

piRNA-mediated regulation of transposon alternative splicing in soma and germline

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Transposable elements can drive genome evolution, but their enhanced activity is detrimental to the host and therefore must be tightly regulated¹. The piwi-interacting small RNAs (piRNAs) pathway is critically important for transposable element regulation, by inducing transcriptional silencing or post-transcriptional decay of mRNAs². Here, we show that piRNAs and piRNA biogenesis components regulate pre-mRNA splicing of *P* transposable element transcripts *in vivo*, leading to the production of the non-transposase-encoding mature mRNA isoform in germ cells. Unexpectedly, we show that the piRNA pathway components do not act to reduce *P*-element transposon transcript levels during *P-M* hybrid dysgenesis, a syndrome that affects germline development in *Drosophila*^{3,4}. Instead, splicing regulation is mechanistically achieved in concert with piRNA-mediated changes to repressive chromatin states, and relies on the function of the Piwi-piRNA complex proteins Asterix/Gtsf1⁵⁻⁷ and Panoramix/Silencio^{8,9}, as well as Heterochromatin Protein 1a (Su(var)205/HP1a). Furthermore, we show that this machinery, together with the piRNA *Flamenco* cluster¹⁰, not only controls the accumulation of Gypsy retrotransposon transcripts¹¹ but also regulates splicing of Gypsy mRNAs in cultured ovarian somatic cells, a process required for the production of infectious particles that can lead to heritable transposition events^{12,13}. Our findings identify splicing regulation as a new role and essential function for the Piwi pathway in protecting the genome against transposon mobility, and provide a model system for studying the role of chromatin structure in modulating alternative splicing during development.

Main text

Hybrid dysgenesis is a syndrome that affects progeny in a non-reciprocal fashion, being normally restricted to the offspring of crosses in which males carry transposable elements but which females lack (Fig. 1a)^{3,14}. In *Drosophila*, the dysgenic traits triggered by the *P*-element DNA transposon (Fig. 1b)⁴ are restricted to the germline and include chromosomal rearrangements, high rates of mutation, and sterility³. The impairment is most prominent when hybrids are grown at higher temperatures, with adult dysgenic females being completely sterile at 29°C (Fig. 1c)¹⁵. Despite the severe phenotypes, little is known about the development of germ cells during *P-M* dysgenesis. To address this, we characterized germline development in the progeny obtained from reciprocal crosses between *w¹¹¹⁸* (*P*-element-devoid strain) and *Harwich* (*P*-element-containing strain) at 29°C. In non-dysgenic progeny, germline development progressed normally throughout embryonic and larval stages (Fig. 1d,e), leading to fertile adults (Fig. 1c). While the development of dysgenic germline cells was not disturbed during embryogenesis, germ cells decreased in number during early larval stages, leading to animals with no germ cells by late larval stages (Fig. 1d,e). These results indicate that the detrimental effects elicited by *P*-element activity are triggered early on during primordial germ cell (PGC) development in dysgenic progeny, leading to premature germ cell death.

Maternally deposited small RNAs cognate to the *P*-element are thought to provide the ‘*P*-cytotype’ by conferring the transgenerationally inherited ability to protect developing germ cells against *P*-elements¹⁶. Small RNA-based transposon regulation is typically mediated either by transcriptional silencing or post-transcriptional clearance of mRNAs,

both of which result in a decrease in transposon mRNA accumulation². To understand how maternally provided small RNAs control *P*-elements in germ cells, we focused our analysis on embryonic PGCs sorted from 4- to 20-hours-old embryos generated from reciprocal crosses between *w¹¹⁸* and *Harwich*. Surprisingly, the accumulation of *P*-element RNA as measured by quantitative RT-PCR (RT-qPCR) showed no significant change in dysgenic PGCs when compared to non-dysgenic PGCs (ORF0-3; Fig. 1f). This indicates that ‘*P*-cytotype’ small RNAs exert their function by means other than regulating *P*-element mRNA levels.

P-element activity relies on production of a functional *P*-element transposase protein, the expression of which requires pre-mRNA splicing of three introns (IVS1-3; Fig. 1b)¹⁷. To analyze *P*-element RNA splicing in germ cells during hybrid dysgenesis, we designed primers that specifically anneal to spliced mRNA transcripts. The accumulation of spliced forms for the first two introns (IVS1 and IVS2) did not show significant changes in dysgenic PGCs when compared to non-dysgenic PGCs. By contrast, the accumulation of spliced transcripts for the third intron (IVS3) was substantially increased in dysgenic germ cells (Fig. 1f). Given that the overall accumulation of *P*-element mRNA showed no changes, our results indicate that the maternally provided ‘*P*-cytotype’ can negatively regulate *P*-element IVS3 splicing and therefore inhibits the production of functional *P*-transposase in germ cells.

Analysis of publically available small RNA sequencing data from 0- to 2-hours-old embryos laid by *Harwich* females¹⁶ indicated that two classes of small RNAs cognate to

the *P*-element are maternally-transmitted: small interfering RNAs (siRNAs, 20-22nt long) and piRNAs (23-29nt long; Extended data Fig. 1). To test the role of distinct small RNA populations on *P*-element expression, we analyzed mutants uniquely affecting each small RNA biogenesis pathway in the *Harwich* background. Mutations disrupting siRNA biogenesis components Dicer-2 (*dcr2*) and Argonaute 2 (*Ago2*), or mutations ablating components of the piRNA biogenesis pathway, such as the Argonautes *piwi*, *aubergine* (*aub*), and *Argonaute 3* (*ago3*), as well as the RNA helicase *vasa* (*vas*) and *spindle E* (*spnE*), did not affect *P*-element mRNA accumulation in adult ovaries as measured by RT-qPCR (ORF0-3; Fig. 2a). However, mutations disrupting piRNA biogenesis, and not the siRNA pathway, led to a strong and specific increase in the accumulation of IVS3 spliced mRNAs (Fig. 2a,b). RNA sequencing (RNA-seq) analysis on poly (A)-selected RNAs from *aub* and *piwi* mutant adult ovaries confirmed the specific effect on IVS3 splicing (Fig. 2c-e; Extended data Fig. 2a-e). To examine transposon expression *in tissue*, we performed RNA Fluorescent In Situ Hybridization (FISH) using probes specific for the *P*-element and for the Burdock retrotransposon, a classic target of the germline piRNA pathway⁶. In mutants affecting piRNA biogenesis, increased abundance of Burdock RNA was readily observed in germline tissues, with most of the signal accumulating close to the oocyte (Fig. 2f). By contrast, we did not detect a difference in *P*-element RNA FISH signal in piRNA biogenesis mutants compared to control. Nuclear RNA foci observed in nurse cells were of similar intensity and number regardless of the genotype, and cytoplasmic signal showed no detectable difference. Therefore, our results indicate that in germ cells, piRNAs modulate specifically IVS3 splicing. This regulation is reminiscent of the well-documented mechanism that restricts *P*-element activity to

germline tissues, which involves the expression of a host-encoded RNA binding repressor protein that negatively regulates IVS3 splicing in somatic tissues¹⁷.

In somatic tissues, *P*-element alternative splicing regulation is mediated by the assembly of a splicing repressor complex on an exonic splicing silencer (ESS) element directly upstream of IVS3¹⁷⁻¹⁹. To test whether the *P*-element IVS3 and flanking exon sequences were sufficient to trigger the piRNA-mediated splicing regulation in germ cells, we used a transgenic reporter system for IVS3 splicing in which a heterologous promoter (*hsp83*) drives the expression of an IVS3-*lacZ-neo* fusion mRNA specifically in the germline (Fig. 3a)²⁰. Using RT-qPCR, we analyzed the F1 progeny from reciprocal crosses between *w¹¹¹⁸* and *Harwich* flies in the presence of the *hsp83*-IVS3-*lacZ-neo* reporter (to avoid developmental defects, F1 progeny were raised at 18°C; for details see methods). The fraction of spliced mRNAs produced from the transgenic reporter was substantially increased in dysgenic compared to non-dysgenic adult ovaries (Fig. 3b; Extended data Fig. 3), in agreement with previously reported results²⁰. Most importantly, genetic experiments confirmed that the repression of IVS3 splicing in germ cells relies on piRNA biogenesis, as the splicing repression observed with this reporter in non-dysgenic progeny was specifically abolished in adult ovaries of *aub* and *vas* mutants (Fig. 3c; Extended data Fig. 3).

Mechanistically, piRNA-mediated splicing regulation may be achieved through direct action of piRNA-complexes on target pre-mRNAs carrying the IVS3 sequence or indirectly by piRNA-mediated changes in chromatin states. Piwi-interacting proteins such

as Asterix/Gtsf1 (Arx) and Panoramix/Silencio (Panx) are dispensable for piRNA biogenesis but are essential for establishing piwi-mediated chromatin changes, possibly by acting as a scaffold to recruit histone modifying enzymes and chromatin-binding proteins to target loci^{8,9}. To test the role of these chromatin regulators on *P*-element splicing, we performed germline-specific RNAi knockdown experiments in the *Harwich* background. Similar to what was observed for the piRNA biogenesis components (Fig. 2), germline knockdown of Arx and Panx showed no change in *P*-element RNA accumulation, but a strong and specific effect on IVS3 splicing in adult ovaries (Fig. 3d). The same pattern on IVS3 splicing was observed in the germline knockdown of the Heterochromatin Protein 1a/Su(var)205 (HP1a) and Maelstrom (Mael; Fig. 3d), both of which act downstream of Piwi-mediated targeting to modulate chromatin structure^{21,22}. The same genetic requirement for Panx for IVS3 splicing control was also confirmed when using the transgenic IVS3 splicing reporter (Fig. 3c; Extended data Fig. 3), further indicating that piwi-mediated chromatin changes at the target locus play a role in IVS3 splicing regulation. At target loci, Piwi-complexes are known to mediate the deposition of the classic heterochromatin mark histone H3 lysine 9 trimethylation (H3K9me3)²². To directly assess the effect of piRNA-targeting on *P*-element chromatin marks, we performed H3K9me3 chromatin immunoprecipitation on adult ovaries followed by sequencing (ChIP-seq) or quantitative PCR on the progeny from reciprocal crosses between *w¹¹¹⁸* and *Harwich* (to avoid developmental defects, ChIP was performed on F1 progeny raised at 18°C; for details see methods). This analysis revealed a significant and specific loss of global H3K9me3 levels over *P*-element insertions in dysgenic progeny when compared to non-dysgenic progeny (Extended data Fig. 4a-d).

To analyze the chromatin structure at individual *P*-element insertions, we used DNA-seq data to identify all euchromatic insertions in the *Harwich* strain (Supplementary Table 1; Extended data Fig. 5) and RNA-seq analysis to define transcriptionally active insertions (Extended data Fig. 6). At transcriptionally active *P*-element euchromatic insertions, spreading of H3K9me3 into the flanking genomic regions was readily observed in non-dysgenic progeny, but was completely absent in dysgenic offspring (Fig. 3e,f; Extended data Fig. 4e-g). Similarly, a significant loss of H3K9me3 modification levels was also observed over the IVS3 transgenic reporter in dysgenic progeny when compared to non-dysgenic progeny (Extended data Fig. 4h). Interestingly, euchromatic insertions with no evidence of transcriptional activity were devoid of H3K9me3 signal in both non-dysgenic and dysgenic crosses (Extended data Fig. 4i-j), providing further evidence for a model initially suggested in yeast²³ and more recently proposed for *Drosophila* and mammals, in which H3K9me3 deposition by piRNA-complexes would require transcription of the target loci^{22,24}. Mechanistically different from the well-described somatic repression, our results uncovered the existence of an unexpected piRNA-mediated, chromatin-based mechanism regulating IVS3 alternative splicing in germ cells (see also Supplementary Note 1).

To expand our analysis, we searched the literature for other cases of transposon splicing regulation. *Drosophila* Gypsy elements are retrotransposons that have retrovirus-like, infective capacity due to their Envelope (Env) protein (Fig. 4a)^{12,13}. These elements are expressed in somatic ovarian cells, where they are regulated by the *flamenco* locus¹⁰, a

well-known piRNA cluster that is a soma-specific source of anti-sense piRNAs cognate to Gypsy¹¹. Interestingly, it has been shown that mutations in *flamenco* not only elicited the accumulation of Gypsy RNA, but also modulated pre-mRNA splicing, favoring the production of the Env protein mRNA and therefore germline infection¹⁰. To test whether the piRNA pathway, in addition to its role in regulating the accumulation of Gypsy RNA¹¹, is also responsible for modulating the splicing of Gypsy elements in somatic tissues, we analyzed publically available RNA sequencing data from poly (A)-selected RNAs extracted from Ovarian Somatic Cell (OSC) *in vitro* cultures^{7,9,25}. Our analysis indicates that *piwi* knockdown was sufficient to modulate *Gypsy* splicing, favoring the accumulation of *env*-encoding mRNA (Fig. 4b,c). In agreement with a chromatin-mediated regulation of alternative splicing, RNAi depletion of Arx, Panx, HP1a, Mael, as well as knockdown of the histone linker H1 was sufficient to favor Gypsy splicing, recapitulating the effect caused by Piwi depletion (Fig. 4c; Extended data Fig. 7). Interestingly, this was also the case for the H3K9 methyltransferase Setdb1, but not for the H3K9 methyltransferases Su(var)3-9 and G9a, indicating specific genetic requirements (Fig. 4c; Extended data Fig. 7). Taken together, our results indicate that the piRNA pathway, through its role in mediating changes in chromatin states, regulates the splicing of transposon pre-mRNAs in both somatic and germline tissues.

Using *P-M* hybrid dysgenesis as a model, we have uncovered splicing regulation elicited by chromatin changes as a previously unknown mechanism by which the piRNA pathway protects the genome against the detrimental effects of transposon activity. Splicing control at piRNA-target loci is likely to be mechanistically different from what has been

observed for germline piRNA clusters given the low enrichment of the HP1-homolog Rhino (HP1D) protein, which is required for piRNA cluster RNA processing²⁶, over the endogenous *P*-element insertions in the Harwich genome or over the transgenic IVS3 splicing reporter in non-dysgenic and dysgenic progeny (as measured by ChIP-qPCR; extended data Figure 8). Because small RNA-based systems leading to chromatin mark changes at target loci are pervasive in eukaryotes²³, we expect this new type of targeted regulation to be of significance in settings far beyond the scope of the piRNA pathway and *Drosophila*. Indeed, small RNA-guided DNA methylation over the LINE retrotransposon *Karma* was recently shown to modulate alternative splicing in oil palm, disrupting nearby gene expression and ultimately affecting crop yield²⁷. In this context, small RNA-based control of chromatin structure may be critically important in genomes with a high content of intronic transposon insertions, such as the human genome, by providing a mechanism to suppress exonization of repeat elements²⁸. While the means by which piRNA-mediated changes in chromatin states could regulate alternative splicing remains to be determined, it is tempting to speculate that piRNA pathway components do so by co-transcriptionally modulating interactions between RNA polymerase II and the spliceosome^{29,30}.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.L. (Ruth.Lehmann@med.nyu.edu) or F.K.T. (fk319@cam.ac.uk).

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Figure Legends

Figure 1. *P*-element DNA transposon splicing is regulated in germ cells during hybrid dysgenesis. **a**, *P-M* hybrid dysgenesis crossing scheme. *P*-cytotype designates animals containing *P*-elements, and *M*-cytotype those lacking them. *Harwich* strain contains *P*-element while *w¹¹¹⁸* strain does not. **b**, Diagram of *P*-element DNA transposon. Arrowheads represent terminal inverted repeats; boxes, exons (ORF0-3); inverted triangles, introns (IVS1-3); ATG, start codon; TGA and TAA, stop codons. **c**, Fertility of non-dysgenic (N.D.) and dysgenic (D.) flies as measured by the number of [F2] adult progeny originated from single [F1] female crosses. (n=20 independent crosses). ***p < 0.0001 (Mann–Whitney–Wilcoxon test). **d**, Number of germ cells during embryonic and larval stages in non-dysgenic (green) and dysgenic (red) [F1] progeny. Dots represent means ± standard deviation. Stage 5 (st.5): complete germ cell count; Stage 15 (st.15) and later stages: germ cell count/gonad. Embryo st.5 (n=28 embryos); embryo st.15 (n>30 gonads); larva L1 (n>10 gonads); larva L2 (n=7 gonads); larva L3 (n>10 gonads). **e**, Representative confocal images of germ cell development in non-dysgenic and dysgenic progeny during embryonic and larval stages. Embryos and larvae were stained for: Vasa (germ cells, green); DAPI (DNA, blue); Phalloidin (F-Actin, red);

embryo stages); 1B1 (somatic cells and spectrosomes, red; larval stages). Pole cell formation, ~1.5h After Egg Laying (AEL); Embryo Stage 15: 10-12h AEL; First Instar larva: 22-48h AEL; Second Instar larva: 48-72h AEL; Third Instar larva: 72-120h AEL. Scale bars, 20 μ M. Experiments were repeated three or more times with similar results. **f**, RT-qPCR analysis on FACS-sorted primordial germ cells (GFP⁺-PGCs) from 4- to 20-hours-old embryos generated from reciprocal crosses between *w¹¹¹⁸* and *Harwich*. Results are represented as means of fold changes in dysgenic GFP⁺-PGCs in relation to non-dysgenic GFP⁺-PGCs \pm standard deviation (n=2 independent biological replicate experiments).

Figure 2. piRNAs, but not siRNAs, modulate P-element splicing in germ cells. All analyses were performed in a *Harwich* background. **a**, RT-qPCR analysis on piRNA- (*piwi*, *aub*, *ago3*, *spnE*, and *vas*) and siRNA-biogenesis (*dcr2* and *ago2*) mutant adult ovaries. Results are presented as means of fold changes in mutants in relation to respective heterozygote \pm standard deviation (n \geq 2 independent biological replicate experiments). **b**, Ethidium bromide-stained gel displaying RT-PCR reactions with primers flanking the *P*-element IVS3 intron in piRNA- and siRNA-biogenesis mutants. Size scale in base pairs (bp) is presented for each gel. Experiments were repeated two times with similar results. For gel source data, see Supplementary Figure 1. **c**, Density plots for normalized strand-specific mRNA steady-state levels (measured by RNA-seq and represented as reads per million, RPM) over consensus *P*-element sequence (top diagram) in *aub*/⁺ heterozygous (yellow, top plot) and *aub* mutant (blue, bottom plot) adult ovaries. The number and position of split-reads (represented by arcs that connect

exons) observed for IVS1, IVS2, and IVS3 splicing junctions is shown below each density plot. Experiments were repeated two times with similar results. **d-e**, Percentage of splicing for *P*-element IVS1, IVS2 (**d**), and IVS3 (**e**). Splicing was quantified using RNA-seq analysis in *aub*/+ heterozygous (yellow), *aub* mutant (blue), *piwi*/+ heterozygous (beige), and *piwi* mutant (purple) adult ovaries. Percentage of splicing was calculated as the number of split-reads for each splicing junction normalized to the total number of reads mapping to the same junction. Results are represented as means \pm standard deviation (n=2 independent biological replicate experiments). **f**, Representative confocal projections of RNA-FISH signal (grayscale) showing the accumulation of sense RNA for Burdock and *P*-element transposons in heterozygous and mutant egg chambers. Bottom panels depict projections of representative nurse cell nuclei (purple dotted line) for the same genotypes. Scale bars, 20 μ M. Experiments were repeated two or more times with similar results.

Figure 3. piRNA-mediated chromatin changes regulate *P*-element splicing. **a**, Diagram of IVS3 transgenic reporter²⁰: white box, germline-specific *hsp83* promoter; ATG, codon start; gray box, nuclear localization signal; purple, *P*-element fragment containing IVS3 (inverted triangle) and exonic flanking sequences (boxes); blue box, *LacZ*; green box, *neo*. **b**, RT-qPCR analysis using adult ovaries of [F1] progeny originated from reciprocal crosses between *Harwich* strain and *w¹¹¹⁸* flies carrying the IVS3 reporter. Bars represent the percentage of spliced IVS3 reporter transgenic transcripts, determined by the ratio between IVS3 spliced transgenic RNA (quantified using primers that specifically anneal to spliced transgenic transcripts) and total IVS3

reporter transgenic mRNA (quantified using primers that anneal within the LacZ coding sequence). Results are presented as means of spliced transgenic RNA (percentage) \pm standard deviation (n=2 independent biological replicate experiments). **c**, RT-qPCR analysis in *aub/+* heterozygous, *aub* mutant, *vas/+* heterozygous, *vas* mutant, *panx/+* heterozygous, and *panx* mutant, carrying the transgenic IVS3 reporter. All experiments were performed in a *Harwich* background (n \geq 2 independent biological replicate experiments). Results are represented as in **(b)**. **d**, RT-qPCR analysis on adult ovaries of germline knockdowns (KD) targeting piRNA pathway components involved in chromatin targeting (*piwi*, *arx/Gstf1*, *Panx/Silencio*, *Su(var)205/HP1a*, and *mael*). Results are presented as means of fold changes in germline KDs in relation to controls (*white* or *mCherry* germline KDs) \pm standard deviation. All analyses were performed in a *Harwich* background (n \geq 2 independent biological replicate experiments). **e-f**, Genome browser view of two of the *P*-element insertions showing transcriptional activity. Normalized RNA-seq and H3K9me3 ChIP signals are presented in gray and blue, respectively. The gray bar crossing the plots represents *P*-element chromosomal insertion site. Annotation is at bottom: purple boxes, coding exons; pink boxes, untranslated regions (UTR); purple lines, introns; gray box, *P*-element insertion. View showing *P*-element insertion into *Bacc/CG9894* (**e**) and *Lk6* (**f**) genes.

Figure 4. piRNA-mediated chromatin targeting machinery modulates splicing of the Gypsy retrotransposon in Ovarian Somatic Cells (OSC). **a**, Diagram of Gypsy retrotransposon: gray boxes, long terminal repeats; white boxes, coding sequences; red inverted triangle, splicing that generates *env* mRNA. **b**, RNA-seq signal (RPM) at the

Gypsy splicing donor and acceptor sites in representative control (GFP KD) and *piwi* KD conditions. Raw data sets from Sienski et al, 2015. Experiments were repeated three times with similar results. **c**, Percentage of splicing for Gypsy splicing donor and acceptor sites as determined by RNA-seq analysis performed in OSC cells KDs of piRNA machinery components. Bars represent the number of split-reads for the *env* splicing donor and acceptor junctions normalized to the total number of sense Gypsy reads mapping to the same junction. Results are represented as means. With the exception *arx/Gst1*, *Su(var)205/HP1a*, *mael*, and *H1*^{7,25}, experiments were repeated two or more times. Raw data sets from Ohtani et al, 2013⁷; Sienski et al, 2015⁹; Iwasaki et al, 2016²⁵.

Methods

No statistical methods were used to predetermine sample size. Experiments were neither intentionally randomized nor intentionally ordered. Investigators were not blinded to allocation during experiments and outcome assessment.

Drosophila stocks, genetics, and husbandry

Drosophila melanogaster stocks used: *w*¹¹¹⁸ (R. Lehmann); *Harwich* (Bloomington Drosophila Stock Center, BDSC# 4264); *w*; *P_{nos}::egfp-moe::nos-3'UTR*³¹, *w*; *hsp83-IVS3-LacZ-neo/TM3 Sb*²⁰, *w*; *nos-GAL4::VP16*³², *P{UAS-Dcr-2.D}1 w*¹¹¹⁸ (BDSC# 24646), *w*¹¹¹⁸; *piwi*¹/*CyO*, *w*⁺ (BDSC# 43637), *w*¹¹¹⁸; *piwi*²/*CyO*, *w*⁺ (BDSC# 43319), *w*¹¹¹⁸; *aub*^{QC42} *cn*¹ *bw*¹/*CyO*, *P{sevRas1.V12}FKI* (BDSC# 4968), *aub*^{HN2} *cn*¹ *bw*¹/*CyO* (gift from P. Macdonald), *bw*¹; *st*¹ *ago3*¹²/*TM6B*, *bw*¹; *st*¹ *ago3*¹³/*TM6B* (gifts from P.

Zamore), *ru¹ st¹ spn-E¹ e¹ ca¹/TM3 Sb¹ Ser¹* (BDSC# 3327), *w*;; *FRT82B spn-E^{100.37} e¹/TM3 Sb* (R. Lehmann), *vas^{PH165}/CyO³³, w¹¹¹⁸; Df(2L)osp29, Adh^{UF} osp²⁹ pr¹ cn¹/CyO, P{sevRas1.V12}FK1* (BDSC# 3078), *w¹¹¹⁸; CG9754^[m1-9]/CyO* (VDRC# v313502; *Panx/Silencio* mutant from J. Brennecke ⁹), *w¹¹¹⁸; CG9754^[m3-4]/CyO* (VDRC# v313500; *Panx/Silencio* mutant from J. Brennecke ⁹), *w^{m4h};; ago2⁴¹⁴/TM6b* (gift from R. Carthew), *w¹¹¹⁸;; Df(3L)BSC558/TM6c Sb* (BDSC# 25120), *dcr2^{L811fsX}, P{GMR-w.IR}; Df(2R)BSC45/CyO* (gifts from E. Lai), *aub*-shRNA (Vienna Drosophila Resource Center, VDRC# v313413), *piwi*-dsRNA (VDRC# v101658), *arx/Gtsf1*-dsRNA (VDRC# v40408), *Panx/Silencio*-dsRNA (VDRC# v102702), *mael*-dsRNA (VDRC# v100907), *Su(var)205/HP1a*-shRNA (BDSC# 36792), *white*-dsRNA (dsRNA control, VDRC# v30033), *mCherry*-shRNA (shRNA control, BDSC# 35785).

Lab-strain chromosomes carrying mutations and transgenes of interest were individually transferred to the *Harwich* background through a series of backcrosses. The genotypes of the resulting *Harwich*-derived stocks, which are characterized by single chromosome permutations, are listed in Supplementary Table 2. Prior to further use, the ability of the *Harwich*-derived stocks to induce dysgenic ovarian phenotype in [F1] progeny was confirmed by dysgenesis testcrosses using five *w¹¹¹⁸* females and five males from each of the *Harwich*-derived stocks. Testcrosses were performed at 29°C on standard medium in at least two biological replicates. Ovary phenotype in [F1] progeny was scored after dissection and results are summarized in Supplementary Table 2.

For the fertility test described in Figure 1c, non-dysgenic and dysgenic [F1] progeny females were individually mated to two *w¹¹¹⁸* males. Parental flies were discarded after 3 days of mating, and number of [F2] adult progeny was assessed 12 days after that. All

other crosses are described in Supplementary Table 3 and were performed in at least two biological replicates. Germline RNAi knockdowns using UAS-dsRNA lines were performed in the presence of the *P{UAS-Dcr-2.D}1* transgene, as previously described²¹. Unless stated otherwise, all crosses were performed at 29°C on standard medium and experiments were performed with 3- to 5-day-old adult female flies. ChIP-seq, RNA-seq analysis, and IVS3 transgenic reporter analysis with [F1] progeny originated from reciprocal crosses between *w¹¹¹⁸* and *Harwich* were performed at 18°C to minimize confounding effects derived from dysgenesis-induced developmental defects. Unlike dysgenesis-triggered developmental defects, which are only observed in flies grown in temperatures >25°C¹⁵, *P*-element splicing regulation is not temperature-sensitive as verified by RT-PCR, RT-qPCR, and RNA-seq analyses performed at 18°C (Extended data Figure 6).

Immunofluorescence

Hand-devitellinized embryos and dissected larval ovaries were immunostained as previously described^{34,35}. Samples were mounted in Vectashield® media containing DAPI (Vector Laboratories). Primary antibodies: rabbit anti-Vasa serum (1:5000, R Lehmann) and mouse anti-1B1 (1:20, DSHB). Alexa Fluor 488- (Life Technologies) and Cy3-conjugated (Jackson ImmunoResearch) secondary antibodies were used at a 1:500 dilution. Phalloidin-TRITC (Sigma) was used at 1:250 (from 20µM stock). Fluorescent images were acquired with Plan-Apochromat 20X/NA0.8 and Plan-Apochromat 40X/NA1.4 (oil immersion) objectives on a Zeiss LSM 780 confocal microscope.

RNA FISH

RNA FISH was performed as previously described³⁶. Custom Stellaris® RNA FISH Probes were designed using the Stellaris® RNA FISH Probe Designer (Biosearch Technologies) and labeled with CALFluor590 and Quasar670 to detect consensus *P*-element (GenBank X06779:996..3902) and Burdock (GenBank U89994) sense mRNA sequences, respectively. FISH probes are composed of 20-nt-long oligo pools, as listed in Supplementary Table 4. Fluorescent images were acquired with a Plan-Apochromat 40X/NA1.4 (oil immersion) objective on a Zeiss LSM 780 confocal microscope.

FACS sorting of PGCs

GFP⁺-PGCs were isolated from 4h- to 20h-old embryos by FACS as previously described³⁷. Reciprocal crosses to obtain non-dysgenic and dysgenic embryos were performed with stocks carrying the *P_{nos}::egfp-moe::nos-3*'UTR transgene³¹, as described in Supplementary Table 3. GFP⁺-PGCs were FACS sorted (MoFlo cell sorter) into Trizol reagent for subsequent RNA extraction.

RNA isolation and analyses

Total RNA from FACS sorted germ cells or dissected adult ovaries was isolated using Trizol reagent (Invitrogen) and quantified by Qubit (Invitrogen). Contaminating DNA was removed using RQ1 DNase (Promega). Reverse transcription was performed using random primers and SuperScript III Reverse Transcriptase (Invitrogen). Transcript levels were assessed by quantitative PCR (qPCR) using LightCycler® 480 SYBR Green I Master 2X (Roche) in a Roche LightCycler® 480 machine. Results were normalized to

the mean value obtained for three control genes (CG7939 [Rp49, also known as RpL32]; CG8187; CG8269 [Dmn]) with invariant expression in a range of tissues and developmental stages, as previously described³⁸. For splicing analysis on gel, PCR reactions were performed in RT-qPCR-normalized cDNA to non-saturating conditions (exponential phase) using LightCycler® 480 SYBR Green I Master 2X (Roche) and a Roche LightCycler® 480 machine, and PCR products were separated in an ethidium bromide-stained, 2.5% agarose gel. Note that this is not a quantitative analysis, as amplification of smaller DNA fragments may be disproportionately favored in PCR reactions targeting fragments of different length. All primers are listed in Supplementary Table 5.

Poly(A)-selected RNA-sequencing (RNA-seq) analysis was performed on 2.5µg of RNA purified from adult ovaries using the NEBNext® Poly(A) mRNA Magnetic Isolation Module and the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®. Libraries were multiplexed using the NEBNext® Multiplex Oligos for Illumina® and sequenced in paired-end, 100-nt-long reads on an Illumina HiSeq 2500.

PCR on genomic DNA

Genomic DNA was extracted from *w¹¹¹⁸* and *Harwich* pools containing 20 adult female flies each, using the DNeasy Blood & Tissue kit (QIAGEN) and following manufacturer's protocol for insects. PCR was conducted on 2ng of total DNA using Q5 Hot Start High-Fidelity DNA Polymerase (NEB). PCR products were separated in an ethidium bromide-stained, 1% agarose gel. To structurally characterize *P*-element insertions, PCR products were purified using the QIAquick PCR purification kit

(QIAGEN) and sequenced using traditional Sanger sequencing. Primers are listed in Supplementary Table 5.

ChIP

ChIP was performed as previously described³⁹, using 100-200 dissected adult ovaries as starting material and the anti-H3K9me3 (Abcam, ab8898) or the anti-rhino⁴⁰ (guinea pig; kind gift from W. Theurkauf) antibody. Prior to immunoprecipitation, 5% of sonicated chromatin was separated to serve as ‘input’. After reverse-crosslinking, immunoprecipitated (IP) and input DNA was extracted using QIAquick PCR Purification Kit (QIAGEN) and used for qPCR reactions as previously described or library preparation for sequencing. ChIP-qPCR results were normalized to the input and compared with signal at the *42AB* locus. Primers used for ChIP-qPCR are listed in Supplementary Table 5. For ChIP-seq, IP and input libraries were prepared using NEBNext[®] Ultra[™] II DNA Library Prep Kit for Illumina[®]. Libraries were multiplexed using the NEBNext[®] Multiplex Oligos for Illumina[®] and sequenced in paired-end, 50-nt-long reads on an Illumina HiSeq 2500. ChIP experiments were performed in two biological replicates. qPCR measurements were performed in at least two technical replicates on each sample.

Computational analysis

Small RNA analysis

Small RNA reads extracted from the NCBI Gene Expression Omnibus (GEO) database accession number GSE13081¹⁶ were mapped to the consensus *P*-element sequence, as previously described¹¹.

RNA-seq analysis

Paired-end (100-nt-long reads) RNA-seq data generated in this study were mapped to the *Drosophila melanogaster* genome (dm3) and the FlyBase and Repbase transposon consensus database using the piPipes package (version 1.5.0; <https://github.com/bowhan/piPipes>), following the RNA-seq pipeline as previously described⁴¹. Briefly, libraries were first aligned to ribosomal RNA using Bowtie2⁴². Non-rRNA reads were then mapped to the transcriptome and transposon consensus using Bowtie2, and transcript abundance was quantified using eXpress⁴³. Analyses were performed with two samples, each with two biological replicates, using the piPipes RNA-seq dual-library mode⁴¹.

Genome-wide analysis of differentially spliced mRNAs was performed using the Junction Usage Model (JUM) package^{44,45} (<https://github.com/qqwang-berkeley/JUM>; version 1.3.2) with default parameters and following the JUM manual. Briefly, RNA-seq data were mapped to the *Drosophila* genome (dm3) using STAR⁴⁶ in two-pass mode, following instructions in the STAR manual (<https://github.com/alexdobin/STAR>). STAR output is then used by JUM to construct statistical models and to quantify splicing from raw counts mapped to each identified junction⁴⁴. Each comparison was performed with two samples, each with two biological replicates. Junctions showing >2-fold changes

between samples and with p -adjusted < 0.05 were considered as differentially spliced. JUM analysis was performed using a minimal of five mapping reads as a filter for valid junctions.

Splicing of *P*-element and Gypsy transposons was defined manually according to the method used in the JUM package⁴⁴. To do so, RNA-seq data was initially analyzed on the Galaxy web-based platform (www.usegalaxy.org)⁴⁷. After the removal of Illumina adaptors and other low quality sequences using the ‘Trim Galore!’ package, stranded RNA-seq data were mapped to consensus *P*-element (GenBank X06779:996..3902) and Gypsy (Genbank M12927) sequences using TopHat⁴⁸, allowing for up to 3 mismatches. To calculate percentage of splicing, TopHat output was used to define the number of split-reads mapping to a junction (N1), as well as the number of non-split-reads fully and uniquely contained within the interval surrounding the same exon-intron junction (N2). This interval is centered of the exon-intron boundary and its size is defined based on the length of RNA-seq, where size is equal to [2x length of RNA-seq reads] minus 4. Percentage of ‘splicing in’ is then defined by the $(N1/(N1+N2))$ ratio multiplied by 100. Fold changes are calculated based on percentage of splicing values. Quality of split-read alignments was visually inspected using IGV browser (version 2.3.77)⁴⁹, and low-quality alignments containing < 3 nucleotides overlapping one side of the splice junction were discarded. For *P*-element splicing analysis, biological replicates generated in this study were individually analyzed and used to calculate average of percentage of splicing and standard deviation. For Gypsy analysis, raw RNA-seq data sets from OSC cells were retrieved from GEO database, accession numbers GSE47006⁷, GSE74097⁹, and

GSE81434²⁵. Given the distance between Gypsy *env* donor and acceptor sites (4982bp), percentage of splicing for each exon-intron boundary was individually calculated as described above. Data obtained for the same tested conditions were pooled whenever possible.

Analysis of transcriptionally active *P*-element insertions was performed on the Galaxy web-based platform using RNA-seq data from non-dysgenic and dysgenic progeny and previously described method to identify non-reference transposon insertions using split-reads⁵⁰. Briefly, paired-end RNA-seq data (100-nt long reads) were mapped to the *Drosophila* genome (dm3) using Bowtie2. Next, unmapped reads were aligned to the *P*-element consensus sequence using Bowtie2, with --sensitive-local settings to obtain soft clip alignments. Reads with <20 nt mapping to *P*-element extremities were removed, and the remaining reads were then treated to remove all *P*-element-derived sequences and mapped to the *Drosophila* genome (dm3) using UCSC genome Browser BLAT Search (<http://genome.ucsc.edu/cgi-bin/hgBlat>). The number of RNA-seq split-reads mapping to a given locus in relation to total number of split-reads (excluding singletons) was used to determine the relative expression of individual insertions.

ChIP-seq analysis

ChIP-seq analysis was performed on the Galaxy web-based platform following previously described methods⁴¹. Briefly, Illumina adaptors and other low quality sequences were removed using the ‘Trim Galore!’ package. ChIP-seq input and IP filtered data were mapped to the *Drosophila* genome (dm3) and transposon consensus

sequences using Bowtie2, using the $-k = 1$ parameter. For the *Drosophila* genome, peak calling and signal normalization (Poisson p -value [ppois] correction) was performed using MACS2⁵¹. Heatmaps were generated using SeqPlots, using default parameters (<http://przemol.github.io/seqplots/>). For transposon consensus sequences, input-corrected H3K9me3 occupancy was normalized to the total number of genome-mapped aligned reads and represented as reads per million (RPM).

DNA-seq analysis

Identification of *P*-element insertions in the *Harwich* strain was performed using the input data from ChIP-seq and the Genome-seq pipeline provided in the piPipes package⁴¹, as previously described⁵². Briefly, genomic sequencing data were first mapped to the *Drosophila* genome (dm3) using BWA⁵³. Then, using the consensus *P*-element sequence (GenBank X06779:996..3902) as template, TEMP⁵⁴ was used to locate *P*-element insertions in the genome. This analysis was individually performed in four independent repeats, provided by two biological replicates of each progeny originated from reciprocal crosses between w^{1118} (*P*-element-devoid strain) and *Harwich*. Shared insertions identified in all four experiments were used to reconstruct the set of *P*-element euchromatic insertion existing in the *Harwich* strain (Supplementary Table 1). Euchromatic coordinates were obtained from Sienski et al, 2012²².

Data availability

Sequencing data generated during the current study are available in the NCBI Gene Expression Omnibus repository under accession number GSE103582.

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Extended data Legends

Extended data Figure 1. Size distribution of small RNAs matching to *P*-elements in small RNA libraries obtained from *Harwich* ovaries (orange) or from 0-2h old embryos laid by *Harwich* females (purple). Analysis was performed on data originally published in Brennecke *et al.*, 2008¹⁶. siRNA (20-22nt long) and piRNA (23-29nt long) populations are indicated.

Extended data Figure 2. *P*-element mRNA steady-state levels do not change in piRNA mutants in comparison to respective heterozygous. a-b, Scatterplots showing the expression of genes (gray dots) and transposons (blue dots), as measured by RNA-seq analysis (expressed in fragments per kilobase per million fragments, FPKM, \log_{10}), in

aub/+ heterozygous vs. *aub* mutant adult ovaries (**a**) and *piwi*/+ heterozygous vs. *piwi* mutant adult ovaries (**b**) comparisons. *P*-element expression is shown in green. Transposons with RNA abundance changes >10-fold are outlined in red. Experiments were repeated two times with similar results. **c-d**, Genome-wide analysis of splicing changes in *aub*/+ heterozygous vs. *aub* mutant adult ovaries (**c**) and *piwi*/+ heterozygous vs. *piwi* mutant adult ovaries (**d**) comparisons. Quantification of splicing changes was performed using RNA-seq data and the JUM method^{44,45}. Results are expressed as log₂ fold changes in splicing (mutant/heterozygous). Gray dots represent individual splice junctions identified, sorted by fold change values. Green dots represent splice junctions with statistically significant changes in heterozygous vs. mutant comparisons (adjusted *p*-value < 0.05). Fold changes for *P*-element splice junctions (IVS1, IVS2, and IVS3) are presented in red. Note that ~70% of genic splice junctions showing statistically significant changes in mutant comparisons (green dots) are located in the same chromosome as the inducing mutations (2nd chromosome), and may be due to genetic background differences. **e**, Density plots for normalized strand-specific mRNA steady-state levels (measured by RNA-seq and represented as reads per million, RPM) over consensus *P*-element sequence (top diagram) in *piwi*/+ heterozygous (beige, top plot) and *piwi* mutant (purple, bottom plot) adult ovaries. The number and position of split-reads (represented by arcs that connect exons) observed for IVS1, IVS2, and IVS3 splicing junctions is shown below each density plot. Experiments were repeated two times with similar results.

Extended data Figure 3. ‘P-cyotype’ as well as piRNA pathway components involved in piRNA biogenesis and targeting regulate the splicing of the IVS3 transgenic reporter *in vivo*. **a.** Ethidium bromide-stained gel displaying RT-PCR reactions with primers flanking the transgenic reporter IVS3 intron. Analysis was performed with adult ovaries of non-dysgenics and dysgenic flies grown at 18°C, or with adult ovaries of heterozygous and mutants for the piRNA components *aub*, *vas*, and *Panx/Silencio*. Mutant analyses were performed in a *Harwich* background, at 29°C. Size scale in base pairs (bp) is presented for each gel. Control reactions omitting Reverse Transcriptase (RT-) are also presented. Diagram of IVS3 transgenic reporter (as in Figure 3a) and primers (arrows) used in RT-PCR reactions are depicted in the top of the ethidium bromide-stained gel. Experiments were repeated two or more times with similar results. For gel source data, see Supplementary Figure 1. **b.** RT-qPCR analysis using adult ovaries of [F1] progeny carrying the IVS3 reporter, probing spliced (IVS3 splicing, quantified using primers that specifically anneal to spliced transgenic transcripts) and total (*LacZ*, quantified using primers that anneal within the *LacZ* coding sequence) IVS3 reporter transgenic mRNA levels. [F1] progeny was originated either from reciprocal crosses between *Harwich* strain and *w¹¹¹⁸* flies, or in *aub/+* heterozygous, *aub* mutant, *vas/+* heterozygous, *vas* mutant, *panx/+* heterozygous, and *panx* mutant. Results are presented as mean-fold changes in the mutants (or in dysgenic) in relation to the respective heterozygous siblings (or non-dysgenic) \pm standard deviation (n \geq 2 independent biological replicate experiments).

Extended data Figure 4. Loss of H3K9me3 in dysgenic progeny is restricted to transcriptionally active *P*-elements. **a-b**, Density plots for normalized H3K9me3 ChIP-seq signals (represented as reads per million, RPM) over consensus *P*-element (**a**) and Het-A (**b**) sequences in non-dysgenic (grays) and dysgenic (red) progeny. Experiments were performed with adult ovaries of flies grown at 18°C. Experiments were repeated two times with similar results. **c-d**, H3K9me3 ChIP-qPCR analysis on *P*-element (**c**) and controls (**d**) in non-dysgenic (grays) and dysgenic (red) progeny. Bars represent means of H3K9me3 signal, normalized to a control genomic region in the *42AB* locus (42AB-3), \pm standard deviation (n=2 independent biological replicate experiments). **d**, Und and Dmn are negative controls. **e-f**, Genome browser view of two of the *P*-element insertions showing transcriptional activity. Normalized RNA-seq and H3K9me3 ChIP signals are presented in gray and blue, respectively. Gray bar crossing the plots represents *P*-element insertion site. Chromosome coordinates are at top. Annotation is at bottom: purple boxes, coding exons; pink boxes, untranslated regions (UTR); purple lines, introns; gray box, *P*-element insertion. View showing *P*-element insertion into CG5953 (**e**) and mir-14/CR43651 (**f**) genes. Experiments were repeated two times with similar results. **g-h**, ChIP-qPCR analysis on sequences flanking transcriptionally active *P*-element insertions (**g**) and on the IVS3 transgenic reporter (**h**) in non-dysgenic (grays) and dysgenic (red) progeny. Bars represent means of H3K9me3 signal, normalized to a control genomic region in the *42AB* locus (42AB-3), \pm standard deviation (n=2 independent biological replicate experiments). **h**, Diagram of IVS3 transgenic reporter is presented below the graph. Black rectangles indicate regions probed by ChIP-qPCR. **i-j**, Heatmaps depicting normalized H3K9me3 levels obtained by ChIP-seq analysis within the 6kb flanking *P*-

element insertions in non-dysgenic (left panels) and dysgenic (right panels) progeny. Experiments were repeated two times with similar results. **i**, 4 *P*-element insertions with evidence of transcriptional activity. **j**, 59 *P*-element insertions with no evidence of transcriptional activity. All experiments were performed with ovaries of adult progeny from non-dysgenic and dysgenic crosses grown at 18°C.

Extended data Figure 5. Characterization of euchromatic *P*-element insertion in Harwich strain. **a**, Ethidium bromide-stained gels displaying genomic PCR reactions with 25 primer sets flanking *P*-element insertions uncovered by DNA-seq analysis. PCR reactions were performed with genomic DNA extract from *w¹¹¹⁸* and *Harwich* pools containing 20 adult females each. Size scale in base pairs (bp) is presented on the right side of each gel. Amplicon sizes representing absence (no insertion) or presence (truncated or full-length elements) of *P*-element insertion are indicated on the left of each gel. Targeted insertion and chromosome localization is displayed in the bottom of each gel. Experiments were repeated two times with similar results. For gel source data, see Supplementary Figure 1. **b**, Schematic representation of five structurally different elements regrouping 24 *P*-element insertions characterized by DNA sequencing in the Harwich strain. Elements size, as well as a list of respective insertions is indicated on the right of each diagram. Arrowheads represent terminal inverted repeats; boxes, exons; inverted triangles, introns; ATG, start codon; TGA and TAA, stop codons. Dashed lines represent internal deletions.

Extended data Figure 6. Analysis of *P*-element expression and splicing in adult ovaries of non-dysgenic and dysgenic progeny grown at 18°C. **a**, Scatterplot showing the expression of genes (gray dots) and transposons (blue dots), as measured by RNA-seq analysis (expressed in fragments per kilobase per million fragments, FPKM, \log_{10}), in adult ovaries of non-dysgenic vs. dysgenic progeny grown at 18°C. *P*-element expression is shown in green. Genes containing *P*-element insertion in Harwich strain are depicted in purple. **b-c**, Percentage of splicing for *P*-element IVS1, IVS2 (**b**), and IVS3 (**c**) splicing junctions as determined by RNA-seq analysis in non-dysgenic (green) and dysgenic (red) adult ovaries. Bars represent percentage of splicing, calculated as the number of split-reads for each splicing junction normalized to the total number of reads mapping to the same junction. Results are represented as means \pm standard deviation (n=2 independent biological replicate experiments). **d**, Ethidium bromide-stained gel displaying RT-PCR reactions with primers flanking the *P*-element IVS3 intron in adult ovaries of non-dysgenic and dysgenic progeny grown at 18°C, as well as *aub*/+ heterozygous and *aub* mutant grown at 29°C. Size scale in base pairs (bp) is presented for each gel. As shown, experiments were repeated three times with similar results. For gel source data, see Supplementary Figure 1. **e**, RT-qPCR analysis testing accumulation of IVS3 spliced mRNA on non-dysgenic and dysgenic progeny (ovaries) grown at 18°C. Results are expressed as means of percentage of expression relative to controls \pm standard deviation (n=3 independent biological replicate experiments). **f**, Genome-wide analysis of splicing changes in in adult ovaries of non-dysgenic vs. dysgenic progeny grown at 18°C. Quantification of splicing changes was performed using RNA-seq data and the JUM method^{44,45}. Results are expressed as \log_2 fold changes in splicing (dysgenic/non-

dysgenic). Gray dots represent individual splice junctions identified, sorted by fold change values. Green dots represent splice junctions with statistically significant changes in heterozygous vs. mutant comparisons (adjusted p -value < 0.05). Fold changes for P -element splice junctions (IVS1, IVS2, and IVS3) are presented in red. **g**, Analysis of RNA-seq data obtained from non-dysgenic (green) and dysgenic (red) progeny (adult ovaries) to identify transcriptionally active P -element insertions. Reads partially mapping to the P -element extremities were subsequently mapped to the *Drosophila* genome. Results are expressed as percentage of reads mapping to a given genomic location in relation to total reads. Bars represent means \pm standard deviation (n=2 independent biological replicate experiments). All experiments were performed with ovaries of adult progeny from non-dysgenic and dysgenic crosses grown at 18°C.

Extended data Figure 7. Analysis of Gypsy splicing in OSC cells. **a**, Diagram of Gypsy retrotransposon, as in Figure 4a. **b**, RNA-seq signal (RPM) at the Gypsy splicing donor and acceptor sites in representative control (GFP KD) and KDs of *arx/Gst1*, *Panx/Silencio*, *Su(var)205/HP1a*, *mael*, *histone H1*, *SetDB1/egg*, *Su(var)3-9*, *G9a*. Data for double KDs of *SetDB1/egg + Su(var)3-9*, *SetDB1/egg + G9a*, and *Su(var)3-9 + G9a* are also shown. With the exception *arx/Gst1*, *Su(var)205/HP1a*, *mael*, and *H1*^{7,25}, experiments were repeated two times with similar results. **c**, Percentage of splicing for Gypsy donor and acceptor splicing sites as determined by RNA-seq analysis performed in OSC cells double KDs of H3K9 methyltransferases. Bars represent the number of split-reads for the *env* donor and acceptor splicing junctions normalized to the total number of sense Gypsy reads mapping to the same junction. Results are represented as means.

Experiments were repeated two times with similar results. **d**, Coverage of Gypsy consensus sequence by RNA-seq data. Coverage was calculated as: [number of reads matching Gypsy consensus sequence x read length in nucleotides]/Length of consensus Gypsy sequence in nucleotides (7469nt). Raw data sets from Ohtani et al, 2013⁷; Sienski et al, 2015⁹; Iwasaki et al, 2016²⁵.

Extended data Figure 8. *rhino* ChIP-qPCR analysis in adult ovaries of non-dysgenic and dysgenic progeny grown at 18°C. *rhino* ChIP-qPCR analysis on *P*-element (**a**), controls (**b**), sequences flanking transcriptionally active *P*-insertions in the Harwich strain (**c**), and transgenic IVS3 splicing reporter (**d**) in non-dysgenic (grays) and dysgenic (red) progeny. Bars represent means of Rhino signal, normalized to a control genomic region in the *42AB* locus (42AB-3), \pm standard deviation (n=2 independent biological replicate experiments). **b**, Dmn, Und, and flam are negative controls; 42AB is a positive control. **d**, Diagram of transgenic IVS3 splicing reporter is presented below the graph. Black rectangles indicate regions probed by ChIP-qPCR. All experiments were performed with ovaries of adult progeny from non-dysgenic and dysgenic crosses grown at 18°C.

Figure 1

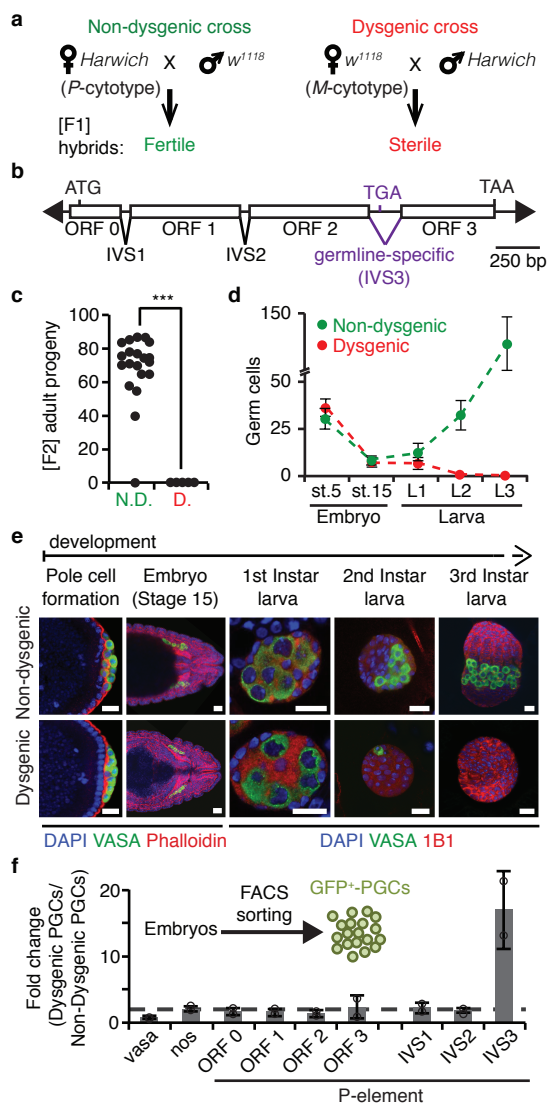


Figure 2

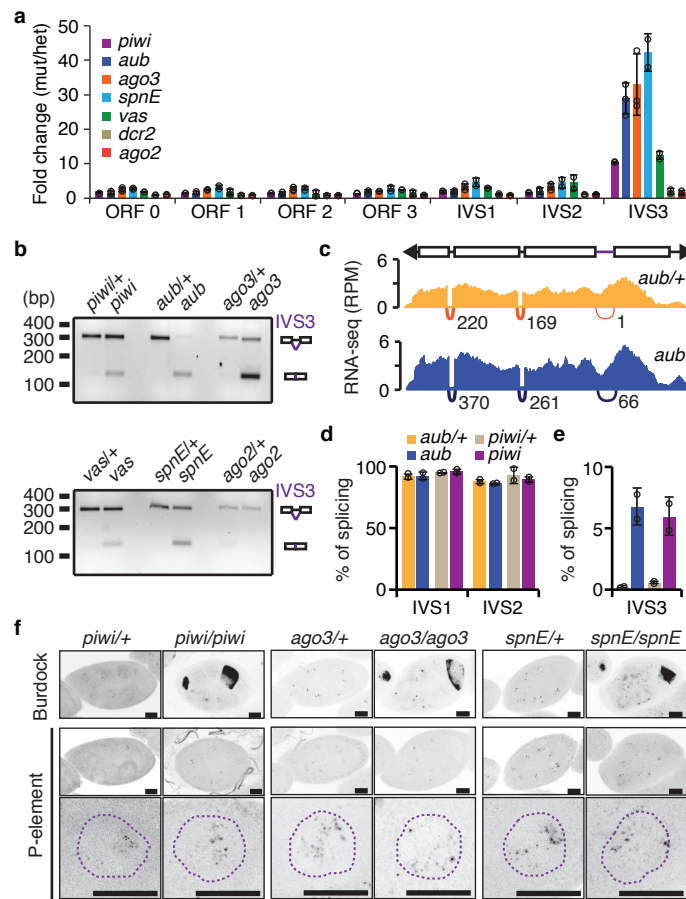


Figure 4

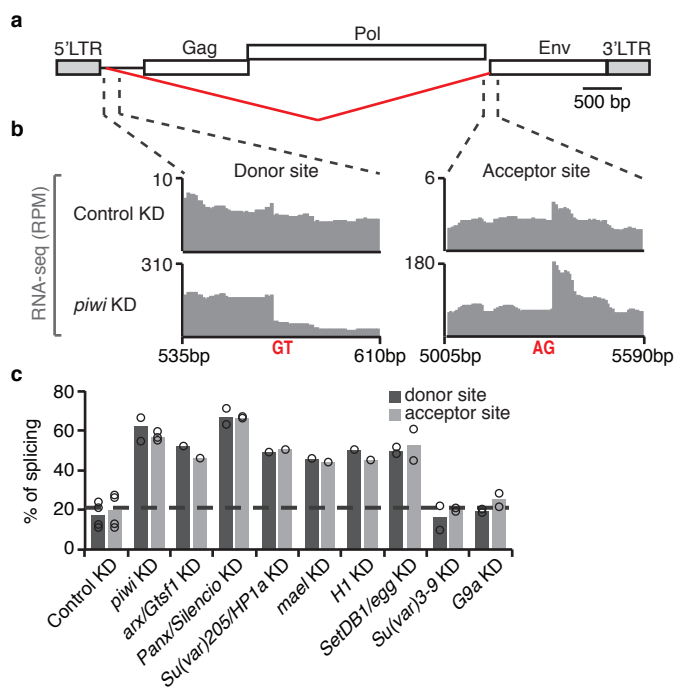
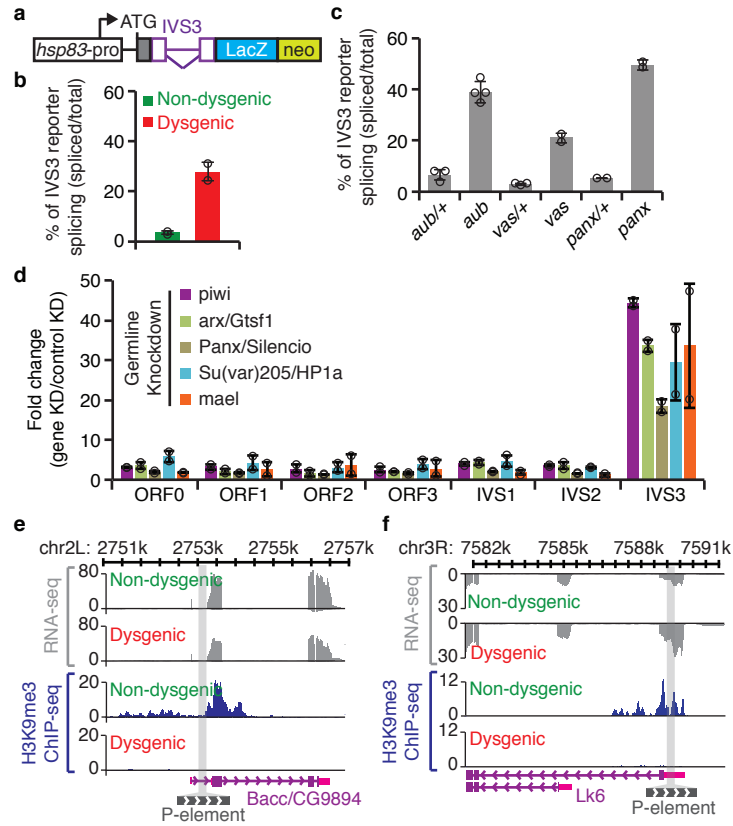
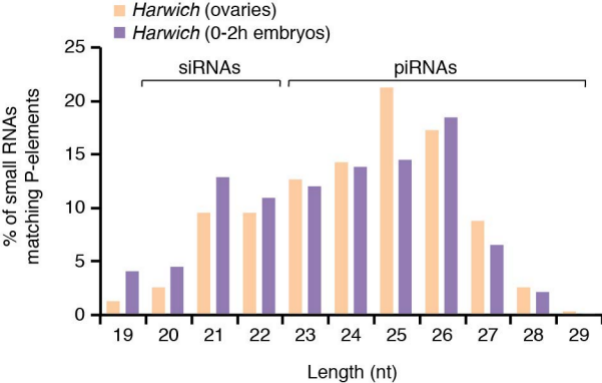
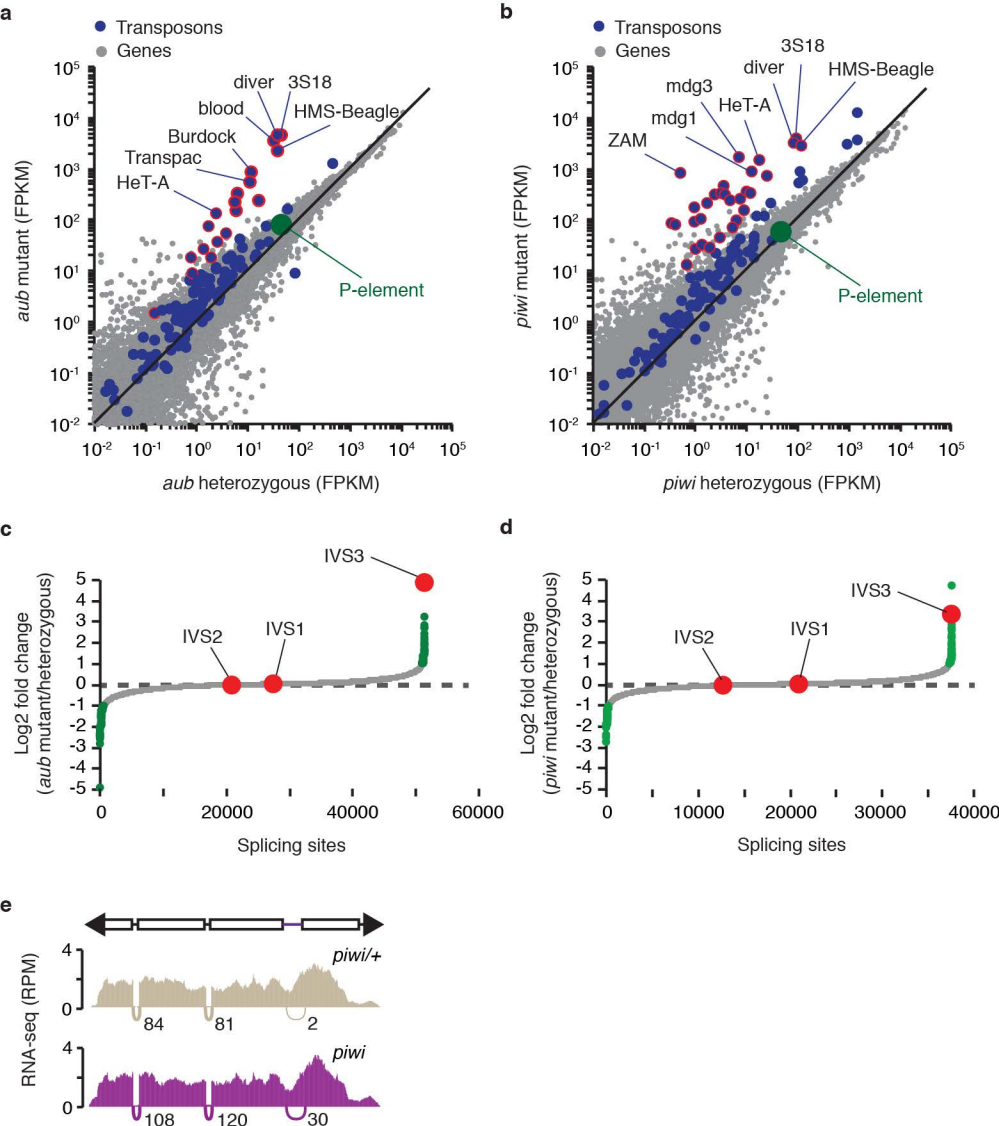
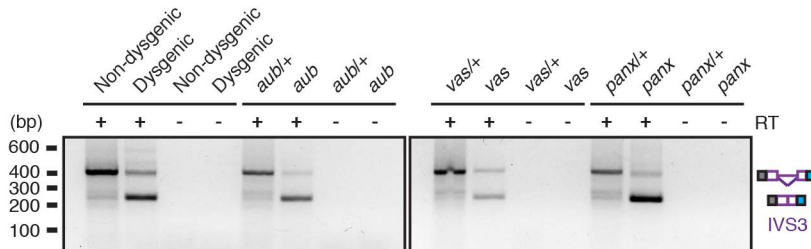
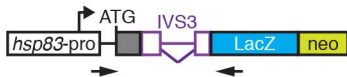
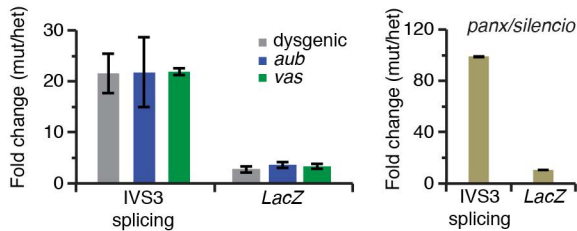


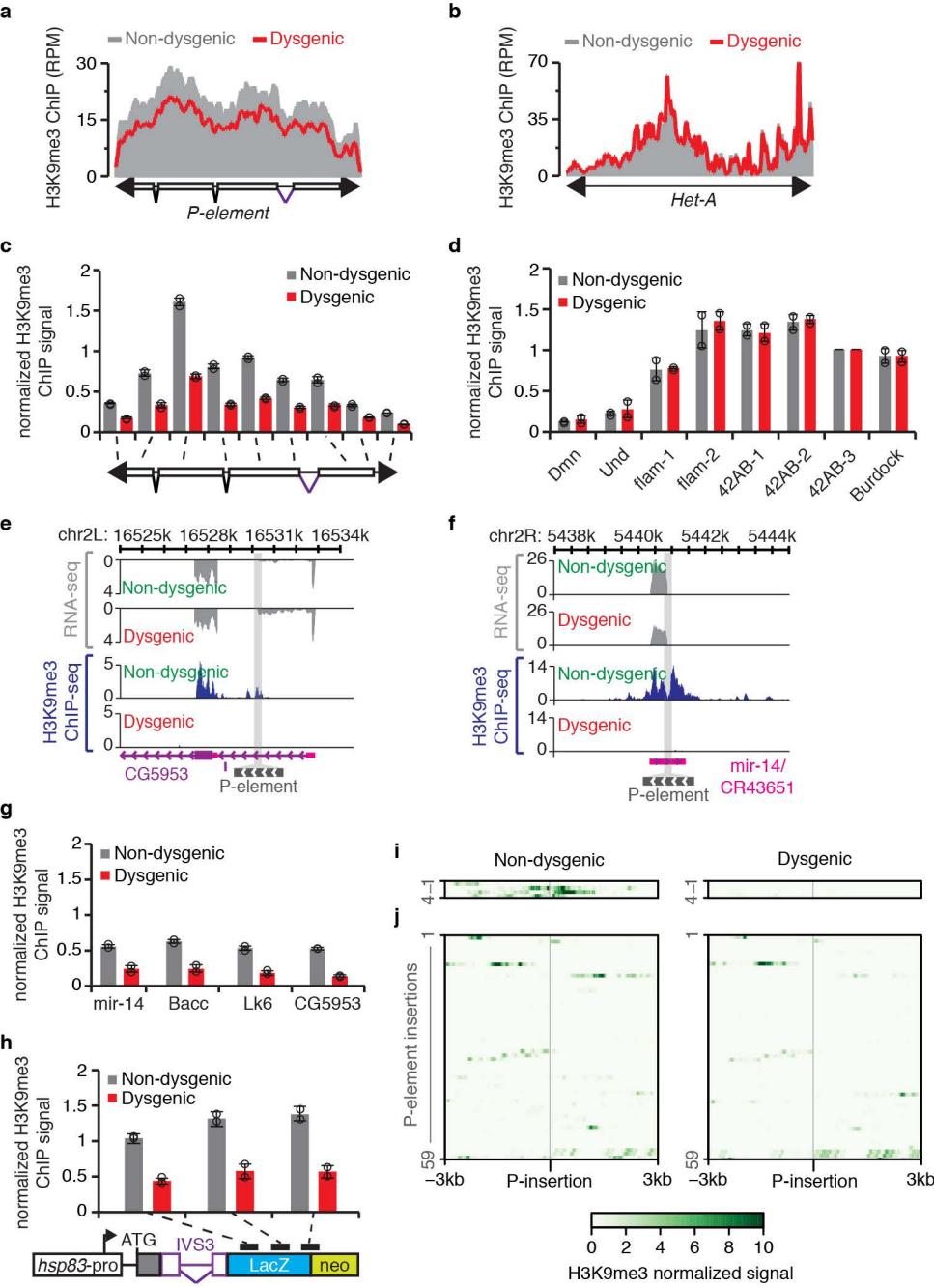
Figure 3

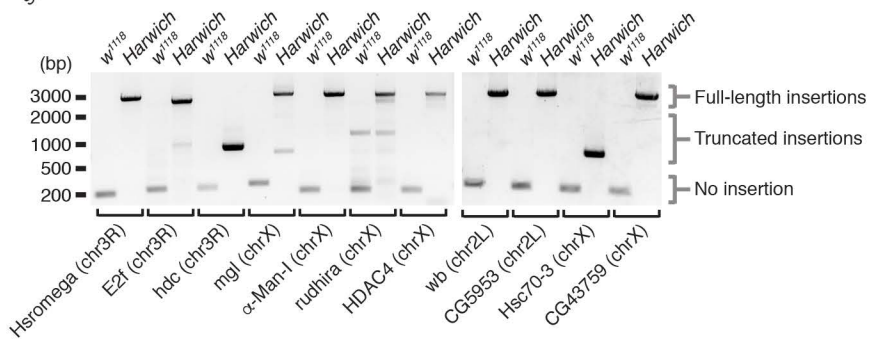
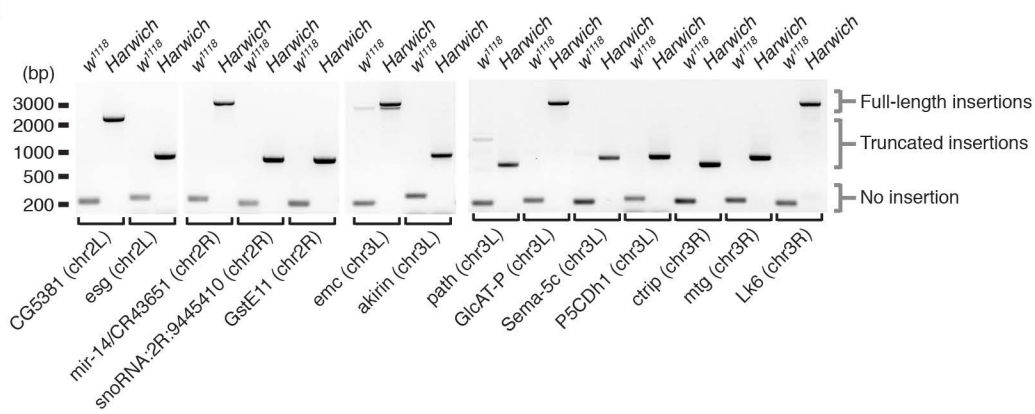




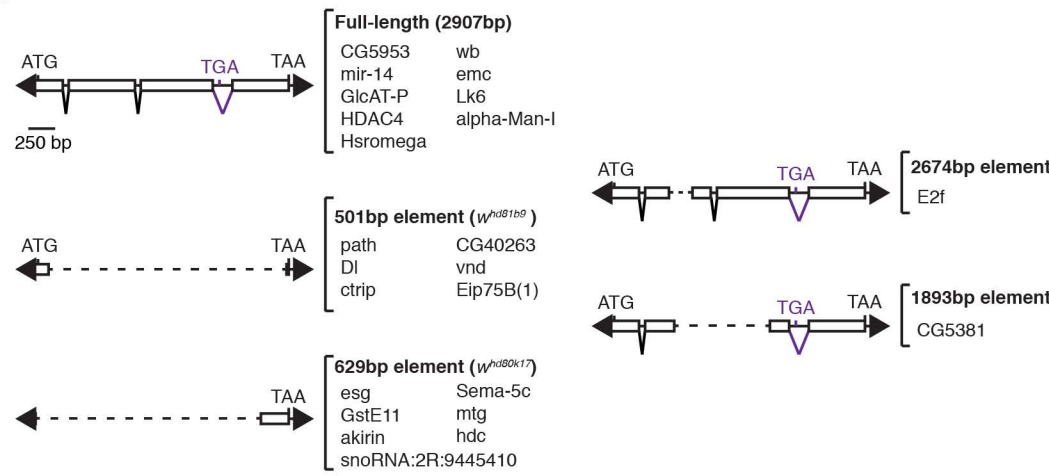


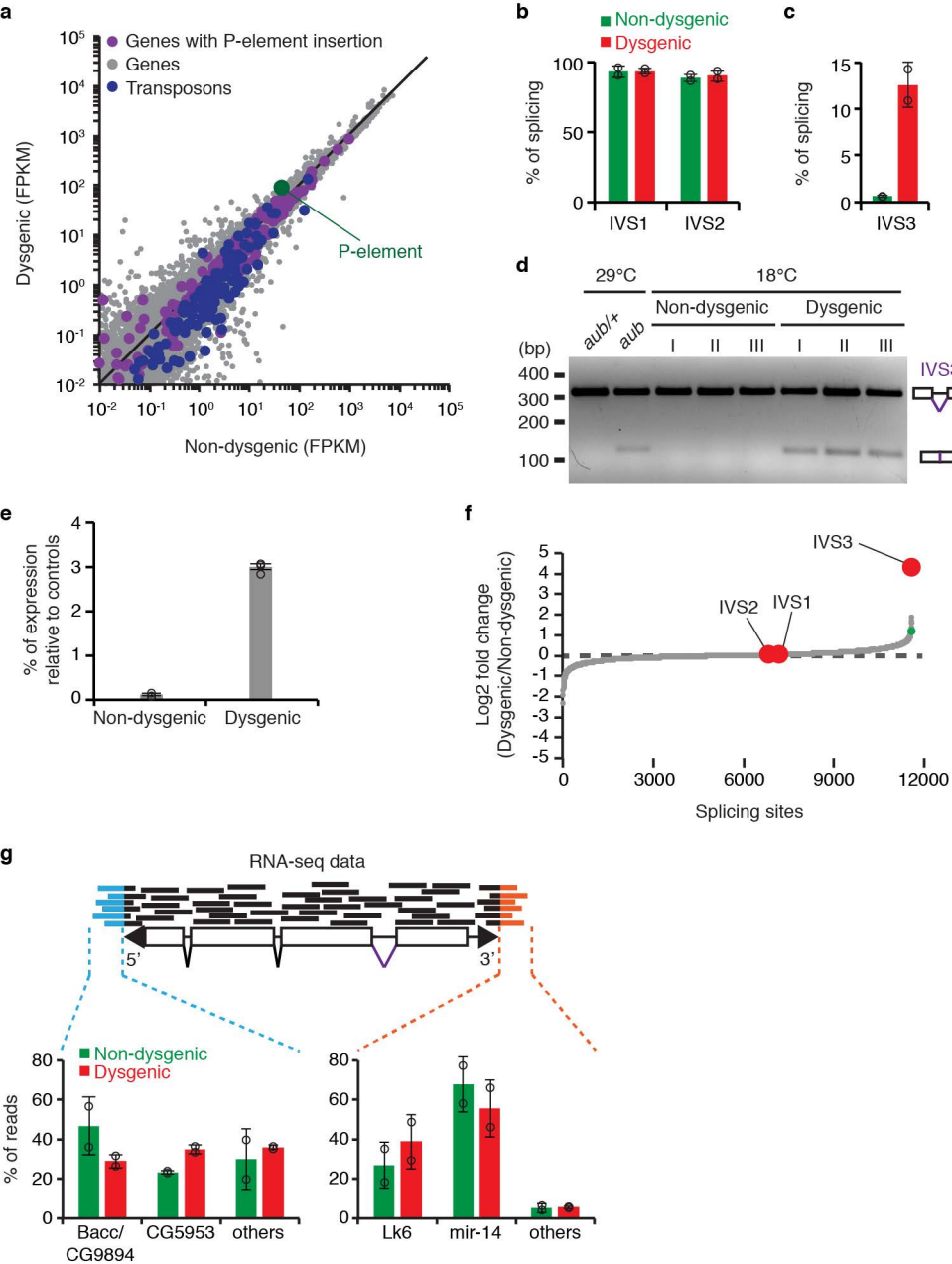
a**b**

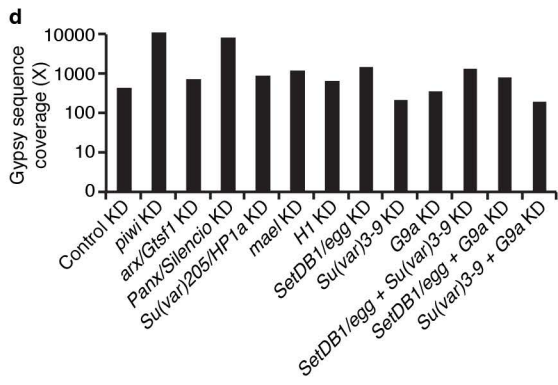
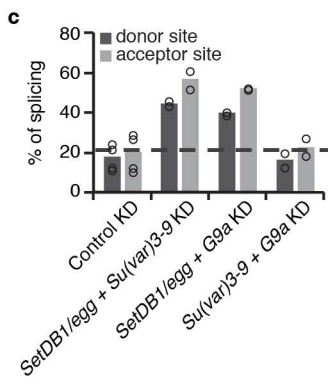
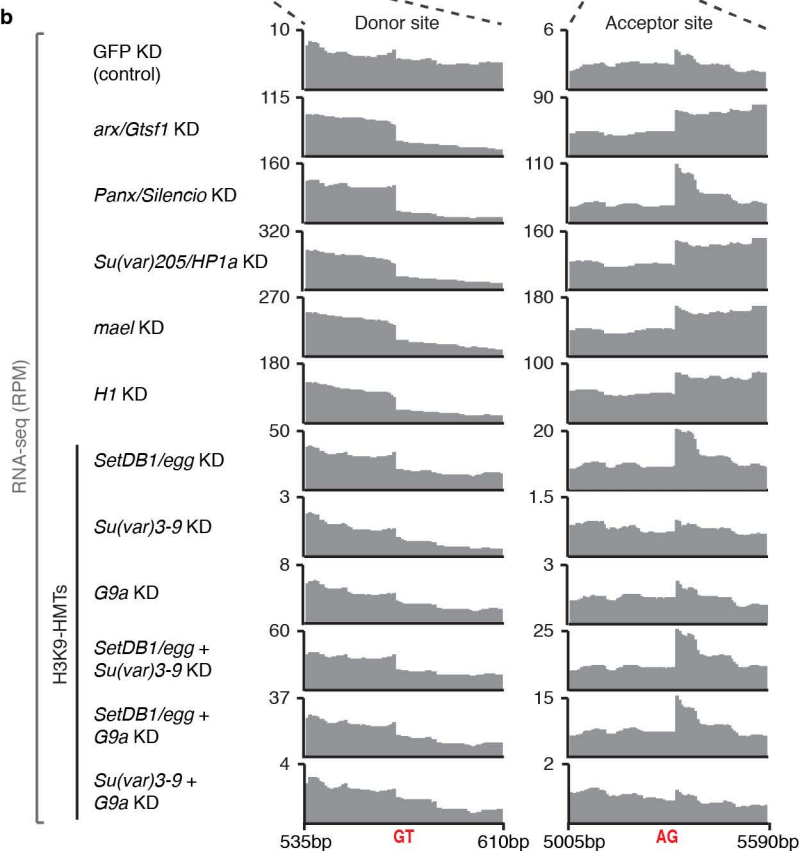
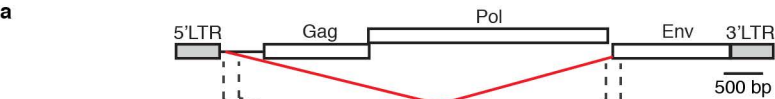


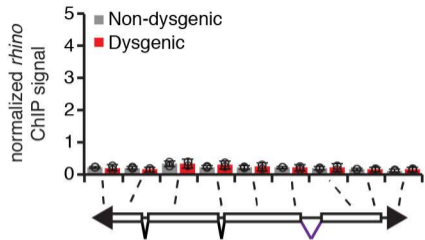
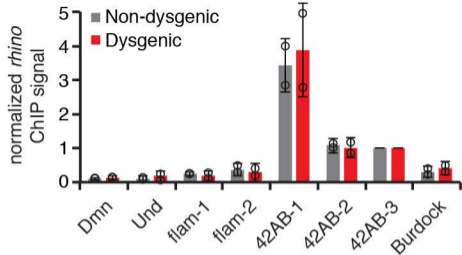
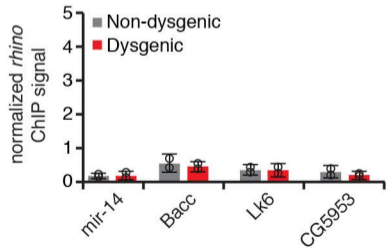
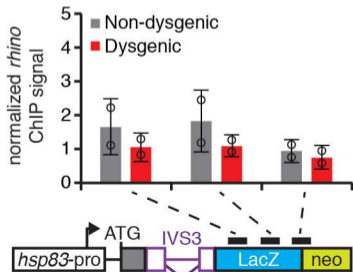


b







a**b****c****d**

Supplementary Information

Supplementary Figure 1. Gel source images.

Supplementary Table 1. Euchromatic *P*-element insertions in *Harwich* strain, as determined by DNA-seq analysis.

Supplementary Table 2. Description of *Harwich*-derived stocks.

Supplementary Table 3. List of crosses.

Supplementary Table 4. RNA FISH probes.

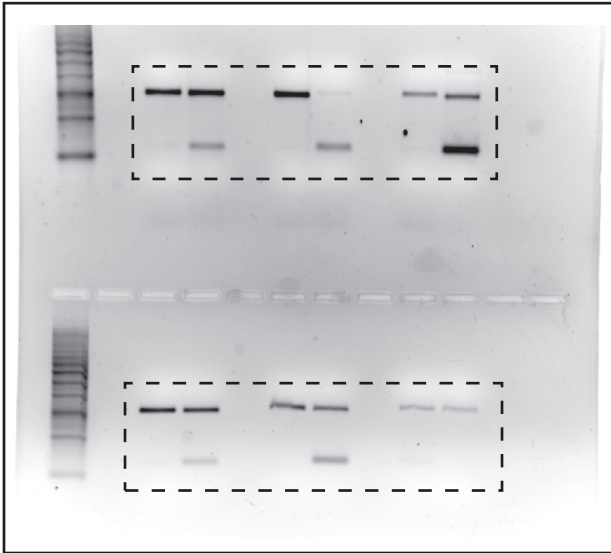
Supplementary Table 5. List of primers.

Supplementary Note 1

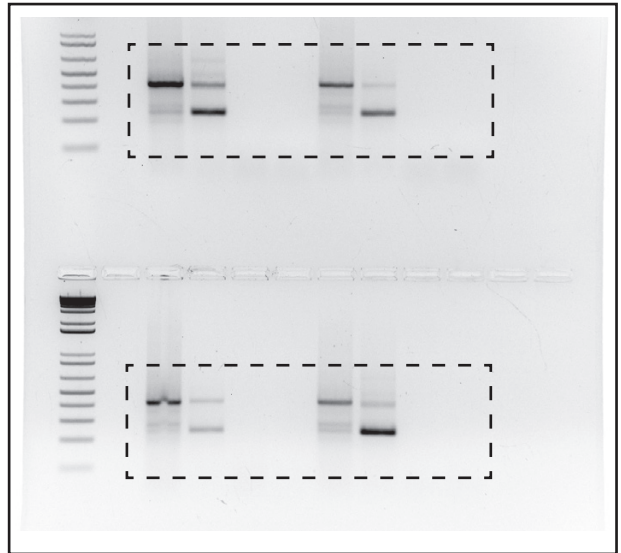
In contrast to Gypsy, for which splicing changes are accompanied with mRNA accumulation changes in piRNA mutants (Figure 4, Extended data Figure 7), *P*-element mRNA steady-state levels under dysgenic conditions or in piRNA pathway mutants in the Harwich background are not up-regulated despite loss of K9me3 marks (Figure 2-3; Extended data Figure 2,4, and 6). We interpret this result as consistent with the lack of sufficient promoter and enhancer activity of the endogenous *P*-elements and with the fact that the expression of cellular genes containing *P*-element insertions is invariable regardless to the methylation status of the resident element (Figure 3e-f, Extended data Figure 4e-g and 6a). Overall, the IVS3-*lacZ-neo* reporter, which is under the control of the HSP83 promoter, behaves similarly to the endogenous *P*-element insertions (i.e. changes are restricted to splicing regulation). Total transcript steady-state levels show a slight change in dysgenic crosses, as well as *aub* and *vas* mutants, with large changes being restricted to IVS3 splicing (Extended data Figure 3). In relation to the Panx/Silencio

mutant analyses, we observed that the effect on RNA levels and splicing was disproportionately affected, with RNA levels changing ~10-fold and splicing level changes of ~100-fold (Extended data Figure 3b). These data strongly suggest that the piRNA pathway mediates chromatin-dependent regulation of alternative splicing and that is largely independent of its previously described effects on the regulation of RNA transcription and RNA stability.

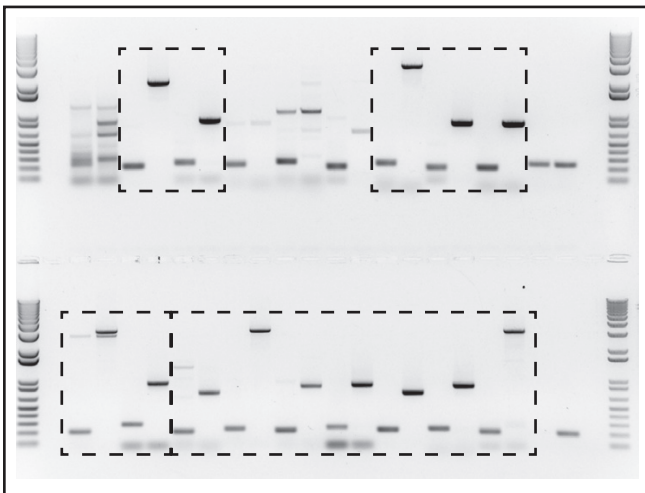
Figure 2b



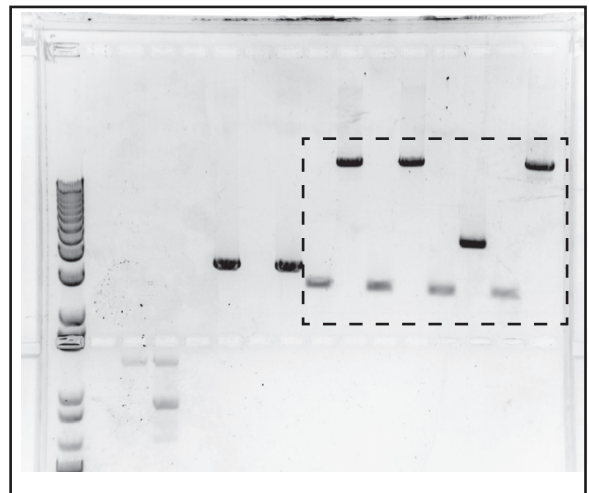
Extended data Figure 3



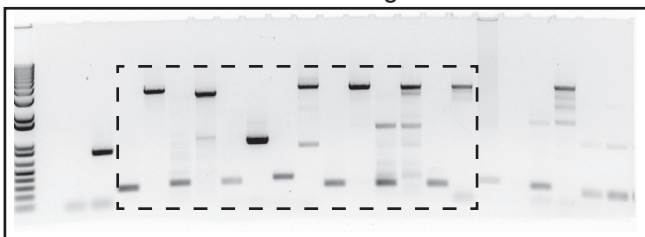
Extended data Figure 5a



Extended data Figure 5a



Extended data Figure 5a



Extended data Figure 6d

