



**A nuclear targeting approach enables
efficient transgenesis in the milkweed bug *Oncopeltus
fasciatus***

**Yu Shirai^{1 *}, Yousuf Hashmi^{1,2}, Ashley Watts^{1,3}, Jonchee A. Kao^{4,5},
Cassandra G. Extavour^{1,4,6 *}**

1. Department of Organismic and Evolutionary Biology, Harvard University,
Cambridge, MA, USA

2. Present address: Committee on Regeneration, Development, and Stem Cell Biology,
University of Chicago, Chicago, IL, USA

3. Present address:

4. Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA,
USA

5. Present address: Department of Applied Biosciences, Graduate School of Agriculture,
Kyoto University, Kyoto, Japan

6. Howard Hughes Medical Institute, Chevy Chase, MD, USA

* Correspondence: Yu Shirai, Cassandra G. Extavour

Email: shiraiyu@fas.harvard.edu, extavour@oeb.harvard.edu

Abstract

Insects show extreme diversity and have long intrigued biologists. Recent technological advancements, such as gene editing and transgenesis, should in principle enable the use of almost any insect species for biological research. However, in practice, species-specific challenges remain and conditions must be optimized carefully. Here, using the milkweed bug *Oncopeltus fasciatus*, we first generate a useful eye- and body-color mutant strain by using CRISPR/Cas9-mediated genome editing. Then we use this strain to develop an efficient *piggyBac*-mediated transgenesis system using a nuclear targeting approach. We show that incorporating 1× and 3× nuclear localization signals (NLS) into *piggyBac* mRNA substantially enhances overall transformation efficiency in *O. fasciatus*. Taking advantage of both the useful mutant strain and the efficient transgenesis system, we successfully integrated multiple expression cassettes ranging from 1.8 to 7.2 kb, including attP strains for phiC31-mediated site-specific integration and histone-labelled strains for live fluorescence imaging. We further provide evidence that the Q system, a binary expression system, is functional in this species, paving the way for future sophisticated genetic manipulations including functional assays for *cis*-regulatory elements. Together, our results not only expand the genetic toolkit of *O. fasciatus* as a comparative model insect, but also provide a practical framework for developing efficient transgenesis in other non-traditional model organisms.

Keywords

CRISPR/Cas9; Transgenesis; *piggyBac*; Q-system; Reporter assay; *Oncopeltus fasciatus*

Introduction

Insects are one of the most diverse groups of animals on Earth (Stork, 2017). Their remarkable beauty, adaptations, and diversity have long fascinated biologists. Many biological questions about insect diversity and evolution cannot be addressed using the long-standing arthropod model *Drosophila melanogaster* alone (Clark et al., 2019; Kao et al., 2026; Truman et al., 2026). Beyond the *Drosophila melanogaster* paradigm, recent technological advancements have enabled us to use functional genetics to investigate many non-traditional model organisms, opening new avenues for studies of the evolution of developmental mechanisms (Gudmunds et al., 2022). Comparative studies are essential to understand the evolutionary history of insects. Establishing genomic and biological resources across multiple species has thus become a critical investment in cell and developmental biology.

The large milkweed bug, *Oncopeltus fasciatus* (Lygaeidae, Hemiptera) has a long history of comparative developmental studies (Chipman, 2017; Feir, 1974). Hemiptera is a large and diverse order of insects that is phylogenetically important for understanding insect evolution, as it is an outgroup to Holometabola, the clade of insects that undergo complete metamorphosis (Misof et al., 2014). In the mid-twentieth century, *O. fasciatus* was widely used as a model organism across many different contexts, including embryology, physiology, and histology (Feir, 1974). However, the rise of *D. melanogaster* genetics largely displaced *O. fasciatus*, along with other insect species (Chipman, 2017). Approximately two decades ago, taking advantage of its ease of rearing and high sensitivity to RNAi-mediated gene perturbation, *O. fasciatus* began to re-emerge as an evo-devo model, particularly for the study of segmentation and body patterning (Angelini and Kaufman, 2005; Angelini et al., 2005; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Liu and Kaufman, 2005; Liu and Patel, 2009; Liu and

Patel, 2010). More recently, the development of CRISPR/Cas9-mediated targeted mutagenesis (Reding and Pick, 2020) and knock-in methods (Shirai et al., 2026) has further expanded its utility as a comparative model organism for functional genetics.

Since transgenesis is a powerful genetic tool for investigating gene function and *cis*-regulatory elements (Gregory et al., 2016; Handler, 2002), deploying this tool in *O. fasciatus* is essential for establishing it as a more versatile model organism. We focus here on a hyperactive variant of the piggyBac transposase called hyPBase (Yusa et al., 2011), which has been successfully applied across multiple insect species (Chen and Palli, 2021; Gonzalez-Sqalli et al., 2024; Hart et al., 2023; Heryanto et al., 2022; Inada et al., 2025; Kou et al., 2023; Ohde et al., 2025), consistently outperforming the original *piggyBac* transposase in overall efficiency (Eckermann et al., 2018; Ohde et al., 2025; Otte et al., 2018).

Here, we describe an efficient *piggyBac*-mediated transgenesis system in *O. fasciatus*. First, we generate a useful eye- and body-color mutant strain by crossing the *Of-vermilion* (*Of-v*) mutant (Reding et al., 2023) with a newly generated CRISPR strain carrying a mutation in *Of-xanthine dehydrogenase* (*xdh1*), the ortholog of *D. melanogaster rosy*, enabling easier detection of fluorescent signals through the adult cuticle. Using this double mutant strain, we develop a highly efficient *piggyBac*-mediated transgenesis system by incorporating additional SV40 nuclear localization signals (NLSs) (Kalderon et al., 1984) into hyPBase. Furthermore, we provide evidence that Q system mediated-binary expression (Potter et al., 2010) is functional in *O. fasciatus*. Together, our results substantially expand the experimental possibilities of *O. fasciatus* as a comparative genetic model organism and provide a practical framework for implementing efficient transgenesis in other non-traditional model organisms.

Results

Establishment of a useful eye- and body-color mutant strain in *O. fasciatus*

Many successful insect transgenesis systems rely on detection of a dominant marker, often expression of a fluorescent protein, in the eye (Berghammer et al., 1999). However, detecting this marker expression can be challenging if the animal's eyes are darkly pigmented. In the case of *O. fasciatus*, the wild-type eye color is black. Therefore, we aimed to generate a mutant strain with a lighter eye color to facilitate the detection of fluorescent signals, such as GFP or RFP, derived from transgenesis. Previous studies have attempted to generate such a strain: one study reported that the *O. fasciatus white* (*Of-w*) gene, commonly used to provide a suitable background for detection of a dominant rescue construct in other insects, caused lethality when disrupted (Reding and Pick, 2020), while another study established strains mutant for *Of-v*, an ommochrome pathway gene, displaying dark red eye color (Reding et al., 2023). However, we were not certain whether the eyes of the *v* mutants were light enough to detect fluorescent signals. In 1970, Peter Lawrence reported *O. fasciatus* mutants named "*red eye (re)*", "*white body (wb)*", and "*cream body (cb)*" (Lawrence, 1970). *re* mutants had red eyes, and the described phenotypes of *re* mutants are generally consistent with that of *Of-v* mutants (Reding et al., 2023), suggesting that *re* mutants lack ommochrome pigments. By contrast, *wb* and *cb* mutants were reported as having both a whitish yellow body color, and these described phenotypes are comparable to the loss of orange body coloration observed upon systemic RNAi of pteridine pathway genes (Liu, 2016; Reding et al., 2023). Importantly, double mutants of *re* and either *wb* or *cb* showed eye color distinct from that of the single mutants (Lawrence, 1970), and maternal RNAi of *punch*, a pteridine pathway gene, resulted in

eye-color defects in embryos (Liu, 2016). Together, these results suggested that both ommochrome and pteridine pigments might contribute to eye color formation in *O. fasciatus*. Based on these findings, we decided to target the *O. fasciatus* ortholog of the pteridine pathway gene *xdh1* (*Of-xdh1*, Accession No. OFAS027123), whose *D. melanogaster* ortholog is the *rosy* gene (Keith et al., 1987). *Of-xdh1* has been implicated in body coloration in *O. fasciatus* based on systemic RNAi-mediated functional analysis (Reding et al., 2023).

Of-xdh1 is predicted to encode a 1341 amino acid pteridine pathway enzyme containing 2Fe-2S, flavin adenine dinucleotide (FAD) binding, and molybdopterin binding domains, as determined by InterProScan (Fig. 1A). To generate a homozygous mutant *Of-xdh1* strain (Fig. 1B), we designed a guide RNA targeting the region immediately downstream of the start codon (Fig. 1A), hoping to generate an indel and frameshift resulting in a premature stop codon and eliminating the functional domains of this enzyme. Following injection of Cas9/gRNA ribonucleoprotein complex into 54 eggs, 17 of 18 hatchlings (94.4%) showed a clear body color difference in the injected generation (G₀) compared to wild type (WT), enabling selection of potential mutants (Fig. 1C). After three rounds of crossing, we established a homozygous strain named *xdh1*^{Δ2}, carrying a two base pair deletion allele introducing a premature stop codon, and displaying orange body coloration (Fig. 1D–E and Supplementary Fig. 1).

To investigate the potential involvement of *xdh1* in eye-color formation, we crossed *xdh1*^{Δ2} animals with those from the *Of-v* mutant strain *v*^l (Reding et al., 2023) (Supplementary Fig. 2) to generate a double homozygous mutant strain. We confirmed the mutations at both loci by sequencing. Comparison of eye color among four different strains, wild type, *v*^l, *xdh1*^{Δ2}, and *v*^l *xdh1*^{Δ2}, revealed that the *v*^l *xdh1*^{Δ2} double mutant strain displayed a brighter orange eye color than all the others (Fig. 1E–F and

Supplementary Fig. 3). The double mutant strain also showed a brighter yellowish body color than the others, particularly at early nymphal stages (Fig. 1E, and Supplementary Fig. 3), indicating that both ommochrome and pteridine pigments contribute to eye and body color formation in *O. fasciatus*. Thus, we used this strain for the subsequent transgenesis experiments described in the following sections.

Nuclear targeting allows an efficient hyPBase-mediated transgenesis in *O. fasciatus*

To develop a transgenesis method in *O. fasciatus* using the $v^l\ xdh1^{A2}$ strain (Fig. 2A), we first utilized the *piggyBac* vector *pXL[9×P3-mScarlet-I-SV40T]* (1,775 bp). In this vector, mScarlet-I is placed under the control of the 9×P3 promoter, consisting of nine copies of a Pax6 homeodomain binding site, which drives specific expression in the eyes in many insects (Berghammer et al., 1999; Horn and Wimmer, 2000; Horn et al., 2000; Inada et al., 2025; Ohde et al., 2025; Sheng et al., 1997) (Fig. 2B). We screened for mScarlet-I expression in the eyes of injected animals at both mid-nymphal and adult stages. In the first round of injection with *in vitro* transcribed *hyPBase* mRNA, 5/29 G₀ animals were mScarlet-I-positive at nymphal stages (G₀ somatic transformation rate (STR) = 17.2%), and 6/25 (STR = 24.0%) at adult stages (Fig. 2C–D and Table 1). However, no G₀ animals showed mScarlet-I expression at either stage in a second round of injection using the same batch of reagents (0/70 at nymphal stages and 0/62 at adult stages; STR = 0%) (Fig. 2D and Table 1), suggesting inconsistent transgenesis efficiency with unmodified hyPBase.

Given this inconsistency across technical replicate experiments, we hypothesized that incorporating additional SV40T nuclear localization signals (NLSs) into hyPBase would improve transgenesis efficiency (Fig. 2B). To test this hypothesis, we compared STR values across 0×, 1×, 3× and 5×NLS *hyPBase* mRNA conditions

using the same donor vector. We found that 1× and 3×NLS *hyPBase* mRNA yielded higher and more consistent STR values than 0×NLS. Across two experimental replicates, STR values for 1×NLS ranged from 15.7% (11/70) to 23.1% (18/78) at nymphal stages and from 17.5% (10/57) to 31.8% (20/63) at adult stages; 3×NLS ranged from 15.2% (7/46) to 34.1% (15/44) at nymphal stages and from 20.0% (8/41) to 38.5% (15/40) at adult stages (Fig. 2D and Table 1). By contrast, with 5×NLS we recovered no positive G₀ animals (0/68 at nymphal stages) (Fig. 2D and Table 1). We also noted that screening at adult stages yielded higher STR values than at nymphal stages (Fig. 2D and Table 1), possibly because the eye color of the *v^l xdhI^{Δ2}* strain becomes progressively lighter with development (Fig. 1E–F and Supplementary Fig. 3).

To investigate whether this trend was conserved in another insect species, we injected the same series of *hyPBase* mRNAs with the same donor vector *pXL[9xP3-mScarlet-I-SV40T]* into embryos of the cricket *Gryllus bimaculatus* (Supplementary Fig. 4 and Supplementary Table 1). In contrast to our results from *O. fasciatus* (Fig. 2D and Table 1), we found no differences in efficiency across NLS copy number in *G. bimaculatus* (Supplementary Fig. 4 and Supplementary Table 1), although overall efficiency in *G. bimaculatus* was generally higher than in *O. fasciatus* (Table 1 and Supplementary Table 1). Together, these results indicate that incorporating 1× and 3×NLS into *hyPBase* mRNA may be a practical strategy to improve transgenesis efficiency in species where standard methods are inefficient.

To assess germ line transmission, we backcrossed mScarlet-I-positive G₀ *O. fasciatus* adults with *v^l xdhI^{Δ2}* adults of the opposite sex and counted the number of mScarlet-I-positive G₁ animals emerging from single pair matings (Supplementary Tables 2–4 for detailed results of individual crosses). In positive G₁ animals we detected mScarlet-I expression throughout the compound eyes and in the ocelli (Fig. 2E). G₁

germ line transformation efficiency (GTE) with 0×NLS *hyPBase* mRNA was 16.7% (1/6 crosses), whereas 1× and 3×NLS conditions provided approximate twofold enhancement, yielding 33.3% (9/27 crosses) and 35.0% (7/20 crosses) GTE, respectively (Fig. 2F and Supplementary Tables 2–4).

Evaluation of a 9×P3-EGFP transgenesis marker in *O. fasciatus*

To expand the transgenesis toolkit in *O. fasciatus*, we next tested the donor plasmid *pXL[9×P3-EGFP-SV40T, attP]* (1,838 bp) carrying an EGFP transformation marker driven by the 9×P3 promoter and an attP site for future application of phiC31-mediated site-specific integration (Fig. 3A) (Groth et al., 2004). Two experimental replicates of injection with 3×NLS *hyPBase* mRNA resulted in 15.4% (10/65) and 9.3% (5/54) G₀ STR when screened at nymphal stages (Table 2). As with mScarlet-I expression, we observed EGFP expression in the compound eyes and in the ocelli (Fig. 3B). Among successful backcrosses (12 of 15 EGFP-positive G₀ animals) with animals of the opposite sex from the *v^l xdh1^{A2}* strain, we recovered five EGFP-positive G₁ animals (5/12 crosses; GTE = 41.7%) (Fig. 3C–D and Supplementary Table 5 for detailed results of individual crosses). We then crossed four G₁ adults with animals of the opposite sex of the *v^l xdh1^{A2}* strain and found that three of these crosses showed >72.5% G₂ segregation rates, suggesting that the parent G₁ animals were likely to have more than one transgene (Supplementary Table 5). Using two G₂ animals from one of the crosses which showed a 50% segregation rate, consistent with one insertion, we performed splinkerette PCR (Potter and Luo, 2010) and determined the transgene insertion site by sequencing the resulting PCR products, confirming genomic integration of the transgene (Supplementary Fig. 5).

Attempt to generate a histone-labelled and a membrane-labelled transgenic milkweed bug

Nuclear and plasma membrane markers are practical and useful tools for developmental biology (e.g. Benton et al., 2013; Karapidaki et al., 2026; Nakamura et al., 2010). To try to generate a transgenic strain with ubiquitously constitutively fluorescently labelled histones, we prepared the donor plasmid *pXL[Gbact-H2B-mScarlet3-SV40T]* (2,974 bp), in which H2B-mScarlet3 is driven by the previously characterized *Gryllus bimaculatus* actin promoter (*Gbact*) (Fig. 4A). This promoter can drive ubiquitous constitutive expression and has been widely used in the cricket community (Nakamura et al., 2010; Ohde et al., 2025; Zhang et al., 2002). We reasoned that, just as some *D. melanogaster* promoters retain their function in transgenic insects of other species (Bottino-Rojas and James, 2022), the cricket promoter might retain its function in another hemimetabolous insect. We injected the donor plasmid with 1×NLS *hyPBase* mRNA and screened approximately 100 hatched G₀ animals for mScarlet3 expression (Table 2). We obtained one animal displaying clear mScarlet3 expression through the integument across the whole body and backcrossed it with the *v^l xdh1^{A2}* strain to generate G₁ progeny. We found that 42.4% of G₁ animals (14/33) displayed the same mScarlet3 expression pattern as the G₀ parent (Fig. 4B and Supplementary Table 6). We observed that nuclei with detectable mScarlet3 expression were located underneath epidermal cells, whereas nuclei in overlying epidermal cells did not express detectable mScarlet3 (Supplementary Fig. 6). When we dissected ovaries from G₁ adult females, we detected nuclear mScarlet3 expression in the ovariole muscle sheath but not in the tropharium or follicle cells (Fig. 4C). Similarly, we observed mScarlet3 expression in the testis muscle sheath, but not in sperm cells (Fig. 4D). These expression patterns suggest the hypothesis that the transgene may have integrated in the vicinity of a

muscle-specific enhancer, resulting in enhancer trap-like expression driven by an endogenous regulatory element, rather than the ubiquitous constitutive expression driven by this *Gbact* promoter in other transgenic contexts (Nakamura et al., 2010). Importantly, this result also suggests that the *Gbact* promoter retains basal promoter activity in *O. fasciatus*, as it appears sufficient to support enhancer trap-mediated expression. We used splinkerette PCR to identify the transgene insertion site as being within an intron of an uncharacterized gene locus encoding a protein with a predicted transmembrane domain (Accession No. OFAS019046-RA; Supplementary Fig. 6).

We next aimed to generate a membrane-labelled transgenic strain using the GAP43 membrane localization tag, which has recently been shown to be broadly applicable across a wide variety of animals (Karapidaki et al., 2026). We prepared the donor plasmid *pXL[Gbact-GAP43-mScarlet3-SV40T, 9×P3-EGFP-SV40T]* (3,635 bp), in which membrane-tagged mScarlet3 and EGFP are driven by the *Gbact* and 9×P3 promoters, respectively (Supplementary Fig. 7). We injected this donor plasmid together with 1×NLS *hyPBase* mRNA and screened for EGFP expression in the eyes at nymphal stages. Of 113 screened nymphs, 15 showed EGFP expression (STR = 13.3%) (Table 2). We backcrossed these 15 EGFP-positive G₀ animals with animals of the opposite sex from the *v^l xdh1^{Δ2}* strain, and seven of the crosses successfully yielded G₁ progeny. Of these, three yielded EGFP-positive G₁ animals (GTE = 42.9%) (Supplementary Fig. 7 and Supplementary Table 7). However, we could not detect clear membrane-labelled expression of mScarlet3 in the ovary or testis of any G₁ progeny. We did, however, detect mScarlet3 expression in the eyes of G₁ adults (Supplementary Fig. 7), which we speculate is due to 9×P3 enhancer trapping within the transgene. Together, these results suggest that the *Gbact* promoter is active in *O. fasciatus* but may not be sufficient to drive ubiquitous expression.

Identification of a constitutive and ubiquitous promoter in *O. fasciatus*

To identify a promoter that would drive stable, constitutive, and ubiquitous expression in *O. fasciatus*, we focused on an endogenous actin promoter. Based on a BLAST search using *D. melanogaster* Actin 5c (Act5c; NP_001284915.1), we identified two orthologs in *O. fasciatus*, Act5ca (Accession No. OFAS010390-RA) and Act5cb (Accession No. OFAS010391-RA), which are annotated as tandem loci in the genome (Kataoka et al., 2026). We prepared the donor plasmid *pXL[Ofact-H2B-mScarlet3-SV40T]* (4,650 bp), in which H2B-mScarlet3 is driven by the 2.5 kb upstream of the *Of-act5ca* gene (Fig. 5A). Three experimental replicates of injection with 3×NLS *hyPBase* mRNA resulted in 73.8% (31/42), 82.2% (37/45), and 70.0% (56/80) G₀ STR respectively when screened at nymphal stages (Table 2). All mScarlet3-positive G₀ animals showed some mScarlet3 expression, even if only as dots or patches on their bodies. Notably, a subset of G₀ animals exhibited mScarlet3 expression across more than 50% of their bodies from embryonic stages (Fig. 5B), which was maintained through nymphal stages. Specifically, 9.5% (4/42), 15.5% (7/45), and 5.0% (4/80) of screened nymphs displayed mScarlet3 expression across more than 50% of their bodies (Table 2). We selected nine of these strongly expressing G₀ animals for backcrossing with animals of the opposite sex from the *v^l xdh1^{Δ2}* strain, and seven of the crosses yielded G₁ progeny. Of these, three yielded mScarlet3-positive G₁ animals (GTE = 42.9%) (Supplementary Table 8) displaying ubiquitous mScarlet3 expression throughout the body (a representative image at the fifth nymphal stage is shown in Fig. 5C).

Having successfully established a ubiquitously expressing histone-labelled transgenic *O. fasciatus*, we asked whether the fluorescent signal could be visualized

through the eggshell in live embryos. Visualizing embryogenesis in living animals through the thick, semi-opaque eggshell and optically occlusive yolk has been a challenge in previous studies of *O. fasciatus* (Auman and Chipman, 2018; Panfilio, 2009; Panfilio et al., 2006). We focused specifically on katatrepsis, an essential embryonic movement (Panfilio, 2008). We found that all nuclei of the developing embryo were easily visualized through the eggshell, allowing us to capture the entire process of katatrepsis and appendage elongation over 36 hours (Fig. 5D and Supplementary Videos 1–3).

Evidence for functionality of the Q system in *O. fasciatus*

To investigate whether this transgenesis method enables integration of large DNA payloads, we prepared the donor plasmid *pXL[Ofinv-QF2, 15×QUAS-mScarlet3, 9×P3-EGFP-SV40T]* (7,161 bp). This construct employs the Q system, a binary expression system composed of the transcriptional activator QF2 (a modified version of QF) and its binding site 15×QUAS (Potter et al., 2010; Riabinina et al., 2015) together with a 2.4 kb region upstream of the segment polarity gene *Of-invented* (*Of-inv*) (Accession No. OFAS025056), and the 9×P3 promoter-driven EGFP transformation marker (Fig. 6A). Injection of the donor plasmid with 1×NLS *hyPBase* mRNA into 192 eggs resulted in 50% (24/48) of G₀ nymphs survived showing mScarlet3 expression on their bodies at the second nymphal instar stage (Table 2, Fig. 6B–D, and Supplementary Fig. 8). Notably, one of these 24 individuals displayed intense expression that partly appeared to align with thoracic segment boundaries. This individual also exhibited a segmental fusion phenotype (#1; Fig. 6B), which we speculate could be due to the strong activity of the *Of-inv* 2.4 kb regulatory region. At later nymphal stages, we observed strong mScarlet3 expression along the lateral edge of thoracic segments in a second individual

323 (#2; Fig. 6C), broad mScarlet3 expression across the dorsal abdomen in a third (#3; Fig.
324 6D), and small patches and dots of expression in others (Supplementary Fig. 8). In
325 individual #3(Fig. 6D), clear segmental stripes of mScarlet3 expression were detected in
326 the ventral abdomen (Fig. 6D), suggesting that *Of-inv* is expressed in post-embryonic
327 development.

328

Discussion

We have established a system for efficient *piggyBac*-mediated transgenesis in the milkweed bug *O. fasciatus*. We show that efficiency is improved by the incorporation of NLS sequences into the hyPBase transposase. We also created a double mutant strain that facilitates more efficient screening with fluorescent 9×P3-driven transformation markers. This efficient and practical system enabled us to introduce multiple transgenic constructs carrying expression cassettes ranging from 1.8 to 7.2 kb in size into the *O. fasciatus* genome. Furthermore, we demonstrated proof of principle feasibility of the Q system-mediated binary expression system, expanding the genetic toolkit available in *O. fasciatus*.

CRISPR-mediated mutagenesis enabled us to revisit a classic study for the first time in half a century. Lawrence (1970) reported eye- and body-color mutants induced by ethyl methanesulfonate (EMS) treatment during the golden age of *O. fasciatus* research. Based on the descriptions of the phenotypes of these mutants (1970) and other studies (Liu, 2016; Reding et al., 2023), we focused on manipulating genes belonging to the pteridine pathway to generate a body-color mutant, and successfully established the v^l *xdhl*^{Δ2} double mutant strain showing body- and eye-color defects (Fig. 1), consistent with that of the previously reported mutants. Although a recent study using RNAi-mediated functional analysis did not reveal a role for *xdhl* in eye-color formation (Reding et al., 2023), our results provide evidence that *xdhl* is involved in both body- and eye-color formation (Fig. 1). This discrepancy may be attributable to the often transient and incomplete nature of RNAi-mediated knockdown compared to a genomic loss-of-function allele. We further showed that both mScarlet-I and EGFP expressions can be detected through the eyes of the v^l *xdhl*^{Δ2} strain (Figs. 2, 3). This property will

also be useful for developing future methods for transgenesis and targeted genomic integration, including CRISPR/Cas9-based knock-in (Matsuoka et al., 2025).

Another key finding is that we enhanced the overall efficiency of *piggyBac*-mediated transgenesis in *O. fasciatus* by incorporating SV40T NLS sequences into *hyPBase*. We found that 1× and 3×NLS increased both STR and GTE compared to 0× and 5×NLS conditions in *O. fasciatus* (Fig. 2), even though *piggyBac* transposase itself carries an intrinsic nuclear localization signal (Keith et al., 2008). A similar nuclear targeting approach may also be usefully applied to future phiC31-mediated site-specific integration in *O. fasciatus*, as a previous study demonstrated that the efficiency of phiC31 integrase is markedly improved by C-terminal incorporation of NLS in cultured mammalian cells (Andreas et al., 2002).

Interestingly, our results showed that 5×NLS completely abolished the transgenesis activity with no positive animals recovered, suggesting that an excessive number of NLS may be detrimental to transposase function in *O. fasciatus*. By contrast, all NLS-tagged *hyPBase* mRNAs functioned equally efficiently in *G. bimaculatus* (Supplementary Fig. 4), indicating that this nuclear targeting approach did not provide additional benefit in this species. We speculate that this species-specific difference may reflect differences in the timing of cellularization or/and biochemical properties between the two species. It would be interesting for future studies to investigate species-specific activity profiles of NLS-tagged *hyPBase* mRNAs across a broad range of insect species.

Identifying a suitable endogenous promoter driving ubiquitous and constitutive expression can be challenging in non-traditional insect models. Here we found that a promoter (*Gbact*) that drives ubiquitous, constitutive expression in a cricket species (Nakamura et al., 2010) did not drive such expression in *O. fasciatus*. Instead, the cricket promoter appeared to drive tissue-specific expression patterns, possibly due to

capture by an endogenous *O. fasciatus* enhancer (Fig. 4). In this case, the *Gbact* promoter appeared to function as a basal promoter. Similarly, in the case of GAP43-mScarlet3 driven by *Gbact*, we observed no clear membrane signals in ovaries or testes across multiple insertions (Supplementary Fig. 7). Although we cannot formally exclude the possibility that these expression patterns reflect position effects at the specific insertion sites, the consistent absence of ubiquitous expression across multiple independent insertions suggests that the *Gbact* promoter itself may not drive sufficiently strong ubiquitous expression in *O. fasciatus* to overcome the influence of nearby *O. fasciatus* enhancers. This observation is similar to one reported previously for the beetle *Tribolium castaneum*, wherein endogenous promoters were required to drive the desired expression patterns in transgenic animals (Schinko et al., 2010). To address this challenge, we identified the endogenous *O. fasciatus Act5c* promoter as one able to drive ubiquitous, constitutive expression, using an and used it to successfully establish multiple histone-labelled transgenic strains (Fig. 5). Together, these transgenic strains and our exploration of heterologous and endogenous promoters provide valuable genetic and experimental resources for future work, facilitating comparative developmental studies in *O. fasciatus*.

Finally, we provided evidence that the Q system, a binary expression system, can function successfully in *O. fasciatus*, providing the potential for transcriptional amplification of *cis*-regulatory element activity. One of the G₀ nymphs we obtained with strong transgene expression exhibited a segmental fusion phenotype (Fig. 6), which we speculate could be due to excessive *Of-inv* promoter activity; this animal died before late nymphal stages. This could mean that maintaining moderate transcriptional expression levels of some constructs may be important for viability.

402 Taken together, our results have substantially expanded the functional genetic
403 toolkit of *O. fasciatus* as a comparative model insect for Evo-Devo research. By
404 combining a visibly light-colored mutant background, NLS-tagged hyPBase activity,
405 and the Q system-based binary expression platform, we have established a versatile and
406 practical framework for functional genetics in this species. These technological
407 advances position *O. fasciatus* as an increasingly powerful system for addressing
408 fundamental biological questions in development and evolution. Moreover, the nuclear
409 targeting approach for improving overall transgenesis efficiency will offer a scalable
410 framework that may benefit other non-model organisms, broadening the scope of
411 comparative genetics in Evo-Devo studies beyond established model species.

412

Materials and Methods

Animal husbandry

Oncopeltus fasciatus was maintained on sunflower seeds at 28–30 °C and 60% relative humidity with 12L:12D as described previously (Ewen-Campen et al., 2011). v^I mutant *O. fasciatus* were kindly provided by Katie Reding and Leslie Pick (University of Maryland, College Park, MD, USA). A *Gryllus bimaculatus* colony derived from the *gwhite* strain at Tokushima University (Niwa et al., 1997) was maintained on cat food (Purina) under identical environmental conditions.

Embryo microinjection

Injection needles were prepared by pulling glass capillaries (Cat#1B100-4, World Precision Instruments) with a Sutter P-97 Flaming/Brown micropipette puller. In *O. fasciatus*, eggs were collected within four hours after egg laying and injections were completed within two hours after egg collection. Two glass microscope slides were taped together with double-sided tape to create a ledge with exposed double-sided tape, against which embryos were aligned. Eggs were submerged in tap water for 20-40 minutes to reduce the inner pressure for ease of injection. Injected eggs were kept at 28 °C. In *G. bimaculatus*, eggs were collected within two hours after egg laying and injection procedures were completed within two hours after egg collection. Eggs were aligned in injection plates with wells made of 2% agarose dissolved in MilliQ water. Injected eggs were kept at 28°C until the desired stage.

CRISPR-Cas9-mediated targeted mutagenesis

Alt-R S.p. Cas9 Nuclease V3 (Cat# 1081059) and tracrRNA (Cat# 1072533) were chemically synthesized by Integrated DNA technologies (IDT). *Of-xdh1* crRNA was designed to target the coding sequence using CRISPR Optimal Target Finder (Gratz et al., 2014). Synthetic crRNA was provided by IDT. Cas9 ribonucleoprotein complex (RNP) was prepared according to IDT instructions. Briefly, tracrRNA and crRNA were resuspended to 100 μ M in Nuclease-Free Duplex Buffer (Cat# 11-01-03-01). 5 μ l of each RNA were mixed, heated to 95°C for five minutes, and allowed to cool to room temperature to form 50 μ M gRNA duplex. 3 μ l gRNA duplex and 2 μ l Cas9 protein were mixed and incubated at room temperature for ten minutes to form Cas9/gRNA RNP at roughly 25 μ M. Finally, RNP was diluted tenfold in 1 $\times$ PBS with 0.05% phenol red.

Donor plasmid construction

The *pXL[Gbact-H2B-EGFP]* vector was kindly provided by Taro Nakamura (National Institute of Basic Biology, Okazaki, Japan) and used as a backbone vector for all *piggyBac*-mediated transgenesis in this study. The plasmid encoding an insect codon-optimized variant of mScarlet-I, *pUC[9 $\times$ P3-mScarlet-I-SV40T]*, was kindly provided by Takaaki Daimon (Kyoto University, Kyoto, Japan). Using these constructs, the donor plasmids *pXL[9 $\times$ P3-mScarlet-I-SV40T]* and *pXL[9 $\times$ P3-EGFP-SV40T, attP]* were assembled. To generate *pXL[Gbact-H2B-mScarlet3-SV40T]* and *pXL[Gbact-GAP43-mScarlet3-SV40T, 9 $\times$ P3-EGFP-SV40T]*, the plasmid 5_*T7-GAP43-mScarlet3* (Addgene #225932) was used in addition to the plasmids described above. To generate *pXL[Ofact-H2B-mScarlet3-SV40T]*, the 2.5 kb region upstream of *Of-act5ca* was amplified from *O. fasciatus* genomic DNA, and other sequences were obtained from *pXL[Gbact-H2B-mScarlet3-SV40T]*. To generate *pXL[Ofinv-QF2, 15 $\times$ QUAS, 9 $\times$ P3-EGFP]*, the 2.4 kb

region upstream of *Of-inv* was amplified from *O. fasciatus* genomic DNA, and QF2 and QUAS sequences were obtained from plasmids *pBAC-DsRed-QF2-hsp70* (Addgene #104876) and *PBAC-ECFP-15×QUAS_TATA-SV40* (Addgene #104875), respectively. Nucleotide sequences of all donor plasmids are provided in Supplementary Data 1.

mRNA preparation

The expression vector for mammalian codon-optimized hyPBase (*pCMV-HA-hyPBase*) (Yusa et al., 2011) was kindly provided by Takahiro Ohde (Kyoto University, Kyoto, Japan). Based on this plasmid, 1×, 3×, or 5× NLS were generated using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Cat#E0554S) according to the manufacturer's protocol. Amino acid sequences of all hyPBase variants are provided in Supplementary Data 2. For *hyPBase* mRNA synthesis, the plasmid was linearized with BbsI (New England Biolabs, Cat#R0539S), and capped mRNA was transcribed using the mMESSAGE mMACHINE T7 Transcription kit (Thermo Fisher Scientific, Cat#AM1344), followed by lithium chloride purification.

Genomic DNA extraction and genotyping

For genotyping, genomic DNA was extracted from a single leg of animals. Legs were homogenized in squish buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 25 mM NaCl, 200 µg/ml Proteinase K), incubated at 60°C for two hours, and then at 95°C for three minutes to inactivate Proteinase K. Genomic PCR was performed using Phusion HF DNA polymerase (New England Biolabs Cat#M0530L). Targeted mutagenesis was detected using the Alt-R Genome Editing Detection Kit (IDT, Cat#1075932), and fixed alleles were confirmed by Sanger sequencing. For splinkerette PCR, genomic DNA was prepared using the DNeasy & Tissue kit (QIAGEN, Cat#69506).

Splinkerette PCR

Genomic DNA was extracted from two G₂ animals carrying *9×P3-EGFP-SV40T*, *attP* and two G₂ animals carrying *Gbact-H2B-mScarlet3-SV40T*. Extracted genomic DNA was digested with BstYI (New England Biolabs, Cat#R0523S), followed by self-ligations. Two rounds of splinkerette PCR were performed using KOD-One (TOYOBO, Cat#KMM-101NV). All procedures for splinkerette PCR were performed as described previously (Potter and Luo, 2010). Primers are listed in Supplementary Table S9.

Screening transgenic animals

Nymphs and/or adults were anesthetized with CO₂ and screened for fluorescence using a Zeiss Axio Zoom V16 stereomicroscope. Images were captured using a Zeiss Axical 512 color camera or HAMAMATSU C11440-22CU digital camera.

Nuclear and F-actin staining

Ovaries were dissected from transgenic G₁ adults and fixed with 4% formaldehyde in 1× PBS for 30 minutes at room temperature, followed by three washes with PBT (1× PBS with 0.1% Triton X-100). Samples were incubated with 5 ng/μl Hoechst 33342 and Alexa Fluor 488-phalloidin (1:200; Thermo Fisher Scientific, Cat#A12379) in PBT at room temperature for one hour or at 4°C overnight. Samples were washed four times over one hour with PBT. After washing, samples were mounted in 100% glycerol. Images were taken using a Nikon CSU-W1 spinning disk inverted confocal microscope.

Live imaging

Appropriately staged eggs were collected under a Zeiss Axio Zoom V16 stereomicroscope and attached to a standard 90 mm polystyrene Petri dish using heptane glue. Live imaging was performed on a Nikon CSU-W1 spinning disk inverted confocal microscope with 10× air objective at 29°C. Image stacks comprising approximately 40 optical sections (4 µm z-steps) were taken at 10 minute intervals.

Acknowledgements

We thank Tarun Kumar and Daniella Haber for helping with animal husbandry, Suhrid Ghosh for assistance with live imaging, and all members of the Extavour lab for discussion. Y.S. is supported by the Human Frontier Science Program (HFSP) Fellowship LT0048/2025-L (DOI: <https://doi.org/10.52044/HFSP.LT00482025-L.pc.gr.231571>). Y.S. was a Japan Society for the Promotion of Science (JSPS) Overseas Research Fellow. This study was supported by NSF award IOS-2220747, NIH award 1R01GM143611, and funds from the Howard Hughes Medical Institute (HHMI) to C.G.E., who is an HHMI investigator.

Author contributions

Conceptualization: Y.S.; Methodology: Y.S.; Validation: Y.S.; Formal analysis: Y.S.; Investigation: Y.S., Y.H., A.W., J.A.K.; Resources: Y.S., Y.H., A.W., J.A.K.; Data curation: Y.S.; Writing - original draft: Y.S.; Writing - review & editing: Y.S., Y.H., A.W., J.A.K., C.G.E.; Visualization: Y.S.; Supervision: Y.S., C.G.E.; Project administration: Y.S., C.G.E.; Funding acquisition: Y.S., C.G.E.

Disclosures

The authors declare no competing or financial interests.

References

- Andreas, S., Schwenk, F., Küter-Luks, B., Faust, N. and Kühn, R.** (2002). Enhanced efficiency through nuclear localization signal fusion on phage phiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. *Nucleic Acids Res.* **30**, 2299–2306.
- Angelini, D. R. and Kaufman, T. C.** (2005). Functional analyses in the milkweed bug *Oncopeltus fasciatus* (Hemiptera) support a role for Wnt signaling in body segmentation but not appendage development. *Dev Biol* **283**, 409–423.
- Angelini, D. R., Liu, P. Z., Hughes, C. L. and Kaufman, T. C.** (2005). Hox gene function and interaction in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). *Dev Biol* **287**, 440–455.
- Auman, T. and Chipman, A. D.** (2018). Growth zone segmentation in the milkweed bug *Oncopeltus fasciatus* sheds light on the evolution of insect segmentation. *BMC Evol. Biol.* **18**, 178.
- Benton, M. A., Akam, M. and Pavlopoulos, A.** (2013). Cell and tissue dynamics during *Tribolium* embryogenesis revealed by versatile fluorescence labeling approaches. *Development* **140**, 3210–3220.
- Berghammer, A. J., Klingler, M. and Wimmer, E. A.** (1999). A universal marker for transgenic insects. *Nature* **402**, 370–371.

557 **Bottino-Rojas, V. and James, A. A.** (2022). Use of Insect Promoters in Genetic
558 Engineering to Control Mosquito-Borne Diseases. *Biomolecules* **13**, 16.

559 **Chen, X. and Palli, S. R.** (2021). Hyperactive piggyBac Transposase-mediated
560 Germline Transformation in the Fall Armyworm, *Spodoptera frugiperda*. *J. Vis.*
561 *Exp. : JoVE*.

562 **Chipman, A. D.** (2017). *Oncopeltus fasciatus* as an evo-devo research organism.
563 *genesis* **55**, e23020.

564 **Clark, E., Peel, A. D. and Akam, M.** (2019). Arthropod segmentation. *Development*
565 **146**, dev170480.

566 **Donoughe, S. and Extavour, C. G.** (2016). Embryonic development of the cricket
567 *Gryllus bimaculatus*. *Dev. Biol.* **411**, 140–156.

568 **Eckermann, K. N., Ahmed, H. M. M., KaramiNejadRanjbar, M., Dippel, S.,**
569 **Ogaugwu, C. E., Kitzmann, P., Isah, M. D. and Wimmer, E. A.** (2018).
570 Hyperactive piggyBac transposase improves transformation efficiency in diverse
571 insect species. *Insect Biochem. Mol. Biol.* **98**, 16–24.

572 **Ewen-Campen, B., Shaner, N., Panfilio, K. A., Suzuki, Y., Roth, S. and Extavour,**
573 **C. G.** (2011). The maternal and early embryonic transcriptome of the milkweed bug
574 *Oncopeltus fasciatus*. *Bmc Genomics* **12**, 61.

575 **Feir, D.** (1974). *Oncopeltus Fasciatus*: A Research Animal. *Annu. Rev. Èntomol.* **19**,
576 81–96.

577 **Gonzalez-Sqalli, E., Caron, M. and Loppin, B.** (2024). The white gene as a
578 transgenesis marker for the cricket *Gryllus bimaculatus*. *G3: Genes, Genomes,*
579 *Genet.* **14**, jkae235.

580 **Gratz, S. J., Ukken, F. P., Rubinstein, C. D., Thiede, G., Donohue, L. K.,**
581 **Cummings, A. M. and O'Connor-Giles, K. M.** (2014). Highly Specific and
582 Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in *Drosophila*.
583 *Genetics* **196**, 961–971.

584 **Gregory, M., Alphey, L., Morrison, N. I. and Shimeld, S. M.** (2016). Insect
585 transformation with piggyBac: getting the number of injections just right. *Insect Mol*
586 *Biol* **25**, 259–271.

587 **Groth, A. C., Fish, M., Nusse, R. and Calos, M. P.** (2004). Construction of
588 Transgenic *Drosophila* by Using the Site-Specific Integrase From Phage ϕ C31.
589 *Genetics* **166**, 1775–1782.

590 **Gudmunds, E., Wheat, C. W., Khila, A. and Husby, A.** (2022). Functional genomic
591 tools for emerging model species. *Trends Ecol Evol* **37**, 1104–1115.

592 **Handler, A. M.** (2002). Use of the piggyBac transposon for germ-line transformation
593 of insects. *Insect Biochem Molec* **32**, 1211–1220.

594 **Hart, T., Frank, D. D., Lopes, L. E., Olivos-Cisneros, L., Lacy, K. D., Tribble, W.,**
595 **Ritger, A., Valdés-Rodríguez, S. and Kronauer, D. J. C.** (2023). Sparse and
596 stereotyped encoding implicates a core glomerulus for ant alarm behavior. *Cell* **186**,
597 3079-3094.e17.

598 **Heryanto, C., Mazo-Vargas, A. and Martin, A.** (2022). Efficient hyperactive
599 piggyBac transgenesis in Plodia pantry moths. *Front. Genome Ed.* **4**, 1074888.

600 **Horn, C. and Wimmer, E. A.** (2000). A versatile vector set for animal transgenesis.
601 *Dev Genes Evol* **210**, 630–637.

602 **Horn, C., Jaunich, B. and Wimmer, E. A.** (2000). Highly sensitive, fluorescent
603 transformation marker for Drosophila transgenesis. *Dev. Genes Evol.* **210**, 623–629.

604 **Inada, K., Ohde, T. and Daimon, T.** (2025). Efficient transgenic system for the
605 firebrat *Thermobia domestica* utilizing hyPBase and G0 founder prescreening. *Gene*
606 **955**, 149449.

607 **Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E.** (1984). A short
608 amino acid sequence able to specify nuclear location. *Cell* **39**, 499–509.

609 **Kao, J. A., Rivard, E. L., Kapoor, R. R. and Extavour, C. G.** (2026). The dynamic
610 evolution of panarthropod germ cell specification mechanisms. *Development* **153**,.

611 **Karapidaki, I., Handberg-Thorsager, M., Momose, T., Yasuo, H., Genikhovich, G.,**
612 **Assaf, S., Deleau, C., Pang, Y., Pavlich, C., Lohmann, B., et al.** (2026). Targeting
613 the cell membrane in established and emerging model organisms. *Development* **153**,.

614 **Kataoka, K., Sanno4, R., Gaczorek, T., Bhattarai, U. R., Ito, Y., Inoue, S., Yura,**
615 **K., Asahi, T., Ylla, G., Mito, T., et al.** (2026). Chromosome-scale genome
616 assembly and annotation of the two-spotted cricket *Gryllus bimaculatus* (Orthoptera:
617 Gryllidae). *G3: Genes, Genomes, Genet.* jkag036.

618 **Keith, T. P., Riley, M. A., Kreitman, M., Lewontin, R. C., Curtis, D. and**
619 **Chambers, G.** (1987). Sequence of the Structural Gene for Xanthine Dehydrogenase
620 (rosy Locus) in *Drosophila melanogaster*. *Genetics* **116**, 67–73.

621 **Keith, J. H., Fraser, T. S. and Fraser, M. J.** (2008). Analysis of the piggyBac
622 transposase reveals a functional nuclear targeting signal in the 94 c-terminal
623 residues. *BMC Mol. Biol.* **9**, 72–72.

624 **Kou, Z., Luo, X., Jiang, Y., Chen, B., Song, Y., Wang, Y., Xu, J., Tomberlin, J. K.**
625 **and Huang, Y.** (2023). Establishment of highly efficient transgenic system for black
626 soldier fly (*Hermetia illucens*). *Insect Sci.* **30**, 888–900.

627 **Lawrence, P. A.** (1970). Some new mutants of the Large Milkweed Bug *Oncopeltus*
628 *fasciatus* Dall. *Genet. Res.* **15**, 347–350.

629 **Liu, J.** (2016). Unraveling the molecular mechanisms of aposematic pigmentation in
630 *Oncopeltus fasciatus*.

631 **Liu, P. Z. and Kaufman, T. C.** (2004a). hunchback is required for suppression of
632 abdominal identity, and for proper germband growth and segmentation in the
633 intermediate germband insect *Oncopeltus fasciatus*. *Development* **131**, 1515–1527.

634 **Liu, P. Z. and Kaufman, T. C.** (2004b). Krüppel is a gap gene in the intermediate
635 germband insect *Oncopeltus fasciatus* and is required for development of both
636 blastoderm and germband-derived segments. *Development* **131**, 4567–4579.

637 **Liu, P. Z. and Kaufman, T. C.** (2005). even-skipped is not a pair-rule gene but has
638 segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband
639 insect. *Development* **132**, 2081–2092.

640 **Liu, P. Z. and Patel, N. H.** (2009). Segmentation in the intermediate germband insect,
641 *Oncopeltus fasciatus*. *Dev Biol* **331**, 456–457.

642 **Liu, P. Z. and Patel, N. H.** (2010). giant is a bona fide gap gene in the intermediate
643 germband insect, *Oncopeltus fasciatus*. *Development* **137**, 835–844.

644 **Matsuoka, Y., Nakamura, T., Watanabe, T., Barnett, A. A., Tomonari, S., Ylla, G.,**
645 **Whittle, C. A., Noji, S., Mito, T. and Extavour, C. G.** (2025). Establishment of
646 CRISPR/Cas9-based knock-in in a hemimetabolous insect: targeted gene tagging in
647 the cricket *Gryllus bimaculatus*. *Dev. (Camb., Engl.)* **152**, dev199746.

648 **Misof, B., Liu, S., Meusemann, K., Peters, R. S., Donath, A., Mayer, C., Frandsen,**
649 **P. B., Ware, J., Flouri, T., Beutel, R. G., et al.** (2014). Phylogenomics resolves the
650 timing and pattern of insect evolution. *Science* **346**, 763–767.

651 **Nakamura, T., Yoshizaki, M., Ogawa, S., Okamoto, H., Shinmyo, Y., Bando, T.,**
652 **Ohuchi, H., Noji, S. and Mito, T.** (2010). Imaging of Transgenic Cricket Embryos
653 Reveals Cell Movements Consistent with a Syncytial Patterning Mechanism. *Curr*
654 *Biol* **20**, 1641–1647.

655 **Niwa, N., Saitoh, M., Ohuchi, H., Yoshioka, H. and Noji, S.** (1997). Correlation
656 between Distal-less Expression Patterns and Structures of Appendages in
657 Development of the Two-Spotted Cricket, *Gryllus bimaculatus*. *Zool Sci* **14**, 115–
658 125.

659 **Ohde, T., Nakamura, T., Mito, T. and Daimon, T.** (2025). Highly efficient hyPBase-
660 mediated transgenesis facilitates gain-of-function analysis in the cricket *Gryllus*
661 *bimaculatus*. *Insect Biochem. Mol. Biol.* **184**, 104405.

662 **Otte, M., Netschitailo, O., Kaftanoglu, O., Wang, Y., Page, R. E. and Beye, M.**
663 (2018). Improving genetic transformation rates in honeybees. *Sci. Rep.* **8**, 16534.

664 **Panfilio, K. A.** (2008). Extraembryonic development in insects and the acrobatics of
665 blastokinesis. *Dev. Biol.* **313**, 471–491.

666 **Panfilio, K. A.** (2009). Late extraembryonic morphogenesis and its zen RNAi -induced
667 failure in the milkweed bug *Oncopeltus fasciatus*. *Dev Biol* **333**, 297–311.

668 **Panfilio, K. A., Liu, P. Z., Akam, M. and Kaufman, T. C.** (2006). *Oncopeltus*
669 *fasciatus* zen is essential for serosal tissue function in katatrepsis. *Dev. Biol.* **292**,
670 226–243.

671 **Potter, C. J. and Luo, L.** (2010). Splinkerette PCR for mapping transposable elements
672 in *Drosophila*. *PloS one* **5**, e10168.

673 **Potter, C. J., Tasic, B., Russler, E. V., Liang, L. and Luo, L.** (2010). The Q System:
674 A Repressible Binary System for Transgene Expression, Lineage Tracing, and
675 Mosaic Analysis. *Cell* **141**, 536–548.

676 **Reding, K. and Pick, L.** (2020). High-Efficiency CRISPR/Cas9 Mutagenesis of the
677 white Gene in the Milkweed Bug *Oncopeltus fasciatus*. *Genetics* **215**, 1027–1037.

678 **Reding, K., Lê, M. and Pick, L.** (2023). Genome editing of the vermilion locus
679 generates a visible eye color marker for *Oncopeltus fasciatus*. *Sci. Rep.* **13**, 4188.

680 **Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M. N., Luo, L. and Potter, C.**
681 **J. (2015).** Improved and expanded Q-system reagents for genetic manipulations. *Nat.*
682 *Methods* **12**, 219–222.

683 **Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch,**
684 **T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012).** Fiji: an open-
685 source platform for biological-image analysis. *Nat. Methods* **9**, 676–82.

686 **Schinko, J. B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M., Klingler, M.,**
687 **Wimmer, E. A. and Bucher, G. (2010).** Functionality of the GAL4/UAS system in
688 *Tribolium* requires the use of endogenous core promoters. *Bmc Dev Biol* **10**, 53.

689 **Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D. S. and Desplan, C. (1997).**
690 Direct regulation of rhodopsin 1 by Pax-6/eyeless in *Drosophila*: evidence for a
691 conserved function in photoreceptors. *Genes Dev.* **11**, 1122–1131.

692 **Shirai, Y., Kao, J. A., Kumar, T., Matsuda, N., Nakagawa, R., Nureki, O.,**
693 **Extavour, C. G. and Daimon, T. (2026).** HUH-tagged Cas9 as a platform for
694 efficient ssODN-mediated knock-in via embryo and adult injection in insects.
695 *Commun. Biol.* **9**, 514.

696 **Stork, N. E. (2017).** How Many Species of Insects and Other Terrestrial Arthropods
697 Are There on Earth? *Annu. Rev. Entomol.* **63**, 1–15.

698 **Truman, J. W., Suzuki, Y. and Riddiford, L. M. (2026).** Juvenile Hormone and the
699 Evolution of Insect Metamorphosis. *Integr. Comp. Biol.* **66**, icag053.

700 **Yusa, K., Zhou, L., Li, M. A., Bradley, A. and Craig, N. L.** (2011). A hyperactive
701 piggyBac transposase for mammalian applications. *Proc. Natl. Acad. Sci.* **108**, 1531–
702 1536.

703 **Zhang, H., Shinmyo, Y., Hirose, A., Mito, T., Inoue, Y., Ohuchi, H., Loukeris, T.**
704 **G., Eggleston, P. and Noji, S.** (2002). Extrachromosomal transposition of the
705 transposable element Minos in embryos of the cricket *Gryllus bimaculatus*. *Dev*
706 *Growth Differ* **44**, 409–417.

707

708

709

Figure Legends

Figure 1. Establishment of a useful double mutant strain in *O. fasciatus*. (A) *xdh1* predicted protein structure. The N-terminal orange square indicates the gRNA target site. Protein domains were predicted using InterProScan. FAD, flavin adenine dinucleotide. (B) Schematic of the experimental workflow and genetic crosses performed to establish the homozygous *xdh1* mutant strain. (C) Representative images of wild type (WT) and *xdh1* G₀ mutant hatchlings (dorsal view). Scale bar, 500 μ m. (D) gRNA target site and mutant allele of *xdh1*. (E) Representative images of nymphs (dorsal view) and close-up views of eye coloration (dorsal view) in WT, *v^l*, *xdh1^{Δ2}*, and *v^l xdh1^{Δ2}*. Arrowheads indicate eye color. Black scale bar, 3 mm; white scale bar, 1 mm. (F) Representative images of adults (lateral view) and close-up views of eye coloration (ventral view) in WT, *v^l*, *xdh1^{Δ2}*, and *v^l xdh1^{Δ2}*. Arrowheads indicate eye color. Black scale bar, 3 mm; white scale bar, 1 mm. Anterior is up in (C), (E), and (F).

Figure 2. Investigation of a nuclear targeting approach for hyPBase-mediated transgenesis in *O. fasciatus*. (A) Schematic of the experiments to identify G₀ mScarlet-I-positive animals and establish stable transgenic lines. (B) Schematic of the *piggyBac* donor vector *pXL[9×P3-mScarlet-I-SV40T]* and *hyPBase* mRNA carrying 0×, 1×, 3×, or 5×NLS. (C) Representative images of G₀ mScarlet-I-positive adults. Scale bar, 1 mm. (D) Mean G₀ somatic transformation rate (STR) across *hyPBase* mRNA carrying 0×, 1×, 3×, or 5×NLS. Each dot represents an independent injection experiment. (E) Representative images of G₁ mScarlet-I-positive nymphs and adults. Arrowheads indicate eyes. Scale bar, 1 mm. (F) Mean G₁ germ line transformation efficiency (GTE). The height of each bar represents the mean proportion of mScarlet-I-positive animals

among G₁ progeny from an individual cross. Each dot represents an individual backcross that yielded G₁ progeny. Red numbers indicate the number of crosses yielding heritable mutant alleles and the corresponding percentages.

Figure 3. Establishment of multiple attP strains using 3×NLS *hyPBase* in *O.*

***fasciatus*.** (A) Schematic of the *piggyBac* donor vector *pXL[9×P3-EGFP-SV40T, attP]* and *hyPBase* mRNA carrying 3×NLS. (B) Representative images of G₀ EGFP-positive adults. Arrowheads indicate eyes. Scale bar, 1 mm. (C) Representative images of G₁ EGFP-positive adults. Arrowheads indicate eyes. Scale bar, 1 mm. (D) G₁ germ line transformation efficiency (GTE). Each bar represents the proportion of EGFP-positive animals among G₁ progeny from an individual cross. Each dot represents an individual backcross that yielded G₁ progeny. Green numbers indicate the number of crosses yielding heritable transgenes and the corresponding percentages.

Figure 3. Establishment of multiple attP strains using 3×NLS *hyPBase* in *O.*

***fasciatus*.** (A) Schematic of the *piggyBac* donor vector *pXL[9×P3-EGFP-SV40T, attP]* and *hyPBase* mRNA carrying 3×NLS. (B) Representative images of G₀ EGFP-positive adults. Arrowheads indicate eyes. Scale bar, 1 mm. (C) Representative images of G₁ EGFP-positive adults. Arrowheads indicate eyes. Scale bar, 1 mm. (D) G₁ germ line transformation efficiency (GTE). Each bar represents the proportion of EGFP-positive animals among G₁ progeny from an individual cross. Each dot represents an individual backcross that yielded G₁ progeny. Green numbers indicate the number of crosses yielding heritable transgenes and the corresponding percentages.

Figure 4. Establishment of the tissue-specific histone-labelled strain using 1×NLS *hyPBase* in *O. fasciatus*. (A) Schematic of the *piggyBac* donor vector *pXL[Gbact-H2B-mScarlet3-SV40T]* and *hyPBase* mRNA carrying 1×NLS. (B) Representative images of G₁ mScarlet3-positive adults (ventral view). Anterior is up. Scale bars, 1 mm (whole-body image) and 100 μm (close-up images). (C) Representative brightfield image (top) and confocal images (bottom) of an ovariole from a G₁ transgenic adult, co-stained for DNA (blue) and F-actin (green). The yellow box in the brightfield image indicates the approximate region shown in the confocal images. Confocal images are single optical sections. In the superficial focal plane, H2B-mScarlet3 expression (magenta) is detectable in muscle sheath nuclei. In the deep focal plane, H2B-mScarlet3 expression is not detectable in the tropharium nuclei. Scale bars, 300 μm (brightfield image) and 50 μm (confocal images) (D) Representative brightfield image (left) and confocal images (right three panels) of a testis of G₁ transgenic adult testis co-stained for DNA (blue) and F-actin (green). H2B-mScarlet3 expression (magenta) was detected in muscle sheath nuclei, but not in sperm cells. Scale bars, 300 μm (brightfield image) and 50 μm (confocal images). Anterior is to the left in (C) and (D).

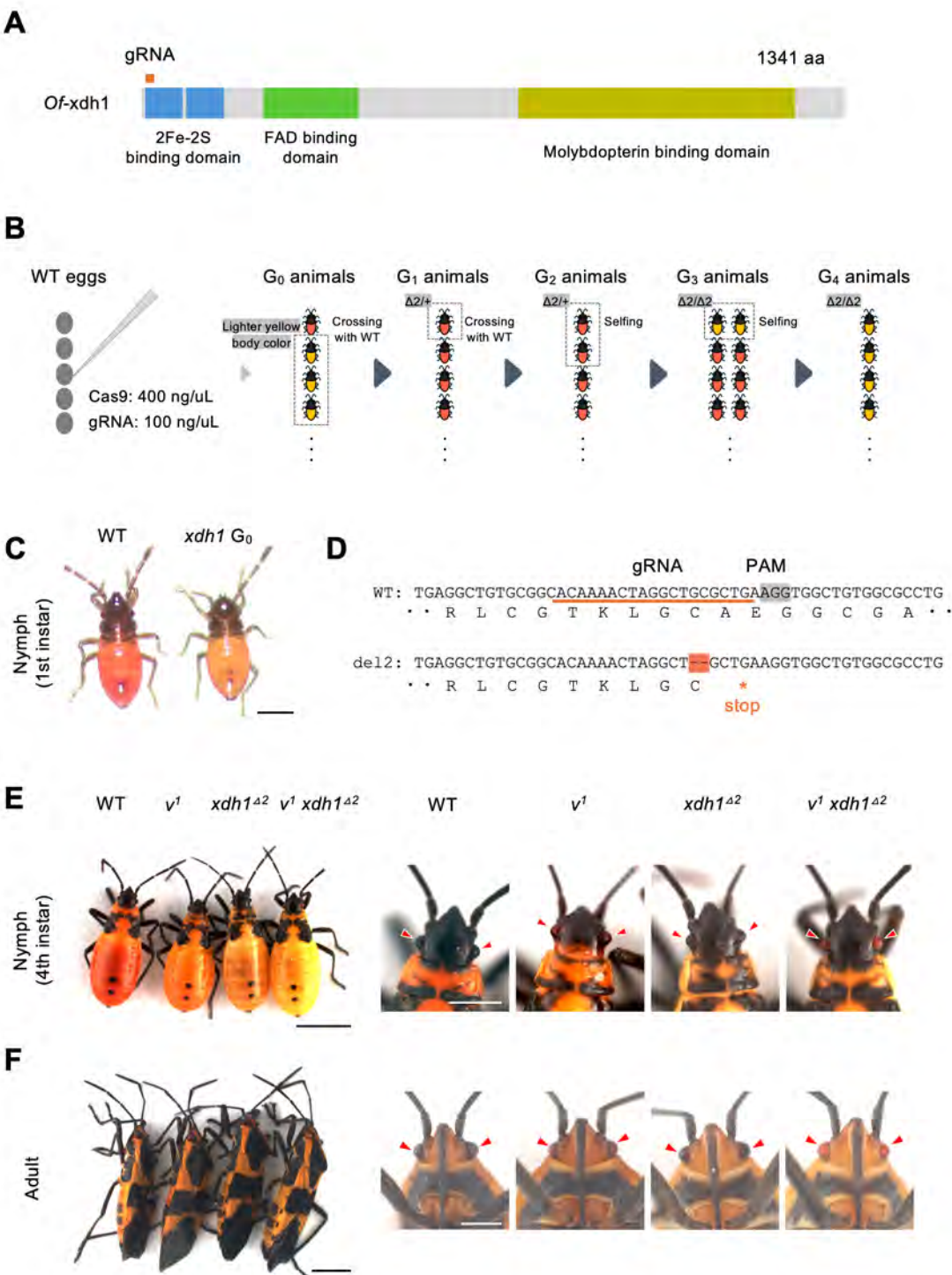
Figure 5. Establishment of the ubiquitous histone-labelled strain using 3×NLS *hyPBase* in *O. fasciatus*. (A) Schematic of the *piggyBac* donor vector *pXL[Ofact-H2B-mScarlet3-SV40T]* and *hyPBase* mRNA carrying 3×NLS. (B) Representative images of G₀ mScarlet3-positive adults (lateral view). Anterior is to the right. Scale bar, 300 μm. (C) Representative images of G₁ mScarlet3-positive nymphs (dorsal view). Anterior is up. Scale bar, 3 mm. (D) Time series of embryonic development of mScarlet3-positive G₂ animal (ventral view), visualized with the "Green Fire Blue" relative intensity lookup table in Fiji (Schindelin et al., 2012). Maximum-intensity projections of a

ventro-lateral view of the same embryo are timed relative to the first time point shown at the far left (0 min). Anterior is up. Scale bar, 100 μ m.

Figure 6. Proof of principle of the Q system using 1 $\times$ NLS *hyPBase* in *O. fasciatus*.

(A) Schematic of the *piggyBac* donor vector *pXL[Ofinv-QF2, 15 $\times$ QUAS-mScarlet3-SV40T, 9 $\times$ P3-EGFP-SV40T]* and *hyPBase* mRNA carrying 1 $\times$ NLS. (B–D)

Representative images of G₀ mScarlet3-positive nymphs. Yellow arrow indicates the putative segmentation defect. Yellow arrowheads indicate the segmentally iterated abdominal expression of mScarlet3. Scale bars, 500 μ m (B) and 1 mm (C, D). Anterior is up in (B–D).



797

798 **Figure 1. Establishment of a useful double mutant strain in *O. fasciatus*.**

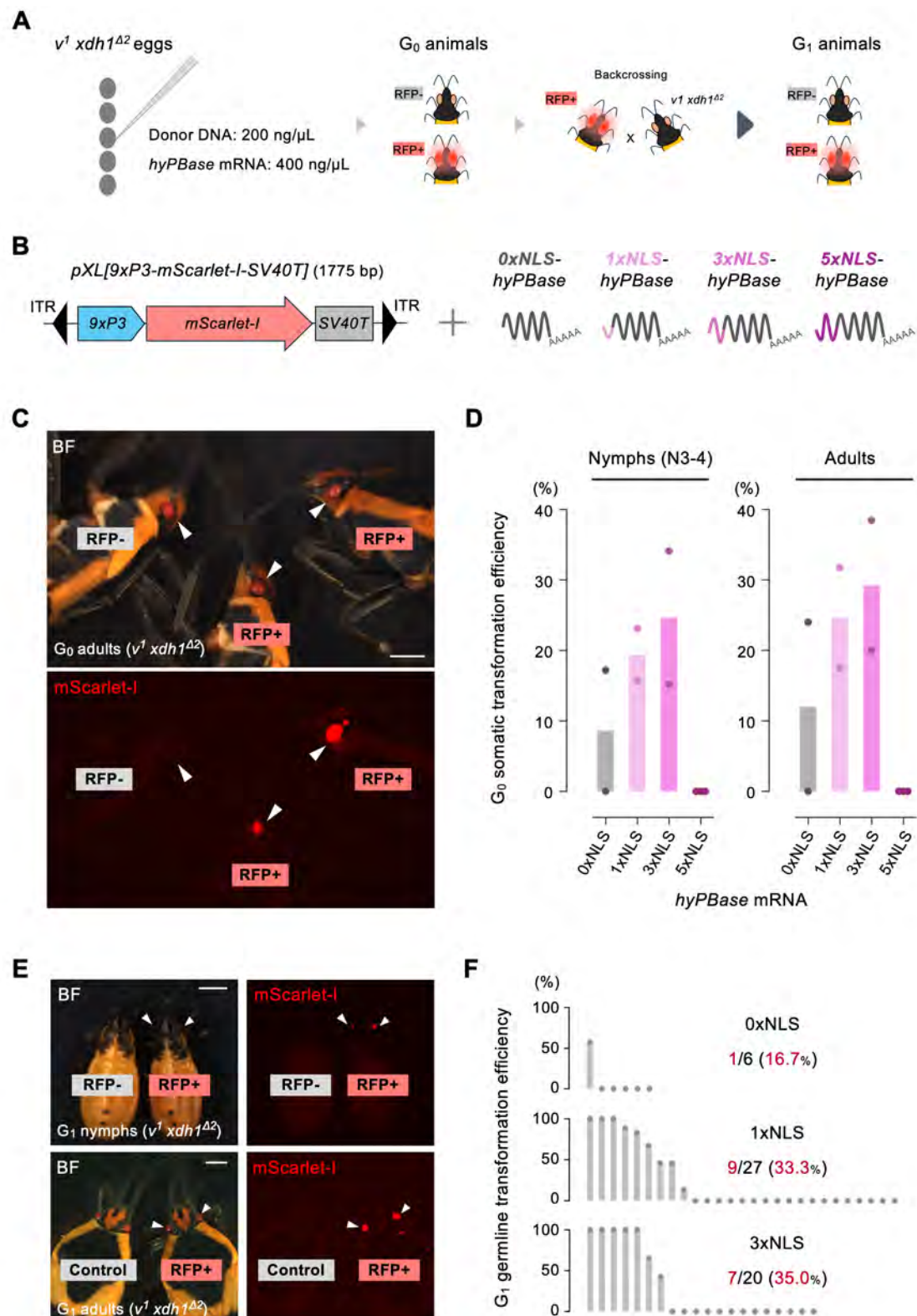
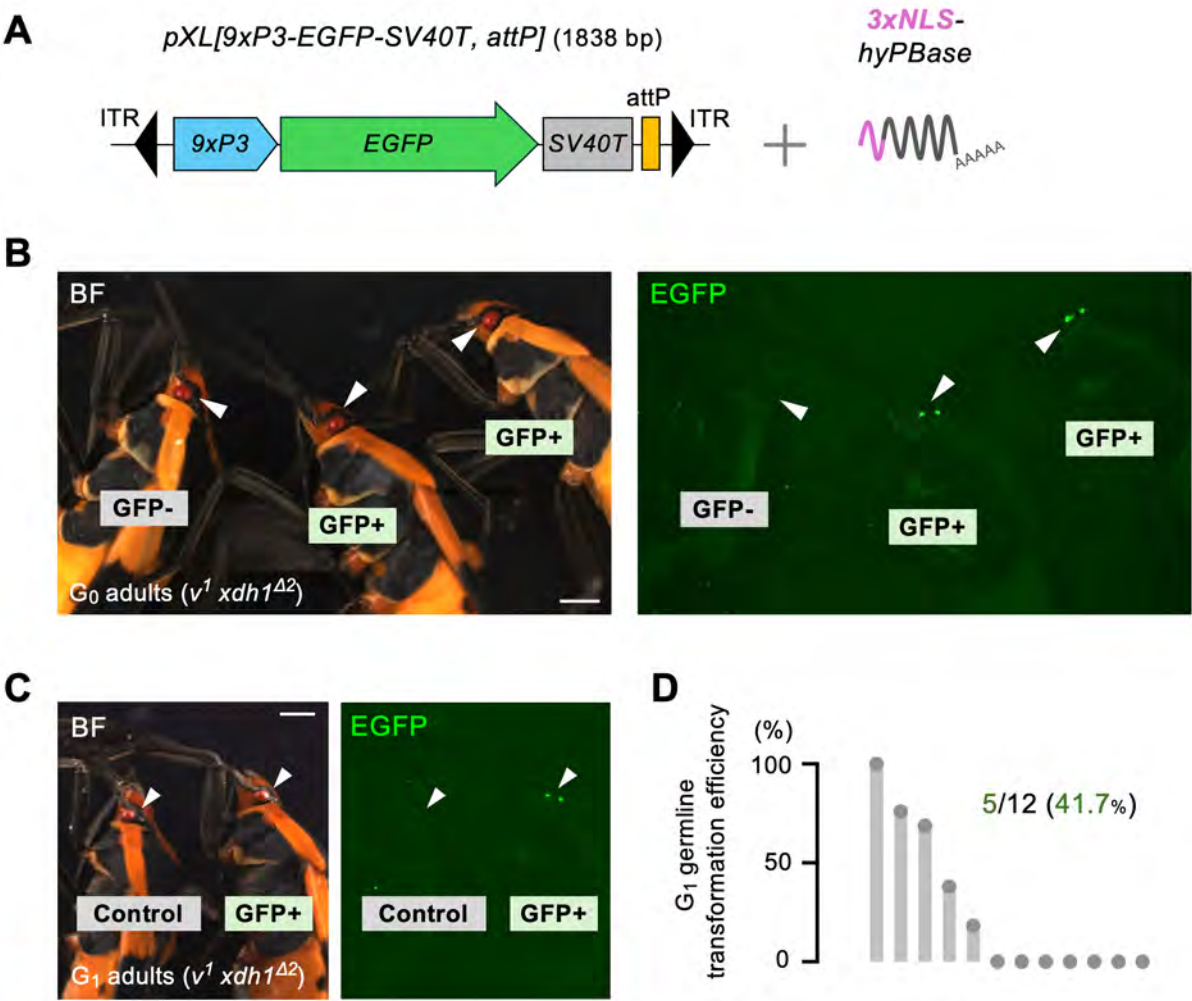


Figure 2. Investigation of a nuclear targeting approach for hyPBase-mediated transgenesis in *O. fasciatus*.

801



802 **Figure 3. Establishment of multiple attP strains using 3×NLS *hyPBase* in *O.***
803 *fasciatus*.

804

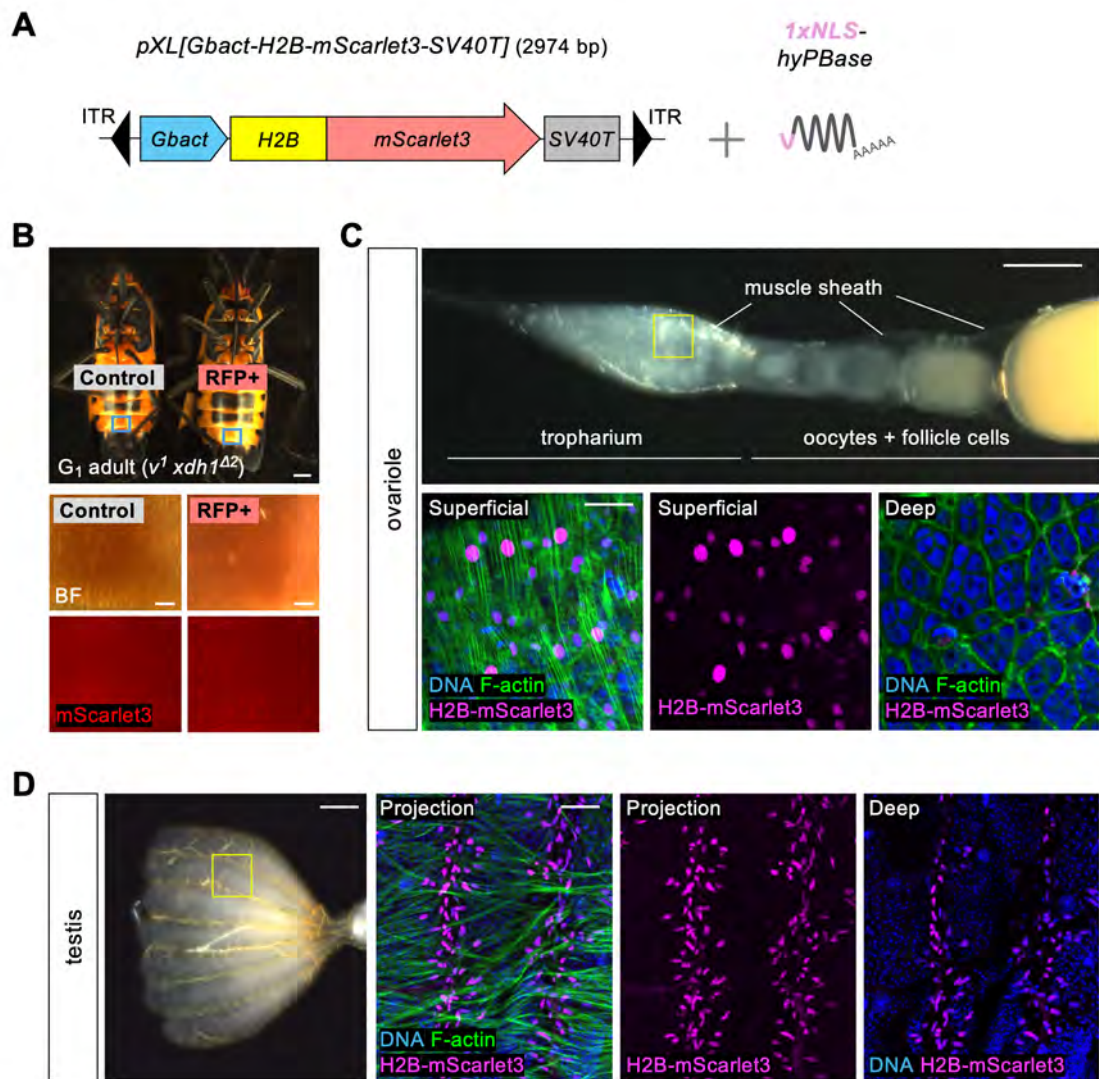


Figure 4. Establishment of the tissue-specific histone-labelled strain using $1\times NLS$ *hyPBase* in *O. fasciatus*.

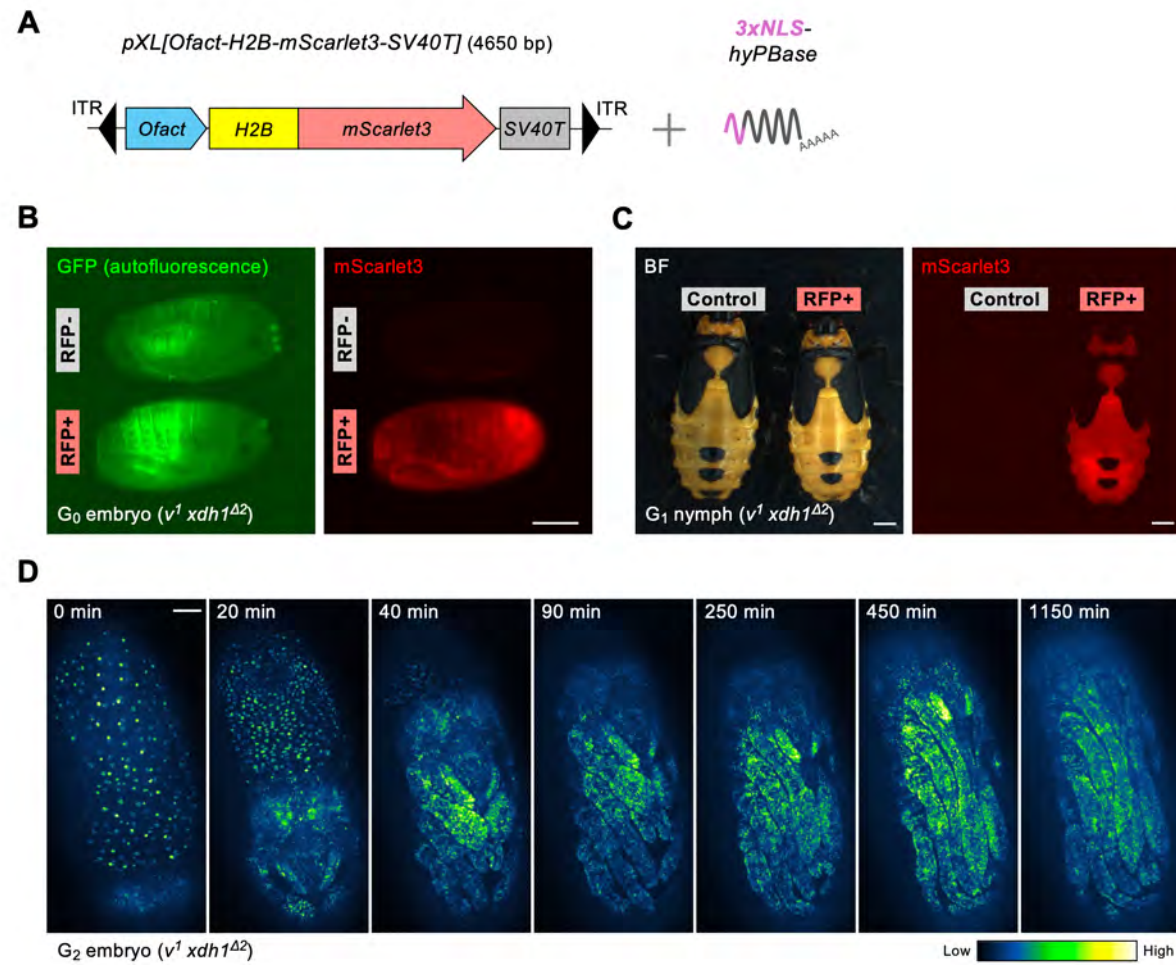


Figure 5. Establishment of the ubiquitous histone-labelled strain using 3×NLS *hyPBase* in *O. fasciatus*.

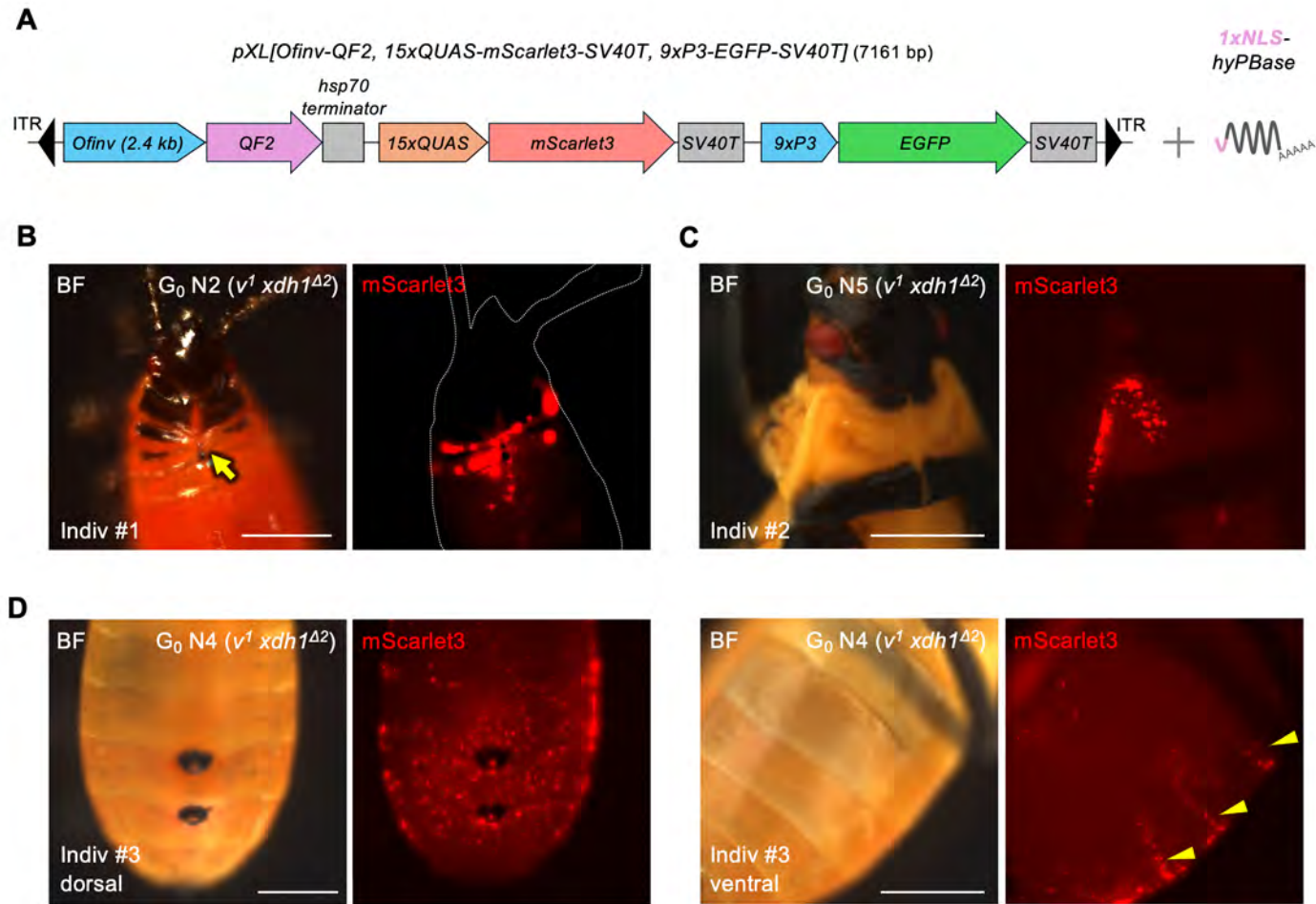


Figure 6. Proof of principle of the Q system using 1×NLS *hyPBase* in *O. fasciatus*.

Tables

Table 1. Detailed results of *piggyBac*-mediated transgenesis experiments with *hyPBase* mRNA carrying 0×, 1×, 3×, or 5×NLS in *O. fasciatus*. Each row shows the results of an independent experiment. All G₀ nymphs and adults reached each respective stage were screened. Percentages shown in parentheses indicate somatic transformation rates (STR). Nymphs were screened at stages 3 to 5.

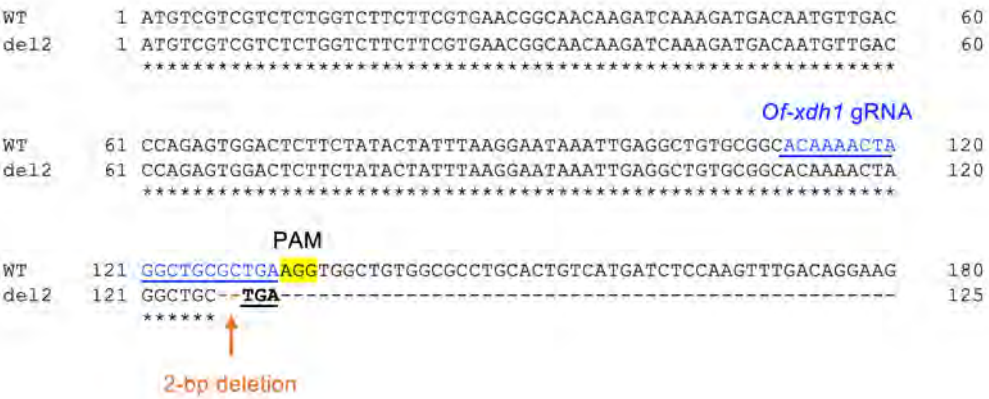
Donor plasmid	Transposase (mRNA)	Injected eggs	% injected eggs survived to nymphal screening	Screened G0 nymphs	mScarlet-I + nymphs	mScarlet-I - nymphs	% injected nymphs survived to adult screening	Screened G0 adults	mScarlet-I + adults	mScarlet-I - adults
pXL[9xP3-mScarlet-I-SV40T]	0xNLS-hyPBase	135	21.5%	29	5 (17.2%)	24	86.2%	25	6 (24.0%)	19
pXL[9xP3-mScarlet-I-SV40T]	0xNLS-hyPBase	246	28.5%	70	0	70	88.6%	62	0	62
pXL[9xP3-mScarlet-I-SV40T]	1xNLS-hyPBase	170	45.9%	78	18 (23.1%)	60	80.8%	63	20 (31.8%)	43
pXL[9xP3-mScarlet-I-SV40T]	1xNLS-hyPBase	185	37.8%	70	11 (15.7%)	59	81.4%	57	10 (17.5%)	47
pXL[9xP3-mScarlet-I-SV40T]	3xNLS-hyPBase	161	28.6%	46	7 (15.2%)	39	89.1%	41	8 (20.0%)	33
pXL[9xP3-mScarlet-I-SV40T]	3xNLS-hyPBase	218	20.1%	44	15 (34.1%)	29	90.8%	40	15 (38.5%)	25
pXL[9xP3-mScarlet-I-SV40T]	5xNLS-hyPBase	130	34.6%	45	0	45	86.7%	39	0	39
pXL[9xP3-mScarlet-I-SV40T]	5xNLS-hyPBase	80	7.5%	6	0	6	83.3%	5	0	5
pXL[9xP3-mScarlet-I-SV40T]	5xNLS-hyPBase	73	23.3%	17	0	17	94.1%	16	0	16

Table 2. Detailed results of *piggyBac*-mediated transgenesis experiments with NLS-tagged hyPBase in *O. fasciatus*. Each row shows the results of an independent experiment. All G₀ nymphs and adults reached each respective stage were screened. Percentages shown in parentheses indicate somatic transformation rates (STR). FP, fluorescent protein. For experiments using donor plasmid pXL[Ofact-H2B-mScarlet3-SV40T], numbers in bold indicate FP + nymphs that had detectable fluorescence in >50% of their body area. Nymphs were screened at nymphal stages 3 to 5, except for the experiment using donor plasmid pXL[Ofinv-QF2, 15xQUAS-mScarlet3-SV40T, 9xP3-mScarlet-I-SV40T], which were screened at nymphal stages 2 to 5.

Donor plasmid	Transposase (mRNA)	Injected eggs	% of injected eggs survived to nymphal screening	Screened G0 nymphs	FP + nymphs	FP - nymphs
pXL[9xP3-EGFP-SV40T,attP]	3xNLS-hyPBase	245	26.5%	65	10 (15.4%)	55
pXL[9xP3-EGFP-SV40T,attP]	3xNLS-hyPBase	122	44.3%	54	5 (9.3%)	51
pXL[Gbact-H2B-mScarlet3-SV40T]	1xNLS-hyPBase	~300	~33.3%	~100	1	~100
pXL[Gbact-GAP43-mScarlet3-SV40T, 9xP3-mScarlet-I-SV40T]	1xNLS-hyPBase	341	33.1%	113	15 (13.3%)	98
pXL[Ofact-H2B-mScarlet3-SV40T]	3xNLS-hyPBase	104	40.4%	42	31, 4 (73.8%, 9.5%)	11
pXL[Ofact-H2B-mScarlet3-SV40T]	3xNLS-hyPBase	175	25.7%	45	37, 7 (82.2%, 15.5%)	8
pXL[Ofact-H2B-mScarlet3-SV40T]	3xNLS-hyPBase	179	44.7%	80	56, 4 (70.0%, 5.0%)	24
pXL[Ofinv-QF2, 15xQUAS-mScarlet3-SV40T, 9xP3-mScarlet-I-SV40T]	1xNLS-hyPBase	192	25.0%	48	24 (50.0%)	24

Supplementary Figures

A



B

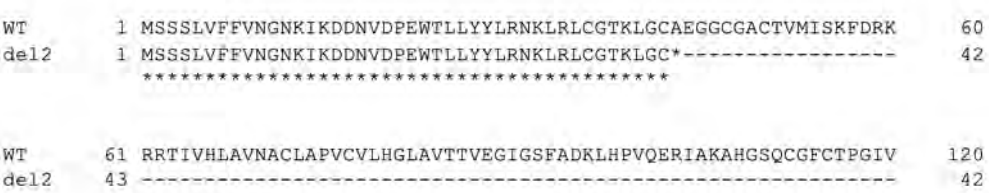


Figure S1. Nucleotide and amino acid sequence of *Of-xdh1* in the *xdh1*⁴² mutant.

(A) Alignment of the nucleotide sequences of *Of-xdh1* around the gRNA target site of WT and *xdh1*⁴² strains. Note the presence of a 2-bp deletion in the *xdh1*⁴² allele (shown in orange). The premature stop codon induced by this deletion is indicated in bold black. (B) Alignment of the amino acid sequences of *Of-xdh1* of the WT and *xdh1*⁴² strains. The 2-bp deletion caused a frameshift mutation in the *xdh1*⁴² allele.

WT 1 ATGGAGCCCGACAGCAATTGCAGATCTTCCGAGATCCAGAA-ATGTGGAGAGCAAAGTGG 59
v1 1 ATGGAGCCCGACAGCAATTGCAGATCTTCCGAGATCCAGAAGATGTGGAGAGCAAAGTGG 60

1-bp insertion

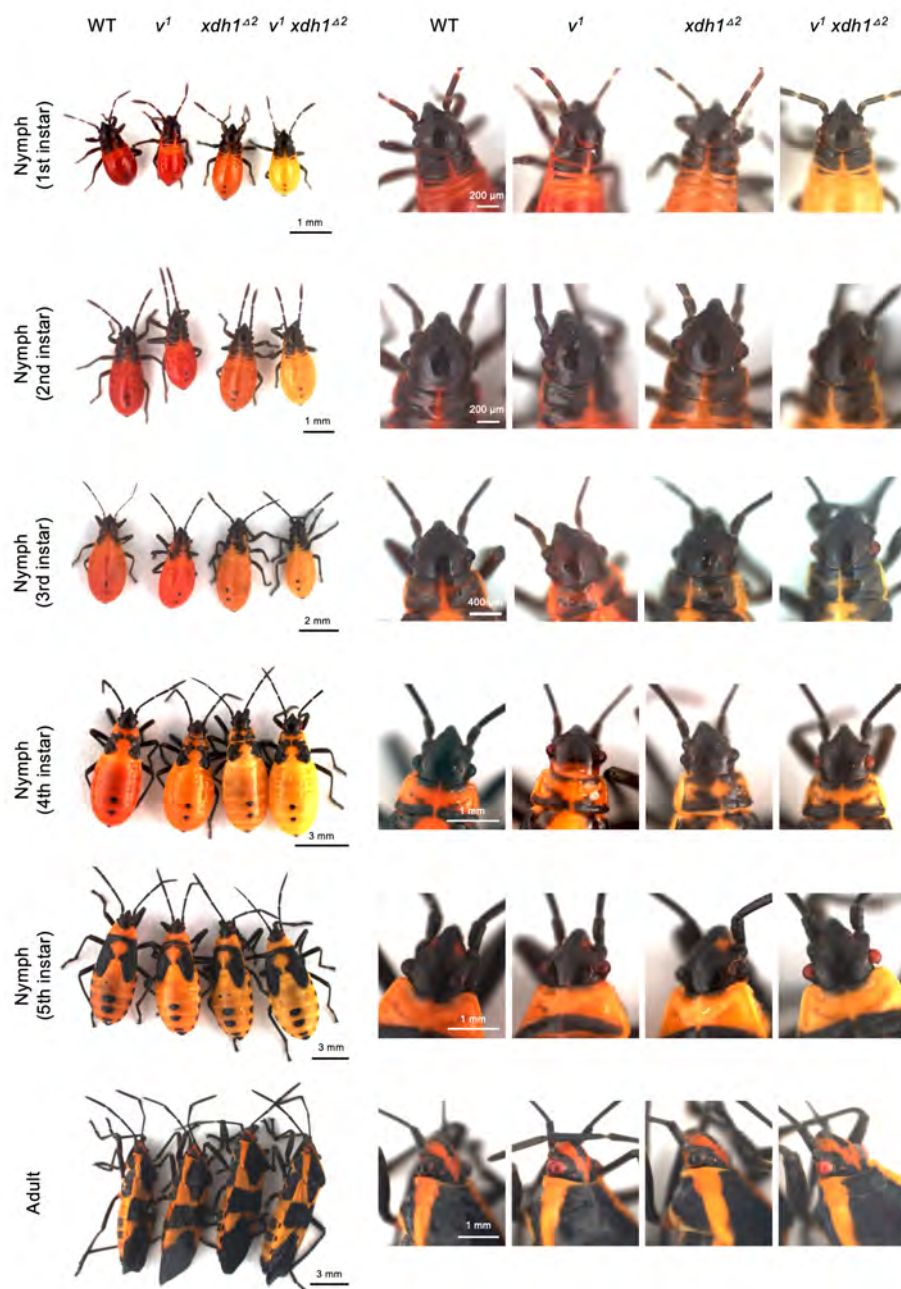
WT 60 AATGCTGTATGGGGATTACCTCCAACCTTGACAAAATACTGAAGGCCCAAAGATTGCTGAG 119
v1 61 AATGCTGTATGGGGATTACCTCCAACCTTGA----- 90

WT	WT	WT	WT	WT	WT	WT	WT
1	1	61	121	181	241	301	361
MEPDSNCRSSEIQKCGEQSGMLYG DY LQLDKILKAQRL LSESNNATVMDEHLFIVTHQGN	MEPDSNCRSSEIQK MWR AK WN AVWGL PPT *-----	KLNSTQEMTNEGLNERQTL DVVKRLNRIVLILKLLVEQVMILETMTPLDFMEFRDYLCPA	SGFQSAQFRLIENKLG VKTEHRVRSFQHYSTVFERDKEALNEIKKSETEQSLADLIEQWL	ERTPGLDENGDFDFWGKYIKNVEFLLQHYYEKAMKSNSETEKTKLA EYEKKKTIFNSVLN	KDMHDLFVQRGERRFTHKAFQGAIMITFYRDEPRFSQPHQIL TLLMDIDSLVTKWRLCSK	TWINYYNDKYK VLLISDRYKCFDLFNLSSFLLPRNFIPPLSSSVRNQINIHTWNTKKQT	EDHEED*
60	29	120	180	240	300	360	366
v1	v1	v1	v1	v1	v1	v1	v1
29	29	29	29	29	29	29	29

Figure S2. Nucleotide and amino acid sequence of *Of-v* in the v^I mutant. (A) Alignment of the nucleotide sequences of *Of-v* around the gRNA target site of wild type (WT) and v^I strains. Note the presence of a 1-bp insertion in the v^I allele (shown in red). The premature stop codon induced by this insertion is indicated in bold black. Further details are described in (Reding and Pick, 2023). (B) Alignment of the amino acid sequences of *Of-v* of the WT and v^I strains. The 1-bp insertion caused a frameshift mutation in the v^I allele (shown in red).

893

894



895

896 **Figure S3. Comparisons of phenotypes of wild type (WT), v^1 , $xdh1^{42}$, and $v^1 xdh1^{42}$**
 897 **throughout postembryonic development.** All images of nymphs and close-up views
 898 (bottom right) are shown in dorsal view; low-magnification adult image (bottom left) is
 899 shown in lateral view. Anterior is up in all images.

900

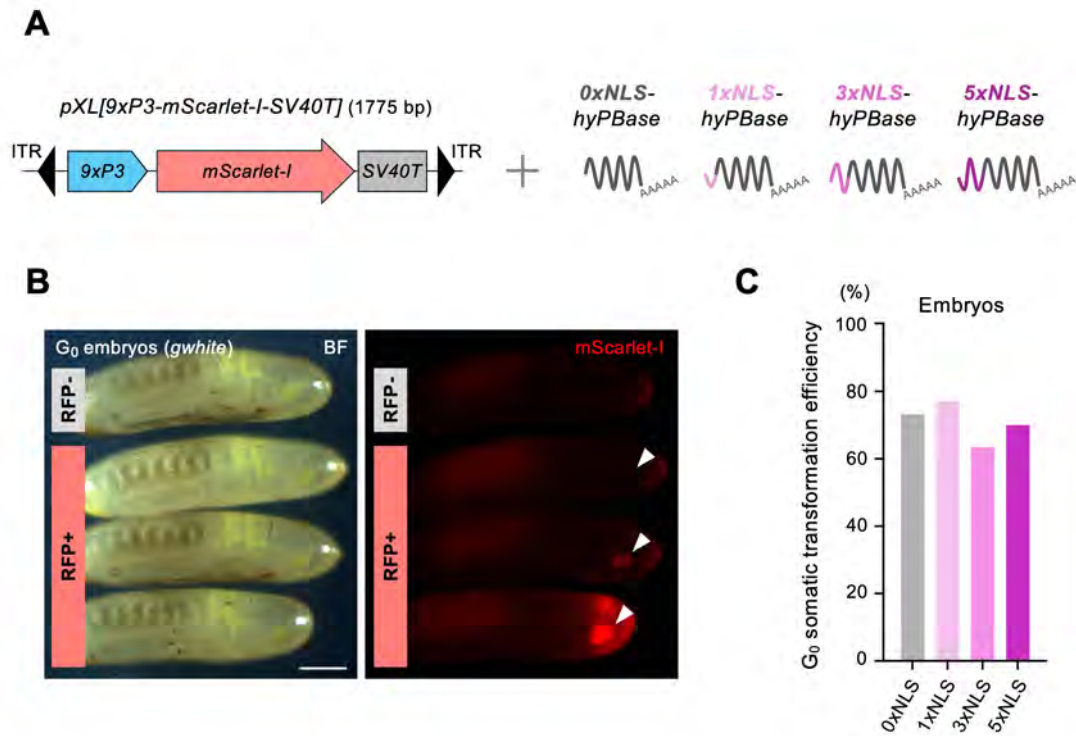


Figure S4. Investigation of a nuclear targeting approach for *piggyBac*-mediated transgenesis in *G. bimaculatus*. (A) Schematic of the *piggyBac* donor vector *pXL[9×P3-mScarlet-I-SV40T]* and *hyPBase* mRNA carrying 0×, 1×, 3×, or 5× NLS. (B) Representative images of G₀ RFP-positive embryos (embryonic stage >13) (Donoughe and Extavour, 2016). Scale bar, 500 μm. Arrowheads indicate eyes. Anterior is to the right. (C) G₀ somatic transformation rate (STR) across 0×, 1×, 3×, or 5×NLS *hyPBase* conditions.

Integration site in 9xP3-EGFP, attP strain 5A

TATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAATTAAAATTGCGTGGTACGATTGT
TAATCTACAGTTTACCCGGACACTAAATTACAATTTTACGTTTATTGGCCGAAAATAGTTGTTTTTCTAGACAA
AA

Scaffold407: 476862-476763 (pident = 100%)

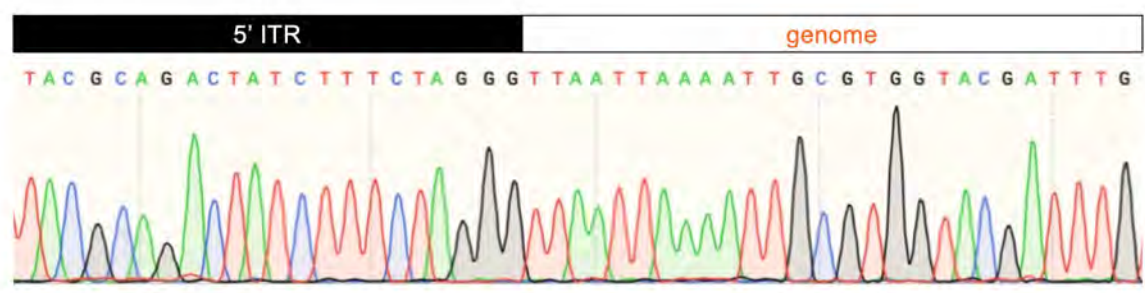


Figure S5. Nucleotide sequences around the transgene integration site in the 9xP3-EGFP, attP strain in *O. fasciatus* as determine by splinkerette PCR.

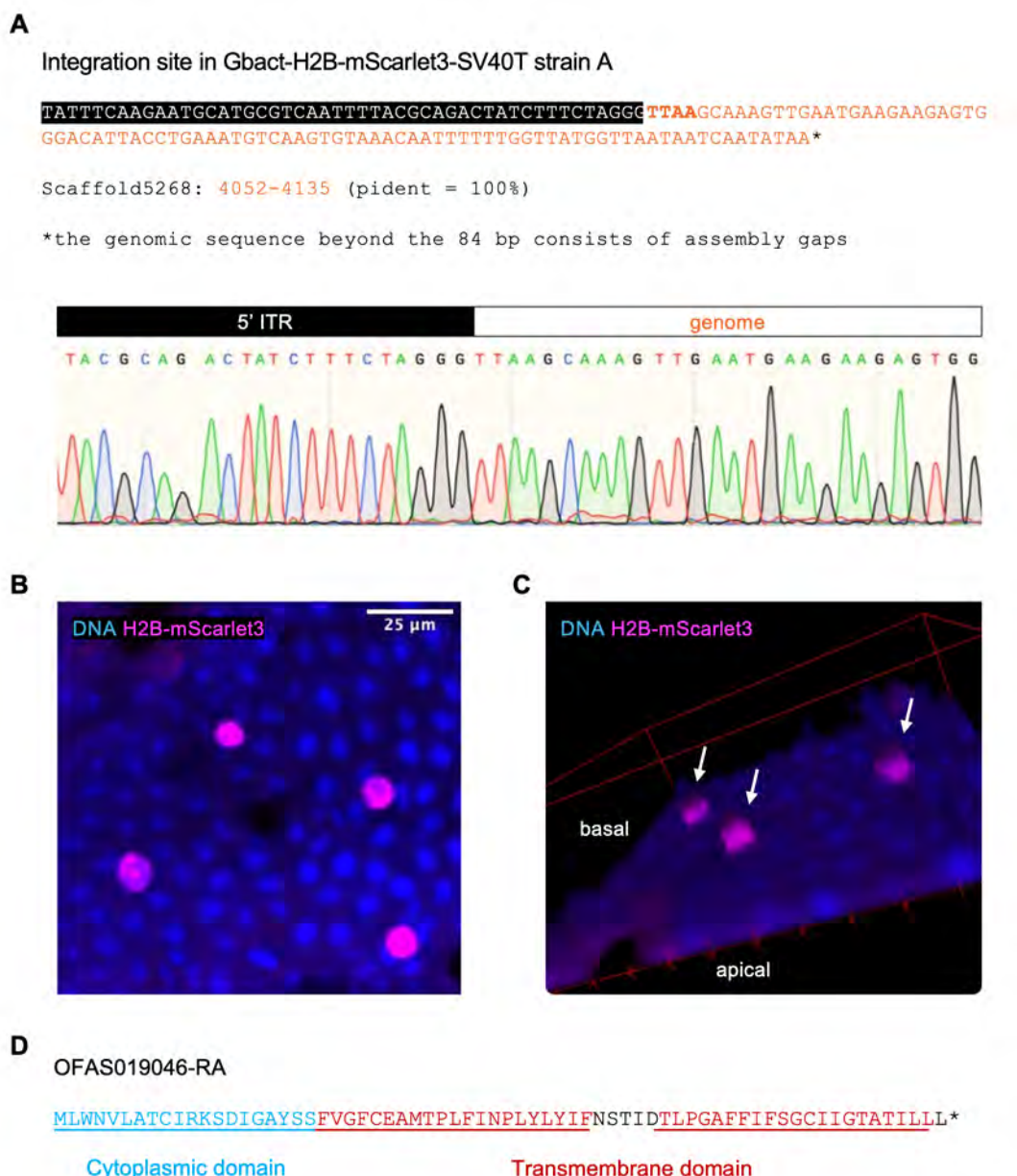


Fig S6. Additional information on the *H2B-mScarlet3-SV40T* strain in *O. fasciatus*.

(A) Injection and crossing experiments used to generate the *H2B-mScarlet3-SV40T* strain. (A) Nucleotide sequences around the transgene integration site in the *H2B-mScarlet3-SV40T* strain as determined by splinkerette PCR. (B) Confocal image of the abdominal integument of the *H2B-mScarlet3-SV40T* strain co-stained for DNA.

Epidermal nuclei were not labelled with H2B-mScarlet3. (D) 3D reconstruction of the abdominal integument of the *H2B-mScarlet3* strain co-stained for DNA, showing that the histone-labelled nuclei with H2B-mScarlet3 were located underneath the epidermal nuclei. (E) Amino acid sequences of the uncharacterized protein (OFAS019046-RA) encoded by the locus containing the transgene insertion site.

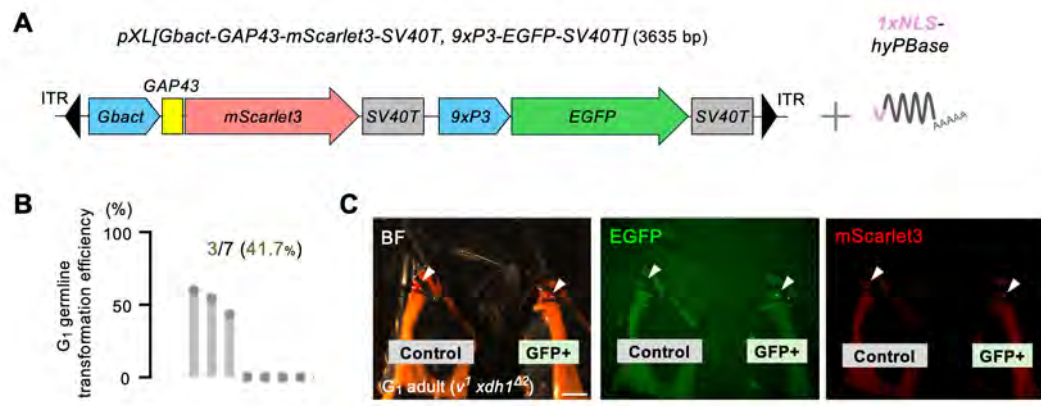


Figure S7. Attempt to generate a membrane-labelled strain using 1xNLS *hyPBase* in *O. fasciatus*. (A) Schematic of the *piggyBac* donor vector *pXL[Gbact-GAP43-mScarlet3-SV40T, 9xP3-EGFP-SV40T]* and *hyPBase* mRNA carrying 1xNLS. (B) G₀ somatic transformation rate (STR). (C) G₁ germ line transformation efficiency (GTE). Each bar represents the proportion of EGFP-positive animals among G₁ progeny from an individual cross. Each dot represents an individual backcross that yielded G₁ progeny. Green numbers indicate the number of crosses yielding heritable transgenes and the corresponding percentages. (D) Representative images of G₁ EGFP-positive adults. Weak mScarlet3 expression in their eyes was also observed. (E) Results of G₁ crossing experiments. Each row indicates the result of a single-pair mating.

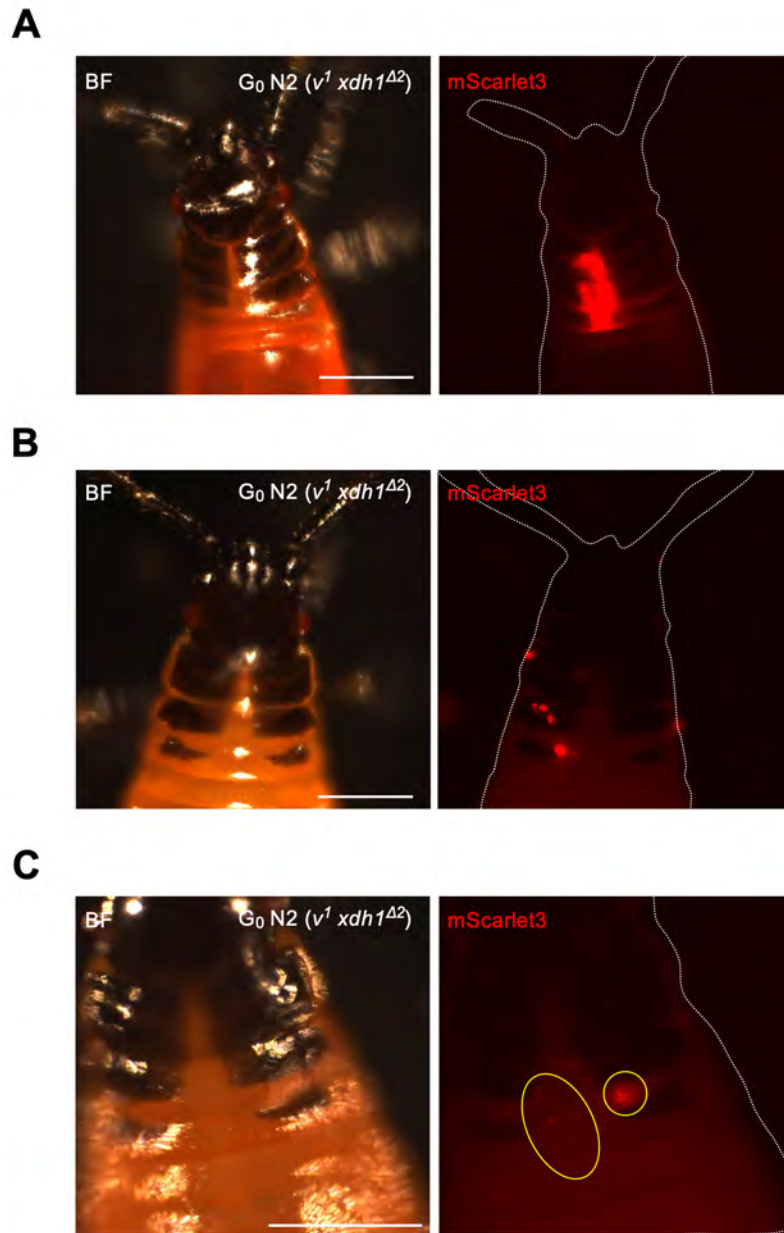


Figure S8. Additional data on Q system application using 1×NLS *hyPBase* in *O. fasciatus*. (A–C) Representative images of G₀ mScarlet3-positive nymphs. White dotted lines indicate animal boundaries. Yellow dotted circles indicate mScarlet3 expression. Scale bars, 500 μm. Anterior is up in (B–D).

Supplementary Tables

Table S1. Detailed results of piggyBac-mediated transgenesis experiments with hyPBase mRNA carrying 0×, 1×, 3×, or 5×NLS in *G. bimaculatus*. Each row shows the results of an independent experiment. Percentages shown in parentheses indicate somatic transformation rates (STR).

Donor plasmid	Transposase (mRNA)	Injected eggs	% of injected eggs survived to screening	Screened G0 embryos	mScarlet-I + nymphs	mScarlet-I - nymphs
pXL[9xP3-mScarlet-I-SV40T]	0xNLS-hyPBase	194	69.6%	135	99 (73.3%)	36
pXL[9xP3-mScarlet-I-SV40T]	1xNLS-hyPBase	194	70.1%	136	105 (77.2%)	31
pXL[9xP3-mScarlet-I-SV40T]	3xNLS-hyPBase	190	72.1%	137	87 (63.5%)	50
pXL[9xP3-mScarlet-I-SV40T]	5xNLS-hyPBase	192	67.7%	130	91 (70.0%)	39

Table S2. Results of crossing experiments with 0×NLS-hyPBase. F, female. M, male. GTR, germ line transformation rate.

mRNA		donor plasmid					
0xNLS-hyPBase#1		9xP3-mScarlet-I-SV40T					
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)
1	+	M	<i>v¹ xdh1^Δ</i>	60	0	60	0
2	+	F	<i>v¹ xdh1^Δ</i>	57	0	57	0
3	+	M	<i>v¹ xdh1^Δ</i>	57	0	57	0
4	+	F	<i>v¹ xdh1^Δ</i>	54	31	23	57.4
5	+	F	<i>v¹ xdh1^Δ</i>	57	0	57	0
6	+	F	<i>v¹ xdh1^Δ</i>	13	0	13	0

988 **Table S3. Results of crossing experiments with 1×NLS-hyPBase.** F, female. M, male. GTR, germ line transformation rate.
989

mRNA	donor plasmid						
1xNLS-hyPBase#1	9xP3-mScarlet-I-SV40T						
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)
1	+	F	<i>v¹ xdh1^{Δ2}</i>	55	0	55	0
2	+	F	<i>v¹ xdh1^{Δ2}</i>	45	0	45	0
3	+	F	<i>v¹ xdh1^{Δ2}</i>	64	0	64	0
4	+	F	<i>v¹ xdh1^{Δ2}</i>	52	0	52	0
5	+	F	<i>v¹ xdh1^{Δ2}</i>	53	44	9	83.0
6	+	M	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA
7	+	M	<i>v¹ xdh1^{Δ2}</i>	24	11	13	45.8
8	+	M	<i>v¹ xdh1^{Δ2}</i>	50	0	50	0
9	+	F	<i>v¹ xdh1^{Δ2}</i>	34	0	34	0
10	+	F	<i>v¹ xdh1^{Δ2}</i>	43	0	43	0
11	+	F	<i>v¹ xdh1^{Δ2}</i>	61	54	7	88.5
12	+	M	<i>v¹ xdh1^{Δ2}</i>	39	18	21	46.2
13	+	M	<i>v¹ xdh1^{Δ2}</i>	51	0	51	0
14	+	M	<i>v¹ xdh1^{Δ2}</i>	52	35	17	67.3

15	+	M	$v^1 xdh1^{42}$	48	0	48	0
16	+	M	$v^1 xdh1^{42}$	26	0	26	0
17	+	M	$v^1 xdh1^{42}$	62	0	62	0
18	+	M	$v^1 xdh1^{42}$	46	6	40	13.0
19	+	M	$v^1 xdh1^{42}$	50	0	50	0
20	+	M	$v^1 xdh1^{42}$	sterile	0	0	NA

mRNA		donor plasmid					
1xNLS-hyPBase#2		9xP3-mScarlet-I-SV40T					
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)
1	+	M	<i>v¹ xdh1^{Δ2}</i>	51	0	51	0
2	+	F	<i>v¹ xdh1^{Δ2}</i>	55	0	55	0
3	+	F	<i>v¹ xdh1^{Δ2}</i>	60	60	0	100.0
4	+	F	<i>v¹ xdh1^{Δ2}</i>	56	0	56	0
5	+	F	<i>v¹ xdh1^{Δ2}</i>	52	0	52	0
6	+	M	<i>v¹ xdh1^{Δ2}</i>	50	0	50	0
7	+	F	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA
8	+	F	<i>v¹ xdh1^{Δ2}</i>	50	50	0	100.0

9	+	M	$v^1 xdh1^{42}$	53	53	0	100.0
10	+	M	$v^1 xdh1^{42}$	51	0	51	0

991 **Table S4. Results of crossing experiments with 3×NLS-hyPBase.** F, female. M, male. GTR, germ line transformation rate.
992

mRNA		donor plasmid						
3xNLS-hyPBase#1		9xP3-mScarlet-I-SV40T						
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)	
1	+	M	<i>v¹ xdh1^{Δ2}</i>	60	0	60	0	
2	+	M	<i>v¹ xdh1^{Δ2}</i>	55	0	55	0	
3	+	M	<i>v¹ xdh1^{Δ2}</i>	3	3	0	100.0	
4	+	F	<i>v¹ xdh1^{Δ2}</i>	51	33	18	64.7	
18	+	M	<i>v¹ xdh1^{Δ2}</i>	61	0	61	0	
19	+	M	<i>v¹ xdh1^{Δ2}</i>	1	0	1	0	
20	+	M	<i>v¹ xdh1^{Δ2}</i>	57	0	57	0	
41	+	M	<i>v¹ xdh1^{Δ2}</i>	30	0	30	0	

mRNA	donor plasmid							
3xNLS-hyPBase#2	9xP3-mScarlet-I-SV40T							
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)	
18	+	M	$v^1 xdh1^{42}$	51	0	51	0	
19	+	M	$v^1 xdh1^{42}$	sterile	0	0	NA	
20	+	M	$v^1 xdh1^{42}$	57	0	57	0	
21	+	M	$v^1 xdh1^{42}$	68	0	68	0	
22	+	M	$v^1 xdh1^{42}$	64	64	0	100.0	
23	+	M	$v^1 xdh1^{42}$	36	0	36	0	
24	+	F	$v^1 xdh1^{42}$	34	0	34	0	
25	+	F	$v^1 xdh1^{42}$	23	0	23	0	
26	+	F	$v^1 xdh1^{42}$	died	0	0	NA	
27	+	M	$v^1 xdh1^{42}$	45	0	45	0	
28	+	M	$v^1 xdh1^{42}$	sterile	0	0	NA	
29	+	F	$v^1 xdh1^{42}$	50	50	0	100.0	
30	+	F	$v^1 xdh1^{42}$	54	23	31	42.6	
31	+	F	$v^1 xdh1^{42}$	51	51	0	100.0	
40	+	F	$v^1 xdh1^{42}$	11	11	0	100.0	

993
994

995 **Table S5. Results of crossing experiments for attP strains.** The integration site was determined for the line derived from G1 individual 8A. F, female. M,
996 male. GTR, germ line transformation rate.
997

mRNA	donor plasmid						
3xNLS-hyPBase#1	9xP3-EGFP-SV40T;attP						
G0 indiv#	EGFP expression	sex	crossed with	Screened G1 animals	GFP+	GFP-	GTR (%)
1	+	F	<i>v¹ xdh1^{Δ2}</i>	49	0	49	0
2	+	F	<i>v¹ xdh1^{Δ2}</i>	41	0	41	0
3	+	F	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
4	+	F	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
5	+	F	<i>v¹ xdh1^{Δ2}</i>	25	19	6	76.0
6	+	M	<i>v¹ xdh1^{Δ2}</i>	26	0	26	0
7	+	M	<i>v¹ xdh1^{Δ2}</i>	44	0	44	0
8	+	F	<i>v¹ xdh1^{Δ2}</i>	42	16	26	38.1
9	+	M	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA
10	+	M	<i>v¹ xdh1^{Δ2}</i>	5	5	0	100.0
G1 indiv#	EGFP expression	sex	crossed with	Screened G2 animals	GFP+	GFP-	G2 segregation rate (%)
5A	+	F	<i>v¹ xdh1^{Δ2}</i>	33	27	6	81.8
8A	+	F	<i>v¹ xdh1^{Δ2}</i>	64	32	32	50.0

mRNA		donor plasmid					
3xNLS-hyPBase#2		9xP3-EGFP-SV40T;attP					
G0 indiv#	EGFP expression	sex	crossed with	Screened G1 animals	GFP+	GFP-	GTR (%)
1	+	F	<i>v¹ xdh1^{Δ2}</i>	48	0	48	0
2	+	M	<i>v¹ xdh1^{Δ2}</i>	31	0	31	0
3	+	F	<i>v¹ xdh1^{Δ2}</i>	45	31	14	68.9
4	+	M	<i>v¹ xdh1^{Δ2}</i>	39	0	39	0
5	+	M	<i>v¹ xdh1^{Δ2}</i>	33	6	27	18.2
G1 indiv#	EGFP expression	sex	crossed with	Screened G2 animals	GFP+	GFP-	G2 segregation rate (%)
3A	+	M	<i>v¹ xdh1^{Δ2}</i>	44	37	7	84.1
3B	+	F	<i>v¹ xdh1^{Δ2}</i>	51	37	14	72.5

Table S6. Results of crossing experiments for Gbact-H2B-mScarlet3 strain. F, female. GTR, germ line transformation rate.

mRNA	donor plasmid							
1xNLS-hyPBase	Gbact-H2B-mScarlet3-SV40T							
G0 indiv#	mScarlet-I expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)	
1	+	F	<i>v¹ xdh1^{Δ2}</i>	30	14	16	46.7%	

002 **Table S7. Results of crossing experiments for Gbact-GAP43-mScarlet3;9xP3-EGFP strains.** F, female. M, male. GTR, germ line transformation rate.
003

mRNA	donor plasmid						
1xNLS-hyPBase	Gbact-GAP43-mScarlet3-SV40T;9xP3-EGFP-SV40T						
G0 indiv#	EGFP expression	sex	crossed with	Screened G1 animals	GFP+	GFP-	GTR (%)
1	+	M	<i>v¹ xdh1^{Δ2}</i>	45	27	18	60.0
2	+	M	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
3	+	M	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
4	+	M	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
5	+	M	<i>v¹ xdh1^{Δ2}</i>	35	0	35	
6	+	M	<i>v¹ xdh1^{Δ2}</i>	36	0	36	
7	+	M	<i>v¹ xdh1^{Δ2}</i>	33	0	33	
8	+	F	<i>v¹ xdh1^{Δ2}</i>	42	23	19	54.8
9	+	F	<i>v¹ xdh1^{Δ2}</i>	35	0	35	
10	+	F	<i>v¹ xdh1^{Δ2}</i>	died	0	0	NA
11	+	F	<i>v¹ xdh1^{Δ2}</i>	44	19	25	43.2
G1 indiv#	EGFP expression	gender	crossing with	Screened G2 animals	GFP+	GFP-	G2 segregation rate (%)
1A	+	M	<i>v¹ xdh1^{Δ2}</i>	56	28	28	50.0
1B	+	M	<i>v¹ xdh1^{Δ2}</i>	46	21	25	45.7

004

8A	+	M	<i>v</i> ¹ <i>xdh1</i> ^{Δ2}	45	23	22	51.1
----	---	---	---	----	----	----	------

005 **Table S8. Results of crossing experiments for Ofact-H2B-mScarlet3 strain.** F, female. M, male. GTR, germ line transformation rate.
006

mRNA		donor plasmid						
3xNLS-hyPBase		Ofact-H2B-mScarlet3-SV40T						
G0 indiv#	RFP expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)	
1	+	F	<i>v¹ xdh1^{Δ2}</i>	20	0	20	0	
2	+	F	<i>v¹ xdh1^{Δ2}</i>	32	17	15	53.1	
3	+	F	<i>v¹ xdh1^{Δ2}</i>	20	0	20	0	
4	+	F	<i>v¹ xdh1^{Δ2}</i>	49	0	49	0	
G1 indiv#	RFP expression	sex	crossed with	Screened G2 animals	RFP+	RFP-	G2 segregation rate (%)	
1A	+	F	<i>v¹ xdh1^{Δ2}</i>	50	32	18	64.0	
1B	+	M	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA	
1C	+	M	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA	
1D	+	M	<i>v¹ xdh1^{Δ2}</i>	31	14	17	45.2	
1E	+	F	<i>v¹ xdh1^{Δ2}</i>	sterile	0	0	NA	
1F	+	F	<i>v¹ xdh1^{Δ2}</i>	46	24	22	52.2	

mRNA	donor plasmid						
3xNLS-hyPBase	Ofact-H2B-mScarlet3-SV40T						
G0 indiv#	RFP expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)
1	+	F	<i>v¹ xdh1⁴²</i>	30	0	30	0.0
2	+	M	<i>v¹ xdh1⁴²</i>	51	10	41	19.6
G1 indiv#	RFP expression	sex	crossed with	Screened G2 animals	RFP+	RFP-	G2 segregation rate (%)
1A	+	F	<i>v¹ xdh1⁴²</i>	53	26	27	49.1
1B	+	M	<i>v¹ xdh1⁴²</i>	sterile	0	0	NA
1C	+	M	<i>v¹ xdh1⁴²</i>	55	33	22	60.0

mRNA	donor plasmid						
3xNLS-hyPBase	Ofact-H2B-mScarlet3-SV40T						
G0 indiv#	RFP expression	sex	crossed with	Screened G1 animals	RFP+	RFP-	GTR (%)
1	+	M	<i>v¹ xdh1⁴²</i>	died	0	0	NA
2	+	M	<i>v¹ xdh1⁴²</i>	died	0	0	NA
3	+	M	<i>v¹ xdh1⁴²</i>	no data	3	no data	no data
G1 indiv#	RFP expression	sex	crossed with	Screened G2 animals	RFP+	RFP-	G2 segregation rate (%)
3A, 3B, 3C	+	F (3)	<i>v¹ xdh1⁴²</i>	63	32	31	50.8

008 **Table S9. Primers used in the study.**
009

Name	Sequence (5'- to -3')	Purpose
Of-xdh1-F1	GGCTAACTTCCTAATGGTGGGT	Genotyping of <i>Of-xdh1</i>
Of-xdh1-R1	CTGCCATAGCAGAAAGAAAGGT	Genotyping of <i>Of-xdh1</i>
SPLNK-BOT	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGTGCGACACTAGTGG	Annealing of splinkerette PCR
SPLNK-GATC-TOP	GATCCCACTAGTGTGCGACACCAGTCTCTAATTTTTTTTTTCAAAAAA	Annealing of splinkerette PCR
SPLNK#1	CGAAGAGTAACCGTTGCTAGGAGAGACC	1st PCR of splinkerette PCR
SPLNK#2	GTGGCTGAATGAGACTGGTGTGCGAC	2nd PCR of splinkerette PCR
5'SPLNK-PB#1	ACCGCATTGACAAGCACG	1st PCR of splinkerette PCR
5'SPLNK-PB#2	CTCCAAGCGGCGACTGAG	2nd PCR of splinkerette PCR
5'SPLNK-PB-SEQ	CGACTGAGATGTCCTAAATGC	Sequencing for splinkerette PCR

010
011

Supplementary Video Legends

Supplemental Video 1. Ventral view of a developing *O. fasciatus* embryo showing ubiquitous expression of H2B:mScarlet driven by the endogenous actin promoter identified herein. Over this 36 hour time course, katatrepsis (second inversion of the embryonic axes such that the head of the embryo moves from the posterior of the egg (right) to the anterior of the egg (left) (Panfilio, 2008)), and appendage elongation occur. The Green Fire Blue look up table implemented in Fiji (Schindelin et al., 2012) shows the highest intensity pixels in white, intermediate intensity pixels in green, and the lowest intensity pixels in black (see Figure 5D). [Download Link](#)

Supplemental Video 2. Dorsal view of a developing *O. fasciatus* embryo showing ubiquitous expression of H2B:mScarlet driven by the endogenous actin promoter identified herein. Over this 36 hour time course, the end of anatrepsis (first inversion of the embryonic anteroposterior axis such that the head of the embryo moves from the anterior of the egg (right) to the posterior of the egg (left)), and the whole of katatrepsis (second inversion of both anteroposterior and dorsoventral embryonic axes relative to those of the egg) occur (Panfilio, 2008). The Green Fire Blue look up table implemented in Fiji (Schindelin et al., 2012) shows the highest intensity pixels in white, intermediate intensity pixels in green, and the lowest intensity pixels in black (see Figure 5D). [Download Link](#)

Supplemental Video 3. Lateral view of a developing *O. fasciatus* embryo showing ubiquitous expression of H2B:mScarlet driven by the endogenous actin promoter identified herein. Over this 36 hour time course, the end of anatrepsis and the whole of

1037 katatrepsis (Panfilio, 2008) occur as in Supplemental Video 3. From this lateral
1038 viewpoint, emerging muscular contractions of the embryo are apparent beginning at
1039 approximately 30 hours, the elongating appendages on the ventral side of the embryo
1040 (bottom), and the dorsal movement (towards the top) of the lateral ectoderm that is
1041 required for dorsal closure, are evident. The Green Fire Blue look up table implemented
1042 in Fiji (Schindelin et al., 2012) shows the highest intensity pixels in white, intermediate
1043 intensity pixels in green, and the lowest intensity pixels in black (see Figure 5D).
1044 [Download Link](#)

pXL[9xP3-mScarlet-I-SV40T] = 1775 bp

TTAA CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT
TTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTT
ACATACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAACAAAACTCA
AAATTTCTTCTATAAAGTAACAAAACCTTTATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTGTGA
ATTGAGATCTCCGGGGATCTAATTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATTAATT
CAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATTAATTCAATTAGAGACTAATTCAATTAGA
GCTAATTCAATTAGGATCCAAGCTTATCGATTTTGAACCCCTCGACCCCGGAGTATAAATAGAGGCGCTT
CGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATA
AACAAGCGCAGCTGAACAAGCTAAACAATCGAAGAATCAAAATGGTGTCTAAGGGAGAGGCTGTCATCAA
GGAATTCATGCGCTTCAAGGTGCACATGGAGGGATCAATGAACGGTCACGAGTTCGAAATCGAGGGAGAA
GGAGAGGGCCGTCCTTACGAAGGTACCCAGACTGCCAAGCTGAAGGTCACCAAGGGTGGCCCACTGCCTT
TCTCCTGGGACATCCTGAGCCCCAGTTCATGTACGGATCCCGCGCTTTCCTAAGCACCCCGCGACAT
CCCAGACTACTACAAGCAGAGCTTCCCAGAGGGTTTCAAGTGGGAACGTGTGATGAACCTCGAGGACGGA
GGTGCTGTGACCGTCACTCAGGACACCTCCCTGGAAGACGGCACTCTGATCTACAAGGTGAAGCTGAGGG
GTACCAACTTCCCTCCCGACGGCCAGTCATGCAGAAGAAGACCATGGGATGGGAGGCTAGCACTGAAAG
ACTGTACCCTGAGGACGGCGTCTGAAGGGAGACATCAAGATGGCCCTGAGGCTGAAGGACGGCGGAAGA
TACCTGGCTGACTTCAAGACCACTTACAAGGCCAAGAAGCCTGTGCAGATGCCCGGTGCCTACAACGTCG
ACAGGAAGCTGGACATCACCTCCCACAACGAGGACTACACTGTGGTCTGAACAGTACGAGCGCTCTGAAGG
CCGTCACTCAACTGGTGGCATGGACGAAGTGTACAAGTAAAACTTGTTTATTGCAGCTTATAATGGTTAC
AAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGT
CCAAACTCATCAATGTATCTTAAGATCTTCTAGATATCGGATCCCGGGGGTTCGACGGTATCGATAAGCTT
GATA TCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAAGTAGAACATGAAATAACAATATAAT
TATCGTATGAGTTAAATCTTAAAGTCACGTAAAGATAATCATGCGTCATTTTGACTCACGCGGTTCGTT
ATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAG
ATGTCCTAAATGCACAGCGACGGATTTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCG
TCAATTTTACGCAGACTATCTTTCTAGGGTTAA

piggybac ITR: 237* bp(L), 306 bp(R)

* 1 adenine deletion

Pax-6 homeodomain binding site: 11 bp x 9

Drosophila hsp70 promoter (minimal): 125 bp

mScarlet-I: 696 bp

SV40 polyadenylation signal: 122 bp

pXL[9xP3-EGFP-SV40T;attP] = 1838 bp

TTAA CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT
TTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTT
ACATACTAATAATAAATTCACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAACAAAACTCA
AAATTTCTTCTATAAAGTAACAAAACCTTTATCGAATTCCTGCAGCCCGGGGATCCACTAGTTCTGTGA
ATTCAGATCTCCGGGGATCTAATTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATTAATT
CAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATTAATTCAATTAGAGACTAATTCAATTAGA
GCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGCGCTT
CGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATA
AACAAGCGCAGCTGAACAAGCTAAACAATCGAAGAATCAAAATGGTGAGCAAGGGCGAGGAGCTGTTTAC
CGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAG
GGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTTCATCTGCACCACCGGCAAGCTGCCCCGTGC
CCTGGCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAA
GCAGCACGACTTCTTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGA
AGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAA
CGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAG
GACGGCAGCGTGCAGCTCGCCGACCCTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGC
CCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGT
CCTGCTGGAGTTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAACTTGTTT
ATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTCAC
TGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGATCTTCTAGATATCGGATCCCCGTG
CCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGGGGTTCGACGGTATCGATAAGCTTGATATCT
ATAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTA
TGAGTTAAATCTTAAAGTACACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTGCTTATAGTTC
AAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCT
AAATGCACAGCGACGGATTTCGCGCTATTTAGAAAGAGAGCAATATTTCAAGAATGCATGCGTCAATTT
TACGCAGACTATCTTTCTAGGGTTAA

piggybac ITR: 237* bp(L), 305 bp(R)

* 1 adenine deletion

Pax-6 homeodomain binding site: 11 bp x 9

Drosophila hsp70 promoter (minimal): 125 bp

EGFP: 717 bp

SV40 polyadenylation signal: 122 bp

phage ϕ C31 attP: 42 bp

pXL[Gbact-H2B-mScarlet3-SV40T] = 2974 bp

TTAAACCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT
TTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTATTATATTTACACTT
ACATACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAATAAAAAAACAAAACTC
AAAATTTCTTCTATAAAGTAACAAAACCTTTTATCGAATTCCTGCAGCCCGGGGGATCCACTAGCGCTACC
GGACTCAGATCTCGAGCAGTTCCTTCAAATGTTCAAAATAGTATTAGTTTTTGTGTACGCCTAAATTTCTT
GAGAATGTTGTTTAACTTTTATTTTCTGACAAATGTTAAATCATTGCTGTTTTTTTTTAGCAGACACTTT
TCGAAGCTTGTGTTTTGATATCTCGTTGTAAAGTAAATTCATTTATAAGTTCTCATTGACTTTTAACCGT
TTTTAGCCGGCATGTTCCGGTAGGGAGCGTTGGTTCCACGCTGCTGGCTCGATACCATATATGGTAAAGA
AAGCTGCCATTGGATGAGAAGCATGCCCTTATTAGTTGGTGGTGGGGTAGGGTAGGCATGGGAGGGGCA
GGGCCTCCGTAGTATATTACTCATAGCCGGCTCCCGGCTTGAATCAGTCTGGTGCTAGTTCTCGTACTG
TGAAGATAGCAGTGTGCGCTGCTGCGTTTTTGTACATATTACATTGTAAGTTTCTAGTGCTTCTCGAAG
ATACTTTGTGAATTTGTGTTACGAGCATTTGTGCCATGTGGGATATCAGCACAATTTTTCGTGTGGATTT
TCGCGTATGTGCTTGTGAGCAGTTAGGGCGGGGCAACCGGCTTCGTCCACACTCCCAAGGATTTTCGCCTT
ATTTGGCGGGTGTGTTAGGGTCTAGCCACTGAATATGGGCGGTGCCTGTGTAGTGGTTGCCGGTGTG
GGGCTTAGTCTTGTCAATGGGCGGGAAACCGGTGTGGGGAAACTAGTAGCGAATTGGATGGTTAACTAGT
TTACTCGTGCTACGGTTCTCTTAATTTTTTTTTTTTATTTCTTGCAGTACCCGTAAACTCAACTACTAACCG
AATTTCTGCAGGTCGACATGCGCGCCCAAGACTAGCGGAAAGGCCGCGAAGAAGGCCGGTAAGGCCCAAGG
AATATCTCCAAGGGAGACAAGAAGAAGAAGCGCAAGAGGAAGGAAAGCTACGCCATCTACATCTACAAGG
TGTTGAAGCAGGTCCATCCTGACACCGGCATCTCCAGCAAGGCGATGAGCATCATGAACAGTTTTGTCAA
TGACATTTTCGAGCGCATCGCCGCCGAGGCTTCCCGCCTGGCGCACTACAACAAGCGCTCTACCATCACC
TCGCGGGAGATCCAGACCGCTGTGCGTCTCCTTCTGCCCGGGCAGCTGGCGAAGCAGCTGTGACGGAAG
GCACTAAGGCTGTCAACCAAGTACACCAGCTCCAAGCAAGGATCCACCGGTGCGCCACCATGGATAGCACCGA
GGCAGTGATCAAGGAGTTCATGCGGTTCAAGGTGCACATGGAGGGCTCCATGAACGGCCACGAGTTCGAG
ATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAGGGTGACCAAGGGTG
GCCCCCTGCCCTTCTCCTGGGACATCCTGTCCCTCAGTTTCATGTACGGCTCCAGGGCCTTCACGAAGCA
CCCCGCCGACATCCCCGACTACTGGAAGCAGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC
TTCGAGGACGGCGGCGTGTGTCCGTGGCCCAGGACACCTCCCTGGAGGACGGCACCTGATCTACAAGG
TGAAGCTCCGCGGCACCAACTTCCCTCCTGACGGCCCCGTAATGCAGAAGAAGACAATGGGCTGGGAAGC
ATCCACCGAGCGGTGTACCCCGAGGACGTGCTGCTGAAGGGCGACATTAAGATGGCCCTGCGCCTGAAG
GACGGCGGTGCTACCTGGCGGACTTCAAGACCACCTACAGGGCCAAGAAGCCCGTGAGATGCCCCGGCG
CTTTCAACATCGACCGCAAGTTGGACATCACATCCACAACGAGGACTACACCGTGGTGAACAGTACGA
ACGCTCCGTGGCCCGCCACTCCACCGGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACAT
TTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGC
AATTGTTGTTGTTAACTTGTTTATTGTCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCT
ACAAATAAAGCATTTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCG
TAAATTGTAAGCGTTAATATTTTGTAAATTCGCGTTAAATTTTGTAAATCAGCTCATTTTTTAACC
AATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTGTTCC
AGTTAGATCTTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCCCTCGAGGTCGACGGTATC
GATAAGCTTGATACTCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAAGTAGAACATGAAATAA
CAATATAATTATCGTATGAGTTAAATCTTAAAGTACGTAAGATAATCATGCGTCATTTTGACTCAC
GCGGTGCTTATAGTTCAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCG
GCGACTGAGATGTCTTAAATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGA
ATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAA

piggybac ITR: 238 bp (L), 305 bp (R)

Gryllus actin promoter: 826 bp

Gryllus H2B: 369 bp

mScarlet3: 672 bp

SV40 polyadenylation signal: 122 bp

pXL[Gbact-gap43-mScarlet3-SV40T;9xP3-EGFP-SV40T] = 3635 bp

TTAAACCCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT
TTTATCGGCTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTT
ACATACTAATAATAAATTC AACAAACAATTTATTTATGTTTATTTATTTATTA AAAAAAAAAACAAAACTC
AAAATTTCTTCTATAAAGTAACAAAAC TTTTATCGAATTCCTGCAGCCCGGGGGATCCACTAGCGCTACC
GGACTCAGATCTCGAGCAGTTCCCTCAAATGTTCAAATAGTATTAGTTTTTGTACGCCTAAATTTCTT
GAGAATGTTGTTTAACTTTTTATTTTCTGACAAATGTTAAATCATTGCTGTTTTTTTAGCAGACACTTT
TCGAAGCTTGTGTTTTGATATCTCGTTGTAAAGTAAATTCATTTATAAGTTCTCATTGACTTTTAACCGT
TTTTAGCCGGCATGTTCCGGTAGGGAGCGTTGGTTCCACGCTGCTGGCTCGATAACCATATATGGTAAAGA
AAGCTGCCATTGGATGAGAAGCATGCCCTTATTAGTTGGTGGTGGGGGTAGGGTAGGCATGGGAGGGGCA
GGGCTCCGTAGTATATTACTCATAGGCCGGCTCCCGGCTTGAATCAGTCTGGTGCTAGTTCTCGTACTG
TGAAGATAGCAGTGTGCGCTGCTGCGTTTTTGTACATATTACATTGTAAGTTTCTAGTGCTTCTCGAAG
ATACTTTGTGAATTTGTGTTACGAGCATTTGTGCCATGTGGGATATCAGCACAATTTTTCTGTGTGGATTT
TCGCGTATGTGCTTGTGAGCAGTTAGGGCGGGGCAACCGGCTTCGTCCACACTCCCAAGGATTTTCGCCTT
ATTTGGCGGGTGTTTGCTTAGGGTCTAGCCACTGAATATGGGCGGTGCCTGTGTAGTGAGTTGCCGGTGTG
GGGCTTAGTCTTGTCAATGGGCGGGAAACCGGTGTGGGGAAACTAGTAGCGAATTGGATGGTTAACTAGT
TTACTCGTGCTACGGTTCTCTTAATTTTTTTTTTTTATTTCTTGCAGTACCCGTAAACTCAACTACTAACCG
AATTCTGCAGGTCGACATGCTGTGCTGTATGAGAAGAACC AAACAGGTTGAAAAGAATGATGAGGACCAA
AAGATCATGGATAGCACCGAGGCAGTGATCAAGGAGTTCATGCGGTTCAAGGTGCACATGGAGGGCTCCA
TGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAA
GCTGAGGGTGACCAAGGGTGGCCCCCTGCCCTTCTCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGC
TCCAGGGCCTTCACGAAGCACCCCGCCGACATCCCCGACTACTGGAAGCAGTCCTTCCCCGAGGGCTTCA
AGTGGGAGCGCGTGATGAAC TTCGAGGACGGCGGCGCTGTGTCCGTGGCCCAGGACACCTCCCTGGAGGA
CGGCACCTGATCTACAAGGTGAAGCTCCGCGGCACCAACTTCCCTCCTGACGGCCCCGTAATGCAGAAG
AAGACAATGGGCTGGGAAGCATCCACCGAGCGGTTGTACCCCGAGGACGTCGTGCTGAAGGGCGACATTA
AGATGGCCCTGCGCTGAAGGACGGCGGTGCTACCTGGCGGACTTCAAGACCACCTACAGGGCCAAGAA
GCCCCGTGCAGATGCCCGGCGCTTTCAACATCGACCGCAAGTTGGACATCACATCCCACAACGAGGACTAC
ACCGTGGTGGAACAGTACGAACGCTCCGTGGCCCCGCCACTCCACCGGCTAAAGCGGCCGCGACTCTAGAT
CATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGAAC
CTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAA
GCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTACTGCATTCTAGTTGTGGTTTGTCAAACT
CATCAATGTATCTTAAGGTTCTGTGAATTCAGATCTCCGGGGATCTAATTCAATTAGAGACTAATTCAAT
TAGAGCTAATTCAATTAGGATTAATTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATTA
TTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCTCGA
CCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACAC
GTCGCTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCGAAGAATCAAATG
GTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACG
GCCACAAGTTTACGCTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCAT
CTGCACCACCGCAAGCTGCCCGTGGCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGC
TTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCC
AGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGA
CACCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG
CTGGAGTACAAC TACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGA
ACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCTACCAGCAGAACCCCC
CATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGAC
CCCAACGAGAAGCGCGATCACATGGTCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGG
ACGAGCTGTACAAGTAAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAA
TTTCACAAATAAAGCATTTTTTTTACTGCATTCTAGTTGTGGTTGTCCAAACTCATCAATGTATCTTGG
TACCGGGCCCCCCCCCTCGAGGTGACGGTATCGATAAGCTTGATACTCTATAACAAGAAAATATATATATAA

TAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTATGAGTTAAATCTTAAAAGTCACG
TAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCAT
TGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTTCGCG
CTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGT
TAA

piggybac ITR: 238 bp(L), 305 bp(R)

Gryllus actin promoter: 826 bp

GAP43 fragment (palmitoylation sites): 60 bp

mScarlet3: 672 bp

SV40 polyadenylation signal: 122 bp

Pax-6 homeodomain binding site: 11 bp x 9

Drosophila hsp70 promoter (minimal): 125 bp

EGFP: 717 bp

SV40 polyadenylation signal: 121 bp

pXL[Ofact-H2B-mScarlet3-SV40T] = 4650 bp

TTAA**CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT**
TTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTATTATATTTACACTT
ACATACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAATAAAAAACAAAACTC
AAAATTTCTTCTATAAAGTAACAAAACCTTTTATCGAATTCCTGCAGCCCGGGGGATCCACTAGCGCTACC
GGACTCAGATCTCGAGCGGGGAGATTTCTCGCCTTTCCTTAGTGAGGAGTGGAGAGAGACTGTGGATATA
TATATATATATCCACACGTACACATTTATATATGGTAGATAATATCTCTAAGAATTTGATTCTTTGACTG
CACGCCACCGACATGCCAACCATCCAGAGGTTAATTATATTTAGGAAATTTGGTTTAGGAATCCTTAAAA
ATAAACATTTGTTAAAACCTCGACATAGACTTCCTTCTTGATTCTTATACTTGACACTACATTGTGGTTA
ATTTAAGGTTGCTCGGGGAAAATCATTCAGACATGTAATAAAATAGAAACGGATTTTGAACCTAATTTCT
GTTTACAGTTAGATAAAGAATTGTATTGCTATATAATGTACACACTGGACTGTCTGTGCCTGTGCTTATC
CTAGTAAATCGGCTTAACTAATAACCCTTCTTATAGAGGTAGATTGGTTGAGAAGATGACTTTTCGTTTCC
AACATTAAAACAGAAACAAAATTACTTCTGTTTGGGTACTTATATAAAACCTGTAAGAAAGCGAACCATA
ATAAGCTGTCAAGAAGAGCCCGGACCACAATGCCTAGGTCAGGTAGAAGCTGGTAGAGAGATTTGGCAGA
GGGCAGACCCATTGATGAGAGAATGGGCATGAATGGGAGTTTTAAAGGGAATCGGGAGAGTTTTAGAGCC
TCAGGGATAGGGACTCTCGCGTCAGACGACATTTTCGACAGGGGCTTAACGGAATTGCACCAGGAATTAGG
GATATTGTAGGGGACGGACGGGAGACTTGGAGGGACTCTCAGCGCTACTAGGATTCGGAAATATTTCCAG
TGACAGAACGGGAAATGGACTCCGCCTGCCTGCAAATGCATGTTCAATAATAAAAACTGGCTGAGAGATA
TTTACAAAGGCGGCTCTTACTAAATGCCGCGAGCTGGAGGGATTTCTCCCTCGCCGCGGGCTCCGCCAA
TCAGCGGGCTCCATCCGTTTTATGCCATATATGGTAAGAGCGGCGGTGAGGCCGCTGGTGGGGGAAGG
CGGGTCCACCTCCCTCCTCCCCCGACTCCCGGCTTTTCCCCCTCACAGCTATACCATACTTAACCCAA
TTGTTGTTATAATTTTTTTTAATTACTCATTTTTTTCCCATAAAAAACTGTATCGTGAAATCCGCTACCG
GGAAGGCGTATTATCAGCGGAATAGTGAAGTATTTGTGAGTGGCGGAGGAGCGACACCCCCCGGATCGG
GCGAGTCCACTCTCCTCCTGGAGACCAACGGCTCAATCCTCCTTCACTCCCGTCAATTGTGTGATAGTGT
ACGCATTGGCTCTCGCCTCCTTCGTCCAGTAAAGGTAAGTCTTGCAGAGAAATCTCACTATTTCCCTACAC
TTACAGTAAATACTCTACATCCCTACTCTTCTAATAGTACTAACCGTTTGTATATTGATATTTAAAGTGA
AGGGGCCGACTTTACTCCCGAATGAGTGCCGGTAGGACCTCGCAAACGTGTTGCCTTCGTTTTTCATTCT
CTCGTCTCTAAGTAAATTTAACTCCGGTATTTCTGGGACGTCCACAGCCAGAATTAACTTGGATCTCTT
CCAATATCTATGTTTAGGGAATGGTTTACTACGGTAGCGCCATTGACTGGAGCGCTATTTGTGTACTGAT
GAGAGTAGAGATGGCTCCTGGCCTATCTTGCCCTACCCCAAGTGTGGCGGTAAATCAGTTTTACCGGCTC
TTTTAGGCCGGAATTGTGTATTCCCTCGACCGTACTGCCATATAAGGAGATGTGCAGAGAGAGGGCGTG
AGGGGCGGGGGCCGGTGGAGGGGGACGGCCTATATCACCTCGGGACCCCTGCCCAAGCCACCAGTCCGA
CTTCACAGCTCAAACAGTACTCTGCGTACTATTTATTTATTTATTTTAAATAAGTAAGTGAAGTCTCAAT
AACCTCTTAAAGAGTGGGTGTGGATTATAGTGCTACTTATATTAATCTATTAGTGCTCATCAGTGAATCG
TGTTTTATGTGATATGAGTGATTCAAAGTGAACTTAATGGATTTACAATTAAATCTTCAGGGTCAGATT
ATAGCATTTGGTCAAGCTTCCCGAGAGGTAAATTTTTCCCTATTCCCTCTAGGCCAATTGGATTTTAA
TCAAGCATCCAATTGTCAAATTAATTCATCTTATAGCTACTGGAAATATCTGGAATTATGTGAAACTCA
TTCAGTAAAACCCCCCATTTATTTAACATGTGTTATAACTTAGTTGTATTTTAAATCACATGTACACTGA
CTTCAATTCACACAGGATGCCTGTAAAGTGATTTATTTAGTATTTTTTTTTTACAATATTTAGAATATTT
TCAAATTATGTACTGTAAAATGCTTGACTATCTGTTGTTCTGAAACCCCTGAAGGCACTCTCTGATTTTA
TGGATACCTTCATAGGATTTTAATTTTAGTTTTTGTTTTTTCAGCTTATTTAACTAACCCAAAACAAATT
CTGCAGGTCGAC**ATGCCCGCCCAAGACTAGCGGAAAGGCCGCGGAAGAAGGCCGGTAAGGCCCGAGAAGAATA**
TCTCCAAGGGAGACAAGAAGAAGAAGCGCAAGAGGAAGGAAAGCTACGCCATCTACATCTACAAGGTGTT
GAAGCAGGTCCATCCTGACACCGGCATCTCCAGCAAGGCGATGAGCATCATGAACAGTTTTGTCAATGAC

ATTTTCGAGCGCATCGCCGCCGAGGCTTCCCGCCTGGCGCACTACAACAAGCGCTCTACCATCACCTCGC
GGGAGATCCAGACCGCTGTGCGTCTCCTTCTGCCCCGGCGAGCTGGCGAAGCACGCTGTCAGCGAAGGCAC
TAAGGCTGTCACCAAGTACACCAGCTCCAAGAAAGGATCCACCGGTCGCCACCATGGATAGCACCGAGGCA
GTGATCAAGGAGTTCATGCGGTTCAAGGTGCACATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCG
AGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAGGGTGACCAAGGGTGGCCC
CCTGCCCTTCTCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAGGGCCTTCACGAAGCACCCC
GCCGACATCCCCGACTACTGGAAGCAGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCG
AGGACGGCGGCGCTGTGTCCGTGGCCCAGGACACCTCCCTGGAGGACGGCACCTGATCTACAAGGTGAA
GCTCCGCGGCACCAACTTCCCTCCTGACGGCCCCGTAATGCAGAAGAAGACAATGGGCTGGGAAGCATCC
ACCGAGCGGTTGTACCCCAGGACGTCGTGCTGAAGGGCGACATTAAGATGGCCCTGCGCCTGAAGGACG
GCGGTGCTACCTGGCGGACTTCAAGACCACCTACAGGGCCAAGAAGCCCGTGCAGATGCCCGGCGCTTT
CAACATCGACCGCAAGTTGGACATCACATCCACAACGAGGACTACACCGTGGTGGAACAGTACGAACGC
TCCGTGGCCCGCCACTCCACCGGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGT
AGAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATT
GTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAA
ATAAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCGTAAA
TTGTAAGCGTTAATATTTTTGTTAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATA
GGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTT
AGATCTTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCTCGAGGTCGACGGTATCGATA
AGCTTGATACTCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAAT
ATAATTATCGTATGAGTTAAATCTTAAAAGTCACGTAAGATAATCATGCGTCATTTTGACTCACGCGG
TCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGA
CTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGC
ATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAA

piggybac ITR: 238 bp (L), 305 bp (R)

Oncopeltus actin promoter: 2502 bp

Gryllus H2B: 369 bp

mScarlet3: 672 bp

SV40 polyadenylation signal: 122 bp

pXL[Ofinv2.4kb-QF2;15xQUAS-mScarlet3-SV40T;9xP3-EGFP-SV40T] = 7161 bp

TTAA**CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTAAAATTGACGCATGTGT**
TTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTATTATATTTACACTT
ACATACTAATAATAAATTCACAAAACAATTTATTTATGTTTATTTATTTATTAATAAAAAACAAAACCTC
AAAAATTTCTTCTATAAAGTAACAAAACCTTTTA**TCGAATTCCTGCAGCCCGGGGGATCCACTAGCGCTACC**
GGACTCAGATCTCGAGATGATGAGGTGAAGTGTACTCGAAGTAAGTGGACAGGGCGAGGGTGCTGTGGTC
TTGTCAACAATTCTCTTTCTCACACCCACATTTTCATCAGTTCTTCACTGTGTCTCATAAGATGCTTAATG
CTTAAGTATGCTTAGGCCACATCAAAGCTCTGTAAATAAATGTGCTTTGCGCTTTGTGATAAGGCTTTGC
ATGACTTAAATACTAATATATATAAAAAGAAAACATAATATTCGCAATACTTTGCGTTATTTCTATTTT
GTGTGTTTGTGAGTGTTGACTACTCTCATTCTTCCTCCTGTTATTAATATATATATATTTAATATATG
ACTTTATATATTATATACATATAATTTCTAATTTCCGATTGCGTGATTAGACAAAATGTGAATCGCTAGC
GTTAGACAACATATTGCCGACTGCACCACGTATGCCGCCATGTGTATCCTTGGAAGCGTGTTTCGCTCCT
TGGTCTTCCTAGCTCTTCCTTCCAGTTATCTTTATCTTCAGAGCTTCCTGTCTTATCAAACCTCAACATAA
TTTCAGCAAGACAACACACACTAAATATTGGACTTTTCCATCTTTCACATTTGCAAATCTTCACTACCAA
TATGTCATACCAATGGCATCATAAATTAACCTGTTGTAAGACTCGATGCCCTCTGAAATTGTCAACTAA
TTTCCTGTGGTTTCCATTTTCCTTGATAGGAATAATACACCTTCCACAATTAGATAATTATGGTATTATA
CATATAAACCTACACAGAGCTATCTATTGAAGTACAGATTATTTTAAGTTTAGTCTTTTGTGATGGGCTA
ATCCACGAGGCTGACTGACGTAATTTGGTTTCATATCCTACATAATTATCGGACGTGATTTGGGTAGGTC
AATATTAGGAAAACCAATATTATATATAAATCATTTTAACTTCTTTTTTTTTTTTTTAAAGTCGTGGTAC
AAGCGTGAATATATTGATTTCTTGTAGGAAATGTACTAAAATCGAATAATCGTATTTATAACAGTTGCCA
TAAAGTTGAAGTAACTGAATAAGTGTAATAATTCAATTCATTACATTTAATGTATAATAACATTTTATAGAG
AGTAAGGCACTAAAATTGTAGCTGTGCGGTTGAGCGAACCAACTAACAGGTAAAATTCAAATAAAGCAC
TTCATTGCAAATAGGGGGAGGGGGAGGGGGAGAGATTTACAGGAGAAACAAATAGAATGCTCGCAGGA
ATCGTGTTTATTATGGTAGGTGTATAGGACTAAAATCTTATCTACAAAAGTGCATTATATTGTAACAGTT
TAATATCCACTGACTTCCAGGACCTCCTTGAGTCATCGGAATGATACAGCTTTATTTTATTTTCAATTTAG
AAAATATTTATGGGAAAATAAATTGTTTCCAGACAGGATAGCTTGCAATGGATTGAGGATAATAAACT
TGTTCTACTGTACTGTGTGTTTCATGATTTCGAAGTTAGGACAAAATCAAACCTTGGGATCAGCGCCGCG
TACTTGATATAAATTACATTATGTGAAATCTGTTGAAAACGATTAATTGTATGGTCTTCTAAAGAAGGAA
ATGATATTTTATAGCCTCCCATCTGAATTGGAAGGGCCAGTCAACGGAGGTTGCTTTCCATACCAAATTT
ATATTAATTGCAATTGTCAACTTCTTAAATTGGACGTAATGAATATTACTTCCCCCTACTAAAGGGGTGTT
GATGGTAATCGCAACCCTCGATGAATACGATTGCTCAAAGTGTCTATTGTCTTAGGCTGTTGAGCTCCTA
AAAAAGAAAGGCTGTCAATCGCATTCATTAGCAACGAAAATGATTCTAACTGGATACATAACCACACAGG
ACGAGAAAGACCAACTATTTCTTCCTGCGAAAAGAATATTTAGAGGCTAATTTGATTACATTCTTCATTC
AAAGAGAAAACCTGATTTCCATCAAAATTAAAAATTAAGTGTGACAACTCAACGAATTGATATGTAATC
GATGATACACATCGATGATAGTCTTCCACATAGATAATTAATTCATGAGTGAGAGGGCGTGAGTGAGGG
GAAGGGAGGGGAGGAGAGGCGCGGTAAAGTGCATAATCATTTTCATCAGTCCTTAATAAACATATAGTGGGT
GGGAGGGGCCGCGGATATATGTAATCTGGCTAATAACTTGATCTGGAGGGGCGGTGGTGGGGGAGCAGG
GGAGGGGAGCCATCGCGGGGCGAGGCCGCTGAGGGGGAGGTGGGCACCCTGATTGGCCGAGAGCGGGGAG
TGGGCGGCGGTGAGGGGGCGGGGCCTTGTCGGACACGCCACCGCCCCCTCCCCACCACCGCACAGCGCT
CTCCCGACTTTGAAATCTTAGTTAGAAGCTGTCAGCCGTTGACAGAAGCTCTCAATCGGATGTAGTGAGG
CGAGGCCGGCCAACATGCCACCCAAGCGCAAAACGCTTAACGCTGCGGCTGAGGCTAACGCTCATGCCGA
CGGACACGCCGACGGAAACGCCGACGGACACGTGGCCAATACGGCCGCTCCTCGAATAATGCGAGGTTTC
GCTGATCTCACTAACATCGATACTCCGGGTCTGGGACCCACAACCTACGACCCTGCTCGTGGAACAGCAC
GCTCAAAGCGTCAACGAGTGTCCCGCGCATGCGACCAGTGCCGTGCAGCCCGAGAGAAATGCGACGGAAT
ACAGCCTGCGTGTTTCCCGTGCGTTTCCCAGGGAAGTCTGCACTTATCAGGCTTCGCCGAAAAAGAGG
GGAGTTCAAACCGGTTATATTTCGTACGCTGGAGCTCGCCCTCGCCTGGATGTTTGAAAATGTGCGCGGTT
CCGAAGATGCCTTGCATAACCTCCTCGTCCGTGACGCCGACAAGGATCAGCTCTGCTCGTTGGTAAAGA
TTCGCCGGCTGCCGAGCGACTCCATGCCCGTTGGGCTACTAGCCGTGTCAATAAGAGCATTACCCGCCTC
CTCCGTGAGTTGGAGCTCCCTCCTACCGCCACGGCTACGGCCTCGATAATGCCGCACGTGATGGAGCAGC
CTCTCAGTACCAGCATTAACCCCGTCAACGACCGCTTCAACGGTATTTCCAACCCCACTCCGTATAACTC
CGATGCAGCTCTCGATGCTATCACTCAGACCAACGATTATGGAAGCGTAAATACACATGGTATCCTCTCT

ACTTACCCGCCACCGGCTACGCACCTTAATGAAGCTTCCGTCGCTCTCGCTCCCGGTGGCGCCCCCCCCC
GACCGCCTCCTCCGTATGTTGACAGCACGACCAATCACCCGCCGTACCACTCGAATCTGGTTCCAATGGC
GAACTTTGGTTACTCGACCGTTGATTACGATGCCATGGTTGACGATTTGGCTAGCATTGAATACACGGAC
GCTGTGGATGTCGACCCACAGTTTATGACCAATCTGGGATTTCGTTCTGGATGTAACCTCTCCGACATTA
ATACATACGAACAGTGATGACGTCATCGATACCGTCGACTAAAGCCAAATAGAAAATTATTCAGTTCCT
GGCTTAAGTTTTTTAAAAGTGATATTATTTATTTGGTTGTAACCAACCAAAAGAATGTAAATAACTAATAC
ATAATTATGTTAGTTTTAAGTTAGCAACAAATTGATTTTAGCTATATTAGCTACTTGGTTAATAAATAGA
ATATATTTATTTAAAGATAATTGCGTTTTTTATTGTCAGGGAGTGAGTTTGCTTAAAACTCGTTTAGATC
CCGGTCACGTGTCACCTGGGTCAGTGGGTAATCGCTTATCCTCGGATAAACAATTATCCTCACGGGTAATC
GCTTATCCGCTCGGGTAATCGCTTATCCTCGGGTAATCGCTTATCCTTGGGTAATCGCTTATCCTCGGAT
AAACAATTATCCTCACGGGTAATCGCTTATCCGCTCGGGTAATCGCTTATCCTCGGGTAATCGCTTATCC
TTGGGTAATCGCTTATCCTCGGATAAACAATTATCCTCACGGGTAATCGCTTATCCGCTCGGGTAATCGC
TTATCCTCGGGTAATCGCTTATCCTTCACGTTGGGACTCAGTGAGGAGGACCTGAATTCCTGCAGCCCG
AGCGGAGACTCTAGCGAGCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAA
ACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACA
ATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAATTAAAGTAACCAGCAACCAAGTAAATCAACTGCAAC
TACTGAAATCTGCCAAGAAGTAATTATTGAATACAAGAAGAGAACTCTGAATAGGGAATTGGGAATTCGT
TAACAGATCGGGGGATCAATTTCGTTAACATGGATAGCACCGAGGCAGTGATCAAGGAGTTTCATGCGGTT
AAGGTGCACATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCT
ACGAGGGCACCCAGACCGCAAGCTGAGGGTGACCAAGGGTGGCCCCCTGCCCTTCTCCTGGGACATCCT
GTCCCCCTCAGTTCATGTACGGCTCCAGGGCCTTCACGAAGCACCCCGCCGACATCCCCGACTACTGGAAG
CAGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGCTGTGTCCGTGG
CCCAGGACACCTCCCTGGAGGACGGCACCCCTGATCTACAAGGTGAAGCTCCGCGGCACCAACTTCCCTCC
TGACGGCCCCGTAATGCAGAAGAAGACAATGGGCTGGGAAGCATCCACCGAGCGGTTGTACCCCGAGGAC
GTCGTGCTGAAGGGCGACATTAAGATGGCCCTGCGCCTGAAGGACGGCGGTGCTACCTGGCGGACTTCA
AGACCACCTACAGGGCCAAGAAGCCCGTGAGATGCCGGCGCTTCAACATCGACCGCAAGTTGGACAT
CACATCCCACAACGAGGACTACACCGTGGTGGAACAGTACGAACGCTCCGTGGCCCGCCACTCCACCGGC
TAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAA
CCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTTATTGCA
GCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTACTGCAAT
CTAGTTGTGGTTTTGTCCAACTCATCAATGTATCTTAAGGTTCTGTGAATTCAGATCTCCGGGGATCTAA
TTCAATTAAGAGACTAATTTCAATTAAGAGCTAATTTCAATTAAGGATTAATTTCAATTAAGAGCTAATTTCAATTA
GAGCTAATTTCAATTAAGGATTAATTTCAATTAAGAGCTAATTTCAATTAAGAGCTAATTTCAATTAAGGATCCAAG
CTTATCGATTTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAA
TTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCT
AAACAATCGAAGAATCAAAATGGTGAGCAAGGGCGAGGAGCTGTTTACCAGGGGTGGTGCCATCCTGGTC
GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG
GCAAGCTGACCCCTGAAGTTCATCTGCACCACCGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACCAC
CCTGACCTACGGCGTGAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCC
GCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCG
CCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAACGTCTATATCATGGCCGACAAG
CAGAAGAAGCGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGAGCTCGCCG
ACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCAC
CCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCTGCTGGAGTTTCGTGACCGCC
GCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAACTTGTATTATGCAGCTTATAATGGTTACA
AATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTGTC
CAAACCTCATCAATGTATCTTGGTACCGGGCCCCCCTCGAGGTTCGACGGTATCGATAAGCTTGATACTCTA
TAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTAT
GAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCA
AAATCAGTGACACTTACCGCATTGACAAGCACGCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTA

AATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTT
ACGCAGACTATCTTTCTAGGGTTAA

piggybac ITR: 238 bp (L), 305 bp (R)

Oncopeltus invected 2.4-kb upstream: 2435 bp

QF2: 1050 bp

hsp70 terminator: 257 bp

15xQUAS: 290 bp

Drosophila hsp70 promoter: 241 bp

mScarlet3: 672 bp

SV40 polyadenylation signal: 122 bp

Pax-6 homeodomain binding site: 11 bp x 9

Drosophila hsp70 promoter (minimal): 125 bp

EGFP: 717 bp

SV40 polyadenylation signal: 121 bp

Data File 2

>0xNLS-hyPBase

MPRSPEFAATMGSSLDDEHILSALLQSDELVGEDSDSEVSDHVSEDDVQSDTEEAFIDEVHEV
QPTSSGSEILDEQNVIEQPGSSLASNRILTLPQRTIRGKNKHCWSTSKPTRRSRVSALNIVRSQ
RGPTRMCRNIYDPLLCFKLFFTDEIISEIVKWTNAEISLKRRESMTSATFRDTNEDEIYAFFGI
LVMTAVRKDNHMSTDDLFDRLSMVYVSVMSRDRFDLIRCLRMDDKSIRPTLRENDVFTPVVK
IWDLFHQCINQYTPGAHLTIDEQLLGFRGRCPFRVYIPNKPSKYGIKILMMCDSGTKYMINGM
PYLGRGTQTNGVPLGEYYVKELSKPVHGSCRNITCDNWFTSIPLAKNLLQEPYKLTIVGTVRSN
KREIPEVLKNSRSRPVGTSMFCDGPLTLVSYKPKPAKMVYLLSSCDEDASINESTGKPQMVMY
YNQTKGGVDTLDMCSVMTCRKTNRWPMALLYGMINIACINSFIIYSHNVSSKGEKVQSRKKF
MRNLYMGLTSSFMKRLEAPTLKRYLRDNISNILPKEVPGTSDDSTE**EPVMKKRTYCTYCPSKI**
RRKASASCKKCKKVICREHNIDMCQSCF

EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI = predicted endogenous NLS

>1xNLS-hyPBase

M**PKKKRKV**PRSPFEAATMGSSLDDEHILSALLQSDDELVGEDSDSEVSDHVSEDDVQSDTEEAF
IDEVHEVQPTSSGSEILDEQNVIEQPGSSLASNRILTLPQRTIRGKNKHCWSTSKPTRRSRVSA
LNIVRSQRGPTRMCRNIYDPLLCFKLFFTDEIISEIVKWTNAEISLKRRESMTSATFRDTNEDE
IYAFFGILVMTAVRKDNHMSTDDLFDRLSMVYVSVMSRDRFDFLIRCLRMDDKSIRPTLREND
VFTPVRKIWDLFIHQCIQNYTPGAHLTIDEQLLGFRGRCPFRVYIPNKPSKYGIKILMMCDSGT
KYMINGMPYLGRGTQTNGVPLGEYYVKELSKPVHGSCRNITCDNWFTSIPLAKNLLQEPYKLT
VGTVRSNKREIPEVLKNSRSPVGTSMFCFDGPLTLVSYKPKPAKMVYLLSSCDEDASINESTG
KPQMVMYYNQTKGGVDTLDMCVMTCRKTNRWPMALLYGMINIACINSFIIYSHNVSSKGEK
VQSRKKFMRNLYMGLTSSFMRKRLEAPTLKRYLRDNISNILPKEVPGTSDDSTE**EPVMKKRTYC**
TYCPSKIRRKASASCKKCKKVICREHNIDMCQSCF

PKKKRKV = SV40 NLS

EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI = predicted endogenous NLS

>3xNLS-hyPBase

M**PKKKRKVPKKRKVPKKRKV**PRSPFEAATMGSSLDDEHILSALLQSDDELVGEDSDSEVSDH
VSEDDVQSDTEEFIDEVHEVQPTSSGSEILDEQNVIEQPGSSLASNRILTLPQRTIRGKNKHC
WSTSKPTRRSRVSALNIVRSQRGPTRMCRNIDPLLCFKLFFTDEIISEIVKWTNAEISLKRRE
SMTSATFRDTNEDEIYAFFGILVMTAVRKDNHMSTDDLFDRSLSMVYVSVMSRDRFDFLIRCLR
MDDKSIRPTLRENDVFTPVRKIWDLFIHQCIQNYTPGAHLTIDEQLLGFRGRCPFRVYIPNKPS
KYGIKILMMCDSGTKYMINGMPYLGRGTQTNGVPLGEYYVKELSKPVHGSCRNITCDNWFTSIP
LAKNLLQEPYKLTIVGTVRSNKREIPEVLKNSRSRPVGTSMFCFDGPLTLVSYKPKPAKMVYLL
SSCDEDASINESTGKPQMVMYYNQTKGGVDTLDQMCSVMTC SRKTNRWPMALLYGMINIACINS
FIIYSHNVSSKGEKVQSRKKFMRNLYMGLTSSFMRKRLEAPTLKRYLRDNISNILPKEVPGTSD
DSTE**EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI**CREHNIDMCQSCF

PKKKRKVPKKRKVPKKRKV = 3x SV40 NLS

EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI = predicted endogenous NLS

>5xNLS-hyPBase

M**PKKKRKVPKKRKVPKKRKVPKKRKVPKKRKVP**PRSPFEAATMGSSLDDEHILSALLQSDDELVGEDSDSEVSDHVSEDDVQSDTEEAFIDEVHEVQPTSSGSEILDEQNVIEQPGSSLASNRILTLPQRTIRGKNKHCWSTSKPTRRSRVSA LNIVRSQRGPTRMCRN IYDPLLCFKLFFTDEI ISEI VKWTNAEISLKRRESMTSATFRDTNEDEIYAFFGILVMTAVRKDNHMSTDDLFDRSLSMVYVSVMSRDRFDFLIRCLRMDDKSIRPTLRENDVFTPVRKIWDLFIHQCIQNYTPGAHLTIDEQLLGFRGRC PFRVYIPNKPSKYGIKILMMCDSGTKYMINGMPYLG RGTQTNGVPLGEYYVKELSKPVHGS CRNITCDNWFTSIPLAKNLLQEPYKLTIVGTVRSNKREIPEVLKNSRSRPVGTSMFCDGPLTLVSYKPKPAKMVYLLSSCDEDASINESTGKPQMVMYYNQTKGGVDTL DQMC SVMTCSRKTNRWPM ALLYGMINIACINSFIIYSHNVSSKGEKVQSRKKFMRNLYMGLTSSFMRKRLEAPTLKRYLRDNISNILPKEVPGTSDDSTE**EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI**CREHNIDMCQSCF

PKKKRKVPKKRKVPKKRKVPKKRKVPKKRKVP = 5x SV40 NLS

EPVMKKRTYCTYCPSKIRRKASASCKKCKKVI = predicted endogenous NLS

>0xNLS-minos

MVRGKPISKEIRVLIRDYFKSGKTLTEISKQLNLPKSSVHGVIQIFKKNGNIENNIANRGRTSA
ITPRDKRQLAKIVKADRRQSLRNLASKWSQTIGKTVKREWTRQQKLSIGYGIFYKAKEKPLLTLR
QKKKRLQWARERMSWTQRQWDTIIFSDEAKFDVSVGDTRKRVIRKRSETYHKDCLKRTTKFPAS
TMVWGCMSAKGLGKLHFIEGTVNAEKYINILQDSLPSIPKLSDCGEFTFQQDGASSHTAKRTK
NWLQYNQMEVLDWPSNSPDLSPINIWWLMKNQLRNEPQRNISDLKIKLQEMWDSISQEHCKNL
LSSMPKRVKCVMQAKGDVTQF

>3xNLS-minos

M**PKKKRKVPKKKRKVPKKKRKV**RGKPISKEIRVLIRDYFKSGKTLTEISKQLNLPKSSVHGVI
QIFKKNGNIENNIANRGRTSAITPRDKRQLAKIVKADRRQSLRNLASKWSQTIGKTVKREWTRQ
QLKSIGYGIFYKAKEKPLLTLRQKKKRLQWARERMSWTQRQWDTIIFSDEAKFDVSVGDTRKRV
IRKRSETYHKDCLKRTTKFPASTMVWGCMSAKGLGKLHFIEGTVNAEKYINILQDSLPSIPKLS
DCGEFTFQQDGASSHTAKRTKNWLQYNQMEVLDWPSNSPDLSPINIWWLMKNQLRNEPQRNIS
DLKIKLQEMWDSISQEHCKNLLSSMPKRVKCVMQAKGDVTQF

PKKKRKVPKKKRKVPKKKRKV = 3x SV40 NLS