

## RESEARCH ARTICLE

## TECHNIQUES AND RESOURCES

# Targeted transgene integration overcomes variability of position effects in zebrafish

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**ABSTRACT**

Zebrafish transgenesis is increasingly popular owing to the optical transparency and external development of embryos, which provide a scalable vertebrate model for *in vivo* experimentation. The ability to express transgenes in a tightly controlled spatio-temporal pattern is an important prerequisite for exploitation of zebrafish in a wide range of biomedical applications. However, conventional transgenesis methods are plagued by position effects: the regulatory environment of genomic integration sites leads to variation of expression patterns of transgenes driven by engineered *cis*-regulatory modules. This limitation represents a bottleneck when studying the precise function of *cis*-regulatory modules and their subtle variants or when various effector proteins are to be expressed for labelling and manipulation of defined sets of cells. Here, we provide evidence for the efficient elimination of variability of position effects by developing a PhiC31 integrase-based targeting method. To detect targeted integration events, a simple phenotype scoring of colour change in the lens of larvae is used. We compared PhiC31-based integration and Tol2 transgenesis in the analysis of the activity of a novel conserved enhancer from the developmentally regulated neural-specific *esrrga* gene. Reporter expression was highly variable among independent lines generated with Tol2, whereas all lines generated with PhiC31 into a single integration site displayed nearly identical, enhancer-specific reporter expression in brain nuclei. Moreover, we demonstrate that a modified integrase system can also be used for the detection of enhancer activity in transient transgenesis. These results demonstrate the power of the PhiC31-based transgene integration for the annotation and fine analysis of transcriptional regulatory elements and it promises to be a generally desirable tool for a range of applications, which rely on highly reproducible patterns of transgene activity in zebrafish.

**KEY WORDS:** Zebrafish, Integrase, Transgenesis, Tol2, Enhancer, Position effects

**INTRODUCTION**

Transgenesis is one of the fastest growing technologies in the zebrafish model system. ZFIN, the Zebrafish Model Organism Database (Bradford et al., 2011), lists 10,867 transgenic zebrafish

lines. Transgenic zebrafish are produced for a variety of reasons, from *in vivo* labelling of cells and tissues to tissue-specific cell ablation, analysis of gene function, protein dynamics and localisation, generation of disease models through mis-expression of disease-associated genes, or manipulation of gene activities for developmental and physiological analysis (e.g. Gilmour et al., 2002; Langenau et al., 2005; Curado et al., 2008; Wyart et al., 2009; Hans et al., 2011).

With the publication of the ENCODE project and the discovery of the pervasiveness of predicted non-coding *cis*-regulatory modules (CRMs) in vertebrate genomes, emphasis is put on the functional validation of these computationally and biochemically predicted elements. Small laboratory fish models, such as zebrafish and medaka, with external development of optically transparent embryos and relatively easy transgenesis stand to make a major contribution to this area (Rada-Iglesias et al., 2011; Bernstein et al., 2012) and can meet the high-throughput capabilities demanded by the vast genomics data generated by next generation sequencing technologies (e.g. Gehrig et al., 2009). For functional analysis, predicted CRMs are often placed in front of a minimal promoter driving fluorescent protein expression. The construct is injected into zebrafish embryos and expression of the fluorescent reporter is used as a readout of CRM activity (reviewed by Ishibashi et al., 2013). In most cases, the CRM-fluorescent reporter cassette is placed into a Tol2 transposon vector to facilitate genomic integration of the transgene (Kawakami, 2004; Fisher et al., 2006; Kwan et al., 2007). However, the regulatory environment of the locus of the transgene integration may influence the expression of the transgenic reporter, leading to substantial variability of reporter expression through ectopic enhancement or silencing. These observations are collectively termed position effects (Jaenisch et al., 1981; Wilson et al., 1990; Rossant et al., 2011). Throughout this manuscript we use the term ‘variability of position effects’, to describe expression variation among transgenic lines with different integration sites to distinguish from ‘position effect variegation’ (PEV), a phenomenon characterised by variable changes (silencing) of a transgene among cells of an organism (Schotta et al., 2003). Variability of position effects are utilised in enhancer trapping, or enhancer detection, in which the regulatory landscape of the genome is probed by a weak expression cassette (Wilson et al., 1990; Balciunas et al., 2004; Parinov et al., 2004; Ellingsen et al., 2005; Davison et al., 2007; Scott et al., 2007; Asakawa et al., 2008). In most, if not all, other applications, where predesigned and controlled expression of transgenes is intended, random transgene integration and position effects are major obstacles. Such negative observations are often quoted in scientific meetings and discussions but are seldom published. We have found that 29 out of 30 lines generated with eight constructs using Tol2 transgenesis contained substantial variation of gene expression among lines with the same construct, probably caused by position effects (Y.H. and F.M., unpublished).

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Furthermore, flanking an expression cassette by insulator elements does not seem to reduce position effects (Grajevskaja et al., 2013). Taken together, the consequence of variable position effects is that often a large number of transgenic lines have to be generated with a single DNA construct to be able to obtain information on tissue-specific activity or to conclude correctly on the function of a tested *cis*-regulatory element.

Site-specific transgene integration using PhiC31 integrase has the potential to limit variability of position effects, leading to much faster and more precise analysis of putative CRMs in the zebrafish model system. Thus, site-specific integration would enable more efficient and high-throughput analysis of putative CRMs with increased reproducibility. Importantly, a robust targeted integration system would aid in the exploitation of the transgenic zebrafish model in the functional analysis of single nucleotide polymorphism (SNP) variants in CRMs identified in large numbers by recent genome-wide disease association studies (Ragvin et al., 2010; Maurano et al., 2012), particularly in those cases in which the SNP was expected to cause only subtle, quantitative changes in gene expression (e.g. Gaulton et al., 2010).

Several methods for site-specific integration of transgenes may be applied in zebrafish. Cre and FLP recombinases have been used for site-specific integration of transgenes in animal models (reviewed by Branda and Dymecki, 2004), but transgene integration potential of these recombinases has not yet been tested in zebrafish. A transcription activator-like effector nuclease (TALEN)-based method for site-specific transgene integration has been described but suffers from very low efficiency of germline transmission (Zu et al., 2013).

Based on successful application in the mouse and especially the fruit fly (Olivares et al., 2002; Groth et al., 2004; Venken and Bellen, 2012), we decided to develop a PhiC31-based method for analysis of enhancer activity in zebrafish. The natural function of PhiC31 integrase is to facilitate the integration of the phage genome into the bacterial host genome (Kuhstoss and Rao, 1991). PhiC31 integrase binds to two recognition sites, attP and attB, and exchanges DNA strands between them (Smith et al., 2004). Lister (Lister, 2010) demonstrated that PhiC31 integrase can facilitate intramolecular excision of a DNA sequence flanked by attP/attB sites in zebrafish (Lister, 2010; Lister, 2011). More recently, targeted integration of a transgene by PhiC31-mediated cassette exchange and insertion of circular plasmid were demonstrated in the genome of zebrafish (Hu et al., 2011; Mosimann et al., 2013).

We have developed a simple phenotype-based strategy using eye colour change to monitor site-specific integration of a transgene into the zebrafish genome. We have reproducibly achieved an ~10% germline transmission rate with site-specific transgenesis using PhiC31. Compared with Tol2-mediated random integration, PhiC31-mediated site-specific integration resulted in reduced variability and aided in the detection of the tissue-specific activity of a novel neural enhancer from the locus of the *esrrga* gene. A modified phenotyping strategy was also developed to detect enhancer function by the analysis of targeted, mosaic transgene activity in microinjected founder embryos.

## RESULTS

### ***In vivo* detection system for targeted integration of reporter constructs in zebrafish**

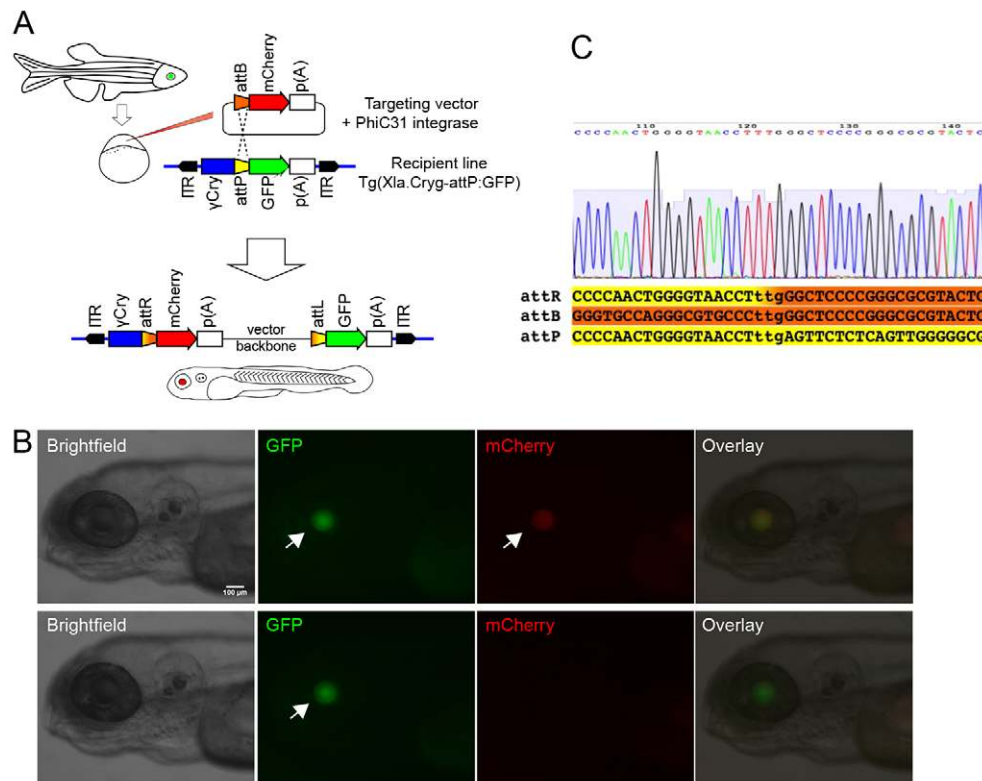
We have adapted a site-specific integration system with PhiC31 integrase consisting of two components: 'recipient transgenic lines' containing docking attP site(s) in the genome, and targeting plasmids containing attB site(s) (Thorpe and Smith, 1998). In mouse and human cells, integrations were observed to also occur into 'pseudo'

attP sites, i.e. endogenous sequences with similarity to the attP site (reviewed by Keravala and Calos, 2008). To facilitate screening for desired site-specific integrations, we designed a phenotypic selection based on fluorescent reporter colour change in the lens (Fig. 1A). Our recipient transgenic lines [Tg(Xla.crygc:attP-GFP)] contain a lens-specific crygc:GFP cassette (Davidson et al., 2003) with an attP site placed between the lens-specific *Xenopus laevis gamma-crystalline* promoter and GFP (henceforth crygc:attP-GFP for brevity). To monitor site-specific integration, our targeting vectors contain an attB site followed by a red fluorescent reporter (either mRFP or mCherry). Site-specific integration into crygc:attP-GFP docking site is expected to produce a crygc:attR-Red recombinant site, which can be scored by red fluorescence in the lens. Random integration of this attB-Red targeting vector into the genome is extremely unlikely to result in RFP expression.

Two different targeting site vectors were constructed: a *Tol2*-based vector pTol2/Xla.crygc:attP-GFP (pDB896) containing miniTol2 arms and a *Sleeping Beauty*-based vector pT2/Xla.crygc:attP-GFP (pKW1) (Fig. 1A). Docking site vectors were co-injected into zebrafish embryos with *in vitro*-transcribed *Tol2* (Balciunas et al., 2006) or *SB100X* (Mátés et al., 2009) mRNA. Injected embryos positive for lens GFP fluorescence at 3 dpf were raised to adulthood. Founder adults were crossed and their embryos were screened for lens GFP fluorescence at 3-5 days post-fertilisation (dpf). GFP-positive embryos were raised to establish the F1 generation. Five different founders produced GFP-positive progeny in Tol2-mediated transgenesis and three different founders produced GFP-positive embryos for SB-mediated transgenesis (supplementary material Table S1). We molecularly characterised the five genomic loci harbouring the docking site by inverse PCR and Southern blotting (supplementary material Fig. S1 and Table S1) and confirmed linkage of the identified integration event with GFP fluorescence (*tpl102*, *tpl103*, *tpl104*, *uobL6* and *uobL12*). The docking loci represent a variety of genomic landscapes: 700-kb intergenic region 100 kb away from nearest gene (*uobL6*), middle of a 100-kb intergenic region (*uobL12*), 5-kb intergenic region with two divergently transcribed genes (*tpl102*), and within a gene (*tpl103* and *tpl104*) (supplementary material Table S1 and Fig. S2). Third generation homozygous individuals of recipient lines *uobL6* and *uobL12* have been outcrossed and resulting offspring were raised to adulthood without notable reduction in fecundity and viability, whereas incrosses of the lines *tpl102*, *tpl103* and *tpl104* did not lead to developmental abnormalities in their offspring up to 6 dpf, arguing against mutagenicity of the docking site transgenes (data not shown).

### **High frequency of PhiC31-mediated targeting detected by lens reporter activity**

Next, we investigated whether we can visualise PhiC31 integrase-mediated insertion of a targeting plasmid containing an attB site into an attP site in zebrafish using an *in vivo* detection system of reporter activity in the lens. *In vitro*-transcribed PhiC31 mRNA was injected along with attB-Red constructs into one-cell zebrafish embryos from the Tol2-based transgenic recipient line Tg(Xla.crygc:attP-GFP). When transgenic embryos containing the docking site were co-injected with PhiC31 mRNA and the targeting construct pJET-attB-mCherry, up to 66.7% of normal developing larvae were RFP-positive in one or both lenses ( $n=258$ , Fig. 1B). By contrast, 0% ( $n=302$ ) of recipient transgenic embryos injected with targeting vector DNA without PhiC31 mRNA were RFP-positive in the lens, indicating that the eye colour change was due to integrase-mediated recombination events at docking sites in the recipient transgenic



**Fig. 1.** *In vivo* detection system for targeted integration of reporter constructs in zebrafish. (A) Schematic of PhiC31 targeted integration system. Transgenic embryos containing an attP docking site have a green lens due to the *gamma-crystalline* promoter driving a GFP reporter. ITR labels recognition sequences of either Tol2 or Sleeping beauty transposases used in generating the recipient transgenic lines with docking site. Injection of a circular plasmid with attB and a red reporter (targeting vector) into recipient line eggs leads to eye colour change upon PhiC31-targeted integration in larvae. (B) Detection of eye colour change upon PhiC31-mediated integration in the transgenic recipient Tg(Xla.crygc:attP-GFP)*uobL6* line. Top row shows a transgenic larva with PhiC31-mediated recombination of the attB-mCherry cassette into the attP-GFP docking site. Bottom row shows transgenic sibling from the recipient line without targeted integration. Side views on cranial end of 5 dpf larvae with anterior to the left. Scale bar: 100 µm. Arrows indicate reporter expression in the lens. (C) Sequence of the *tp102* recipient docking site with germline integration of targeting construct *tnnT2*:attB-mRFP. Sequence shows the attR site in the crygc:attR-Red recombination product. The three lower case nucleotides denote the recombination site.

locus. We confirmed integrase-dependent transgene integration by PCR amplification and sequencing of the attL and attR sites, which arise by recombination of attP and attB sites (Fig. 1A,C).

In accordance with previously published observations (Hu et al., 2011), we observed that PhiC31 RNA *in vitro* transcribed from a pT3TS (Hyatt and Ekker, 1999) or pCS2+ based vectors was highly toxic to zebrafish embryos (supplementary material Table S2). A significant percentage of abnormal/dead embryos was observed using as little as 15 pg of integrase RNA (supplementary material Table S2). It was recently demonstrated that addition of *nanos1* 3' UTR to PhiC31 mRNA leads to improved embryo survival and germline transmission of recombination events in zebrafish (Hu et al., 2011). Indeed, we observed improved survival upon injection of PhiC31-*nos1*-3'UTR compared with PhiC31 without *nanos1* 3' UTR (supplementary material Table S2). Injection of PhiC31-*nos1*-3'UTR mRNA did not substantially reduce the viability of primordial germ cells (supplementary material Table S2). However, co-injection of PhiC31-*nos1*-3'UTR into SB-based recipient lines resulted in varying percentage of lens RFP-positive embryos. We observed 48% (33/69), 14% (6/43) and 36% (9/25) RFP-positive embryos upon injection into *tp1102*, *tp1103* and *tp1104*, respectively (data not shown). RFP-positive embryos were raised to adulthood, outcrossed and resulting embryos were screened for lens RFP. Rates of site-specific

germline transgenesis are ~10%, and they slightly varied between experiments using different docking loci and different types of integrase RNA (supplementary material Table S3).

#### Identification of enhancer-specific expression features obtained in various genomic loci

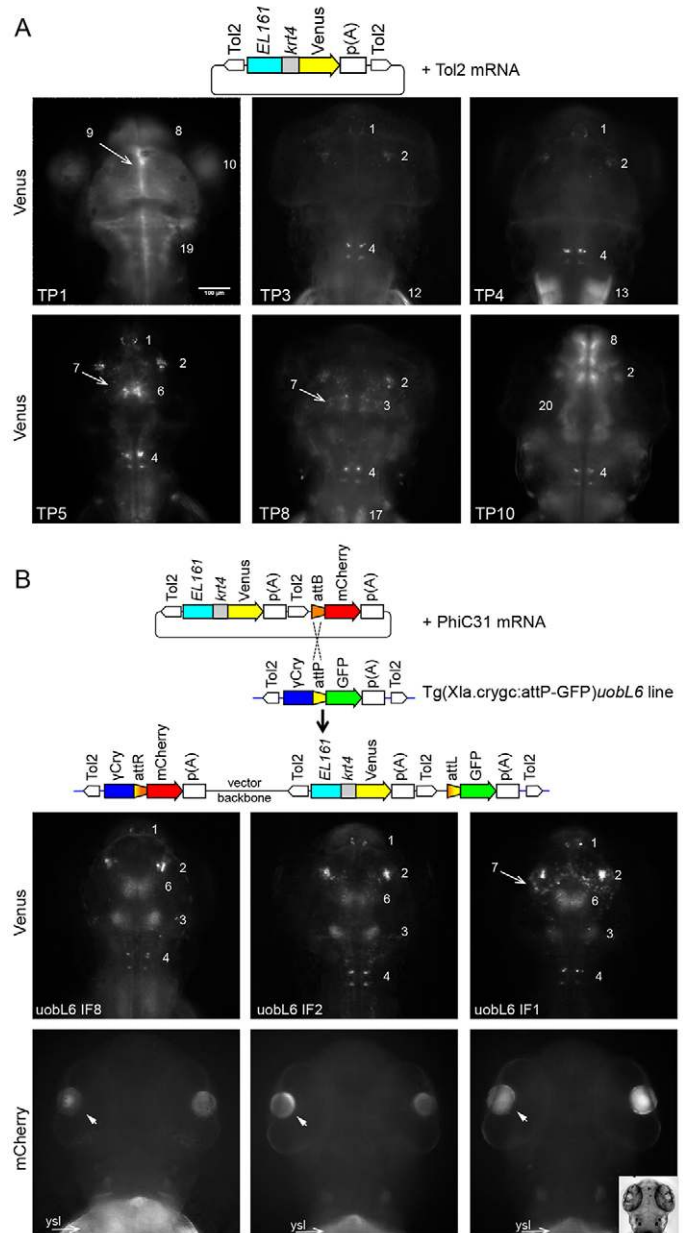
To test if site-specific integration would help in reducing the variability of position effects in experiments aiming to characterise enhancer-driven tissue-specific reporter gene activity, we analysed reporter activity driven by a putative highly conserved enhancer after integration into the genome either by Tol2 or by PhiC31 integrase. In order to select candidate enhancers, we have identified highly conserved syntenic sequences with >80% sequence identity between the zebrafish and human genome (see Materials and Methods). They do not overlap any known exon of annotated coding or noncoding genes. We choose EL161 for this study, which resides in the last intron of the *esrrga* gene in zebrafish (supplementary material Fig. S3). Based on the conserved synteny and conservation around the *esrrga* gene, we postulated that EL161 may regulate *esrrga* expression (Bardet et al., 2004; Thisse et al., 2004). In a preliminary study, EL161, when linked to the *krt4* gene minimal promoter with minimal transcriptional activity (Gehrig et al., 2009) was able to activate reporter transgenes in the brain; however, the expression was highly variable due to position effects (our unpublished data).



The Tol2/EL161-krt4:YFP cassette containing the EL161 element was then used with both Tol2- and PhiC31-mediated transgenesis. Tol2-mediated transgenesis is expected to integrate only the Tol2/EL161-krt4:YFP cassette into the genome (Fig. 2A, top), whereas PhiC31-mediated transgenesis integrates the whole vector containing Tol2/EL161-krt4:YFP and attB:mCherry (Fig. 2B, top). We screened F1 embryos of 20 founders injected with Tol2 by fluorescence microscopy, and recovered ten different YFP expression patterns, suggesting multiple integration sites and that Tol2/EL161-krt4:YFP is highly susceptible to position effects (Fig. 2A). We screened the offspring of all 29 adult founders from *uobl6* and *uobl12* injected with PhiC31 integrase and showing eye colour change during larval development. Four of these founders gave progeny with red lenses together with YFP expression pattern, indicating site-specific integration events (Fig. 2B; supplementary material Tables S3, S4). Notably, the three founders with targeted integration into the *uobl6* line showed remarkable similarity in their expression patterns, in contrast to Tol2-mediated transgene integrations, which were characterised by widely varying patterns (Fig. 2; supplementary material Table S5).

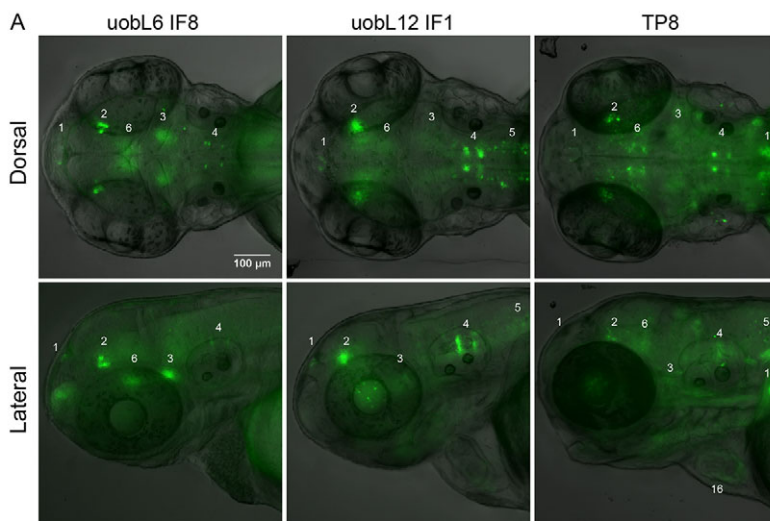
To evaluate the specific reporter activity driven by EL161 element and to dissect enhancer-driven activity from position effects, we have identified distinct expression domains and analysed the frequency of their occurrence in targeted and random transgene integration loci. We have identified a total of 20 domains of activity labelled 1-20 (Figs 2, 3; supplementary material Figs S4, S5). The PhiC31 integrase-mediated expression activity was present in seven distinguishable expression domains in the neural tube (Fig. 3). Six out of seven expression domains were shared between the three lines with targeting events in the same L6 docking site and only one additional domain (Domain 7) registered in one line indicating mild variation (Fig. 3B). By contrast, Tol2 lines showed a total of 21 expression domains with a variation between seven and 12 domains per pattern, which included a variety of tissues such as somatic muscle, pectoral fins and heart besides neural tube activity (Fig. 3B). This result indicates that integrase-mediated targeting of a single locus (in *uobl6*) led to reduced variability of expression patterns induced by a neural enhancer among transgenic lines.

The expression domains 1-6 were shared among *uobl6* and *uobl12* integration loci and were present in eight out of ten Tol2-mediated transgene loci, which together suggest autonomous enhancer activity in these neural domains. This result demonstrates that the shared expression patterns are unlikely to be due to position effects at the *uobl6* locus and are autonomous property of the targeting transgene (Fig. 3A,B). Notably, these six shared expression domains, including epiphysis, diencephalic and hindbrain nuclei and tegmentum, overlap with expression domains of the *esrrga* gene (supplementary material Fig. S6) (also see www.zfin.org) (Bardet et al., 2004), suggesting that EL161 may contribute to the activity of *esrrga* in these neural subdomains. Low level of variability was still observed in these transgenic lines, at least in part explained by variation in focal planes of imaging as well as by potential differences in developmental stage of the individuals imaged. For stage-dependent variation of transgene activity, see supplementary material Fig. S7. The variability of expression patterns among PhiC31-mediated targeted integrants was significantly lower than those found among Tol2-mediated transgene integrants (likelihood ratio=15.0, DF=1,  $P=0.0001$ ). Taken together, the low variability of transgene activity observed in PhiC31-targeted integration events demonstrate



**Fig. 2. Variability of position effects on reporter activity is sharply reduced among PhiC31 integrase-mediated transgenic lines compared with Tol2 transgenesis.** (A) Schematic of the pTol2/EL161-krt4:Venus construct injected with Tol2 transposase mRNA. Below are examples of expression patterns of transgenic F1 larvae (3 dpf). TP indicates Tol2-mediated expression patterns. Numbers refer to domains of Venus expression activity (listed in Fig. 3B). (B) Schematic indicates the recombination of the targeting plasmid pattB-mCherry, Tol2/EL161-krt4:Venus into the attP docking site of the transgenic recipient line Tg(Xla.crygc:attP-GFP)*uobl6*. Larvae from three different founders (IF1, IF2, IF8) targeted with PhiC31 integrase are shown. Venus expression domains are labelled as in Fig. 3B. Lens activity driven by mCherry (arrowheads in bottom row) demonstrates PhiC31-mediated integration. Insert in bottom right shows brightfield image of the head region. Arrows labelled 'ysl' in red channel indicate auto-fluorescence of the yolk syncytial layer. Dorsal views of larvae head are shown. Scale bar: 100  $\mu$ m.

superior reproducibility and robustness of enhancer-driven expression patterns compared with that obtained by transposase-mediated integration.



**Fig. 3. Reproducible Venus expression patterns upon targeted integration of EL161 enhancer construct demonstrate *cis*-regulatory function in the brain.** (A) Brain-specific enhancer effect of a transgene inserted in different integration sites. Domains of Venus activity are specified in B. Dorsal (top) and lateral (bottom) views of the head of 3 dpf F1 transgenic larvae. Scale bar: 100  $\mu$ m. (B) Domains of Venus reporter expression and their distribution among targeted (PhiC31 integrase) and randomly (Tol2 transposase) transgenic lines. IF, integrase-injected founders; IP, integrase-mediated expression patterns; TF, Tol2-injected founders; TP, Tol2-mediated expression patterns. Blue depicts expression domains overlapping with *esrrga* activity, whereas red colour indicates additional domains. Light blue indicates weak expression.

**B**

Domains of expression	Integrase lines				Tol2 lines													
	uobL6		uobL12		TF1		TF2		TF3		TF4							
	IF1	IF2	IF8	IF1	IP1	IP2	IP3	IP4	TP1	TP2	TP3	TP4	TP5	TP6	TP7	TP8	TP9	TP10
1 Epiphysis																		
2 Dorsal diencephalon																		
3 Tegmentum																		
4 Hindbrain neurons (4)																		
5 Posterior hindbrain/Spinal cord																		
6 Ventral diencephalon																		
7 Dorsal diencephalic neurons																		
8 Telencephalon																		
9 Ventricular zone																		
10 Lens																		
11 Cerebellum																		
12 Pectoral fins																		
13 Myotomes																		
14 Yolk syncytial layer																		
15 Branchial arches																		
16 Heart																		
17 Glomerulus																		
18 Pronephric ducts																		
19 Central nervous system 1																		
20 Central nervous system 2																		

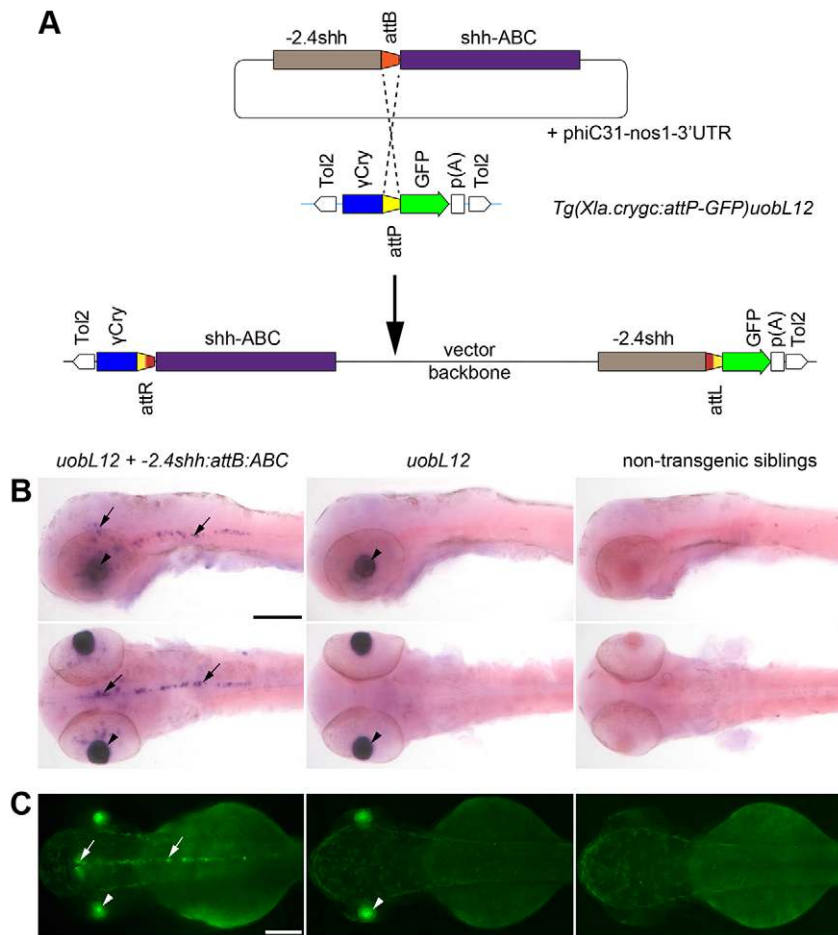
### Transient transgenesis strategy for rapid evaluation of enhancer activity upon PhiC31-mediated targeting

Transient transgenic zebrafish with mosaic reporter activity have often been used for the analysis of enhancer function because they allow high-throughput analysis of *cis*-regulatory elements (Gehrig et al., 2009; Rada-Iglesias et al., 2011). Because transient transgenesis is likely to suffer from variability, we tested whether the PhiC31-based integration strategy could also be applied for analysis of enhancer activity in mosaic transgenic embryos. To ensure that only targeted integration events are detected by reporter activity, we have designed a modified targeting strategy (Fig. 4), whereby an attB site is cloned adjacent to an enhancer-promoter combination, and can only lead to GFP activity driven by these *cis*-regulatory elements when PhiC31 integrase targets the donor construct upstream to the GFP in the recipient line (Fig. 4A). A set of well-characterised embryonic midline-specific enhancers, ar-A, B and C in introns 1 and 2 and ar-D in the upstream sequence of the promoter from the *sonic hedgehog* (*shh*) gene (Müller et al., 1999; Hadzhiev et al., 2007), were joined to the attB with or without mCherry reporter. These donor vectors were injected with PhiC31o-nos1-3'UTR integrase mRNA into embryos of in-cross of *uobL12* recipient line, in which the multicopy docking site maximised the probability of targeting by PhiC31 integrase. Expression of *gfp* in tissues [floor plate, ventral brain, retina (Shkumatava et al., 2004)] in which *shh* enhancers are active was detected by whole-mount *in situ* hybridisation in 52.0% ( $n=25$ ) and 46.2% ( $n=91$ ) of donor construct-injected embryos with or without

mCherry, respectively at high-pec stage with green lens. No embryos without lens reporter ( $n=5$  and  $n=23$ , respectively) showed any *gfp* expression (Fig. 4B,C; supplementary material Table S6). These results indicate that *gfp* expression driven by *shh* regulatory elements was due to landing at the docking site. Expression was highly specific to the *shh* pattern, only 15.4% ( $n=13$ ) and 6.3% ( $n=16$ ) of analysed embryos with *shh* pattern showing ectopic *gfp* activity in a small number of cells (data not shown) and no embryos showing ectopic activity without *shh* pattern, respectively. The activity of the GFP reporter protein was also detected by fluorescence albeit at lower frequency, 3.8% ( $n=53$ ) and 4.4% ( $n=91$ ) of lens GFP-positive embryos [3.8% ( $n=53$ ) and 4.4%, ( $n=91$ ), respectively], compared with 0% ( $n=23$ ) of lens GFP-negative embryos (Fig. 4C). These results together demonstrate a proof of principle application for enhancer testing in mosaic transgenic zebrafish embryos using PhiC31 integrase-mediated transgenesis.

### DISCUSSION

We tested the ability of PhiC31 integrase to site-specifically integrate transgenes into the zebrafish genome. We devised a simple phenotypic screening strategy based on change of lens fluorescence, and demonstrated germline transmission of site-specific integration rates in >10% of injected founders using different targeting transgenes, different recipient lines and two different versions of *in vitro* transcribed PhiC31 transposase. The simple phenotyping tool based on lens colour change provides an easily detectable measure



**Fig. 4. Transient transgenesis strategy for rapid evaluation of enhancer activity upon PhiC31-mediated targeting.** (A) Schematic of PhiC31-targeted integration system, designed for *cis*-regulatory element analyses in transient transgenic embryos. '-2.4 shh', 2.4 kb upstream region of *shh* gene; 'shhABC', *shh* complete introns 1 and 2, with ar-A, ar-B, ar-C enhancers (Müller et al., 1999; Ertzer et al., 2007). (B) Detection of reporter activity in embryos injected with construct containing the *shh* *cis*-regulatory elements by *in situ* hybridisation (B) and fluorescence microscopy (C). Arrows indicate activity in *shh* expression domains, resulting from successful integration of the *shh cis*-regulatory cassette in to the attP site of *uobL12*, whereas arrowheads indicate the activity of the *crygc* promoter in *uobL12* recipient line. Scale bars: 150 µm.

of targeted integration events, yet is restricted to the lens with little interference with imaging anatomical features commonly studied by reporter expression-based assays. We chose the crystalline promoter for screening integrants because it is representative of a specialised class of promoters characteristic of highly active tissue-specific genes with no interaction with long-range enhancers (reviewed by Lenhard et al., 2012). As expected, no interference was detected from the *crystallin* promoter on the tissue-specific enhancer construct inserted in the vicinity.

We have demonstrated a reproducible expression pattern driven by a predicted transcriptional enhancer using targeted integration of an expression cassette. The observed expression domains appeared to match broadly with expected activity of *esrrga*, the predicted target gene for the enhancer. Given that enhancers can act at distances as far as 1 Mb (reviewed by Krivega and Dean, 2012), unambiguous identification of the target gene(s) remains challenging. Nevertheless, we were able to identify several *esrrga* expression domains with reproducible activity, which were attributable to the enhancer in independent genomic integration sites. Tol2 transgenics also showed several of the enhancer-specific patterns but were mostly coupled to a variety of additional ectopic patterns. It is unlikely that the additional patterns are due to Tol2 sequences as the PhiC31 donor vector also included them.

*uobL6* appeared to be an excellent landing site for the analysis of the EL161 enhancer; however, this may not be the case for other CRMs. It is expected that genomic context and enhancer promoter interaction specificity (Gehrig et al., 2009) will affect different CRMs to varying degrees. To identify optimal sites for a broad

range of CRMs, more landing sites will need to be generated and evaluated in the future. Towards this long-term goal we have established and molecularly characterised five different recipient lines. The five 'docking' loci cover a variety of locations with respect to potential position effects, including intragenic as well as large (0.7 and 0.1 Mb) intergenic regions, and provide a variety of genomic contexts for future analyses of position effects. Many pseudo-att sites may exist in the genome (Hu et al., 2013). Integration of the donor plasmid into a pseudo-att site is expected to yield YFP fluorescence in the absence of RFP fluorescence. We observed 100% concordance between red lens colour and YFP expression pattern, which indicates that such non-specific events are extremely rare, consistent with a recent publication by Mosimann and colleagues (Mosimann et al., 2013).

We chose insertion of full donor constructs as opposed to the previously published strategy of cassette exchange (Hu et al., 2011). Cassette replacement has the advantage that the vector backbone is not integrated, eliminating the potential impact of bacterial vector backbone on transgene expression. However, the advantage of the insertion strategy applied here lies in the simplicity of the transgene design and the fact that there appears to be no size limit for the sequence to be integrated (Venken et al., 2009), making it potentially applicable for analysis of large vectors such as bacterial artificial chromosomes to test regulatory function of super-enhancers, CRMs acting at large distance and multiple CRMs acting synergistically (Kikuta et al., 2007; White et al., 2008).

In accordance with previously published work, we find that wild-type PhiC31 integrase RNA is toxic to zebrafish embryos (Hu et al.,



2011). Application of PhiC31-nos1-3'UTR resulted in substantially increased embryo survival and somewhat reduced activity in somatic tissues, similarly to previously published work (Hu et al., 2011). Interestingly, application of PhiC31-nos1-3'UTR did not result in a significant increase in germline transmission of targeting events. It is also reasonable to expect that different recipient loci may have different accessibility for PhiC31-mediated integration. In this light, the fact that our site-specific transgenesis rate was reproducible using different recipient lines, different targeting vectors and two different integrase expression vectors is very encouraging for future reproducibility of this method.

We have demonstrated in a proof of principle experiment that a PhiC31 integrase-mediated targeting strategy can also aid in generating transient transgenic embryos with reproducible patterns of mosaic expression of transgene reporter driven by tissue-specific *cis*-regulatory elements. Transient transgenics are immensely useful for fast and high-throughput analysis of a large number of potential *cis*-regulatory modules (Woolfe et al., 2005; Gehrig et al., 2009; Bernstein et al., 2012). The targeted integration system described herein will improve the reliability and thus the utility of transient transgenesis-based enhancer validation data. The targeting strategy presented here has the advantage of unambiguous identification of integration events by tissue-specific activity of the GFP reporter. However, it is suboptimal for fluorescence microscopy potentially owing to reduced translational efficiency of the reporter when preceded by an attR site. Future experiments will be required to test alternative integration strategies that do not impact on translational efficiency (e.g. a system whereby reconstitution of mRNA splicing occurs upon targeted integration) and/or test potentially more sensitive, enzymatic reporters, such as *lacZ*.

It should be noted that reproducibility of transgene expression offered by site-specific integration has a much broader range of potential application than functional characterisation of CRMs. It will enable investigators to first integrate a cassette containing a fluorescent reporter into a specific locus and determine precise spatiotemporal expression pattern of the transgene. Subsequently, a cassette in which the fluorescent reporter has been replaced with an effector transgene (e.g. Cre recombinase, calcium channel rhodopsin, calcium sensor, nitroreductase or dominant-negative signalling protein) integrated into the very same locus will provide highly reproducible spatiotemporal expression activity. Furthermore, site-specific integration will simplify comparison of phenotypic effects of several different transgenes expressed in the same spatiotemporal pattern at comparable levels.

During the revision of this manuscript, a similar report has been published showing enhancer testing with PhiC31 integrase-mediated integration in the medaka (*Oryzias latipes*), signifying the importance of this technology in vertebrate model organisms (Kirchmaier et al., 2013).

## MATERIALS AND METHODS

### Generation of plasmid vectors for PhiC31-mediated integration system in zebrafish

We generated two recipient vectors with an attP docking site for the PhiC31 integrase. pTol2/Xla.crygc:attP-GFP (pDB896) vector contains miniTol2 arms (Balciunas et al., 2006), ~390 bp of the *X. laevis gamma-crystallin* promoter (Davidson et al., 2003), an 83 bp attP integrase recognition site (Thorpe and Smith, 1998), GFP coding sequence and a SV40 pA tail. pT2/Xla.crygc:attP-GFP (pKW1) is identical to the Sleeping Beauty vector pT2/gCry:GFP (pDB387) (Davidson et al., 2003), but has an 83 bp attP site inserted between *Bam*HI and *Kpn*I sites. The targeting vector pJET-attB-mCherry was constructed by joining an 84 bp attB sequence (Thorpe and Smith, 1998) to the mCherry coding sequence with polyA tail by PCR. This

PCR product was then cloned into pJET1.2 using a blunt-ended cloning kit (Thermo Scientific). The targeting vector *tmt2*(3.2)-attB:mRFP contains a heart-specific 3.2 kb *tmt2* promoter followed by 83 nucleotide attB site and monomeric RFP (mRFP) (Campbell et al., 2002) for potential phenotypic screening of heart RFP activity indicating random or illegitimate integration events). The pCS2+PhiC31 plasmid was generated by amplifying PhiC31 coding sequence from plasmid pPhiC31o (Addgene) using the following primers: F: 5'-AAAAGGATCCATGGATACCTACGCCGGAG-3'; R: 5'-AAATTCTCGAGTCACACTTTCCGCTTTTCTTAG-3'. This sequence was then cloned into pCS2+ vector using *Bam*HI and *Xho*I sites. pCS2+PhiC31 plasmid was linearised with *Nor*I (New England Biolabs) and this product was used to synthesise PhiC31 mRNA using the *in vitro* transcription kit mMESSAGE mMACHINE (Ambion), according to the manufacturer's instructions. The pT3TS-PhiC31 (pRG3) plasmid contains an integrase open reading frame from pCS2+PhiC31 cloned between *Bgl*II and *Spe*I sites of *in vitro* transcription vector pT3TS (Hyatt and Ekker, 1999). The plasmid was linearised using *Xba*I and *in vitro* transcribed using mMESSAGE mMACHINE (Ambion) *in vitro* transcription kit and T3 RNA polymerase. After RNA synthesis and DNase digestion, RNA was purified using RNeasy MiniElute RNA purification kit (Qiagen). pCS2+PhiC31-nos1-3'UTR plasmid was a kind gift from Dr Shannon Fisher (University of Pennsylvania, PA, USA). The mRNA for microinjection was made as described by Hu et al. (Hu et al., 2011), except that after synthesis and DNase digestion, RNA was purified using RNeasy MiniElute RNA purification kit (Qiagen). Constructs described in this article will be made available through Addgene.

### Identification conservation analysis and cloning of *esrrga* enhancer

The zebrafish (*Zv9*) and human (GRCh37) repeated masked version of the genomes were downloaded from the Ensembl FTP site (Flicek et al., 2013). The Ensembl gene annotations (coordinates of the genes and the exons and homology relationships) were downloaded from BioMart using R and the Bioconductor package BiomaRt (ensembl mart version 60) (Durinck et al., 2005; Durinck et al., 2009). The zebrafish genome was aligned against the human genome using blastn (Camacho et al., 2009) from the blast+ suite (version 2.2.24) with a word\_size parameter of 50 bp. The resulting conserved regions were mapped according to the overlapping/flanking genes. Sixteen conserved elements showing the following features were considered: (1) minimum 500 bp length, (2) minimum 80% identity, (3) overlapping and/or directly flanking at least one pair of homologous genes between zebrafish and human and (4) presence of the element in the same genomic context (intronic or intergenic) in the two species. The 794 bp conserved element EL161 from the last intron of the *esrrga* gene was selected from the list and amplified by PCR from zebrafish genomic DNA using the oligos 5'-CATCCTAAATTTGGTGTCTCTCTG-3' (forward) and 5'-TGCGTCAACAAGATGAGATGAAC-3' (reverse) and cloned upstream of the minimal promoter from the *keratin 4* (*krt4*) gene and a Venus reporter gene (Gehrig et al., 2009) using Gateway Multisite System (Urasaki et al., 2006; Roue et al., 2007) resulting in the vector pTol2/EL161-krt4:Venus. For PhiC31 integrase-mediated experiments, the attB-mCherry cassette from pJET-attB-mCherry vector was cloned into the *Nor*I site of pTol2/EL161-krt4:Venus construct using PCR In-Fusion System (Clontech), according to the manufacturer's instructions. The primers used for amplifying attB-mCherry cassette were: F: 5'-AGTTCTAGAGCGGCCGATTGACGGTCTCGAAGCCG-3' and R: 5'-ACCGCGGTGGCGGCCGAAAAACCTCCACACCTCCC-3'. The resulting vector was named pattB-mCherry, Tol2/EL161-krt4:Venus.

### Production and microinjection of zebrafish fertilised eggs

Fertilised zebrafish eggs were generated by natural crossings. The zygote stage embryos were injected with either with 3 nl of water solution containing 8.3 ng/μl DNA and 8.3 ng/μl of PhiC31 integrase transcribed from pT3TS or with 15 ng/μl of DNA and 15 ng/μl of PhiC31 integrase transcribed from pCS2+ vector. After injections, embryos were incubated in embryo water containing 0.1 g/l of Instant Ocean salts or E3 medium at 28–29°C (Westerfield, 1993).

### Fluorescence reporter expression analysis

Transgenic embryos and larvae [60–96 hours post-fertilisation (hpf)] were imaged using Olympus ScanR high content screening microscope (Olympus) as described (Gehrig et al., 2009). Anaesthetised embryos were oriented in an agarose template made with a brass mould (Peravali et al., 2011). Stacks containing 100 slices of 4 µm thickness were taken, out of focus slices were manually removed and maximum projections were made using an extended depth of field plug-in for ImageJ software (Forster et al., 2004). Expression domains were identified by comparing to anatomies and gene expression data in ZFIN database (www.zfin.org). Statistical analysis of variation of expression domains was carried out by binomial regression using a generalised linear mixed-effects model.

### Whole-mount *in situ* hybridisation

Whole-mount *in situ* hybridisation was carried out as previously described (Thisse and Thisse, 2008). Digoxigenin (DIG)-labelled antisense probes were produced by PCR using the following primers: Venus probe: F: 5'-CAAGGGCGAGGAGCTGT-3' and R: 5'-AACTCCAGCAGGACCATGT-3'; *esrrga* probe: F: 5'-CACTAACGGACAGCGTAAATCA-3' and R: 5'-TCTCCAGTTCATGCTCTTG-3'; GFP probe: F: 5'-ACACTGT-CACTACTTTCGC-3' and R: 5'-AACTCAAGAAGGACCATGTG-3'. Embryos were mounted in glycerol and imaged on an AxioScope microscope (Carl Zeiss).

### Analysis of *shh* enhancer activity in transient transgenic embryos

The reporter constructs *-2.4shh:attB:mCherry:ABC* and *-2.4shh:attB:ABC* were generated by replacing the GFP:poly(A) cassette from the *-2.4shh:gfp:ABC* vector (Ertzer et al., 2007) with PCR-amplified *attB:mCherry:p(A)* or *attB* cassettes from the *pJET-attB-mCherry* vector, using *XhoI/NotI* restriction sites. About 2 nl of injection solution, containing 20 ng/µl reporter plasmid DNA, 15 ng/µl PhiC31nos mRNA and 0.1% Phenol Red, was injected into zygotes of the landing lines. The injected embryos were analysed for reporter expression by fluorescence microscopy and whole-mount *in situ* hybridisation using GFP DIG-labelled probe at 48 hpf. The reporter-positive embryos identified by the *in situ* hybridisation were categorised according to number and type of positive cells in relation to the expected pattern of reporter construct activity.

### Analysis of primordial germ cell (PGC) survival

PGC numbers were assessed at 24 hpf in embryos injected with PhiC31 and PhiC31o-nos1-3'UTR mRNA (15 and 30 ng/µl, respectively) by anti-Vasa antibody staining (kind gift from Holger Knaut, Skirball Institute, NY, USA) as previously described (Nusslein-Volhard and Dahm, 2002). Antibodies used were anti-Vasa (rabbit, 1:500) (Knaut et al., 2000) and secondary antibody conjugated with HRP (goat, 1:150; A10260, Life Technologies). Detection was carried out with DAB Peroxidase Substrate Kit (Vector Laboratories) following the manufacturer's instructions.

### Molecular analysis of integration events

Genomic DNA was prepared from batches of lens GFP-positive and lens GFP-negative embryos from an F1 or F2 outcross of Tg(pT2/Xla.crygc:attP-GFP)*tpl102*, Tg(pT2/Xla.crygc:attP-GFP)*tpl103*, Tg(pT2/Xla.crygc:attP-GFP)*tpl104*, and from an F3 outcross of Tg(Xla.crygc:attP-GFP)*uobL6* and Tg(Xla.crygc:attP-GFP)*uobL12* lines. To map pT2/Xla.crygc:attP-GFP integrations in lines *tpl102*, *tpl103* and *tpl104* integrations, genomic DNA was digested in two separate reactions with *TaqI*, and a mix of *BamHI/BclI/BglIII* restriction enzymes. The digested DNA was ligated overnight as described (Hermanson et al., 2004; Clark et al., 2011). Amplification of genomic sequences flanking pT2/Xla.crygc:attP-GFP was achieved by carrying two nested PCR reactions. For *TaqI*-digested DNA, the 5' end of the construct was amplified using the primer pairs LP1 (5'-GTGTCATGCACAAAGTAGATGTCC-3') and GFPE3 (5'-TGAGTAAAGGAGAAGAAGCTTTTCAC-3') in the first PCR reaction followed by the second PCR reaction with LP2 (5'-ACTGACTTGCCAAAACTATTGTTTG-3') and GFPE1a (5'-TTCAGTGGAGTTGTCCCAATTCTTG-3'). Similarly, the 3' end of the construct was amplified using

nRP1 (5'-CTAGGATTAATGTCAGGAATTGTG-3') and SBR1 (5'-ACACTCAATTAGTATTTGGTAGCAT-3') primers in the first PCR, and RP2 (5'-GTGAGTTAAATGTATTTGGCTAAG-3') and SBR2 (5'-CATTGCCTTAAATGTTAACTTG-3') in the second PCR reaction. For *BamHI/BclI/BglIII*-digested DNA, the 5' end of the construct was amplified with LP1 (see above) and SBF1 (5'-AATTCCAGTGGGTCA-GAAGTTTACA-3'), followed by second PCR with LP2 (see above) and SBF2 (5'-GTTGACTGTGCCTTAAACAGCTTG-3') primers. The 3' end of the construct was isolated using nRP1 and GFPR1 (5'-CAAGAGTCCATGCCGAA-3') as well as RP2 (see above) and GFPR2 (5'-TTGCGAGATACCCAGATCATA-3') in first and second PCRs, respectively. To map Tg(Xla.crygc:attP-GFP)*uobL6* and *uobL12* integrations, genomic DNA was digested in three separate reactions: *TaqI*, *NlaIII*, and *BamHI/BclI/BglIII*. After ligation, inverse PCRs were carried out to amplify the 3' end of the construct only using the same primers for all three differently digested genomic DNA sources: Tol2-R3 (5'-ACTGGGCATCAGCGCAATCAATTG-3') and S1/3' No1 (5'-GTTG-TTAACTTGTATTGCAGCTT-3'), as well as Tol2-R4 (5'-ATAATACT-TAAGTACAGTAATCAAG-3') and S1/3' No2 (5'-CAAACATCA-ATGTATCTTATCAT-3') in first and second PCRs, respectively. In addition to the transposase-mediated integration recovered in Tg(Xla.crygc:attP-GFP)*uobL12*, this line also has a non-transposase-mediated integration even of pTol2/Xla.crygc:attP-GFP. Transgenic lines with integrase docking sites will be made available from the zebrafish stock centres at the Zebrafish International Resource Center (ZIRC) and the Karlsruhe Institute of Technology (KIT).

### Southern blotting

DNA from GFP-positive and GFP-negative embryos from the five recipient lines was digested with *PvuII* and *PstI*. Specificity and completeness of digestions was verified by carrying out a parallel digestion with added plasmid DNA. Digested DNA was separated on 1% agarose gel and transferred to Whatman SPC Nytran membrane sheets (ThermoFisher Scientific). The GFP probe was produced by carrying out a PCR using primers GFP-F3 (5'-TGAGTAAAGGAGAAGAAGCTTTTCAC-3') and S1/3No2 (5'-CAAACATCAATGTATCTTATCAT-3'), then digesting the PCR product with *PstI* and purifying it with ThermoFisher Fermentas Gel Extraction Kit. The PCR fragment was then labelled using DECAPrimeII DNA labelling kit (Ambion) and alphaP32 dCTP (Perkin Elmer) and purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare).

To analyse the copy number of the *uobL12* transgenic recipient line, the nylon membrane was exposed overnight, blocked by light for 30 minutes and transferred to a Storage Phosphor Screen (Molecular Dynamics). It was scanned on a Typhoon 9200 Variable Mode Imager (Amersham Biosciences) with Typhoon Scanner Control software (v5.0) and analysed using ImageQuant 5.1.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

D.B. and F.M. conceived the study, J.A.R., I.M.-E., Y.H., D.B. and F.M. designed experiments, J.A.R., I.M.-E., K.J.S., K.T.W., Y.H., E.K.M., J.B. and D.B. performed experiments, R.S. and E.S. performed computational analyses. J.A.R., I.M.-E., Y.H., J.B., D.B. and F.M. analysed the data, D.B. and F.M. wrote the manuscript with input from J.A.R., I.M.-E., Y.H. and J.B.

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### Supplementary material

Supplementary material available online at  
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