Prevalent fast evolution of genes involved in heterochromatin functions

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Abstract

Heterochromatin is a gene-poor and repeat-rich genomic compartment ubiquitously found in eukaryotes. Despite its low transcriptional activity, heterochromatin plays important roles in maintaining genome stability, organizing chromosomes, and suppressing transposable elements (TEs). Given the importance of these functions, it is expected that the genes involved in heterochromatin regulation would be highly conserved. Yet, a handful of these genes have been found to evolve rapidly. To investigate whether these previous findings are anecdotal or general to genes modulating heterochromatin, we compiled an exhaustive list of 106 candidate genes involved in heterochromatin functions and investigated their evolution over both short and long evolutionary time scales in *Drosophila.* Our analyses found that these genes exhibit significantly more frequent evolutionary changes, both in the forms of amino acid substitutions and gene copy number variation, when compared to genes involved in Polycomb-based repressive chromatin. While positive selection drives amino acid changes within both structured domains with diverse functions and irregular disordered regions (IDRs), purifying selection may have maintained the proportions of IDRs. Together with the observed negative associations between rates of protein evolution of these genes and genomic TE abundance, we propose an evolutionary model where the fast evolution of genes involved in heterochromatin functions is an inevitable outcome of the unique molecular features of the heterochromatin environment, while the rapid evolution of TEs may be an effect rather than cause. Our study provides an important global view of the evolution of genes involved in this critical cellular domain and provides insights into the factors driving the distinctive evolution of heterochromatin.

Introduction

Heterochromatin, first discovered as the heavily stained chromosomal regions that remain condensed throughout the cell cycle (Heitz 1928), is a distinct cytological domain that is conserved across eukaryotic cells (Liu et al. 2020). While a small fraction of heterochromatin was found to be cell-type specific, or “facultative”, the majority of these chromosomal blocks remain condensed across different cell types and are known as “constitutive” heterochromatin (Heitz 1928). Constitutive heterochromatin (referred to as “heterochromatin” for simplicity hereafter) is usually located around centromeres and telomeres (Janssen et al. 2018), and its underlying DNA is depleted of functional genes and mainly composed of repetitive sequences, including satellite repeats (Peacock et al. 1978) and transposable elements (TEs) (Hoskins et al. 2007; Hoskins et al. 2015). Accordingly, heterochromatin is oftentimes assumed to be functionally inert and nicknamed the “dark matter” of the genome. Yet, studies have found heterochromatin playing critical roles in many chromosomal functions, such as maintaining genome stability (Peng and Karpen 2009; Janssen et al. 2018), mediating chromosome segregation (Dernburg et al. 1996; Karpen et al. 1996; Bernard et al. 2001), and ensuring proper DNA repair of repeat-rich sequences (Janssen et al. 2016; Kendek et al. 2021). Not surprisingly, disruption of heterochromatin functions has been linked to various diseases (Hahn et al. 2010), aging progression (Villeponteau 1997; J.-H. Lee et al. 2020), and cancer (Janssen et al. 2018).

The critical functions and inter-species conservation of heterochromatin domains would naturally lead to the expectation that genes modulating the function of heterochromatin should be highly conserved. Surprisingly, however, a handful of these genes show non-neutral evolution of their amino acid sequence and rapid turnover of gene copy number, both of which are consistent with a history of positive selection. The evolution of some of these genes was of interest due to them being a core structural component of heterochromatin, such as Heterochromatin Protein 1a (HP1a) (Levine et al. 2012; Helleu and Levine 2018), and its paralogs (Vermaak et al. 2005; Ross et al. 2013). The rest of the genes were studied for other research questions, including the evolution of small RNA pathways (Obbard et al. 2006; Obbard et al. 2009), transcription factors (Kasinathan et al. 2020), or germline stem cells (Flores et al. 2015), as well as speciation (Barbash et al. 2003). Because satellite repeats (Henikoff et al. 2001) and TEs (Cosby et al. 2019), both enriched in heterochromatin, can defy Mendel’s law and increase their transmission to the next generation, an arms race between heterochromatin and host genes suppressing such selfish behaviors has been a common theme explaining the rapid evolution of these genes (e.g., (Vermaak et al. 2005; Satyaki et al. 2014; Helleu and Levine 2018; Brand and Levine 2021)). Further supporting such conjecture, the composition, abundance, and location of repetitive sequences in heterochromatin are found to diverge rapidly even between closely related species (Wei et al. 2014; Wei et al. 2018; Kim et al. 2021; de Lima and Ruiz-Ruano 2022).

However, this handful of genes represents only the tip of the iceberg of genes involved in modulating heterochromatin functions. The unique and essential functions of heterochromatin depend on its specific enrichment of repressive di- and tri-methylation at histone H3 Lysine 9 (H3K9me2/3, (Allshire and Madhani 2018)), and the “reader” of these marks, HP1a, which

serves as the foundational structural component of heterochromatin (Eissenberg and Elgin 2014) and leads to DNA compaction (Verschure et al. 2005) and transcriptional suppression (Li et al. 2003). Interestingly, the binding of HP1a to H3K9me2/3 further recruits “writers” of H3K9me (histone methyltransferase, (Schotta 2002)), and this positive feedback mechanism between readers and writers leads to the propagation of such repressive epigenetic marks that is independent of the underlying DNA sequence (Allshire and Madhani 2018). This unique ability of heterochromatin to “spread” is best exemplified by the Position Effect Variegation (PEV) first discovered in *Drosophila* (Muller 1930)—the mosaic expression of euchromatic genes translocated to heterochromatin-proximal regions due to the spreading of repressive H3K9me2/3 from heterochromatin (Girton and Johansen 2008; Elgin and Reuter 2013). By studying mutants that either enhance or weaken PEV, many genes involved in heterochromatin functions were identified. These include not only the structural (e.g., (Shaffer et al. 2006)) and enzymatic (e.g., (Czermin et al. 2001)) components for heterochromatin, but also those that antagonize the initiation and/or maintenance of heterochromatin (e.g., writers of antagonizing histone modifications; (Bao et al. 2007)). Still other genes were discovered through their co-localization with heterochromatin domain either cytologically (e.g., (Swenson et al. 2016)) or epigenomically (e.g., (Alekseyenko et al. 2014)), and were subsequently identified to be involved in heterochromatin function.

Given the large numbers and vastly diverse functional roles of genes involved in heterochromatin function, we conducted an expansive survey to determine whether the previously reported rapid evolution of a small subset of these genes is anecdotal or a common feature of proteins involved in the functions of this unique chromatin environment. To do so, we compiled an exhaustive list of candidate genes that have been shown, or are likely, to be involved in heterochromatin functions, including PEV modifiers, histone-modifying enzymes influencing H3K9me2/3 enrichment, and genes whose protein products localize to heterochromatin. Our investigation finds that these genes evolve exceptionally fast, a pattern that is not general to genes interacting with other repressive chromatin markers and suggests a constitutive heterochromatin-unique mechanism. We further dissect the domains and protein properties targeted by positive selection and specifically test the premise that the rapid evolution of genes involved in heterochromatin functions could be driven by fast-changing repetitive sequences. Based on our findings, we propose an evolutionary model where the rapid changes in these genes are the unavoidable consequence of the molecular features of heterochromatin, and that the evolution of repetitive sequences may be the consequence, instead of the cause. Our study provides an important global view of the evolution of genes involved in this critical cellular domain and sheds light on the drivers behind the unique evolution of heterochromatin.

Results

Identification of candidate genes involved in heterochromatin functions

With the goal of identifying the global evolutionary patterns for genes involved in the functions of heterochromatin, we selected candidate genes based on three criteria to maximize inclusivity (**Table S1**). The first category contains genes demonstrated to modulate PEV (Girton and Johansen 2008; Elgin and Reuter 2013; Swenson et al. 2016). Genes whose mutants weaken PEV and therefore heterochromatin functions are known as Suppressors of Variegation (*Su(var)*) while mutants that augment PEV are Enhancers of Variegation (*E(var)*). Here, we included 59 and seven previously identified *Su(var)s* and *E(var)s* respectively.

The second category focuses on histone-modifying enzymes that can influence the enrichment levels of H3K9me2/3. Enrichment of H3K9me2/3 requires "eraser" proteins that first remove antagonizing active marks, such as H3K9ac (deacetylases, (Czermin et al. 2001)) and H3K4me3 (demethylase, (Rudolph et al. 2007)). This is followed by writer proteins depositing H3K9me2/3 (H3K9 methyltransferases, (Schotta 2002)). We consider these histone-modifying enzymes to enhance H3K9me2/3 enrichment and thus heterochromatin functions. On the contrary, erasers for H3K9me2/3 (H3K9 demethylase, (Herz et al. 2014)) as well as writers for acetylation (acetyltransferase, (Kuo and Andrews 2013)) and S10 kinase (Deng et al. 2008) are known to antagonize the deposition and maintenance of H3K9me2/3. H4K20me3 is another conserved hallmark of heterochromatin (Schotta et al. 2004) and we included the corresponding methyltransferases. In total, our list contains 16 and seven histone-modifying enzymes that enhance or weaken heterochromatin functions respectively.

The last category includes genes whose protein products co-localize with heterochromatin, suggesting they play roles in this subnuclear compartment. We surveyed the literature for cytology-based evidence of co-localization with HP1a through either immunofluorescence (e.g., (Greil et al. 2007)) or live imaging (e.g., (Swenson et al. 2016)) as well as epigenomics through Chromatin Immunoprecipitation (ChIP, e.g., (Alekseyenko et al. 2014; Kasinathan et al. 2020)). Because a previous comprehensive survey found that some HP1a-binding proteins identified through Immunoprecipitation-Mass Spectrometry (IP-Mass Spec) are not necessarily enriched in heterochromatin cytologically (Swenson et al. 2016), genes implicated in HP1a-binding but lacking localization evidence are not included in our analysis. Hereafter, we referred to this largest category with 63 genes as "heterochromatin proteins."

In total, we studied the evolution of 106 genes involved in heterochromatin functions, which we termed "heterochromatin-related genes" hereafter (see **Figure 1A** for the number of genes in each category and **Table S1** for a full list of genes and references). It is worth noting that 43 candidate genes belong to more than one category because these three categorizations are not mutually exclusive (**Table S1**). We consider genes in categories of *Su(var)s*, histone modifying enzymes enhancing H3K9me2/3 or H4K20me3 enrichment, or heterochromatin proteins as "mediating" heterochromatin functions, while those defined as *E(var)s* or histone modifying enzymes weakening H3K9me2/3 enrichment as "antagonizing" heterochromatin functions. To determine whether the evolutionary histories of heterochromatin-related genes are exceptional, we compared them with Polycomb group (PcG) genes (Kassis et al. 2017), whose protein products are enriched at facultative heterochromatin. Even though also generally associated with transcriptional suppression, facultative heterochromatin is restricted to

developmentally regulated genes in euchromatin and is enriched for another type of repressive histone modification (H3K27me3) (Bannister and Kouzarides 2011; Bell et al. 2023). Polycomb genes thus allowed us to determine whether the exceptional evolutionary histories, if any, of heterochromatin-related genes are due to their involvement in a repressive chromatin environment or specifically for heterochromatin structure.

Heterochromatin-related genes experience prevalent positive selection for the protein sequences across short and long-time scales

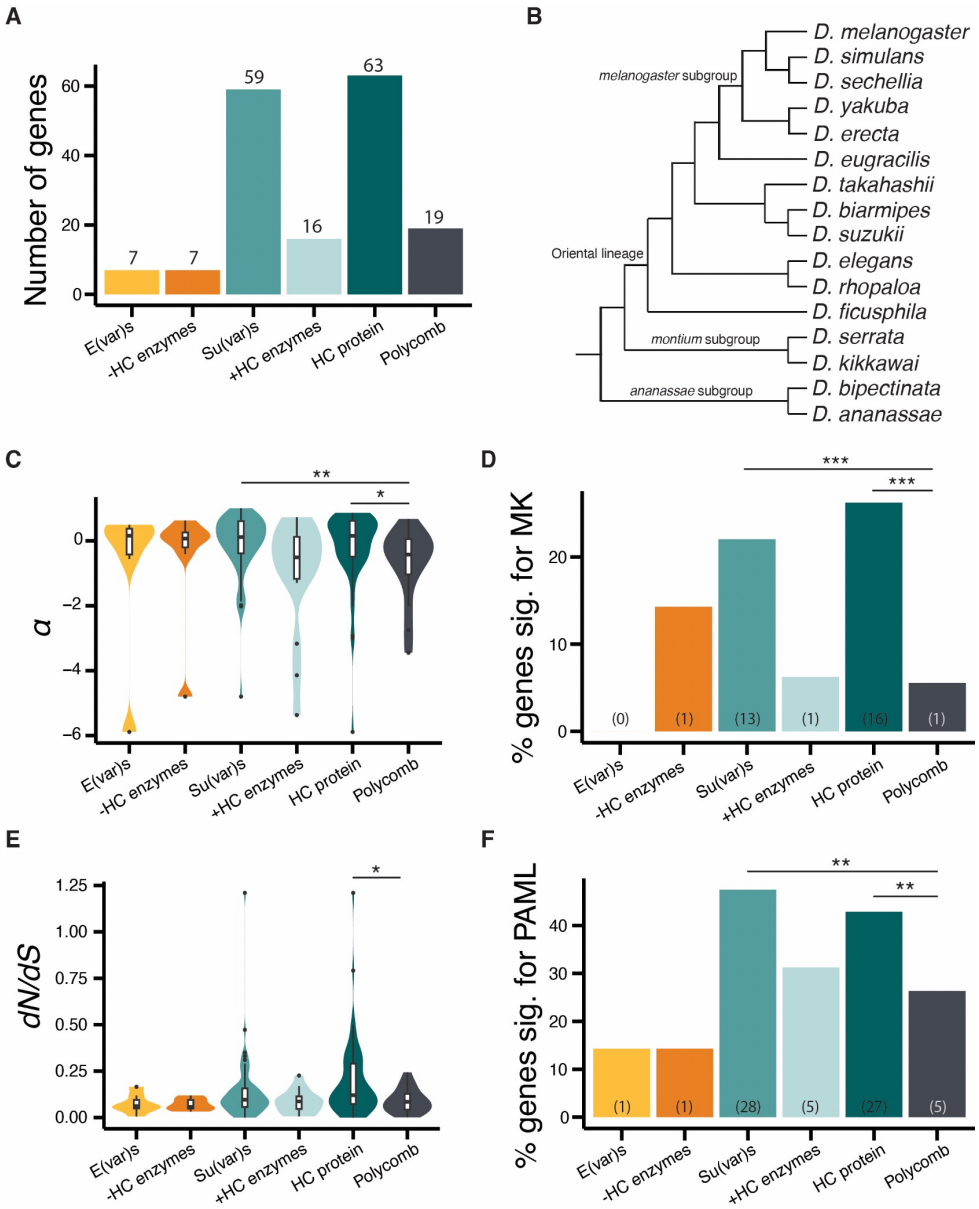
We performed evolutionary genetic tests to identify heterochromatin-related genes with signatures of positive selection at two time scales—contrasting the polymorphism within *D. melanogaster* and divergence between *D. melanogaster* and *D. simulans* (~5 million years divergence) under the framework of unpolarized McDonald-Kreitman tests (MK tests, (McDonald and Kreitman 1991)), and phylogenetic analysis by maximum likelihood (PAML) tests (Yang 2007) using 16 *Drosophila* species (see **Figure 1B** for studied species) with an estimated divergence time of ~25 million years (Suvorov et al. 2022) (see Materials and Methods).

On a shorter evolutionary time scale, we first estimated the proportion of amino acid substitutions that might have been driven by positive selection (α, (Smith and Eyre-Walker 2002)). Heterochromatin-related genes, as a group, have significantly larger α than those of Polycomb (*Mann-Whitney U tests, p* = 0.0319*, median* = 0.0875 (heterochromatin-related genes) and -0.424 (Polycomb)). Breaking down our candidate genes according to functions revealed that all categories, except for *E(var)s*, have a significantly larger α than Polycomb control genes (**Figure 1C**). These observations suggest that heterochromatin-related genes experienced prevalent positive selection. Consistently, we identified that 21 out of 104 (20.1%) heterochromatin-related genes with sufficient polymorphism data to perform MK tests have evidence of adaptive evolution (rejection of the null hypothesis and an excess of nonsynonymous fixed differences between species). This is a significantly larger proportion than that of the Polycomb genes (5.56%; *Fisher's Exact Test*, $p = 0.191$; *Binomial test*, $p < 10^{-7}$, odds ratio = 4.30) and all genes in the genome (8.03%, *Fisher's Exact Test*, $p = 10^{-5}$; *Binomial test*, $p = 10^{-5}$, odds ratio = 2.90), while we found no difference in this proportion between Polycomb genes and all genes (*Fisher's Exact Test* and *Binomial test*, $p = 1$). Specifically, *Su(var)* (22.0%) and heterochromatin proteins (26.2%), both of which are expected to mediate heterochromatin functions, have much larger proportions of genes with evidence of positive selection than Polycomb (**Figure 1D**). Indeed, candidate genes mediating heterochromatin functions, as a group, show significantly more prevalent evidence of adaptive evolution than the Polycomb (20.2%, *Fisher's Exact Test*, $p = 0.190$; *Binomial test*, $p < 10^{-7}$, odds ratio = 4.27). It is worth noting that the number of codons involved in the MK tests is similar across categories of genes (*Mann-Whitney U tests, p* > 0.05 for all comparisons), suggesting that these observations are unlikely driven by differences in the statistical power of the MK tests.

On a longer evolutionary time scale, we estimated the relative rates of amino acid substitution (nonsynonymous divergence/synonymous divergence, *dN/dS*) across 16 studied *Drosophila* species. While there is an overall trend that heterochromatin-related genes have larger *dN/dS* ratios than the Polycomb control genes (medians = 0.0970 (heterochromatin-related genes) *v.s.* 0.0852 (Polycomb controls)), the difference is not statistically significant

(*Mann-Whitney U test, p* = 0.225) and only the category of "heterochromatin protein" has a statistically larger *dN/dS* ratio than that of the Polycomb genes (**Figure 1E**). To test whether the changes in *dN/dS* ratios of heterochromatin-related genes over the phylogenetic tree might have been driven by positive selection, we compared the log likelihood for two alternative models estimating *dN/dS* at each site*:* the null model that assumes *dN/dS* ratio is beta-distributed and not greater than one (M8a) and the alternative model in which *dN/dS* ratio is allowed to be greater than one (M8 models; (Yang 2007)). A significantly larger likelihood of the alternative model is consistent with a history of positive selection acting on the gene (Swanson et al. 2003). We found that the substitution patterns of 42 heterochromatin-related genes (39.6%) better fit the alternative model, suggesting prevalent positive selection acting on them. This proportion is significantly larger than that of Polycomb genes (26.3%; *Fisher's Exact Test*, *p* = 0.314; *Binomial test*, *p* = 0.00276, odds ratio = 1.83) and a set of randomly sampled genes (24.8% of 1,000 randomly selected genes, *Fisher's Exact Test*, *p* = 0.00159; *Binomial test*, *p* = 0.000975, odds ratio = 1.99), while there was no difference in this proportion between the Polycomb and random genes (*Fisher's Exact Test*, *p* = 0.795; *Binomial test*, *p* = 0.796). Similar to observations made on a short evolutionary time scale, *Su(var)s* and heterochromatin proteins have stronger evidence of positive selection compared to Polycomb and other genes (**Figure 1F**), as do all genes mediating heterochromatin functions (41.4%, *Fisher's Exact Test*, *p* = 0.305; *Binomial test*, *p* = 0.00125, odds ratio = 1.97). Interestingly, while histone-modifying enzymes that enhance heterochromatin, as a group, do not exhibit exceptional rates of protein evolution, likely due to the low statistical power associated with its small number of genes, *all* methyltransferases for H3K9me2/3 (*Su(var)3-9, egg, G9a*) show accelerated rates of protein evolution (**Table S1**), strongly suggesting the likely source of positive selection (see Discussion). Overall, we found that heterochromatin-related genes, especially those mediating heterochromatin functions, experienced prevalent positive selection on their protein sequence at both short- and long-time scales. This observation is likely driven by the involvement of these genes in functions specific to the heterochromatin environment, instead of interactions with repressive epigenetic marks.

Figure 1. Positive selection on protein sequences of heterochromatin-related genes found by evolutionary tests at both short- and long- evolutionary time scales. (A) Barplot showing the number of genes in each category of heterochromatin-related genes and the Polycomb control genes. Those expected to mediate or antagonize heterochromatin functions are in hues of green and orange respectively. -HC enzyme: histone-modifying enzymes weakening H3K9me2/3 enrichment; +HC enzyme: histone-modifying enzymes enhancing H3K9me2/3 enrichment. (B) Phylogenetic tree for the species included in the study. Note that the branch lengths are not to scale. (C and E) Violin plots for α, the proportion of amino acid substitutions fixed by positive selection inferred by the MK test (C) and *dN/dS* ratio, the relative rates of nonsynonymous substitutions (E), for different categories of genes. Numbers of significant genes are in parentheses. (D and F) Barplots showing the proportion of genes with significant MK tests (D) and accelerated rates of *dN/dS* driven by positive selection (F). *Mann-Whitney Test* (C and E) and *Binomial test* (D and F): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$



Rapid evolution of heterochromatin-related genes is also exhibited as changes in gene copy number

In addition to changes in amino acid sequences, the rapid evolution of genes can be in the form of changes in gene copy number (Hastings et al. 2009), and dramatic turnover of gene copy number for a small set of heterochromatin-related genes was previously reported (Levine et al. 2012; Lee et al. 2017; Helleu and Levine 2018). Changes in gene copy number may hold particular significance for heterochromatin functions—several enzymatic and structural proteins of heterochromatin have been shown to exhibit dosage-dependent effects (Elgin and Reuter 2013). Accordingly, changes in gene copy number of heterochromatin-related genes could have immediate functional consequences.

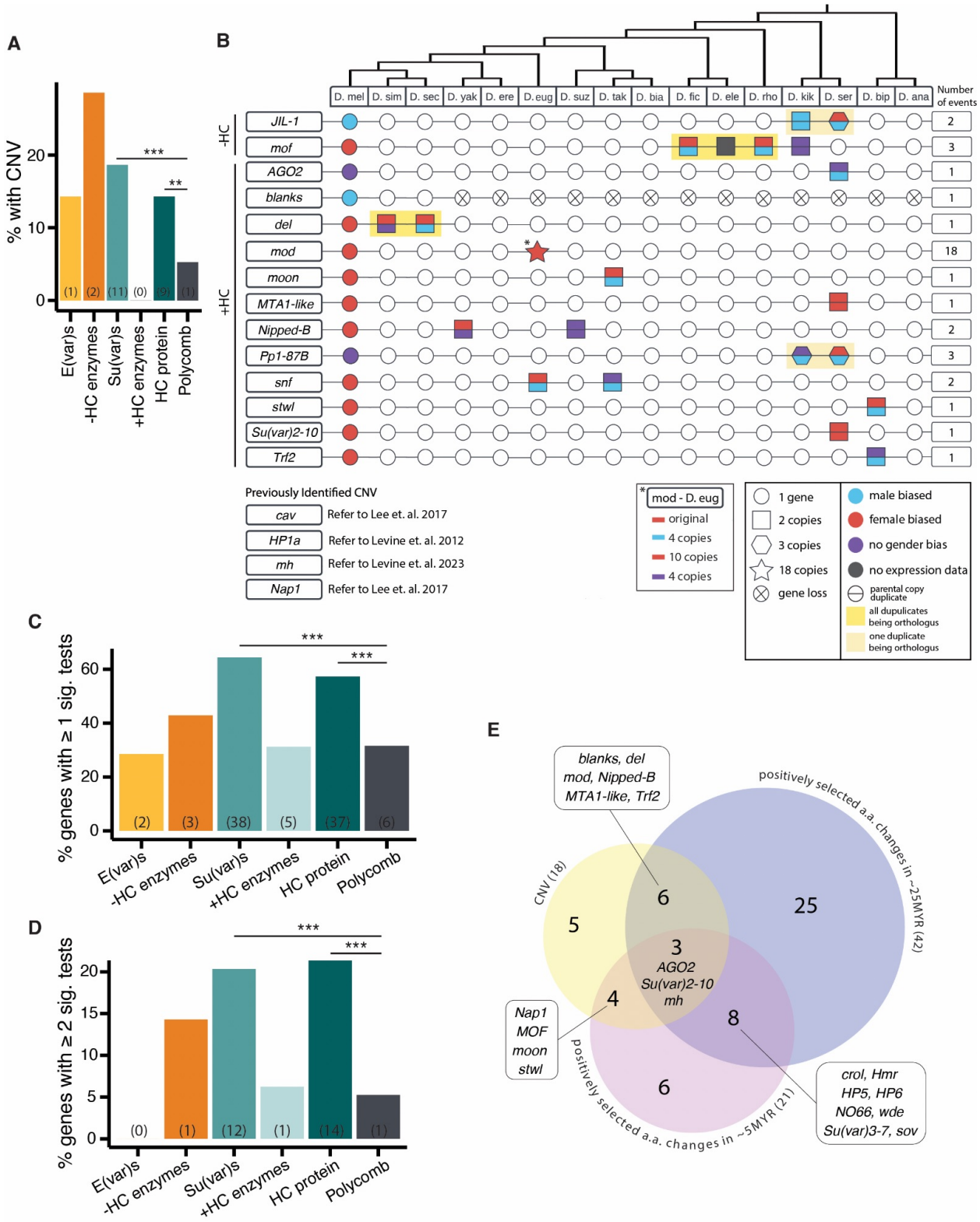
We performed reciprocal *BLAST* searches using *D. melanogaster* amino acid sequences as queries to identify homologs and paralogs in other species, followed by manual curations (see Materials and Methods). With these, we found 18 heterochromatin-related genes having differences in gene copy number among 16 *Drosophila* species studied (17.0% of the heterochromatin-related genes), with 17 genes having gains of copies and one gene loss when compared to *D. melanogaster.* Similar to our analyses for the evolution of amino acid sequences (**Figure 1**), the proportion of heterochromatin-related genes with copy number variation (CNV) is significantly more than that of Polycomb genes (5.26%, *Fisher's Exact Test*, $p = 0.302$, *Binomial test*, $p < 10^{-5}$, odds ratio = 3.66). *Su(var)* genes and heterochromatin proteins again exhibit exceptionally large proportions of genes with CNV (**Figure 2A**). Several of these have been studied before (e.g., *HP1a* (Levine et al. 2012), *Cav* and *Nap-1* (Lee et al. 2017), and *mh* (Brand et al. 2023)) and our following discussions mainly focused on those identified by our study.

By using synteny to infer the orthologous relationships between duplicates in different species (see Materials and Methods), we found that most observed CNVs for heterochromatin-related genes involve a single or two duplication events (**Figure 2B and Table S2**). The most notable exception is the nucleolin homolog, *mod,* which has 18 duplications in *D. eugracilis* alone. *mod* plays important roles in morphogenesis (Graba et al. 1994) and spermatogenesis (Park et al. 2023) and has been shown as a dosage-dependent *Su(var)* (Garzino et al. 1992), suggesting these identified CNVs could readily alter heterochromatin functions. In addition, we observed complex duplication/loss events leading to the CNV observed in *mof*, an acetyltransferase that influences H3K9me2/3 enrichment (Feller et al. 2015) and is known to be involved in dosage compensation (Hilfiker et al. 1997). In addition to the duplication happening on the lineage leading to *D. kikawai,* there is likely a duplication event of *mof* in the common ancestor of the oriental linage, followed by a subsequent loss in the subsets of lineages, leading to the paraphyletic presence of a *mof* duplicate.

Duplicated genes are commonly found to have male-biased expression, which is suggested as a resolution to sexual genetic conflicts (Gallach and Betrán 2011). We are interested in examining whether duplicates of heterochromatin-related genes exhibit similar trends of sex-biased expression. By using publicly available transcriptome data for the species studied, we categorized the parental ortholog and duplicated paralog (with respect to *D. melanogaster*) as male-biased, female-biased, or unbiased in expression (see Materials and Methods). With the exception of *MTA-like* and *Su(var)2-10*, most identified duplicates do indeed show male-biased expression, irrespective of whether the parental copy is male or female-

biased in expression (**Figure 2B**). Interestingly, for *mod* in *D. eugracilis*, the parental copy has a female-biased expression, while the 18 duplicates exhibit a mixture of male-biased, female-biased, and unbiased expression. Another worth-mentioning case is *del,* which is part of a germline complex enabling the transcriptions of TE-targeting small RNAs from loci in pericentromeric heterochromatin (Mohn et al. 2014) and shows female-biased expression. While the *del* duplicate in *D. simulans* shows male-biased expression, the *orthologous* duplicated copy in the sister species *D. sechellia* has no biases in expression. In summary, the rapid evolution of heterochromatin-related genes, when compared to Polycomb genes, is also reflected in their changes in gene copy number.

Figure 2. Rapid evolution of heterochromatin-related genes manifested in multiple forms. (A) Barplot showing the proportion of heterochromatin-related genes and Polycomb control genes with CNV. The numbers of genes with CNV are in parentheses. (B) Summary of the duplication and loss events (the numbers of events are denoted by shape) as well as sex-specific expression biases (represented by colors) for heterochromatin-related genes identified to have CNV. Shapes split into two halves represent the expression biases for the parental copy (top half) and the duplicated copy (bottom half). The shaded yellow denotes the ortholog relationship between identified duplicates. (C and D) Barplots showing the proportion of genes found to be under positive selection and/or fast evolve with at least one (C) or at least two (D) conducted evolutionary tests. The numbers of genes in each category are shown in parentheses. (E) Venn diagram depicting the number of genes identified to have exceptional evolution with one of the three evolutionary tests as well as their overlaps, with those identified by more than one test listed. a.a. = amino acid. *Binomial tests*: $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.


A
% with CNV
E(var)s
-HC enzymes
Su(var)s
+HC enzymes
HC protein
Polycomb
B
D. mel
D. sim
D. sec
D. yak
D. ere
D. eug
D. suz
D. tak
D. bia
D. fic
D. ele
D. rho
D. kik
D. ser
D. bip
D. ana
Number of events
-HC
+HC
JIL-1
mof
AGO2
blanks
del
mod
moon
MTA1-like
Nipped-B
Pp1-87B
snf
stwl
Su(var)2-10
Trf2
Previously Identified CNV
cav
Refer to Lee et. al. 2017
HP1a
Refer to Levine et. al. 2012
mh
Refer to Levine et. al. 2023
Nap1
Refer to Lee et. al. 2017
mod - D. eug
original
4 copies
10 copies
4 copies
1 gene
2 copies
3 copies
18 copies
gene loss
male biased
female biased
no gender bias
no expression data
parental copy duplicate
all dupulicates being orthologus
one duplicate being orthologus
C
% genes with ≥ 1 sig. tests
D
% genes with ≥ 2 sig. tests
E
blanks, del
mod, Nipped-B
MTA1-like, Trf2
positively selected a.a. changes in ~25MYR (42)
CNV (18)
positively selected a.a. changes in ~5MYR (21)
AGO2
Su(var)2-10
mh
Nap1
MOF
moon
stwl
crol, Hmr
HP5, HP6
NO66, wde
Su(var)3-7, sov

Multiple heterochromatin-related genes rapidly evolve on both time scales on both protein sequences and gene copy number

Our above observations suggest that heterochromatin-related genes, *as a group*, experienced prevalent positive selection on both short and long evolutionary time scales and on both amino acid sequences and gene copy number. We investigated the similarities and differences in genes with evidence of rapid evolution for the three evolutionary tests we conducted, which may provide information for the possible source of positive selection.

We first examined the number of genes with evidence of rapid evolution in *any one of our tests* (positive selection on protein sequence on a short time scale, positive selection on protein sequence on a long time scale, and changes in gene copy number) and found that the proportion of these genes is significantly larger for heterochromatin-related genes than that of Polycomb control genes (53.8% vs 31.6%, *Fisher's Exact test*, $p = 0.0861$, *Binomial test*, $p < 10^{-6}$, odds ratio = 2.50). Consistent with analyses conducted separately for different evolutionary tests, *Su(var)s* and heterochromatin proteins have an especially large proportion of genes with evidence of rapid evolution in any one of the tests (**Figure 2C**). We also found that the differences in the proportion of genes with evidence of rapid evolution between gene categories become even more dramatic when considering genes with exceptional evolution for *more than one test* (**Figure 2D**), suggesting some heterochromatin-related genes may be recurrent targets for positive selection and/or exhibit rapid evolution in multiple forms.

To identify genes that repeatedly show evidence of rapid evolution in our tests, we counted the number of heterochromatin-related genes with multiple evidence of rapid evolution (**Figure 2E**) and compared that to null expectations. For all combinations of evolutionary tests, heterochromatin-related genes are at least 1.5 times more likely (odds ratio > 1.5) to show more than one evidence of positive selection than by chance (positive selection on protein sequence on short *v.s.* long evolutionary time-scale, *Fisher's Exact Test, p* = 0.217, *odds ratio* = 1.90, *Binomial tests*, $p = 0.362$; CNV *v.s.* positive selection on protein sequence on a short evolutionary scale, *Fisher's Exact Test, p* = 0.0463, *odds ratio* = 3.316, *Binomial tests*, $p = 0.0933$; CNV *v.s.* positive selection on protein sequence on a long evolutionary scale, *Fisher's Exact Test, p* = 0.429, *odds ratio* = 1.658, *Binomial tests*, $p = 0.436$). Three genes show evidence of rapid evolution for *all* three tests: *Ago2*, the core effector in endogenous siRNA silencing pathway that silences TEs in somatic cells (Ghildiyal et al. 2008), *Su(var)2-10*, which critically links piRNA targeting of TE sequences and the transcriptional silencing of TEs (Ninova et al. 2020), and *mh*, whose evolution is the focus of a recent study (Brand et al. 2023). Other heterochromatin-related genes with more than one evidence of rapid evolution (**Figure 2E**) include those related to silencing of TEs (e.g., *del, moon, sov, Trf2, wde*), dosage compensation (e.g., *Su(var)3-7*), maintenance of chromatin structure (e.g., *Nipped-B*), and structural components of heterochromatin (e.g., *HP5*). While most of these genes are either *Su(var)* or heterochromatin proteins, several histone-modifying enzymes were also found to undergo positive selection with multiple evolutionary tests, such as the H3K4me3 demethylase *NO66* and acetyltransferase *mof*. These observations suggest that positive selection recurrently acts on heterochromatin-related genes with diverse functions.

Positive selection targets both ordered domains and intrinsically disordered regions encoded by heterochromatin-related genes

To identify the potential evolutionary drivers for the observed rapid evolution of heterochromatin-related genes, we investigated the locations of positive selection within each rapidly evolving gene. We identified windows with evidence of adaptive evolution by performing sliding MK tests and located sites with high Bayes Empirical Bayes (BEB) posterior probability of positive selection under the PAML framework (Yang et al. 2005). Locations of these windows or sites with evidence of positive selection were then contrasted with the annotated and/or predicted domains of heterochromatin-related genes (see Materials and Methods). We found that signatures of positive selection are present in domains with diverse functions (**Figure 3**) and several of them are especially pertinent to heterochromatin functions. These include chromo domains that directly interact with histone methylation (in *HP6, rhi, Kis*) and Jmjc demethylase domain in *NO66*, the H3K4me3 demethylase. We also found signatures of positive selection located within various nucleic acid binding domains (e.g., C2H2 type and UBZ4-type Zinc-Finger DNA binding domain) and domains mediating protein-protein interactions (e.g., BESS domains).

In addition to structured domains with well-characterized functions, proteins also contain regions that lack fixed three-dimensional structures and, because of that, are expected to be less evolutionarily constrained. Known as intrinsically disordered domains (IDRs), these protein regions are found to harbor more amino acid and indel polymorphisms than structured domains (Brown et al. 2002; Khan et al. 2015). Yet, IDRs are increasingly appreciated for playing critical roles in protein functions (Forman-Kay and Mittag 2013). In particular, they are frequently found in proteins enriched in phase-separated cellular compartments (Nott et al. 2015; Pak et al. 2016; Lin et al. 2017), and IDR-mediated phase properties have been suggested to be critical for heterochromatin functions (Larson et al. 2017; Strom et al. 2017). Accordingly, we investigated whether IDRs in heterochromatin-related genes may also have signatures of positive evolution by using flDPnn (Hu et al. 2021) to predict IDRs in *D. melanogaster* protein sequences. Interestingly, we found that signatures of rapid evolution also frequently fall within IDRs (**Figure 3**), and, for multiple rapidly evolving heterochromatin-related genes, fast-evolving sites/windows only fall within IDRs, but not other structured domains (**Figure 3**, right column).

The observed prevalence of signatures of positive selection falling within IDRs prompted us to investigate whether varying IDR properties of heterochromatin-related genes contribute to the evolutionary differences between gene categories. For each gene of each species, we estimated the proportion of amino acid sequences encompassing IDRs (% of IDRs). The percentage of IDRs for *D. melanogaster* proteins does not differ between heterochromatin-related genes and Polycomb controls, even for heterochromatin proteins whose protein products localize to heterochromatin and are expected to have higher IDR content (Guthmann et al. 2019) (*Mann-Whitney U test,* $p > 0.05$ for all tests **Figure 4A**). This unexpected finding could result from the fact that Polycomb proteins were also shown to exhibit phase separation mechanisms (Tatavosian et al. 2019) and may thus similarly have high IDR content. Contrary to the expectation that IDRs are less evolutionarily constrained, the proportion of IDRs does not differ between heterochromatin-related genes with or without evidence of positive selection (*Mann-Whitney U test,* $p > 0.05$; **Figure 4B**).

We also estimated the phylogenetic signals (Blomberg and Garland Jr 2002) of % of IDRs across studied species to assess whether the evolution of IDR content deviates from that of random expectation (Brownian motion of trait evolution) given the phylogenetic tree (topology and branch length, see Materials and Methods). A small *Blomberg's K* (Blomberg et al. 2003),

our chosen index for phylogenetic signal, suggests that the evolution of the focused trait deviates from null expectation, which could result from either rapid trait evolution or convergent evolution (Kamilar and Cooper 2013). On the contrary, a large *K* indicates a strong phylogenetic signal, and stabilizing selection on the focused trait is a possible driver (Kamilar and Cooper 2013). We found no difference in *K* values between heterochromatin-related genes and the Polycomb control (*Mann-Whitney U test, p* = 0.485; **Figure 4C**), indicating that IDR content of heterochromatin-related genes evolves similarly with genes interacting with other kinds of repressive chromatin. Intriguingly, there is neither difference in *K* value between heterochromatin-related genes with and without evidence of positive selection over long evolutionary time scales (*Mann-Whitney U test, p* = 0.175; **Figure 4D**). Also, we found no correlations between *K* value and *dN/dS* for heterochromatin-related genes (*Spearman rank correlation tests, rho* = -0.00532, *p* = 0.953). Even more, several heterochromatin-related genes under positive selection have large *K* (e.g., heterochromatin proteins *D1, HP5*, and *XNP* and H3K9me2/3 methyltransferase *egg*; **Table S1**). In summary, positive selection acts on both structured domains with diverse functions as well as IDRs of heterochromatin-related genes, while stabilizing selection may have maintained the IDR content in the face of rapidly changing amino acid sequences.

Figure 3. Signatures of positive selection fall within various domains of heterochromatin-related genes. Genes with evidence of positive selection on protein sequences are categorized according to which types of domains the signatures of positive selection fall within. Annotated and/or predicted structured domains are shown as horizontal lines with the following functional categories: nucleic acid binding (orange), protein-protein interaction (dark blue), histone modification binding (yellow), and other domains (purple). IDRs are marked with gray. Highlighted windows or sites represent those under positive selection as identified by sliding MK tests (green) and BEB inferences (light blue). Note that genes with no positively selected windows/sites overlapping with either type of domain were not shown.

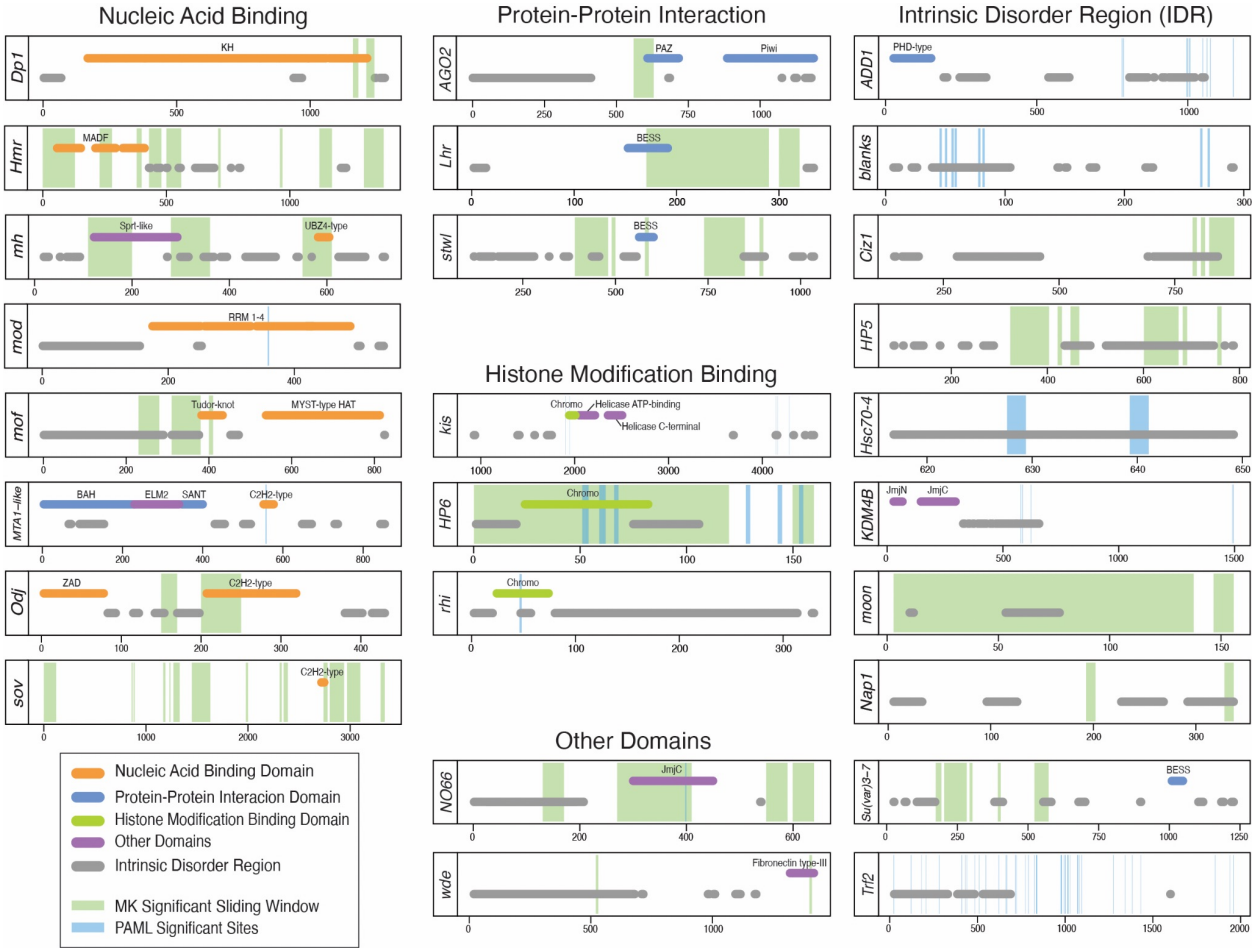
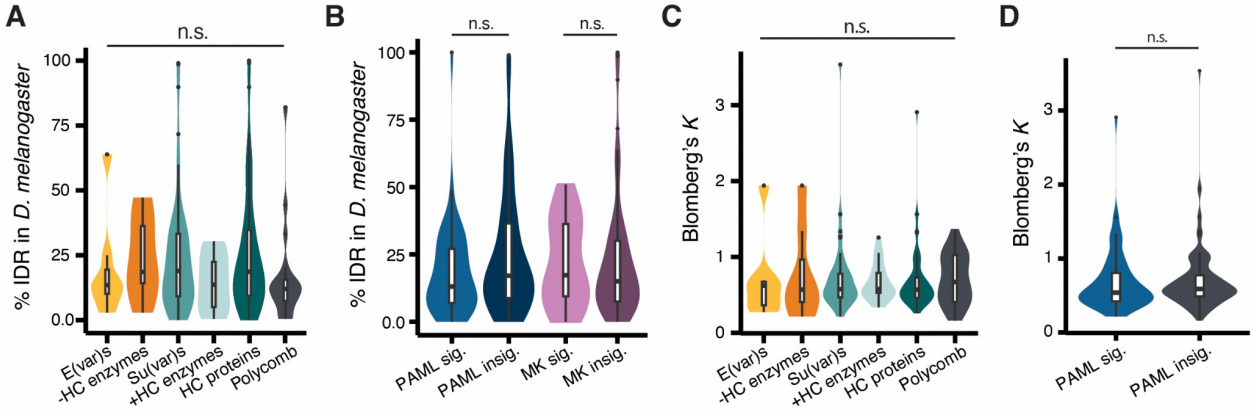


Figure 4. Evolution of IDR content of heterochromatin-related genes
(A and B) Violin plots comparing the % of IDR in *D. melanogaster* between categories of heterochromatin-related genes and the Polycomb control (A) and between heterochromatin-related genes with and without evidence of positive selection over both long or short evolutionary time scales (B). (C and D) Violin plots comparing *Blomberg's K* across species for categories of heterochromatin-related genes and the Polycomb control (C) and between heterochromatin-related genes with and without evidence of positive selection over a long evolutionary time scale (D). *Mann-Whitney U test*: *n.s. p* > 0.05.



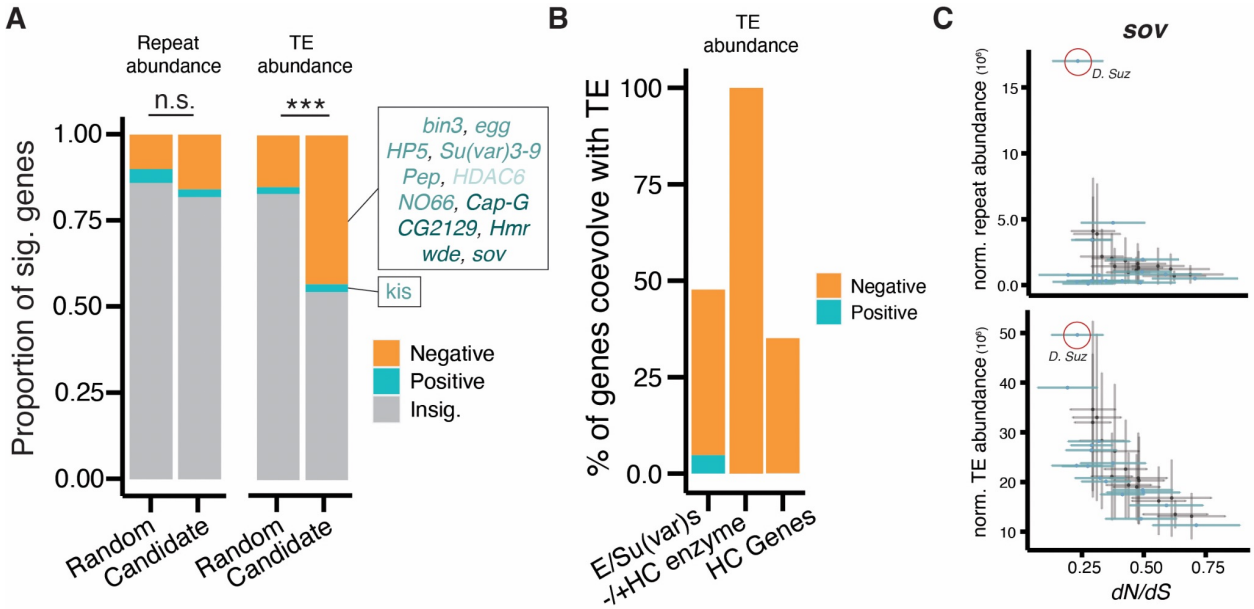
Rates of protein evolution of heterochromatin-related genes significantly associate with genomic TE abundance

The antagonistic interaction with repetitive sequences enriched in heterochromatin was suggested as the driver for the rapid evolution of a handful of heterochromatin-related genes (Vermaak et al. 2005; Satyaki et al. 2014; Helleu and Levine 2018). To test whether this conjecture may be broadly applicable to heterochromatin-related genes, we estimated the correlation between rates of protein evolution (*dN/dS*) of heterochromatin-related genes under positive selection and the abundance of heterochromatic repetitive sequences while correcting for phylogenetic nonindependence (using methods developed in (Lartillot and Poujol 2011)). We performed Illumina sequencing with PCR-free library preparation to avoid the sequencing bias against AT-rich sequences, which is commonly found in repetitive sequences (Wei et al. 2018), and estimated the genomic abundance of simple repeats and TEs, which should be dominated by the DNA content of heterochromatin (see Materials and Methods).

Among the 42 genes with evidence of positive selection over the long evolutionary time scale, we found 45.2% of them having *dN/dS* ratio significantly correlate with TE abundance across species, a proportion that is much higher than that of randomly selected genes (15.0%, *Fisher's Exact test*, $p = 0.000222$, *Binomial test*, $p < 10^{-7}$, **Figure 5A, Table S3**). Intriguingly, 95% of these correlations are in the negative direction, which indicates that these genes evolved faster in species with lower TE abundance. We also examined this proportion separately for each category of heterochromatin-related genes (**Figure 5B**). Quite strikingly, even though only a small number of histone-modifying enzymes were found to have evidence of positive selection (26.1%), *all* of their rates of protein evolution were significantly associated with TE abundance (**Figure 5B**). These include all three H3K9me2/3 methyltransferase (*egg, G9a*, and *Su(var)3-9*), *NO66* (H3K4me3 demethylase), *KDM4B* (H3K9me2/3 demethylase), and *HDAC6* (zinc-dependent deacetylase) (**Table S3**).

In stark contrast to analysis focusing on TE abundance, only 16.67% of heterochromatin-related genes with evidence of positive selection show a significant correlation between *dN/dS* and the abundance of simple repeats, a proportion that is not significantly different from randomly sampled genes (**Figure 5A**, *Fisher's Exact Test* and *Binomial test*, both $p > 0.05$). While examining the identified associations between simple repeats and *dN/dS* between species, we noticed that *D. suzukii*, a species with an unusually high abundance of simple repeats (Paris et al. 2020), might have driven many of the observed correlations (e.g., **Figure 5C**). Accordingly, we conducted a similar analysis without *D. suzukii* and found only one gene (2.38% of the genes) still shows a significant correlation between *dN/dS* and the abundance of simple repeats. On the contrary, the correlation between rates of protein evolution and TE abundance largely holds with and without this species (30.95% of the genes; **Table S3**). Overall, we found that the rates of protein evolution of heterochromatin-related genes negatively correlated with the abundance of TEs, but not simple repeats, across species, suggesting a possible source of selective force shaping the evolution of these genes (see Discussions).

Figure 5. Prevalent negative associations between *dN/dS* of heterochromatin-related genes and genomic TE abundance. (A) Stacked bar plots showing the proportion of positively selected genes whose *dN/dS* correlates with repeat abundance (left) or TE abundance (right) for heterochromatin-related genes and randomly sampled genes. The color of the genes in the side box represents the gene's category: *Su(var)s* (green), histone-modifying enzyme enhancing heterochromatin (light green), heterochromatin protein (dark green). (B) Bar plots showing the proportion of positively selected heterochromatin-related genes whose *dN/dS* significantly coevolve with TE abundance for different gene categories. (C) X-Y plots showing the associations between *dN/dS* (X-axis) and abundance of repetitive sequences (Y-axis; top: simple repeats, bottom: TEs) for an example gene (*sov*). *D. suzukii*, the outlier species with exceptionally abundant simple repeats, is circled. Blue points are estimates for the extant species while gray points are for internal nodes, with 95% confidence intervals. *Binomial test: n.s.* $p > 0.05$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.



Discussion

Heterochromatin is a highly conserved cellular compartment with essential functions across complex eukaryotes (Allshire and Madhani 2018; Janssen et al. 2018). Nevertheless, our evolutionary analyses revealed that genes involved in heterochromatin function are highly labile, exhibiting prevalent evidence of rapid evolution both in the forms of amino acid substitutions and gene copy number changes at both short and long evolutionary time scales. Importantly, the rapid evolution of these genes is likely driven by functions specific to constitutive heterochromatin, instead of mechanisms general to proteins interacting with repressive chromatin. Evidence of positive selection on protein evolution is especially prominent for heterochromatin-related genes that should enhance heterochromatin function and, strikingly, *all* three methyltransferases responsible for the enrichment of H3K9me2/3, the characteristic histone modification of heterochromatin, are under positive selection. Our further characterization for the various aspects of the evolution of heterochromatin-related genes provided an important avenue to identify the possible source of selective forces acting on heterochromatin, which we discussed below.

Close examinations of the signatures of positive selection of heterochromatin-related genes found them not only located within structured domains with well-known functions, but also inside unstructured IDRs (**Figure 3**). IDRs were recently found to be critical for the phase properties and thus functions of heterochromatin (Larson et al. 2017; Strom et al. 2017), and our observation suggests that positive selection could also act on heterochromatin functions mediated by such properties. Interestingly, despite the previous suggestions that IDRs are evolutionarily less constrained (Brown et al. 2002; Khan et al. 2015), the proportion of IDR sequences in positively selected heterochromatin-related genes is similar to other candidate genes (**Figure 4B**). Even more, we identified strong phylogenetic signals that are consistent with the presence of stabilizing selection preserving the proportion of IDRs even for heterochromatin-related genes under strong positive selection. Such findings may indicate that the proportion of IDRs, but not the underlying amino acid sequence, is evolutionarily constrained and plays an essential role in the function of studied heterochromatin-related genes. Indeed, IDRs with rapidly evolving sequences were found to possess conserved molecular features (Moesa et al. 2012; Zarin et al. 2019), with some of them experimentally demonstrated to be functionally equivalent (Zarin et al. 2017). This raises another question *why,* in need of maintaining the IDR content of heterochromatin-related genes, do the underlying amino acid sequences rapidly evolve (see below)?

Several lines of evidence suggest that TEs, but not simple repeats, must be involved in the prevalent rapid evolution of heterochromatin-related genes. First, the associations between rates of protein evolution of heterochromatin-related genes and repeat abundance were mainly observed for TEs. In addition, many heterochromatin-related genes under positive selection have functions related to TE suppression. In particular, the interactions between the protein products of several of these genes (e.g., *del, moon, rhi, Trf2*, and *sov* (Klattenhoff et al. 2009; Mohn et al. 2014; Andersen et al. 2017; Andreev et al. 2022)) and repressive H3K9me2/3 are responsible for licensing piRNA clusters, which are TE-enriched loci generating the majorities of piRNAs targeting TEs and located within pericentromeric heterochromatin. Similar to the rapid

evolution of the DNA and protein components of centromeres (i.e., the centromeric drive hypothesis, (Henikoff et al. 2001; Malik et al. 2002)), changes in heterochromatic TE sequences may alter their interactions with proteins encoded by heterochromatin-related genes. Consequently, selection may favor evolutionary changes in these proteins that revert such DNA-protein interaction to its original state, with further changes in TE sequences starting another cycle of this interaction and ultimately driving the fast evolution of the genes. However, only some, but not all, signatures of positive selection fall within nucleic acid binding domains (**Figure 3**) and the majority of the positively selected heterochromatin-related genes lack sequence specificity.

What might have driven the fast evolution of heterochromatin-related genes then? The genomic autoimmunity hypothesis (Blumenstiel et al. 2016) provides a plausible explanation—molecular mechanisms suppressing TE activities are expected to be constantly juggling between maximization of TE silencing while minimizing inadvertent off-target suppression of functional genes, and this alternating selective pressure could drive the rapid evolution of genes involved. Under this model, for example, variants of heterochromatin-related genes that enhance the generation of piRNAs from piRNA clusters, and thus strengthen TE silencing, may lead to the inadvertent production of piRNAs that target other functional elements, a possibility with empirical supports (Andersen et al. 2017; Kelleher 2021). Then, selection will instead favor variants increasing the strigency of this licensing to avoid fitness costs of the off-target effect. This repeated alternation of selective targets could accordingly drive the rapid evolution of heterochromatin-related genes.

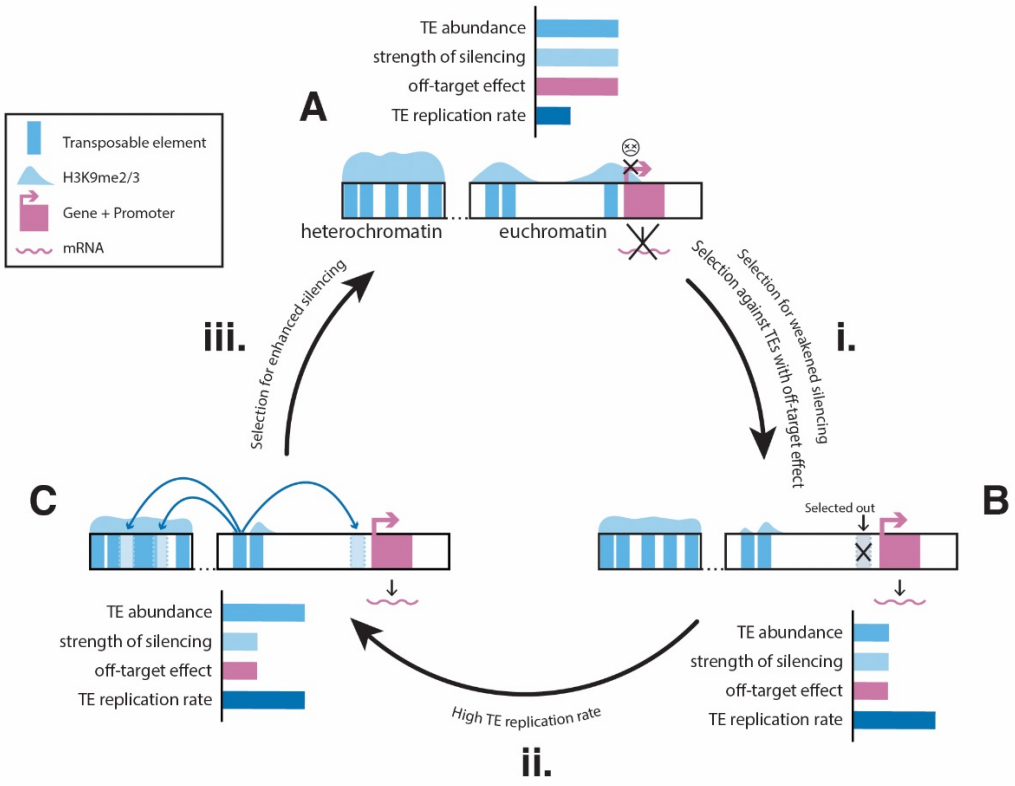
The genomic autoimmunity hypothesis is even more suitable to explain the rapid evolution of heterochromatin-related genes involved in modulating the intrinsic and unique molecular properties of heterochromatin—spreading. The positive feedback between writers and readers of H3K9me2/3 promotes the propagation of repressive chromatin marks (Allshire and Madhani 2018; Bell et al. 2023), and the extent of this spreading depends on the concentrations of readers and writers at the suppressed loci (Locke et al. 1988). Heterochromatin-mediated silencing thus needs to be carefully balanced to prevent inadvertent silencing of functional elements while maintaining sufficient suppression at the euchromatin-heterochromatin boundaries. Interestingly, such a balance needs to be maintained not only at the ends of chromosomes (around pericentromeric and subtelomeric heterochromatin), but also around epigenetically silenced, H3K9me2/3-enriched TEs in the euchromatic genome. The spreading of repressive marks from silenced euchromatic TEs into functional genes is widely documented (reviewed in (Choi and Lee 2020)) and has been inferred to impair individual fitness (Hollister and Gaut 2009; Lee 2015; Lee and Karpen 2017; Huang et al. 2022). Several genes known to modulate these off-target effects of TE silencing are found to be under positive selection in our analysis (e.g., *wde, Su(var)2-10*). Furthermore, histone-modifying enzymes, which are directly involved in the reader-writer positive feedback loops, should frequently be caught in cycles of alternating selection for enhanced or weakened TE epigenetic silencing and thus rapidly evolve (**Figure 6**). Indeed, we found that *all* three writers for H3K9me2/3 show evidence of rapid evolution (*Su(var)3-9, egg, G9a*) and *all* histone-modifying enzymes with accelerated rates of protein evolution have evidence of coevolving with genomic TE abundance (**Table S3**). In

addition, the deleterious off-target effects of TE silencing could result from the long-range spatial interactions between H3K9me2/3-enriched euchromatic TEs and pericentromeric heterochromatin, which is mediated by phase separation mechanisms and is selected against (Y.C.G. Lee et al. 2020). Repeated alteration in selective pressure to ensure these proteins confer sufficient phase properties for proper heterochromatin functions while avoiding such off-target effects may similarly drive rapid changes in protein sequences while preserving the IDR content of heterochromatin-related genes, as we have observed.

Intriguingly, we found the associations between TE abundance and rates of protein evolution of heterochromatin-related genes to be predominantly negative, which might be contrary to intuition. Nevertheless, this broad pattern can be explained by our proposed model that augments the genomic autoimmunity hypothesis (**Figure 6**). In addition to imposing selection *for* variants weakening heterochromatin-mediated silencing, rampant off-target effects from strong TE silencing can result in strong selection *against* individual TE copies due to the spreading of repressive marks to TE-adjacent functional sequences, leading to reduced TE abundance (**Figure 6A**, (Huang et al. 2022)). Upon the fixation of variants that weaken heterochromatin-mediated silencing (**Figure 6B**), the resultant weakened maintenance of heterochromatin at epigenetically silenced TEs could lead to increased rates of TE replication, giving rise to more TE insertions in both euchromatin and heterochromatin (**Figure 6C**). The relative strength of these two processes then determines whether genomic TE abundance increases or decreases while heterochromatin-related genes evolve through cycles of alternating selection targets. Selection coefficients for the harmful off-target effects of TE epigenetic silencing are yet to be estimated, but they could be strong if such effects perturb the expression of nearby vital genes (e.g., (Coronado-Zamora and González 2023); **Figure 6i**) or disrupt global 3D genome organizations (Y.C.G. Lee et al. 2020). On the other hand, replication rates of *Drosophila* TEs are generally low (10^{-5}~10^{-4}; (Charlesworth and Langley 1989; Adrion et al. 2017; Wang et al. 2023); **Figure 6ii**) and likely weaker than selection removing TEs through the off-target effects, leading to negative associations between genomic TE abundance and rates of protein evolution. It is worth noting that many other processes, such as recent demographic changes (e.g., (Mérel et al. 2021)), also contribute to between-species differences in TE abundance and, if they correlate with the evolution of heterochromatin-related genes, similar associations could arise in the absence of the proposed model.

Our observed prevalent positive selection acting on heterochromatin-related genes points out the high lability of the molecular feature of this functionally conserved genomic compartment. Instead of the usually assumed “arm race” with the changing repeatome, we proposed that the selective pressure of heterochromatin-related genes might mainly come from a need to maintain a delicate balance of its unique ability to “suppress” and to “spread,” which also consequently influence the evolutionary dynamics of TEs. Signatures of positive selection identified here could serve as an entry point to further investigate how the delicate balance of heterochromatin-mediated silencing may be conferred by vastly changing components between species (e.g., (Rosin and Mellone 2016; Parhad et al. 2017)), providing a fruitful opportunity to further dissect the molecular mechanisms shaping heterochromatin functions and evolution.

Figure 6. Model for the correlated evolution of heterochromatin-related genes and genomic TE abundance. (A) When heterochromatin-mediated silencing is strong, heterochromatin formed at epigenetically silenced euchromatic TEs are well maintained, resulting in the low replicative activity of TEs. Additionally, there will be strong off-target effects from silenced euchromatic TEs, which could lead to (1) positive selection favoring variants of heterochromatin-related genes that weaken heterochromatin-mediated silencing and (2) negative selection removing TEs mediating the off-target effects (i). (**B**) Due to selection removing TEs with off-target effects, the average TE load in the population reduces. At the same time, upon the fixation of variants of heterochromatin-related genes that weaken silencing, the maintenance of TE epigenetic silencing decreases, leading to not only reduced occurrence of off-target effects from TE epigenetic silencing, but also reduced silencing of TEs. The replicative activity of TEs thus increases (ii). **(C)** Increased TE activity results in increased TE abundance in both euchromatin and heterochromatin, which can drive selection for variants of heterochromatin-related genes that enhance heterochromatin-mediated silencing. The relative strength of i (decrease in TE abundance through selection) and ii (increase in TE abundance through TE replication) determines whether there are positive or negative associations between TE abundance and protein evolution of heterochromatin-related genes across species.



Materials and Methods

Evolutionary analyses of protein sequences

Coding sequences and genome annotations for 16 studied species (**Figure 1B**) were downloaded from NCBI, with GenBank ID listed in **Table S4**. Because we compiled the list of candidate genes based on *D. melanogaster* literature, we used orthologous information from OrthoDB (Kuznetsov et al. 2023)(last accessed 12/10/2022) to retrieve one-to-one orthologs for *D. melanogaster* candidate genes. For genes without one-to-one ortholog according to OrthoDB, we performed *BLAST* search (see below). The retrieved coding sequences were translated to amino acid sequences, aligned using Clustal Omega (version 1.2.4; (Sievers et al. 2011)), and converted back to codon alignments using PAL2NAL (version 14; (Suyama et al. 2006)).

We performed unpolarized McDonald-Kreitman (MK) tests (McDonald and Kreitman 1991) using polymorphism within a *D. melanogaster* Zambian population (197 strains, (Lack et al. 2015)) and divergence between *D. melanogaster* and *D. simulans.* Following the recommendation of (Lack et al. 2015), we treated genomic regions with non-African ancestry or identity-by-descent as missing data, and only included sites with at least 100 non-missing alleles. To count the number of nonsynonymous and synonymous changes, we used the mutational paths that minimize the number of amino acid changes. Codons with more than two alleles, considered both within species polymorphism and between species divergence, were excluded. Genes with fewer than 100 codons were excluded from the analysis due to insufficient statistical power. A gene is deemed under positive selection if the MK test, whose significance was assessed using Fisher's Exact test, rejected the null hypothesis and the ratio of nonsynonymous to synonymous changes is greater for between-species substitutions than for within-species polymorphism. Sliding window MK tests were performed with windows of 100 codons and 10-codon steps.

We conducted phylogenetic analysis by maximum likelihood (PAML) (Yang 2007) using 16 species to identify candidate genes experiencing positive selection over a long evolutionary time scale. We compared two site models, M8a (*dN/dS* ratio is beta-distributed and not greater than one) and M8 (*dN/dS* > 1), and determined the significance using likelihood ratio tests. Species tree reported in (Suvorov et al. 2022) was used. Sites with > 0.95 BEB posterior probability of coming from the site class with *dN/dS* > 1 (Yang et al. 2005) are considered as under recurrent adaptive evolution.

Evolutionary analysis for gene copy number

To identify genes with varying gene copy number, we first used a genome-wide, high throughput search with liberal parameters to identify many potential candidates, followed by careful manual curations. We first used tblastn and reciprocal blastx (Camacho et al. 2009) to search for homologous and paralogs of candidate genes in studied species using *D. melanogaster* amino acid sequence as queries, with the following parameter: e-value < 10^{-2}, amino acid identity > 20%, and blast score > 10. We required the best reciprocal blastx hit to be the original *D. melanogaster* query. Each potential CNV was manually validated using reciprocal best blast with more stringent criteria (e-value < 10^{-5}), and orthologs and paralogs were distinguished

using synteny of flanking genes. We further examined the expression levels of candidate CNVs using RNA-seq exon coverage tracks of NCBI Data Viewer and removed those with no expression. Several duplicates identified in *D. suzukii* were filtered due to redundant contigs. For gene loss, we followed the procedures detailed in (King et al. 2019) to confirm the true absence of a gene.

To examine the sex-biased expression, we deemed a gene male-biased if the log2 fold change of the ratio of male and female expression (TPM or FPKM) is >1, female-biased if such value is < -1, and otherwise unbiased. For *D. melanogaster,* we used Insect Genome database (http://www.insect-genome.com/Sexdb/, last retrieved November, 2023) and FlyAtlas2 RNA-seq data (Krause et al. 2022) for whole adult males and whole adult females. For the other 15 species, we mapped publicly available transcriptome datasets to public genome assemblies with gene annotations (**Table S5**) to the genome assemblies using HiSAT2 (v2.2.1 with parameters –exon and –ss to specify the exon positions and splice sites; RRID:SCR_015530; (Kim et al. 2019)), and estimated the expression levels usingStringtie (v2.1.4 with parameters -dta -G to specify annotation files; RRID:SCR_016323; (Kovaka et al. 2019)). Candidate duplicates identified by manual curation but have no annotation or show no expression were excluded from the analysis.

Domain annotations

We used UniProt (The UniProt Consortium 2023) to annotate known structured domains within the *D. melanogaster* allele of heterochromatin-related genes. For IDRs, we used flDPnn (Hu et al. 2021), which performed well in the previous benchmark study (Necci et al. 2021), and the predicted binary index for IDRs was used to annotate such regions for all studied species. To detect the phylogenetic signal of the % of IDR, we computed *Blomberg's K* statistic using the phylosig function from phytools R package (Blomberg et al. 2003; Revell 2012). The tree structure and branch lengths were obtained from (Suvorov et al. 2022) and generated by treeio R package (Wang et al. 2020).

Analyses of repetitive sequences and their coevolution with candidate genes

DNA for each studied species was extracted from 40 females using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol. To avoid PCR amplification bias during the preparation of sequencing libraries, which was found to skew the quantification of simple repeats (Wei et al. 2018), extracted DNA was prepared into Illumina sequencing library with PCR-free protocol and sequenced with 150bp paired-end reads by Novogene (Sacramento, CA).

We applied K-seek (Wei et al. 2014) to the Illumina data to estimate the abundance of satellite repeats, which are predominantly located in heterochromatin. TE abundance was estimated as the total TE reads from the Illumina sequencing data, with the assumption that TEs in the heterochromatin mainly originated from jumping events of euchromatic TEs and TE abundances in the heterochromatin and euchromatin are highly correlated. To minimize TE annotation bias across species, repetitive sequences from each of the 16 genomes (**Table S4**) were identified using RepeatMasker (version 4.1.0; http://www.repeatmasker.or/) with the Dfam database

(Storer et al. 2021), using the command “RepeatMasker -q [genome sequence file] -species drosophila -e hmmer”. TE sequences annotated as LTR, LINE, DNA element, and Unknown category were obtained from all 16 genomes to create a master TE library. Illumina reads from each species were mapped to the library with bwa-mem (version 0.7.17; (Li and Durbin 2009)) and viewed by samtools (version 1.15.1, (Li 2011)). The total number of reads mapped to the library, regardless of mapping quality, was considered the number of TE reads. In order to compare the abundance of simple satellites and TEs across samples/species, these estimates need to be normalized. Illumina reads from each species were mapped to its repeat-masked genome using bwa-mem, with positions with mapping quality less than 30 filtered (using samtools -q 30). The median read depth for the unmasked regions for each sample was then used to normalize the read counts for simple satellites and TEs.

We used Coevolve (Lartillot and Poujol 2011) to estimate the correlation between *dN/dS* and repeat abundance (satellite repeats or TEs) given the tree structure. For each gene, the analysis was performed in duplicate to assess convergence (relative difference < 0.1) and a burn-in of 300 with at least 3,000 MCMC chains to get the final estimated correlation between *dN/dS* and repeat abundance. Because the number of Polycomb genes with significant PMAL tests is small, we instead used 100 randomly selected genes to contrast heterochromatin-related genes.

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