Published in final edited form as:

Dev Dyn. 2011 September ; 240(9): 2101–2107. doi:10.1002/dvdy.22699.

ΦC31 integrase mediates efficient cassette exchange in the zebrafish germline

Gui Hu¹, Mary G. Goll^{2,‡}, and Shannon Fisher^{1,*}

¹University of Pennsylvania, Department of Cell and Developmental Biology, Philadelphia, PA 19104

²Carnegie Institution for Science, Department of Embryology, Baltimore, MD 21218

Abstract

Site specific recombinases (SSRs) are powerful tools for genome manipulation, used in diverse organisms including *Drosophila melanogaster*, mouse, *Arabidopsis*, zebrafish, and human cultured cells. The integrase from the bacteriophage ΦC31 belongs to the large serine family of integrases, and in contrast to other widely used SSRs such as Cre and Flp, recombination is directional and therefore irreversible. We have developed a vector system for recombinase mediated cassette exchange (RMCE) in the zebrafish, allowing swapping of the coding sequence in an integrated transgene. Utilizing codon-optimized ΦC31 integrase RNA bearing the 3'UTR from the *nanos1* gene, we replaced the *egfp* coding sequence of an integrated reporter transgene with mCherry coding sequence. Recombination was achieved at high efficiency in both somatic cells and in the germline. We demonstrate an effective approach to RMCE, increasing the repertoire of tools available to manipulate the zebrafish genome.

Keywords

ΦC31 integrase; recombinase-mediated cassette exchange; zebrafish

Introduction

Multiple site-specific recombinases (SSRs) have been identified from bacteriophage and plasmids, and adapted as tools for genome manipulations in eukaryotes. These enzymes catalyze recombination events, typically the integration of the phage or plasmid into the host genome, between specific short sequences. The most widely used are Cre, from the P1 bacteriophage, and FLP, from the *S. cerevisiae* 2μ plasmid; both are members of the tyrosine family of recombinases and have similar biochemical properties and activities. More recently, the integrase from the bacteriophage ΦC31 has been adapted for use in a wide number of organisms, and has proven especially useful in *D. melanogaster*. In the recombination events mediated by Cre and FLP, the recognition sequences (*LoxP* and *FRT* respectively) are homologous, and the reactions therefore are reversible. In contrast, the recombination mediated by ΦC31 integrase is directional; the *att* sites recognized by the integrase are heterologous, and the hybrid sequences created by recombination cannot be used by the integrase as substrates. This property may make certain types of reactions, such

*Address correspondence to: Shannon Fisher, 1213 BRB II/III, 420 Curie Blvd., University of Pennsylvania, Philadelphia, PA 19104, sfisher4@upenn.edu.

‡Current address:

Sloan-Kettering Institute, Program in Developmental Biology, New York, NY 10065

as recombinations between an integrated transgene and an exogenous plasmid, proceed more efficiently, since even in the presence of large excess of plasmid the reaction is irreversible.

An increasing number of tools are being developed to facilitate manipulations of the zebrafish genome, including efficient methods for transgenesis (Kawakami et al., 2000; Thermes et al., 2002; Moens et al., 2008), forward genetic approaches to induce or screen for mutations in specific genes (Doyon et al., 2008; Meng et al., 2008; Moens et al., 2008), and the use of the Cre/LoxP system to conditionally activate transgenes (Langenau et al., 2005; Pan et al., 2005; Thummel et al., 2005). However, having another SSR that could be practically used in zebrafish would allow the development of additional applications, particularly in a genetic background where Cre was also being used. In *D. melanogaster*, systems employing Φ C31 allow high frequency transgenesis (Groth et al., 2004; Fish et al., 2007), particularly for very large transgenes (Venken et al., 2006); provide well-characterized universal “docking sites” for transgenes, to avoid position effects (Markstein et al., 2008); and carry out recombinase-mediated cassette exchange (RMCE), in which a portion of an integrated transgene is swapped for a sequence on a targeting plasmid (Bateman et al., 2006). All of these applications require efficient intermolecular recombination between an integrated transgene and an exogenous plasmid. Two groups have recently described the use of Φ C31 in zebrafish, and demonstrated activity in somatic (Lu et al., 2010) and germ cells (Lister, 2010). In both cases, the reaction catalyzed was an intramolecular recombination event within an integrated transgene, resulting in excision of part of the transgene and activation of a second coding sequence. These reactions are similar to those typically mediated by the Cre/LoxP system, and will allow conditional activation of transgenes, particularly useful in conjunction with Cre. Here we report the use of Φ C31 integrase to efficiently mediate intermolecular recombination in zebrafish somatic and germ cells. This makes feasible the development of systems, analogous to those used in *D. melanogaster*, for RMCE (as we demonstrate here), and also for facilitating transgene integration into well-characterized chromosomal sites.

Results and Discussion

As part of an ongoing large-scale screen for enhancer sequences, we have analyzed many putative enhancers via transgenesis in zebrafish, by assessing their ability to drive tissue-specific expression of *egfp* in conjunction with a heterologous minimal promoter. The general approach and the vector we use for analysis have been previously described (Fisher et al., 2006a; Fisher et al., 2006b). Through this analysis, we have generated and characterized many transgenic lines with tissue-specific patterns of *egfp* expression. It would greatly increase the utility of these lines if the *egfp* coding sequence could be easily replaced, for example with that for another fluorescent protein or for Cre, which would be expressed in the identical pattern. Therefore, we sought to design a system to accomplish recombinase-mediated cassette exchange (RMCE) in the zebrafish, using the Φ C31 integrase; our strategy for vector design is illustrated in Figure 1.

We created the target transgene, intended to be integrated chromosomally, by modification of our standard enhancer analysis vector. To replace the entire coding sequence of the integrated transgene, two recombination events, at two flanking pairs of *att* sites, are required. For the *att* sites, we used the minimal sequences shown to have full recombination activity, as assayed in cultured human cells (Calos, 2006). The Φ C31 *att* sites are fairly short, less than 40 bp, and are likely to have little or no effect on transgene expression. However, to avoid any potential interference, we placed the *attP* sites outside of the *cFos* minimal promoter and polyadenylation sequence. To verify that enhancer function and transgene expression were unchanged, we incorporated a previously characterized enhancer, from upstream of the mouse *Sox10* gene, into the modified construct. The enhancer regulates

a distinct pattern of expression, in neural crest and oligodendrocytes (Antonellis et al., 2008). Following Tol2-mediated transgenesis, both the mosaic expression and that following germline transmission were indistinguishable from the original construct lacking the *attP* sites (data not shown).

Two groups have recently reported successful use of Φ C31 integrase in the zebrafish (Lister, 2010; Lu et al., 2010). However, in both cases the integrase mediated a single, intramolecular recombination event. For RMCE, two intermolecular recombinations must take place, and it is possible that the frequency will be lower and different optimal conditions will be required. To maximize integrase activity in the zebrafish, we used a version of the coding sequence (Φ C31o) that had been codon-optimized for the mouse (Raymond and Soriano, 2007). To test the feasibility of the required recombination and the activity of the integrase, we performed injections of the target transgene, with the *Sox10* enhancer driving *egfp*, together with the *mCherry* (*mCh*) targeting plasmid, Φ C31o integrase RNA, and Tol2 transposase RNA. Although the *mCh* plasmid does contain a minimal promoter, and could be expressed if it integrates near a genomic regulatory sequence, we anticipate such an event would be quite rare, since the plasmid lacks the Tol2 repeat sequences. Only if the expected recombinations, mediated by Φ C31 integrase, took place would *mCh* fall under control of the *Sox10* enhancer and display the appropriate tissue-specific expression. When all four components were injected, we frequently saw evidence of recombination in somatic cells, as seen by *mCh* expression in expected cell types, including neural crest and derivatives (Fig. 2A), often coexpressed with *egfp*. Such expression was not observed in the absence of Φ C31o integrase RNA (not shown). The resulting fish were raised, and with high frequency they transmitted the recombined transgene, expressing *mCh* under the control of the *Sox10* enhancer (Table 1). Clearly the Φ C31 integrase is capable of carrying out the necessary recombinations in the zebrafish embryo. However, we cannot distinguish in this assay whether the recombination took place episomally or after integration of the target transgene.

To test the efficiency of recombination with an integrated transgene as a substrate, we isolated fish with the target transgene integrated chromosomally. Their GFP+ progeny were injected with the *mCh* targeting plasmid and Φ C31o integrase RNA. When >20 pg of Φ C31o RNA was injected per embryo, we began to observe a high frequency of abnormal embryos, and at 40 pg, most injected embryos were dead by 10 hours (data not shown). Therefore, we performed these injections with 20 pg RNA per embryo. As before, we saw evidence of *mCh* expression, and therefore recombination, in the appropriate somatic cells (not shown). In *D. melanogaster*, it has been shown that the frequency of recombination can vary at different chromosomal locations (Bischof et al., 2007). Therefore, we performed similar injections with fish carrying two other *attP* target transgenes, containing enhancers from the human *AGGRECAN* (*ACAN*) or *TWIST1* loci. In these fish, we also observed somatic cells within the appropriate cell-specific pattern switch expression from *egfp* to *mCh* (Fig. 2B and data not shown).

To test for germline transmission of the recombination event, the *Sox10:egfp* embryos displaying mosaic *mCh* expression were raised to adulthood. We identified four independent adult founders that transmitted the altered transgene to a fraction of their progeny, which showed the full *Sox 10*-regulated pattern of *mCh* expression (Fig. 2E–H; Table 1; Table S1). Successful transmission of recombination was also observed for an additional founder carrying the *TWIST1:egfp* transgene. (Figure 1I–L; Table 1). However, the percentage of injected embryos that showed germline recombination as adults was low, making it impractical for a routine procedure. To attempt to increase the efficiency of recombination in the germline, we performed similar injections using Φ C31o integrase RNA altered by addition of the 3'UTR from the zebrafish *nanos1* gene (*nos3'UTR*). This sequence enriches

heterologous RNAs in the germline precursor cells of the zebrafish embryo (Koprunner et al., 2001), and we anticipated it would increase the effective concentration, and activity, of the integrase in the germline. GFP⁺ embryos were injected as above, with the targeting plasmid and the altered integrase RNA. We still observed some appropriate somatic expression of *mCh* in the injected embryos, suggesting that while the integrase RNA was enriched in the germ cells, it was not exclusively localized there. As in the previous injections, we selected injected embryos with somatic expression to raise; however, these typically comprised more than half of the viable embryos, so this selection was not likely to increase the frequency of identifying founders substantially. The injected embryos were raised, and a high percentage transmitted the expected expression pattern of mCherry to their progeny (Table 1; Table S1). Overall, we identified 37 founders from 74 screened adults in fish injected with the Φ C31o-*nos3*'UTR RNA, compared to 5/110 for the RNA lacking the *nos3*'UTR, indicating that inclusion of the 3'UTR substantially increased the frequency of recombination events in the germline.

Many founders gave rise to individual embryos expressing both *egfp* and *mCh* in an identical pattern. In some cases, these are presumably the result of founders transmitting multiple independent insertions of the *attP* construct, as is frequently observed with Tol2-mediated transgenesis. It is also possible that the entire targeting plasmid could insert *via* recombination at a single *attP* site, creating an unresolved intermediate, as has been described during RCME in *Drosophila* (Bateman et al., 2006). We tested individual GFP⁺/mCh⁺ progeny from four different adults, and verified the presence of plasmid backbone in progeny from two of the adults (data not shown). The presence of backbone is consistent with insertion of the entire targeting plasmid. We are currently testing whether additional injections of Φ C31o RNA can induce the second recombination event in the unresolved intermediates, as is the case in *Drosophila* (Bateman et al., 2006).

Following a complete RCME event, the *mCherry* cassette will be flanked by *attR* sites. Additionally, from a founder with only one genomic insertion of the original transgene, we should obtain progeny with either *egfp* or *mCh* expression, adding up to a total of approximately 50% of embryos in an outcross clutch. To verify complete recombination, we analyzed embryos from one such founder in more detail. We amplified portions of the transgenes from genomic DNA isolated from individual GFP⁺ or mCh⁺ embryos. In each pair of primers, one was designed to hybridize with either the *egfp* or *mCh* coding sequence; only the appropriate specific bands were detected from each sample (Fig. 3B). The amplified fragments were sequenced; from the GFP⁺ embryos, the unaltered *attP* site was present between the *egfp* coding and downstream sequences, while in the mCh⁺ embryos, *attR* sequences, produced by recombination, were found on both sides of the *mCherry* cassette (Fig. 3C, D).

To map the locations of the *egfp* and *mCh* transgenes, we performed linker-mediated PCR on DNA from individual GFP⁺ and mCh⁺ embryos from the same founder. From each embryo we obtained a single amplified band, indicating the presence of only one Tol2 transgene. The sequence flanking the Tol2 arm was too short to place unambiguously in the genome, but was identical for the GFP⁺ and mCh⁺ embryos, indicating that in each embryo the Tol2 transgene is at the same genomic location. Together with the above data, showing that the *mCh* cassette is flanked by *attR* sites, we can conclude that the *mCh* cassette replaced the *egfp* cassette in the original transgene *via* RMCE.

As with other SSRs, such as Cre and Flp, Φ C31 integrase can mediate intramolecular recombinations resulting in excision of a portion of a transgene. It has previously been reported that it can carry out this type of recombination in zebrafish (Lister, 2010; Lu et al., 2010), making it a viable alternative to Cre for applications such as long-term lineage

tracing or targeted misexpression of a gene. As transgenic lines expressing Φ C31 integrase in specific tissues are made and become available to the community, the utility of these approaches will increase. Here, we report for the first time efficient intermolecular recombination, mediated by Φ C31 integrase, in the zebrafish germline. Although the frequency was low in our initial experiments, the use of integrase RNA altered by addition of the *nanos1* 3'UTR greatly increased the recombination frequency in the germline. We found that nearly one half of injected fish were transmitting a recombined transgene, to as many as 18% of their progeny, making this a highly efficient and very practical approach to alter integrated transgenes. In our system, the sequence replaced by RMCE is relatively small (~1 kb). However, the native Φ C31 bacteriophage is >43 kb, and in *Drosophila* inserts >100 kb can be inserted *via* recombination at a single *att* site (Venken et al., 2006). There is less data on RMCE, but at least 5 kb sequences can be inserted with high efficiency (Bateman et al., 2006). If the same is true in zebrafish, it will expand the applications for which Φ C31-mediated recombination can be used.

Because the embryos carrying the integrated target transgene were derived via Tol2-mediated transgenesis, many of them were carrying multiple independent insertions, as could be seen from the total transmission frequencies of transgenic embryos, which were >50% for many fish (Table S1). In this scenario, it is difficult to determine if there are differences in efficiency of Φ C31-induced recombination at different chromosomal locations, although it has been described in *Drosophila* (Bischof et al., 2007). In the future, it would be highly beneficial in zebrafish to isolate a set of characterized insertion sites with integrated *attP* sequences that support stable transgene expression and high recombination frequencies. Such a collection could facilitate several applications, including high-frequency transgenesis with large constructs, and the quantitative comparison of expression levels among multiple transgenes, such as when testing mutated versions of enhancer sequences.

Experimental Procedures

Maintenance of zebrafish

Wild-type fish of the AB strain were maintained by standard husbandry practices (Westerfield, 1995), and in accordance with institutional guidelines.

Constructs for transgenesis

We modified our previously described vector for enhancer analysis, pGW-cFos-*egfp* (Fisher et al., 2006a; Fisher et al., 2006b) by the addition of two Φ C31 *attP* sites, flanking the *cFos* minimal promoter, *egfp* coding sequence, and poly-adenylation sequence. Insertion of the sites was achieved through PCR-mediated, ligase-independent mutagenesis (Chiu et al., 2004). The primers used to insert the two *attP* sites are listed in Table S2. After the separate insertion of the two *attP* sites, the vectors were digested with *Xho*I and *Cla*I and the resulting fragments ligated to obtain the final vector with two *attP* sites. To create the transgene target for *in vivo* tests, we inserted via the Gateway cassette a previously described enhancer from upstream of the mouse *Sox10* gene (Antonellis et al., 2008), which regulates robust expression in the neural crest and oligodendrocytes of transgenic fish.

The targeting plasmid consisted of *attB* sites flanking the *cFos* minimal promoter, *mCherry* coding sequence, and poly-adenylation sequence, in a pBluescript vector backbone. It was constructed in the following steps: 1) The *attB* sites were inserted separately into pBluescript, as above, using the *attB1* and *attB2* primers listed in Table S2. 2) The two *attB*-containing plasmids were cut with *Not*I and *Sal*I, and the two *attB*-containing bands ligated to obtain a plasmid with both *attB* sites. 3) The *egfp* coding sequence was replaced in the original pGW-cFos-*egfp* plasmid through PCR-mediated mutagenesis, using the *mCh*

primers listed in Table S2. 4) From the resulting vector, the entire *cFos-mCherry*-pA cassette was amplified and inserted into the *attB*-containing pBluescript plasmid, using the *cFos* primers in Table S2. *Zebrafish injections and transgenesis*. A coding sequence for Φ C31 integrase, codon-optimized for expression in mouse (Raymond and Soriano, 2007) was cloned into the pCS2+ plasmid to allow efficient *in vitro* transcription of RNA for injections. For some experiments, the RNA was further modified by addition of the 3'UTR from the zebrafish *nanos1* gene, which enriches transcripts in the germ cells of the early embryo (Koprunner et al., 2001).

For *in vivo* testing of integrase activity between episomal plasmids, the target transgene (~12 pg) and mCherry donor plasmid (~6 pg) were injected into embryos at the one-cell stage, together with RNAs encoding the Tol2 transposase and Φ C31o integrase (20 pg each); RNA and DNA were prepared, and injections performed, as previously described (Fisher et al., 2006a; Fisher et al., 2006b). To test RCME on an integrated transgene, fish carrying only the target transgene were generated by standard Tol2-mediate transgenesis and screened for germline transmission. The resulting GFP+ embryos were injected with the mCherry donor plasmid (10 pg) and Φ C31o integrase RNA (20 pg), either with or without the *nanos1* 3'UTR. *PCR genotyping*. A PCR assay was designed to distinguish the original target transgene (containing *egfp*) from the product of recombination (containing *mCherry*), using the primers listed in Table S2; the expected size of amplification products were 968 bp (*egfp*F-Tol2); 1172 bp (*egfp*R-*Sox10*); 1157 bp (*mCherry*F-Tol2); and 1230 bp (*mCherry*R-*Sox10*). DNA was isolated from individual embryos using standard protocols, subject to PCR, and the PCR products cloned for sequencing.

Linker-mediated PCR mapping

To map the insertion sites of the target transgene in the genome, we employed linker-mediated PCR, essentially as described previously (Davison et al., 2007; Akitake et al., 2011), using genomic DNA prepared from individual embryos. The insert-specific primers are complementary to the Tol2 arms (Table S2), and hybridize to both the original *egfp* transgene and to the locus altered by recombination with the *mCh* donor plasmid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Haibo Zhang for helpful input on cloning strategies; Marnie Halpern for useful discussions and thoughtful comments on the manuscript; and Paula Roy and Liping Sun for excellent fish care. The work was supported by a grant from the National Human Genome Research Institute (R01HG005039) to SF, and MGG was supported by the Damon Runyon Cancer Research Foundation as a Damon Runyon Fellow (DRG#1945-07) and by a grant from the National Institute of Child Health and Human Development (R01HD058530) to Marnie Halpern.

References

- Akitake CM, Macurak M, Halpern ME, Goll MG. Transgenerational analysis of transcriptional silencing in zebrafish. *Dev Biol.* 2011; 352:191–201. [PubMed: 21223961]
- Antonellis A, Huynh JL, Lee-Lin SQ, Vinton RM, Renaud G, Loftus SK, Elliot G, Wolfsberg TG, Green ED, McCallion AS, Pavan WJ. Identification of neural crest and glial enhancers at the mouse *Sox10* locus through transgenesis in zebrafish. *PLoS Genet.* 2008; 4:e1000174. [PubMed: 18773071]
- Bateman JR, Lee AM, Wu CT. Site-specific transformation of *Drosophila* via ϕ C31 integrase-mediated cassette exchange. *Genetics.* 2006; 173:769–777. [PubMed: 16547094]

- Bischof J, Maeda RK, Hediger M, Karch F, Basler K. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc Natl Acad Sci U S A*. 2007; 104:3312–3317. [PubMed: 17360644]
- Calos MP. The phiC31 integrase system for gene therapy. *Curr Gene Ther*. 2006; 6:633–645. [PubMed: 17168696]
- Chiu J, March PE, Lee R, Tillett D. Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res*. 2004; 32:e174. [PubMed: 15585660]
- Davison JM, Akitake CM, Goll MG, Rhee JM, Gosse N, Baier H, Halpern ME, Leach SD, Parsons MJ. Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. *Dev Biol*. 2007; 304:811–824. [PubMed: 17335798]
- Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol*. 2008; 26:702–708. [PubMed: 18500334]
- Fish MP, Groth AC, Calos MP, Nusse R. Creating transgenic *Drosophila* by microinjecting the site-specific phiC31 integrase mRNA and a transgene-containing donor plasmid. *Nat Protoc*. 2007; 2:2325–2331. [PubMed: 17947973]
- Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS. Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science*. 2006a; 312:276–279. [PubMed: 16556802]
- Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS. Evaluating the biological relevance of putative enhancers in zebrafish using transposon-mediated transgenesis. *Nature Protocols*. 2006b; 1:1297–1305.
- Groth AC, Fish M, Nusse R, Calos MP. Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics*. 2004; 166:1775–1782. [PubMed: 15126397]
- Kawakami K, Shima A, Kawakami N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proc Natl Acad Sci U S A*. 2000; 97:11403–11408. [PubMed: 11027340]
- Koprunner M, Thisse C, Thisse B, Raz E. A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes Dev*. 2001; 15:2877–2885. [PubMed: 11691838]
- Langenau DM, Feng H, Berghmans S, Kanki JP, Kutok JL, Look AT. Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 2005; 102:6068–6073. [PubMed: 15827121]
- Lister JA. Transgene excision in zebrafish using the phiC31 integrase. *Genesis*. 2010; 48:137–143. [PubMed: 20094996]
- Lu J, Maddison LA, Chen W. PhiC31 integrase induces efficient site-specific excision in zebrafish. *Transgenic Res*. 2010
- Markstein M, Pitsouli C, Villalta C, Celniker SE, Perrimon N. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nat Genet*. 2008; 40:476–483. [PubMed: 18311141]
- Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol*. 2008; 26:695–701. [PubMed: 18500337]
- Moens CB, Donn TM, Wolf-Saxon ER, Ma TP. Reverse genetics in zebrafish by TILLING. *Brief Funct Genomic Proteomic*. 2008; 7:454–459. [PubMed: 19028802]
- Pan X, Wan H, Chia W, Tong Y, Gong Z. Demonstration of site-directed recombination in transgenic zebrafish using the Cre/loxP system. *Transgenic Res*. 2005; 14:217–223. [PubMed: 16022392]
- Raymond CS, Soriano P. High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. *PLoS ONE*. 2007; 2:e162. [PubMed: 17225864]
- Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly JS. I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mech Dev*. 2002; 118:91–98. [PubMed: 12351173]
- Thummel R, Burket CT, Brewer JL, Sarras MP Jr, Li L, Perry M, McDermott JP, Sauer B, Hyde DR, Godwin AR. Cre-mediated site-specific recombination in zebrafish embryos. *Dev Dyn*. 2005; 233:1366–1377. [PubMed: 15977183]

Venken KJ, He Y, Hoskins RA, Bellen HJ. P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science*. 2006; 314:1747–1751. [PubMed: 17138868]
Westerfield, M., editor. *The Zebrafish Book*. Eugene, OR: University of Oregon Press; 1995.

Figure 1. Strategy for recombinase-mediated cassette exchange

Enhancer sequences are PCR-amplified and cloned into the *attP*-*cFos*-*egfp* vector via Gateway™ recombination to generate the target transgene, which is introduced into the germline through Tol2-mediated transgenesis. The donor plasmid, containing the *mCherry* (*mCh*) coding sequence flanked by inverted *attB* sites, is injected into transgenic embryos together with ΦC31o RNA. The expected recombination event will bring *mCh* expression under control of the specific enhancer sequence.

Figure 2. *In vivo* activity of Φ C31 integrase in somatic and germ cells

A) Embryos injected with the *Sox10:egfp* enhancer analysis vector and *mCh* donor plasmid, together with RNAs encoding Tol2 transposase and Φ C31o integrase, show mCh⁺ cells in the same tissues as the GFP⁺ cells seen with injection of the analysis vector alone. Fluorescently labeled cells are detected in tissues that are typically positive using the *Sox10* enhancer, including the oligodendrocytes of the spinal cord (arrowheads) and glia associated with the lateral line nerve (arrow). B–D) Embryos carrying an integrated *egfp* transgene with an enhancer from the *ACAN* gene (A) were injected with the donor plasmid and Φ C31o RNA. Injected embryos display mCh⁺ cells overlapping the Aggrecan pattern of expression in the notochord at 1 dpf (B), demonstrating appropriate recombination in somatic cells. E–L) Φ C31o mediates recombination in germ cell precursors. Embryos carrying the integrated *Sox10:egfp* transgene were injected with the *mCh* targeting plasmid and Φ C31o RNA, and the resulting fish raised to adulthood. In the progeny of some injected fish, *mCh* expression (G, H) replaced the specific *egfp* expression pattern (I, J), indicative of RMCE in germ cells of the injected fish. Fish carrying an integrated transgene with an enhancer from *TWIST1* were similarly injected with targeting plasmid and Φ C31o RNA; from one founder, embryos with *mCh* expression in the complete pattern were obtained (I, J), as well as progeny with the original *egfp* expression (K, L).

Figure 3. Recombination creates hybrid *attR* site at integrated locus

A) The position of primers used for validation of the recombination event is indicated, on the original *egfp* transgene and on the locus after successful RCME with the *mCh* targeting plasmid. B) PCR genotyping: Lane 1, DNA ladder; 2, GFP+ embryo, *egfpF* and Tol2 primers; 3, mCh+ embryo, *egfpF* and Tol2 primers; 4, mCh+ embryo, *mChF* and Tol2 primers; 5, GFP+ embryo, *mChF* and Tol2 primers. DNA from the mCh+ embryo was also amplified with the *mChR* and *Sox10* primers, and gave the expected size band (not shown). C) Shown are the sequences of the minimal *attP* site, on the target transgene; the *attB* site, on the donor plasmid; and the hybrid *attR* site created at the integrated locus by ΦC31 recombination. Note the underlined triplet in all three sequences, the actual site of recombination. D) Sequence traces from genotyping. From the GFP+ embryo (top) the original *attP* site was recovered; from the mCh+ embryo, sequence of the hybrid *attR* site was obtained on both the 3' (middle) and 5' (bottom) ends of the *mCherry* cassette.

Table 1

Frequency of recombination. Number of adults that transmitted progeny where *mCh* expression replaced *egfp* expression was observed under different conditions. In **II** and **III**, injections were performed into embryos carrying the integrated target transgene, as evidenced by specific *egfp* expression. In the second column, separate lines indicate results from screening different stocks, derived from separate injection experiments. In the third column, the range shown is the lowest to the highest frequency, for individual founder fish, at which transmission of specific *mCh* expression was observed.

Injection mix	Founders/adult fish screened	Donor cassette transmission frequency
I. Target plasmid + donor plasmid + Tol2 transposase RNA + Φ C31 integrase RNA	3/12 5/40 3/12	5–20%
II. Donor plasmid + Φ C31o integrase RNA	2/32 1/34 1/30 1/14	1.6–15%
III. Donor plasmid + Φ C31o- <i>nos1</i> -3'UTR RNA	12/36 25/38	0.3–37%