Live Analysis of Position Effect Variegation Reveals Different Modes of Action for *HP1* and *Su(var)3-9*

by

Farah J. Bughio and Keith A. Maggert

Department of Cellular and Molecular Medicine, University of Arizona, Tucson, AZ 85724

To whom correspondence may be addressed. Email: kamaggert@email.arizona.edu

ABSTRACT

Position Effect Variegation (PEV) results from the juxtaposition of euchromatic and heterochromatic components of eukaryotic genomes, silencing genes near the new euchromatin/heterochromatin junctions. The degree of silencing is itself heritable through S-phase, giving rise to distinctive random patterns of cell clones expressing the genes intermixed with clones in which the genes are silenced. Much of what we know about epigenetic inheritance in the soma stems from work on PEV aimed at identifying the components of the silencing machinery and its mechanism of inheritance. Despite identifying two central gene activities – the *Su*(*var*)*3-9* histone H3-Lysine-9 methyltransferase and the *Su*(*var*)*205*/*HP1* methyl-H3-Lysine-9 binding protein – their role in PEV has been inferred from terminal phenotypes, leaving considerable gaps in understanding how PEV behaves through development. Here, we investigate the phenotypes of *Su*(*var*)*3-9* and *Su*(*var*)*205/HP1* mutations in live developing tissues. We discovered that mutations in *Su*(*var*)*205*/*HP1* compromise the initial establishment of PEV in early embryogenesis. Later gains of heterochromatin-induced gene silencing are possible, but are unstable and lost rapidly. In contrast, mutations in *Su*(*var*)*3-9* exhibit robust silencing early in development, but fail to maintain it through subsequent cell divisions. Our analyses show that while the terminal phenotypes of these mutations may appear identical, they have arrived at them through different developmental trajectories. We discuss how our findings further challenge existing models for epigenetic inheritance of heterochromatin-induced gene silencing.

INTRODUCTION

Position Effect Variegation (PEV) was first observed in *Drosophila* as random "ever-sporting" patterns of *white*$^{+}$ gene expression in individual ommatidia of the compound eye (1). Genes undergoing PEV did so because genome rearrangements (*e.g.*, chromosome inversions, transpositions) created new heterochromatin-euchromatin breakpoints (2). Rearrangements brought normally-euchromatic genes into juxtaposition with heterochromatin and normally heterochromatic-genes with euchromatin (3-5). In both cases, newly juxtaposed genes were repressed in some cells but in others they were not, resulting in random and compelling patterns of expression in otherwise genetically-identical cells.

The current view of PEV derives mostly from work on heterochromatin-induced gene silencing as euchromatic reporter genes are placed near heterochromatin (6). In these cases, it is envisioned that heterochromatin proximity induces silencing on the reporter gene when heterochromatin forces the acquisition of methylation of Lysine-9 of Histone H3 (H3K9) near the breakpoint, including in those nucleosomes that package the reporter gene. A necessary component of this view is that heterochromatin-induced gene silencing "spreads" from the heterochromatin to juxtaposed DNA, regardless of its sequence, bringing heterochromatin's intrinsic repression to closely-linked genes. Close analysis of H3K9 methylation status on genes linked to breakpoints indicate an increase in H3K9 methylation on them, however the "spreading" appears discontinuous, focused at gene promoters (7-8). The manifest silencing

itself is also discontinuous, occasionally "skipping over" one or more genes (9). These studies indicate that the phenomenon of PEV is best envisioned as multiple separable phenotypes: the mechanism of silencing brought about by heterochromatin, the degree to which "spreading" may occur, and whether/how a gene can escape silencing.

Because the patterns of gene silencing in each cell are inherited through cell division – leading to the classical clonal patterns of expression in PEV – it is believed that PEV is necessarily epigenetic. In fact, spreading itself demands it to be so: it is not just the silencing that is inherited through S-phase, but specifically the extent of spreading from the heterochromatin source. In two different cell lineages, heterochromatin may repress only closely-linked genes, or it may repress those genes hundreds of kilobases away, and this information indicating how far spreading may proceed is the key epigenetic component of PEV.

Genetic screens for suppressors of PEV (*i.e.*, second-site mutations that alleviate the silencing of heterochromatin, returning a variegating allele to a more *wild-type* expression state), uncovered many major protein components of heterochromatin (10). The gene product of the *Drosophila Su*(var)*3-9* locus is a Histone Methyltransferase capable of methylating Histone H3K9. The H3K9 methylation recruits Heterochromatin Protein 1 (HP1), the gene product of the *Su*(*var*)*205* locus. It is HP1 that effects gene silencing. Mutations in either *Su*(*var*) gene derepresses genes being silenced by heterochromatin, and in fact these are the two strongest genic modifiers of PEV yet described, restoring expression of most alleles to *wild-type*.

The accepted model for PEV and the roles of *Su*(*var*)*3-9* and *Su*(*var*)*205* are based on dominant loss of function phenotypes in terminally-differentiated tissues, most always the expression of the *white*$^{+}$ gene in adult ommatidial pigment cells or the expression of the *yellow*$^{+}$ gene in adult bristles or abdominal cuticles. However, there are many trajectories that silencing can take before arriving at an endpoint in the eyes or cuticles of adult organisms. We were inspired by Dr. Janice Spofford's still-superb and still-prescient review of PEV from 1976 (2), "*In flies, the mosaic phenotypes* [PEV] *are not expressed until the final cell divisions; descendants of known single cells have not been sampled sequentially during the history of a cell lineage, and the degree of reversibility of the inactive state and the consequent identification of the time of inactivation remain moot*" [we certainly hope that Dr. Spofford used the word "moot" in 1976 in it's now less-common meaning of "*subject to debate, dispute, or uncertainty*" (11)]. Her point is excellent as the times at which silencing is set, when it's lost, and when *Su*(*var*)*205* and *Su*(*var*)*3-9* act on PEV are still not clear.

We recently developed the Switch Monitoring (SwiM) System to monitor gains and losses of heterochromatin-induced gene silencing in a living PEV model (12). We embedded a ubiquitously-expressed gene encoding *gal80*, the yeast *gal4*-specific transcriptional repressor, in heterochromatin causing it to undergo PEV. Simultaneous use of a ubiquitously-expressed yeast *gal4* transactivator allowed us to monitor PEV by assaying *gal4* activity (*i.e.*, *Gal4* in the absence of *Gal80*). We employed the dual fluorescent reporter *G-TRACE* lineage tracing system (13) to identify those cells in which *gal80* was repressed, expressed, or those in which *gal80* had undergone rounds of gains or losses ("switches") of heterochromatin-induced gene

silencing. The SwiM System proved to be remarkably rich, as we could infer the histories of switching in individual cell clones/lineages by analyzing the presence/absence and intensities of GFP and RFP fluorescence from the *G-TRACE* component, the size of like-expressing clones, the patterns within clones, and the proximity of clones to those of other expression patterns (12). Using SwiM we previously concluded that PEV is highly dynamic through development, specifically that Spofford's "*reversibility of the inactive state*" was very high, arguing against the stable maintenance of heterochromatin-induced gene silencing through epigenetic mechanisms. Rather, as Spofford predicted, the final "mosaic" phenotypes of PEV are not expressed until the final cell divisions although we could enumerate rich and varied trajectories during their development.

Since the accepted model of PEV is based largely on the phenotypes and biochemical activities of *Su*(*var*)*3-9* and *HP1*, namely their necessity as mediators of epigenetic information through S-phase, it became important to analyze mutations using the SwiM System. In this work, we do so. We extend our previous work on PEV by showing that the classically-used mutations in the *Su*(*var*)*3-9* histone methyltransferase and *Su*(*var*)*205* methyl-histone binding protein exert their effects on PEV at distinct times in development. Heterozygous *Su*(*var*)*205*^{5} mutants exhibit an almost complete loss of silencing in early embryogenesis, and the lack of silencing persists until adulthood. Individual cells rarely acquire silencing, and those that do lose it again probably within the same cell cycle. We could only detect these rare events indirectly, in contrast to *wild-type* organisms where gains of silencing are relatively common. In contrast to mutants of *Su(var)205*, heterozygous*Su*(*var*)*3-9*^{1} mutants are able to well-establish

silencing early in development, but we observed a progressive loss of silencing through development. Specifically, even at late stages, mutants in *Su*(*var*)*3-9* are better able to establish silencing, but it is rapidly lost. Analysis of both mutant conditions further supports our developing ideas that PEV is not an epigenetic phenomenon, but is instead mediated by non-chromosome-bound factors that differ between cells and cell clones.

Results and Discussion

The expressivity of PEV is a wide spectrum

The *gal80* component of the SwiM System contains a *white*$^{+}$ transgene that can be scored in adult eyes, which allowed us to easily estimate the variation of silencing occurring at the heterochromatic *gal80*/*white*$^{+}$ locus within the population of isogenic organisms. We measured the amount of pigmentation in 104 *w*/*Y*; *P*{*white*$^{+mC}$=*tubP-GAL80*^{ts}}$^{10\text{-}PEV\text{-}80.4}$/+ adult eyes, dependently scoring both left and right eyes, and dividing them into categories of pigment (Figure 2). We observed that about one-third of the flies had near-absent *white*$^{+}$ pigmentation, indicating robust heterochromatin-dependent gene silencing (Figure 2A, D). The remainder had intermediate levels of silencing and expressed classical patterns of *white*$^{+}$ PEV (Figure 2B), and few flies (~5%) had a high level of *white*$^{+}$ pigmentation (Figure 2C), indicating organisms with weak heterochromatin-induced gene silencing at the *white*$^{+}$/*gal80* locus.

The high variability in studies of PEV makes many assessments fraught: proportions of cells experiencing silencing versus expression vary too much between individuals to produce robust or meaningful statistical descriptions, and population measurements (*e.g.*, pigment extractions) lose meaningful information (*e.g.*, the patterns of expression). One of the pronounced benefits of the SwiM System is the ability to infer individual trajectories of silencing through development, and thereby more-completely understand the breadth of histories of heterochromatin-induced gene silencing.

In contrast to the pronounced variability between individuals, the variability in silencing within individuals was minimal (Figure 2E; examples of high, medium and low silencing are shown in Figures 2F-H', respectively). We individually dissected eye/antennal and wing imaginal discs and compared the degree of silencing and expression as revealed by the SwiM System. We found a strong correlation between degree of expression or silencing in all four discs of individuals. This validates the use of SwiM to understand silencing through development, and extends our analysis of eye discs in our previous work to wing discs in this work. By shifting to SwiM System analysis of wing discs we abrogate concerns about the influence of the eye-specific enhancer/promoter combination of the *mini-white* gene in the *P{white*$^{+mC}$*=tubP-GAL80*^{ts}*}*$^{10\text{-}PEV\text{-}80.4}$ component of the SwiM System.

Switch Monitoring System analysis of Position Effect Variegation in wild-type organisms

We analyzed the *wild-type* patterns of heterochromatin-induced gene silencing in multiple wing imaginal discs dissected from *P{white*$^{+mC}$*=tubP-GAL80*^{ts}*}*$^{10\text{-}PEV\text{-}80.4}$*/+; P{white*$^{+mC}$*=Act5C-GAL4}17bFO1/G-TRACE* animals. The patterns provided us a baseline to understand how establishment and maintenance of silencing manifest in comparison to mutations in *Su*(*var*)*205* and *Su*(*var*)*3-9* animals, which are expected to alter these characteristics.

In organisms *wild-type* for components of heterochromatin, expression of RFP and GFP in fluorescent clones fell broadly into three categories (Figure 3A-B). First, clones expressing

bright RFP and GFP fluorescence indicated cells in which silencing of *gal80* was ongoing (Figure 3C-D). In these cells, the absence of *gal80* due to silencing allowed the *gal4*-dependent RFP to express, and the *gal4*-dependent *FLP* rearrangement to permanently activate the GFP transgene. The relative brightness of RFP and GFP indicated that the silencing of *gal80* had been ongoing long enough to allow both RFP and GFP accumulation, corresponding to Figure 1B, between developmental time points 4 and 5, "RG" type. It is possible that some of these cells had very recently lost heterochromatin-induced gene silencing at the *gal80* locus because the perdurance of RFP is on the order of 6-8 hours, which defines the temporal limits of our Switch Monitoring analyses (12).

Second, there were many clones of cells which express GFP and some low level of RFP (Figure 3E-F). We interpret these to be cells which had sufficient heterochromatin-induced gene silencing of *gal80*, followed afterward by a loss of that silencing. This first allowed *gal4*-dependent activation of RFP and GFP, but the following silencing of *gal80* caused the loss of RFP expression (Figure 1B, developmental time point 5, "rG" type). In these cells, the loss of silencing must have happened recently relative to observation, since the diminishing RFP had not entirely degraded at the time of observation (*i.e.*, those cells are between Figure1B, developmental time points 5 and 6).

Third, we observed some contiguous clones of cells expressing GFP but apparently no RFP (Figure 3G-H). These were cells in which silencing had been established, as in the second category, but then lost and enough time allowed to pass to allow full derepression of *gal80*, *gal80*'s subsequent repression of *gal4*, and decay of *gal4*-dependent RFP mRNA and protein

(Figure 1B, developmental time point 6, "G" type). We find support for this interpretation because over-manipulation of bright/contrast often revealed a very small amount of RFP in some of these cells (Figure 3I-J).

Apart from these three broad categories of clones, there were cells that appeared to be clonally-related that were intermixes of the first and second fluorescent phenotypes (Figure 3K-L). The lineage-tracing properties of the SwiM System indicate a possible life-history for the cells in these clones. We interpret that a single ancestor cell, or multiple cells in the same clone, established silencing of *gal80* (causing them to express RFP and GFP), then silencing was lost in a subset of cells (switching those to GFP alone) as the clone continued to expand. We note that the intensity of GFP in these were more-or-less equal, further supporting our interpretation that the initial establishment of silencing was a single or synchronous event much earlier in the lineage.

Some cells expressed RFP alone, and often these cells were near each other (Figure 3M-N), as we have commented on previously. These are individual cells in which silencing had not been established in early embryogenesis or the first two larval instars, but had become established within the last few hours prior to observation (Figure 1B, developmental time point 2, "r" cells). Then, as now, we interpret these cells to be evidence of gains of heterochromatin-induced gene silencing. Moreover, that these cells are individual suggests a non-S-phase-dependent switch in silencing state, and their proximity to like-acting cells suggests a non-heritable mechanism for heterochromatin-induced silencing within common clones. Related to this ongoing instability, those clones that are GFP, or occasionally lone GFP-expressing cells

within non-fluorescent clones (Figure 3O-P), indicate that individual cells may gain and subsequently lose silencing while their cousin cells remain un-silenced.

Finally, non-fluorescent clones or cells can only come from complete and ongoing failure to silence *gal80* because, if *gal80* were to be repressed at any time in development, the cell and its descendent should be permanently labelled by GFP expression. This category of cells – present but not common in *wild-type* – is the one we expect to find more frequently in conditions compromising the establishment of early heterochromatin-induced gene silencing.

Su(var)205 **mutation, reducing HP1, impacts early establishment and later maintenance**

To understand the developmental impact of reduced HP1 function on PEV, we created flies heterozygous for the amorphic *Su(var)205*^{5} allele in conjunction with the Switch Monitoring System. We dissected and analyzed *P{white*$^{+mC}$*=tubP-GAL80*^{ts}*}*$^{10\text{-}PEV\text{-}80.4}$*/Su(var)205*^{5}; *P{white*$^{+mC}$*=Act5C-GAL4}17bFO1/G-TRACE* 3rd instar larval imaginal discs.

For the most part, we saw a dramatic expansion of non-fluorescent clones relative to *wild-type*, covering the majority of every disc that we analyzed (Figures 4A-B). These cell clones had persistent *gal80*-mediated repression of the *gal4*-controlled components of the *G-TRACE* lineage marker, indicating that heterochromatin-induced gene silencing of *gal80* had never been experienced in most cells of the organism. This includes the two particularly critical phases of heterochromatin function, one acting early (peri-fertilization, identified by us (12)) and one later (peri-gastrulation, identified by Eissenberg (14)). Both of these phases were strongly

compromised in *Su*(*var*)*205* heterozygotes, but so was silencing outside of these phases, indicating that *Su*(*var*)*205* mutation has effects throughout development and not just at specific stages of tissue determination and differentiation. Our findings here are therefore consistent with others', identifying HP1 as a dose-dependent structural component of heterochromatin function, but our results also assertively establish that HP1 has a continuous requirement.

Despite the reduction of HP1 and the dramatic decrease in most cells' ability to establish repression, some cells were capable of both establishing and maintaining silencing. These GFP and RFP expressing cells were much-less numerous in *Su*(*var*)*205* heterozygotes, but were nonetheless consistently observable in analyzed discs (Figure 4C-D). As in *wild-type* discs, the cells capable of continued silencing were generally found near each other in clones. Also as in *wild-type*, these robustly RFP-expressing cells were intermixed with GFP-expressing cells that had no or low levels of RFP. We interpret this to indicate that some cells within these clones gained silencing late in development, during or after clonal expansion, but then later lost that silencing.

In contrast to *wild-type* however, different clones in *Su*(*var*)*205* mutants differed in the intensities of RFP and GFP expression from each other, which we believe to mean they had established silencing at different points in development. Whereas in *wild-type*, such clones are uniform in their GFP expression, they are not in *Su*(*var*)*205* mutants. This indicates that as these clones develop, they undergo much more frequent switching, gaining and losing silencing repeatedly.

The propensity to lose any acquired silencing was also evident by the many large clones of GFP expression, some uniformly brighter and some uniformly dimmer, with no evident RFP-expression (Figure 4E-F and 4G-H). The former we believe to be cells descended from ancestors that experienced one or few early gains of silencing followed promptly by complete loss of silencing, permanently tagging all descendent cells with GFP despite the absence of any perduring RFP. The latter we believe to have had a similar history, although having happened more recently, after the clone had expanded. Consistent with this, we could find individual cells within the latter type of clone that were both RFP-expressing and brighter-GFP expressing, although we cannot rule out that those cells have undergone a now second round of silencing. The presence of GFP expressing cells with no detectable RFP further shows that silencing may be established in *Su*(*var*)*205* heterozygotes, but with an increased likelihood of losing that silencing. This is generally evident in comparison to discs from wild-type organisms (Figure 3) which had more-uniformly bright GFP.

From these behaviors, we can predict that gains of silencing should be rare or nonexistent. As we showed in previous work, recent gains are easily detected as RFP expression without any detectable GFP expression (Figure 1B, r phase). We could not identify any cells within otherwise non-fluorescent clones that had detectable RFP that were not also brightly-expressing for GFP. We did, however, note some isolated GFP cells within non-fluorescent clones (Figure 4I-J). We interpret these as cells that were silenced for a short enough time so that RFP could not rise to a detectable level, but nonetheless expressed

sufficient FLP to catalyze the permanent activation of ubiquitous GFP expression. These lone GFP cells, as far as we can ascertain, are unique to this genotype.

By every cell type we could analyze, we believe that *Su*(*var*)*205* mutation acts by rendering cells less capable of establishing silencing *and* less capable of maintaining it. These defects in establishing silencing – non-fluorescent cells from ongoing defects starting in early embryogenesis, and green fluorescent cells from defects any time henceforth – are responsible for the well-characterized near-complete suppression of PEV in adult tissues. The SwiM System, however, allows us to clearly delineate very different life histories of these cells on their way to an ultimately common phenotype.

Within the cells that could, at least sporadically, establish silencing, there were clear differences in the variation of intensities for RFP and GFP when comparing HP1 mutant and *wild-type* clones. The former had higher variation despite the clone sizes being smaller. This condition is predicted if in *Su*(*var*)*205* mutants cells are more likely to switch from silenced to non-silenced *and* from non-silenced to silenced more frequently. We proposed in our previous work that such rapid switches might underlie the overall suppression by mutation in *Su*(*var*)*205*. We also believe that rapid switches are responsible for the types of patterns of adult PEV – patches versus salt-and-pepper. Large clonal patches of expression would be the result of lower instability (lower switch rates leading to larger clones of consistently-acting cells), while the smaller salt-and-pepper spots of PEV would be the result of higher instability (higher switch rates leading to smaller clones with higher variability between cells) (12).

***Su*(*var*)*3-9* mutation, reducing a key heterochromatic HMT, preferentially affects maintenance of PEV**

Heterozygous loss-of-function mutants of the *Su*(*var*)*3-9* gene, which encodes the H3K9 histone methyltransferase (HMT), largely do not exhibit a loss of the initial establishment of heterochromatin-induced gene. This is evident from the overall expression of GFP (Figure 5A-B) in discs taken from *P{white*$^{+mC}$*=tubP-GAL80*^{ts}*}*$^{10\text{-}PEV\text{-}80.4}$*/G-TRACE*; *P{white*$^{+mC}$*=Act5C-GAL4}17bFO1/Su*(*var*)*3-9*^{1} individuals.

Although the discs resembled *wild-type* in the number of GFP-expressing cells and clones, the variation of GFP expression was higher than we saw in *wild-type*. For example, we observed clones of low GFP expression within encircling clones that had a relatively higher amount of GFP fluorescence (Figure 5C-D). This condition is likely due to a more-recent gain of silencing than in the outlying clones of cells. Moreover, the GFP expression in all GFP-expressing clones of *Su*(*var*)*3-9* individuals were more heterogeneous in their fluorescence than in *wild-type*. Some individual cells had undetectable GFP, while those cells which did express GFP, even within the same clone, varied from moderate to bright intensities of GFP fluorescence (Figure 5E-F). This variation was true whether the expression of GFP in a clone was high or low on average. The only way we can envision such a situation is as above in *Su*(*var*)*205*/+ mutants, as extremely-brief periods of silencing producing a dip in *gal80*, and activation of RFP and *FLP* for a short window of time, insufficient to accumulate RFP but sufficient to permanently activate GFP. We therefore interpret the cell-by-cell variation in these

patches as extremely short-lived silencing of the *gal80* followed by infrequent genome rearrangement by the few *FLP* molecules in cells of those clones.

Non-fluorescent cells were more plentiful than in *wild-type*, although not to the same degree as *Su*(*var*)*205* mutants. Non-fluorescent clones arise from cells in which *gal80* silencing is never established at any time in development. Their existence indicates failure to establish silencing in early embryogenesis, but also indicates a persistent inability to ever establish silencing even later. Non-fluorescent cells in *Su*(*var*)*3-9* mutants are less numerous than those in *Su*(*var*)*205* mutants indicating that reduction of HMT has a less severe phenotype than reduction of HP1. In SwiM, the combination of GFP- and non-fluorescing cells are expected to represent those cells of adults in which the variegating gene would be expressed. It is clear to see the ultimately identical suppression-of-variegation phenotypes for *Su*(*var*)*205* and *Su*(*var*)*3-9* mutants even at these earlier stages, and also that they reach those terminal phenotypes through different trajectories.

Related to the observation above, currently-silenced (GFP and RFP-expressing) cells were less numerous than in wild-type, but more prevalent than in *Su*(*var*)*205*/+. This is again consistent with the instability that comes from reducing HMT function. Further, those clones of cells that did express RFP exhibited stochasticity and high variation in RFP amount (Figure 4C-D). On the whole, cell-by-cell variable RFP expression, clone-by-clone variable GFP expression, and increased frequency of non-expressing clones that contain individual GFP-expressing cells all support the same interpretation: heterozygous *Su*(*var*)*3-9* mutants have a decrease in the ability to establish silencing and/or an increased likelihood to lose it should it be established.

In contrast, however, to *Su*(*var*)*205* mutants, *Su*(*var*)*3-9* mutants appear well-capable of establishing silencing, but by the time of observation most cell clones have again lost it. Although both *Su*(*var*)*205* and Su(var)3-9 produce equally derepressed phenotypes as adults, they seem to arrive at that terminal phenotype in different ways. *Su*(*var*)*205* mutants fail to ever establish silencing in most cells. Those few cells in which silencing is established is lost through development, and establishment or reacquisition of silencing is rare or non-existent. *Su*(*var*)*3-9* mutants do establish silencing quite well, however they are unable to retain it. Further, once lost, silencing is rarely, if ever, re-established.

The presence of any RFP cells in either mutant condition was itself a surprise given the terminal and well-characterized phenotypes in the eyes of adult PEV-expressing flies (*e.g.*, *white*$^{mottled-4}$, (10)) who also bear these mutations. We believe that the ability for any cell to establish silencing, even transiently, must mean that there is at some point sufficient gene activity even though the gene dose has been halved. We imagine that the levels of HP1 and HMT fluctuate normally, thus in some cells through development there is ample silencing activity, while in others there is not. In a *wild-type* condition, this leads to PEV phenotypes. In mutants reducing HP1 or HMT, the fluctuations still occur, but at a lower average level below a threshold required to established heterochromatin-induced gene silencing. Still, a few cells by means of these fluctuations do rise above the threshold and establish silencing but, more often than not and perhaps ultimately inevitably, they lose it again.

Further Thoughts on Epigenetic Regulation and PEV

Our data from analyzing *wild-type* discs further support our previous conclusion, that what we observe as PEV is the final outcome of a life-long series of dynamic switches, from silenced to derepressed and back again. This developmental dynamism in heterochromatin-induced gene silencing challenges the prevailing notion of epigenetic silencing. In the current understanding, the epigenetic information that directs silencing is the same as the mechanism for silencing, as H3K9 is thought to be copied to both daughter chromatids in S-phase and then to enact silencing in the subsequent G1-phase. Our data are not easily reconciled with that view because the losses of silencing we observe would also cause an erasure of the epigenetic memory. Rather, we view heterochromatin-induced gene silencing as constant establishment with no epigenetic "maintenance" phase through S-phase. This view corresponds to others' work in *Drosophila* and *S. cerevisiae* that have shown that liberation of a variegating gene from juxtaposition to heterochromatin immediately and completely derepresses it (15-16).

Finally, our extended analysis here of mutants that compromise known components of the H3K9 heterochromatin silencing mechanism show that decreased silencing leads to increased fluctuations in instability. We reason that this should be interpreted as a need for constant levels of gene products from both *Su*(*var*)*205* and *Su*(*var*)*3-9* because the functions of those heterochromatin components are themselves dynamic, either through transient interactions, through competing activities that act to counteract their roles, or from fluctuations in their expression level or activity. One, or all, of these factors may predominate the correlation with silencing. We do not see them as mutually exclusive and, in fact, they may be

different aspects of the same mechanism. For example, at lower concentration of HP1, as in mutants, we expect longer times in which H3K9 methylated histones are not bound by HP1, leading to derepression. Similarly, ubiquitously-acting H3K9 demethylases (members of the KDM family) act in opposition to HMT activity. This could explain why an enzyme shows dose-sensitivity, because its activity is in constant competition with other enzymes. While not supported specifically by any experiment of which we are aware, this possibility would account for the results we see with our SwiM analysis.

The SwiM System has allowed us to answer some of Dr. Janice Spofford's question. We have discovered that PEV is a highly dynamic process, with each gene undergoing repeated rounds of silencing and derepression; the degree of reversibility is quite high. More, there is no single time of inactivation, but instead a constant need to re-establish it (17). There are times in development during which this re-establishment is more difficult, and these correspond to periods more sensitive to genetic mutation and, perhaps, to environmental perturbations.

FIGURE LEGENDS

Figure 1.

Schematic of the Switch Monitoring (SwiM) System. **(A)** The SwiM System includes ubiquitously-expressed *gal4* activator (teal ActP-*GAL4*) and *gal80* repressor (pink TubP-*GAL80*) genes, the latter transposed into the heterochromatin under study. *Gal4* activity in the absence of *Gal80* activity is monitored by the multi-component *G-TRACE* lineage tracer (13). *Gal4* transactivates a UAS-RFP (red uas-RFP) transgene directly, and thus reports on repression of the *gal80* gene. *Gal4* also transactivates the FLP site-specific recombinase (blue uas-FLP) which catalyzes the removal of a STOP cassette and permanent ubiquitous activation of GFP (converting the inactive white UbiqP-(FRT)-STOP-(FRT)-GFP to the active green UbiqP-(FRT)-GFP and the RT)-STOP-(F extrachromosomal circle which is lost through mitosis). **(B)** Expression of *gal4* (teal) and switches in silencing of *gal80* (pink and black) lead to RFP and GFP fluorescence. The comparison of RFP and GFP fluorescence levels are indicative of whether silencing is intact, compromised, or has switched. 1-6 indicate hypothetical developmental timepoints that are discriminated by the RFP/GFP fluorescence levels. "ø" indicates no fluorescence; "r" indicates low RFP fluorescence with no GFP fluorescence at the onset of gal80 silencing; "Rg" occurs later as RFP reaches maximal levels and GFP begins to build up; "RG" indicates robust expression during times in which *gal80* is silenced; "rG"

indicates that silencing has been lost and the RFP is decaying while GFP expression persists; "G" indicates that silencing is no longer existent leaving just the persistent GFP.

Figure 2.

Extent of PEV (silencing) is correlated within individuals, but variant within populations. **(A-C)** Paired left and right eyes of representative categories of PEV expressed by *w/Y*; *P*{*white*+mC=*tubP-GAL80*^{ts}}$^{10\text{-}PEV\text{-}80.4}$/+ flies, showing that silencing within an individual affects the entire organism similarly (12). (A) shows complete silencing (*i.e.*, "0" expression), (B) shows mid-level ("40-60%"), and (C) shows near *wild-type* expression ("90-100%"). **(D)** Histogram of data taken from scoring 52 *w/Y*; *P*{*white*+mC=*tubP-GAL80*^{ts}}$^{10\text{-}PEV\text{-}80.4}$/+ flies. **(E)** Dissected eye and wing imaginal discs were categorized for expression, scoring the fraction of discs expressing GFP, RFP, or the overlap. Average fluorescence categories of eye discs (X-axis) correlate well with average fluorescence categories of wing discs (Y-axis) from the same individual. Non-parametric analysis cannot discriminate any difference between eye and wing fluorescences, indicating they come from indiscriminable populations (χ^2 = 51.1; P = 0.43). Non-parametric regression was close unity (Kendall's robust line-fit, b = 0.91 (18)). **(F-H)** Images of representative eye-antennal imaginal discs (F-H) and wing discs (F'-H') taken from the same organisms. (F) shows robust silencing of the *gal80* component of the SwiM System, (G) shows medial level, and (H) shows poor silencing.

Figure 3.

Switch Monitoring System analysis of PEV in wild-type organisms. **(A-B)** Whole-mount wing imaginal discs showing RFP and GFP fluorescence as revealed by SwiM System analysis. A-RFP, A-GFP, B-RFP, and B-GFP show inverted monochrome separations of the red and green color channels. Images in (A) and (B) are two discs taken from separate individuals and are both presented and analyzed to show the common features of the patterns revealed by SwiM System analysis. Pink letters indicate location of blow-ups shown in (C-P). **(C-D)** show regions of imaginal discs in which silencing of *gal80* is ongoing, appearing as robust RFP and GFP fluorescence. **(E-F)** show regions that have undergone relatively recent losses of silencing of *gal80*, evident by diminished RFP and robust GFP. (E-RFP) shows the inverted monochrome RFP separation of (E), adjusted for bright-contrast to show low levels of RFP. **(G-H)** show regions that, like (E-F), have lost silencing and express GFP but not RFP. These regions must have lost silencing relatively long before observation as RFP has had time to decay. (G-RFP) and (H-RFP) show inverted monochrome RFP separations, adjusted for bright-contrast to show the very-low levels of RFP (triangles). **(I-J)** show regions that, like (G) and (H), have no obvious RFP. However the inverted monochrome RFP separations, (H-RFP) and (J-RFP), reveal extremely low levels of RFP in some cells (triangles). **(K-L)** show regions regions intermixed with bright and dim RFP within fields of uniform GFP. **(M-N)** show individual cells expressing RFP and no detectable GFP. These represent cells that have recently acquired gene silencing of the *gal80* gene. **(O-P)** show isolated GFP-expressing cells, as confirmed by the (O-GFP) and (P-GFP)

inverted monochrome GFP separations. Separations for (I), (J), (O), and (P) were manipulated by decreasing brightness and increasing contrast to reveal low expression.

Figure 4.

Switch Monitoring System analysis of PEV in *Su(var)205*/+ organisms. **(A-B)** Whole-mount wing imaginal discs showing RFP and GFP fluorescence as revealed by SwiM System analysis. A-RFP, A-GFP, B-RFP, and B-GFP are inverted monochrome separations of the RFP and GFP color channels. Images in (A) and (B) are two discs taken from separate individuals and are presented and analyzed to show the common features of the patterns revealed by SwiM System analysis. Pink letters indicate location of blow-ups shown in (C-J). **(C-D)** show regions with GFP and RFP expression, indicating ongoing silencing of *gal80*. Note in these regions that the variability of GFP and RFP is higher than in discs from wild-type organisms (Figure 2). **(E-H)** show regions with variable GFP expression and little RFP within single clones. Those in (E) and (F) were presumably earlier in their gains and losses of silencing, while those in (G) and (H) were more recent in their gains and losses of silencing. **(I-J)** show individual cells expressing GFP but not RFP (triangles). Such cells indicate ongoing variation, here thought to be short-lived gains of silencing followed by almost-immediate loss.

Figure 5.

Switch Monitoring System analysis of PEV in *Su(var)3-9*/+ organisms. **(A-B)** Whole-mount wing imaginal discs showing RFP and GFP fluorescence as revealed by SwiM System analysis. A-RFP, A-GFP, B-RFP, and B-GFP are inverted monochrome separations of the RFP and GFP color channels. Images in (A) and (B) are two discs taken from separate individuals and are presented and analyzed to show the common features of the patterns revealed by SwiM System analysis. Pink letters indicate location of blow-ups shown in (C-J). **(C-D)** show regions with comparatively low GFP within clones with higher GFP expression, highlighting variation in the timing of GFP activation even within single clones of cells. **(E-J)** show regions with high variation – within the indicated clones and between the clones shown.

Materials and Methods

Strains and husbandry

wild-type flies were *yellow*^{1} *white*^{67c23}. The main SwiM System strain was maintained as *y*^{1} *w*^{67c23}; *P*{*white*$^{+mC}$=*tubP-GAL80*^{ts}}10-PEV-80.4; *P*{*w*$^{+mC}$=*Act5C-gal4*}17bF01/TM6B, Tb1. For *wild-type* SwiM analysis, male SwiM flies were crossed to virgins of the *G-TRACE* strain, either genotype *w**; *P*{*w*$^{+mC}$=*UAS-RedStinger*}6, *P*{*w*$^{+mC}$=*UAS-FLP.Exel*}3, *P*{*w*$^{+mC}$=*Ubi-p63E(FRT.STOP)Stinger*}15F2 or *w**; *P*{*w*$^{+mC}$=*UAS-RedStinger*}4, *P*{*w*$^{+mC}$=*UAS-FLP1.D*}JD1, *P*{*w*$^{+mC}$=*Ubi-p63E(FRT.STOP)Stinger*}9F6/*CyO*. We saw no difference in these two strains and the choice was based on linkage of the two *Su*(*var*) mutations. *Su*(*var*)*205* was *w*^{m4}; *Su*(*var*)*205*^{5}/*CyO*, *Cy*, and *Su*(*var*)*3-9* was *w*^{m4h}; Su(*var*)*3-9*^{1}/*TM3*, *Sb*^{1}. Flies were maintained in glass vials, fed "Karpen- Eckert" medium (30g/L yeast extract, 55 g/L corn meal, 11.5 g/L agar, 72 mL/L dark molasses, 6 mL/L propionic acid, 2.4 g/mL tegosept), and raised at 25°C at 80% humidity; manipulation was done after etherization.

Photography

Images of whole flies were taken with a Sony a7iii camera back attached to a Nikon SMZ-1500 microscope, illuminated with a Peak Plus Tactical LED Flashlight. Flies were cradled in a 90° angle constructed of two mirrors to simultaneously image both eyes.

Dissection, Microscopy, and Fluorescence Detection

Larval imaginal discs were dissected from wandering third instar larvae in 1X PBS, and were visualized and photographed using a Zeiss AxioZoom.v16 equipped with series 00, 38HE, or 74HE filter cubes. Images were processed for bright-contrast using the "Best" algorithm of the Zeiss ZEN (version 2.3-blue) software. Black-on-white separations were made in Adobe Photoshop CC (version 20.0.4) and images were cropped using Photoshop or Apple Pages (version 11.2).

Statistical Analysis

Data in Figure 2E were analyzed for goodness-of-fit using a non-parametric chi-square test because these are categorical data. Regression was done using Kendall's robust line-fit method according to (18).

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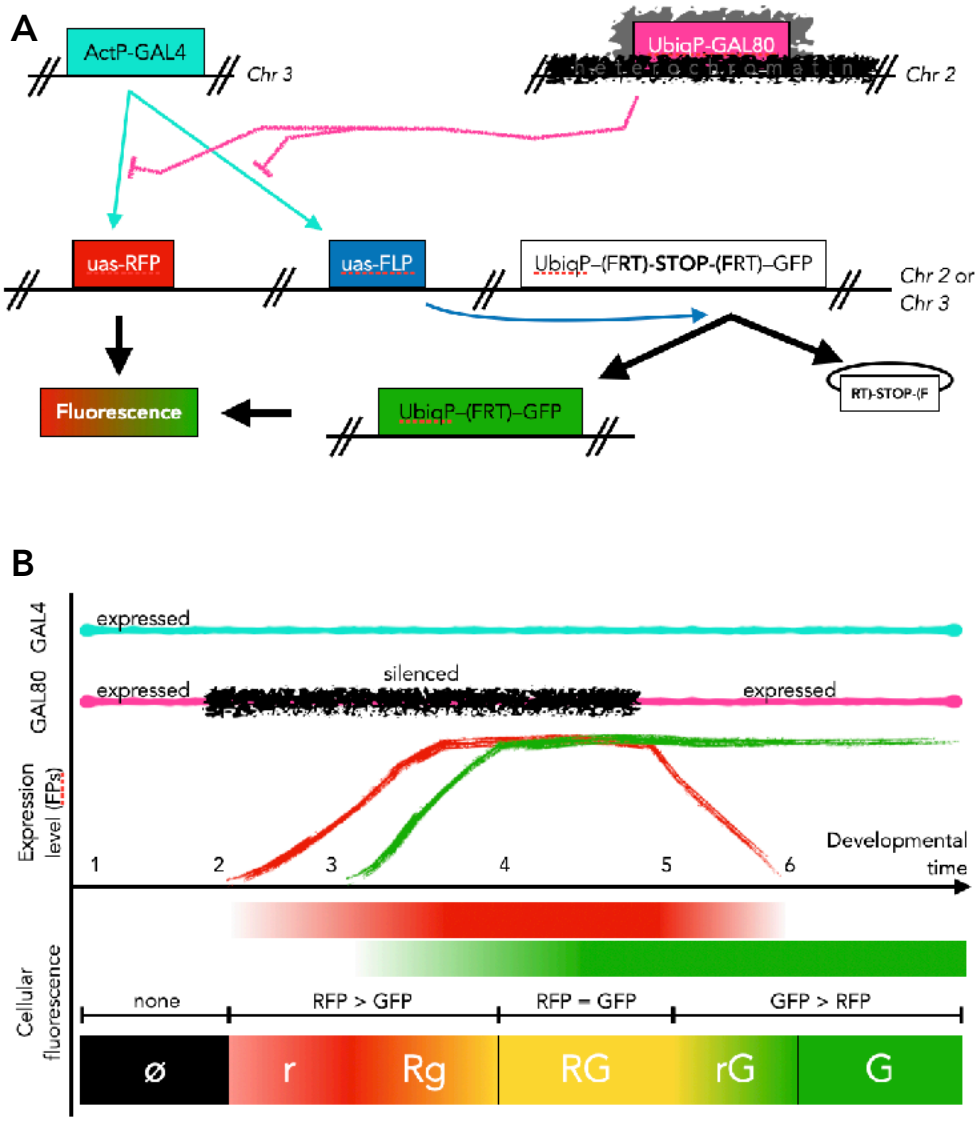
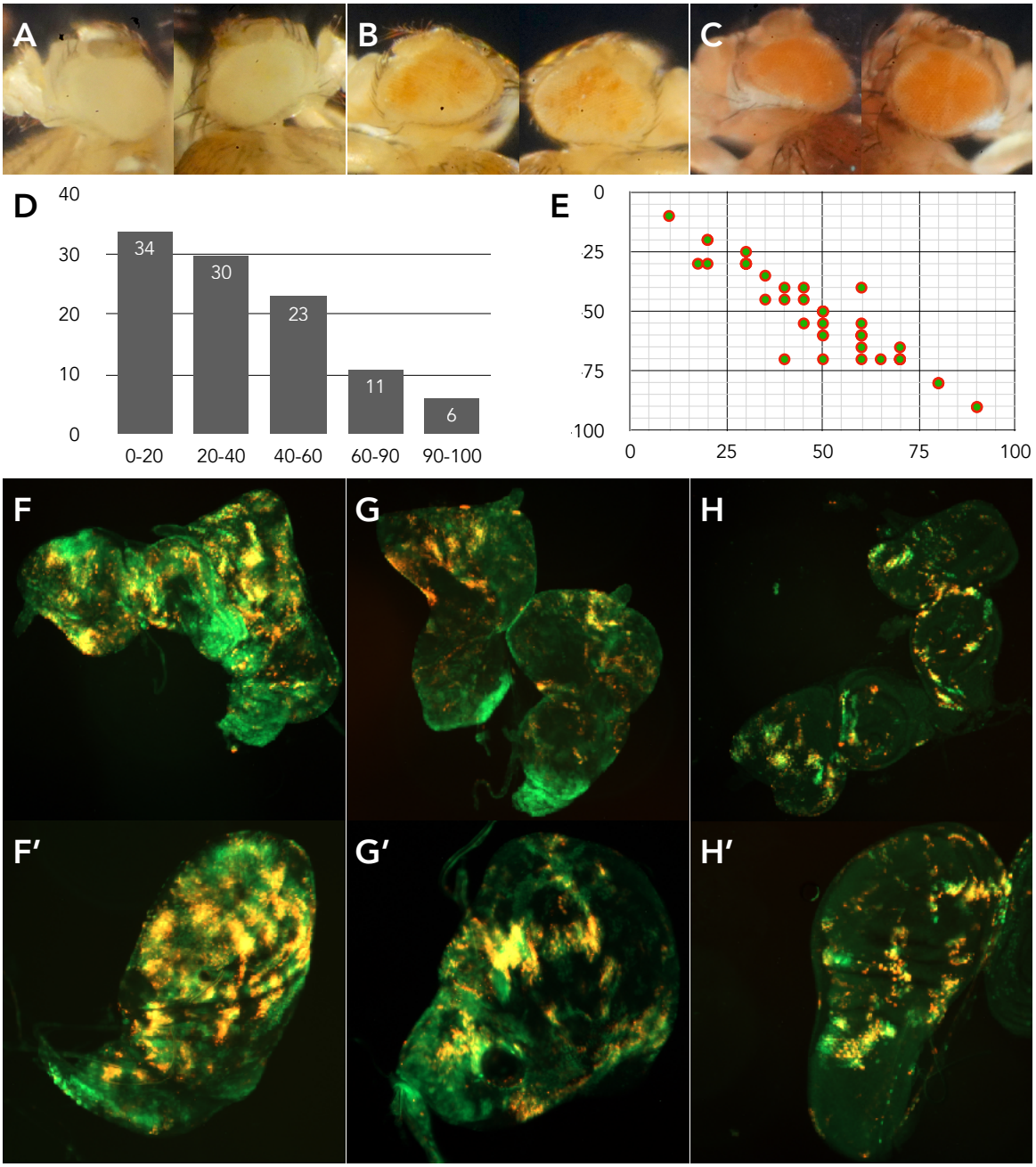


Bughio and Maggert
Figure 1



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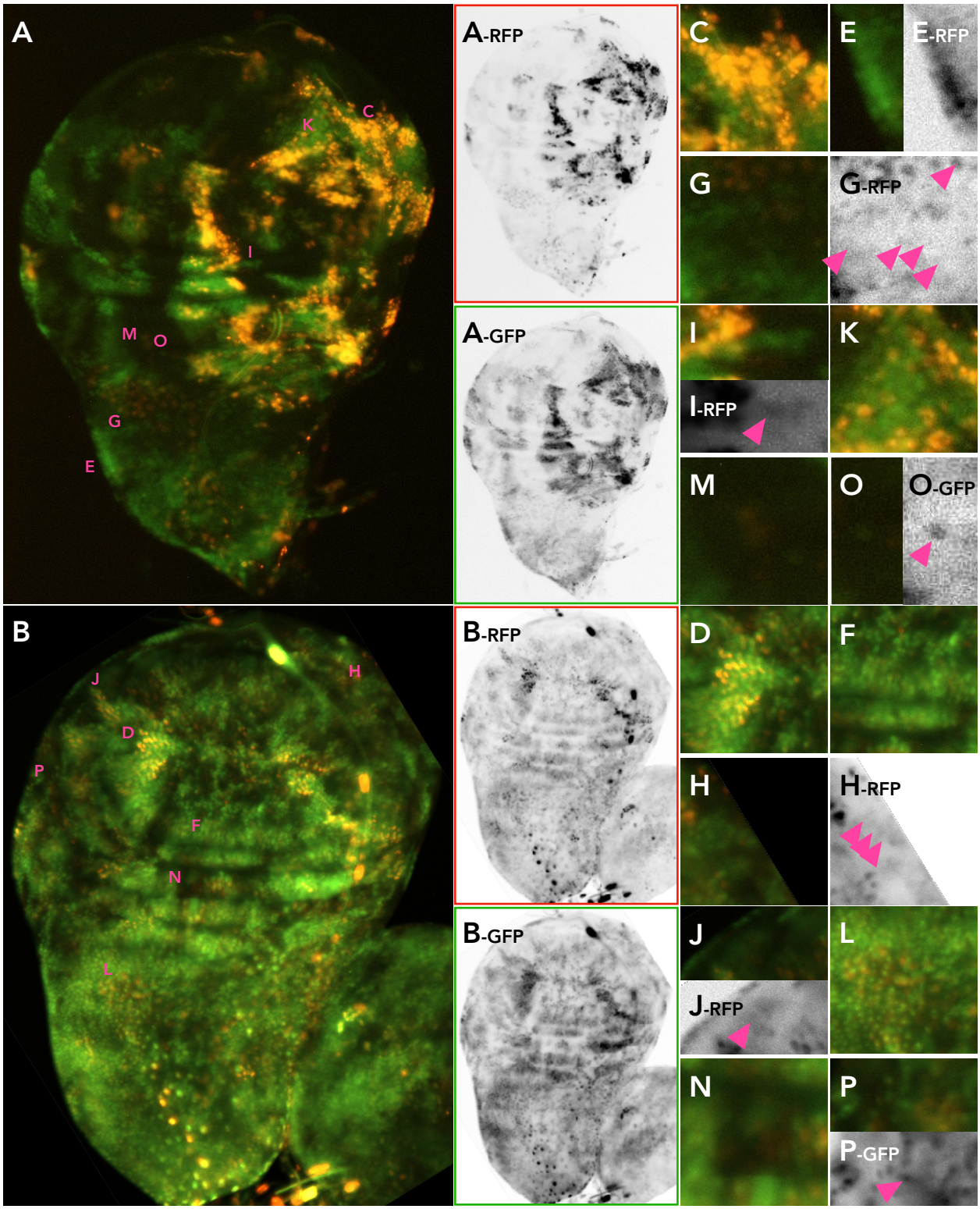

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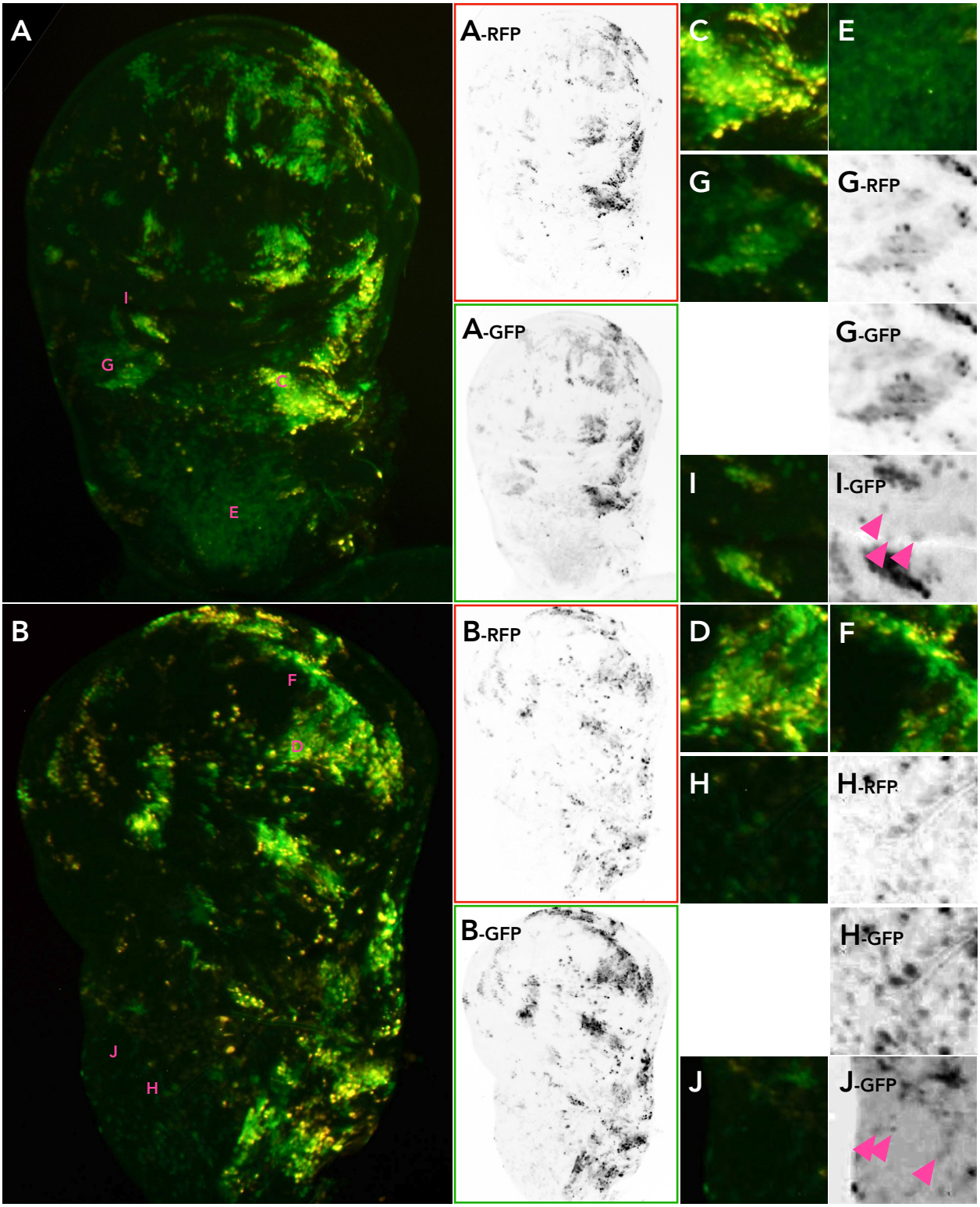

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Figure 4

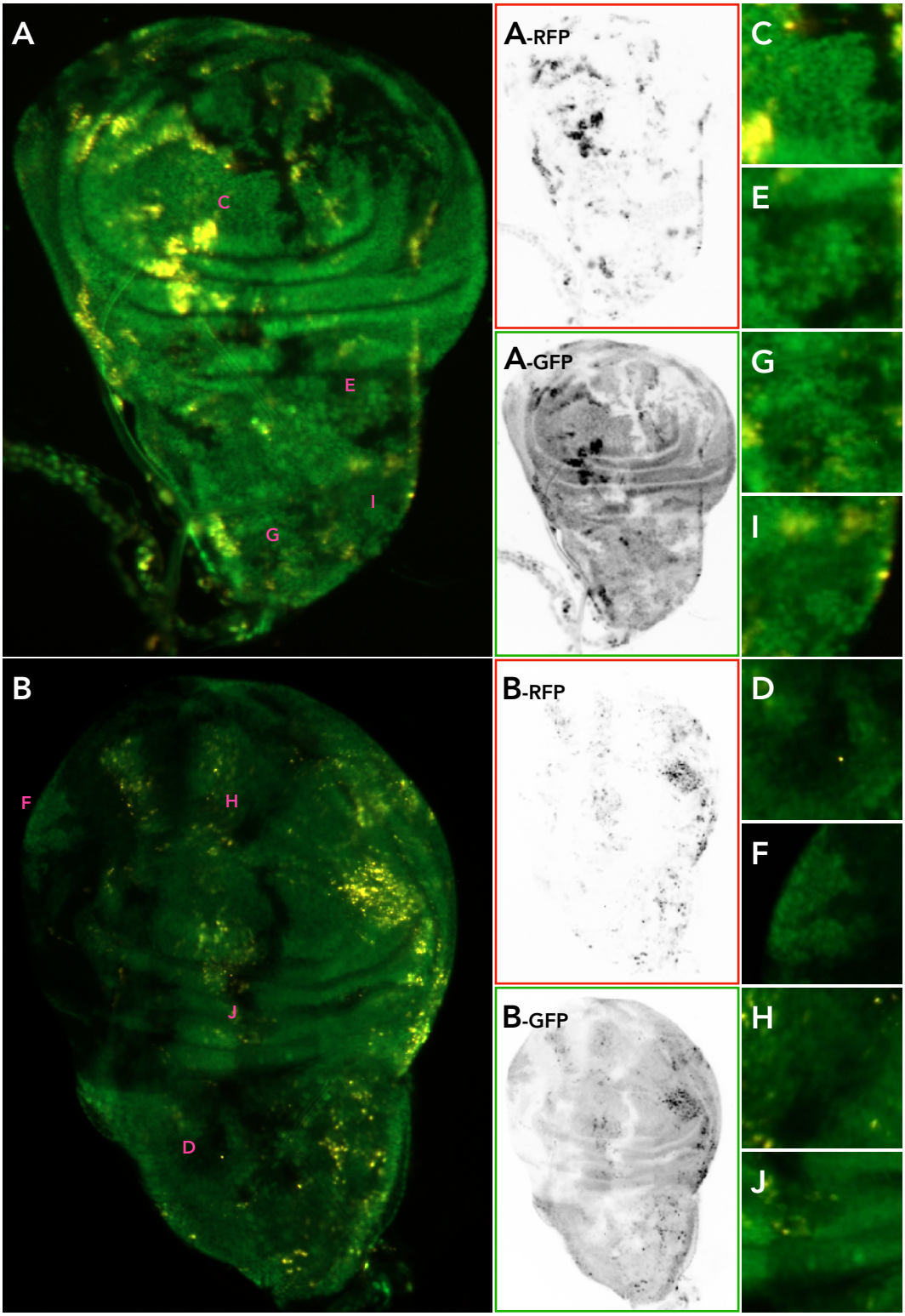

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Figure 5