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The cellular basis of hybrid dysgenesis and *Stellate* regulation in *Drosophila*

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During normal tissue development, the accumulation of unrepaired cellular and genomic damage can impair growth and ultimately leads to death. To preserve cellular integrity, cells employ a number of defense mechanisms including molecular checkpoints, during which development is halted while dedicated pathways attempt repair. This process is most critical in germline tissues where cellular damage directly threatens an organism's reproductive capacity and offspring viability. In the fruit fly, *Drosophila melanogaster*, germline development has been extensively studied for over a century and the breadth of our knowledge has flourished in the genomics age. Intriguingly, several peculiar phenomena that trigger catastrophic germline damage described decades ago, still endure only a partial understanding of the underlying molecular causes. A deeper reexamination using new molecular and genetic tools may greatly benefit our understanding of host system biology. Among these, and the focus of this concise review, are hybrid dysgenesis and an intragenomic conflict that pits the X and Y sex chromosomes against each other.

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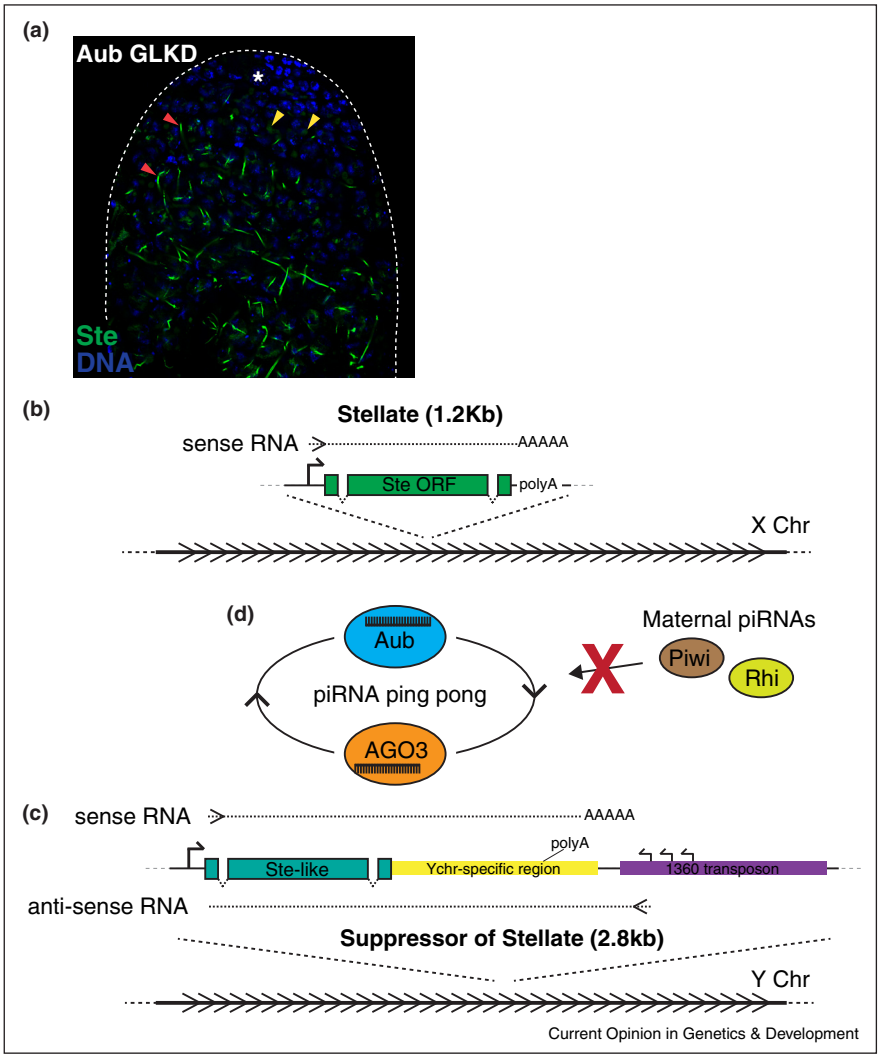
Stellate gene regulation by the piRNA pathway in *Drosophila* testes

The *Drosophila* X chromosome contains intriguing sets of tandem repeats of a gene called *Stellate* (*Ste*) [1], which when overexpressed leads to the accumulation of nuclear and cytoplasmic protein that ultimately aggregates into crystalline structures visible by both immunofluorescence [2] and phase contrast [3] (Figure 1a). The *Ste* gene encodes a putative β subunit of casein kinase II [4,5] and *Ste* repeat loci reside in distinct hetero-chromatic and eu-chromatic loci in numbers ranging from 10 to 400 [2–7] (Figure 1b). Depending on the number of *Ste* copies found in the genome, crystal formation can present as either needle or star structures [3,5] and is linked to meiotic chromosome condensation and segregation defects during primary spermatogenesis, leading to at least partial sterility [8–11]. Ste protein aggregation was initially observed in flies lacking a Y chromosome (XO), which develop into phenotypic, yet sterile, males [3]. This led to the postulation and subsequent discovery of a paralogous, repetitive locus on the Y-chromosome that serves as the key regulator of *Ste* expression and the maintenance of developmental robustness in the *Drosophila* testes.

Initially identified as the *crystal* (*cry*) locus [12,13], *Suppressor of Stellate (Su(Ste))* [4] is a *Ste*-related transcriptional unit repeated approximately 80 times on the Y chromosome [1,14,15]. The repeating *Su(Ste)* units are 2800nt in length and consist of a non-coding *Ste*-homologous region, a Y chromosome-specific AT-rich segment, and the insertion of a 1360 DNA transposable element [14] (Figure 1c). The *1360* transposon is highly correlated with heterochromatic loci [16], and may influence chromatin state at the repetitive *Su(Ste)* locus. Since *Ste* promoter activity is elevated specifically in the testes germline [17,18], there appears to have been an evolutionary pressure towards heightened expression. Since *Su(Ste)* shares sequence similarity to *Ste*, it is likely that *Su(Ste)* evolved from *Ste* and acquired the ability to maintain germline integrity and serve as a checkpoint ensuring proper Y segregation by regulating the overexpression of *Ste*. The key to *Su(Ste)*'s capacity to regulate *Ste* overexpression appears to be the *1360* transposon insertion, which provides a promoter that transcribes opposite of the still-present 5′ Ste-homologous region promoter. Together, these two promoters produce sense and antisense RNA transcripts [19], a key first step in RNA interference (RNAi). This led to the discovery that *Su(Ste)* double-stranded non-coding RNAs serve as the precursors to repeat-associated small interfering RNAs (rasi-RNAs), which regulate *Ste* expression through the piwi-interacting RNA (piRNA) pathway [19–21].

In the *Drosophila* testis germline, Aub and AGO3, two of the three argonaute proteins of the Piwi clade, act to regulate transposable element and gene expression, including *Ste* [22,23]. In addition to post-transcriptionally

Figure 1



The *Stellate* silencing system. **(a)** Stellate protein crystals form in germline RNAi knockdowns (GLKD) of *Aub* (Dapi staining of DNA is in blue, Stellate is green, testis is outlined in a white dotted line, * indicates the germline stem cell hub, yellow arrows show nuclear Stellate accumulation, and red arrows show crystals). **(b)** *Stellate* gene repeats on the X chromosome are transcribed in the sense orientation and are poly-adenylated. **(c)** *Su(Ste)* repeats on the Y chromosome are transcribed in sense and antisense orientations, and these transcripts serve as precursors to piRNAs produced during ping pong between Aub and AGO3 **(d)**.
Partially adapted from [19].

regulating target RNAs, Aub and AGO3 act to increase piRNA levels through an adaptive amplification loop termed ping-pong [24,25] (Figure 1d). Here, Aub is loaded with small RNAs complementary to the target to be silenced (transposon transcripts or in this case, *Ste*), it slices the target RNA, and further processing leads to the production of a new piRNA, which is loaded into AGO3. AGO3 is now licensed to recognize antisense transcripts complementary to the original target transcript (in this case likely derived from *Su(Ste)*), again inducing cleavage and production of an additional piRNA to be loaded into Aub.

Because relatively few *Su(Ste)* piRNAs bound to Aub and AGO3 display ping-pong characteristics [26••], it has been questioned whether ping-pong acts to regulate *Ste* and amplify piRNA populations. However, *Su(Ste)* piRNAs from total testes RNA show robust ping-pong signatures ([26••] and unpublished data). Furthermore, other genes known to support ping-pong in the ovary are required for *Ste* regulation, including *spn-E*, *armi*, *zuc* and *squ* [11,22,27,28]. Also, many of these factors are found in pING-bodies of the testes [29], which associate with germcell nuage, a subcellular RNA-processing structure where many piRNA factors localize. This indicates that

piRNA-based regulation of *Ste* most likely depends upon ping-pong, but possibly in a manner diverged from that occurring in the ovary.

In support of a specialized system of ping-pong based regulation in the testis, several key factors essential for piRNA trans-generational inheritance in the ovary are missing in males. In particular, to carry-on the ping-pong cycle from one generation to the next, Piwi protein uses maternally synthesized piRNAs to mark ping-pong loci (Figure 1d). These loci are bound by the heterochromatin protein 1 (HP1) homolog Rhino (Rhi), which then licenses transcripts into the Aub/AGO3 ping-pong cycle [30,31,32•,33•]. Interestingly, while *Su(Ste)* piRNAs display clear ping-pong signatures, Piwi and Rhi are dispensable for *Ste* silencing [31]. This indicates that the initial steps in piRNA-based regulation of *Ste* are fundamentally different from those occurring in the ovarian germline. Additionally, while maternally deposited piRNAs trigger ping-pong amplification in newly forming germ cells [30,34•,35•], there is no evidence that *Su(Ste)* piRNAs generated in the male can be provided to the egg by the sperm. Thus, it will be interesting to more closely examine eu-chromatic and hetero-chromatic *Ste* transcript fate, and how sense and antisense *Su(Ste)* transcripts enter the amplification loop (Figure 1b,c).

There has not been a systematic approach to investigate male/testes-specific factors and their role in Ste gene regulation, which will most likely uncover additional layers of specialization in the male. Taken together, it is clear that the ping-pong and piRNA silencing system has adopted a unique mechanism to control Ste gene expression in *Drosophila* testes. This system may be more similar to mammalian piRNA pathways, which likely do not depend upon a maternal contribution and are not solely tasked with regulating transposable element activity.

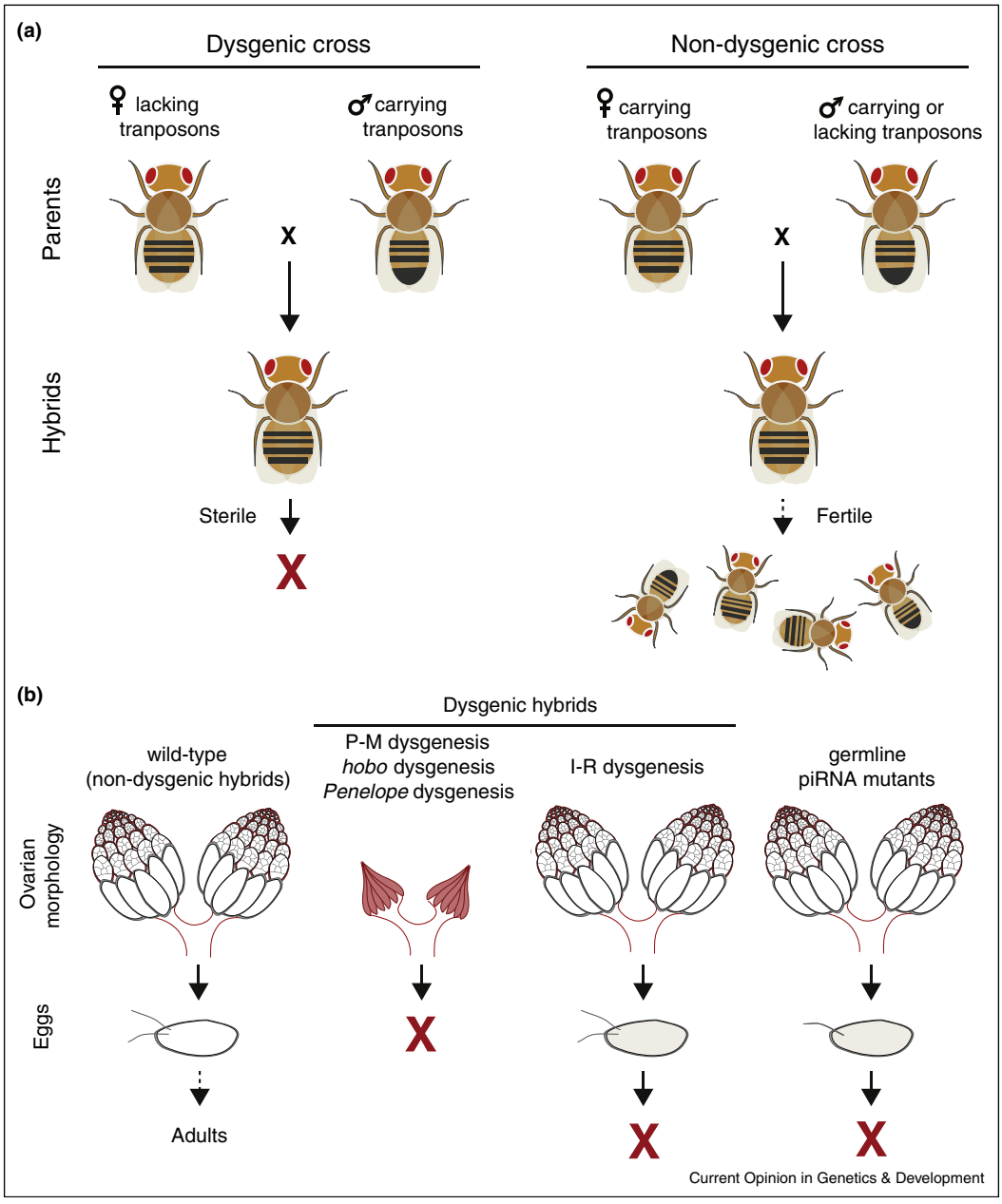
Hybrid dysgenesis

Hybrid dysgenesis is a syndrome that affects the offspring of crosses involving different strains of the same species and is commonly triggered by enhanced activity of transposable elements. Dysgenic aberrant traits are mainly restricted to the germline and can include chromosomal rearrangements, increased recombination frequency, high mutability, chromosome-transmission-rate distortion, and sterility [36]. Independent dysgenic systems were described in *Drosophila*, each relying on the activity of specific families of transposable elements such as the *P*-element (P-M system; [36]), the *I*-element (I-R system; [37,38]), *hobo* [39], and *Penelope* (dysgenesis in *Drosophila virilis*; [40]). In each system, dysgenesis is only observed in the offspring produced by crosses in which males carry transposable elements that females lack (Figure 2a). These and other observations led to a model whereby maternally inherited factors (also referred to as 'cytotype') protect the progeny against the activation of paternally inherited transposons.

The P-M dysgenesis is by far the most characterized hybrid dysfunction in *Drosophila* [41,42]. In fact, the molecular dissection of its causal agent led to the development of a series of tools that have been the basis of the *Drosophila* molecular genetics revolution [43,44]. In parallel, work focusing on the maternally inherited factors and the mechanisms required for the protection against *P*-element activation revealed a central role for the germline-specific RNAi machinery on transposon regulation. Early studies revealed that a typical *P*-strain contains about 40–60 *P*-insertions, a third of these consisting of complete, full-length elements. Remarkably, genetic analysis soon indicated that while dysgenesis relies on the activity of paternally inherited full-length elements, the maternally provided protective state (also known as '*P*-cytotype') does not strictly require active elements in the maternal genome [45,46]. In fact, even truncated *P*-elements inserted in a subtelomeric heterochromatin region (known as Telomere Associated Sequences or 'TAS') at the tip of the X chromosome were alone sufficient to recapitulate most aspects of the germline regulation provided by the original *P*-cytotype. For instance, these include maternal inheritance of *P*-cytotype, repression of *P*-elements *in trans*, and the protection against hybrid dysgenesis [47,48]. Transgenic insertions at the X-chromosome 'TAS' were then extensively used to dissect many aspects of cytotype inheritance, notably revealing that repression *in trans* is dependent on sequence homology and is particularly sensitivity to mutations affecting chromatin-associated proteins and germline-specific RNAi factors [49••,50–52].

Similarly to Ste regulation in *Drosophila* testis, the molecular nature of the maternally inherited cytotype was brought about by the discovery of germline piRNAs [20]. While other mechanisms of *P*-element regulation such as the maternal inheritance of the *P*-element-derived repressor protein have been described, it is now clear that the preeminent mode of cytotype regulation falls on piRNAs [53]. These small RNAs are mostly cognate to transposons and other repeats, and are encoded by specific genomic regions found at peri-centromeric and subtelomeric regions such as the 'TAS' regions [24]. Remarkably, mutations impairing the piRNA pathway lead to male and female sterility in flies and are associated with the de-repression of many transposon families, including the *I*-element [22,54–56]. In every dysgenic system tested so far, the ability to provide a protective cytotype was directly correlated with an over-accumulation of piRNAs cognate to the causal transposon [30,54,57]. In addition, germline-accumulated small RNAs and their bound partners Aub and Piwi, are maternally deposited into embryos, a process that is thought to fulfill the transgenerational character of the cytotype

Figure 2



Hybrid dysgenesis and its effects in germline development. **(a)** Females lacking transposable elements such as the *P*-element, the *I*-element, *hobo*, or *Penolope*, produce sterile hybrid offspring when crossed to males containing one of these transposons (dysgenic cross). On the other hand, when females carry respective transposable elements in their genomes, their offspring is fertile regardless of the paternal transposon contribution (non-dysgenic cross). **(b)** Disruption of germline development varies depending on the underlying dysgenic systems or mutations affecting the germline-specific branch of the piRNA pathway. P-M dysgenesis, as well as *hobo*-induced and *Penelope*-induced dysgenesis, induce gonadal atrophy and are characterized by impaired egg production. Ovary morphology is mostly unaffected in I-R dysgensis or in piRNA mutants. In these cases, sterility results from the arrest of embryonic development. Egg patterning defects, as well as persistent double strand breaks are mostly clear in piRNA mutants. Embryonic lethality induced by I-R dysgenesis is caused by catastrophic meiosis of the maternal pronuclei [59].

[30,56–58]. Indeed, recent studies revealed that maternally provided piRNAs are sufficient to trigger homology-dependent RNA processing as well as to promote chromatin changes in the next generation [34•,35•].

In contrast to hybrid dysgenesis repression, for which a pervasive molecular model has been consolidated over the last decade, little is known about how transposon activation causes sterility. The prevailing notion evokes

the disruption of genome integrity by enhanced transposition as a general path leading to sterility. However, marked developmental differences are observed not only depending on the dysgenic system but also in comparison to piRNA mutants (Figure 2b), suggesting these may trigger distinct developmental checkpoints. For instance, oogenesis seems mostly undisturbed in *I*-element dysgenesis, and subtle defects are only observed during karyosome formation [59•]. While this and the ultimate embryonic lethality are reminiscent of piRNA mutants, oogenesis in *I*-element-induced dysgenesis is not associated with other piRNA mutant landmarks, such as persistent double-strand breaks, formation of dynein aggregates, and egg patterning defects [59•,60,61]. On the other hand, *P*-element-induced, *hobo*-induced, and *Penelope*-induced dysgenesis are characterized by gonadal atrophy and germ cell loss [36,40,62,63] leading to adult females that are unable to produce eggs. While dysgenic phenotypes may be influenced by the nature of the causal transposon (i.e. expression level or transposition competency), the basis of the observed developmental differences remains to be determined. Thus, the genetic and molecular dissection of hybrid dysgenesis-induced phenotypes is likely to shed light into yet uncharacterized host mechanisms required for accessing and preserving cellular and genomic integrity during germline development.

Conclusion

Given the severity of developmental phenotypes in both Stellate misregulation and hybrid dysgenesis, there remain many outstanding questions to be answered. In the case of Stellate regulation, it will be key to determine the fate of sense and antisense derived transcripts, their processing by the piRNA machinery and the specialization of the pathway acting in the testis. Hybrid dysgenesis has such severe developmental consequences that cannot simply be explained by transposon activation alone, so again, important work is yet to show the precise causes of dysgenic phenotypes. With advances in molecular genetic tools including CRISPR and tissue specific RNAi, and the ease of generating high throughput sequencing data, there exists an opportunity to revisit these long standing phenomena to provide novel perspective to germ line function.

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