Reexamining the *P*-Element Invasion of *Drosophila melanogaster* Through the Lens of piRNA Silencing

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ABSTRACT Transposable elements (TEs) are both important drivers of genome evolution and genetic parasites with potentially dramatic consequences for host fitness. The recent explosion of research on regulatory RNAs reveals that small RNA-mediated silencing is a conserved genetic mechanism through which hosts repress TE activity. The invasion of the *Drosophila melanogaster* genome by *P* elements, which happened on a historical timescale, represents an incomparable opportunity to understand how small RNA-mediated silencing of TEs evolves. Repression of *P*-element transposition emerged almost concurrently with its invasion. Recent studies suggest that this repression is implemented in part, and perhaps predominantly, by the Piwi-interacting RNA (piRNA) pathway, a small RNA-mediated silencing pathway that regulates TE activity in many metazoan germlines. In this review, I consider the *P*-element invasion from both a molecular and evolutionary genetic perspective, reconciling classic studies of *P*-element regulation with the new mechanistic framework provided by the piRNA pathway. I further explore the utility of the *P*-element invasion as an exemplar of the evolution of piRNA-mediated silencing. In light of the highly-conserved role for piRNAs in regulating TEs, discoveries from this system have taxonomically broad implications for the evolution of repression.

KEYWORDS transposable element; piRNA; hybrid dysgenesis; *P* element

TRANSPOSABLE elements (TEs) are ubiquitous genetic entities that populate almost all genomes. Particularly in eukaryotes, these mobile genetic elements selfishly spread throughout the genome and achieve staggering copy numbers, making them major players in genome evolution. “Gigantic” genomes—particularly in plants—are often predominantly TEs, indicating an important role for TEs in genome expansion (Vitte and Panaud 2005; Vitte and Bennetzen 2006). Additionally, TEs fuel structural genome evolution by generating inversions, duplications, and translocations through nonhomologous recombination; and by inducing double-stranded breaks that are repaired by non-homologous end joining (Lemaitre *et al.* 2008; Zichner *et al.* 2013; Grandaubert *et al.* 2014; Sarilar *et al.* 2014; Startek *et al.* 2015). Finally, TE insertions influence the architecture

of eukaryotic gene expression by acting as *cis*-regulatory elements (Kunarso *et al.* 2010; Lynch *et al.* 2011).

Like any other type of mutation, TE insertions are occasionally adaptive alleles that provide a selective advantage for their host (Daborn 2002; Aminetzach *et al.* 2005; González *et al.* 2008). Additionally, some “domesticated” TE families perform important cellular functions, such as the retrotransposons that form *Drosophila* telomeres (reviewed in Silva-Sousa *et al.* 2012). However, these rare examples of beneficial TEs, together with their evolutionary success of TEs in populating host genomes, belie their parasitic nature. TEs rely partially or completely on host-derived proteins for replication, thereby acting as sponges for critical host-cellular machinery (Nuzhdin 1999; Yang and Nuzhdin 2003; Pasyukova *et al.* 2004). TE propagation, furthermore, introduces new insertions nearly randomly throughout the genome, many of which disrupt gene function (Spradling *et al.* 1999; Dupuy *et al.* 2001). Once inserted, TEs continue to be mutagenic by producing structural rearrangements (reviewed in Hedges and Deininger

2007). The combination of random insertion and structural rearrangement may explain why TE deregulation has been associated with the onset and progression of certain classes of tumors (Vilà *et al.* 2003; Howard *et al.* 2008; Belancio *et al.* 2010).

Perhaps in response to these manifold and multifaceted fitness costs, host genomes have acquired mechanisms to regulate TE activity. In eukaryotes this is especially true in the germline, where TE-associated mutations can be transmitted to offspring. Recent studies have revealed that small RNA-mediated silencing pathways provide a conserved and critical strategy for TE control, acting as genomic immune systems that regulate endogenous TE families (reviewed in Creasey and Martienssen 2010; Saito and Siomi 2010; Blumenstiel 2011). Analogous to the vertebrate adaptive immune system, these pathways are challenged to recognize and acquire regulation of new parasites as TEs frequently invade novel host genomes through horizontal transfer (Gilbert *et al.* 2010; Thomas *et al.* 2010). The mutational and selective mechanisms that fuel the evolution of small RNA-mediated TE regulation, however, remain poorly understood.

A stunning example of horizontal transfer and the subsequent evolution of TE regulation is provided by the invasion of the *Drosophila melanogaster* genome by *P* elements in the mid-20th century. These DNA transposons rapidly spread through natural populations of *D. melanogaster*, with repression evolving almost concurrently with invasion (Kidwell 1983; Anxolabéhère *et al.* 1988). Until recently, the molecular mechanism(s) that underlie *P*-element repression baffled *Drosophila* geneticists. Although early studies demonstrated that *P* elements are partially regulated by self-encoded repressor proteins, this autoregulation does not explain the very strong germline repression exhibited by many wild-derived strains (Robertson and Engels 1989; Misra and Rio 1990; Gloor *et al.* 1993; Misra *et al.* 1993; Jensen *et al.* 2008). The recent discovery of the Piwi-interacting RNA (piRNA) pathway, a conserved silencing mechanism that controls germline TE activity in both metazoans and ciliates (reviewed in Aravin *et al.* 2007; Mani and Juliano 2013), provided the critical missing piece to the puzzle: the activity of *P* elements in *D. melanogaster* is also regulated by piRNAs (Brennecke *et al.* 2008; Jensen *et al.* 2008; Khurana *et al.* 2011).

This review integrates 40 years of research on the invasion and regulation of *P* elements in *D. melanogaster* genomes, with recent insights into piRNA-mediated silencing that have emerged over the last decade. I describe both repressor protein and piRNA-mediated regulation, and relate these mechanisms to the genetic architecture of repression that is observed among wild-derived strains. I further integrate empirical observations of repression in natural populations and wild-derived genotypes, with theoretical predictions about the evolution of TE regulation. I conclude by highlighting features of the *P*-element invasion and host-genome response that exemplify broader patterns in the evolution TE regulation.

P-element Molecular Genetics and Self-Encoded Regulation

P elements were one of the first families of metazoan transposons to be studied, and the genetic and molecular requirements for their transposition have been exceptionally well characterized (reviewed in Rio 1991, 2002; Castro and Carareto 2004). Here I review literature on the molecular genetics of *P* elements, with particular focus on their regulation through self-encoded repressor proteins.

***P*-element structure and transposition**

Full-length *P* elements (2.9-kb long, Figure 1A) consist of a protein-coding gene sandwiched between multiple inverted repeats at the 5' and 3' termini (O'Hare and Rubin 1983). The coding gene has four exons, 0, 1, 2, and 3 (Karess and Rubin 1984), which are alternately spliced to produce two proteins (Laski *et al.* 1986; Rio *et al.* 1986). Removal of all three introns produces a transcript encoding an 87-kDa transposase enzyme, which is required for transposition (Laski *et al.* 1986; Rio *et al.* 1986). By contrast, transcripts that retain the intron between exons 2 and 3 (intervening sequence 3, IVS3) encode a 66-kDa protein with no transposase activity (Laski *et al.* 1986; Rio *et al.* 1986).

P transposase catalyzes the mobilization of *P* elements through a nonreplicative “cut and paste” mechanism, in which they excise from one genomic location and insert into another (Engels *et al.* 1990). Full-length elements transpose autonomously because they encode this enzyme. Internally-deleted elements do not encode the transposase; however, these nonautonomous elements can be mobilized in *trans* if transposase is furnished by a full-length element elsewhere in the genome (O'Hare and Rubin 1983). Multiple sequences at the 5' and 3' ends of *P* elements are required for their mobilization. In particular, a 10-bp consensus sequence at either end of the element is bound by *P* transposase (Figure 1B) (Kaufman *et al.* 1989; Beall and Rio 1997) and is required for efficient transposition (Mullins *et al.* 1989).

P-element transposition occurs predominantly in the germline, with estimated transposition rates ranging from 10^{-1} to 10^{-3} (new insertions/element/genome) (Eggleston *et al.* 1988; Robertson *et al.* 1988; Berg and Spradling 1991; Kimura and Kidwell 1994). By contrast, *P*-element activity is rare in somatic tissues, where estimated excision rates are more than two orders of magnitude lower than in germline cells (Engels 1979a). Germline-specific transposition is regulated by alternative splicing of the *P*-transposase messenger RNA (mRNA). While fully-spliced, transposase-encoding transcripts predominate in the germline, somatic transcripts generally retain IVS3 and encode the 66-kDa protein (Laski *et al.* 1986; Rio *et al.* 1986). Modified *P* elements with a deletion of IVS3 (*P*{ Δ 2-3}) are sufficient to bring somatic excision to a level similar to that observed in the germline, consistent with a predominant role for splicing regulation in repressing *P* activity in the soma (Laski *et al.* 1986). However, *in vivo* assays reveal that when somatic activity is restored

Figure 1 Structural features of autonomous and nonautonomous *P* elements. (A) Full-length *P* elements include terminal inverted repeats (black) and four exons (gray) (O'Hare and Rubin 1983; Karess and Rubin 1984; Laski *et al.* 1986; Rio *et al.* 1986). Regions of exon 0 and 1 that encode a site-specific, DNA-binding domain are indicated in dark gray (Lee *et al.* 1996). ▽ indicates the position of the 10-bp consensus recognized by *P* transposase (Kaufman *et al.* 1989). Stop signs indicate the locations of stop-codons in IVS3-retaining and full-length transcripts, which encode repressor and transposase proteins, respectively (Laski *et al.* 1986). (B) The 10-bp consensus sequence recognized by *P*-transposase protein overlaps the TATA box (black letters) near the 5' terminus of the element, and interferes with recruitment of RNA polymerase II (Kaufman *et al.* 1989; Kaufman and Rio 1991). (C) The structure of type I and type II repressor-encoding *P* elements. The jagged 3' terminus of the type I elements reflects variation in the deletion breakpoints among elements.

by *P*{ Δ 2-3}, transgenes encoding the 66-kDa protein reduce the somatic excision rate, suggesting that the protein product acts as a secondary repressor of somatic transposition (Robertson and Engels 1989; Misra and Rio 1990).

Germline regulation by repressor proteins

Although IVS3-retaining transcripts are predominantly produced in the soma, they also occur at lower frequency in the female germline, as does the 66-kDa repressor protein (Misra and Rio 1990; Roche *et al.* 1995). Furthermore, some internally-deleted *P* elements encode truncated transposase proteins that, similar to the 66-kDa protein, act as repressors of transposition. Unlike full-length *P* elements, however, the production of repressor proteins from internally-deleted elements does not depend on alternative splicing.

Repressor proteins vary in structure and sequence, but are broadly placed into two classes. Type I repressors, which include the 66-kDa protein, are encoded by transcripts that include exons 0–2 and at least the first nine nucleotides of IVS3 (Figure 1C) (Gloor *et al.* 1993). By contrast, type II repressors are much shorter, and are encoded by transcripts that include only exon 0 and portions of exon 1 (Figure 1C) (Gloor *et al.* 1993; Andrews and Gloor 1995). Notably, although *P* elements encoding type II repressors have been observed in natural populations (Black *et al.* 1987; Jackson *et al.* 1988; Itoh and Boussy 2002; Itoh *et al.* 2007; Fukui *et al.* 2008), type I repressors are only known to arise from full-length elements (Misra and Rio 1990) and deletion variants produced by mutational analysis (Robertson and Engels 1989; Gloor *et al.* 1993).

Transgenes encoding both type I (Robertson and Engels 1989; Misra and Rio 1990; Rasmusson *et al.* 1993) and type II repressors (Gloor *et al.* 1993; Simmons *et al.* 2002; Jensen *et al.* 2008) reduce the excision rate of *P* elements *in vivo*. Although the precise mechanism of repressor protein action remains unclear, they are posited to act as competitive inhibitors of *P* transcription. The 10-bp consensus sequence that is recognized by *P* transposase overlaps the TATA box at the *P* promoter (Kaufman *et al.* 1989), causing *P* transposase to

inhibit the binding of transcription factor IID and subsequent RNA polymerase recruitment (Figure 1B) (Kaufman and Rio 1991). The site-specific binding domain of *P* transposase is found in both type I and type II repressors (Figure 1), and type II repressors are empirically known to retain binding affinity (Kaufman *et al.* 1989; Lee *et al.* 1996, 1998). Therefore, repressor proteins could also act as competitive inhibitors of transcription.

Transcriptional regulation is supported by *in vivo* assays. Type II repressors reduce the activity of germline-expressed *P*-*LacZ* reporters (Lemaitre *et al.* 1993), and both types of repressors reduce the expression of *singed-weak*, a *P*-element insertion allele of the germline-expressed gene *singed* (Roiha *et al.* 1988; Robertson and Engels 1989; Misra *et al.* 1993; Paterson *et al.* 2007). *In vitro* assays further suggest that type II repressors directly repress transposition through competitive or negative interactions with full-length *P* transposase (Lee *et al.* 1996, 1998).

A Primer on piRNA-Mediated Silencing

The piRNA pathway strongly represses *P* elements and other TEs in the *D. melanogaster* germline (Brennecke *et al.* 2008; reviewed in Senti and Brennecke 2010). Similar to other RNA-mediated silencing pathways, piRNA-mediated silencing relies on both small guide RNAs (piRNAs) that target silencing, and proteins that enforce silencing. The piRNA pathway performs diverse biological functions in addition to TE regulation, which are reviewed comprehensively elsewhere (Mani and Juliano 2013). However, the role of the piRNA pathway in regulating TEs is particularly clear in *Drosophila*, where piRNAs are derived overwhelmingly from TEs, and mutations in piRNA pathway components cause dramatic upregulation of TE transcripts, DNA damage, and sterility (Chen *et al.* 2007; Lim and Kai 2007; Li *et al.* 2009; reviewed in Senti and Brennecke 2010; Klenov *et al.* 2011; Rozhkov *et al.* 2013). Here, I outline key features of piRNA-mediated TE regulation in *Drosophila* germlines, with a particular focus on females, who play a pivotal role in transmitting piRNA-mediated silencing to offspring.

Figure 2 TE regulation via piRNA-mediated silencing in the female germline. Most piRNA clusters are transcribed bidirectionally, and the resulting piRNA transcripts (large solid arrows) are processed into mature piRNAs (small solid arrows) in germline cytoplasm (Brennecke *et al.* 2007; Klattenhoff *et al.* 2009). piRNA-Piwi complexes establish transcriptional silencing in the nucleus. piRNA-Aub complexes implement post-transcriptional silencing of TEs in the cytoplasm. Aub-mediated transcriptional silencing also initiates the ping-pong amplification loop, which produces sense (black) and antisense (gray) piRNAs (Gunawardane *et al.* 2007). Full-length TE transcripts from euchromatic TE insertions enhance ping-pong mediated piRNA production by acting as additional sources of precursor piRNAs (Brennecke *et al.* 2008; Shpiz *et al.* 2014). Maternally-deposited piRNA-Aub and piRNA-Piwi complexes (pink) feed forward ping-pong amplification and piRNA cluster transcription (Brennecke *et al.* 2008; Le Thomas *et al.* 2014a). TE insertions of a targeted TE family, such as the P-element family, are represented in red.

piRNAs target transcriptional and post-transcriptional silencing of TEs

In *Drosophila*, the piRNA pathway implements genome-wide silencing of TEs in male and female germlines, as well as in the somatic support cells of the ovary (Senti and Brennecke 2010). piRNA-mediated silencing is targeted by TE-derived antisense piRNAs (23–29 nt), which are complementary in sequence to TE-derived mRNAs. Antisense piRNAs act as guides for two Piwi-Argonaute proteins, Piwi and Aubergine (Aub), which enforce silencing of homologous TEs (Figure 2) (Gunawardane *et al.* 2007; Brennecke *et al.* 2007; Yin and Lin 2007).

Piwi and Aub are distinct in their cellular localization, germline specificity, and mechanism of silencing. Aub is a cytoplasmic protein that enforces post-transcriptional silencing exclusively in the germline (Aravin *et al.* 2007; Lim and Kai 2007; Malone *et al.* 2009; Lim *et al.* 2009). Aub can silence target transcripts directly through slicer cleavage of the mRNA (Gunawardane *et al.* 2007), and indirectly by interacting with components of the mRNA-degradation machinery (Lim *et al.* 2009). By contrast, Piwi is a nuclear protein that establishes transcriptional silencing both in the germline and in the somatic follicle cells of the ovary (Klenov *et al.* 2011; Rozhkov *et al.* 2013). Piwi can induce transcriptional silencing both by promoting deposition of the repressive histone methylation mark H3K9me3 (Sienski *et al.*

2012; Huang *et al.* 2013), and by repressing the deposition of the activating mark H3K4me2 (Klenov *et al.* 2014) at TE loci.

piRNAs are derived from specialized heterochromatic loci

piRNA regulation of an individual TE family relies on the presence of at least one representative insertion in a piRNA cluster, a specialized genomic locus that gives rise to piRNAs (Brennecke *et al.* 2007; Zanni *et al.* 2013). piRNA clusters can be exceptionally large (up to 240 kb) and generally harbor insertions from multiple TE and repeat families (Brennecke *et al.* 2007). These clusters are transcribed as long precursors (>1 kb), which are subsequently processed into mature piRNAs (Brennecke *et al.* 2007; Klattenhoff *et al.* 2009). The majority of the 142 annotated piRNA clusters in the *D. melanogaster* genome are located in pericentromeric and subtelomeric heterochromatin (Brennecke *et al.* 2007).

Most piRNA clusters are defined epigenetically by H3K9me3, a mark that is typically associated with constitutive heterochromatin (Rangan *et al.* 2011; Mohn *et al.* 2014). Somewhat counterintuitively, H3K9me3 does not confer a transcriptionally-silenced state to piRNA clusters, but rather acts as a critical activator of piRNA biogenesis by recruiting proteins involved in piRNA cluster transcription and precursor processing (Rangan *et al.* 2011; Le Thomas *et al.* 2014a). The role of H3K9me3 in defining piRNA clusters may explain why euchromatic insertions of TE families, which are transcriptionally silenced by piRNAs through the deposition of H3K9me3, sometimes act like piRNA clusters; producing abundant piRNAs from both genomic strands (Shpiz *et al.* 2014).

Maternally-transmitted piRNAs promote piRNA biogenesis in offspring

While piRNA production from a few clusters appears to be genetically hardwired, most clusters are at least partially dependent on maternally-deposited piRNAs to feed forward piRNA production in offspring germlines (Malone *et al.* 2009; Le Thomas *et al.* 2014a). Maternally-deposited piRNAs are found in complex with Piwi and Aub, which become localized to the primordial germline in developing embryos, thus providing a mechanism for transgenerational silencing (Harris and Macdonald 2001; Megosh *et al.* 2006; Brennecke *et al.* 2007). piRNA-Piwi complexes target nucleosomes that package sequences homologous to the piRNA for H3K9me3 modification, thereby promoting the establishment of piRNA clusters in offspring (Le Thomas *et al.* 2014a). Because H3K9me3 is not a common chromatin mark outside of constitutive heterochromatin, its piRNA-mediated deposition may be particularly important in the establishment of clusters in euchromatic and facultatively heterochromatic environments (Klenov *et al.* 2014; Le Thomas *et al.* 2014a).

Maternally-deposited piRNAs further enhance piRNA production in offspring by initializing the processing of piRNA precursor transcripts. Specifically, maternally-deposited piRNA-Aub complexes are thought to initiate the ping-pong cycle: a

germline-specific feed-forward amplification loop that produces both sense and antisense piRNAs (Gunawardane *et al.* 2007; Brennecke *et al.* 2007; Malone *et al.* 2009; Le Thomas *et al.* 2014a). Ping-pong processing starts when antisense piRNA-Aub complexes identify and transcriptionally-silence sense-piRNA precursor transcripts, or TE-derived mRNA transcripts, by slicer cleavage (Gunawardane *et al.* 2007; Brennecke *et al.* 2007). The resulting cleavage products are further processed into sense piRNAs, which are loaded onto a third Piwi-Argonaute protein, Argonaute-3 (Ago-3) (Brennecke *et al.* 2007; Gunawardane *et al.* 2007). Sense piRNAs complexed with Ago-3 can, in-turn, identify and cleave antisense precursors, thereby producing more antisense piRNAs that can reinitialize the cycle (Brennecke *et al.* 2007; Gunawardane *et al.* 2007).

Although there are multiple mechanisms for piRNA biogenesis (reviewed in Senti and Brennecke 2010), the ping-pong cycle produces the bulk of germline piRNAs and is indispensable for the regulation of many TE families (Klattenhoff *et al.* 2009; Li *et al.* 2009; Malone *et al.* 2009). Ping-pong amplification is thought to represent an adaptive feature of piRNA-mediated silencing. By using TE-derived mRNAs as ping-pong substrates, piRNA production from the most transcriptionally-active TEs is enhanced, thereby increasing regulation of those TE families that are most likely to transpose (Malone and Hannon 2009; Kelleher and Barbash 2013).

P-element Invasion and Hybrid Dysgenesis

The first hints of *P*-element invasion surfaced in the late 1970s and early 1980s, when multiple investigators reported unusual behavior of wild-derived chromosomes, including the presence of male recombination (normally absent in *D. melanogaster*) (Hiraizumi 1971; Hiraizumi *et al.* 1973; Slatko and Hiraizumi 1973, 1975) and unexpectedly high mutation rates (Sved 1973; Green 1977). The F₁ offspring of some intraspecific crosses were subsequently demonstrated to experience hybrid dysgenesis: a germline-specific syndrome that is caused by *P*-element activity (Kidwell and Kidwell 1975; Sved 1976; Kidwell *et al.* 1977; Engels and Preston 1979; Engels 1979b; Schaefer *et al.* 1979). Dysgenic phenotypes include both male recombination and elevated mutation; but also elevated female recombination, segregation distortion, sterility, and gonadal atrophy. Ovarian atrophy in particular provided an easy phenotypic assay for the presence *P* activity, which facilitated the documentation of *P* elements spreading through populations worldwide (Kidwell 1983). It further allows for the detection and genetic dissection *P*-element regulation.

Hybrid dysgenesis is caused by *P*-element activity

The frequency of dysgenic F₁ offspring that are produced by a given cross depends on both the paternal and the maternal genotype. In the traditional nomenclature of *P* element-induced hybrid dysgenesis, there are two types of strains: M and P. When a *P* strain is paternal and an M strain is maternal,

Figure 3 *D. melanogaster* strains differ in their capacity to induce and repress hybrid dysgenesis. (A) Reciprocal crosses between P and M strains differ with respect to hybrid dysgenesis, which is only observed when M strain females are crosses to P-strain males. (B) P, P', Q, M, and M' strains are classified by their ability to induce F₁ female gonadal atrophy paternally when crossed to reference M females, and repress it maternally when crossed to reference P males. The frequencies of ovarian atrophy among F₁ offspring in these two crosses are reported on the x- and y-axes, respectively. Different strain classes are separated by dotted lines. Classes with *P* elements in their genomes are indicated in gray.

the hybrid offspring are dysgenic (Figure 3A). However, in the reciprocal cross, where M-strain males are mated to P-strain females; fully fertile, nondysgenic offspring are produced (Kidwell and Kidwell 1975; Sved 1976; Kidwell *et al.* 1977).

Elevated mutation rates in dysgenic germlines provided for the discovery of *P* elements as mobile genetic entities whose activity induces the hybrid dysgenesis syndrome. Sequence analysis of dysgenesis-induced alleles revealed that the causative mutations were insertions of DNA elements, which exhibited high sequence similarity to each other (Bingham *et al.* 1982). Because these elements were abundant in P-strain genomes, but rare or absent from M-strain genomes, they were referred to as *P* elements (Bingham *et al.* 1982; Rubin *et al.* 1982). Atypical M strains, referred to as M', were observed to harbor *P* elements; however, almost all of those elements had internal deletions and were therefore immobile in the absence of autonomous copies (Bingham *et al.* 1982; Anxolabéhère *et al.* 1985, 1988; Ronsseray *et al.* 1989a; Itoh *et al.* 2001, 2004; Ogura *et al.* 2007; Fukui *et al.* 2008). The demonstration of increased *P*-element transcription and transposition in dysgenic germlines further solidified *P* elements as the molecular cause of hybrid dysgenesis (Eggleston *et al.* 1988; Lemaitre *et al.* 1993; Khurana *et al.* 2011).

Variation in paternal induction and maternal repression

The ability to contribute both paternally and maternally to hybrid dysgenesis exhibits continuous variation in natural populations (Kidwell *et al.* 1983; Kocur *et al.* 1986; Ogura *et al.* 2007). However, it is useful to delineate strains into phenotypic classes based on their binary capacity to produce dysgenic offspring, both paternally and maternally (Figure 3B). Diagnostic crosses are used in which a strain of unknown phenotype is crossed to an P or M reference strain, with the incidence of gonadal atrophy among the F₁ female offspring providing an indicator of the frequency of hybrid dysgenesis. A strain is considered a paternal inducer of dysgenesis if it produces >10% F₁ female gonadal atrophy in crosses with M females, and a maternal repressor of dysgenesis if it produces <10% F₁ female gonadal atrophy in crosses with P males (Kidwell *et al.* 1977).

In P and M strains, paternal induction and maternal repression are coupled: P strains exhibit both, while M strains exhibit neither (Figure 3B). However, two other strain types, Q and P', decouple paternal induction from maternal repression, thereby revealing these phenotypes to be genetically separable. Q strains repress but do not induce dysgenesis, making them fully fertile with P and M strains in both directions of crossing (Kidwell 1979). Reciprocally, P' strains induce but do not robustly repress dysgenesis (Quesneville and Anxolabéhère 1998). The low fertility of P' strains likely explains why they are only very rarely observed in natural populations (Itoh *et al.* 2007; Onder and Kasap 2014).

Population invasion and horizontal transfer

Probing for P-element sequences in strains isolated from different geographic regions at different times (Anxolabéhère *et al.* 1985, 1988); as well as classifying these strains as P, P', Q, or M (Kidwell 1983; Kidwell *et al.* 1983); provided a temporal and biogeographic picture of P-element invasion. P elements most likely invaded *D. melanogaster* in the late 19th or early 20th century (Engels 1992), although the oldest strain harboring P elements was not collected until 1938 in Idaho Falls, United States (Anxolabéhère *et al.* 1988). The oldest P strains are all from the Americas, suggesting that the initial invasion occurred in this region (Kidwell 1983; Kidwell *et al.* 1983; Anxolabéhère *et al.* 1988). After invasion, P elements spread rapidly worldwide; the most-recent P element-free strain was collected in Birsik, Russia in 1974 (Anxolabéhère *et al.* 1988). P elements therefore transitioned from exceptionally rare to ubiquitous in <40 years.

The directional spread of P elements from the Americas is associated with changes in the prevalence of strain types. In the Americas, P strains were predominant by the mid-1970s, although Q strains were also common (Kidwell 1983; Kidwell *et al.* 1983). Similar frequencies of P and Q strains were observed among North American collections from 2001 and 2003 (Itoh *et al.* 2007), indicating temporal stability of strain types. By contrast, populations outside of the Americas were mostly composed of M/M' and Q strains in the early 1970s,

and more recent collections also indicate that many regions have stably maintained this strain composition (Itoh *et al.* 2001, 2004; Onder and Kasap 2014). These geographic differences are consistent with theoretical predictions based on an invasion that started in the Americas. Forward simulation suggests that the initial population invaded by P elements will reach an equilibrium state with a high frequency of P strains, whereas the migration of P element-bearing individuals to neighboring populations will produce more variable strain composition (Quesneville and Anxolabéhère 1998).

At the time of invasion, P elements were absent from the genomes of *D. melanogaster*'s sibling species *D. simulans* and *D. mauritiana* (Brookfield *et al.* 1984), but common among the genomes of its more distant relatives in the *Drosophila* genus (Daniels *et al.* 1984; Clark *et al.* 1994; Clark and Kidwell 1997; Quesneville and Anxolabéhère 1997). In particular, P elements of *D. melanogaster* are almost identical (1 nt substitution) to those of *D. willistoni*, despite >40 million years of divergence between these two species (Daniels *et al.* 1990). The geographic ranges of these two species overlap in Florida as well as in Central and South America, consistent with the American origin of *D. melanogaster* P elements. The original P element that invaded *D. melanogaster*, therefore, was likely horizontally transferred from *D. willistoni*. Although the vector of transfer remains unknown, an early study implicated a parasitic mite that feeds on the cytoplasm of early *Drosophila* embryos, potentially introducing foreign DNA into germline nuclei prior to cellularization (Houck *et al.* 1991). This model is consistent with recent genomic analyses, which increasingly point to host-parasite interactions as vehicles for the horizontal transfer of TEs (Gilbert *et al.* 2010, 2014; Parisot *et al.* 2014).

Dissecting the P Cytotype: From Classical Genetics to piRNAs

The analysis of hybrid dysgenesis revealed not only the invasion of P elements, but also the presence of powerful regulatory factors that arose in natural populations of *D. melanogaster* after invasion. Both P and Q strains provide maternal repression of P element-induced ovarian atrophy, whereas M and M' females leave their offspring susceptible to P-element activity. Early genetic analysis revealed that maternal regulation arises from complex interactions between chromosomal and extrachromosomal (cytoplasmic) factors (Engels 1979b; Sved 1987; Ronsseray *et al.* 1993). Engels (1979b) therefore proposed the term "cytotype" to denote the ability of a fly to repress P-element activity maternally, with P and M cytotypes exhibiting repression and no repression, respectively.

Early studies proposed that the chromosomal and extrachromosomal factors responsible for the P cytotype were repressor-encoding P elements and their gene products (Misra and Rio 1990; Gloor *et al.* 1993; Roche *et al.* 1995). However, recent research has revealed that the piRNA pathway is the molecular basis of cytotype regulation (Reiss *et al.*

2004; Brennecke *et al.* 2008; Jensen *et al.* 2008; Belinco *et al.* 2009; Khurana *et al.* 2011). Indeed, P-cyotype genetics and piRNA-mediated silencing are increasingly synergistic, with classic observations motivating modern molecular experiments and new mechanisms shedding light on old discoveries. Here I relate the genetic architecture of the P cyotype to models of P-element regulation by both repressor proteins and piRNAs.

Two molecular models of P-cyotype regulation

In the original repressor protein model, P-cyotype regulation was attributed to 66-kDa proteins or other type I repressors, which are produced by P-cyotype females and loaded into oocytes (Figure 4A) (Misra and Rio 1990; Gloor *et al.* 1993; Roche *et al.* 1995). The maternally-deposited proteins were proposed to both negatively regulate P-element transposition in the offspring germline and feed forward IVS3 retention, thereby propagating regulation by promoting the production of additional repressor proteins (Misra and Rio 1990; Gloor *et al.* 1993; Roche *et al.* 1995). By contrast, when M cyotype females are crossed to P-strain males, no repressor proteins would be maternally transmitted, abundant transposase would be produced, and dysgenesis would ensue.

In new the piRNA model, P-cyotype regulation is transmitted and enforced by P element-derived piRNAs, which are maternally deposited into oocytes (Figure 4B) (Brennecke *et al.* 2008; Jensen *et al.* 2008). Maternally deposited piRNAs from P-cyotype females are proposed both to target the transcriptional and post-transcriptional silencing of P elements in offspring germlines, and feed forward the production of additional P-derived piRNAs through the ping-pong cycle (Figure 4B). In crosses involving M-cyotype females, the absence of maternally-deposited P-derived piRNAs both leaves the P element unregulated and short circuits piRNA biogenesis.

P cyotype is established by P-element insertions in piRNA clusters

Genetic mapping of the P cyotype associated it with specific chromosomes and, in some cases, individual P-element insertions, clearly implicating the P element in its own regulation. (Engels 1979b; Kidwell 1981; Periquet and Anxolabéhère 1982; Ronsseray *et al.* 1989b, 1991; Marin *et al.* 2000; Stuart *et al.* 2002; Khurana *et al.* 2011). However, P cyotype-conferring elements were observed to be either full length (Ronsseray *et al.* 1991, 1996) or internally deleted (Nitasaka *et al.* 1987; Marin *et al.* 2000; Stuart *et al.* 2002), suggesting that there is no particular mutation in the P element itself that determines repression. Indeed, for two internally-deleted elements, which at their endogenous locus confer the P cyotype, the transgenic introduction of the element to novel genomic sites did not confer repression (Jensen *et al.* 2008). Thus, it is the genomic position of an insertion that determines its regulatory capacity, and not the polypeptide that it encodes.

In particular, repression resulted from independent P-element insertions in cytological position 1A, which resides

immediately proximal to the X-chromosome telomere (Kidwell 1981; Ronsseray *et al.* 1991; Marin *et al.* 2000; Stuart *et al.* 2002). All 1A repressors are located in the same block of telomere-associated sequence (TAS) repeats, the X-TAS, and are therefore referred to as telomeric repressive P elements (TPs) (Ronsseray *et al.* 1996; Marin *et al.* 2000; Stuart *et al.* 2002). Although position effects are explained in the repressor protein model by the capacity of certain genomic sites to facilitate the production of repressor-encoding mRNAs in germlines, the significance of the X-TAS in P-element regulation results from its function as the fourth most prolific piRNA cluster in the *D. melanogaster* genome, producing up to 2.6% of the ovarian piRNA pool (Brennecke *et al.* 2007; Yin and Lin 2007). Furthermore, two TPs have been demonstrated to generate P-derived piRNAs in ovaries (Brennecke *et al.* 2008), and another P-element insertion into an autosomal piRNA cluster has similarly been associated with ovarian piRNA production and P-cyotype repression (Khurana *et al.* 2011). Therefore, the ability of an individual P-element insertion to establish the P cyotype is now attributed to the function of particular genomic regions in producing piRNAs.

Maternal effects are explained by the maternal transmission of piRNAs

The dramatic difference in reciprocal crosses between P and M strains with respect to hybrid dysgenesis implied that the P cyotype is transmitted maternally (Kidwell and Kidwell 1975; Engels 1979b; Eggleston *et al.* 1988). The requirements for maternal transmission were carefully analyzed by examining the capacity of TP females to repress hybrid dysgenesis in their offspring. In crosses between heterozygous (TP/+) females and P-strain males, ovarian atrophy was approximately equally repressed among the female offspring, regardless of their genotype (Figure 5A vs. Figure 5B) (Ronsseray *et al.* 1993; Simmons *et al.* 2007a; Belinco *et al.* 2009). However, only those female offspring that inherit the TP are themselves able to establish maternal repression (Figure 5B). Therefore, in the presence of dysgenesis-inducing, paternally-inherited P elements, ovarian atrophy is determined entirely by the genotype of the mother—a true maternal effect. P-cyotype regulation is not exclusively determined by maternal effects in all assays or tissues, however. For example, although maternal TPs are required to repress P-element excision in the male germline, the TP also influences the phenotype zygotically, and in some cases is zygotically required for repression (Stuart *et al.* 2002).

The repressor-protein model proposed that maternally-deposited repressor proteins, which are loaded into oocytes (Misra and Rio 1990), underlie the maternal effect that characterizes P-cyotype repression. However, transgenic analyses revealed that both type I and type II repressors act zygotically—not maternally—to regulate P-element excision and transcription (Robertson and Engels 1989; Misra and Rio 1990; Lemaitre *et al.* 1993; Simmons *et al.* 2002; Jensen *et al.* 2008). Thus, the transmission genetics of repressor proteins fails to support this hypothesis. The single exception to this rule

Figure 4 Two molecular models of the P cytotype. (A) In the repressor protein model, P females were proposed to deposit repressor proteins into the egg cytoplasm. Repressor proteins would then act to promote IVS3 retention and production of additional repressor protein. By contrast, M females would not promote IVS3 retention in the F₁ germline, thereby allowing for abundant transposase production (Misra and Rio 1990; Gloor *et al.* 1993; Roche *et al.* 1995). (B) In the piRNA model, P females maternally deposit piRNAs (gray arrows), including P-derived piRNAs (red arrows) in complex with Piwi-Argonaute proteins (gray ovals) into oocytes. piRNA-Piwi-Argonaute complexes are proposed to establish piRNA-mediated silencing of P elements in F₁ germlines (Brennecke *et al.* 2008). By contrast, the maternally deposited piRNA pool in M females is lacking in P-derived piRNAs, allowing P transposase to be produced and P elements to mobilize.

exhibits only modest differences between maternal and paternal inheritance, as opposed to the strict requirement for maternal inheritance that characterizes the P cytotype (Misra *et al.* 1993).

In contrast, piRNA-mediated silencing is entirely consistent with the maternal-effect behavior of the P cytotype. The presence of maternal—but not paternal—P-element insertions in piRNA clusters or piRNA production is associated with robust piRNA production in F₁ ovaries (Brennecke *et al.* 2008; Khurana *et al.* 2011). Interestingly, however, F₁ females from at least one dysgenic cross are known to initiate production of P-derived piRNAs from a paternally-inherited piRNA cluster as they age, as well as partially recover their fertility (Khurana *et al.* 2011). Therefore, some genotypes are able to developmentally bypass the requirement for maternally-deposited piRNAs.

Further supporting maternally-deposited piRNAs as the cause of the maternal effect, P-cytotype regulation in offspring is sensitive to the maternal dosage of proteins involved in piRNA biogenesis and silencing. Maternal heterozygosity for loss-of-function mutations in *aub* and *piwi* impairs the repression of P-element activity in offspring (Reiss *et al.* 2004; Simmons *et al.* 2007b, 2010; Belinco *et al.* 2009). Similar sensitivity to maternal dosage is also observed with heterochromatin protein 1 (HP1) (Ronsseray *et al.* 1996; Haley *et al.* 2005; Belinco *et al.* 2009; Simmons *et al.* 2010), which promotes the heterochromatic environment required for piRNA cluster transcription (Moshkovich and Lei 2010; Rangan *et al.* 2011).

Epistasis between repressive and nonrepressive P elements is explained by ping-pong biogenesis of piRNAs

Maternal heterozygosity for a single TP reduces P-element activity in F₁ offspring, with different TP alleles exhibiting different degrees of repression (Ronsseray *et al.* 1998;

Marin *et al.* 2000; Stuart *et al.* 2002; Simmons *et al.* 2007a). However, significantly-stronger maternal repression is seen when the maternal genotype includes both a TP and additional P elements elsewhere in the genome (Figure 5B vs. Figure 5C) (Ronsseray *et al.* 1998; Simmons *et al.* 2007a, 2012, 2014; Belinco *et al.* 2009). TP-dependent maternal repression, therefore, is epistatically enhanced by additional P-element copies, which in isolation do not confer repression. In the repressor protein model, genetic interactions between repressive and nonrepressive elements are explained by the proposed role of repressor proteins in promoting IVS3 retention and additional repressor protein production (Roche *et al.* 1995). However, repressor protein has never been described as a splicing modifier of P-derived transcripts.

In the piRNA model, the ability of nonregulatory P elements to enhance regulation in the presence of a TP is explained by the feed-forward biogenesis of piRNAs through the ping-pong amplification cycle (Figure 2). Because TE-derived mRNAs provide sense transcripts for ping-pong amplification, transcriptionally-active TE insertions outside of piRNA clusters are expected to amplify piRNA production and enhance the robustness of TE silencing (Brennecke *et al.* 2008). Therefore, mRNAs from nonrepressive P insertions would enhance piRNA production that is initiated by insertions in the X-TAS piRNA cluster, thereby establishing stronger regulation. In support of this model, *aub* mutations impair both ping-pong amplification (Li *et al.* 2009; Malone *et al.* 2009) and the enhanced repression exhibited by maternal genotypes combining TPs with nonrepressive P insertions (Belinco *et al.* 2009; Simmons *et al.* 2012, 2014).

Multigenerational, extrachromosomal inheritance is likely mediated by piRNAs

Robust P-cytotype regulation depends on multiple generations of maternal transmission of P-cytotype conferring

Figure 5 Transmission genetics of the P cytotype. Maternal and paternal chromosomes are shown in pink and blue, while offspring assayed for *P*-element excision (males) and gonadal dysgenesis (GD) (females) are shown in black. Note that excision can also be analyzed in female germlines (Stuart *et al.* 2002), however, this assay is not performed as consistently across studies, and therefore is excluded from the figure. Plus signs indicate the amount of *P* activity, with + denoting low activity (strong repression) while +++++ denotes high levels of activity (no repression). Repressive *P*-element insertions in the X-TAS piRNA cluster are denoted by red boxes, while nonrepressive insertions outside of piRNA clusters are denoted by gray boxes. (A) M and M' strains do not repress *P* activity in their offspring (Kidwell *et al.* 1977; Engels 1979a). (B) Females that are heterozygous for telomeric insertions in the X-TAS piRNA cluster provide partial repression of *P* activity that relies on a maternal effect (Ronsseray *et al.* 1993; Simmons *et al.* 2007a; Belinco *et al.* 2009). (C) Enhanced repression of *P* activity is observed when repressive insertions are combined with nonrepressive insertions outside of piRNA clusters (Ronsseray *et al.* 1993; Stuart *et al.* 2002; Simmons *et al.* 2004, 2007a). Additionally, nonrepressive insertions that are cotransmitted through females with repressive insertions acquire modest repression of *P* activity (Simmons *et al.* 2007a; Belinco *et al.* 2009). (D–F) Grand-maternally inherited repressive insertions confer stronger maternal repression (D) of *P*-element activity than grand-paternally inherited repressive insertions (E). However, this is rescued by the presence of a repressive insertion in the grand-maternal genotype (F) (Marin *et al.* 2000; Niemi *et al.* 2004).

elements. The first demonstration of multigenerational effects came from experiments that introduced P-strain chromosomes into an M strain cytoplasm. After two generations, the lines mostly failed to exhibit strong P cytotype, indicating that the mother's genotype does not exclusively determine repression in her offspring (Sved 1987). The requirement for multiple generations of maternal transmission was even more clearly demonstrated by the observation that maternal *TP*s that are themselves maternally inherited (*i.e.*, grand-maternally derived) confer stronger repression of *P*-element activity in offspring than those that are paternally inherited (*i.e.*, grand-paternally derived) (Figure 5D vs. Figure 5E) (Ronsseray *et al.* 1993; Stuart *et al.* 2002; Simmons *et al.* 2004, 2007a). The required grand-maternal effects are extrachromosomal, as the increased *P*-element activity that is associated with the grand-paternal inheritance of a *TP* is partially or completely rescued by the presence of a nontransmitted *TP* in the grand-maternal genotype (Figure 5F) (Ronsseray *et al.* 1993; Niemi *et al.* 2004). Even more intriguing, different *TP* alleles are able to “complement” each other for grand-maternal effects (Marin *et al.* 2000; Niemi *et al.* 2004), revealing that the required extrachromosomal factor is largely interchangeable between different P-cytotype conferring insertions. Although the extrachromosomal factor was originally proposed to be repressor protein, the

exclusively zygotic behavior of repressor protein regulation (Robertson and Engels 1989; Misra and Rio 1990; Lemaitre *et al.* 1993; Simmons *et al.* 2002; Jensen *et al.* 2008) and the absence of a correlation between maternal repressor protein production and maternal repression (Misra *et al.* 1993) precluded this possibility.

The piRNA pool provides an obvious vehicle for the multigenerational, extrachromosomal transmission of P-cytotype regulation. Maternally-deposited piRNAs are known to exhibit extrachromosomal transmission of silencing by feeding forward piRNA production from sequences that are exclusively inherited paternally (de Vanssay *et al.* 2012). Additionally, the disruption of piRNA-mediated silencing has multigenerational effects on P-cytotype regulation, which are easily explained by persisting perturbations in the piRNA pool. The dosage sensitivity of the P cytotype to Aub, Piwi, and HP1 persists for multiple generations after loss-of-function alleles of the genes encoding these proteins are removed from the maternal background (Haley *et al.* 2005; Belinco *et al.* 2009). Reciprocally, the impact of decreased dosage accumulates with increasingly severe loss of P-cytotype repression when *TP*s and loss-of-function alleles for *aub*, *piwi*, and *Su(var)205* (which encodes HP1) are cotransmitted through females for multiple generations (Haley *et al.* 2005; Belinco *et al.* 2009).

Uncovering the Evolution of P-element Regulation in Natural Populations

The acquisition of *P*-element regulation in natural populations is a stunning example of rapid evolution. Armed with a mechanistic understanding of the *P* cytotype and genomic technologies for examining the structure, distribution, and piRNA targeting of *P* elements in natural populations, this classic system is ripe for rediscovery. Below I consider both old and new questions that await investigation.

Repressor proteins or piRNAs?

Although the *P* cytotype is best explained by piRNA-mediated silencing, repressor proteins and piRNAs are not mutually-exclusive mechanisms of *P*-element regulation: they likely coexist within populations and individuals. When transmitted maternally, piRNAs enforce stronger repression of *P*-element activity than repressor proteins (Jensen *et al.* 2008), particularly with respect to ovarian atrophy, which is very rarely reduced by the presence of a repressor-encoding element (Robertson and Engels 1989; Gloor *et al.* 1993; Misra *et al.* 1993). However, repressor proteins could still play a complementary role to that of piRNAs in regulating *P*-element activity. A recent study suggests that maternally-deposited piRNAs enhance zygotic regulation by type II repressor proteins (Simmons *et al.* 2015). Furthermore, zygotic repression is beneficial for the offspring of crosses between *P* element-harboring males and naïve females, where piRNA-mediated regulation is expected to be absent or weak.

P strains, which by definition exhibit maternal-effect silencing, are most likely regulating *P* elements through piRNAs. Given the worldwide prevalence of *P* strains, particularly in American populations where they compose >60% of sampled strains (Kidwell 1983; Itoh *et al.* 2007), it can be inferred that piRNA-mediated silencing is an important regulator of *P*-element activity. Further supporting a role for piRNA-mediated silencing, *P* insertions in cytological position 1A, where the *X-TAS* piRNA cluster resides, are common in natural populations (Ajioka and Eanes 1989; Ronsseray *et al.* 1989b; Biémont *et al.* 1994).

Q strains, which repress *P* activity but do not induce hybrid dysgenesis, are also common worldwide, particularly in Europe (Onder and Kasap 2014), Asia (Itoh *et al.* 2001, 2004), and Oceania (Ogura *et al.* 2007), where *P* strains are comparatively rare. Because *Q* strains by definition do not exhibit a maternal effect in reference crosses (Figure 3B), their regulation of *P* activity cannot be attributed to piRNA-mediated silencing based on their strain classification. Nevertheless, more comprehensive genetic analysis has demonstrated that many *Q* strains regulate *P* elements through a maternal effect (Black *et al.* 1987; Lemaitre *et al.* 1993; Ronsseray *et al.* 1996; Simmons *et al.* 2004). Additionally, multiple *Q* strains have been demonstrated to produce piRNAs (Brennecke *et al.* 2008), and to have *P*-element insertions in the *X-TAS* piRNA cluster (Ronsseray *et al.* 1996; Marin *et al.* 2000; Stuart *et al.* 2002).

Other *Q* strains exhibit *P*-element regulation that is purely zygotic (Jackson *et al.* 1988), a genetic behavior that is more consistent with the action of repressor proteins than piRNAs (Robertson and Engels 1989; Misra and Rio 1990; Lemaitre *et al.* 1993; Simmons *et al.* 2002; Jensen *et al.* 2008). However, strictly zygotic regulation does not necessarily exclude a role for piRNA-mediated silencing. At least one piRNA cluster that is active in the female germline, *cluster 20A*, is not dependent on ping-pong amplification for piRNA production (Zhang *et al.* 2014) and therefore may not rely on maternally-transmitted piRNAs to feed forward silencing. Indeed, while precursors are produced from most piRNA clusters by noncanonical, bidirectional transcription; *cluster 20A* is unidirectionally transcribed in a manner that resembles protein coding genes (Mohn *et al.* 2014; Zhang *et al.* 2014). Thus, *P*-element insertions residing in *cluster 20A* could account for zygotic repression in some *Q* strains. However, *P*-element insertions in cytological position 20A, which harbors *cluster 20A*, are relatively rare in natural populations (Ronsseray *et al.* 1989b; Biémont 1994), which is inconsistent with the reported prevalence of zygotic *P*-element repression (Jackson *et al.* 1988).

The *KP* element, a type II repressor that was originally identified in a wild-derived strain from Krasnodar, Russia, is widespread in extant genomes (Black *et al.* 1987; Jackson *et al.* 1988; Itoh and Boussy 2002; Itoh *et al.* 2004, 2007; Ogura *et al.* 2007; Onder and Kasap 2014) and provides an appealing explanation for the prevalence of zygotic repression. However, evidence that *KP* elements are important repressors of *P* activity in wild-derived genomes remains mixed, despite observations that transgenically expressed *KP* elements confer modest zygotic repression (Simmons *et al.* 2002; Jensen *et al.* 2008). Early studies suggested that the presence of *KP* elements in wild-derived genomes is associated with repression (Black *et al.* 1987; Jackson *et al.* 1988). By contrast, more extensive analyses, which quantified *KP* elements in wild-derived genomes, did not find evidence of a linear relationship between *KP* abundance and the strength of *P*-element regulation (Boussy *et al.* 1988; Itoh and Boussy 2002; Itoh *et al.* 2007; Ogura *et al.* 2007; Fukui *et al.* 2008).

Existing studies of the regulatory role of *KP* elements are limited by two major factors. First, they do not focus on strains that exhibit exclusively zygotic repression, meaning that *KP*-mediated repression and piRNA-mediated repression are confounded. Although weak piRNA-mediated silencing may enhance *KP* repression (Simmons *et al.* 2015), strong piRNA-mediated silencing would most likely swamp the modest impact of *KP* elements when found in the same genetic background. The second limitation of many studies of *KP*-mediated repression is that they often relied on the presence and intensity of a particular restriction fragment size to identify and quantify *KP* elements. With this approach, internally-deleted elements of similar size to *KP*, but not encoding a repressor protein, would obscure the relationship between the abundance of *KP* elements and repression.

Furthermore, quantifying band intensity from gel electrophoresis is not a sensitive measure of copy number.

Population genomics offers the path forward toward examining whether particular structural variants, such as *KP*, contribute to repression in natural populations. The relative genomic abundance of particular variants can be determined simply by aligning short reads to a consensus sequence for the TE family (Kofler *et al.* 2015a), an approach that could easily be used to compare *KP* abundance among different wild-derived genotypes. Furthermore, recent technological advancements promise to greatly enhance our ability to examine structural variation at the level of individual TE insertions (Voskoboynik *et al.* 2013; Kim *et al.* 2014; McCoy *et al.* 2014; Berlin *et al.* 2015; Hall *et al.* 2016), which has historically been prevented by the impossibility of assigning most TE-derived sequencing reads to a particular genomic location. Synthetic long-range sequencing, a new method of sequencing library construction, avoids this problem by employing a hierarchical approach that reduces or eliminates multiply mapping reads, thereby empowering the assembly of individual TE insertions (Voskoboynik *et al.* 2013; McCoy *et al.* 2014). Alternatively, the very long reads generated by single molecule real time sequencing (10–15 kb) generally include the full length of a TE insertion, negating the requirement for assembly entirely (Kim *et al.* 2014; Berlin *et al.* 2015; Hall *et al.* 2016).

Mutation and selection in the rapid evolution of host repression

American populations evolved repression of *P*-element transposition in <40 years (Kidwell 1983). Although such rapid phenotypic change could imply strong directional selection, population genetic theory has historically suggested that in sexually reproducing eukaryotes, TE repression is only weakly beneficial (Charlesworth and Langley 1986; Nuzhdin 1999; Lee and Langley 2012). Although repressor alleles confer an advantage by reducing the occurrence of new deleterious TE insertions, recombination and independent assortment rapidly separate repressors from the chromosomes that they have protected from TE-induced mutational load (Charlesworth and Langley 1986). Therefore, the benefit of repression is ephemeral, and the adaptive evolution of TE repression is expected to be rare. This impact of recombination is expected to be even more pronounced in the presence of maternal repression, because repressor alleles are separated from the fitness effects of new TE insertions by an additional generation.

An important caveat to the argument that recombination limits the benefit of repression by ameliorating mutational load is that *P* elements also impose fitness costs by causing hybrid dysgenesis, and the selective advantage of repressing hybrid dysgenesis would not be limited by recombination. To explore the benefits of repressing hybrid dysgenesis, Lee and Langley (2012) examined the strength of selection on a *P* cytotypic-independent repressor of *P*-element transposition and hybrid dysgenesis during genome invasion with a deterministic model. They observed a sizable selective advantage

for the repressor allele (~0.6%) when hybrid dysgenesis was common. However, their model assumed that all genomes harboring *P* elements exhibit the *P* cytotypic, limiting the time-period during which the repressor is beneficial to the narrow window where *P* cytotypes are common but naïve genotypes also persist in the population. Since the occurrence of *P'* genomes clearly reveal that the presence of active *P* elements is not sufficient to establish repression (Itoh *et al.* 2007; Onder and Kasap 2014), relaxing the assumption that *P*-elements instantly produce *P* cytotypic is justified and would prolong the period during which repressor alleles enhance fitness.

In a complementary model, Lu and Clark (2010) explicitly examined the evolution of piRNA-mediated silencing in a *Drosophila*-like genome through forward simulation. A retrotransposon was allowed to increase in frequency from an initially low copy number (two copies per genome), and repression evolved via random transposition into piRNA clusters. Repressors enjoyed a selective advantage by decreasing the occurrence of dominant lethal TE insertions in an individual's gametes. Their simulations revealed that TE insertions in piRNA clusters segregated at higher allele frequencies when compared to nonrepressive insertions outside of piRNA clusters, suggesting that they were beneficial. Furthermore, they potentially underestimate the impact of selection on *P*-element insertions in piRNA clusters, because the transposition rates that they considered (~10⁻⁴ new insertions/element/genome), and therefore the frequency of dominant lethal insertions that select for repression, are considerably lower than those estimated for *P* elements (10⁻¹–10⁻³) (Eggleston *et al.* 1988; Robertson *et al.* 1988; Berg and Spradling 1991; Kimura and Kidwell 1994). Additionally, the model did not incorporate the benefits of repressing hybrid dysgenesis.

Although the extent to which piRNA or *KP* repressors have been targets of selection in natural populations of *D. melanogaster* remains to be elucidated, numerous repressor alleles have been documented. Thus far, seven unique *P*-element insertion alleles in the X-TAS piRNA cluster have been isolated from natural populations and demonstrated to confer the *P* cytotypic (Ronsseray *et al.* 1996; Marin *et al.* 2000; Stuart *et al.* 2002). Additionally, many *P* cytotypes must be established by insertions into other piRNA clusters, because a worldwide sampling of *P* strains revealed that only ~50% harbor a *P*-element insertion in cytological position 1A (Ronsseray *et al.* 1989b). Similarly, the *KP* structural variant is extremely prevalent in wild-derived genomes (Itoh and Boussy 2002; Itoh *et al.* 2007; Ogura *et al.* 2007; Kofler *et al.* 2015a), however, no single euchromatic *P*-element insertion (*KP* or otherwise) is observed to segregate at high frequency (Zhuang *et al.* 2014; Kofler *et al.* 2015b). A plethora of repressor alleles have therefore arisen in *D. melanogaster* populations since the invasion of the *P* element.

Although traditional models of adaptive molecular evolution propose “hard sweeps”, in which individual adaptive mutations are driven to fixation, the presence of multiple putatively-adaptive alleles does not exclude a role for positive

selection. Recent theory suggests that when the effective population size is large or the mutation rate is high, multiple beneficial alleles can contribute to adaptive evolution through “soft sweeps” (Pennings and Hermisson 2006; Karasov *et al.* 2010). The effective population size of *D. melanogaster* over the last 150 years is thought to be much larger than the historic value ($\sim 10^8$ vs. $\sim 10^6$), which has been proposed to explain the incidence of soft sweeps in an insecticide resistance locus (Karasov *et al.* 2010). Similarly, both *KP* and piRNA-mediated repressor alleles are produced by transposition, and the unrepressed transposition rates of an individual *P* element (10^{-1} – 10^{-3} new insertions/element/generation) (Eggleston *et al.* 1988; Robertson *et al.* 1988; Berg and Spradling 1991; Kimura and Kidwell 1994) is approximately seven orders of magnitude higher than the per-site/per-generation mutation rate for the *D. melanogaster* genome (8.4×10^{-9}) (Haag-Liautard *et al.* 2007). Even though only a subset of new insertions will act as repressors, namely those that occur in piRNA clusters, or *KP* insertions that are expressed in the germline, the mutation rate to repressive insertions is still expected to be quite high. For example, assuming random transposition, $\sim 2.4\%$ of new *P*-element insertions will occur in existing piRNA clusters (Brennecke *et al.* 2007), predicting an overall mutation rate to piRNA-mediated repressor alleles of 10^{-3} – 10^{-5} (new repressors/*P*-element copy/generation). *P* elements also exhibit an insertional bias for the X-TAS repeats (Karpen and Spradling 1992), further elevating the expected mutation rate to piRNA-mediated repressor alleles.

If repressive *P*-element insertions in natural populations are beneficial mutations, it is predicted that their allele-frequency spectrum will include more common variants when compared to that of nonrepressive insertions (Lu and Clark 2010). However, performing this test of selection requires curating these alleles among wild-derived chromosomes, a goal that has been limited by the challenge of examining repetitive elements in short-read sequence data. Although population genomic approaches for TE annotation have improved greatly (Kofler *et al.* 2012; Cridland *et al.* 2013; Zhuang *et al.* 2014; Fiston-Lavier *et al.* 2015; Rahman *et al.* 2015), the repetitive nature of piRNA clusters poses a particular problem for annotating insertions in these genomic regions. Similarly, determining the population frequencies of *KP* insertions requires not just annotating, but also assembling, specific *P* elements in order to localize structural variants to particular insertion sites. The aforementioned new approaches in library preparation and TE assembly offer great promise to overcome these obstacles in the near future (Voskoboinik *et al.* 2013; McCoy *et al.* 2014; Berlin *et al.* 2015), allowing for the detection of positive selection on repressive *P*-element insertions.

Epimutation of piRNA clusters could accelerate the evolution of host repression

Epigenetic mutation, or epimutation, occurs when the phenotypic effect of an allele is altered without changes to its DNA

sequence. Recent studies reveal that piRNAs act as agents of epimutation, promoting regions of the genome that are quiescent with respect to piRNA production to emerge as novel piRNA clusters (de Vanssay *et al.* 2012; Erwin *et al.* 2015). Therefore, in addition to the random insertion of *P* elements into existing piRNA clusters, novel piRNA-producing, *P*-element insertions could also be produced through epimutation; further facilitating the evolution to repression.

piRNA-mediated epimutation is best understood when it occurs between two epialleles, one of which is an active source of piRNAs and other of which is quiescent with respect to piRNA production. In these examples, cotransmission through females of the two alternate alleles initiated piRNA production from the quiescent allele, which then persisted over multiple generations of maternal transmission (Figure 6A; de Vanssay *et al.* 2012; Erwin *et al.* 2015). In *D. melanogaster*, epimutated piRNA cluster alleles have been demonstrated to maintain piRNA production for >50 generations, suggesting that some piRNA cluster epialleles are very stable (de Vanssay *et al.* 2012). While less extensively examined, nonallelic epimutation has also been demonstrated, in which a piRNA cluster in one location epimutates partially-homologous sequences at a second location into a new piRNA cluster epiallele (Figure 6B; de Vanssay *et al.* 2012). Epimutation, therefore, could produce new piRNA clusters.

Although the mechanism remains an area of active research, epimutation of piRNA clusters is mediated by piRNAs themselves in a sequence-specific manner. For a genomic region of interest, the presence of homologous piRNAs in the ooplasm is associated with the deposition of H3K9me3 and the onset of piRNA production (Olovnikov *et al.* 2013; Le Thomas *et al.* 2014a,b; Shpiz *et al.* 2014). Experiments in which tandemly-duplicated arrays of transgenes were epimutated into piRNA clusters suggest that higher-copy arrays are both more likely to undergo epimutation, and more likely to stably maintain piRNA production over successive generations after they have been epimutated, rather than spontaneously reverting to the quiescent state (de Vanssay *et al.* 2012). Therefore, a threshold amount of homologous piRNA may be required to produce and maintain a piRNA cluster epiallele.

Interestingly, some *TPs* have been demonstrated to act as epimutators, converting nonrepressive *P* element-bearing chromosomes from *M'* strains into modest repressors when the two are cotransmitted through the female germline (Simmons *et al.* 2007a; Belinco *et al.* 2009). In the absence of the epimutator *TP*, the acquired repression of *M'* chromosomes persisted over several generations of maternal transmission, but attenuated in strength (Figure 5C, as compared to Figure 5A) (Simmons *et al.* 2007a; Belinco *et al.* 2009). Although the attenuation of the repression conferred by the *M'* chromosomes indicates that these particular epialleles were not stable, it remains possible that an unexplored subset of *P*-element insertions could be converted into stable epialleles, propagating piRNA-mediated repression over many successive generations.

Figure 6 Epimutation of piRNA clusters. (A) In instances of allelic epimutation, a maternally-inherited piRNA cluster epimutates the allelic region on a paternally-inherited chromosome, which was previously quiescent with respect to piRNA production, into a piRNA cluster. (B) In instances of nonallelic epimutation, a maternally-inherited piRNA cluster epimutates a nonallelic region on a paternally-inherited region which was previously quiescent with respect to piRNA production, into a piRNA cluster. In both the allelic and nonallelic examples, the piRNA cluster epi-

allele can be propagated through females for several generations in the absence of the original maternally-inherited epimutator. piRNA production in a particular genomic region is denoted by a ☆. Epimutation is indicated by a dashed arrow connecting two regions or genomic loci. Chromosomes that are inherited paternally or maternally in the female where the epimutation event occurs are indicated in pink and blue, respectively. Multiple successive generations of maternal transmission are indicated by multiple successive arrows.

Even the unstable epimutation exhibited by M' chromosomes has interesting implications for the evolutionary dynamics of *P*-element repression. If *P*-element insertions in stable piRNA clusters (*i.e.*, clusters that do not spontaneously revert to a quiescent state) are mild epimutators, then they could act as a type of reservoir for piRNA-mediated repression in natural populations. With a high-enough frequency of these epimutators in a population, most individuals would be phenotypically repressive, either because they themselves carry a *P*-element insertion in a stable piRNA cluster, or because some maternally-inherited *P* elements in their genomes were recently epimutated into unstable piRNA clusters. These sorts of dynamics could simultaneously increase the frequency of the repressive phenotype in natural populations, and reduce the strength of selection on *P*-element insertions in stable piRNA clusters by lowering their selective advantage relative to epimutable *P* elements that form unstable piRNA clusters.

Broader Implications

The *P*-element invasion into the *D. melanogaster* genome has advanced our understanding of the evolutionary dynamics that occur between TEs and their hosts. In particular, the historical timescale of the *P*-element invasion presents a powerful opportunity to dissect the evolution of repression, with a newly-discovered invasion into *D. simulans* allowing for parallel comparisons in an independent genetic lineage (Kofler *et al.* 2015a; Hill *et al.* 2016). The recent discovery of the piRNA pathway now paves the way for a deeper understanding of how host genomes respond to invasion by novel TEs by revealing the molecular and genetic framework within which repression evolves.

Insights into the evolution of piRNA-mediated silencing that are taken from the *P*-element invasion into *D. melanogaster* are likely to have general implications that extend far outside this particular system. piRNA-mediated regulation of TEs is largely conserved among metazoans (reviewed in Aravin *et al.* 2007; Mani and Juliano 2013). More generally, small RNAs are implicated in TE regulation in almost all taxa, including bacteria (van der Oost *et al.* 2009; Blumenstiel 2011; Barrangou 2015). Therefore, many features of the evolution of *P*-element repression,

such as the production of repressive alleles via transposition into small RNA-encoding genomic regions, and the potential for epimutation, are likely to be of general significance.

An emerging conserved feature of small RNA-mediated silencing of TEs is the presence of genomic regions that, analogous to piRNA clusters, harbor sequences to be targeted for silencing. In *C. elegans*, many of the endogenous small interfering RNAs (siRNAs) that control TEs are produced from clusters of silenced insertions (Ni *et al.* 2014). Similarly, endogenous siRNAs that regulate plant TEs are proposed to derive from degenerate insertions residing in heterochromatin (Lisch and Bennetzen 2011). It is appealing to speculate that the genomic architecture of small RNA-mediated TE silencing, in which large repetitive regions give rise to small RNAs that establish genome-wide regulation, facilitates the evolutionary acquisition of repression. These genomic regions provide a sizable mutational target for the random insertion of an invading TE, while also avoiding the potential for new insertions to disrupt functional sequences like protein-coding genes. If new small RNA-encoding regions often arise through epimutation, this would further accelerate the evolution of silencing.

The maternal transmission of piRNAs and its consequences for TE regulation also has striking parallels in other systems. Five hybrid dysgenesis syndromes have been documented in *Drosophila*, all of which are associated with the exclusive or predominant paternal inheritance of a TE family (Bingham *et al.* 1982; Bucheton *et al.* 1984; Blackman *et al.* 1987; Lozovskaya *et al.* 1990; Hill *et al.* 2016). In three of these cases, the dysgenesis syndrome has been attributed to a paucity of maternally-deposited piRNAs (Blumenstiel and Hartl 2005; Brennecke *et al.* 2008; Chambeyron *et al.* 2008; Rozhkov *et al.* 2010). Outside of *Drosophila*, TE derepression has been observed in interspecific hybrids of both mammals and plants (O'Neill *et al.* 1998; Josefsson *et al.* 2006; Kenan-Eichler *et al.* 2011). Therefore, the uniparental transmission of TE regulation, through small RNAs or other means, may be a general mechanism that allows for TE derepression in the offspring of crosses between divergent lineages. Indeed, in *Arabidopsis*, endogenous siRNAs targeting the *Athila* retrotransposon are generated and transmitted exclusively through male pollen (Slotkin *et al.* 2009).

The piRNA-independent mechanisms of *P*-element regulation, namely alternative splicing of transposase transcripts (Laski *et al.* 1986; Rio *et al.* 1986) and the production of transposon-encoded repressor proteins (Misra and Rio 1990), are also paralleled by other DNA transposons. For example, in *D. mojavensis*, alternative splicing of the *Bari3* transposase-encoding transcript occurs when it is overexpressed, and may dampen transposase production (Palazzo *et al.* 2014). Additionally, many bacterial transposons produce alternative transposase transcripts that encode repressor proteins (de la Cruz *et al.* 1993; Salvatore *et al.* 2001; Bellanger *et al.* 2008). These bacterial transposon repressors are posited to act through competitive inhibition and the formation of nonfunctional multimers (de la Cruz *et al.* 1993; Salvatore *et al.* 2001), as has been proposed for the *KP* repressor protein (Lee *et al.* 1996, 1998). Collectively, therefore, the *P*-element invasion of *D. melanogaster* provides opportunities to examine the concurrent evolution of TE regulation through both small RNA-dependent and independent mechanisms.

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