Parallel Genomic Engineering of Two Drosophila Genes Using Orthogonal attB/attP Sites

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ABSTRACT Precise modification of sequences in the Drosophila melanogaster genome underlies the powerful capacity to study molecular structure-function relationships in this model species. The emergence of CRISPR/Cas9 tools in combination with recombinase systems such as the bacteriophage serine integrase ΦC31 has rendered Drosophila mutagenesis a straightforward enterprise for deleting, inserting and modifying genetic elements to study their functional relevance. However, while combined modifications of non-linked genetic elements can be easily constructed with these tools and classical genetics, the independent manipulation of linked genes through the established ΦC31-mediated transgenesis pipeline has not been feasible due to the limitation to one attB/attP site pair. Here we extend the repertoire of ΦC31 transgenesis by introducing a second pair of attB/attP targeting and transgenesis vectors that operate in parallel and independently of existing tools. We show that two syntenic orthologous genes, CG11318 and CG15556, located within a 25 kb region can be genomically engineered to harbor attPTT and attPCC sites. These landing pads can then independently receive transgenes through ΦC31-assisted integration and facilitate the manipulation and analysis of either gene in the same animal. These results expand the repertoire of site-specific genomic engineering in Drosophila while retaining the well established advantages and utility of the ΦC31 transgenesis system.

The amenability of the fruitfly’s genome to targeted manipulation in combination with the vast phenotyping repertoire for this model species has enabled the precise interrogation of gene product functions. Several methodological advances have facilitated the use of directed genomic engineering in the fly. The advent of homologous recombination strategies enabled the exact targeting of genomic sequences in the fly genome, yet the stochastic nature of the occurrence of double-strand breaks (DSB) rendered this method a tedious and time-consuming venture (Gong and Golic 2003). Protocols that have made CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) technology available to Drosophila genomic engineering have overcome these limitations, and single or multiple DSBs can since be exactly and efficiently induced at genomic targets (Bassett et al. 2013; Gratz et al. 2013a; Yu et al. 2013; Kondo and Ueda 2013; Ren et al. 2013; Sebo et al. 2014) . A DSB can either be inaccurately repaired by non-homologous end joining creating a palette of insertion-deletion mutations, the exact sequence of which cannot be controlled by the experimenter. Alternatively, in a gene replacement approach two DSBs release a defined genomic fragment that may harbor an entire gene or part of it (Gratz et al. 2013b). The concomitant provision of a DNA template via a homology directed repair (HDR) vector containing homology arms corresponding to the up- and downstream sequences of the released genomic fragment, a selection cassette for the identification of recombinant progeny, and sequences for their further genomic manipulation, offer an elegant means to pre-determine the precise layout of the engineered allele (Gratz et al. 2014; Port et al. 2014).

The incorporation of an attP (phage attachment) recognition site for the Streptomyces ΦC31 phage integrase within HDR vectors has become a standard procedure to replace an endogenous locus in Drosophila (Huang et al. 2009; Gratz et al. 2014). Subsequent integration of matching attB (bacterial attachment) site-encoding plasmids
leading to the generation of (C) attRCC and (D) attLCC sites. Note the CC cross-over dinucleotide (boxed in blue) present in both hybrid sites. Chromatograms display the forward strand nucleotide sequence (upper strand in B), which was confirmed by corresponding reverse strand sequencing (lower strand in B; not shown).

into attP-carrying flies together through germ-line expression of ΦC31 has greatly enhanced the speed, accuracy and reproducibility of fly transgenesis (Groth et al. 2004; Bischof et al. 2007). Once an attP founder fly line is established, ΦC31 transgenesis can be used to generate an unlimited number of allelic variants of the locus by restoring it with modified genomic fragments that contain mutations, in-frame fusions or other modifications of the genetic element of interest. Consequently, ΦC31-mediated transgene insertion enables high-throughput structure-function studies of genes and their products in Drosophila at single nucleotide and single amino acid resolution, respectively.

The analysis of genetic pathways, the study of gene homologs and the requirement of independent genomic modifications in the same animal requires multiple concurrent changes to its genome. However, the one-on-one compatibility of the attB/attP pair restricts the use of the ΦC31 platform to one locus per fly strain. The targeting of multiple attP sites at different genomic positions with non-identical transgenes in the same founder animal is not possible as each attP-landing pad is equally receptive to the insertion event. Consequently, if and where each plasmid integrates is stochastic violating the concept of site-specificity of ΦC31 transgenesis, one of its most compelling features. For non-linked genomic targets this problem can be solved by the manipulation of each locus of interest in an individual parental line, and their subsequent genetic combination through crossing. In contrast, linked loci on the same chromosome are not amenable to this option, particularly if their genetic distance is too small and thus the frequency of meiotic recombination to place them on the same chromosome is impractically low.

Here we present an alternative approach to permit the manipulation and independent genomic engineering of linked loci through established ΦC31 integrase resources. ΦC31 cleaves double-stranded DNA at a central crossover dinucleotide within attB and attP sites generating a matching two-base pair 5’-TT overhang in both (Figure 1A, Table S1). Subsequently, the integrase swaps the half-sites and ligates the reciprocal partners creating hybrid attL and attR sites (Smith et al. 2004a). While the overhangs are essential for the recombination reaction, their sequences are not as long as they remain reverse-complementary to each other (Colloms et al. 2014). We have capitalized on this aspect of ΦC31 integration and adopted a matched attB/attP pair whose crossover dinucleotide consists of two cytosines (Figure 1B, Table S1; here referred to as attBCC/attPCC) instead of the commonly used thymines in standard ΦC31 vectors for Drosophila (Figure 1A; here referred to as attBTT/attPTT). This allowed us to use a selection of established ΦC31 integrase expressing fly strains with high integration efficiency without changes in the integration protocol. Existing plasmids for CRISPR/Cas9-mediated gene replacement and ΦC31 transgenesis were modified to encode the orthogonal attBCC/attPCC pair.

This approach permitted the targeting and subsequent genomic engineering of two homologs of the adhesion GPCR (aGPCR) family (Hamann et al. 2015), CG11318 and CG15556, which are closely linked on a genomic fragment and separated through intervening genes on chromosome III. We show that our approach can be used to sequentially but also simultaneously integrate transgenes in a chromosome endowed with orthogonal attP sites using ΦC31 while maintaining efficiency, specificity and directionality of the targeting procedure. We demonstrate the utility of this approach by obtaining the co-transcriptional gene activity pattern of the CG11318/CG15556 gene pair in Drosophila.

MATERIALS AND METHODS

Molecular reagents

All plasmids engineered herein were modified using restriction enzymes from New England Biolabs. PCRs were conducted using AccuStar DNA Polymerase (Eurogentec), primers and custom DNA fragments were synthesized by MWG Eurofins or Life Technologies. All intermediate and final constructs were DNA-sequenced to ensure no errors were introduced during the cloning procedures. The template genomic DNA used for PCR amplification throughout the study was from our stock of the w1118 strain (Flybase ID: Fbal0018186).

**pHD-mW-attBCC-FRT** (Addgene ID: 115158): The HDR vector contains a combination of principal elements of the pHD-DsRed-attP targeting.

| Table 1 pU6-gRNA plasmid cloning for CG15556 and CG11318 targeting |
|-------------------|-----------------|-----------------|------------------|
| Gene   | Site | chIRNA plasmid | Primers used |
| CG15556 | 5’ cut | pTL633 | t_657F/t_658R |
|        | 3’ cut | pTL634 | t_659F/t_660R |
| CG11318 | 5’ cut | pTL635 | t_661F/t_662R |
|        | 3’ cut | pTL636 | t_663F/t_664R |

**Figure 1** Orthogonal attP and attB site design. (A) Canonical attP/attB vectors contain a central TT dinucleotide at which the ΦC31 integrase-mediated crossover between the two partner sequences occurs. The recombination event leads to the generation of hybrid attR and attL sites as indicated. (B) The orthogonal att site pair contains a CC instead of the TT sequence as cross-over nucleotide in both attP and attB vectors. (C,D) Sanger sequencing of genomic DNA of recombinant fly strains after insertion of attBCC+ transgenes into an attPCC+ landing site confirms that ΦC31 catalyzes the recombination between these non-canonical elements.
and pGX-attP vectors previously published by (Gratz et al. 2014) and (Huang et al. 2009). It harbors two multiple cloning sites on both sides of the replacement/mini-white marker element that are flanked by type IIS restriction sites, AarI (5′-MCS) and SapI (3′-MCS), respectively, to seamlessly insert homology arms for homology directed repair after CRISPR/Cas9-mediated cleavage of genomic sequences (Gratz et al. 2014). The mini-White marker element is flanked by two FRT sites for its subsequent removal by FLP recombinase expression. In addition, the replacement cassette contains a modified attP Fc31 docking site with the central cross-over nucleotides changed from TT to CC (attPCC). This way, pHD-mW-attPCC-FRT with its selection marker, marker removal sites and attP integration elements can be used in parallel and thus in combination with pHD-DsRed-attP.

The vector was generated as follows. A 175 bp DNA fragment containing the 5′-NdeI-attPCC-FRT-BsiWI-BamHI-FRT-SpeI-3′ elements was custom synthesized (pTL706), and inserted into pHD-DsRed-attP at the NdeI/SpeI restriction sites (pTL716) replacing its attP TT-loxP-DsRed-loxP cassette. Subsequently, the resulting plasmid was opened with BsiWI and BamHI and a 3.0 kb fragment of pGE-attB (Huang et al. 2009) containing the GMR-mini-White cassette was inserted therein generating the final pHD-mW-attPCC-FRT (pTL717) replacement vector (Figure S1A).

pGE-attBCC-FRT-mW (Addgene ID: 115159): The attBCC integration vector was constructed by synthesizing a 153 bp DNA fragment containing 5′-SacII-attBCC-FRT-BsiWI-3′ elements (pTL705). This fragment was then inserted into the pGE-attB plasmid (Huang et al. 2009) after a SacII and BsiWI double digest (pTL788; Figure S1B). The central cross-over nucleotides of the attB Fc31 site are changed from TT to CC (attBCC).

pGE-attBHLOX-DsRed (Addgene ID: 115160): A 1.2 kb PCR fragment containing theloxP-3xP3-DsRed selection/integration cassette was amplified off pHD-DsRed-attP (Gratz et al. 2014) using primers tl_814F/tl_815R, the amplicon was cut with BsiWI/BglII and ligated into the 3.1 kb BsiWI/BamHI fragment.
and inserted into spectively. The DNA fragment was double digested with CRISPR/Cas9 cutting sites 5′ (pTL780; Figure S1C).

Aar CG15556 HDR vector: A0.9k b5 tl_768F/tl_769R, which contained CG11318-GAL4 reporter vector: CRISPR/Cas9 cuts was ampli-

Table 3 PCR primers for detection of attB/P recombination events

| Primer          | 5′-3′ sequence                          |
|-----------------|----------------------------------------|
| attL-CC.F       | GGGCGTGCCCAGGAGGTTACC                  |
| attL-CC.R       | ATAGGAACTTCACTACGGCC                   |
| attP-CC.F       | GTGCCCCAAGGTTAAGGC                    |
| attP-CC.R       | TACGGGCCCAGGTTAAGGC                    |
| attP-TT.F       | TACGAAGTTATCTACGCCCC                   |
| attP-TT.R       | CTACGCCCCAGGTTAAGGC                    |
| attR-CC.F       | GGGCGGCCAGGTTAC                      |
| attR-CC.R       | GGGCGGCCAGGTTAC                      |

of pGE-attB (Huang et al. 2009) to generate pGE-attBTT-locP-DsRed (pTL780; Figure S1C).

pU6-gRNAs: CRISPR/Cas9 cutting sites 5′ and 3′ of the CG11318 and CG15556 loci suitable to remove all exons, UTRs and the promoter regions were identified by CRISPR Optimal Target Finder (Gratz et al. 2014) (Table 1). The genomic sequence of all CRISPR/Cas9 cleavage sites were confirmed by DNA sequencing of PCR fragments encompassing the suggested sites prior to cloning. Target-specific sequences for CG11318 and CG15556 gRNAs were synthesized as 5′-phosphorylated oligonucleotides, annealed, and ligated into the BbsI sites of the pU6-BbsI-chirRNA vector (Gratz et al. 2013a).

CG11318 HDR vector: A 0.9 kb fragment encoding the 5′ homology arm was amplified from genomic DNA using primers tl_681F/682R, cut with AarI and inserted into de-phosphorylated AarI-digested pHDDsRed-attP (pTL645). Subsequently, the 1.2 kb 3′ homology arm was PCR-amplified from genomic DNA using primers tl_683F/684R, cut with SapI, and inserted into de-phosphorylated pTL645 to generate the final CG11318 targeting vector pTL650 (attPTT+, loxP+, DsRed+).

CG15556 HDR vector: A 0.9 kb 5′ homology arm fragment was amplified from genomic DNA using primers tl_673F/674R, cut with AarI and inserted into de-phosphorylated AarI-digested pHDDsRed-attP (pTL645). Subsequently, a 1.4 kb 3′ homology arm fragment was amplified from genomic DNA using primers tl_675F/676R, cut with SapI, and inserted into de-phosphorylated pTL645 to generate the final CG15556 targeting vector pTL724 (attPCC+, FRT+, mW+). For primer design see also Figure S1D.

CG11318-GAL4 reporter vector: A 4.6 kb fragment corresponding exactly the genomic CG11318 sequence removed through the CRISPR/Cas9 cuts was amplified off genomic DNA with primers tl_768F/tl_769R, which contained Nofl and Ascl restriction sites, respectively. The DNA fragment was double digested with Nofl and Ascl and inserted into pGE-attBTT-DsRed to generate a wild-type CG11318 rescue vector (pTL784). In order to insert a GAL4.2 transcription factor cassette at the transcriptional start site of CG11318, a 1.6 kb AgeI/NsiI fragment of pTL784 was subcloned into pTL550 (pMCS5 derivative with KanR; MobITec; pTL785). This subclone was outward PCR-amplified using primers tl_824F/tl_825R to generate a 4.6 kb amplicon. An 1.6 kb fragment encoding the optimized GAL4 cassette was amplified off pBPGal4.2-p65d (Pfeiffer et al. 2010) using primers tl_822F/tl_823R. Both PCR fragments were appended with primer-encoded BgIII and Nhel sites on either end, respectively, digested with BgIII/Nhel and ligated generating clone pTL787. A 3.2 kb AgeI/NsiI fragment of this clone was re-transferred into the CG11318 rescue vector pTL784 to construct the final CG11318-GAL4 reporter allele plasmid pTL789 (attBTT+, loxP+, DsRed+).

CG15556 LexA reporter vector: A 4.0 kb fragment corresponding exactly to the genomic CG15556 sequence removed through the CRISPR/Cas9 cuts was amplified off genomic DNA with primers tl_827F/tl_828R, which contained Nofl and Ascl restriction sites, respectively. The DNA fragment was double digested with Nofl and Ascl and inserted into pGE-attBTT (pTL788) to generate a wild-type CG15556 rescue vector (pTL790). In order to insert a LexA transcription factor cassette at the transcriptional start site of CG15556, a 1.4 kb EcoRI fragment of pTL790 was subcloned into pTL550 (pTL791). This subclone was outward PCR-amplified using primers tl_834F/tl_835R to generate a 4.5 kb amplicon, which was appended with a BstEII site and was re-circularized at an AattII site introduced through both primers (pTL792). The so modified 1.4 kb EcoRI fragment of pTL792 was re-introduced into pTL790 generating pTL793. An 1.7 kb fragment encoding the LexA cassette with primer-inserted AattII and BstEII sites was amplified off pBSK-Lexa-VP16-SV40 (Diegelmann et al. 2008) using primers tl_836F/tl_837R, cut with AattII/BstEII and inserted into pTL793 to generate the final CG15556-LexA reporter allele plasmid pTL794 (attBTT+, FRT+, mW+) (Table 2, Table 3).

Table 4 Details for CRISPR/Cas9 targeting of CG15556 and CG11318

| Target gene | gRNA | HDR repair with | Injection into | % Germine transmission | Transgenesis marker | % Marker removal |
|-------------|------|----------------|----------------|------------------------|---------------------|------------------|
| CG15556     | S1, S6 | attPTT (pTL649) | vas-Cas9;* (#55821) | 7 (4/55) | loxP-DsRed-loxP | N.A. |
|             | S1, S6 | attPCC (pTL724) | vas-Cas9;* (#55821) | 7 (4/60) | FRT-mW-FRT | 100 (3/3) |
|             | S1, S6 | attPCC (pTL724) | vas-Cas9; CG11318CO; | 11 (6/55) | FRT-mW-FRT | 100 (3/3) |
| CG11318     | S7, S10 | attPTT (pTL650) ; vas-Cas9;* (#56552) | 1 (7/75) | loxP-DsRed-loxP | 100 (5/5) |

Indicated gRNAs were co-injected with respective HDR vectors (in brackets) encoding attPTT or attPCC landing pad sites into embryos containing germ-line expressing vas-Cas9 transgenes (Bloomington Drosophila Genome Center stock numbers of source strains in brackets). Injected flies were crossed to y w animals and progeny screened for germ-line transmission of w+ or DsRed+ eye markers (founder animals). At least one founder strain per targeting was sequenced to confirm the presence of the desired targeted lesion.
Figure 2  Gene targeting of linked loci CG15556 and CG11318. (A) Genomic position of CG15556 and CG11318 on chromosome III. Note the presence of two intervening genes between the two aGPCR loci. (B) Molecular layout of the two aGPCRs encoded by CG11318 and CG15556, which are marked by the heptahelical (7TM) and GPCR autoproteolysis-inducing (GAIN) domains, the latter of which contains the GPCR proteolysis site (GPS). Ex, extracellular, In, intracellular. (C) Target and potential off-target sequences (indicated with mismatches to the target sequence by lowercase red letters), location on forward/reverse DNA strand and number of potential off-targets (chromosome arm location of off-targets in brackets) for each gRNA used. (D,E) Targeting scheme for CRISPR/Cas9-mediated gene replacement for (D) CG15556 and (E) CG11318. The upper line in each panel depicts the gene structure, while the lower line indicates the dimensions and elements of the replacement cassette with the selection marker (mWhite, 3xP3-DsRed), attP site type (attPCC or attPTT) and recognition sites for the removal through site-specific Flippase or Cre recombinases (FRT, loxP). Dashed lines indicate homology arms of the HDR vector. Note the position of gRNAs that determine the positions of Cas9 cleavage above each gene locus and were used to replace CG15556 (S1, S6) and CG11318 (S7, S10). (F,G) PCR genotyping results on the genomic removal of (F) CG15556 and (G) CG11318. Each lane represents an individual strain recovered after CRISPR/Cas9-targeting. 5′ and 3′ breakpoints in genomic DNA of strains with confirmed CG15556 (magenta bullets; predicted PCR product sizes 5′=1,306 bp; 3′=1,628 bp) and CG11318 (green bullets; predicted PCR product sizes 5′=1,033 bp; 3′=1,343 bp) removal were subsequently sequenced. (H) PCR genotyping results for separate detection of linked CG15556 and CG11318 removal in doubly targeted strains. Note: a different primer pair than employed in (F) was used to detect the presence of the CG11318 deletion (predicted PCR product size = 658 bp).
LAT540, w1118, +; attPCC{CG15556-rescue mW-}; CG15556KO, attPTT
{CG15556-rescue 3xP-DsRed}CG11318KO; (CG15556Rescue
CG11318 Rescue w DsRed)
LAT584, w1118, +; attPCC{CG15556-LexA mW-}CG15556KO, attPTT
{CG11318-p-GAL4 3xP-DsRed}CG11318KO (CG15556LexA
CG11318 GAL4, w DsRed)

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
BL#32223, y1 trichromatic
X; P{y+t7.7 = CaryIP}su(Hw)attP6;;;
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;
et al.

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
C31 integration: (Gift by Dan Hartl, Harvard University.)
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;

FR-T-flanked marker cassettes: BDSC#851, y1 w67c23
P[yr+17.7 = nos-phiC31\int.NLS] X; P[y+17.7 = Cary]su(Hw)attP6;;;
(BSDC#55821, y1 M{vas-Cas9.RFP-}ZH-2A w1118;;;

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
C31 integration: (Gift by Michael Ashburner, University of Cambridge.)
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;

FLP removal of FRT-flanked marker cassettes: BDSC#6419, y1 w1118
P[yr+17.2 = 70FLP\int]/Dp(1;Y)Y2; TM2/TM6C, Sb1; (Gift by Dan Hartl, Harvard University.)

Others strains: GN86, y1 w1; wgSp1/CyO, P[Wee-P-ph0]BaccWec-P20;
P[yr+17.7 = nos-phiC31\int.NLS] X; P[y+17.7 = Cary]su(Hw)attP6;;;
(Gift by Dan Hartl, Harvard University.)

CRISPR/Cas9 gene targeting and Cas9 VK00037/CyO, P{w+mC =T b1}CprCyO-A;;

LD540, w1118, +; attPCC{CG15556-LexA mW-}CG15556KO, attPTT
{CG15556-LexA mW-}CG11318KO, attPTT
{CG11318-p-GAL4 3xP-DsRed}CG11318KO (CG15556LexA
CG11318 GAL4, w DsRed)

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
BL#32223, y1 trichromatic
X; P{y+t7.7 = CaryIP}su(Hw)attP6;;;
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;
et al.

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
C31 integration: (Gift by Michael Ashburner, University of Cambridge.)
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;

CRISPR/Cas9 targeting: BDSC#56552, w1118; PBac{y+mDint2 = vas-
C31 integration: (Gift by Michael Ashburner, University of Cambridge.)
BL#40161, y1 M{vas-phiC31}ZH-2A w1118;;;

CRISPR/Cas9 gene targeting and Cas9 VK00037/CyO, P{w+mC =T b1}CprCyO-A;;

Other strains: GN86, y1 w1; wgSp1/CyO, P[Wee-P-ph0]BaccWec-P20;
P[yr+17.7 = nos-phiC31\int.NLS] X; P[y+17.7 = Cary]su(Hw)attP6;;;
(Gift by Dan Hartl, Harvard University.)

CRISPR/Cas9 gene targeting and Cas9 VK00037/CyO, P{w+mC =T b1}CprCyO-A;;

Other strains: GN86, y1 w1; wgSp1/CyO, P[Wee-P-ph0]BaccWec-P20;
P[yr+17.7 = nos-phiC31\int.NLS] X; P[y+17.7 = Cary]su(Hw)attP6;;;
(Gift by Dan Hartl, Harvard University.)

CRISPR/Cas9 gene targeting and Cas9 VK00037/CyO, P{w+mC =T b1}CprCyO-A;;

RESULTS

Construction of an orthogonal attBC/C-attPCC pair
We have previously investigated structure-function relationships of the neuronal aGPCR homolog latrophilin/dCIRL using genomic engineering of the dCirl locus in Drosophila (Scholz et al. 2015; 2017). In quest of additional potential fly aGPCR homologs we identified two genes on chromosome III that contain open reading frames encoding seven transmembrane-spanning (7TM) and GPCR autoproteolysis-inducing (GAIN) domains, the combination thereof being the molecular tell-tale signature of the aGPCR family (Figure 2B) (Langenhan et al. 2013). CG15556 and CG11318 display high sequence conservation (data not shown) necessitating the construction of single and double knockout animals to account for possible functional redundancy. However, as both genes are closely linked on a 25 kb genomic fragment and separated through two additional genes (Figure 2A) we sought to remove each gene separately through CRISPR/Cas9-assisted homologous recombination.

To allow for later gene-specific rescue and modification of each locus independently through Cas9-mediated integration, we generated a set of vectors encoding an attBC/C-attPCC site pair that can function orthogonally to attBTT/attPTT sites contained in standard genomic engineering vectors (Huang et al. 2019; Gratz et al. 2013a; Gratz et al. 2014): i. A homology-directed repair vector harboring an attPCC site, in which the central crossover dinucleotide was changed from TT to CC (pHD-attPCC-CRT-mW-FRT). To facilitate the selection of recombinant flies that were also targeted with pHD-DsRed-attP TT (Gratz et al. 2014), the plasmid additionally contains a FRT-flanked mini-White selection cassette for removal through FLP recombination expression rendering all main characteristics of the vector orthogonal to pHD-DsRed-attP TT. All other elements including the multiple cloning sites for homology arm insertion are identical to pHD-DsRed-attP TT (Figure S1A).

M, marker lane; gray bullets, unconfirmed strains; 5’/3’ ext = primer location outside the homology arms of the respective HDR vector; dashed lines indicate removal of intervening lanes from gel images.
and recombination sites

F

C31 mediated insertion of

and transgenes using attBTT and attBCC recombination sites

Injected with integration

Targeting precision

Transgenesis

marker

% Marker removal

attBXX

attBTT

Single

Correct

Incorrect

% Germline transmission

Encoding

attBTT

attBCC

CG15556KO attPCC

CG11318KO attPTT

1) CG11318Rescue

attBCC+

FRT-mW

100 (2/2)

CG15556KO attPCC+

CG11318KO DsRed- attPTT+

2) CG15556Rescue

attBTT+

loxP-DsRed

100 (2/2)

CG11318KO attPTT+

CG15556pLexA

1) CG15556pLexA

attBCC+

FRT-mW

100 (1/1)

CG11318pGAL4

2) CG15556pLexA

attBTT+

loxP-DsRed

100 (2/2)

CG11318pGAL4

FRT-mW + loxP-DsRed

100 (2/2)

CRISPR/Cas9 targeting of CG11318 and CG15556 with canonical attPTT and novel attPCC sites

CG11318 and CG15556 were individually targeted through CRISPR/Cas9-mediated homology directed repair (Gratz et al. 2014) (Figure 2C-E). Chimeric guide RNAs (gRNAs) for the gene targeting were selected to completely remove each gene, 5’ and 3’ UTRs and potential promoter regions (Figure 2C). Homology arms were about 1 kb in length for each HDR vector and placed to immediately edge the Cas9 cleavage sites (Figure 2D-E).

In a first round, CG11318 and CG15556 were individually targeted with a standard HDR plasmid (Gratz et al. 2014) replacing the genes with an attPTT docking site, whereas in a separate targeting round of CG15556 an HDR vector encoding the attPCC variant was used. Cre-mediated DsRed cassette removal in CG15556 recombinants and FLP-mediated mini-White excision (Golic and Lindquist 1989) from CG15556-targeted animals was performed yielding single CG11318KO attPTT+ and CG15556KO attPCC+ knockout/knockin founder animals for both aGPCR loci. Subsequent PCR genotyping and sequencing confirmed the correct insertion of the replacement cassettes into the CG15556 (Figure 2F; Table 4) and CG11318 (Figure 2G; Table 4) loci.

To generate a chromosome lacking both genes, we selected a CG11318KO attPTT+ founder strain, crossed it to a vasa-Cas9 background and targeted CG15556 by CRISPR/Cas9 as described above to yield CG15556KO attPCC+; CG11318KO attPTT+ double mutant founders, which were verified by PCR genotyping and sequencing (Figure 2H; Table 4). mW and DsRed markers were subsequently removed from these recombinants through consecutive rounds of Cre and FLP recombinase to yield CG15556KO attPCC+ mW, CG11318KO attPTT+ DsRed- founders (Table 4).

Integration of attBCC transgenes into attPCC landing pads

We next tested whether the novel attBCC/attPCC pair can be used for ΦC31-mediated recombination. We selected a CG15556KO attPCC+-targeted allele and independently injected two constructs carrying cognate attBCC (CG15556KO attBCC+) for ΦC31 mediated integration and recovered 2 and 3 recombinant founder animals, respectively (Table 5). Sequencing of the genomic site of the attBattP recombination for both transgenic integrants confirmed the formation of hybrid attRC and attLC sites on either side of the inserted DNA fragment (Figure 1C,D; Table S1). This demonstrates that the attBCC/attPCC pair with exchanged overlap dinucleotides allows for directional transgene incorporation in Drosophila (Figure 1B).

We continued to remove the mini-White marker cassette by standard FLP expression (Golic and Lindquist 1989) showing that the pHDR-mW-attPCC.FRT and its matching pGE-attBCC-FRT-mW partner vector can be used to complete ΦC31 assisted allele construction including transgenesis marker removal (Table 5).

Orthogonality of attBTT+ and attBCC+ transgene integration

To evaluate the precision at which attPTT and attPCC sites, concomitantly present in the genome, are targeted we injected CG15556KO w-attPCC+, CG11318KO DsRed- attPTT+ embryos expressing ΦC31 in the germline with either CG15556KO attBCC+ or CG11318pGAL4 attBTT reporter vectors, and recovered independent integrants from each injection (Table 5).
After expanding a balanced stock from each of the resultant CG15556pLexA mW+ or CG11318pGal4+ DsRed+ founder animals, genomic DNA was harvested and subjected to PCR genotyping and DNA sequencing to assess into which genomic DNA was harvested and subjected to PCR genotyping and DNA targeting of two loci is feasible in the same animal. To this end we repeated the FC31 recombinations in two regimes:

i. Sequential attPTT/attPCC targeting: We selected single integrant strains of the transcriptional reporters CG11318pGal4 and CG15556pLexA that contained an unoccupied attPCC or attPTT site, respectively, and repeated the FC31 protocol with the other transgenic reporter not present yet. I.e. CG15556KO w- FRT+ attPCC[CG15556pLexA mW+ FRT+], CG11318KO DsRed loxP+ attPTT+ animals were injected with a plasmid carrying CG11318pGal4 attBTT+ DsRed+ loxP+, while CG15556KO w- FRT+ attPCC+, CG11318KO DsRed:loxP+ attPTT[CG11318pGal4 DsRed+ loxP+] embryos received the CG15556pLexA attPCC- mW+ FRT+ vector. Resulting recombinants were selected by the presence of both eye selection markers.

ii. Simultaneous attPTT/attPCC targeting: CG15556KO w- FRT+ attPCC+, CG11318KO DsRed:loxP+ attPTT+ founders were injected with both plasmids as in (i) in the same injection round.

We successfully recovered double recombinants with both regimes (Table 5) demonstrating that the dual targeting of two loci is feasible in succession but also simultaneously, although for the latter expectedly at the expense of efficiency (Table 5). The precision of attPTT+ and attPCC+ transgene integration into their respective genomic landing pads within the same genome was confirmed by PCR genotyping (Figure S2). In addition, we crossed a founder line carrying the linked CG11318pGal4 CG15556pLexA transcriptional reporter alleles to a strain with matching receiver gene expression patterns confirming whole transcriptome microarray and RNAseq datasets made available through the Flyatlas (Chintapalli et al. 2007) and modENCODE (Graveley et al. 2011) projects.

In sum, this indicates that the overlap dinucleotide difference in both attP sites allows for a sufficiently high specificity of cognate attB vector integration using standard FC31 expression and injection protocols, and that both attB/attP pairs function orthogonally to each other. Nonetheless, future projects that will increase the sample size of parallel targetings with this approach are warranted to gain a definitive estimate on the specificity of both attB/attP site pairs.

**Figure 3** Transcriptional expression pattern of CG15556 and CG11318 in the third instar larva. The patterns were obtained through single (A) CG11318-GAL4, (B) CG15556-LexA, and (C) double CG11318-GAL4, CG11318-GAL4 reporter lines in third instar larvae. For clarity the genotype of the reporter carrying parent is given in the lower left corner of each panel. Anterior to the left, posterior to the right. Chevrons, gut; arrowheads, Malpighian tubules; arrow, anal pad; asterisk marks CG15556 expression in neuronal or tracheal profiles in the head. Scale bars = 0.5 mm.

**Discussion**

Here, we demonstrate a simple, easily adaptable and efficient system for the separate and repeated manipulation of two linked genetic loci in *Drosophila* (Figure 4). This protocol capitalizes on the currently most widely used genomic engineering toolkit for this model species, the FC31 integrase assisted transgenesis method (Bischof et al. 2007). Altogether, the results indicate that the specificity, directionality and recombination efficiency of the orthogonal attBCC/attPCC site pair introduced here can be used in conjunction with the canonical attPTT/attPTT system and established FC31 resources to handle two transgenesis targets independently and simultaneously.

This confirms results obtained in *E. coli* using an array of similar orthogonal attB/attP site pairs including the attBCC/attPCC version used here in *Drosophila* (Colomns et al. 2014). The successful recombination
or attB<sup>CC</sup> and attP<sup>CC</sup> elements suggests that also in metazoan cells the parallel alignment of the recombination sites during synopsis of the DNA strands is not influenced by the nature of the crossover nucleotides as long as they are reverse-complementary to each other (Smith et al., 2004b). Likewise, our work further implies that sites carrying asymmetric central overlap dinucleotides (suppliers for widely used yet by academic laboratories and not offered by commercial suppliers for fly transgenesis services) may thus even be combined with non-ϕC31 integrase mediated transgenesis tools, and in addition also be adopted by non-ϕC31 systems to expand their own recombinatorial transgene target logic.

ACKNOWLEDGMENTS

We thank the O’Connor-Giles and Hong labs for sharing plasmids for this study, M. Oppmann K. Heise, S. Lautenschläger for expert technical assistance, and N. Scholz and D. Ljaschenko for comments on this manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft to T.L. (FOR 2149/P01 [LA 2861/4-2] and P03 [LA 2861/5-2], SFB 166/C03, LA 2861/7-1). Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. The authors declare no conflict of interest.

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*Communicating editor: H. Salz*