the oligonucleotide but the 5′ end undergoes error-prone NHEJ[22].

METHODS SUMMARY

TALENs were assembled via the GoldenGate method[13]. For ease of analysis, TALEN recognition sequences flanked a unique restriction site within the targeted gene. TALEN repeat variable di-residues (RVDs) were cloned into a pT3TS[17]-driven TALEN scaffold, and mRNA was injected into single-cell zebrafish embryos. The injected larvae were either molecularly tested or raised for germline mutation analysis. Somatic and germline TALEN-induced mutations were evaluated via PCR and restriction fragment length polymorphisms. To induce HDR events, singe-stranded DNA oligonucleotides with either an *EcoRV* or *mloxP* site were designed with short homology arms around a TALEN target site and were injected into one-cell zebrafish embryos. PCR analysis of modified loci was used to detect the resulting somatic and germline HDR events.

Full Methods and any associated references are available in the online version of the paper.

Received 3 May; accepted 6 September 2012.
Published online 23 September 2012.

1. Huang, P. *et al.* Heritable gene targeting in zebrafish using customized TALENs. *Nature Biotechnol.* **29,** 699–700 (2011).
2. Sander, J. D. *et al.* Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nature Biotechnol.* **29,** 697–698 (2011).

3. Cade, L. *et al.* Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. *Nucleic Acids Res.* **40,** 8001–8010 (2012).
4. Moore, F. E. *et al.* Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). *PLoS ONE* **7,** e37877 (2012).
5. 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).
6. Nasevicius, A. & Ekker, S. C. Effective targeted gene 'knockdown' in zebrafish. *Nature Genet.* **26,** 216–220 (2000).
7. Doyon, Y. *et al.* Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nature Biotechnol.* **26,** 702–708 (2008).
8. Meng, X., Noyes, M. B., Zhu, L. J., Lawson, N. D. & Wolfe, S. A. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nature Biotechnol.* **26,** 695–701 (2008).
9. Foley, J. E. *et al.* Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). *PLoS ONE* **4,** e4348 (2009).
10. Lawson, N. D. & Wolfe, S. A. Forward and reverse genetic approaches for the analysis of vertebrate development in the zebrafish. *Dev. Cell* **21,** 48–64 (2011).
11. Moscou, M. J. & Bogdanove, A. J. A simple cipher governs DNA recognition by TAL effectors. *Science* **326,** 1501 (2009).
12. Boch, J. *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326,** 1509–1512 (2009).
13. Cermak, T. *et al.* Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* **39,** e82 (2011).
14. Bedell, V. M. *et al.* The lineage-specific gene *ponzr1* is essential for zebrafish pronephric and pharyngeal arch development. *Development* **139,** 793–804 (2012).
15. Miller, J. C. *et al.* A TALE nuclease architecture for efficient genome editing. *Nature Biotechnol.* **29,** 143–148 (2011).
16. Mussolino, C. *et al.* A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* **39,** 9283–9293 (2011).
17. Hyatt, T. M. & Ekker, S. C. Vectors and techniques for ectopic gene expression in zebrafish. *Methods Cell Biol.* **59,** 117–126 (1998).
18. Wang, Y. *et al.* Moesin1 and Ve-cadherin are required in endothelial cells during *in vivo* tubulogenesis. *Development* **137,** 3119–3128 (2010).
19. Lawson, N. D. & Weinstein, B. M. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **248,** 307–318 (2002).
20. Traver, D. *et al.* Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. *Nature Immunol.* **4,** 1238–1246 (2003).
21. Chen, F. *et al.* High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nature Methods* **8,** 753–755 (2011).
22. Radecke, S., Radecke, F., Cathomen, T. & Schwarz, K. Zinc-finger nuclease-induced gene repair with oligodeoxynucleotides: wanted and unwanted target locus modifications. *Mol. Ther.* **18,** 743–753 (2010).
23. Thomson, J. G., Rucker, E. B. III & Piedrahita, J. A. Mutational analysis of *loxP* sites for efficient Cre-mediated insertion into genomic DNA. *Genesis* **36,** 162–167 (2003).
24. Doyon, Y. *et al.* Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nature Methods* **8,** 74–79 (2011).

Supplementary Information is available in the online version of the paper.

Acknowledgements State of Minnesota grant H001274506 to S.C.E. and D.F.V.; NIH GM63904 to S.C.E.; NIH grant P30DK084567 to S.C.E. and K.J.C.; NIH grant DK083219 to V.M.B.; Mayo Foundation; NIH DA032194 to K.J.C.; NIH grant R41HL108440 to D.F.C. and S.C.F.; NIH grant GM088424 to J.J.E.; NSF grant DBI0923827 to D.F.V.; General Research Fund (HKU771611, HKU771110, HKU769809M) from the Research Grant Council, The University of Hong Kong and the Tang King Yin Research Fund to A.C.M. and A.Y.H.L. We thank G. Davis for discussion on ssDNA use with custom restriction enzymes, H. J. Fadel for *in vitro* RNA synthesis, and S. Westcot for comments on this manuscript. We thank G. Moulder for help in DNA analyses and members of the Mayo Clinic Zebrafish Core Facility for excellent animal care.

Author Contributions W.T. designed and constructed full-length pT3TS-Tal vectors targeting *Danio rerio crhr1* and *crhr2* against exon sequences provided by K.J.C.; D.F.C. and S.C.F. designed and produced the pT3TS-Tal and GoldyTALEN cloning vectors. D.F.C. synthesized *crhr1* and *crhr2* TALEN mRNA from the pT3TS-Tal vectors. C.G.S. designed and assembled *ponzr1* TALENs and transferred TAL repeats from original *crhr1* and *crhr2* TALEN vectors into GoldyTALEN. T.L.P. and K.J.C. did initial *crhr1* TALEN microinjections. T.L.P. performed initial characterization of *crhr1* mutagenesis efficiency by PCR and restriction fragment length polymorphism analysis. T.L.P. and K.J.C. microinjected *crhr2* TALEN and *loxP* oligonucleotides. T.L.P. performed initial characterization by PCR demonstrating *loxP* integration. R.G.K. fully characterized efficiency and sequence of somatic *loxP* insertions in the *crhr2* locus. K.J.C. screened adult fin clips for *mloxP* integrations into *crhr2*. T.L.P. screened F1 offspring for *mloxP* integrations into *crhr2* and together with J.M.C. cloned and sequenced integration events. K.J.C. designed experiments associated for *crhr1* and *crhr2* modification. K.J.C. selected *loxP* mutant JTZ17 for integration. A.C.M. developed initial zebrafish genetic testing, TALEN cell-free assays, and ssDNA HDR protocols. S.G.P. conducted the cell-free TALEN endonuclease assay. A.Y.H.L. contributed to the design of initial TALEN and ssDNA HDR experiments. Y.W. and J.J.E. conducted biallelic conversion TALEN experiments in somatic and germline tests. D.F.V. and S.C.E. initiated the strategy to use custom restriction enzymes for genome editing in zebrafish. S.C.E. developed the plan for HDR targeting using ssDNAs, conducted overall project design and data analysis, and wrote the initial manuscript text. All authors contributed to manuscript composition. V.M.B. and J.M.C. conducted *ponzr1* and *crhr1* TALEN scaffold comparison experiments. V.M.B. and K.J.C. designed ssDNA oligonucleotides for HDR experiments. V.M.B. and J.M.C. injected and screened the *EcoRV* and *mloxP* HDR experiments at the *ponzr1* locus. J.M.C. ran quantitative data assessments and statistical analyses. V.M.B. and J.M.C. made first drafts and legends of Figs 1, 3 and 5. V.M.B. and J.M.C. conducted fin biopsy analyses of the *ponzr1* locus. V.M.B. completed the analysis of *ponzr1* germline transmission with assistance from J.M.C.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.C.E. (ekker.stephen@mayo.edu).

METHODS

TALEN design. The software developed by the Bogdanove laboratory (https://boglab.plp.iastate.edu/node/add/talen) was initially used to find candidate binding sites as described[13]. Three criteria were used for TALEN design. First, TALEN binding sites were selected that ranged from 15–25 bases in length. Second, the spacer length was initially selected to be 14 to 18 base pairs (bp), but subsequent GoldyTALEN designs were restricted to 15–16 bp. Additionally, when possible TALEN cut sequences were selected around a restriction enzyme centrally located within the spacer. To simplify the TALEN design process, a free, open access software (Mojo Hand) was created and made available online (http://www.talendesign.org). Mojo Hand downloads sequence from NCBI and uses an exhaustive database of commercially available restriction enzymes to identify TALEN binding sites with a restriction enzyme site in the spacer region to simplify downstream analysis (personal communication, Neff *et al.*). Mojo Hand also features a BLAST interface that will search genomes for potential second site effects.

TALEN binding sites and spacer regions. The *ponzr1* TALEN recognition sequences are: left TALEN 5′-GTGAGCACCCAGCGGACCTCCTCT-3′ and right TALEN 5′-ATCAGAACAACAGTCAGAGAT-3′. Between the two binding sites is an 18-bp spacer with a *BstNI* sequence (GGAACCTGGACCACGGGC, *BstNI* underlined). The *crhr1* TALEN recognition sequences are: left TALEN 5′-TGCAACACTGAGCTCTGTAAACCT-3′ and right TALEN 5′-CTGCTGCCGACTGGACCCTGAGGT-3′. Between the two binding sites is a 15-bp spacer with a *BstUI* site (GTCCGCGTGTGGCGA, *BstUI* underlined). The *moesina* TALEN recognition sequences are: left TALEN 5′-ACCCAGAAGACGTTT-3′ and right TALEN 5′-CTTTGAGTGGCCTCCT-3′. Between the two binding sites is a 15-bp spacer with an *XmnI* site (CTGAGGAACTGATTC, *XmnI* underlined). The *ppp1cab* TALEN recognition sequences are: left TALEN 5′-CCACCAGAGAGTAACT-3′ and right TALEN 5′-GCCTCTGTCAACATAGT-3′. Between the two binding sites is a 15-bp spacer with a *BsII* site (ACCTATTTCTGGGAG, *BsII* underlined). The *cdh5* TALEN recognition sequences are: left TALEN 5′-CTCCTCAACATACATACT-3′ and right TALEN 5′-ACAAATGATTCATCTT-3′. Between the two binding sites is a 16-bp spacer with a *HincII* site (GGAGAGTTAGTTGACA, *HincII* underlined). The *crhr2* binding sites are: left TALEN 5′-GTCAAATCTGCAGCTCCACGCTT-3′ and right TALEN 5′-CCTCTGCCTCTGACTCTGT-3′. Between the two binding sites is a 15-bp spacer (CACGCCTCAGCAAAC).

TALEN constructs. TALEN assembly of the RVD-containing repeats was conducted using the Golden Gate approach[13]. Once assembled, the RVDs were cloned into a pT3TS destination vector with the appropriate TALEN backbone to generate mRNA expression plasmids—pT3TS-TAL (pTAL) and pT3TS-GoldyTALEN (GoldyTALEN). *In vitro* transcription of TALEN mRNA was conducted by linearizing the expression plasmids with SacI endonuclease at 37 °C for 2–3 h, transcribing the linearized DNA (T3 mMessage Machine kit, Ambion) and purifying the mRNA by phenol/chloroform extraction (T3 mMessage Machine kit user manual protocol) for injection.

TALEN mutation screening. One-cell embryos were microinjected with 50–400 pg of TALEN mRNA. The dose of each pair of TALENs injected was empirically determined, with up to a threefold difference noted between different TALEN pairs. In each case, conditions were used where over 50% embryos survived post-injection. Genomic DNA for Figs 1, 3 and 4 were collected at 2–4 days post-fertilization from 24–32 individual larvae by incubating in 50 mM NaOH at 95 °C, followed by cooling to 4 °C and adding 1/10 volume 1 M Tris-HCL pH 8.0 (ref. 25). Genomic DNA for Fig. 2 was isolated from groups of 10 larval zebrafish using DNeasy Blood and Tissue kit (Qiagen). Genotyping was conducted using PCR followed by restriction enzyme digest. For *ponzr1*, the primers were 5′-GTTCACACAAAATGTCTCTCAAGTCTCTAAATC-3′ and 5′-AGTGGCCAGTGAGTGTATGTTACCT-3′. For *crhr1* the primers were 5′-CGTGAAAGAGACAGCGAAGGGATTG-3′ and 5′-AGAAACTACCATTGTCACACTGAGCGAAG-3′. The primers for *moesina* were 5′-GTTACGGCTCAAGACGTC-3′ and 5′-CAGGATGCCCTCTTTAAC-3′. The primers for *ppp1cab* were 5′-GATGTTCATGGTCAGTAC-3′ and 5′-TGATTGAGGCACATTCATGG-3′. The primers for *cdh5* were 5′-TTGTTGTCCTTGCAAAGCTG-3′ and 5′-TCTAGAGGATTCGCTGAT-3′. The primers for *crhr2* were 5′-CCCTGATTGTGGAACTTTTCAGAACGTA-3′ and 5′-TGGTTTGGAATTAGTGCAGCATGAGTA-3′. Mutations were assessed by loss of restriction enzyme digestion. To sequence-verify mutations, the gel-purified, uncut PCR products were cloned into the TOPO TA Cloning Kit (Invitrogen).

Analysis of *cdh5*. A *cdh5* morpholino[18] was injected at the 1–4 cell stage into Tg(*fli1:efgp*)^{y1} embryos[19]. The vascular phenotype of the morpholino and the GoldyTALEN-injected embryos were assessed using a confocal microscope. Antibody staining using the anti-Cdh5 antibody[26] was performed as described[18].

Genome editing. For the *ponzr1* locus, a ssDNA oligonucleotide was designed to target the spacer sequence between the TALEN cut sites. The oligonucleotide extends to half the length of the TALEN recognition site. An *EcoRV* site (5′-GATATC-3′) or a modified *loxP* (*mloxP*) site (5′-ATAACTTCGTATAGCATACATTATAGCAATTTAT-3′) was introduced near the centre of the oligonucleotide resulting in a 20-base homology arm on the 5′ end and an 18-base homology arm on the 3′ end. For the *crhr2* locus, the *crhr2* mloxP oligonucleotide (5′-TCAAATCTGCAGCTCCACGCTTCACGCATAACTTCGTATAGCATACATTATAGCAATTTATGCATATCTCCTTTTCTCGAAAAGTAAG-3′) was designed to replace the 3′ TALEN binding site with an *mloxP* site while providing 27 bases of homology at both 5′ and 3′ end. The oligonucleotides were ordered from Integrated DNA Technologies (IDT) and purified using the Nucleotide Removal Kit (Qiagen).

One-cell embryos were microinjected with both the GoldyTALEN mRNA and ssDNA donor. The ssDNA oligonucleotide dose was varied to improve the rate of HDR without increasing toxicity beyond 50% embryonic death post-injection. For the *ponzr1* locus, 50–75 pg of *ponzr1* GoldyTALEN mRNA and 50–75 pg of the ssDNA donor. For the *crhr2* locus, 50 pg of *crhr2* GoldyTALEN mRNA was injected with either 25 pg or 50 pg of *crhr2 mloxP* oligonucleotide. Genomic DNA was isolated as described above. If the embryos were injected with the *EcoRV* oligonucleotide, PCR was conducted using the same primers as listed above and the product was digested using EcoRV. The full-length amplicon from *EcoRV*-positive larvae was cloned into a TOPO TA Cloning Kit (Invitrogen). Colony PCR was used to identify plasmids with *EcoRV*-modified inserts. Those plasmids were subsequently sequenced to confirm *EcoRV* integration and determine details of sequence changes due to HDR. If the embryos were injected with the *mloxP* oligonucleotide, the genomic DNA was amplified using the same forward primer as listed above and a *mloxP* reverse primer, 5′-ATAAATTGCTATAATGTATGCTATACGAAGT-3′, or the same reverse primer as listed above and a *mloxP* forward primer, 5′-ACTTCGTATAGCATACATTATAGCAATTTAT-3′. For sequence analysis, the complete amplicon was produced using the gene-specific primers listed above and cloned (TOPO TA Cloning Kit, Invitrogen). Colony PCR was used to find *mloxP*-positive plasmids. The positive plasmids were sequenced for confirmation of *mloxP* integration.

Injected fish from the same batch of somatically screened embryos were raised. When the fish were at least two months old, fin tissue was obtained using standard protocols pre-approved by Institutional Animal Care and Use Committee guidelines. The fish were anesthetized using Tricaine (approximately 200 $\mu g\,ml^{-1}$). The tail fins were trimmed with a fresh razor blade for each fish to prevent contamination. The most caudal 2–3 mm of fin was biopsied and placed on ice until all fin biopsies were collected. 150 µl of 50 mM NaOH was added to the fin clips before DNA isolation (above). Those fish that maintained somatic modifications were outcrossed to wild-type fish and the embryos were screened for germline mutations. Somatic mutations were determined by RFLP analysis for *EcoRV* integration into *ponzr1*. Quantitative PCR of *mloxP* integrations into the *crhr2* locus were compared to a reference gene, *RPS6Kb1*. Twenty of 53 fish included >0.2% of their DNA containing *mloxP* integrations into *crhr2* (CT of ≤ 10) and were prioritized for screening. For *mloxP* integration into *crhr2*, 42 fish that were not screened by quantitative PCR were also tested for germline transmission and no appreciable difference in germline transmission between these two methods was noted.

The PCR product for germline HDR events were cloned and sequenced. In one clone that contained a sequence insertion along with integration of the *EcoRV* site, the sequencing was more difficult, presumably because the insertion tended to form a hairpin and disrupted the sequencing reaction. To obtain the full sequence, the PCR product was digested with EcoRV and each half sequenced separately. Similar cloning difficulties were observed in some *crhr2* lineages, but not for precise HDR or limited sequence addition.

The sequence addition process using ssDNA oligonucleotides is inherently less efficient than the relatively simpler NHEJ events seen in the GoldyTALEN-alone injected embryos. Therefore, to identify a precise HDR event, more fish will need to be raised and screened. Fin clipping the fish for maintenance of the somatic insertion may be a good indicator of germline transmission. Continued investigation into the mechanism of HDR incorporation in zebrafish will likely increase the efficiency of this technique.

Zebrafish work. The zebrafish work was conducted under full animal care and use guidelines with prior approval by the local institutional animal care committee's approval. *Danio rerio* transgenic lines were described previously: Tg(*fli1:efgp*)^{y1} vasculature[19] and Tg(*gata1:dsred*)^{sd2} red blood cells[20].

Data analysis and statistics. ImageJ was used to quantify the percent GoldyTALEN-modified chromosomes by measuring the intensity of bands post-digestion. For each gel, the background was subtracted and each lane isolated to generate individual intensity plot profiles. A straight line was drawn across the bottom of each plot to eliminate inconsistencies caused by baseline skew. The intensity measurement for each band was added together to determine total intensity. To calculate percent cutting, the intensity of the top band was divided

by the total intensity. A student's *t*-test was used to compare TALEN scaffold cutting efficiencies. To measure the differences between pTAL and GoldyTALEN at two different loci, several whisker plots were constructed (Fig. 1c). The interquartile range (IQR; Q3–Q1) is shown as a box, with the median value (Q2) being the horizontal line within the box. The upper and lower whiskers are the highest and lowest data point within 1.5 times the IQR added or subtracted from Q3 or Q1, respectively.

A similar approach was used to calculate the percent of HDR-converted chromosomes. The intensity of the digested products were added together and divided by the total intensity. The percent of embryos with an HDR signal was determined by dividing the number of embryos with signal by the total number of screened embryos.

Cell-free TALEN restriction endonuclease assay. *In vitro* translation of 2 μg of each TALEN mRNA was conducted using the TNT Quick Coupled Transcription and Translation System (Promega). 5 μg of the *ponzr1* PCR product was included in the assay mix during *in vitro* translation of different TALEN combinations, allowing the translation and *in vitro* nuclease digestion to occur simultaneously. The highest signal was obtained when translation and digestion steps were conducted simultaneously presumably because the TALEN protein is unstable using these *in vitro* conditions. Translation was conducted for 2 h at 30 °C. To further facilitate TALEN *in vitro* nuclease activity, the assay mix was diluted five fold in *in vitro* digestion buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 5% glycerol and 0.5 mg ml^{-1} BSA)[27]. The assay mix was incubated at 30 °C for 4 h. The digested DNA was purified using a PCR Purification kit (Qiagen), concentrated via ethanol precipitation, and separated on a 2% agarose gel. No TALEN mRNA was added to the negative control.

25. Meeker, N. D., Hutchinson, S. A., Ho, L. & Trede, N. S. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *Biotechniques* **43,** 610–614 (2007).
26. Blum, Y. *et al.* Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev. Biol.* **316,** 312–322 (2008).
27. Mahfouz, M. M. *et al.* De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. *Proc. Natl Acad. Sci. USA* **108,** 2623–2628 (2011).