Multi-tissue GAL4-mediated gene expression in all *Anopheles gambiae* life stages using an endogenous polyubiquitin promoter

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ABSTRACT

The ability to manipulate the *Anopheles gambiae* genome and alter gene expression effectively and reproducibly is a prerequisite for functional genetic analysis and for the development of novel control strategies in this important disease vector. However, *in vivo* transgenic analysis in mosquitoes is limited by the lack of promoters active ubiquitously. To address this, we used the GAL4/UAS system to investigate the promoter of the *An. gambiae Polyubiquitin-c* (PUBc) gene and demonstrated its ability to drive expression in mosquito cell culture before incorporation into *An. gambiae* transgenic driver lines. To generate such lines, piggyBac-mediated insertion was used to identify genomic regions able to sustain widespread expression and to create φC31 docking lines at these permissive sites. Patterns of expression induced by PUBc-GAL4 drivers carrying single intergenic insertions were assessed by crossing with a novel responder UAS-mCD8:mCherry line that was created by φC31-mediated integration. Amongst the drivers created at single, unique chromosomal integration loci, two were isolated that induced differential expression levels in a similar multiple-tissue spatial pattern throughout the mosquito life cycle. This work expands the tools available for *An. gambiae* functional analysis by providing a novel promoter for investigating phenotypes resulting from widespread multi-tissue expression, as well as identifying and tagging genomic sites that sustain broad transcriptional activity.

KEYWORDS

Transgenic mosquitoes; GAL4/UAS; *Polyubiquitin*; piggyBac transposon; φC31 docking lines

1 Introduction

Anopheles gambiae mosquitoes are the major vectors of malaria in the sub-Saharan Africa which accounts for most of the global malaria cases and related deaths (WHO, 2016). While a remarkable reduction in malaria incidence and mortality has been achieved in recent years (Bhatt et al., 2015), the spread of resistance to insecticides and antimalarial drugs poses a major challenge, and the latest report suggests that progress has stalled (WHO, 2017). Therefore, innovative methods for malaria control based on genetically-modified mosquitoes open a promising prospective (Hammond et al., 2016). Such strategies are enabled by the growing abundance of genomic and transcriptomic data combined with the ability to effectively reprogram gene expression in *An. gambiae.* Nevertheless, tools for *in vivo* functional analysis to examine the role of genes in affecting a phenotype are limited, and mosquito transgenic analysis remains a demanding task due to the labor-intensive husbandry of continuous lines.

Binary systems such as the GAL4/UAS (Brand and Perrimon, 1993) can be used in insects for ectopic gene expression or silencing in spatially and/or temporally controlled patterns. The GAL4/UAS system comprises two transgenic components, a driver or GAL4 and a responder or UAS, inserted in separate transgenic lines (Fig. 1A). The driver line carries the yeast transcription activator GAL4 under the control of a specific promoter; the responder line contains an Upstream Activating Sequence (UAS) that serves as GAL4 binding site and activates the transcription of a target gene located downstream. Expression occurs only in the progeny resulting from the cross of driver and responder lines in the transcription pattern dictated by the selected promoter. The modular nature of the system offers several advantages compared to linear transgene expression including amplification of gene expression, creation of lines bearing toxic genes, and repeated use of lines for combinational crosses. Since the first applications in *Drosophila melanogaster* (Brand and Perrimon, 1993; Piccin et al., 2001), the GAL4/UAS system has been applied to *Bombyx mori* (Imamura et al., 2003), *Tribolium castaneum* (Schinko et al., 2010), *Aedes aegypti* (Kokoza and Raikhel, 2011), *An. stephensi* (O'Brochta et al., 2012), as well as *An. gambiae* (Lynd and Lycett, 2011) for gene overexpression (Lynd and Lycett, 2012).

However, gene functional analysis using the GAL4/UAS system is restricted by the limited set of functionally-characterized promoters incorporated in *An. gambiae* driver lines. Indeed, while promoters specific for several mosquito tissues have been characterized [midgut (Moreira et al., 2000; Kim et al., 2004; Abraham et al., 2005; Nolan et al., 2011), testes (Catteruccia et al., 2005), salivary glands (Lombardo et al., 2000, 2005), germline (Papathanos et al., 2009), fat body (Volohonsky et al., 2015 and 2017), and olfactory receptors (Riabinina et al., 2016)], to date only the *An. gambiae* carboxypeptidase promoter has been used to establish a midgut-specific GAL4 line (Lynd and Lycett 2012). Most notable is the lack of a characterized endogenous ubiquitous promoter in *An. gambiae*, which greatly limits our understanding of phenotypes resulting from genes expressed in a multi-tissue manner. In an effort to obtain ubiquitous-like expression, promoters of highly conserved housekeeping genes such as *Polyubiquitin* (PUB) have been investigated in insects. Amongst these, the *D.*

melanogaster PUB promoter drives widespread expression in transgenic *D. melanogaster* (Handler and Harrell, 1999), *Anastrepha suspensa* (Handler and Harrell, 2001), *Lucilia cuprina* (Heinrich et al., 2002), and the mosquito *An. albimanus* (Perera et al., 2002). A similar phenotype was described for the *Tribolium castaneum* PUB regulatory region in transgenic beetles (Lorenzen et al., 2002). Finally, the promoter of the *Ae. aegypti* PUB gene drives widespread expression across all mosquito developmental stages (Anderson et al., 2010).

Achieving the desired expression pattern of transgenes, however, does not solely depend on identifying the regulatory region that will produce the intrinsic spatio-temporal pattern required, but it is affected by the genomic environment into which it is inserted. This has been observed previously examining potential 'ubiquitous promoters' in *An. gambiae*, most notably the *α-tubulin-1b* promoter (Lycett et al., 2012) in which a core expression profile in a limited number of tissues was overlaid by unique temporal and spatial expression patterns in each of the 9 isofemale lines examined. Positional effect is a consequence of the essentially random integration mediated by transposons and results in the laborious process of creation of multiple lines and their assessment for appropriate or 'desired' gene expression and fitness (Handler, 2002). In general, defining universally permissive expression sites is challenging as the properties of any DNA sequence are likely to be affected by its relocation in the genome, yet it is desirable as these active sites would represent an established context for future comparative experiments. By including docking sites in the constructs used to define these locations, characterized lines can be established that bear reusable transcriptionally active sites. From such docking strains it is possible to create a great variety of other lines by integrating the target DNA alongside the existing construct or by replacing the existing cassette with a donor cargo via recombinase-mediated cassette exchange (RMCE) (Bateman et al., 2006), the latter having the advantage of integrating only the desired construct without the plasmid backbone. To date, while φC31-mediated integration (Meredith et al., 2011; Pondeville et al., 2014, Volohonsky et al., 2015) and RMCE (Hammond et al., 2016) have been applied to *An. gambiae*, a very limited number of docking strains has been created.

Here, we use the GAL4/UAS system to characterize the regulatory regions of the *An. gambiae Polyubiquitin-c* (PUBc) gene, orthologue of *Ae. aegypti* AAEL003877 (Anderson et al., 2010), as a promising candidate for driving ubiquitous expression in transgenic mosquitoes. Driver lines carrying a PUBc-GAL4 cassette were created using the piggyBac transposon and assessed to identify unique genomic locations able to sustain multi-tissue expression to be exploited for subsequent φC31-mediated integration and cassette exchange. To validate GAL4 expression patterns, a unique UAS-mCD8:mCherry responder line was also created by site-specific integration and used to examine expression patterns in the progeny of crosses to PUBc-GAL4 driver lines.

2 Materials and Methods

2.1 Plasmid construction

To obtain the PUBc-GAL4 driver plasmid, primers were designed from the annotation in the PEST genome. PUBc5 consists of 2005 bp upstream of the predicted start codon and includes the intron, the 5'UTR and the intergenic space that separates PUBc (AGAP001971) from the previous gene (PUBb, AGAP001970). PUBc3 includes the intergenic space (407 bp) between the stop codon of PUBc and the following gene (TSR4 protein, AGAP001972). Plasmid components were individually amplified and ligated into pSLfa1180fa by Gibson cloning (Gibson et al., 2009, 2010) to create pSL-Gyp:PUBc5:GAL4 and pSL-PUBc3:Gyp:attP. The PUBc3:Gyp:attP cassette was then inserted into the BglII/NotI sites of pSL-Gyp:PUBc5:GAL4 to generate pSL-Gyp:PUBc5:GAL4:PUBc3:Gyp:attP. The Gyp:PUBc5:GAL4:PUBc3:Gyp:attP fragment was finally cloned into the AscI sites of pBAC:attP:eCFP:LRIM to replace LRIM and obtain the final plasmid pBAC:attP:eCFP:Gyp:PUBc5:GAL4:PUBc3:Gyp:attP (pPUBc-GAL4). All primers and templates used are reported in Table S1.

The UAS-mCD8:mCherry responder plasmid was designed to carry an attB site for site-specific integration into an attP docking line, an enhanced yellow fluorescent protein (eYFP) driven by the 3xP3 promoter, and a fluorescent mCD8:mCherry membrane marker under the control of 10x upstream activating sequences (UAS). In a first step, attB-EcoRI from pTA-attB (gift from M. P. Calos, Addgene plasmid #18937 (Groth et al., 2000)) was cloned into pBAC[3xP3-eYFPafm] (obtained from E. Wimmer). The cassette 3xP3-eYFPafm-attB was further amplified and cloned into pGEM-T Easy Vector (Promega) to create a recipient plasmid pGEM-T[3xP3-eYFPafm-attB]. A second recipient plasmid pSLfa-MF3 was created by cloning the cassette NotI-10xUAS-MCS-ftzintron-SV40-BamHI (from pMF3, DGRC) into pSLfa (obtained from E. Wimmer). mCD8:mCherry was amplified from pUAS-mCD8:mCherry (obtained from F. Schnorrer) and further cloned into pSLfa-MF3. The AscI-10xUAS-mCD8:mCherry-ftzintron-SV40-FseI cassette was cloned into pGEM-T[3xP3-eYFPafm-attB] to obtain the final plasmid pGEM-T[3xP3-eYFPafm-10xUAS-mCD8:mCherry-ftzintron-SV40-attB] (pUAS-mCD8:mCherry). All primers and templates used are reported in Table S1.

2.2 An. gambiae mosquito cells transfection and luciferase assay

The hemocyte-like *An. gambiae* cell line Sua5.1 (Müller et al., 1995) was used as described in Lynd and Lycett, 2012 to perform cell transfection and luciferase assay using Effectene® Transfection Reagent (Qiagen) and Dual-Luciferase® Reporter Assay System (Promega) respectively. DNA samples consisted of 100 ng of pPUBc-GAL4 driver plasmid, 100 ng of a 14xUAS responder plasmid driving the expression of firefly luciferase (pUAS-Luc) (Lynd and Lycett, 2012), and 2 ng of Actin Renilla plasmid to normalize for efficiency of transfection. The pPUBc-eGFP plasmid (Lycett, unpublished), which expresses cytoplasmic eGFP under the control of a shorter version of the PUBc promoter sequence used in this study, was used to visually monitor transfection efficiency and measure background activity (blank) in the absence of a GAL4 driver. Relative light units (RLU)

146 measurements were obtained from 6 replicate wells, normalized by Renilla activity (RLU_{Luc} / RLU_{Ren})
147 and adjusted for the activity of the blank (RLU_{sample} – RLU_{blank}). One-way ANOVA with Tukey's
148 multiple comparison analysis was performed to determine statistical significant differences ($P < 0.05$).

149 *2.3 Transgenic lines*

150 The PUBc-GAL4 transgenic line was produced using the piggyBac transposon as previously
151 described (Lycett et al., 2012; Lombardo et al., 2009). G3 strain embryos were microinjected with 350
152 ng/µl of GAL4 driver plasmid and 150 ng/µl of transposase helper plasmid pHSP-pBac (Handler and
153 Harrell, 1999). F_0 L1 larvae expressing eCFP transiently in the anal papillae were reared separately
154 and backcrossed in sex-specific cages. F_1 progeny was assessed for eCFP stable inheritance with
155 positive females then backcrossed to wild type and allowed to lay in individual tubes. F_2 isofemale
156 lines were scored for inheritance of the eCFP marker. Lines yielding a percentage of transformants
157 not compatible with a single insertion (*i.e* >50% fluorescent progeny) were discarded, while isofemale
158 lines showing ~50% of transgenic progeny were interbred to create stable lines.

159 The transgenic line UAS-mCD8:mCherry was created by injecting embryos of the E φC31-docking
160 line (Meredith et al., 2011) with 250 ng/µl of UAS-mCD8:mCherry plasmid and 800 ng/µl of mRNA
161 encoding an insect codon optimized mutant φC31 integrase (obtained from A. A. James). Of the F_0
162 adults recovered, pools of 10 females were backcrossed to 50 males, while all males were crossed
163 with 5x females of the E line. Microinjections, screening and stable homozygous line generation were
164 carried out as described by Pondeville et al. (2014).

165 *2.4 Inverse-PCR*

166 Inverse PCR was conducted as described by Lycett et al., 2012. Genomic DNA was extracted from
167 pools of 20 F_2 transgenic adults from selected isofemale lines using Qiagen Genomic tips (Qiagen). 1
168 µg of gDNA was then digested with BfuCI or TaqαI, self-ligated, and PCR was performed to amplify
169 DNA regions flanking the piggyBac arms at the site of insertion (primers ITRL1F and ITRL1R for
170 piggyBac left arm, ITRR1F and ITRR1R for right arm, Table S1). PCR products were sequenced and
171 genomic location of insertions identified using the BLAST tool integrated in VectorBase (Giraldo-
172 Calderón et al., 2015).

173 *2.5 Dissections*

174 Dissections were performed on 2-5-day-old GAL4/UAS adult females in PBS supplemented with
175 EDTA-free protease inhibitor cocktail (Roche). Body parts were incubated in fixing solution (4%
176 paraformaldehyde, 1X PBS pH 7.4, 2 mM $MgSO_4$, 1 mM EGTA) for 30-45 minutes, washed in PBS
177 and mounted on a microscope slide using Vectashield mounting medium (Vector Laboratories). For
178 preparation of abdomens, after removing internal organs, they were incubated in methanol for 2
179 minutes before fixing.

180

3 Results

3.1 An. gambiae PUBc regulatory regions drive expression in mosquito cells

To examine the activity of the regulatory regions surrounding PUBc, the entire intergenic sequences upstream and downstream of the coding region were used to flank the GAL4 gene. This transcription unit was placed between gypsy insulators (Cai and Levine, 1995) to potentially reduce position effect variation, and flanked by inverted φC31 attP repeats to generate RMCE docking sites. Finally, the construct was inserted in a piggyBac transformation vector marked with 3xP3-eCFP (Fig. 1B).

Initially, the ability of PUBc to activate GAL4-mediated expression was investigated in *An. gambiae* SUA5.1 cells. When co-transfected with a responder plasmid expressing UAS-regulated luciferase (pUAS-Luc) (Lynd and Lycett 2012), pPUBc-GAL4 showed ~4000-fold higher activity than control transfections with pUAS-Luc only ($P < 0.003$) (Fig. 1D). Additionally, background activity from a PUBc-eGFP construct without the luciferase gene (blank) was not significantly different to the pUAS-Luc control ($P = 0.16$), indicating limited leakiness in UAS-regulated expression (Fig. 1D).

3.2 Generation and characterization of transgenic lines

3.2.1 PUBc-GAL4 driver lines

The piggyBac transposon was used to create a series of driver lines carrying the PUBc-GAL4 cassette in single, unique genomic locations. From 180 *An. gambiae* G3 strain embryos injected, 113 (63%) larvae hatched, 77 (68%) of which showed eCFP transient expression in the anal papillae. 97 F_0 adults (54%) were obtained and pooled into 6 sex-specific F_0 founder cages (A-F) (Table 1). Transgenic F_1 progeny was recovered from each F_0 cage established from founders showing transient expression (A-D), while no transformants were recovered from negative founders (cages E,F) (Table 1). 21 F_1 transgenic females were backcrossed with wild-type and F_2 progeny from individual females were assessed for inheritance of the fluorescent marker as a proxy for transgene copy number. We identified 14 isofemale lines showing 47-53% transgene inheritance (A2, A3, A6, A7, A8, A10, A11, A12, A15, B3, B8, C1, C2, D2), suggestive of single insertions (Table 1). Upon verification of copy number and location of integration sites by inverse-PCR we found several instances of identical integration sites in distinct isofemale lines from the same founder cage (Fig. S1). Replicate lines were discarded thus overall the final analysis was consistent with the isolation of 7 isofemale lines carrying separate single integration sites (Table 2). Four lines (A8=A15, A10=A12, B3, C1), carried intergenic insertions, one line (A3=A6=A7=A11) carried an insertion within the open reading frame of *Cyp6m4* (AGAP008214), and two (A2 and D2) carried insertions within highly repetitive regions that precluded their exact localization in the genome (Table 2). iPCR failed to amplify the region flanking the piggyBac right arm of lines A8 and A10 and location in these lines was confirmed using primers designed on the genomic DNA flanking the insertion site predicted from the sequencing data obtained from the left arm (Fig. S2). All sites of integration characterized occurred at

piggyBac canonical TTAA integration sites (Table 2). Overall, we achieved a minimum transformation efficiency of 7% (*i.e.* number of independent insertions / total F_0 survivors). All 7 lines were interbred and underwent preliminary assessment of *in vivo* promoter activity in larvae.

3.2.2 UAS-mCD8:mCherry line

The responder plasmid was designed to carry an attB site for φC31-mediated site-specific integration into the E docking line (Meredith et al., 2011), an enhanced yellow fluorescent protein (eYFP) driven by the 3xP3 promoter, and a fluorescent mCD8:mCherry membrane marker under the control of 10x UAS (Fig. 1C). From the 1182 E line embryos injected, 374 F_0 adults (32%) were recovered, 185 females and 189 males, which were backcrossed to individuals of the E line. A total of 23 transgenic F_1 larvae were recovered from the F_0 males only and from which the line was established. Crosses of the UAS-mCD8:mCherry responder line with a carboxypeptidase-GAL4 *An. gambiae* line (Lynd and Lycett 2012) showed the expected adult midgut specific expression of red fluorescence (Fig. S3) in the progeny.

3.3 PUBc drives widespread expression in transgenic An. gambiae mosquitoes

To investigate the pattern of expression driven by PUBc and identify unique insertion sites able to sustain ubiquitous expression, profiles generated by transgenic PUBc-GAL4 isofemales lines (A2, A3, A8, A10, B3, C1 and D2) and mixed male pools were assessed and compared after crossing with the responder line UAS-mCD8:mCherry. Preliminary analysis of the larval expression profiles driven by individual PUBc-GAL4 lines differed in terms of signal intensity and distribution: A10 and D2 derived progeny had similar intense and widespread mCherry signal throughout the whole body; A8 produced symmetrically patterned fluorescent signal along the abdomen that was noticeably weaker than A10 and D2 but with intense reporter expression in the head and mouthparts; A3 and C1 progeny displayed asymmetrical mCherry signal along the abdomen, in the head and mouthparts, whereas in A2 and B3 derived progeny mCherry signal was largely limited to the terminal part of the abdomen, and intense signal in the brain and mouthparts respectively. Unfortunately, these profiles were not documented as images (data not shown). We were unable to detect distinct phenotypes from the mixed male pool progeny (Fig. S4) which were not also observed from the single isofemales lines, and therefore our further analysis focused on female derived lines.

A8 and A10 were chosen for further in depth analysis as they displayed a similar temporal and spatial distribution, yet displayed distinct intensities of reporter gene expression, with A10 being generally higher in most tissues. In larval progeny from both drivers, mCherry fluorescence was evident in the mouthparts, brain, fat body, muscles surrounding the aorta and the heart (Fig. 2A,B), developing thoracic imaginal discs, and central nerve cord (Fig. 2C,D). Signal in A10/UASmCherry (A10/ch) larvae (Fig. 2A) was more intense compared to A8/ch larvae (Fig. 2B), with the exception of the mouthparts. Furthermore, while A10/ch individuals displayed a very bright and widespread signal throughout the larval body (Fig. 2A,C), in A8/ch larvae a distinct symmetrical expression pattern was detected dorsally and ventrally in each abdominal segment (Fig. 2B,D), most likely derived from the

integument. In pupae, the differences in signal intensity and the distinct abdominal pattern of expression were maintained, and strong fluorescence was detected in the developing antennae in both A10/ch (Fig. 2E) and A18/ch pupae (Fig. 2F). At adult stage, mCherry was widespread in intact females (Fig. 2 G,H) and males (Fig. 2 I,J) of both A10 and A8 crosses with A10/ch displaying a brighter signal than A8/ch mosquitoes. Fluorescence was widely detected in the thorax and along the abdomen with bright signal visible through the non-sclerotized areas of the cuticle. In both crosses, fluorescence was detected in all of the appendages including legs, veins of the wings, antennae, palps, and proboscis/labium (Fig. 2G to J). Expression was also robust at the base of the antennae in the pedicel, which hosts the Johnston's organ, and in the brain (Fig. 2G to J). A10/ch adults showed expression in some but not all of the ommatidia, while in A8/ch individuals this was less evident and occurred in fewer ommatidia (Fig. 2G to J).

In dissected abdomens of both crosses expression was detectable in all of the tissues present in the integument including the epidermis, fat body, oenocytes, lateral muscles and nerve cord (Fig. 3A,C). Here, besides displaying a lower signal intensity, A8/ch abdomens showed a mosaic pattern of expression in muscular tissues (Fig. 3C). A comparable level of fluorescence was detected in A10/ch (Fig. 3E) and A8/ch (Fig. 3G) mosquitoes along the majority of trachea surrounding the internal organs including the foregut, midgut, hindgut and Malpighian tubules of unfed females; however, no expression was detectable in the midgut or Malpighian tubule epithelium. PUBc was active in the salivary glands of A10/ch (Fig. 3I) with mCherry fluorescence detected in all lobes, while signal was weaker in A8/ch salivary glands (Fig. 3K). Ovaries (Fig. 3 M,O) of sugar-fed A10/ch females showed bright oviducts and follicles, and fluorescence was also strongly detected 24-48 h after blood meal in the developing oocytes; while fluorescence in A8/ch mosquitoes was weaker (not shown). In male reproductive system, signal was moderately detected in the testes (not shown). Finally, hemocytes attached to tissues and circulating in the hemolymph had detectable mCherry expression (not shown).

4 Discussion

To expand the available tools for functional genetic analysis of *An. gambiae*, we have isolated and characterized the regulatory regions of the *An. gambiae Polyubiquitin-c* gene and validated their ability to drive robust expression in cultured mosquito cells and widespread multi-tissue expression in all life stages of transgenic mosquitoes. This activity was tested in the context of the GAL4/UAS system to allow flexible use of the generated lines in future analysis. As part of the validation, we also established and tested a new UAS reporter construct that yields bright mCherry expression with all of the GAL4 lines so far examined.

The generation of piggyBac GAL4 driver lines was achieved with an efficiency equal to or higher than reported elsewhere using a variety of promoter/effector combinations (Grossman et al., 2001; Kim et al., 2004; Lobo et al., 2006; Lombardo et al., 2009; Lycett et al., 2012; Meredith et al., 2013). This would suggest that any potential toxicity of widespread GAL4 expression does not manifest as

reduced transformation efficiency. Furthermore, as reported elsewhere (Pondeville et al., 2014), transgenic progeny was only obtained from F_0 mosquito pools that had showed transient episomal expression of the transformation marker in larval stages, not only suggesting that effective screening in future could be confined to progeny from these pools, but that concurrent episomal expression of GAL4 in the F_0 is readily tolerated. Homozygous A8 and A10 are viable during inbreeding of the stock colonies, and we have created a true breeding homozygous A10 line that continues to be stable over multiple generations. However, routine observation suggests reduced longevity in relation to the parental G3 line, and although we haven't performed detailed life table analysis, there may be a fitness cost associated with high levels of GAL4 expression achieved in the homozygous A10 line. This will be explored further when the Gal4 driver is removed from these lines by RMCE.

PiggyBac-mediated integration occurs essentially randomly at TTAA genomic sites and can lead to insertion of the transgenic construct in multiple sites of an individual genome (Lombardo et al., 2009). The selection strategy used here aimed at maximizing the identification of lines carrying insertions at single intergenic genomic sites, desirable for docking lines, as well as identifying insertion sites that gave the broadest tissue expression of GAL4, whilst limiting workload. To do so, we performed expression profiling and molecular characterization of insertion site on isofemale lines derived from F_1 females, while F_1 males were used as a backup, crossed in pools to UAS-mCD8:mCherry and screened *en masse* to recover any distinct expression profiles (and thus different insertion sites) not observed in female lines. However, no distinct phenotypes were recovered in the F_2 male progeny, suggesting they originated from the same F_0 individuals as the females.

Although differences in the expression of the 3xP3-eCFP marker from the piggyBac transposon were detected in different F_1 isofemale lines, these were minor and did not readily correlate with the extensive variegation observed in the GAL4 driven mCherry signal, suggesting that not all genomic sites supportive of robust neuronal expression from 3xP3 are capable of sustaining widespread expression driven by other regulatory regions. This is supported by findings in *Ae. aegypti*, where 3xP3 was an unreliable promoter for inferring efficiency of gut specific transgene expression at distinct genomic locations (Franz et al., 2011).

Position variegation derived from our PUB-GAL4 lines also suggests that the inclusion of gypsy insulators flanking the GAL4 cassette did not overcome the influence of nearby effectors and/or chromatin status on transgene expression. Gypsy insulators (Roseman et al., 1993) have been previously reported to repress the action of nearby suppressors in *D. melanogaster* and *An. stephensi* (Sarkar et al., 2006; Carballar-Lejarazú et al., 2013). Yet, no quantitative study has been conducted in *An. gambiae* when the GAL4/UAS system is used. When insulating UAS cassettes in *D. melanogaster*, gypsy insulators have proven useful to mitigate positional effect, but efficiency of insulation was still dependent on genomic locus (Markstein et al., 2008). In the first analysis of GAL4/UAS in *An. gambiae*, gypsy sequences were used to flank the UAS-reporter cassette, and all combinations of carboxypeptidase-GAL4 driver lines crossed to 6 UAS reporter lines drove robust midgut-specific expression regardless of location of insertion (Lynd and Lycett 2012). There may be, thus, a difference in the insulation capacity of gypsy sequences when flanking UAS compared to

GAL4 cassettes or depending on the regulatory regions used. It should be noted though that RMCE will remove the gypsy sequences from these loci, and so depending on the nature of the transgenics being produced it may be necessary to include gypsy sequences in the exchanged cassette to recreate a similar sequence context.

Overall, visual assessment of progeny derived from selected PUBc driver lines (A10 and A8) crosses with UAS-mCD8:mCherry individuals, revealed that the regulatory regions identified for PUBc are active in a variety of tissues and organs in all mosquito life stages examined and presumably in embryonic stages too, as neonate larvae are fluorescent. In adults, these tissues include: eyes, brain, and ventral nerve cord; muscles of the aorta and heart; trachea surrounding the digestive tract and the Malpighian tubules; salivary glands; sugar-fed and blood-fed ovaries and developing oocytes; fat body; appendages (legs, palps, antennae, proboscis); and hemocytes. Tissues where expression was not readily visible include the epithelium of the digestive tract and Malpighian tubules, although it cannot be excluded that expression occurs in these tissues at a level that is not detectable by fluorescence. However, it should be noted that robust midgut epithelium expression of mCherry was observed in crosses with carboxypeptidase-GAL4 lines.

The *An. gambiae* PUBc-derived expression pattern largely overlaps those described for promoter fragments derived from other insect PUB genes (Handler and Harrell, 2001; Heinrich et al., 2002; Perera et al., 2002) and, more specifically, with that of the *Ae. aegypti* PUB promoter described by Anderson et al. (2010) where expression was robust and widespread throughout the mosquito body from larvae to adults. Nevertheless, we found that the A10 line drives strong fluorescence in the salivary glands, while in *Ae. aegypti* these tissues displayed little fluorescence and lacked mRNA signal. Conversely, strong fluorescence was seen in *Ae. aegypti* midguts, whilst it was observed only in the trachea surrounding the digestive tract but not the midgut epithelium in *An. gambiae*. These differences may be due to innate variation in promoter activity from the region selected in the two species or they may be the result of positional effect. Nonetheless, the widespread core expression pattern described here for PUBc is unprecedented for an *An. gambiae* endogenous promoter.

Previous work analyzing a potential endogenous ‘ubiquitous’ promoter directly with an eGFP reporter gene, had indicated that positional effects can be extremely frequent in *An. gambiae* transgenics (Lycett et al., 2012). Each line produced had a core expression profile in the same limited tissues, including nerve cord and chordotonal organs, but different patterns of expression in other tissues were observed in each of the 9 isofemale lines analyzed. In the current study, we reasoned that by using the GAL4/UAS system to generate and follow the expression pattern conferred by putative regulatory regions, then selected GAL4 lines that gave a desired expression pattern could be archived for future functional genetic studies. The two lines thus retained (A10 and A8) will be used to compare different UAS-regulated transgenes and monitor resultant phenotypes based on a consistent, defined and robust expression pattern. If the UAS transgenes are created through φC31 site-directed insertion into the same genomic location (Pondeville et al., 2014), then such work can be performed in similar genetic backgrounds to allow accurate phenotypic comparison.

Since the number of docking strains for φC31 integrase in *An. gambiae* is limited, we also included attP sites into the PUB-GAL4 transformation construct so that these lines can also be used as docking lines for RMCE or integration. Indeed, in the presence of two inverted attP sites, cassette integration also occurs in some individuals as a result of a single crossover event, rather than the double crossover that generates cassette exchange. As such, F_1 progeny can be selected by screening for the presence of single or double markers respectively. RMCE allows complete swapping of transgene cargoes for phenotypic analysis (Bateman et al., 2006). For example, the activity of other promoters could be assessed and directly compared to that of PUBc; similarly, different UAS cassettes could be exchanged into these sites, and then crossed to parental or other GAL4 driver lines. In other instances, integration may be desirable, such as inserting a UAS construct next to the PUBc-GAL4 in order to create stable GAL4/UAS lines without the need of crossing, and thus to readily generate homozygous GAL4/UAS lines. Although the φC31 system may be the most convenient way to exploit these genomic sites, they can also be targeted by CRISPR/Cas9 approaches in which transgenes can be targeted to defined transcriptionally permissive sites.

The A10 and A8 driver lines established as part of this work sustain widespread gene expression at different levels and will prove valuable and versatile tools for gene functional analysis in *An. gambiae* using the GAL4/UAS system. Their potential uses include gain- or loss-of-function of any gene located in corresponding UAS lines. Owing to its multi-tissue expression, the PUBc promoter could provide more reliable expression of transformation markers than 3xP3 and would be useful for efficient mass screening of large numbers of transgenic individuals at lower magnification or by automated screening systems (Marois et al., 2012; Volohonsky et al., 2015). It could also be used to create a transgenic line that stably produces broad expression of Cas9, which can be transiently modified by injection with guide RNAs or crossed with transgenic lines expressing guide RNAs.

Authors contributions

Conceived the work: AA, EP, CB, GJL

Participated in design of experiments AA, EP, AL, CB, GJL

Performed the experiments: AA, EP

Analyzed the data: AA, EP, CB, GJL

Drafted the manuscript: AA

Edited and reviewed manuscript: AA, EP, CB, AL, GJL

Funding

The work was funded through an internal studentship program from the LSTM. Support for AL was in part provided by European Union Seventh Framework Programme FP7 (2007–2013) under grant

403 agreement no. 265660 AvecNet. Support to EP was from an ANR-07-MIME-O25-01 award to CB and
404 from Fondation Roux (Institut Pasteur) fellowship to EP.

405

406 **Conflicts of interest**

407 Authors declare no conflict of interest.

408

409 **Acknowledgments**

410 We thank the *Drosophila* Genomics Resource Center (DGRC), supported by NIH grant
411 2P40OD010949 for supply of MF3 plasmid. We are grateful to Prof. Hilary Ranson (LSTM) for helpful
412 comments on manuscript and mentoring AA during her studies. We are also very thankful for the
413 assistance of Amalia Anthousi (LSTM) in rearing and dissections of mosquitoes, and Nicolas Puchot
414 (Institut Pasteur) for assisting in the production of the UAS:mCherry line.

415

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559

FIGURE CAPTIONS

Figure 1. **Constructs used for transgenic line creation and analysis of cell line activity of PUBc regulatory regions.**

A) Schematic of the GAL4/UAS bi-partite system for ectopic gene expression (adapted from Lynd and Lycett, 2011). The driver construct consists of a promoter that induces expression of the transcription activator factor GAL4. The responder construct contains the GAL4 binding site UAS (Upstream Activating Sequence) located upstream of the gene of interest. **B)** Schematic of the PUBc-GAL4 construct inserted in the driver lines. Grey arrows: piggyBac inverted terminal repeats. P orange arrows: φC31 attP sites for RMCE; 3xP3: neuronal promoter. eCFP: enhanced cyan fluorescent protein. Black arrows: Gypsy insulators. PUBc5': PUBc gene 5' region cloned upstream of the GAL4. PUBc3': PUBc gene 3' region cloned downstream of the GAL4. **C)** Schematic of the responder plasmid driving the 14xUAS-regulated expression of luciferase (Lynd and Lycett, 2012) used to co-transfect mosquito cells. UAS(14x): 14 repeats of the Upstream Activating Sequence. Luc: firefly luciferase gene. **D)** Schematic of the plasmid used to measure background activity (blank). The PUB5' region present in this plasmid is a shorter version of the PUBc promoter sequence incorporated in the driver construct fused to eGFP (enhanced green fluorescent protein) gene and SV40 terminator sequences derived from PTubGFP (Lycett et al, 2012) **E)** Schematic of the responder construct present in the UAS-mCD8:mCherry line obtained by φC31-mediated site-specific integration into the E docking line. Orange R and L boxes: attR and attL sites resulting from the recombination of attB and attP site. UAS(10x): 10 UAS repeats. mCD8:mCherry: mCherry fluorescent marker directed to the cell membrane. **F)** Activity of PUBc regulatory regions in *An. gambiae* SUA5.1 cells. Promoter activity is shown as luciferase expression (Relative Lights Units – RLU) after co-transfection of the driver plasmid pPUBc-GAL4 with the pUAS-Luc responder plasmid. Top bars represent standard error from mean. Significant differences (****: $P < 0.0001$, ns: $P > 0.05$) were calculated using one-way ANOVA with Tukey's multiple comparisons test on six replicates.

Figure 2. Expression profiles driven by the PUBc regulatory regions in A10/mCherry (left) and A8/mCherry (right) whole mosquitoes.

A-B) L3-4 larvae dorsal view. m: mouthparts; b: brain; ms: muscles; fb: fat body. **C-D)** L3-4 larvae ventral view. nc: nerve cord; id: imaginal discs. **E-F)** Female pupae. an: antenna. **G-H)** Adult females ventral view. p: palps; an: antenna; om: ommatidia; l: leg; t: thorax; ab: abdomen. **I-J)** Adult males ventral view. mCherry signal was detected at 650 nm. Numbers show seconds (s) of exposure.

Figure 3. Expression profiles driven by the PUBc regulatory regions in dissected and fixed female tissues from A10/mCherry and A8/mCherry mosquitoes.

PUBc-driven expression in A10/mCherry (A,E,I,M) and A8/mCherry (C,G,K,O) female adult abdomen (A-D), digestive tract (E-H), salivary glands (I-L), ovaries (M-P). A,C,E,G,I,K,M,O represent mCherry signal at 650 nm; numbers show milliseconds (ms) or seconds (s) of exposure. B,D,F,H,J,L,N,P are

602 the corresponding bright fields. fb: fat body; ms: muscles; nc: nerve cord; tr: trachea; f: follicles; ov:
603 oviduct.
604

605 **Table 1.** Selection strategy to isolate stable transgenic driver lines carrying a single
606 insertion of the PUBc-GAL4 cassette.
607

F_0 pool	F_0 adults Number and sex	Transient eCFP expression	Number of F_1 eCFP + Adults (sex)	% eCFP + F_2 larvae (Number eCFP +/total)
A	13 F	+	34 (17 F, 17 M)	A 2 – 49% (84/173) A 3 – 48% (74/154) A 5 – 62% (5/8) A 6 – 42% (80/190) A 7 – 50% (52/103) A 8 – 49% (67/136) A 10 – 47% (65/139) A 11 – 52% (63/121) A 12 – 53% (102/193) A 15 – 51% (93/181)
B	14 M	+	3 (3 M)	B 1 – 73% (8/11) B 2 – 58% (18/31) B 3 – 44% (31/71) B 7 – 59% (69/116) B 8 – 53% (62/116)
C	18 F	+	5 (2 F, 3 M)	C 1 – 47% (28/59) C 2 – 47% (73/155)
D	20 M	+	3 (2 F, 1 M)	D 2 – 46% (53/115)
E	23 M	–	0	N/A
F	9 F	–	0	N/A

608
609 F_0 pool: designation given to cage of surviving F_0 adults; F_0 adults: the number and sex of F_0 adults
610 crossed with wild type; Transient eCFP expression: F_0 adults either displayed (+) or lacked (-) transient
611 eCFP expression at larval stage; Number of F_1 eCFP + Adults: total number and sex of adults derived
612 from eCFP + larvae from F_0 cage cross; % eCFP + F_2 larvae: % of eCFP + larvae derived from
613 isofemale lines originated from F_1 adults.
614 F: female, M: male. N/A: not applicable.
615 Other isofemales: A1: dead (mosquito died without laying eggs). A4, B9: not mated (eggs did not hatch).
616 A9, A13, A14, B4, B5, B6, B10, D1: sterile (mosquito did not lay eggs).
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620

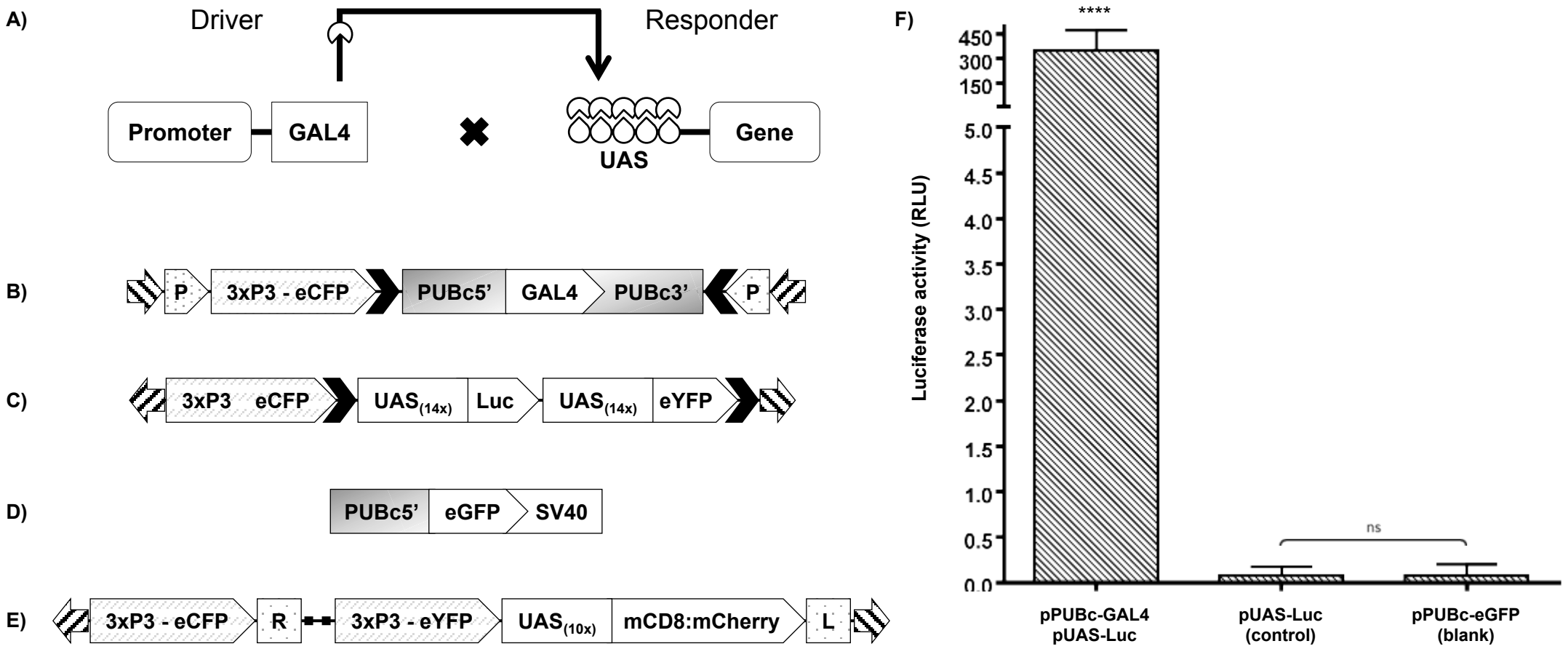
Table 2. Molecular characterisation of transgenic lines carrying single insertions by inverse-PCR.

Line	pBAC arm	Enzyme	Sequence	GenBank ID	Query length	Alignment length	Identity %	PEST Chromosome	Position	
A2	Left Right	TaqαI BfuCI	TTAAATGGATAGCGGTAGCT CCGGTGAGTGCGTAGCTTAA	MG934563 --	795 79	653-6 79	95-96 100	Multiple hits	Undetermined	
A3	Left Right	BfuCI BfuCI	TTAAACTGTTTGCCGGCCGC GTCCTCCAGGTAGTCTTTAA	-- --	75 33	75 31	96 100	3 R	6,934,418 6,934,415	Intragenic AGAP008214
A8	Left Right	TaqαI *	TTAATCTCGGTTCTCGGTAT ATATTTACGCAGATTCTTAA	MG934564 --	323 53	323 42	100 90.5	2 R	32,162,290 32,162,293	Intergenic
A10	Left Right	TaqαI *	TTAAAGAACTGATCAATACA CAAACGCACAGTACCATTAA	MG934565 MG934566	404 368	329** 353	99.1 98.9	2 R	5,816,202 5,816,199	Intergenic
B3	Left Right	BfuCI BfuCI	TTAAAGCTCGTTCATACTCT GAAGCTGTAAAAAGCTTTAA	MG934567 --	221 192	221 191	98.2 95.8	3 R	32,092,431 32,092,428	Intergenic
C1	Left Right	TaqαI BfuCI	TTAAAAGAGATCCCGCGAGG TAGGTCGATGAACTCCTTAA	MG934568 --	652 102	648 102	99.2 100	X	21,936,498 21,936,501	Intergenic
D2	Left	TaqαI	TTAAAGTGCTTTCAACGTTA	MG934569	700	700	98.6-99.4	Multiple hits	Undetermined	

Enzyme refers to the enzyme used for genomic DNA cleavage prior to iPCR; sequence refers to the first 20 bp directly adjacent to the TTAA of the insertion site (--: sequence shorter than 200 bp and thus not submitted to GenBank); PEST Chromosome indicates the chromosome of transgene insertion; position indicates the co-ordinates of insertion with reference to the PEST genome sequence AgamP4 reported in VectorBase (Giraldo-Calderón et al 2015).

* the genomic position flanking this arm was obtained by amplification of genomic DNA using primer sequences deduced from the position of the left arm.

** there is an insertion in the PEST sequence that is not present in A10, Pimperena strain or *An. coluzzii*.


A)
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Promoter
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UAS
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B)
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PUBc5'
GAL4
PUBc3'
P
C)
3xP3 eCFP
UAS(14x)
Luc
UAS(14x)
eYFP
D)
PUBc5'
eGFP
SV40
E)
3xP3 - eCFP
R
3xP3 - eYFP
UAS(10x)
mCD8:mCherry
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F)
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Luciferase activity (RLU)
450
300
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4.0
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1.5
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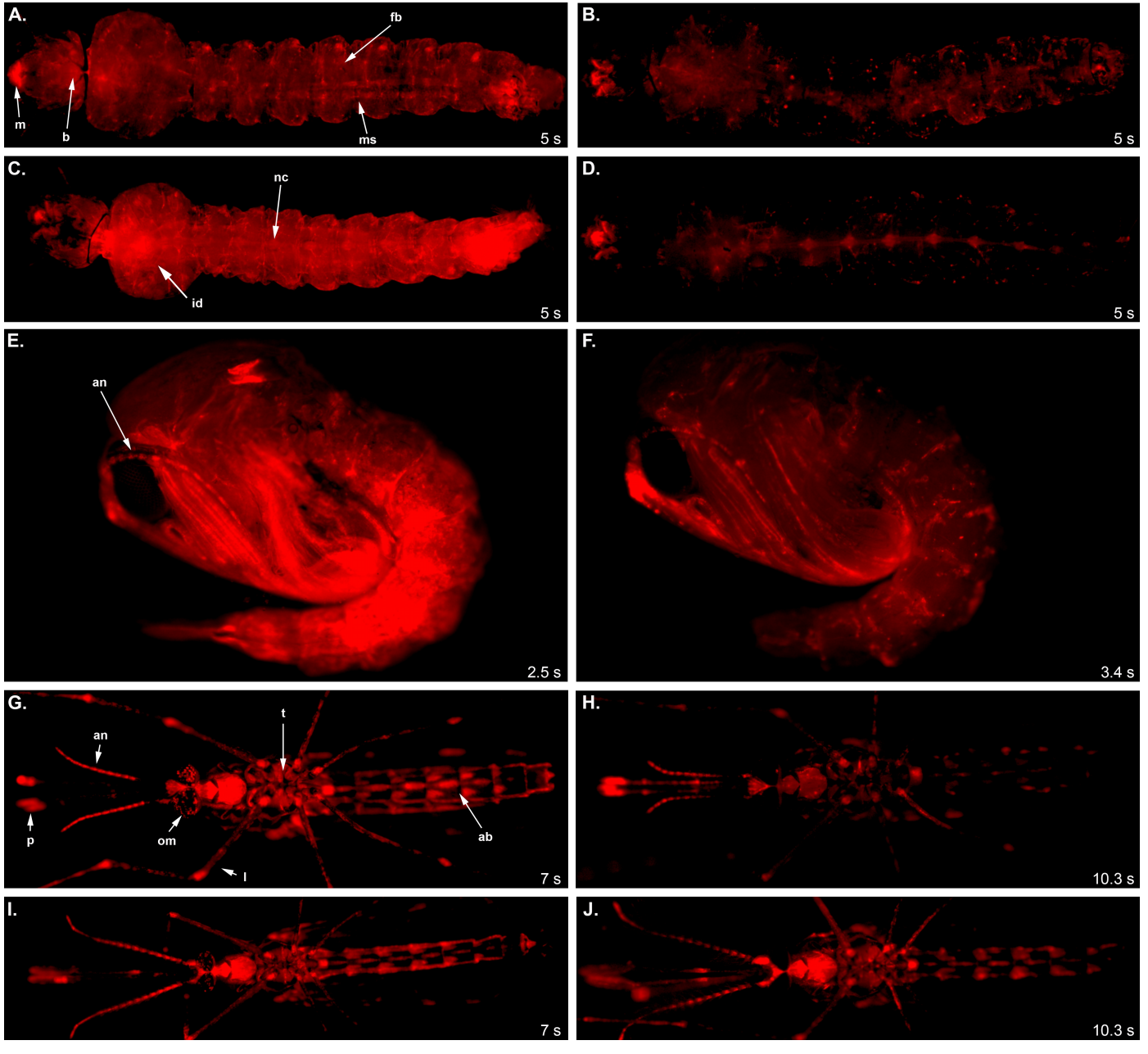
0.5
0.0
pPUBc-GAL4
pUAS-Luc
pUAS-Luc
(control)
pPUBc-eGFP
(blank)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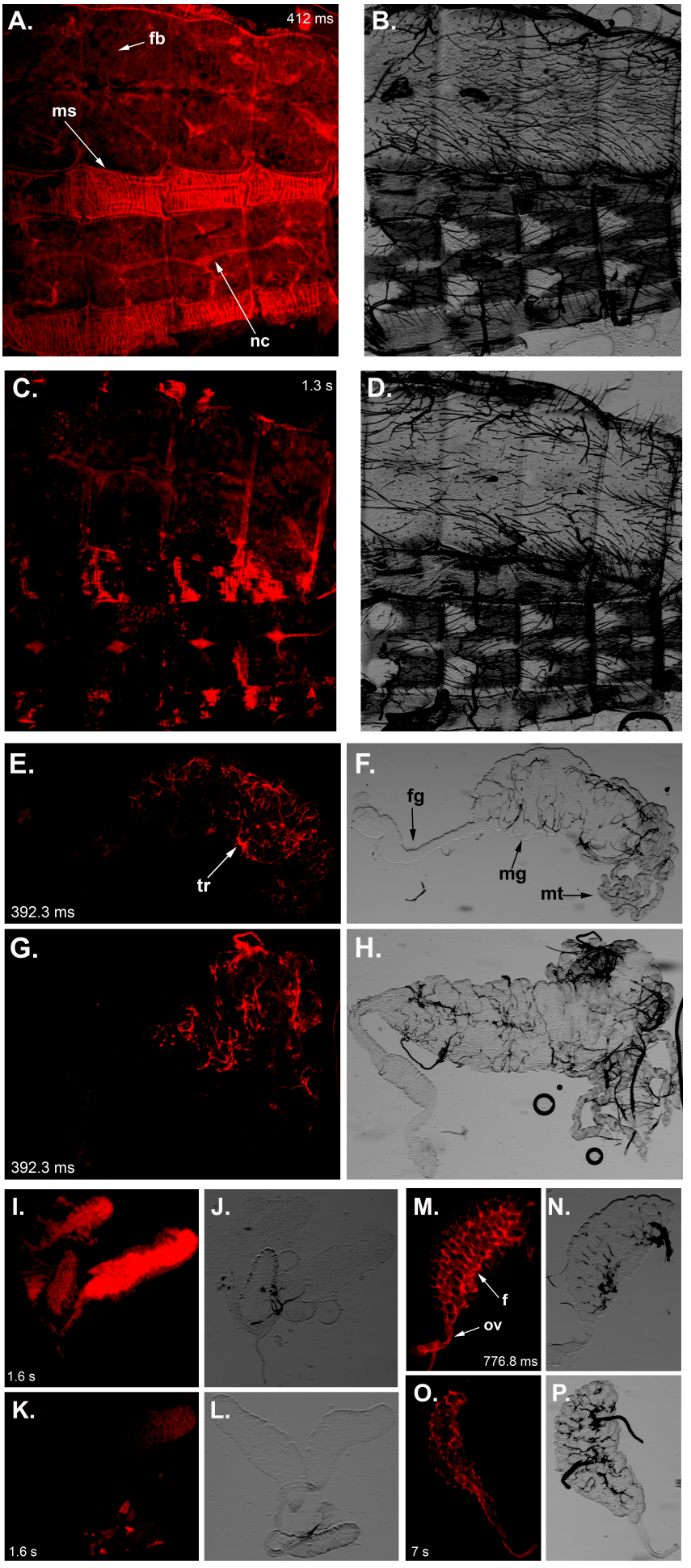
J.
10.3 s


A.
412 ms
fb
ms
nc
B.
C.
1.3 s
D.
E.
tr
392.3 ms
F.
fg
mg
mt
G.
392.3 ms
H.
I.
1.6 s
J.
M.
f
ov
776.8 ms
N.
K.
1.6 s
L.
O.
7 s
P.

SUPPLEMENTARY DATA

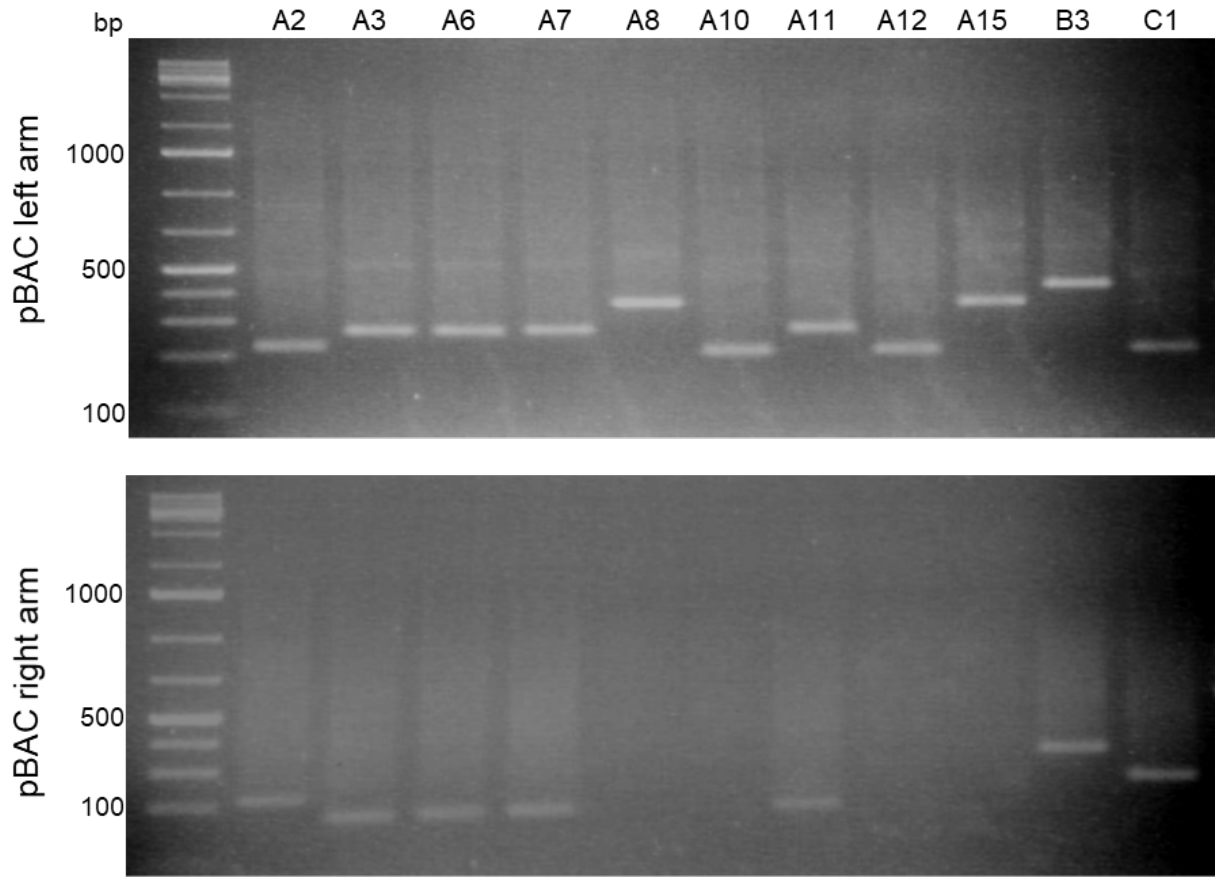


Figure S1. Agarose gel electrophoresis showing results for inverse-PCR on regions flanking the piggyBac arms after BfuCI digestion and self-ligation of genomic DNA isolated from isofemale lines. Among the isofemale lines derived cage A, those sharing the same PCR product size originated from the same F_0 founder individual and thus bear an insertion in the same genomic location. Six different single insertions are shown: 1) A2; 2) A3 = A6 = A7 = A11; 3) A8 = A15; 4) A10 = A12, 5) B3; 6) C1. Ladder is GeneRuler 1 kb Plus (Thermo Scientific).

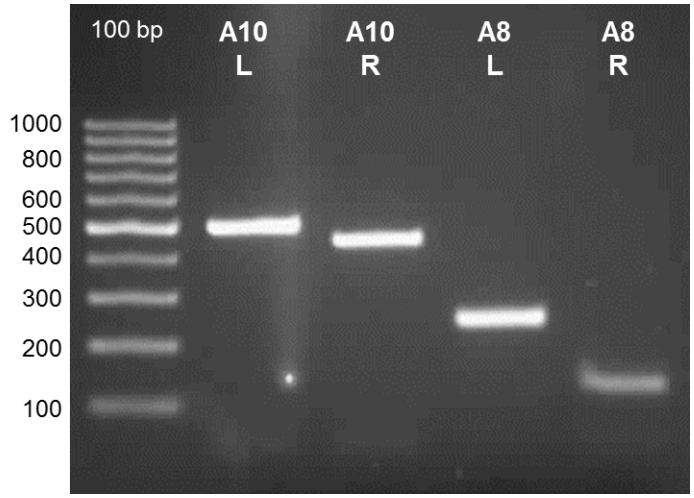


Figure S2. Agarose gel electrophoresis confirming insertion sites in A8 and A10 lines by PCR using a primer designed against regions near the predicted genomic insertion sites and the other annealing to conserved region of the integrated construct. A10: genomic regions external to the left (L) and right (R) piggyBac arms at the insertion site in the A10 line. A8: genomic regions external to the left (L) and right (R) piggyBac arms at the insertion site in the A8 line. Ladder is GeneRuler 100 bp (Thermo Scientific).

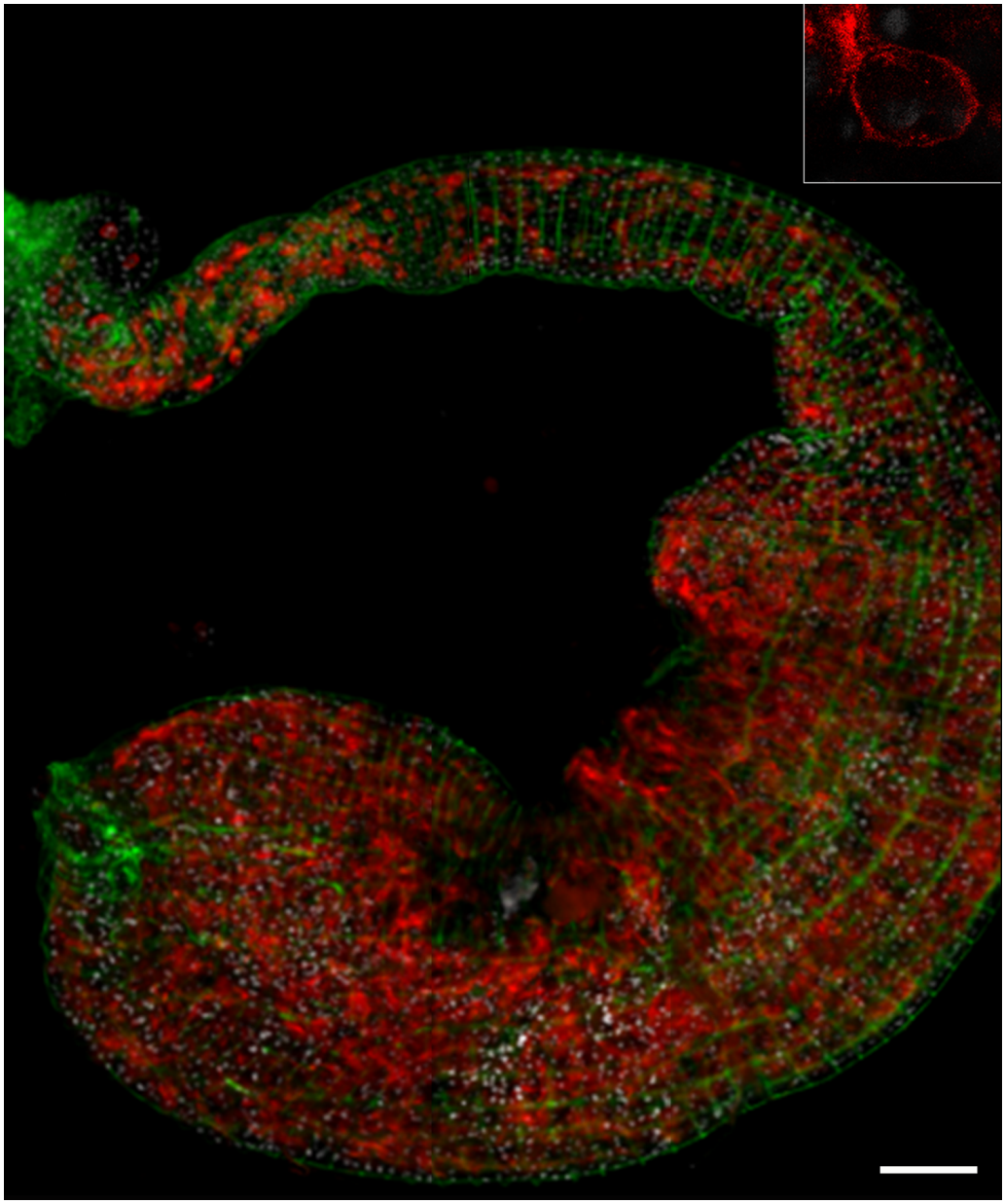


Figure S3. Combined Z stacks from confocal microscopy to include whole midgut from an adult female of the carboxypeptidase-GAL4/mCD8:mCherry line. Fixed midguts were stained with DAPI (white) and phalloidin 488 (green). Red signal shows the endogenous expression of mCD8:mCherry. Scale bar is 250 μm. Inset shows an individual midgut cell expressing mCD8:mCherry at the cell membrane.

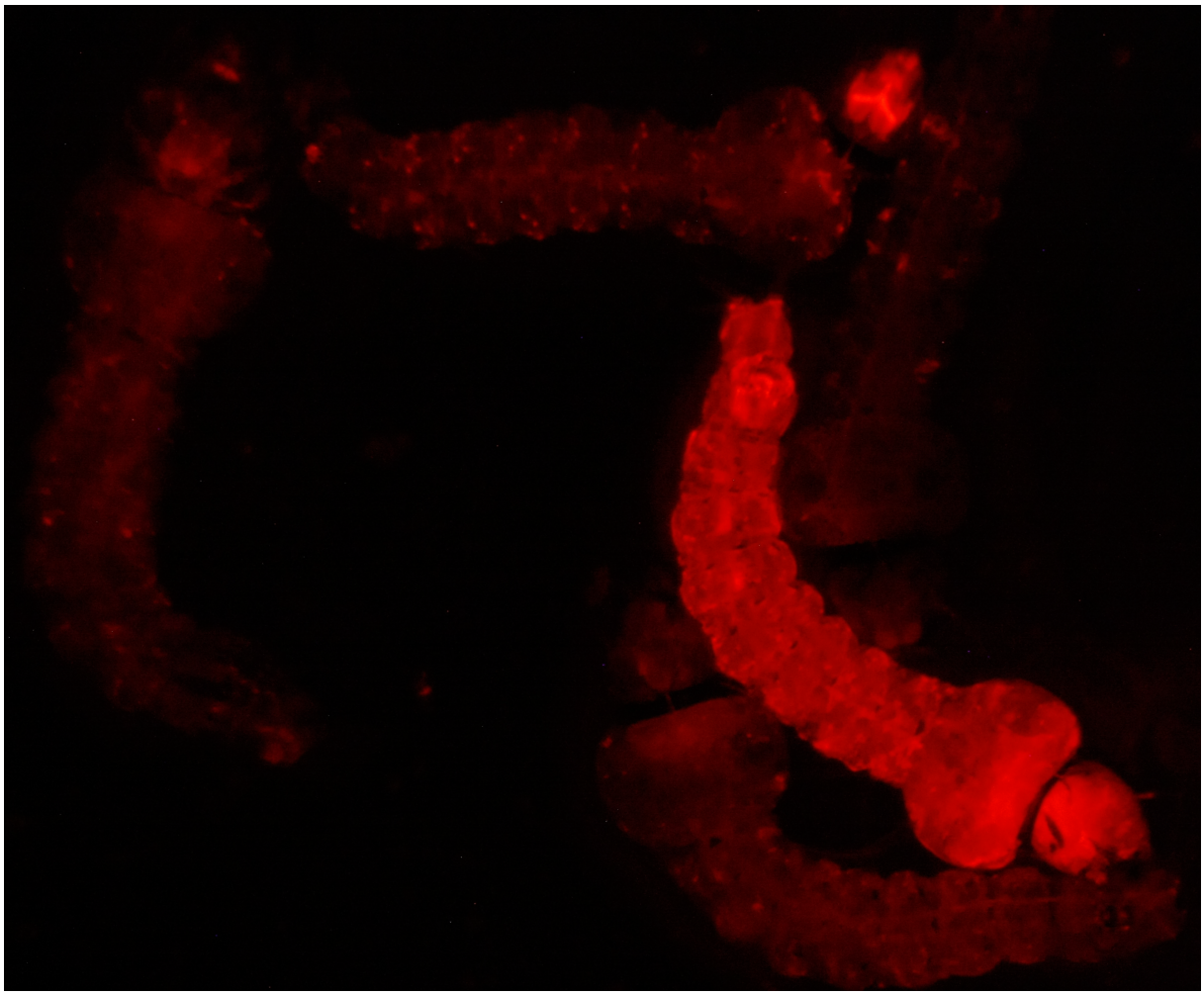


Figure S4. Example of phenotype variegation in the PUBc-GAL4 lines. mCherry phenotypes in L2-3 larvae derived from PUBc-GAL4 F_1 transformant males. These are also representative of the phenotypes observed in the progeny of single laying females.

Table S1. Primers used in this study.

Primer	Fragment	Template	Sequence 5'→3'
pSL-Gypsy1-FW	Gypsy1	attB:UAS:Cyp6m2	GAGGTAATTATAACCCGGGCCCTATATATGACTAGAATTGATCGGCTAAATGGTATGGCA
Gypsy1-PUBc5-RV			GAAATATTTTTTTATTCGCTAGCCAATGTATCTTAACTACTCACGTAATAAGTGTGCG
Gypsy1-PUBc5-FW	PUBc5	Kisumu gDNA	GAGTAGTTAAGATACATTGGCTAGCGAATAAAAAAATATTTCTTAATAATATTCTAAC
PUBc5-GAL4-RV			GAAGACAGTAGCTTCATATTTTATCTGTAAATATAAACGAAAACAAC
PUBc5-GAL4-FW	GAL4	attB:GAL4:DsRed	GTTTTCGTTTATATTTACAGATAAAATATGAAGCTACTGTCTTCTATCGAACAAGCATG
GAL4-pSL-RV			CAGCTGCAGGCGGCCGCCATATGCAAGATCTTTACTCTTTTTTTGGGTTTGGTGGG
pSL-PUBc3-FW	PUBc3	Kisumu gDNA	TAATTATAACCCGGGCCCTATATATGAGATCTTTCGTTGAATAAAGCATATTGAAGCTTC
PUBc3-Gypsy2-RV			AGCCGATCAATTCTAGTCATATGCCGTCGAAATTGTTTTACAATGACAATTTT
PUBc3-Gypsy2-FW	Gypsy2	attB:UAS:Cyp6m2	GTAAAACAATTTCGACGGCATATGACTAGAATTGATCGGCTAAATGGTATGGCA
Gypsy2-attP-RV			GTCAGTCGCGCGAGCGCGCCGCGGCAATGTATCTTAACTACTCACGTAATAAGTGTGCG
Gypsy2-attP-FW	attP	pBAC:attP:eCFP	AGTAGTTAAGATACATTGCCGCGGCGCGCTCGCGCGACTGACGGTCGTAAGCAC
attP-pSL-RV			AGCTGCAGGCGGCCGCCATATGCACGAAGCCCCGGCGGCAACCCTCAGCG
3P3-FW	3xP3-eYFPafm-attB	pBAC[3xP3-eYFPafm]	GTCATCACAGAACACATTTG
attB-RV			CAGGTACCGTCGACGATGTAG
BglII-mCD8-FW	mCD8:cherry	pUAS-mCD8:cherry	TAGCAGCCAGATCTGTCGACGGTATCGATAAGC
XbaI-cherry-RV			TAGCAGCCTCTAGATTACTTGTACAGCTCGTCCATGC
ITRL1F	--	--	ATCAGTGACACTTACCGCATTGACA
ITRL1R	--	--	TGACGAGCTTGTTGGTGAGGATTCT
ITRR1F	--	--	TACGCATGATTATCTTTAACGTA
ITRR1R	--	--	GATGTTTTGTTTTGACGGACCCC