Recombineering-mediated tagging of Drosophila genomic constructs for in vivo localization and acute protein inactivation

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ABSTRACT

Studying gene function in the post-genome era requires methods to localize and inactivate proteins in a standardized fashion in model organisms. While genome-wide gene disruption and over-expression efforts are well on their way to vastly expand the repertoire of Drosophila tools, a complementary method to efficiently and quickly tag proteins expressed under endogenous control does not exist for fruit flies. Here, we describe the development of an efficient procedure to generate protein fusions at either terminus in an endogenous genomic context using recombineering. We demonstrate that the fluorescent protein tagged constructs, expressed under the proper control of regulatory elements, can rescue the respective mutations and enable the detection of proteins in vivo. Furthermore, we also adapted our method for use of the tetracysteine tag that tightly binds the fluorescent membrane-permeable FlAsH ligand. This technology allows us to acutely inactivate any tagged protein expressed under native control using fluorescein-assisted light inactivation and we provide proof of concept by demonstrating that acute loss of clathrin heavy chain function in the fly eye leads to synaptic transmission defects in photoreceptors. Our tagging technology is efficient and versatile, adaptable to any tag desired and paves the way to genome-wide gene tagging in Drosophila.

INTRODUCTION

Drosophila melanogaster is an important model organism in the study of biology and disease. Critical in these studies is a versatile transgenesis platform (1). In Drosophila, efficient transformation systems exist based on P elements (2) and site-specific integration mediated by the integrase from bacteriophage φC31 (3,4). Furthermore, recent advances based on recombineering-mediated gap repair and φC31-mediated site-specific integration allow the cloning and transformation of large genomic DNA fragments of the Drosophila genome (5). A critical subsequent step is to perform gene modification such as tagging the cloned genes with functional proteins or peptides, allowing the detection as well as manipulation of the corresponding tagged proteins in vivo.

Different strategies for tagging Drosophila proteins have been described. Most technologies are based on cloning of cDNA sequences fused to protein and peptide tags that are expressed in vivo using the UAS/GAL4 system (6). While such applications may be suitable to study subcellular distribution of proteins, these strategies lead to over-expression of the tagged genes, and expression is not controlled by the native promoter elements of the studied gene. Existing Drosophila gene-tagging strategies within a genomic context are limited and time-consuming. These strategies employ high-copy plasmids, precluding efficient recombineering (see below), hence relying on conventional cloning, and they are limited to inserts of only up to 20 kb (7,8). An alternative that overcomes these limitations involves protein trapping (9–11). Protein traps are transposons harboring a protein tag flanked by splice acceptor and donor sites such that an intronic
insertion of the transposon in a gene of interest may result in the creation of a fusion protein expressed under control of native elements. Protein traps are undoubtedly a great gene- and protein-discovery tool; however, expression of a functional-tagged protein depends on an intronic in-frame splice event and on insertion of the tag at a location where it does not disrupt functional domains of the protein. In addition, transposon insertions in the genome occur relatively randomly and functional protein trap insertions cannot be obtained for intronless genes, hampering the applicability for straightforward functional analyses of any gene of interest. Hence, while progress has been made towards gene tagging in Drosophila, a universal method that allows for the quick modification of an endogenously expressed gene of interest with a variety of functional tags is not available to researchers using Drosophila as a model.

Here, we describe the development of a proficient gene tagging strategy based on recombineering that may be easily adapted for high-throughput applications. Recombineering avoids the use of restriction enzymes and DNA ligase. Instead, the technology supports the modification of plasmids using PCR products or oligonucleotides that contain the DNA change as recombination templates (12). Hence, integrations such as protein tags can be introduced at will, allowing for the detection and manipulation of the corresponding tagged proteins in vivo. Different paradigms such as positive/negative selectable markers (13) and selectable markers (14) have been developed to facilitate the recombineering process. While these technologies have been adapted to allow for recombineering-mediated tagging of Caenorhabditis elegans (13,14) and mammalian (15) genes, it does not yet exist for tagging D. melanogaster genes.

The method we describe allows for the efficient tagging of any gene of interest in Drosophila transformation vectors with a variety of functional sequences and is based on novel selection cassettes that we created. We describe tagging of several endocytic proteins and demonstrate that these proteins are functional and can be detected in live animals. Furthermore, we also tag clathrin heavy chain (chc) with a tetracysteine tag allowing for the detection as well as acute inactivation of the protein in vivo using FlAsH-mediated fluorescein-assisted light inactivation (FALI) (16,17). Our tagging strategy expands on existing methods in that it not only allows us to study protein expression and localization, but it also enables us to analyze protein function by using acute protein inactivation based on FlAsH-FALI.

**MATERIALS AND METHODS**

**Bacterial transformation, colony PCR, primers, enzymes and plasmid copy number induction**

Bacteria were grown in Luria-Bertani (LB) broth. The following antibiotic concentrations were used: kanamycin (30 μg/ml:K30) for selecting insertion of tag-selection cassettes, ampicillin (100 μg/ml: A100) for attB-P(pacman)-ApR (5) also when tag-selection cassettes were inserted, and tetracycline (10 μg/ml:T10) for the DY380 recombineering strain (18). Antibiotics were obtained from USB. L-arabinose was obtained from Sigma, Bornem, Belgium and used for induction of the Cre recombinease in the EL350 strain (18). Electroporations were performed in 1mm cuvettes using a Biorad Gene Pulser set at 1.8 kV, 200 μF and 25 μF. SOC medium was used in recovery steps after electroporation. Colony PCR screening was performed using the following parameters: a denaturation cycle (94°C for 10 min), 35 amplification cycles (94°C, 30 s; 52°C, 30 s, 72°C, 1 min) and a post-amplification cycle (72°C, 10 min). Primers were obtained from Operon or Eurogentec as salt-free purified; no additional purification steps were required for efficient and correct recombineering. The default screening/sequencing primers used for all tag-selection cassettes are PL452-5′-Seq-R (TAAAGCGCATGCTCCAGACTG) and PL452-3′-Seq-F (GGTGCGCTCTATGGCTTCTGA) (Figure 1A). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Leusden, Belgium. Taq polymerase was obtained from Qiagen, Venlo, The Netherlands or Invitrogen, Merelbeke, Belgium and Pfu polymerase was obtained from Stratagene, La Jolla, CA, USA. Gel and PCR purifications were carried out using the QIAquick Gel Extraction and the PCR Purification Kit (Qiagen). Miniprep plasmid DNA was prepared using the QIAprep Miniprep Kit (Qiagen). P(acman) plasmid copy induction was performed using EPI300 copy-up bacteria (Epicentre, Madison, WI, USA) and CopyControl solution (Epicentre) according to the supplier’s instructions.

**Generation of tag-template vectors harboring the tag-selection cassettes used for N- or C-terminal tagging**

Plasmid backbones of the tag-template vectors are derived from PL452 (19). PL452 and the tag-template vectors were grown in A100 and/or K30. Fluorescent protein tags used in this study are CyPet (20), YPet (20), EGFP (Clontech, Saint-Germain-en-Laye, France) and mDsRed (Clontech). The Flag-4c peptide MDYKDDDDKGS AGSFLNCCPGCCM CE is an artificial peptide fusion between the flag peptide DYKDDDDK and the optimized FlAsH peptide FLNCCPGCCM separated by the peptide linker GSAQS (21).

To create N-tag-template vectors, the 5′ LoxP site was removed from PL452 and re-introduced in another transcriptional reading frame through PCR of the relevant peptide-avoiding stop codons between the introduced tag and the gene of interest (Figure 1A). PCR primers used were tag-KpnI-F and tag-wo-STOP-LoxP-NheI-R (Supplementary Table 1). LoxP containing primes were HPLC purified. Templates used were the relevant tag containing plasmids pBAD33-CyPet (20), pBAD33-YPet (20), pEGFP-N1 (Clontech) and pDsRed-Monomer-N1 (Clontech). The template for N-Flag-4C is an HPLC purified primer (Flag-4C-tag-NEW-wo-STOP) encoding the peptide-avoiding codons rarely used by D. melanogaster (Supplementary Table 1). The resulting PCR fragments were subcloned as KpnI/NheI fragments in PL452, upstream of the kanamycin selection marker, resulting in N-tag-template vectors containing the selection cassettes.
For C-terminal tag fusions, the relevant tag was amplified through PCR using tag-BamHI-F and tag-SacII-R (Supplementary Table 1). Templates used were the same plasmids as for the N-tag-templates except, for C-Flag-4C, we designed an HPLC purified primer (Flag-4C-tag-NEW-with-STOP) encoding the peptide (Supplementary Table 1), again avoiding codons rarely used by *D. melanogaster*. The resulting PCR fragments were subcloned as BamHI/SacII fragments in PL452, downstream of the *kanamycin* selection marker, resulting in C-tag-template vectors containing the selection cassettes. Note that other tag-template vectors can be generated by cloning the tag in PL452 using this strategy.

**Amplification of the tag selection cassettes from the tag-template vectors**

Tag-selection cassettes were PCR amplified from the relevant template vectors using both recombineering primers (GOI-N-tag-F and GOI-N-PL452-R for N-terminal fusions and GOI-C-PL452-F and GOI-C-tag-R for C-terminal fusions) using the following parameters: a
denaturation cycle (94°C for 10 min), 35 amplification cycles [94°C, 30 s; 44-66°C (12 PCR reactions in a gradient), 30 s; 72°C, 2 min] and a post-amplification cycle (72°C, 10 min). The resulting PCR product was gel purified, digested with DpnI to remove left-over methylated template plasmid and PCR purified.

Recombineering with the tag-selection cassettes

All recombineering-mediated gap repair was performed as described (5). Recombineering-mediated tagging with the PCR product encompassing the tag-selection cassette was performed similarly with minor modifications using the DY380 strain (18). More specifically, attB-P(acman)-ApR containing the genomic fragment of interest was transformed in the DY380 strain and selected on LB plates (A100/T10). Note that other P(acman) vectors (5), conditionally amplifiable bacterial artificial chromosome (BAC)’s (22) or other BAC backbones containing different selection markers can be used as long as the antibiotics resistance is adapted to the plasmid used. Single DY380 colonies containing attB-P(acman)-ApR were grown and heat shocked prior to making the cells electroporant and then electroporated with 1 µl of purified PCR product. Potential recombinants were selected on LB plates (A100/K30) at 30°C. Correct recombination events were identified through colony PCR screening using primers GOI-reco-F and PL452-3'Seq-R in one PCR reaction and PL452-3'Seq-F and GOI-reco-R in a second PCR reaction (Figure 1 and Supplementary Table 2). Note that each PCR results in different sized bands depending on an N- or C-tag fusion. Colonies that were positive for both colony PCR assays were processed further. Plasmids were isolated from the DY380 cells and electroporated into EPI300 cells, and plasmid copy number of single colonies was induced as described in Kasprowicz et al. (manuscript submitted for publication). Used genotypes were: w, yw, yw; 4C-chc+, yw; synj+-YPet, w chc1; 4C-chc+, yw GMR-LacZ eyFLP; FRT42D/FRT42D cl2R P[w+] yw GMR-LacZ eyFLP; FRT42D synj'/FRT42D cl2R P[w+].

FlAsH-FALI of chc in photoreceptors

To inactivate the chc in photoreceptors using FlAsH-FALI, we immobilized control w1118 and w chc1; 4C-chc+ flies with ‘liquid Pritt glue’ on a microscope slide. Then flies were microinjected directly underneath the photoreceptor layer with ~0.5 µl of FlAsH reagent (1 µM in HL-3) [4’,5’-bis(1,3,2-dithioarsolan-2-yl) fluorescein, Invitrogen] or HL-3 (110 mM NaCl, 5 mM KCl, 10 mM NaHCO3, 5 mM HEPES, 30 mM sucrose, 5 mM trehalose, 10 mM MgCl2, pH 7.2) and left for 10 min to equilibrate. Microinjections were done with a FemtoJet microinjector (Eppendorf, Hamburg, Germany) using Femtotips of 0.5–0.2 µm (Eppendorf). Immobilized and injected w1118 control and w chc1; 4C-chc+ animals were then illuminated using 500 ± 12 nm epifluorescent light for 5 min (or not illuminated), as indicated. Synaptic transmission between photoreceptors and the rest of the brain was tested by recording electroretinograms (ERG) (see below).

Immunohistochemistry, imaging, behavior test and ERG

To detect expression of tagged proteins, we dissected third instar brains of yw; synj+-YPet larvae and mounted them in vectashield (Vector labs, Peterborough, UK). yw; synj+-YPet larval fillets were also fixed with 3.7% formaldehyde, permeabilized with 0.4% Triton-X100 and labeled with anti-Dlg antibodies (Developmental studies hybridoma bank, Iowa City, USA). Primary antibodies were used at 1:50, Alexa555 conjugated secondary antibodies (Invitrogen) were used at 1:500. Images were taken on a BioRad Radiance confocal microscope (40×) and processed with Photoshop 7.

Expression of 4C-chc+ was detected by incubating third instar fillets in 1 µM FlAsH reagent (in HL-3) for 10 min. Unbound FlAsH was washed away with 1 × ‘Bal buffer’ (Invitrogen) diluted in HL-3 and with HL-3. FlAsH fluorescence was excited at 500 ± 12 nm and imaged with a cooled digital camera (Nikon model DS-2MBwc), using YFP emission filters and a 40× water immersion lens (NA 0.8) on a Nikon Eclipse F1 microscope. Data were processed with Photoshop 7.

Climbing behavior of flies was determined by tapping flies down in an empty vial and counting the number of flies that crossed a 4 cm mark within 5 s. The flying ability was determined by holding flies with forceps by their wings above a circular pad and dropping them. If they fall on the pad they were scored as unable to fly (23).

ERGs were recorded as described (24), except flies were immobilized with ‘liquid Pritt glue’ and we used a green
LED light digitally controlled to present 1 s light pulses. Data were stored on a PC with Clampex 10 and processed with Clampfit and Canvas 7.

RESULTS
Construction of universal template vectors
Our gene tagging strategy is based on a two-step protocol. In a first step, the gene of interest is retrieved in the fly transformation competent-P(acman) vector (5). In a second step, this fragment is modified with an N-tag- or C-tag-selection cassette amplified by PCR from one of the universal tag-template vectors (N-tag or C-tag) (the second step of the procedure is shown in Figure 1A).

The PCR fragment harbors a kanamycin selection marker and a protein tag (Figure 1B). The N-tag- and C-tag-template vectors that we created can be used for N- or C-terminal tagging of the gene of interest with various markers including the green and red fluorescent proteins, EGFP and mRFP, respectively; and the cyan fluorescent protein, that tightly binds the fluorescein derivatives FlAsH or ReAsH (21). To create the tag-template vectors for N-terminal tagging, we combined each of the tags without their termination codon upstream of a LoxP-flanked kanamycin marker (19), ensuring that the LoxP site 3' of the tag and the tag itself are in frame (see Materials and methods section). Similarly, we created tag-template vectors for C-terminal tagging that combine the tags with a termination codon downstream of a LoxP flanked kanamycin marker, again ensuring that the loxP site 5' of the tag and the tag itself are in frame. Note that other N-tag- or C-tag-template vectors can be easily created to accommodate any desired tag (25).

Efficient tagging of several genes
Next, we used the strategy outlined in Figure 1 to create genomic constructs with various tags that allow for the detection or manipulation of the tagged proteins in vivo. In a first step, we used recombineering to retrieve clathrin heavy chain (che), endophilin (endo) and synaptotagmin (synj) in attB-P(acman)-ApB by gap repair (5). We then used tag-template vectors (Figure 1A) to PCR-amplify the tag-selection cassettes with gene specific primers (Figure 1B). These primers are 'recombineering primers' containing ~20 bp annealing homology against the respective tag-selection cassettes (Table 1) and ~50 bp recombineering homology against the gene of interest (Supplementary Table 2). More specifically, we amplified the N-tag-selection cassette from the N-Flag-4C vector to tag che, the C-tag-selection cassette from C-CyPet to tag endo and the C-tag-selection cassette from C-Ypet to tag synj. We then used recombineering to integrate the amplified tag-selection cassettes just downstream of the translational initiation codon of the gene of interest for N-terminal tagging or just upstream of the translational termination codon of the gene of interest for C-terminal tagging and used kanamycin selection to enrich for integration events (Figure 1C). Correct recombination events were identified by PCR, using a second set of gene specific primers (GOI-reco-F and GOI-reco-R) and two default primers specific for the selection cassette (PL452-5'-Seq-R and PL452-3'-Seq-F) (Figure 1D and Table 1). Two PCR's, one with GOI-reco-F and PL452-5'-Seq-R, and a second one with PL452-3'-Seq-F and GOI-reco-R, identify

Table 1. Annealing sequence used to amplify TAG-selection cassettes

| Template vector | Primer name  | Primer sequence (5’ to 3’)|
|-----------------|-------------|---------------------------|
| N-CyPet         | N-CyPet-F   | ATGTCCTAAAGGTGAAGAATTTATTCC |
|                 | N-term-PL452-R | ACTAGTTGGATCCCCCTCGAGGAGGC |
| N-YPet          | N-YPet-F    | ATGTCCTAAAGGTGAAGAATTTATTCC |
|                 | N-term-PL452-R | ACTAGTTGGATCCCCCTCGAGGAGGC |
| N-EGFP          | N-EGFP-F    | ATGGTGAACAGGGCGAGGAGGAGGAG |
|                 | N-term-PL452-R | ACTAGTTGGATCCCCCTCGAGGAGGC |
| N-mDsRed        | N-mDsRed-F  | ATGGACAACACCGAGGAGGCAGGC |
|                 | N-term-PL452-R | ACTAGTTGGATCCCCCTCGAGGAGGC |
| N-Flag-4C       | N-Flag-4C-F | ATGGATTACAAAGGATCCAGGAGGAG |
|                 | N-term-PL452-R | ACTAGTTGGATCCCCCTCGAGGAGGC |
| C-CyPet         | C-CyPet-F   | GCAGCCCAATCTCCGATCATATTC |
|                 | C-term-PL452-F | TTTATTGTACAAATTTCCATAC |
| C-Ypet          | C-Ypet-F    | GCAGCCCAATCTCCGATCATATTC |
|                 | C-term-PL452-F | TTTATTGTACAAATTTCCATAC |
| C-EGFP          | C-EGFP-R    | GCAGCCCAATCTCCGATCATATTC |
|                 | C-term-PL452-F | TTTATTGTACAAATTTCCATAC |
| C-mDsRed        | C-mDsRed-R  | GCAGCCCAATCTCCGATCATATTC |
|                 | C-term-PL452-F | TTTATTGTACAAATTTCCATAC |
| C-Flag-4C       | C-Flag-4C-R | CTACTGGGAGCCCAGTTGCGGCGGCCTC |
|                 | C-term-PL452-F | GTAGCCCAATCTCCGATCATATTC |

Annealing sequence used to amplify selected Tag-selection cassettes for N- (top five vectors) or C-terminal protein fusions (bottom five vectors). To design appropriate primers that can amplify the Tag selection cassettes, left or right homology arm sequences need to be included: Approximately 50 base pairs of gene specific recombineering homology should be included 5' of these primers (see Supplementary Table 2) adding up to 70-bp in total. The gene specific homologies that are to be included are a left homology arm for the forward primers and a right homology arm for the reverse primers.
correct recombination events. Finally, the \textit{LoxP} flanked \textit{kanamycin} selection gene in the integrated selection cassette was removed using \textit{Cre}-recombinase expressing bacteria (Figure 1E) (18). Our strategy leaves an in-frame \textit{LoxP} site behind, that when translated, adds a small unstructured 20 (for N-tagging) or 30 (for C-tagging) amino acid linker (Figure 1F), potentially improving folding and/or function of the tagged protein or the tag itself. Our strategy to tag proteins using tag-selection cassettes amplified from newly created template vectors not only allows identification of correct integration events with minimal effort, but the method may also facilitate large scale efforts to tag multiple \textit{Drosophila} genes simultaneously.

\textbf{Tagged proteins are expressed in vivo}

As illustrated in Figure 2, the tagging strategy resulted in an N-terminally Flag-4C tagged \textit{chc} (Figure 2A), a C-terminally CyPet tagged \textit{endo} (Figure 2B) and a C-terminally YPet tagged \textit{synj} (Figure 2C). To test if the tags are functional \textit{in vivo}, we created transgenic \textit{Drosophila} for the N-terminally Flag-4C tagged \textit{chc} (4C-\textit{che}+) and for the C-terminally YPet tagged \textit{synj} (\textit{synj}+-\textit{YPet}) (Figure 2A and C) using \textit{P}-element-mediated transformation. While the constructs we transformed are sufficiently small to use \textit{P}-element-mediated transformation (<35 kb), the \textit{P}(acman) vectors that we use also contain an \textit{attB} site allowing for \textit{PhiC31}-mediated integration of larger pieces of DNA (5) at defined docking sites in the genome (3–5,26), facilitating high throughput applications.

To test expression of the constructs we performed western blot analysis and found expression of the tagged proteins (data not shown). Furthermore, we also tested expression \textit{in situ}. While third instar \textit{yw} control larvae do not show any fluorescence, larvae harboring the \textit{synj}+-\textit{YPet} construct show YFP fluorescence in the neuropile of the larval ventral nerve cord and at presynaptic neuromuscular junction boutons demarcated by anti-DLG (PSD-95) labeling, in line with the previously reported endogenous localization of Synj (Figure 2D–F).
Similarly, unlike controls labeled with the membrane permeable 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH) (27), w chc+; 4C-chc+ larval fillets show clear fluorescence in the synaptic boutons of 4C-chc+ animals and low levels in muscles, resembling the previously reported endogenous localization of chc (Figure 3C and D) (28). Hence, the tagged constructs are expressed and the proteins localize very similarly to their endogenous counterparts.

**Tagged proteins are functional and do not cause lethality or toxicity**

To determine if the presence of these constructs affects viability, climbing or flight, we performed motor performance assays as shown in Figure 3. While w chc– mutant flies are embryonic lethal (29), w chc+; 4C-chc+ flies that harbor 4C tagged chc are viable and do not show any obvious developmental defects, indicating the 4C-chc+ protein is fully functional. Moreover, when compared to controls (yw or w), flies that harbor the 4C-chc+ and synj+-YPet constructs do not show defects in their ability to climb, negatively geotax, or fly, indicating that the presence of the tagged protein is not toxic (Figure 3A and B). Furthermore, we recorded ERGs (Figure 2C). ERGs are electrophysiological field recordings during a light flash that report on the ability of photoreceptors to depolarize in response to light and to communicate with postsynaptic cells as determined by the presence of ‘on’ and ‘off’ transients (Figure 3D, arrowheads in the ‘control recording’). In line with ERG defects recorded in numerous other mutants that affect synaptic transmission, homozygous mutant eyes for synj+ show no ‘on’ and ‘off’ transients in response to a light flash (24), indicating defects to properly activate the post-synaptic cells of the fly eye (Figure 3D; yw eyFLP; synj+). However, we did not observe significant variation in ERG traces between controls and yw; 4C-chc+ or yw; synj+-YPet flies (Figure 3D), suggesting that expression of the tagged proteins does not significantly affect the physiology of the photoreceptors. Similarly, ERG recordings of w chc+; 4C-chc+ flies are comparable to w controls (Figure 3D). Hence, the data suggest that the tagged constructs are functional and can replace the function of the endogenous gene.

**FlAsH-FALI of chc causes synaptic transmission defects**

To further expand on the usefulness of the technique, we extended our tagging strategy beyond fluorescent proteins and also included the tetracysteine tag that allows the acute inactivation of tagged proteins using FlAsH-FALI (17). The chc is an essential gene, encoding a structural protein thought to be critical for vesicle cycling (30); however, its function in a live organism has not been investigated. To study the function of chc in Drosophila photoreceptors, we used FlAsH-FALI. As shown in Figure 2A, we generated a 4C tagged chc and constructed...
null mutant animals that harbor the 4C-chc rescue construct. To load the 4C-chc expressed in the eyes of these flies with FIAsh, we micro-injected the compound directly underneath the photoreceptor layer (Figure 4A). For controls, we also micro-injected w control flies with FIAsh and we injected physiological solution (HL-3) in w and w chc1; 4C-chc+ flies. Following a 10 min equilibration period, eyes of injected flies were illuminated for 5 min with 500 ± 12 nm epifluorescent light to induce FALI of chc (Figure 4A). We then tested synaptic transmission efficiency by recording ERGs. While most controls injected with HL-3 or FIAsh with or without illumination show normal ‘on’ and ‘off’ transient responses, w chc1; 4C-chc+ flies injected with FIAsh and illuminated lack ‘on’- and ‘off’-transients in response to a 1 s light flash, indicating synaptic communication defects. Hence, our tagging strategy in combination with FIAsh-FALI allowed us to study the effect of chc loss of function, circumventing organismal as well as cell lethality associated with the chc1 mutation and the data furthermore indicates that chc functions at the tonically firing synapses of the fly eye to regulate synaptic transmission.

**DISCUSSION**

Here, we describe an effective strategy to create tagged genomic constructs that can be used to create transgenic *Drosophila*. Our method offers several advantages over existing methods to create tagged genomic constructs for *Drosophila* (7,8). First, the tag is inserted in the endogenous locus allowing native elements to control expression of the tagged protein. Second, the technology relies on recombineering ensuring the precise integration of the tag at the N- or C-terminus and avoiding conventional cloning using restriction enzymes and DNA ligase. Third, the tag-template vectors can also be used to create internal tag fusions useful for proteins that harbor functional domains at their N- or C-terminal ends. Fourth, the technique is faster than creating antibodies against the protein of interest. Finally, while we already created 10 different template vectors with five different tags, the technology can easily be adapted to create protein fusions with other tags harboring different functionalities, including tags for protein or protein–DNA complex purification.

We extended the tagging strategy beyond the standard fluorescent protein tags used to tag genes in other species (14,15). We employed the tetracysteine peptide as a ‘dual purpose tag’, not only allowing for the detection of protein localization but also enabling to acutely inactivate any protein of interest in order to study its function in vivo. While several methodologies exist to study gene function in developed tissues in flies, including MARCM and other clonal analysis tools based on mitotic recombination (31); or tissue-specific expression of RNAi (32), these techniques suffer from the long inactivation time needed to create ‘loss of function tissue’ and concomitant developmental defects due to loss of gene function. FIAsh-FALI on endogenously expressed proteins avoids possible artifacts that could arise from overexpressing 4C-tagged proteins, while allowing quick and local inactivation of the proteins studied. As a proof of principle, we studied the role of chc in synaptic transmission in the eye. While loss of chc would cause developmental and cellular defects (33–35), acute inactivation of chc expressed under native control allowed us to test its role in adult neurons of a live organism. We believe that our strategy will vastly expand the toolkit available to Drosophilists and will enable efficient tagging of large collections of genes with a variety of proteins and peptides.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.
ACKNOWLEDGEMENTS

We are indebted to Hugo Bellen for comments on the article. We also thank Yuchun He for embryo injections, members of the Bellen and Verstreken labs for comments and the Bloomington Drosophila stock center. NCI Frederick and Patrick Daugherty for materials. Plasmids encoding tag-selection cassettes are available for academic research at Addgene: http://www.addgene.org/. Howard Hughes Medical Institute to the Bellen lab, VIB to PV and BAH and the Research fund K.U.Leuven and a Marie Curie Excellence Grant (MEXT-CT-2006-042267) to P.V. Funding to pay the Open Access publication charges for this article was provided by VIB.

Conflict of interest statement. none declared.

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