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Transgenic tools for members of the genus Drosophila with sequenced genomes

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The sequencing of the genomes of 12 Drosophila species has created an opportunity for much in the way of comparative molecular analyses amongst these species. To aid that endeavor, we have made several transformation vectors based on the piggyBac transposon with 3x P3-EGFP and -ECFP transgenic markers that should be useful for mutagenesis and establishing the GAL4/UAS system in these species. We have tested the ability of mini-*white* to be used as a marker for insertional mutagenesis, and have observed mini-*white*-derived pigmentation of the testes sheath in a subset of lines from *D. pseudoobscura* and *D. virilis*. We have incorporated a source of piggyBac transposase into nine Drosophila species, and have demonstrated the functionality of these transposase lines for mobilization of marked inserts in vivo. Additionally, we tested the ability of a *D. melanogaster nanos* enhancer element to drive expression of GAL4 in *D. melanogaster, D. simulans, D. erecta, D. yakuba, D. pseudoobscura* and *D. virilis*. The efficacy of the *nos*-Gal4 transgene was determined by measuring the response of UAS-EGFP*tub* in all six species. Our results show that *D. melanogaster nos*-Gal4 drives expression in other species, to varying degrees, in similar spatiotemporal domains in the ovaries, testes and embryos as seen in *D. melanogaster*. However, expression levels are variable, demonstrating the possible need to use species-specific promoters in some cases. In summary, we hope to provide a set of guidelines and basic tools, based upon this work, for both insertional mutagenesis and GAL4/UAS system-based experiments in multiple species of Drosophila.

Introduction

Drosophila melanogaster has developed into the model organism of choice for many scientists over the past hundred years. Its short life cycle, inexpensive rearing media, simple genetics and ease of transgenesis make it appealing to many laboratories. As a result, people who work on *D. melanogaster* enjoy some of the most sophisticated research tools available today. The genome sequence of *D. melanogaster* was published in 2000;[1] http://www.fruitfly.org/sequence/release5genomic.shtml; in addition, large repositories of mutant strains, transgenic lines, online databases and tools for doing elegant genetic experiments are widely available to those who need them.[2] Clearly, an interesting new direction in this progression of knowledge will be to investigate different outlying phylogenetic branches of the genus Drosophila and perform comparative studies with *D. melanogaster*. One major step in this direction was the sequencing and annotation of 11 additional Drosophila species genomes that were interesting either because of their relationship to *D. melanogaster*, or their relationship to each other.[3,4] This wealth of information can be used to predict functional elements in these species such as regulatory regions or the protein products of genes, just to name two obvious examples.[5] These in silico predictions must be tested in the organism, however; and this requires, among other things, a transgenic system to incorporate tools for conducting such experiments. With a working transgenesis system in place, one can do experiments designed at testing a sequence or protein product from one species by putting it in the environment of another species and vice versa.

Transgenesis is performed in *D. melanogaster* to create new mutant stocks, binary expression systems and gene tagging systems.[6,7] Transgenesis in *D. melanogaster* requires the following framework: a transposon-based vector with cloning sites for the incorporation of DNA of interest, an easily identified reporter to confirm integration of the construct into the genome and a suitable fly stock that contains the genetic background necessary to visually detect the reporter. In recent years, those working on *D. melanogaster* have used the P-element integration system,[8] a mini-*white* transgene[9,10] as a visible eye marker and *white* mutant fly stocks that contain the appropriate mutant background to score for eye color rescue. However, while this system has been refined for *D. melanogaster*, a similar transgenic system is not available for the other Drosophila species.

There is evidence that the system used in *D. melanogaster*, as described above, would not be the ideal choice for experimentation in other Drosophila species. One problem is that while the P-element transposon works well in *D. melanogaster,* it does not appear to work equivalently in other drosophilids, nor

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does this transposable element appear to function efficiently in non-drosophilids.[11-14] In addition, mini-*white*, the visible transformation marker widely used for *D. melanogaster* transformation, may not be well suited for use in other Drosophila species. Mini-*white* has been used in *D. melanogaster* due to its ease of detection and minimal equipment requirements, needing only a dissecting microscope and light source to screen for transgenic flies. However, mini-*white* is not a perfect transformation marker. Position effects can eliminate expression of mini-*white* depending on genome insertion location.[15,16] These position effects would lead to a percentage of insertions being missed in large-scale mutagenesis or enhancer trap screens, if relying upon a visible marker such as mini-*white* to recover novel events. Moreover, if mini-*white* has these shortcomings in *D. melanogaster*, it is not known whether this minigene will work at all in outlying Drosophila species. Scoring of an eye rescue marker also requires a mutant eye phenotype to detect rescue (in this case, white-eyed flies); for many non-model organisms, mutant stocks do not exist that would allow detection of a marker such as mini-*white*. In summary, successful transgenesis in other insects requires a more universal transposition system and a transformation reporter that is visible in a variety of genetic backgrounds.

To this end, researchers using insects other than *D. melanogaster* have worked extensively to create reliable, easily marked and highly efficient transformation systems for their research organism (reviewed in ref. 17). Specifically, piggyBac has proven to be one of the most universally useful transformation systems in insects to date and has been adopted by many research groups to make transgenic stocks (reviewed in ref. 18). For visible markers in insects, eye markers have been developed that can express enhanced green, yellow, blue (*Aequorea Victoria* variants) and red (*Discosoma sp.*) florescent proteins in the eye using the artificial promoter 3xP3.[19-22] 3xP3-EGFP has been shown to work in a wide variety of arthropods, from flies to crustaceans.[15,23] It has also been shown to be less susceptible to position effect suppression than mini-*white* in *D. melanogaster*: in a construct containing both 3xP3-EGFP and mini-*white*, about 20% of insertions were found to be white-eyed but expressed 3xP3-EGFP.[15] But to use the 3xP3-EGFP system, a dissecting scope with a UV light source is necessary; in addition, unlike mini-*white* it is not feasible to use dose sensitivity of expression to detect homozygosity. Thus both the mini-*white* and 3xP3-EGFP markers have potential advantages and disadvantages when it comes to making practical, broadly detectable transgenic lines in the sequenced Drosophila genome species.

In addition to testing markers and making tools for insertional mutagenesis, we also wanted to test the GAL4/UAS system in a subset of Drosophila species. The GAL4/UAS system is the most widely used binary expression system used in *D. melanogaster*.[24] GAL4/UAS has also been demonstrated to work in the lepidopteran silkworm *Bombyx mori*.[25,26] However, the GAL4/UAS system has not been tested in the 11 sequenced Drosophila genome species outside of *D. melanogaster*. Furthermore, it is not known how a *D. melanogaster*-specific promoter or enhancer will act in other species. On one hand, making one construct for all species and simply testing the promoter or enhancer used would be experimentally efficient. On the other hand, using a promoter or enhancer native to one species and examining expression in other species may give unexpected expression patterns, or may not express at all.[27-29] To test this, we made a set of GAL4/UAS constructs in piggyBac. We chose the *D. melanogaster nanos* promoter/enhancer to compare expression patterns in a chosen subset of species. We cloned a UAS-EGFP*tub* responder in piggyBac for our *nos*-Gal4 experiments and examined the ovaries, testes and embryos of *D. melanogaster*, *D. simulans*, *D. erecta*, *D. yakuba*, *D. pseudoobscura* and *D. virilis*.

Given the predicted functionality of the piggyBac system and the potential usefulness of both the 3xP3-EGFP and mini-*white* markers, we had a framework for the construction of transformation vectors for the sequenced Drosophila genome species. Within this group, the piggyBac transposon and 3xP3-EGFP marker have been tested in *D. melanogaster*, *D. simulans*, *D. pseudoobscura* and *D. virilis*[21] and therefore should work efficiently in all Drosophila species. However, mini-*white* expression has not been tested in any of these species except *D. melanogaster* (originally observed in ref. 30) *D. simulans*/*D. mauritiana* hybrids[31] and *D. virilis*.[32] To this end, we needed to examine the following: (1) does mini-*white* work at all in the subset of sequenced genome species that we chose for this project and (2) what fraction, if any, of transgenic flies would express 3xP3-EGFP but have white eyes? For the purposes of insertional mutagenesis, having both mini-*white* expressing and white-eyed transgenic lines could be useful. In theory, an insertional mutagenesis screen could be performed using a mini-*white* and 3xP3-EGFP expressing line and the recovery of white-eyed, 3xP3-EGFP fly lines would show that the transposon had mobilized and reinserted. The reciprocal would also be true: a white-eyed, 3xP3-EGFP fly line could be mobilized with a transposase source and mini-*white* expressing lines could be recovered.

The 12 annotated Drosophila genomes enable scientists to perform extensive in silico analyses and predictions on gene function, gene regulation, enhancer functionality and so on. This work describes the production and testing of vectors and transgenesis system components in many of the sequenced Drosophila species to achieve the next major step in the progression of testing computer-based models. We use the piggyBac transposon, mini-*white* and 3xP3-EGFP transformation markers and GAL4/UAS system to demonstrate proof-of-principle functionality of transgenesis in 10 of the 12 sequenced Drosophila species.

Results and Discussion

For an overview of the phylogenetic relationship amongst the ten sequenced Drosophila genome species used in this work, with a summary of the transgenic fly lines generated in this project, see **Figure 1**. For simplicity, piggyBac GAL4/UAS lines will be referred to as *nos*-Gal4, UAS-EGFP*tub* and UAS-dsRed.

3xP3-EGFP and mini-*white* expression in Drosophila species. pBac{GreenEye} was injected into the Drosophila species to examine the efficacy of transformation in each species, to determine the relative frequency of mini-*white* and 3xP3-EGFP expression and demonstrate the usefulness of the

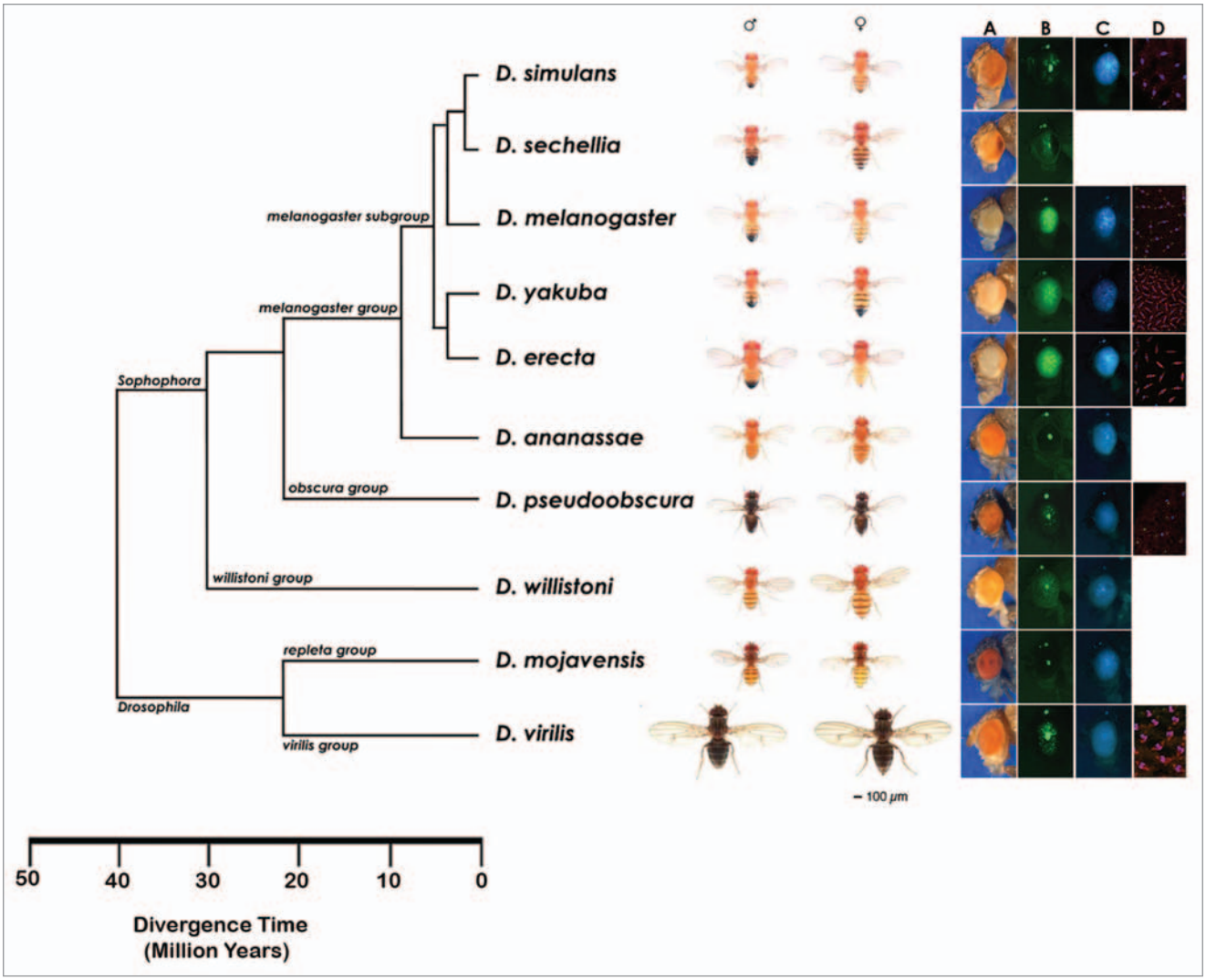


Figure 1. Pictorial representation of the ten chosen sequenced Drosophila genome species used for transgenesis. Phylogenetic tree is shown on the left (http://flybase.org/static_pages/species/sequenced_species.html). Male and female flies were photographed by Nicolas Gompel. Fly eyes and confocal images on the right represent transgenic lines produced in this project. Columns (A and B): pBac{GreenEye}. Column (C): pBac{5pBlueEye}. Column (D): *nos*-Gal4=>UAS-EGFP*tub*. Scale bar applies to images of adult flies only.

endogenous piggyBac transposase fly lines (pBac{5pBlueEye}). pBac{GreenEye} transgenic lines have been recovered in *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae, D. willistoni*, *D. mojavensis*, *D. pseudoobscura* and *D. virilis* (**Fig. 1**). Representative injection data with relative numbers of mini-*white* expression/non-expression are shown in **Table 3**. Many species were relatively easy to transform; exceptions to this were *D. sechellia*, *D. ananassae* and *D. willistoni*. It was expected that *D. sechellia* would be more difficult to work with, given the tendency of females to hold eggs until well after cellularization, which is too late for transformation injections.[48] *D. ananassae* and *D. willistoni* perform poorly in culture in our lab, so it was not surprising that transformation efficiency was low in these species. In an attempt to recover transformants in *D. willistoni,* we used a *D. willistoni* pBac{5pBlueEye} line to test for the ability of a resident source of transposase to increase transformation efficiency. Indeed transformation levels increased dramatically as compared to using helper plasmid alone (the lack of transformants in the *white D. willistoni* stock shown in **Table 3** reflects what was typical for that species). Given this result, it would be advantageous to use the piggyBac transposase lines for injections, despite the inconvenience of subsequently having to eliminate this source of transposase to establish a stable stock.

Transformant lines in all species express 3xP3-EGFP; however, subsets of lines in some species do not express mini-*white* (**Table 3**). In addition to the variation in eye color that is normally seen with mini-*white* rescue in *D. melanogaster*, patterning and/or speckling is occasionally seen in the pigmented mini-*white* expressing eyes of other species (some examples are represented in **Fig. 1**). While 3xP3-EGFP appears to be less susceptible to position effect than mini-*white* in all the species examined in this project, we have demonstrated that mini-*white* can be used in all species, eliminating an absolute requirement for EGFP screening. Doing so however, will unfortunately result

in a lower recovery of transgenic lines. This problem is at least partially ameliorated by the fact that using mini-*white* for stock construction in other species due to the fact that one copy of the transgene can be distinguished from two. This has also been seen in *Tribolium castaneum*; Lorenzen et al.[49] found that two copies of a *Tc. vermilion* transgene were distinguishable from one, while the same was not true for 3xP3-EGFP. Since there are no balancer chromosomes or sufficient visible markers for genetically mapping transgenic insertions in the sequenced Drosophila genome species, mini-*white* expression can facilitate making "selection-free" homozygous stocks that cannot usually be selected for on the basis 3xP3-EGFP expression levels. Nevertheless, 3xP3-EGFP has also proven useful as a marker, for reasons complimentary to those of mini-*white*. Thus while 3xP3-EGFP does display a range of position effects,[15,50] it appears less prone to these effects than mini-*white* and is therefore a more reliable marker for the recovery of transformant lines. An added benefit is that 3xP3-EGFP is easy to detect in Drosophila at many time points in development, including the embryonic, larval and pupal stages.

Given these results, we conclude that transformation is feasible in the Drosophila species chosen for this work; however, more needs to be done with species that have characteristics that are particularly ill-suited to transformation, such as *D. sechellia* (having few embryos available at the right stage for these experiments) or in our hands *D. willistoni* which we found difficult to culture. We also find that in some cases where transformation rates are low, using fly stocks that carry an endogenous source of piggyBac transposase can increase transformation success. Finally, we conclude that utilizing both mini-*white* and 3xP3-EGFP as transformation markers provides the best overall transformation recovery and experimental utility and gives more broadly useful transgenic lines than using either marker alone.

pBac{5pBlueEye} piggyBac transposase lines and enhancer trapping. There is a specific advantage to having both 3xP3-EGFP and mini-*white* in the same transformation vector. It has been shown previously in *D. melanogaster* that about 20% of all insertions containing both transgenic markers do not express mini-*white* but do show accumulation of EGFP;[15] this number can be as high as 50% using other promoters.[16] Our results are in keeping with these previous findings, although the percentages vary among the species. White-eyed, 3xP3-EGFP expressing lines can be useful when doing experiments such as mobilization for insertional mutagenesis or "enhancer sniffing." It is difficult to detect transposition events using mini-*white* eye color change alone. If a mini-*white*-negative, 3xP3-EGFP-positive line were used as the initial "mutator", flies could theoretically be screened for mobilization of the insert by the presence of mini-*white* expression in the eye of progeny flies.

To test this possibility we used pBac{5pBlueEye} fly stocks of several species, with or without heat shock, to act as "jumpstarters" testing for their ability to mobilize pBac{GreenEye}. **Figure 2** shows mosaic eye patterns in the offspring of pBac{GreenEye} (both mini-*white* expressing and white-eyed) crossed to pBac{5pBlueEye} in *D. melanogaster, D. simulans, D. yakuba, D. erecta, D. pseudoobscura* and *D. virilis*. Subsequent isolation of mini-*white* expressing fly lines (or the reciprocal) that were different from the original line in *D. melanogaster*, *D. pseudoobscura* and *D. virilis* demonstrates that these insertions mobilized and reinserted elsewhere in the genome (data not shown). In addition, an insertion in *D. virilis* that was male lethal was also mobilized in this fashion, giving rise to new lines that were male viable (data not shown).

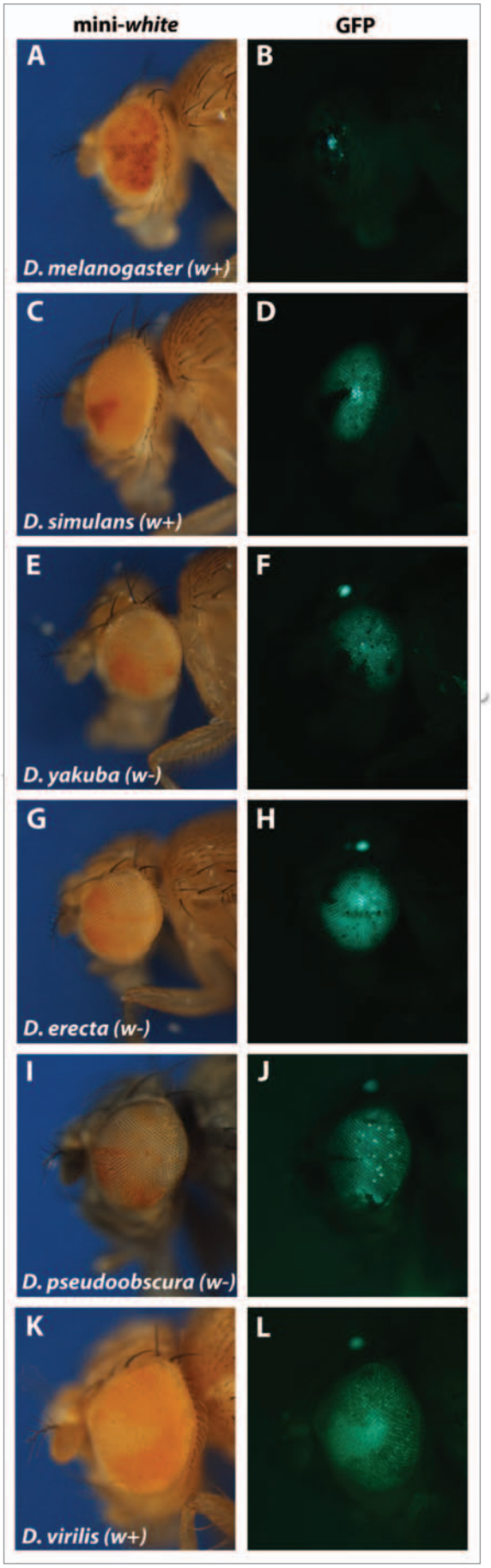
mini-*white* expression in the testis sheath of *D. pseudoobscura* and *D. virilis*. In *D. melanogaster*, the enhancer region responsible for expression in the testis sheath is absent from mini-*white*.[30,51] Our mini-*white* construct was taken from the P{W8} vector and utilizes the *hsp70* promoter to drive expression.[9] This mini-*white* transgene has been used extensively for P-element transformation in *D. melanogaster*, but expression outside of eye expression in *D. melanogaster* has not been reported. Moreover, we did not observe pigmented testes in any of the *D. melanogaster* transgenic lines generated in this work. In *D. pseudoobscura* and *D. virilis*, most of the transgenic lines produced that had mini-*white* pigmented eyes had non-pigmented testes, consistent with what is observed in *D. melanogaster* (**Fig. 3A–F**). However, a small number of transgenic lines with pigmented eyes also had pigmented testes in these two species (**Fig. 3G–O**). In one *D. virilis* line, although mini-*white* eye expression was uniform, expression in the testis was specific to only one short region of the entire testis (**Fig. 3M and O**), reminiscent of position effect patterning events in the eye. Overall, testis pigmentation by mini-*white* in *D. pseudoobscura* and *D. virilis* was seen in a variety of constructs, including *nos*-Gal4, UAS-EGFP*tub* and PBac{GreenEye}. Although statistically insignificant, it was noted that mini-*white* testes expression in a white-eyed, 3xP3-EGFP fly line was never observed in any species.

Cryptic enhancers may reveal themselves when complex regulatory regions of genes are taken out of their native environment and expressed as transgenic reporters.[34,52] We posit that there is likely a cryptic enhancer present in the mini-*white* construct used here that is capable of driving mini-*white* expression in the testes of *D. pseudoobscura* and *D. virilis*. It also appears that this enhancer is sensitive to position effect, as not all lines express in the testes and some lines have a restricted pattern of expression. Additionally, we note that unique lines such as those with pigmented testes recovered in *D. pseudoobscura* and *D. virilis* could be used in a manner similar to the w^- EGFP$^+$ and w^+ EGFP$^+$ lines described above, i.e., they could be mobilized and their progeny screened for absence of pigmentation.

GAL4/UAS lines generated in Drosophila species. The GAL4/UAS system is the most widely used binary expression system in *D. melanogaster* for targeted expression experiments aimed at investigating enhancer functionality, gene function and trans-species experiments, to name a few examples.[24,53] In order to test its potential use outside of *D. melanogaster*, we performed a proof-of-principle test of the GAL4/UAS system in a subset of the Drosophila sequenced genome species, to potentially create a useful set of fly lines and vectors for the fly community.

The gene *nanos* (*nos*) is required in *D. melanogaster* during oogenesis, in the male and female germline and is also required for posterior identity in embryos.[54,55] The regulatory region of *nanos* coupled with the enhanced transcriptional activity of GAL4-VP16 [56] has been used to make a maternally expressed

Figure 2. Flies shown are offspring of the cross pBac{GreenEye} X pBac{5pBlueEye} in various species. Mini-*white* mosaic expression is shown in the left column; 3xP3-EGFP expression is shown in the right column. After the species name it is noted whether the parental line expresses mini-*white* (w^+) or is white eyed (w^-). Note how elevated expression of mini-*white* masks the expression of EGFP.



GAL4 driver in *D. melanogaster*. This construct has been used extensively by researchers that study a wide range of stem cell, germline and oogenesis related genes and biological processes.[39] This P-element *nos*-Gal4 driver is commonly used in combination with a UAS-EGFP-tagged α-*tubulin84B*, which labels microtubules in the germarium, some egg chambers, blastoderm-stage embryos and the hub region of the testes[38,57] (and this work). In addition to using such UAS responders as UAS-EGFP*tub* to label proteins that are normally present, ectopic expression experiments or live imaging can be performed with UAS responders that make other EGFP tagged proteins.[58] We therefore chose this GAL4/UAS combination for testing the efficacy of the *nos*-Gal4 driver by making transgenic lines of *nos*-Gal4 and UAS-EGFP*tub* in six of the ten chosen Drosophila species: *D. melanogaster*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. pseudoobscura* and *D. virilis*. (UAS-dsRed lines were also generated in *D. melanogaster*, *D. pseudoobscura* and *D. virilis* as controls). Since the expression pattern of the *D. melanogaster nos* enhancer in the other species could not be predicted, ovaries, testes and embryos produced by *nos*-Gal4-UAS=>EGFP*tub* females were stained and expression patterns were observed for these six species.

***nos*-Gal4=>UAS-EGFP*tub* expression during gametogenesis in Drosophila species.** Ovaries from *nos*-Gal4=>UAS-EGFP*tub* F_1 females were examined using confocal microscopy. In *D. melanogaster*, EGFP can be detected in the germarium; expression is weak in early egg chambers, but becomes more robust at stage 7 (**Fig. 4A and B**). Expression patterns are similar within the melanogaster subgroup, but differ slightly from one another (**Fig. 4A–H**). *D. simulans* EGFP expression was high at stage 7, but was not observed in the germarium (**Fig. 4D**). *D. yakuba* egg chambers expressed EGFP at earlier stages (**Fig. 4F**) and had weak expression in the germarium (not shown). *D. erecta* expressed EGFP at high levels in the germarium and early stage egg chambers, with expression levels dropping before stage 6 (**Fig. 4H**). *D. pseudoobscura* has no detectable expression of EGFPTub in egg chambers (**Fig. 4I and J**). *D. virilis* expresses high levels of EGFP in both the germarium and subsequent egg chambers (**Fig. 4K and L**). In immunostained confocal micrographs of multiple *D. virilis* line combinations, we observed a variety of expression patterns, from the perduring expression in ovarioles described and shown here to more restricted patterns and/or levels of expression (not shown).

Within the melanogaster subgroup, *nos*-Gal4 drives EGFPTub expression in the hub region of the testes, consistent with the expression pattern seen with *D. melanogaster* (**Fig. 5A–H**). *D. yakuba* shows an unexpected discrepancy between fixed tissue staining and live image (**Fig. 5F** with inset), possibly due to the failure of our protocol to permeate the testes sheath during fixation, resulting in weak staining. *D. pseudoobscura* testes

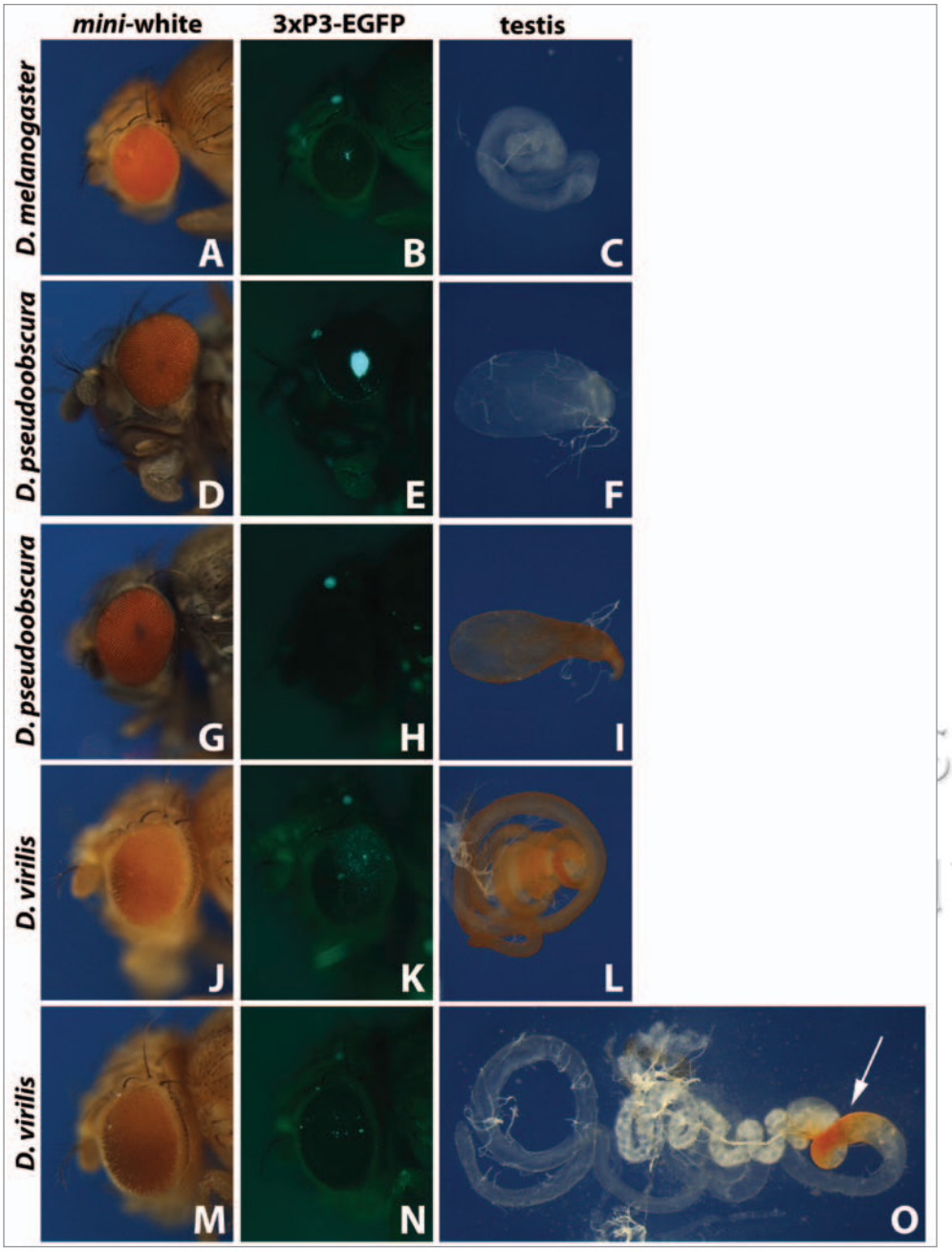


Figure 3. In *D. pseudoobscura* and *D. virilis*, mini-*white* is expressed in the testes sheath in a subset of mini-*white* expressing lines. (A and B) *D. melanogaster* expresses mini-*white* in the eyes in the majority of transgenic lines recovered and always expresses 3xP3-EGFP in the eye. (C) A testis from the same *D. melanogaster* fly line; mini-*white* expression has never been observed in the testes of our piggyBac *D. melanogaster* transgenic lines. (D–I) *D. pseudoobscura* transgenic lines may or may not express mini-*white* in the testis sheath. (F) shows a testis from a line that expresses mini-*white* and 3xP3-EGFP in the eye (D and E); no pigmentation is seen in the testis sheath. Conversely, (I) shows a pigmented testis from another line that expresses mini-*white* and 3xP3-EGFP in the eye (G and H). (J–O) *D. virilis* transgenic lines may or may not express mini-*white* in the testis sheath (lack of testis pigmentation in *D. virilis* mini-*white* expressing lines not shown) and patterned pigmentation is sometimes seen in the testis. (J and K) *D. virilis* line expressing mini-*white* in a graded fashion in the eye; 3xP3-EGFP eye expression is partially masked by the pattern of mini-*white* pigment. (L) Testis sheath pigmentation seen in the same *D. virilis* line. (M and N) *D. virilis* transgenic line expresses mini-*white* in the eye, while also expressing 3xP3-EGFP; (O) mini-*white* from this *D. virilis* line is only expressed in one portion of the testis sheath (arrow).

expression is barely above background (**Fig. 5I and J**). Although *D.* virilis nos-Gal4=>UAS-EGFPtub testes also expressed EGFP in the hub region (**Fig. 6B**), cell masses of unknown origin expressed EGFP throughout the testes (**Fig. 6C and D**). A series of controls were performed to determine whether the masses were associated with the GAL4 cross, antibody staining, or some other factor (see Materials and Methods). Examination of the *white D. virilis* stock shows that these cell masses are present in the working stock (**Fig. 6A**) and are not a result of the GAL4/UAS components or the cross itself, albeit the cross does result in EGFP expression in the cell masses. Recent work exploring histopathology in flies has revealed similar cell masses in the testes of aging *D. melanogaster* males,[59] raising the possibility that this *D. virilis* mutant fly line has some defect or mutation related to aging and/or tumorigenesis. Although the GAL4/UAS system components tested here work well in *D. virilis* (**Fig. 6G**), another *white* line that does not have this defect should be used for all future transgenics in *D. virilis*.

With the exception of *D. pseudoobscura, nos*-Gal4 driven expression of UAS-EGFP*tub* is found in similar patterns to those seen in *D. melanogaster* in the ovaries and testes of the species tested here. We do note that varying levels of expression are observed both among lines recovered and between species; for future work, multiple lines should always be tested to optimize expression levels. Since multiple lines were tested in *D. pseudoobscura* and gave similar low levels of expression, the problem does not appear to be related to position effect for this particular species. This demonstrates that there may be instances where a

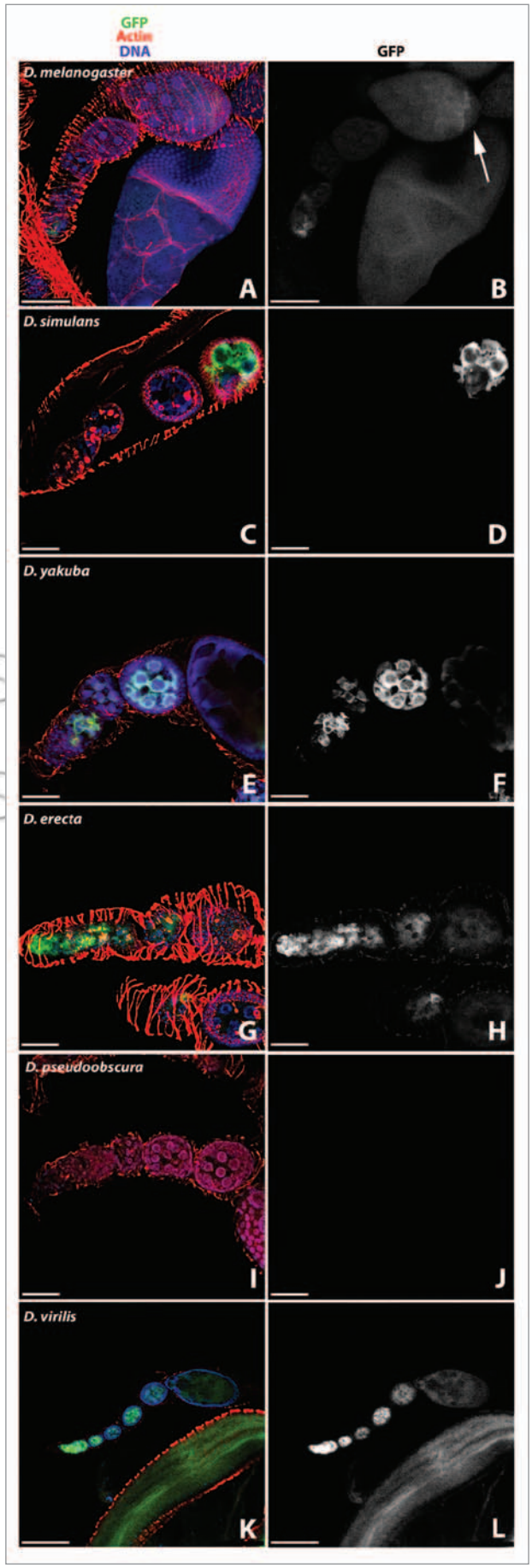


Figure 4. piggyBac *nos*-Gal4 displays unique expression patterns in the ovaries of Drosophila species. (A, C, E, G and I) Confocal images of *nos*-Gal4=>UAS-EGFP*tub* Drosophila species ovarioles labeled for EGFP (green), Actin (rhodamine-phalloidin, red) and DNA (blue). (B, D, F, H, J and L) Corresponding EGFP channel shown in grayscale. (A and B) *D. melanogaster nos*-Gal4 expression can first be detected in the germarium; early egg chambers show low levels of EGFP, but expression levels rise noticeably at stage 7 (arrow). (C and D) *D. simulans* has no detectable levels of EGFP expression from *nos*-Gal4 until stage 7 (arrow). (E and F) *D. yakuba* expresses EGFP weakly in the germarium (not shown), is detectable in early egg chambers, expresses strongly at stage 6 and becomes diluted in the later stage egg chambers. (G and H) *D. erecta* shows a similar pattern of expression, but expresses in the germarium at higher levels than *D. yakuba.* (I and J) *D. pseudoobscura* does not express detectable levels of EGFPTub with this driver in the ovary. (K and L) *D. virilis* germarium and egg chambers express high levels of EGFPTub in this particular *nos*-Gal4=>UAS-EGFP*tub* cross. Scale bar is 50 μm for (A and B); 25 μm for (C–J); 100μm for (K and L).

species-specific promoter/enhancer region must be used to achieve the desired expression pattern and/or levels.

nos-Gal4-UAS-EGFPtub expression patterns in Drosophila species F_2 embryos. In addition to the above analyses we wanted to determine the functionality of the GAL4/UAS system components for investigating maternal loading of the early embryo. When *nos*-Gal4 is crossed to a UAS-EGFP*tub* line, the resulting F_2 embryos should express EGFP-tagged microtubules during syncytial development. Embryos laid by *nos*-Gal4=>UAS-EGFP*tub* F_1 females were examined in the six species for which transgenic lines were available (**Fig. 7**). EGFP staining marks microtubules in all six species, although staining is weak in *D. pseudoobscura* (**Fig. 7E**). The melanogaster subgroup species express levels of EGFPTub in embryos comparable to that seen in *D. melanogaster* (**Fig. 7A–D**). *D. pseudoobscura* embryos do not visibly express EGFP, but EGFP-marked microtubules can be detected in pre-cellularized embryos when immunostained for EGFP (**Fig. 7E**). *D. virilis* embryos express high levels of EGFP, as seen in **Figure 7F**; however, staining reactions using our protocols do not give high quality images. *D. virilis* embryos contain more yolk material than the embryos of other species examined (R. Eisman, personal observations; this work); additionally, there could be an abundance of cytoplasmic EGFP due to the relatively high activity of the *nos*-Gal4 driver in this species.

Materials and Methods

Dual element jumpstarter/mutator piggyBac vector. *pBac{5pBlueEye} = pBac(hsp70-transposase)::(3xP3-ECFP).* This construct has two components: a 3xP3-ECFP marked source of hsp70 driven *piggyBac* transposase and an *hsp27*-EGFP enhancer trapping construct carrying the marker 3xP3-dsRed, which can be used to detect the presence of the insert in the absence of EGFP expression (original construct = pBac•(hsp70-transposase)::(3xP3-ECFP)::pBac•(hsp27-EGFP)::(DsRed-3xP3)pBac) (Kuwayama, Nishikawa, Yaginuma, Niimi; submitted). Note that the entire construct contains three pBac repeats. The two at the outside are in inverted orientation and are utilized if the entire construct is inserted into the genome.

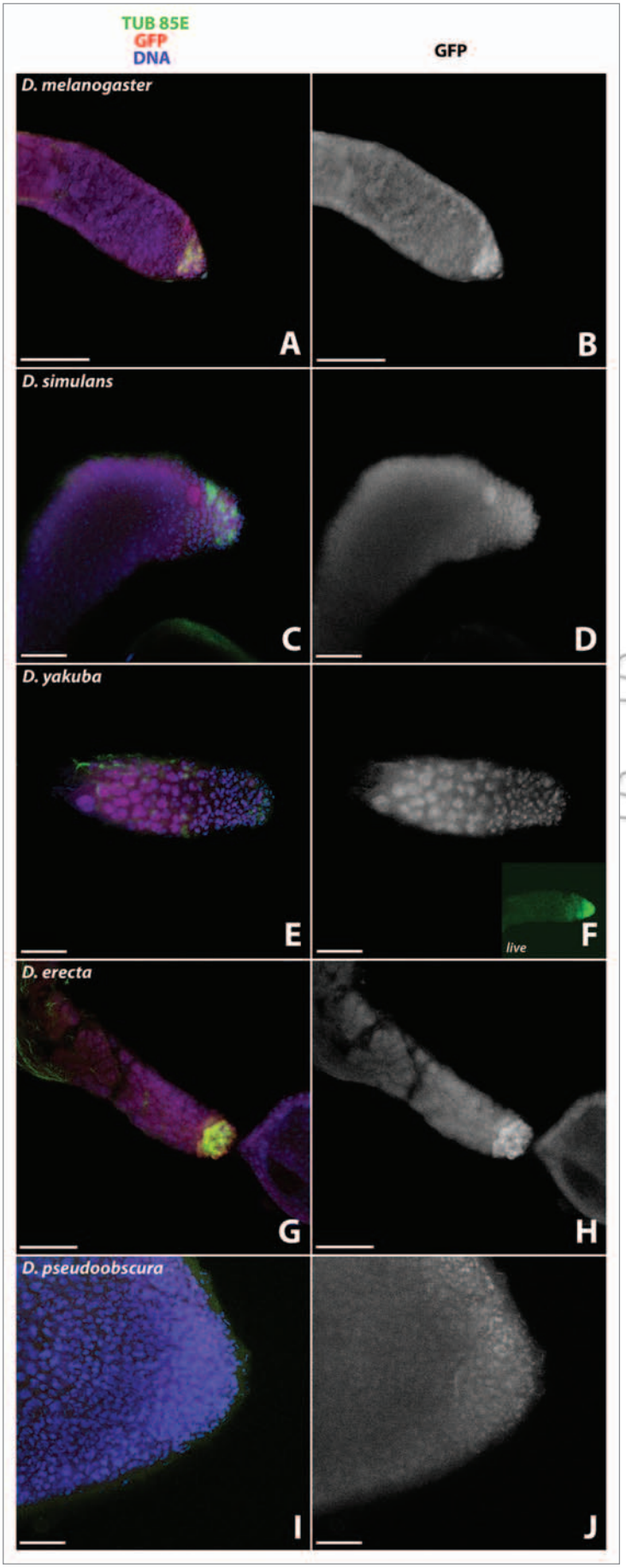


Figure 5. piggyBac *nos*-Gal4 expresses in the hub region of most Drosophila species testes. (A, C, E, G and I) Confocal images of *nos*-Gal4=>UAS-EGFP*tub* Drosophila species testes labeled for α-Tubulin 85E (green), EGFP (red) and DNA (blue). (B, D, F, H and J) Corresponding EGFP channel shown in grayscale. (F) *D. yakuba* includes an inset of live EGFP expression, which staining of *D. yakuba* testes does not accurately depict. Morphology and expression patterns in the melanogaster subgroup are similar; *D. pseudoobscura* testes are morphologically different and express low levels of EGFP in the hub region compared to the melanogaster subgroup and *D. virilis* (Fig. 6). Scale bar is 75 μm for (A and B); 25 μm for (C–F, I and J); 50 μm for (G and H).

The resultant flies have both blue and red fluorescent eyes and carry both halves of the vector. The third pBac repeat resides between the (hsp70-transposase)::(3xP3-ECFP) component and the (hsp27-EGFP)::(DsRed-3xP3) half. It is oriented such that it is inverted relative to the right end repeat but is in the same orientation as the left end repeat. Thus after recovery of animals carrying the entire insert, expression of the transposase can act on the pBac•(hsp27-EGFP)::(DsRed-3xP3)pBac component, excise it and leave behind the pBac•(hsp70-transposase)::(3xP3-ECFP) half. Since this latter portion has only one pBac end it will be stably integrated even in the presence of active transposase and will be detectable by having only blue fluorescence. Transposition of the pBac•(hsp27-EGFP)::(DsRed-3xP3) pBac half will produce flies with only red fluorescence and it was hoped that the *hsp27*-EGFP could be used for enhancer trapping. However, after several screening experiments with the enhancer trapper component it was determined that the *hsp27* promoter was not useful for this purpose (data not shown). The fly lines containing this half of the dual construct were therefore discarded. However, the insertionally stable (single piggyBac end) 3xP3-ECFP piggyBac transposase lines were retained and have been used for the rest of this project; they will be referred to as pBac{5pBlueEye}.

Construction of piggyBac vectors. All of the vectors described below are available from the DGRC (https://dgrc.cgb.indiana.edu/). Maps of the following constructs are provided in the **Supplementary Materials**. Sequences are available on request.

pBac{GreenEye} = pBac(3xP3-EGFPafm)::MCS::(pW8 mini-*white*). This construct provides a transposable insert with both mini-*white* and 3xP3-EGFP eye markers and a polylinker for inserting cloned fragments that contain open reading frames complete with regulatory elements and poly(A$^+$) signal. pBac (3xP3-EGFPafm)[20] was cut with *Not*I and cohesively ligated to the double-stranded linkers 5'-GGC CTG AGC GAT CGC TAA-3' and 5'-GGC CTA AGC GAT CGC TCA-3' that contain cohesive ends to the *Not*I site. This modification eliminated the *Not*I recognition sequence (outside the future polylinker location) while adding an *Sgf*I site to that position (pBacns). The mini-*white* gene was removed from the P{W8} vector[9] as a 4.2 Kb *Spe*I fragment and inserted into the shuttle vector BSTM (unpublished vector; available from the DGRC https://dgrc.

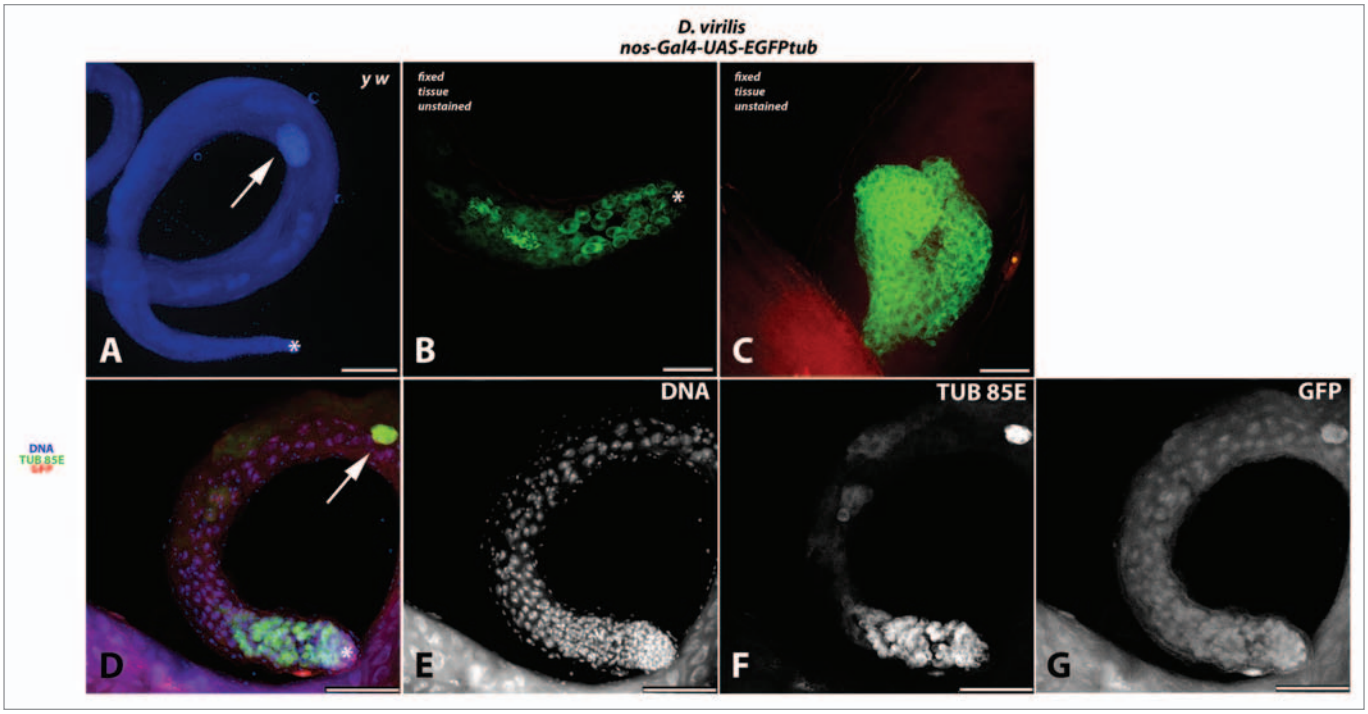


Figure 6. piggyBac *nos*-Gal4 expresses in the hub region of *D. virilis* and is also expressed in cell masses that are unique to the *y w D. virilis* fly line. Hub is marked with an asterisk in (A, B and D). (A) Fixed testis of the *D. virilis y w* line used for transgenesis, stained with DAPI. Arrow is pointing to large cell mass in testis. (B and C) Fixed, unstained testes of *D. virilis nos*-Gal4=>UAS-EGFP*tub*. (B) shows EGFP expression in the hub of the testis. (C) *nos*-Gal4=>UAS-EGFP*tub* expression is also seen in cell masses. Fluorescing cell masses are observed in fixed testis tissue, similar to what is seen when looking at the same genotype live (not shown). (D) Confocal image of *nos*-Gal4=>UAS-EGFP*tub* testis, including hub, labeled for DNA (blue), α-Tubulin 85E (green) and EGFP (red). Arrow points to cell mass in this testis. (E–G) Corresponding channels are shown in grayscale. Scale bar is 75 μm for (A); 25 μm for (B and C); 50 μm for (D–G).

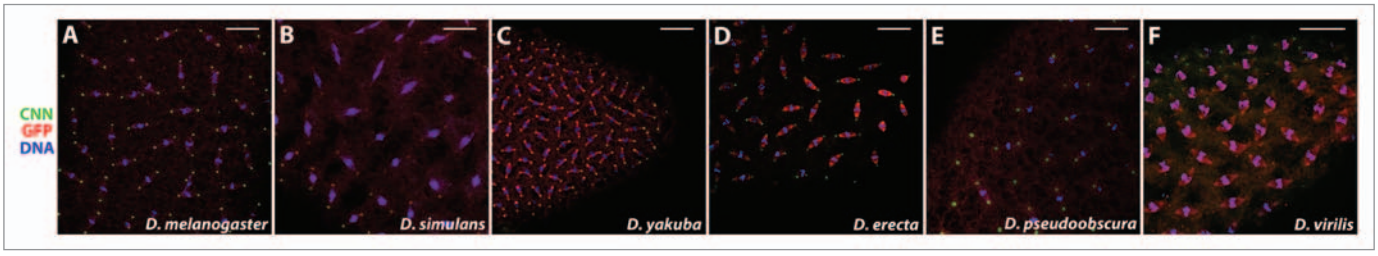


Figure 7. EGFPTub can be detected in F_2 embryos from *nos*-Gal4=>UAS-EGFP*tub* mothers in Drosophila species. Triple stains of pre-cellularized embryos are shown for each species tested. (A) *D. melanogaster* embryos look similar to staining done with P-element constructs (not shown). (B) *D. simulans* embryos contain more cytoplasmic EGFPTub; this could be due to higher expression levels of the *nos*-Gal4 driver in this species. (C) *D. yakuba* also has higher levels of cytoplasmic EGFPTub in embryos, but spindles are well labeled. (D) *D. erecta* expression looks clean compared to other species. (E) *D. pseudoobscura* expression is weak; spindles are scarcely visible above background. (F) *D. virilis* EGFP expression is clearly seen in the spindles, but high yolk content in embryos gives suboptimal staining results with our fixation/staining protocol. Scale bar is 25 μm.

cgb.indiana.edu/) to make MW-BSTM. Mini-*white* was then removed from BSTM with *Fse*I and inserted into the same site in the pBacns vector to generate MW-pBacns. The distal *Fse*I site was removed with an *Fse*I partial digest, T4 DNA polymerase and ligation to generate pBac{GreenEye}.

UASpBacFPN, UASpBacNPF = pBac(3xP3-EGFP-afm)::K10-MCS-UAS::(pW8-mini-*white*). These vectors function as base constructs for the insertion of ORFs that will be expressed when Gal4 is present. FPN and NPF in the construct symbols indicate a multiple cloning site in forward and reverse orientation, respectively. First, a 1.5 kb *Asc*I K10 poly(A^+) fragment was inserted into pBacns (described above) at the *Asc*I site. Then a 4.8 kb *Fse*I UAS-MW module was constructed the following way: a 0.5 kb *Asc*I, *Bam*HI GAGA-UAS fragment was made from pUASP[33] by PCR with the following primers:

5'-GGC GCG CCA CAT ACT AGA ATT GGC CGC TCT AG-3' and 5'-GGA TCC GGC TAT CGA CTC GAC CCT TG-3'.

This GAGA-UAS fragment contains two GAGA sites followed by 14 repeats of the Gal4 UAS binding sequence. A 0.15

Table 1. Summary of culture data for various Drosophila species

Species†	Optimal temperature	Scaffold for pupae	Supplement to bloomington food	Days to egg laying	Generation time (days) at 25°C
D. melanogaster	25°C	no	none	<2	9
D. simulans	25°C	optional	none	<2	8
D. erecta	25°C	yes	none	<2	13
D. yakuba	25°C	yes	none	<2	9
D. sechellia	25°C	optional	*Morinda citrifolia*	<2	9
D. ananassae	25°C	yes	none	2	11
D. willistoni	25°C	yes	Opuntia powder	2	12
D. mojavensis	29°C	optional	Opuntia-banana medium	7–10	11 at 29°C
D. pseudoobscura	20°C	no	Opuntia powder	<2	21 at 20°C
D. virilis	25°C	no, but add rayon if media liquifies	none	5–7	16

†Culture data for these species was based on the *white* stocks used for injections. For stock numbers, refer to Table 2.

kb *Bam*HI, *Asc*I *hsp70* minimal promoter was generated by PCR from the HZW vector[34] with the following primers:

5'-GGA TCC GGC GAA AAG-3' and 5'-GGC GCG CCT TTA AAT CGA TTG TTT AGC TTG TTC-3'. The resulting 0.7 kb *Asc*I fragment was fused to the 4.2 kb *Spe*I MW in BSTM at the *Asc*I site. This 4.8 Kb *Fse*I UAS-MW module was inserted into the *Fse*I site of the K10-pBacns construct to generate the UAS-MW-pBac intermediate. A region of the newly introduced polylinker was deleted by cutting with *Sbf*I and *Pme*I followed by treatment with T4 DNA polymerase and ligation to create UAS-MW-pBacT4. Then the proximal *Fse*I site was removed by an *Fse*I partial digest, T4 DNA polymerase and ligation to generate UAS-MW-pBacNPF. UAS-MW-pBacFPN (reverse poly-linker orientation) was generated by cutting UAS-MW-pBacNPF with *Not*I and *Fse*I and then inserting the cohesive ended linker generated from the following primers:

5'-CGG CCG CTT AAT TAA GGC-3' and 5'-GGC CGC CGG CGA ATT AAT TCC GGC CGG-3'.

pBac{GreenEye.UASdsRed} = pBac(3xP3-EGFPafm)::K10-dsRed-UAS::(pW8-mini-*white*). This construct functions as a UAS-dsRed Gal4 responder. A 0.25 kb *Pst*I AD fragment was generated from an *Antp* cDNA[35,36] by PCR with the following primers:

5'-ATA CTG CAG TTT CAA AAT CAA AAT TG-3' and 5'-ATA CTG CAG CTA AAT ATA CTT TGA ACA C-3'. A 0.8 kb *Apa*I dsRed2 fragment (Clontech) was made by PCR with the following primers:

5'-GGG CCC CGC CAC CAT GGC CTC CTC-3' and 5'-GGG CCC TAC AGG AAC AGG TGG TGG CGG-3'. A 1.1 kb *Pac*I AD-dsRed2 fragment was generated by fusing the 0.25 kb *Pst*I AD ribosomal entry site from *Antp* exon D[37] to the 0.8 kb *Apa*I dsRed2 fragment in BSTM. pBac{GreenEye.UASdsRed} was constructed by inserting the 1.1 kb *Pac*I AD-dsRed orf into the *Pac*I site of UAS-MW-pBac (above).

pBac{GreenEye.UAStubEGFP} = pBac(3xP3-EGFPafm)-::K10-EGFP-Tub-UAS::(pW8-mini-*white*). This construct functions as a UAS-EGFP*tub*-84B Gal4 responder. pBac{GreenEye.UAS*tub*EGFP} was made by inserting the 2.6 kb *Not*I GfpS65C::alphaTub84B fragment[38] into the *Not*I site of UASpBacNPF (above).

pBac{GreenEye.nosGal4} = pBac(3xP3-EGFPafm)::nos-Gal4-::(pW8-mini-*white*). This construct serves as a Gal4 driver in the *nanos* expression pattern.[39] The pBac{GreenEye.*nos*Gal4} construct was made by inserting the 5.1 kb *Not*I *nanos*::Gal4::VP16 fragment[39] into the *Not*I site of MW-pBacns (above).

Drosophila species media and culture. All Drosophila species were grown on standard Bloomington Stock Center media (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm) except for *D. mojavensis*. These were grown on a modified Bloomington media enriched with Opuntia, banana and additional ingredients. Flies did notably better on fresh food (1- to 2-days-old) as opposed to older, drier food. All information on media and culture conditions is summarized in **Table 1**. More details on the culture of each species are included in the **Supplementary Materials**.

Injection of Drosophila species embryos and transformation rates. Initially, injections were performed in all Drosophila species in a manner similar to *D. melanogaster*. Since many species had different culture requirements and embryos were subject to desiccation, different strategies were adopted for some species to improve transformation rates. The specific details for culture and injection-related information can be found in the **Supplementary Materials**. General information on injections, subsequent fly culture and transformation rates follow.

Cages for embryo collections were made with 1- to 2-day-old adults (5- to 10-day-old adults in the case of *D. mojavensis* and *D. virilis* due to their longer maturation times after eclosion). Genesee large and small fly cages (http://www.flystuff.com/general.php) with molasses agar plates coated with yeast paste (except for *D. mojavensis*, which laid well on banana-Opuntia medium soft plates with no yeast paste) were used for embryo collections. Embryos were washed with distilled water, collected onto a cover slip and aligned pair wise with spaces in between the pairs to prevent hypoxia. (Note: yeast paste can form a hard shell on embryos if not cleaned completely and will break injection needles).

Table 2. Summary of transgenic Drosophila species fly lines generated for distribution†

Species	White eyed stock††	FlyBase ID	pBac(3x P3-EGFPafm):: MCS::(pW8 mini-*white*) = pBac{GreenEye}	pBac(hsp70-transposase):: (3x P3-ECFP) = pBac {5pBlueEye}	pBac(3x P3-EGFPafm):: nos-Gal4::(pW8-mini-*white*) = pBac{GreenEye. nosGal4}	pBac(3x P3-EGFPafm):: K10-EGFP-Tub-UAS::(pW8-mini-*white*) = pBac{GreenEye. UAStubEGFP}	pBac(3x P3-EGFPafm):: K10-dsRed-UAS:: (pW8-mini-*white*) = pBac {GreenEye. UASdsRed}
D. melanogaster	w^{1118} (3605) or w^{1} (2390)	FBst0003605 or FBst0002390	√	√	√	√	√
D. simulans	14021-0251.195	FBst0201374	√	√	√	√	
D. erecta	14021-0224.06	FBst0203613	√	√	√	√	
D. yakuba	14021-0261.02	none	√	√	√	√	
D. sechellia	14021-0248.30	none	√				
D. ananassae	k-aa131	none					
D. willistoni	14030-0811.33	FBst0201586	√	√			
D. mojavensis	15081-1352.05	FBst0200740	√	√			
D. pseudoobscura	14011-0121.12	FBst0200044	√	√	√	√	√
D. virilis	15010-1051.45	FBst0200544	√	√	√	√	√

†All transgenic species lines can be obtained from the Species Stock Center at UC San Diego; *D. melanogaster* lines can be obtained from the Bloomington Stock Center. ††*D. melanogaster* lines can be obtained from the Bloomington Stock Center; all other species except for *D. ananassae* can be obtained from the Species Stock Center at UC San Diego.

After embryos were aligned and air-dried until adhered to the cover slip, they were cleared with 400-weight halocarbon oil and injected. All Drosophila species were injected as described previously.[40] To prevent mold, bacterial infections and dehydration, cover slips were embedded in fresh bottles (for *D. mojavensis*, banana-Opuntia food was used) with damp Kim-wipes® so that larvae could develop in an ideal environment with minimal handling. G_0 adults from each construct injected were then mated together and F_1 progeny were screened for the appropriate transformation marker. DNA was prepared using Qiagen kits; the ratio of construct to helper was 600 μg:400 μg/ml for most injections performed.[41]

For many of these species, this is the first reported attempt at germ line transformation. For most constructs, transformation rates were low; attempts at improving animal husbandry, obtaining timely embryo collections and increasing post-injection survival rates all occurred while doing injections. The most successful culture conditions are described in this work; however, there is room for improvement for each sequenced genome species. **Table 3** gives examples of typical transformation rates for each species and also gives a breakdown of mini-*white* expressing/white-eyed ratio. Since sib-matings were performed with G_0 adults and their progeny screened for transformants, differences in eye color or pattern were used to identify unique insertions. Thus some recovered transformants could be duplicate insertions further reducing our estimated rates of transformation. Due to the small numbers of transformants obtained in most experiments, the purpose of these data is to demonstrate proof of principle only.

Fly strains used. Two *white* mutants were used for *D. melanogaster* injections: w^{1118} (BSC 3605) and w^{1} (BSC 2390). White-eyed strains of *D. simulans*, *D. sechellia*, *D. willistoni*, *D. mojavensis*, *D. pseudoobscura* and *D. virilis* were obtained from the Species Stock Center (https://stockcenter.ucsd.edu/info/welcome.php). A *white* stock of *D. ananassae* came from the Kyorin-fly Stock Center (http://kyotofly.kit.jp/cgi-bin/kyorin/index.cgi). *D. erecta* and *D. yakuba white* mutants were generated in this work (**Table 2**).

Mutagenesis of *D. erecta* and *D. yakuba*. *D. erecta* and *D. yakuba white* mutants were obtained by performing EMS mutagenesis as described.[42] For both species, 400 mutagenized males were crossed to 800 virgin females in 40 bottles and subcultured daily for five days. Progeny were then mated together and their offspring, in turn, were screened for white-eyed males. The first screen yielded two *D. erecta* white-eyed fly stocks. A total of three mutagenesis screens were performed for *D. yakuba* before two *white* strains were obtained in the last screen, which was quadrupled in number (1,600 mutagenized males were crossed to 3,200 females in 160 bottles). The mutants generated from these screens were donated to the Drosophila Species Stock Center (https://stockcenter.ucsd.edu/info/welcome.php).

Table 3. Representative transformation rates and mini-*white* expression in Drosophila species

Species or stock	Construct injected	G_0 adult survivors	No. of transformants (GFP$^+$)	Mini-*white*$^+$	White eyes
D. melanogaster	Pbac{GreenEye.nosGal4}	188	11	9	2
D. simulans	Pbac{GreenEye}	144	11	11	0
D. erecta	Pbac{GreenEye}	31	11	8	3
D. yakuba	Pbac{GreenEye}	99	12	7	5
D. sechellia	Pbac{GreenEye}	68	3	3	0
D. ananassae	Pbac{GreenEye}	78	3	1	2
D. willistoni	Pbac{GreenEye}	36	0	0	0
D. willistoni Pbac{BlueEye}	Pbac{GreenEye}	30	11	8	3
D. mojavensis	Pbac{GreenEye}	74	13	2	11
D. pseudoobscura	Pbac{GreenEye.UASdsRed}	60	3	3	0
D. virilis	Pbac{GreenEye.nosGal4}	107	12	9	3

GAL4/UAS system in Drosophila species. All GAL4/UAS experiments were performed at 25°C. For the six species in which the GAL4/UAS system was tested, up to four different lines or combinations of lines were crossed for driver and responder when possible. For some constructs, only one or two transformants were recovered; in addition, some lines were homozygous lethal or sterile and were not tested.

Location of all generated fly stocks and their nomenclature. *D. melanogaster* stocks generated in this project are available from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/). All other Drosophila species stocks can be found at the Drosophila Species Stock Center (https://stockcenter.ucsd.edu/info/welcome.php). Only a subset of homozygous viable lines were kept for any given construct and all homozygous lethal or sterile lines were discarded. A summary of generated lines is listed in **Table 2**. Few transgenic lines were generated in *D. ananassae*; none were homozygous viable and therefore were not submitted to the Species Stock Center and were discarded.

Since many species carry the same construct, a simplified nomenclature was adopted. The name of each construct is listed in bold in the top row in **Table 2**. When lines were recovered, they were given this common name, followed by a species abbreviation and a line number. For example, the first *D. simulans* transgenic line carrying pBac{GreenEye} was named "pBac{GreenEye} Dsim1."

Tissue fixation and immunohistochemistry. Cages for collecting embryos from *nos*-Gal4=>UAS-EGFP*tub* females were constructed as above. Two-hour (four-hour for *D. virilis*) embryo collections were dechorionated and fixed as previously described[43] with the exception that embryos were fixed for 10 minutes instead of 12 minutes. Fixed embryos were stored at -20°C in MeOH and were stained within two weeks after fixation.

Ovaries were fixed as described in Protocol 4.2, page 75: "Hand Dissection of Egg Chambers" of Drosophila Protocols.[44] Staining was performed immediately after fixation.

Testes were fixed as follows: testes were dissected in Ringer's solution and kept there for no longer than 30 minutes. Ringer's solution was then removed and testes were fixed in 280 μl PBS/120 μl 10% paraformaldehyde/500 μl heptane with violent shaking for ten minutes. Fix was removed and testes were washed in increasing concentrations of MeOH (25%, 50%, 75% and 100%). After removing the 100% MeOH wash, testes were stored in 97% MeOH/15 mM EGTA at -20°C. All staining reactions were performed within two weeks of fixation. Testes that were labeled with rhodamine-phalloidin were not taken through the MeOH washes; after fixation, testes were washed three times with PBS, then twice for ten minutes with PBTB and stained as usual. Autofluorescing *D. virilis nos*-Gal4=>UAS-EGFP*tub* testes cell masses were observed after rehydrating testes of this genotype that had been fixed with the MeOH series and stored at -20°C for up to three weeks.

Immunostaining for all tissues was performed as described[45] with the exception that primary incubation was carried out in 30 μl normal donkey serum and 470 μl PBTB. All tissues were mounted in 90% glycerol with 0.2 mM n-Propyl-Gallate/10% PBS.

For confocal microscopy, TOTO3 (Molecular Probes) was used at 1:500 to observe DNA. Monoclonal EGFP antibody (Santa Cruz Biotechnology, Inc.: sc-9996), was used to detect expression of EGFPTub at 1:50. Guinea pig Cnn antibody[46] was used at 1:500. Rhodamine-phalloidin (Molecular Probes) was utilized at 1:100 to label Actin in ovaries and testes. Tubulin antibodies used were E7 ascites (DSHB, Iowa) and rabbit polyclonal Tub85E[47] at dilutions of 1:1,000 and 1:100, respectively. 5% Dimethylsulfoxide was added to testes during primary incubation. In order to identify cell masses in *y w D. virilis* testes, DNA staining with DAPI (Sigma-Aldrich) was performed on fixed testes at a 1:1,000 dilution.

Microscopy and imaging. Mini-*white*, EGFP and CFP expression in fly eyes was photographed using a Leica MZ16 motorized dissecting scope with attached Qimaging digital camera and Image-Pro Plus software. Live ovaries, testes and embryos, as well as DAPI stained testes, were observed and photographed on a Zeiss Axiophot with a Nikon DXM1200 digital camera and ACT-1 software. Screening for mini-*white* negative/EGFP expressing transformant flies, CFP expressing flies and imaging embryos and pupae utilized a Nikon SMZ1500 dissecting scope with GFP, RFP and CFP filters. This scope also has a Nikon DXM1200 digital camera and ACT-1 software. Images of immunostained embryos, ovaries and testes were obtained using a Leica TCS confocal microscope.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/HoltzmanFLY4-4-Sup.pdf

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