A toolkit to generate inducible and interconvertible *Drosophila* transgenes

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ABSTRACT

The existence of three independent binary systems for conditional gene expression (Gal4/UAS; LexA/LexAop; QF/QUAST) has greatly expanded versatile genetic analyses in the fruit fly *Drosophila melanogaster*; however, the experimental application of these tools is limited by the need to generate multiple collections of non-interchangeable transgenic fly strains for each inducible gene expression system. To address this practical limitation, we developed a multipurpose modular vector that contains the regulatory elements from all three binary systems, enabling Gal4-, LexA- or QF-dependent expression of transgenes. Our methods also incorporate DNA elements that facilitate independent site-specific recombination and elimination of regulatory UAS, LexAop or QUAST modules with spatial and temporal control, thus offering unprecedented possibilities and logistical advantages for *in vivo* genetic modulation and efficient interconversion of transgenic fly lines.

MAIN

The inducible Gal4/UAS gene expression system revolutionized genetic experimentation in fruit flies¹. This binary genetic tool facilitates gene expression in larval and adult fly tissues with cellular specificity and temporal control¹. The Gal4/UAS system relies on the production of the yeast Gal4 transcription factor from gene-specific regulatory enhancers and adjacent promoters¹. The Gal4 protein can bind to cognate DNA upstream activating sequences (UAS), thereby inducing transcription of any DNA sequence of interest inserted downstream of the UAS¹. The Gal4/UAS system has been instrumental in deciphering many biological processes, and has inspired the development of analogous conditional binary gene expression tools such as the LexA/LexAop and QF/QUAST systems^{2,3}. The use of these bipartite gene expression tools, singly or in combinations, has greatly broadened conditional genetics in fruit flies⁴. Currently, each of these gene expression tools requires the generation of independent transgenic fly lines, limiting experimental interchangeability and combinatorial usage, as well as generating cost inefficiencies related to the production and maintenance of multiple transgene collections.

To circumvent these limitations, we have developed a multi-purpose modular vector (MV, hereafter) that incorporates the gene-activating regulatory sequences for three binary gene expression systems, upstream of a multi-cloning site (Fig. 1a). If successful, this design should enable the *in vivo* expression of an inserted DNA sequence within the MV, dependent on the specific transactivator chosen. To demonstrate the flexible functionality of the MV, we inserted a transgene encoding a chimeric form of Dronc (*Drosophila* ortholog of Caspase2/9) which was fused at its carboxy-terminus to a modified Green Fluorescent Protein labelled with a myc immunoepitope (Dronc-GFP-myc, hereafter) (Supplementary Fig.1a). This engineered GFP variant contained an intrinsic Dronc-cleavage site (TETD), which after enzymatic excision leads to a GFP rearrangement and green fluorescent emission (Supplementary Fig.1a). This fusion construct strategy was based on a GFP template containing a specific cleavage site for effector caspases (DEVD), which successfully showed terminal caspase activation in mammalian and *Drosophila* tissues^{5,6}. To validate the features of our MV plasmid and the *Dronc-GFP-myc* transgene, *Drosophila* cells expressing Gal4 under the regulation of an inducible a metallothionein promoter (pMT-Gal4 S2R⁺ cells, Methods) were transfected with the MV-*Dronc-GFP-myc* plasmid. As indicated by anti-myc immunostaining, the addition of copper sulphate induced expression of the *Dronc-GFP-myc* transgene in the pMT-Gal4 transfected cells (Supplementary Fig.1b, c). However, as expected, the GFP fluorescence was only detectable within a subset of transfected cells showing signs of apoptosis (Supplementary Fig.1c). These results

confirmed the ability of the UAS repeats in the MV to induce transgene expression, as well as the green fluorescence emission upon Dronc activation in apoptotic cells.

Next, we generated a transgenic fly line incorporating the MV-*Dronc-GFP-myc* in a specific attP-tagged genomic locus⁷, which was subsequently intercrossed with independent fly lines expressing different transcriptional activators (*decapentaplegic-lexA dpp-lexA*⁸; *spalt-Gal4*, *sal-Gal4*⁹; or *Hedgehog-QF*, *Hh-QF*¹⁰). Each of these intercrosses generated F1 larval progeny and restricted Dronc-GFP-myc production in specific cells expressing Gal4, LexA and QF, respectively (Fig.1b-d). Furthermore, combinations of two transcriptional activators (*dpp-lexA/Hh-QF*; *dpp-lexA/sal-G4*) in a single transgenic fly facilitated the simultaneous, additive expression of *Dronc-GFP-myc* in the corresponding cellular domains (Fig. 1e,f). We observed higher levels of myc immunostaining and more abundant GFP signal in *dpp-lexA* cells compared to *sal-Gal4* expressing cells after irradiation (Fig. 1f, g). These results demonstrated that the MV allows the expression of a transgene under the control of either separate or combined bipartite gene expression systems. We speculate that enhanced spatial and temporal precision will be achievable by incorporating Gal80 or QS, the respective repressors of Gal4/LexA or of QF⁴. These MV features can streamline genetic configurations for investigating gene function in cells with different origins, and for studying inter-organ cell communication.

Drosophila genetics has benefited from three independent mechanisms of sequence specific genomic DNA recombination, especially the Flippase/FRT (Flp/FRT) system¹¹. This system capitalises on Flp DNA recombinase activity that targets Flp-recognition sites (FRT sequences)¹¹. Recently, directed mutagenesis of the 'wild type' Flp enzyme (hereafter, Flp^{WT}) and the FRT-recognition sites generated the mFlp⁵/mFRT 7.1 system¹². Another alternative, the Cre/LoxP system, relies on Cre-mediated recombination of LoxP-recognition motifs¹³. Because no cross-reactivity has been reported between these three recombination systems^{12,14}, gene regulatory elements in the MV were flanked by specific pairs of these recombination sequences (Fig. 1a) to investigate if sequence-specific excision of gene-regulatory sequences could be achieved. Specifically, the UAS sites were flanked by FRT (Flp sensitive), the QUAST repeats by loxP (Cre sensitive) and the LexAop site by mFRT 7.1 (mFlp⁵ sensitive: Fig.1a). Upon expression of the cognate recombinase (Flp^{WT}, Cre or mFlp⁵), we observed successful and specific excision of the corresponding regulatory components from the genome (Fig. 2a-c; Methods).

Next, we assessed whether gene *cis*-regulatory elements could be excised in the germline of a founder MV fly strain; if so, this would provide a means to efficiently generate derivative strains with permanently altered gene induction capacities. We intercrossed the MV-*Dronc-GFP-myc* founder line with flies expressing Flp^{WT} from a *Beta2-tubulin* promoter

(*BTub2*, hereafter) (Fig. 2d) to direct highly efficient FRT-dependent recombination in the male germline^{15,16}. F1 male progeny containing both transgenes (MV-*Dronc-GFP-myc* and *Btub2-Flp^{WT}*) were subsequently intercrossed with females harbouring both the *sal-Gal4* and *dpp-lexA* driver transgenes (Fig. 2e). Remarkably, 90% (9/10) of F1 male progeny generated larvae which did not express the *Dronc-GFP-Myc* construct in the *sal-Gal4* domain but retained the expression in the *dpp-lexA* territory (Fig. 2f). These results demonstrated that most of the males expressing *Flp^{WT}* in the germline permanently excised the UAS regulatory repeats without compromising the LexA-binding sites. Comparable results were obtained when Cre was expressed under the regulation of the *Btub2* promoter (Fig. 2d, 2f). Unexpectedly, we observed that 60% of the larvae generated from F1 males expressing Cre-recombinase showed also compromised activation of the MV transgene in the posterior cells of the *SalG4* territory. However, this was a transient interference between Cre and the Gal4/UAS activation system, since the subsequent outcross of the *Btub2-Cre* chromosome generated flies which exhibited the expected expression of MV-*Dronc-GFP-Myc* (Fig. 2h).

Unlike the results obtained when using *Flp^{WT}* or Cre, we found that intercrosses using flies expressing *Btub2-mFlp⁵* only affected expression of the *Dronc-GFP-myc* construct in *lexA*-transcribing cells in approximately 2% of analysed larvae (n=15 larvae per intercross: see Methods). These results are likely due to a lower recombination frequency mediated by *mFlp⁵* in the male germline¹⁶. Despite this potential limitation, our findings confirm the suitability of the MV to efficiently obtain new fly strains starting from a single founder MV-line through a simple intercross strategy (Fig. 2e). Moreover, it does not compromise with the combined usage of new MV fly strains with the previously created UAS fly repositories.

In summary, we have generated a next-generation modular vector that offers unprecedented experimental opportunities, whilst providing enhanced versatility to conduct targeted genetics in *Drosophila*. This expansion of the fruit fly genetic toolkit allows development of interconvertible fly collections with a substantial reduction of logistical costs, including experimenter time and effort. Resulting lines retain specific gene regulatory elements of interest and can be rapidly interconverted through a simple scheme of intercrosses to allow altered inducible transgene regulation.

FIGURE LEGENDS

Fig. 1. Features of the new Modular Vector (MV).

a) Schematic showing the different features incorporated into the new Modular-Vector (MV). **b-e)** The MV-Dronc-GFP-myc construct (anti-GFP, gray) expressed in several cellular domains of the wing disc, either separately (b-d) or in combination (e); Patched expression indicating the boundary between anterior and posterior cell of the wing disc is shown in red. **f)** The MV-Dronc-GFP-myc construct expressed in the *Sal-Gal4* and *dpp-LexA* cellular domains at the same time (anti-myc, red; *dpp* domain highlighted by the white bracket) in wing discs 4 hours after irradiation; Dronc activation upon GFP cleavage is shown in green (cyan arrowhead), and Dapi labels the DNA (blue). **g)** Transversal cross section of the wing disc shown in f; notice the differences in GFP signal and myc expression between the different wing areas. **h)** Quantification of red fluorescent signal intensity in the picture shown in h; the black bracket indicates the *dpp*-expression domain. Scale bars represent 50 μm in the entire figure.

Fig.2. Somatic and germline elimination of specific gene regulatory components included in the MV.

a-c) Clonal elimination from the genome of UAS (a), LexAop (b), and QUAS (c) binding sites upon random exposure to wild type Flippase (a), mFlippase5 (b) and Cre (c), respectively; anti-GFP is used to detect the expression of MV-Dronc-GFP-myc (green) and Dapi (blue) labels the DNA; red arrowheads indicate the lack of expression of MV-Dronc-GFP-myc in the expression domain of the different drivers (Gal4, LexA and QF). Compare the pattern of expression of the MV-Dronc-GFP-myc construct (green) within this figure to the pattern previously shown in Fig.1c-e. Notice that the lexOP-Cre transgene is basally expressed in random cells without LexA. **d)** Schematic showing the configuration of the new plasmids that express the different recombinases under the regulation of *Btub2*. **e)** Genetic scheme of intercrosses to permanently eliminate any of the gene regulatory components in the male germline, in order to create new transgenic flies containing a subset of the activating binding sites. **f)** Frequency excision in the germline of each gene regulatory element upon exposure to different recombinases; individual male crosses were set up to obtain the frequency of excision. **g-h)** Induced expression of the MV-Dronc-GFP-myc construct (gray) under the regulation of *Sal-Gal4* and *Hh-QF* drivers after removing the QUAS binding sites using the interconversion scheme described in f; Patched expression is shown in red. Notice the expression gaps (arrowhead) in the posterior cells of the *Sal-Gal4* domain in g, whereas MV-Dronc-GFP is continuously expressed in h after outcrossing the *Btub-Cre* chromosome. Scale bars represent 100 μm in the entire figure.

Supplementary Fig.1 Features of the Dronc-GFP-myc caspase activation reporter.

a) Schematic showing the main features of the Dronc-GFP-myc construct before and after Dronc activation; notice that Dronc activation initiates the appearance of GFP signal in apoptotic cells. **b, c)** Induced expression of MV-Dronc-GFP-myc in pMTGal4-S2R+ expressing cells before (b) and 3 hours after treatment with Staurosporine (2 µg/ml) (c). Anti-myc immunostaining labels the S2R+ cells expressing the MV-Dronc-GFP-myc construct (red), the appearance of green fluorescence indicates the activation of Dronc as a result of the cleavage in the GFP template (green); Dcp-1 immunostaining indicates apoptosis (blue) and Dapi labels the DNA (gray).

MATERIAL AND METHODS

Molecular cloning

All PCRs were performed with Q5 High-Fidelity polymerase from New England Biolabs (NEB, M0492L). Standard subcloning protocols were used to generate all the DNA plasmids (see details below). Genomic DNA was extracted via standard protocols was used as a template to amplify different DNA fragments. Transgenic flies harbouring the new transgenes were obtained by attP/attB PhiC31-mediated integration. Some of the transgenes were generated by Bestgene Inc, whilst others were generated in house by Sangbin Park. Fly strains generated will be deposited at the Bloomington Stock Centre. Whilst resources are transferred to Bloomington, reagents will be provided upon request.

Generation of the Module Vector (MV)

The different modules included in the vector were first designed *in silico* using the SnapGene software. Each module contains 5 repeats of each upstream regulatory sequence type, followed by an hsp70 minimal promoter. Each module was also flanked by specific recombination sites (see diagram Figure 1). This fragment was synthesized as a large DNA fragment by GENEWIZZ and subcloned in the PUC57 vector. The modular construct was then extracted from PUC57 as a SpeI-KpnI fragment and subcloned into a UAS-attB-w+ vector previously digested with NheI-KpnI. PmeI, XhoI, KpnI and BamHI are potential unique restriction sites available for conventional cloning downstream of the gene regulatory elements (see diagram Figure 1).

Generation of Beta2-Tubulin Flippase 5 and Cre transgenic flies

We extracted genomic DNA and amplified a Beta2-tubulin promoter via PCR using the following primers:

Forward primer Beta2-tubulin promoter:

5' ttattatccctagggcagctgtggactcctcattgtagg 3'

Reverse primer Beta2-tubulin promoter:

5' aaatttaaatctgcaggcgccgcgaattcaagcttcgccccttttcacaccg 3'

Convenient restriction sites for cloning were placed at the 5' (AvrII) and 3' (NotI and EcoRI) end of the PCR product. We then digested a UAS-attB-w+ vector with NheI and EcoRI, thus replacing the UAS repeats with the Beta2-tubulin promoter but keeping the rest of the plasmid backbone. The PCR was digested with AvrII and EcoRI and ligated with the aforementioned backbone, creating an intermediate plasmid (Beta2-tubulin-attB plasmid) suitable to subclone the cDNA of the Flippase-5 and Cre. The Flippase5 cDNA was amplified via PCR from a plasmid vector kindly provided by Iris Salecker using the following primers:

Forward primer Flippase-5:

5' attacagttGCGGCCGCGatgccacaattgatattatgtaaacacc 3'

Reverse primer Flippase-5:

5' AAtATAaaggcctTctagattatgcgtctattatgtagg 3'

The NotI and StuI restriction sites were conveniently placed in the PCR product of the Flippase-5 to facilitate the cloning in the Beta2-tubulin-attB plasmid. Before ligation, the Beta2-tubulin-attB plasmid and the Flippase-5 PCR product were digested with NotI and StuI.

To generate the Beta2-tubulin-CreV5-attB-w+ vector, we replaced the UAS repeats included in a UAS-Crev5-attB-w+ plasmids previously generated in the laboratory by the Beta2-Tubulin minimal promoter. To that end and prior ligation, we digested the UAS-Crev5-attB-w+ plasmid and the Beta2-Tubulin minimal promoter PCR product with NheI-EcoRI. Notice that the Cre cDNA was tagged with the V5 epitope.

MV-Dronc-GFP-Myc

The generation of the Dronc-GFP-Myc construct was generated following the cloning strategy described in a previous manuscript of the laboratory¹⁷; however, we used a wildtype form of Dronc as a template instead of a catalytically inactive version. The final construct was extracted from the Actin vector as a NotI-blunt and XhoI fragment. This was finally subcloned into the MV vector previously digested with PmeI-XhoI. Sequence of the plasmid will be provided upon request until the vector is deposited in a public repository.

lexAop-CreV5

We created a CreV5 PCR product using as a template the pcDNA3.1-CMV-CFP;UBC-Cre25nt plasmid¹⁷ and the following primers:

Forward primer 1:

5' ATTATT GCGGCC GCATGT CCAATT TACTGA CCGTAC ACC 3'

Reverse primer 1:

5' ATAAAT GCTAGC CTACGT AGAATC GAGACC GAGGAG AGGGTT AGGGAT
AGGCTT ACCATC GCCATC TTCCAG CAG 3'

The PCR product was digested with NotI and NheI and subcloned into a LexOP⁸ vector previously linearised with NotI and XbaI. The new plasmid was inserted on the attP-landing site of the X Chromosome 9753 using PhiC31-mediated integration.

Fly Husbandry and full description of genotypes

All fly strains used are described at www.flybase.bio.indiana.edu unless otherwise indicated. Primary *Drosophila* strains and experiments were routinely maintained on Oxford fly food at 25 °C.

Full Genotype Description

Fig. 1

1b: *Sal-Gal4/MV-Dronc-GFP-myc*

1c: *MV-Dronc-GFP-myc/+; dpp-lexA/+*

1d: *MV-Dronc-GFP-myc/+; Hh-QF/+*

1e: *Sal-Gal4/MV-Dronc-GFP-myc; Hh-QF/+*

1f-g: *Sal-Gal4/MV-Dronc-GFP-myc; dpp-lexA/+*

Fig. 2

2a: *hs-Flp^{1,22}; Sal-Gal4/MV-Dronc-GFP-myc*

2b: *MV-Dronc-GFP-myc/+; dpp-lexA/hs-mFlp5*

2c: *lexOP-CreV5; MV-Dronc-GFP-myc/+; Hh-QF/+*

2g: *Sal-Gal4/MV-Dronc-GFP-myc; Hh-QF/Btub2-CreV5*

2h: *Sal-Gal4/MV-Dronc-GFP-myc^{QUAS-}; Hh-QF/+*

Immunohistochemistry

Third instar larvae were dissected on ice-cold PBS. The larvae were then fixed and immunostained following standard protocols (fixing in PBS 4% paraformaldehyde, washing in PBT 0.3% (0.3% Triton X-100 in PBS)). Primary antibodies used in our experiments were: anti-GFP (goat, 1:400; Abcam, ab6673); anti-Ptc (1:200, Hybridoma Bank, Apa1); anti-myc (rat 1:200, Abcam ab206486, wing discs immunostainings); anti-myc (goat 1:200, Abcam ab206486, wing discs immunostainings). The following secondary antibodies were diluted in 0.3% PBT from (Molecular Probes) and used to detect the primary antibodies: anti-goat Alexa 488, anti-goat Alexa 555, anti-mouse Alexa 555 and anti-mouse Alexa 647, anti-rabbit Alexa 647. The secondary antibodies were always used at the standard concentration 1:200. DAPI was added to the solution with the secondary antibodies for labelling the DNA in the

nuclei (1:1000; Thermo Scientific 62248). After incubation for 2h with the secondary antibodies, samples were washed in PBT several times and mounted in Vectashield.

Imaging of wing discs

Confocal imaging of wing imaginal discs was performed using the Olympus Fluoview FV1200 and the associated software. 55 focal planes were taken per wing disc using the 40x lens. Acquired images were processed using automated Fiji/ImageJ. Generally, Z-stacks were projected, and the channels split. The Reslice function was used to obtain transversal sections of the images. Figures were produced using Adobe photoshop CC2018.

Generation of genetic mosaics

Larvae *yw hs-Flp1.22; MV-Dronc-GFP-myc/Sal-Gal4* were heat shocked for 25 mins at 37°C 24-48h after egg laying and dissected at the end of LIII.

Larvae *w; MV-Dronc-GFP-myc/+; dpp-LexA/hs-mFlp5* were heat shocked for 60 mins at 37°C 48-72h after egg laying and dissected at the end of LIII. We have noticed that the recombination efficiency using the *hs-mFlp5* is lower than the WT and a longer heat-shock treatment is needed to generate the genetic mosaics.

Larvae *lexAop-CreV5; MV-Dronc-GFP-myc; Hh-QF* were dissected at the end of LIII without giving any heat-shock since we have noticed that the *lexAop-CreV5* shows basal expression in tissues. After heat-shock treatment (if needed), the larvae were maintained at 25°C until dissection at the end of LIII stage.

Irradiation protocol

Larvae were exposed to a source of γ-ray to induce apoptosis. Time exposure was adjusted to administer a dose of 1500 rads to the samples.

Cell culture, transfection and Imaging

Stable S2R+pMTGal4 cells were seeded to 80% confluency on coverslips 3hrs prior transfection. Transfection was carried out using 1μg of MV-Dronc-GFPmyc expressing plasmid DNA per well in a 6 well-plate according to the manufacturer protocol (Effectene, Qiagen). Cells were grown for 2 days in Schneider's medium supplemented with 100μM copper sulphate for the induction of the Gal4 protein. Apoptosis was induced for 3hrs with 2μg/ml of staurosporine (SIGMA; S5921) in serum-free Schneider's medium. Cells were washed, fixed in 4% paraformaldehyde for 10min and processed for immunofluorescence microscopy using the primary antibodies, anti-myc (goat 1:200, Abcam ab9132, cell culture immunostaining) and anti-cleaved Dcp-1 (1:200; Cell Signalling 9578). The following secondary antibodies were diluted in 0.3% PBT from (Molecular Probes) and used to detect

the primary antibodies: anti-goat Alexa 555, anti-rabbit Alexa 647. The secondary antibodies were always used at the standard concentration 1:200. DAPI was added to the solution with the secondary antibodies for labelling the nuclei (1:1000; Thermo Scientific 62248). After incubation for 2h with the secondary antibodies, samples were washed in PBT several times and mounted in Vectashield. The imaging was performed using the an Evos M7000 system (60x, oil).

CONTRIBUTIONS

L.A.B-L. was responsible for the initial conception of the work and the design of all the plasmids described in the manuscript. The empty MV, the *Dronc-GFP-myc* construct and *Beta2-tubulin-Cre* plasmids were generated by L.A.B-L. The experiments shown in Figure 1 and 2 were performed by L.A.B-L. F.W. generated the *MV-Dronc-GFP-Myc* and *Beta2-tubulin-mFlippase-5* plasmids and completed the experiments shown in Supplementary Figure 1. C.H. generated the *lexOP-CreV5* plasmid. Many of the transgenic flies shown in the study were generated by S.P. and Bestgene Inc. The initial *in vivo* validation of the *MV-Dronc-GFP-Myc* construct was made by C.H., A.G., K.R., I.A., Y.S., M.B., S.L., E.S. and G.A. as part of a summer course in molecular biology and fly genetics for undergraduate students. S.K. was responsible for establishing and coordinating the execution of the aforementioned summer course. S.K., FW and L.A.B-L. wrote the manuscript and prepared all the figures of the manuscript. All co-authors have provided useful criticisms and commented on the manuscript before submission.

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