

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 FIAsh 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 Φ C31 (3,4). Furthermore, recent advances based on recombineering-mediated gap repair and Φ C31-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

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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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 can not 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 tetracycline tag allowing for the detection as well as acute inactivation of the protein *in vivo* using FIAsh-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 FIAsh-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(acman)-Ap^R (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 recombinase in the EL350 strain (18). Electroporations were performed in 1 mm cuvettes using a Biorad Gene Pulser set at 1.8 kV, 200 Ω and 25 µFD. 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 (GGTGGGCTCTATGGCTTCTGA) (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-Layne, France) and mDsRed (Clontech). The Flag-4C peptide MDYKDDDDKGS AGSFLNCCPGCCMEP is an artificial peptide fusion between the flag peptide DYKDDDDK and the optimized FIAsh peptide FLNCCPGCCMEP separated by the peptide linker GSAGS (21).

To create N-tag-template vectors, the 5' *LoxP* site was removed from PL452 and re-introduced in another translational reading frame through PCR of the relevant tag-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 primers 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.

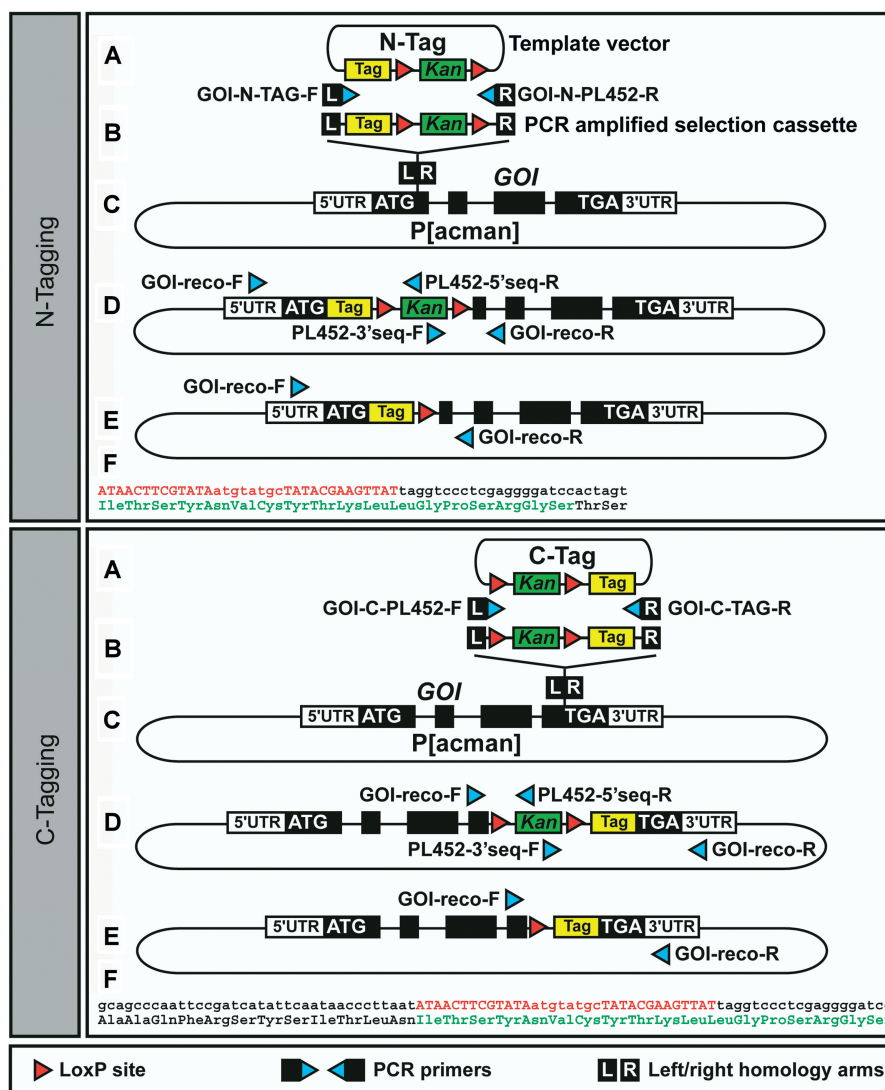


Figure 1. Tag-selection cassettes and strategy to insert N- (Top) or C- (Bottom) terminal protein tags in P(acman) rescue fragments for *Drosophila* transgenesis. (A) N-tag- and C-tag-template vectors, containing tag and a *kanamycin* (*Kan*) selection marker, were generated and can be used to create N- or C-terminal protein fusions of a gene of interest. (B) Using the N- or C-tag-template vectors (A) and the primers indicated in (B) tag-selection cassettes are PCR amplified. The 70-bp primers harbour 20-bp homology to the tag-template vectors (blue arrowhead) required for PCR amplification and 50-bp homology to the target region (black box with left homology arm, L; and right homology arm, R). These 50-bp homology arms are required for recombineering. (C) The PCR fragment is subsequently transformed in recombination competent bacteria that harbour P(acman) containing the gene of interest, previously obtained by gap repair. (D) Recombination between the homology arms in the PCR fragment and the GOI occurs and recombination events are selected using *kanamycin*; correct recombination events are identified by PCR using GOI-reco-F/PL452-5'Seq-R in one PCR reaction and PL452-3'Seq-F/GOI-reco-R in a second PCR reaction. (E) Finally, the *Kan* cassette is removed using Cre recombinase expressing bacteria and verified by PCR using primers GOI-reco-F and -R. In each case, the final product is a gene fusion between the tag and gene of interest, with an in-frame *LoxP* site as linker. Fusion protein expression is controlled by endogenous elements. (F) N- and C-terminal protein fusions harbor a 20 and 30 amino acid peptide linker, respectively. Remaining *LoxP* site is indicated in red and the corresponding amino acid residues are indicated. Amino acids identical in both peptide linkers are indicated in green.

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-template vectors 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)-Ap^R 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)-Ap^R were grown and heat shocked prior to making the cells electrocompetent, 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 PL452-5'-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 using CopyControl solution as described above. Correct integration of the tag-selection cassette was confirmed by restriction digestion and DNA sequencing.

Next, P(acman) plasmids that harbor a correctly inserted selection cassette were transformed in EL350 cells (18) where Cre expression was previously induced with 0.1% L-arabinose for 1 h as described (19). Cells were plated at 10⁻⁴ and 10⁻⁶ dilutions on LB plates (A100) at 30°C and Cre-mediated excision of the *kanamycin* gene was identified by colony PCR using GOI-reco-F and GOI-reco-R primers. Plasmids where the *kanamycin* gene was removed were isolated from the EL350 background, electroporated into EPI300 cells, and plasmid copy number of single colonies was induced as described earlier. Correct excision of the *kanamycin* gene of the tag-selection cassette was also confirmed by plating cells on LB plates (K30) and determining lack of growth, and by restriction digestion and DNA sequencing.

P element transformation and genetics

Embryos obtained from a cross between *yw* virgins and *yw*; *Ki Δ2-3* males were injected with 300–500 ng/µl DNA in 10 mM Tris-HCl (pH 8.0). Both Flag-4C N-terminally tagged *chc* (*4C-chc*⁺) and YPet C-terminally tagged *synj* (*synj*⁺-YPet) were injected and insertions were mapped to

chromosomes using standard genetics. Both *4C-chc*⁺ and *synj*⁺-YPet used in this study are inserted on the third chromosome. A *chc* null mutation (*w chc*¹) obtained from the Bloomington stock center was crossed with *4C-chc*⁺ to determine if the tagged *chc* is functional as described in Kasprowicz *et al.* (manuscript submitted for publication). Used genotypes were: *w, yw, yw*; *4C-chc*⁺, *yw*; *synj*⁺-YPet, *w chc*¹; *4C-chc*⁺, *yw GMR-LacZ eyFLP*; *FRT42D/FRT42D cl2R P{w⁺}*, *yw GMR-LacZ eyFLP*; *FRT42D synj*¹/*FRT42D cl2R P{w⁺}*.

FLAsH-FALI of chc in photoreceptors

To inactivate the *chc* in photoreceptors using FLAsH-FALI, we immobilized control *w*¹¹¹⁸ and *w chc*¹; *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 *w*¹¹¹⁸ control and *w chc*¹; *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 and yellow fluorescent proteins, CyPet and Ypet, respectively (20). In addition, we also created tag-template vectors harboring an artificial 'Flag tag-optimized tetra-cysteine peptide' fusion, that tightly binds the fluorescein derivatives FAsH 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 (chc)*, *endophilin (endo)* and *synaptojanin (synj)* in *attB*-P(acman)-Ap^R 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 *chc*, 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 PCRs, 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	ATGTCTAAAGGTGAAGAATTATTC
	N-term-PL452-R	ACTAGTGGATCCCCTCGAGGGAC
N-YPet	N-YPet-F	ATGTCTAAAGGTGAAGAATTATTC
	N-term-PL452-R	ACTAGTGGATCCCCTCGAGGGAC
N-EGFP	N-EGFP-F	ATGGTGAGCAAGGGCGAGGAG
	N-term-PL452-R	ACTAGTGGATCCCCTCGAGGGAC
N-mDsRed	N-DsRed-F	ATGGACAACACCCGAGGACGTC
	N-term-PL452-R	ACTAGTGGATCCCCTCGAGGGAC
N-Flag-4C	N-Flag-4C-F	ATGGATTACAAGGATGACGAC
	N-term-PL452-R	ACTAGTGGATCCCCTCGAGGGAC
C-CyPet	C-term-PL452-F	GCAGCCCAATTCCGATCATATTC
	C-CyPet-R	TTATTTGTACAATTCATCCATAC
C-YPet	C-term-PL452-F	GCAGCCCAATTCCGATCATATTC
	C-YPet-R	TTATTTGTACAATTCATCCATAC
C-EGFP	C-term-PL452-F	GCAGCCCAATTCCGATCATATTC
	C-EGFP-R	TTACTTGTACAGCTCGTCCATG
C-mDsRed	C-term-PL452-F	GCAGCCCAATTCCGATCATATTC
	C-DsRed-R	CTACTGGGAGCCGGAGTGGCGGGCCTC
C-Flag-4C	C-term-PL452-F	GCAGCCCAATTCCGATCATATTC
	C-Flag-4C-R	TTAGGGTCCATGCAGCAG

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.

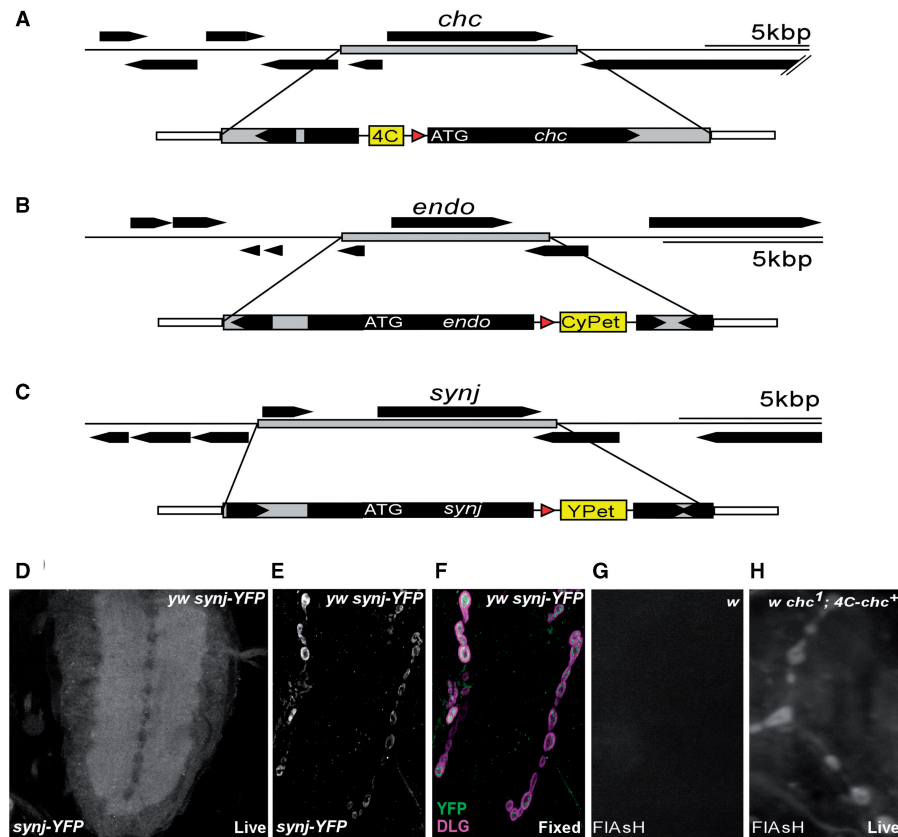


Figure 2. Fusion proteins generated using the tagging strategy are expressed. (A–C) Overview of transgenic strategies resulting in P(acman) containing Clathrin heavy chain (*chc*) N-terminally tagged with a FLAG-4C tag (*4C-chc*⁺) (A), Endophilin (*endo*) C-terminally tagged with a CyPet fluorescent protein (endo-CyPet) (B) or Synaptojanin (*synj*) C-terminally tagged with a YPet fluorescent protein (*synj-YPet*) (C). Corresponding fragments obtained by gap repair from respective BAC's are indicated; tag is in yellow, *LoxP* site is red. (D) Synaptojanin, a synaptic protein involved in endocytosis is enriched in the neuropile of dissected live third instar larval ventral nerve cords, in line with the endogenous localization of synaptojanin. (E–F) Synaptojanin (E, green in F) is also detected at the presynaptic side of neuromuscular junction boutons and is surrounded by post-synaptic DLG labeling (magenta). (G–H) Ectopically provided *chc*, expressed from the *4C-chc*⁺ transgene, is detected using FIAsh reagent at live neuromuscular junction preparations incubated with FIAsh. Non-bound FIAsh was washed away and fluorescence was detected using YFP optics. While wild types (*w*) (G) treated with FIAsh do not show significant fluorescence, transgenic *chc* (H) is ubiquitously expressed but significantly enriched in synaptic boutons, similar to endogenously expressed *chc*.

correct recombination events. Finally, the *LoxP* flanked *kanamycin* selection gene in the integrated selection cassette was removed using *Cre*-recombinase expressing bacteria (Figure 1E) (18). Our strategy leaves an in-frame *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 *Drosophila* genes simultaneously.

Tagged proteins are expressed *in vivo*

As illustrated in Figure 2, the tagging strategy resulted in an N-terminally Flag-4C tagged *chc* (Figure 2A), a C-terminally CyPet tagged *endo* (Figure 2B) and a C-terminally YPet tagged *synj* (Figure 2C). To test if the

tags are functional *in vivo*, we created transgenic *Drosophila* for the N-terminally Flag-4C tagged *chc* (*4C-chc*⁺) and for the C-terminally YPet tagged *synj* (*synj*⁺-YPet) (Figure 2A and C) using *P*-element-mediated transformation. While the constructs we transformed are sufficiently small to use *P*-element-mediated transformation (<35 kb), the P(acman) vectors that we use also contain an attB site allowing for ΦC31-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 *in situ*. While third instar *yw* control larvae do not show any fluorescence, larvae harboring the *synj*⁺-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)

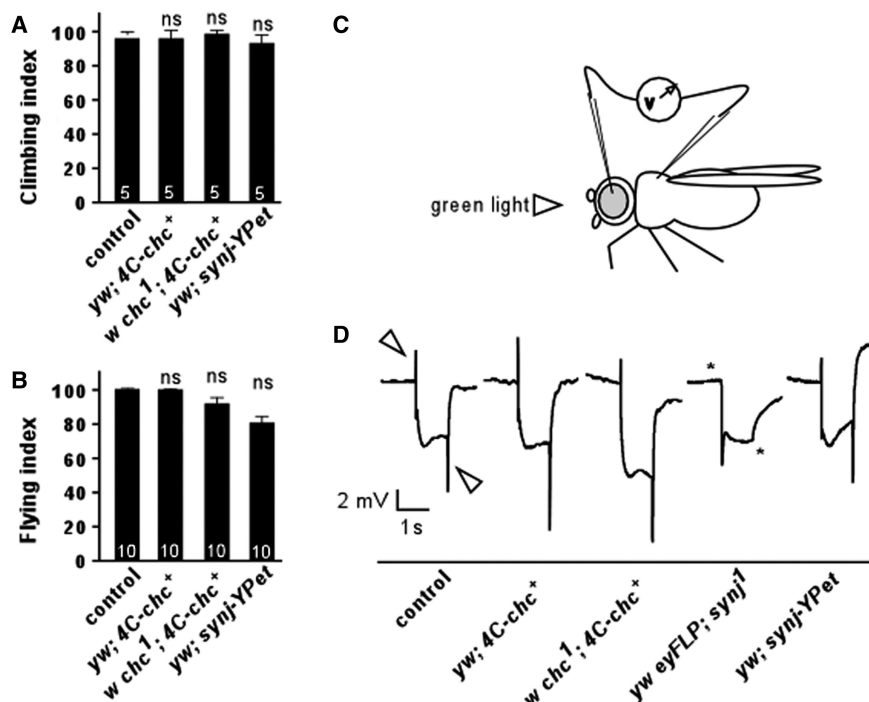


Figure 3. Fusion proteins generated using the tagging strategy are functional. (A–B) Transgenic *Drosophila* that harbor P(acman) containing Clathrin heavy chain N-terminally tagged with a Flag-4C tag (*4C-chc*⁺) or Synaptojanin C-terminally tagged with a YPet fluorescent protein (*synj-YPet*) are fully viable and show no obvious behavioral defects, including climbing behavior after being tapped down (A) and flight ability (B). (C) Schematic diagram of ERG recordings. ERG are field recordings of the retina layer in response to a light flash. (D) A normal ERG response shows a depolarization in response to turning light on and a repolarization in response to turning light off. In addition, activation of the post-synaptic cells located under the photoreceptors also generates an ‘on’ transient and an ‘off’ transient that coincides with turning light on or off, respectively (arrowheads). While ERGs of flies with defective endocytosis in their photoreceptors show no on and off transients (asterisk), the ERGs of flies with *4C-chc*⁺ or *synj-YPet* constructs are indistinguishable from controls.

(24). Similarly, unlike *w* controls labeled with the membrane permeable 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FIAsh) (27), *w chc*¹; *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*¹ mutant flies are embryonic lethal (29), *w chc*¹; *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 post-synaptic 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*¹ 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*¹). 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*¹; *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.

FIAsh-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 tetracycline tag that allows the acute inactivation of tagged proteins using FIAsh-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 FIAsh-FALI. As shown in Figure 2A, we generated a 4C tagged *chc* and constructed

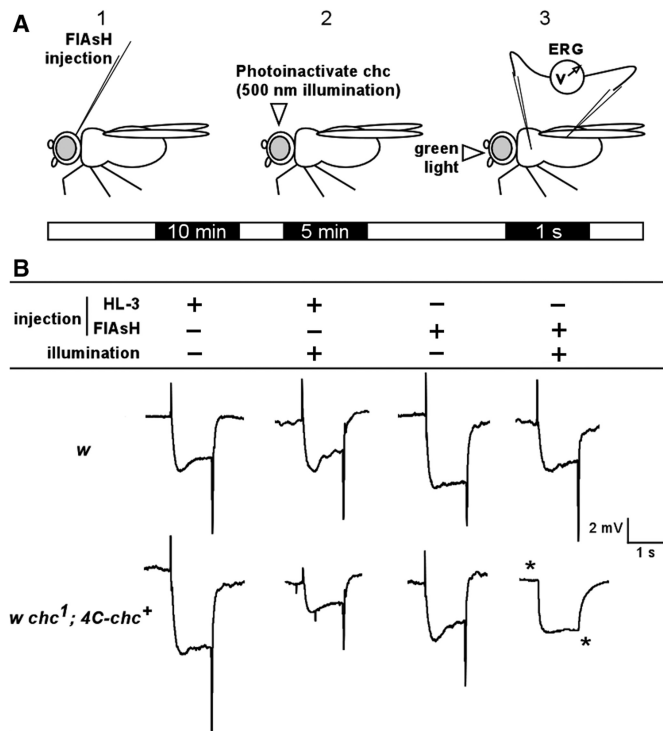


Figure 4. FIASH-FALI of 4C-chc in photoreceptors leads to defects in synaptic transmission. (A) Strategy to inactivate 4C-chc using FIASH-FALI. (i) Flies are immobilized on a microscope slide with Pritt glue and microinjected with FIASH (diluted in HL-3) or with HL-3 (controls) underneath the photoreceptor layer. Injected flies are left to equilibrate and (ii) chc is subsequently photoinactivated by illuminating the entire eye for 5 min with 500 ± 12 nm epifluorescent light using a $10\times$ lens. ERGs in response to a 1 s green light pulse are recorded by placing a recording electrode on the eye and a reference electrode in the thorax. (B) ERGs of FIASH or HL-3 injected *w* (top) or *w chc¹; 4C-chc⁺* (bottom) flies with or without illumination with 500 ± 12 nm light. Note that 'on' and 'off' transients are apparent in all conditions, except when chc in *w chc¹; 4C-chc⁺* animals is photoinactivated using FIASH-FALI (asterisks). For all conditions, at least 10 flies were injected and their ERG recorded (flies that did not survive injection were excluded). While injection of FIASH (and not HL-3) specifically leads to loss of 'on' and 'off' transients upon light inactivation, injection of eyes with FIASH or with HL-3 also leads to a somewhat smaller depolarization of the photoreceptor layer in response to a light pulse. As the ERG depolarization of flies with HL-3 or FIASH injection is not significantly different; *t*-test >0.05 , the data indicate that injection per se leads to a depolarization defect. (Average: $2.8 \text{ mV} \pm 0.53 \text{ mV}$ for HL-3 injected flies versus $4.2 \text{ mV} \pm 0.8 \text{ mV}$ for FIASH injected flies; $7.1 \text{ mV} \pm 0.9 \text{ mV}$ for uninjected flies).

chc¹ 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.

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