A rapidly evolving genomic toolkit for *Drosophila* heterochromatin

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Heterochromatin is the enigmatic eukaryotic genome compartment found mostly at telomeres and centromeres. Conventional approaches to sequence assembly and genetic manipulation fail in this highly repetitive, gene-sparse, and recombinationally silent DNA. In contrast, genetic and molecular analyses of euchromatin-encoded proteins that bind, remodel, and propagate heterochromatin have revealed its vital role in numerous cellular and evolutionary processes. Utilizing the 12 sequenced *Drosophila* genomes, Levine et al.[1] took a phylogenomic approach to discover new such protein "surrogates" of heterochromatin function and evolution. This paper reported over 20 new members of what was traditionally believed to be a small and static Heterochromatin Protein 1 (HP1) gene family. The newly identified HP1 proteins are structurally diverse, lineage-restricted, and expressed primarily in the male germline. The birth and death of HP1 genes follows a "revolving door" pattern, where new HP1s appear to replace old HP1s. Here, we address alternative evolutionary models that drive this constant innovation.

HP1: A Flashlight for the Dark Matter of Eukaryotic Genomes

The human genome sequence is not complete.[2] Neither are the *Mus musculus*,[3] *Drosophila melanogaster*[4] nor *Arabidopsis thaliana*[5] genomes. Indeed, "complete genome sequence" assemblies from many eukaryotes may be missing up to 30% of nuclear-encoded DNA. The unassembled genome compartment is mostly comprised of heterochromatin—packed with satellites and transposable elements, but relatively sparse in protein-coding genes and recombination events. The challenges of assembling repetitive DNA and genetic mapping to regions of low recombination, combined with a mistaken perception that heterochromatin harbors few functional elements or no genes, has contributed to decades of scientific neglect. With the advent of new sequencing technologies and painstaking efforts of large consortia of researchers (e.g., the *Drosophila* Heterochromatin Genome Project[4]), this slight is being slowly corrected with significant advances in heterochromatin function and evolution.

Despite renewed interest in the study of heterochromatin DNA sequence, most insights into heterochromatin function have emerged from genetic and molecular studies of euchromatin-encoded proteins that affect heterochromatin properties, especially the expression of proximal genes in the phenomenon of position-effect-variegation (PEV).[6] These and subsequent studies led to the awareness that heterochromatin participates in many essential cellular processes, including chromosome segregation,[7] genome defense,[8] and gene regulation,[9] transforming the scientific community from disinterest to broad appreciation of heterochromatin's biological significance. The resulting picture revealed that rather than representing a sea of functionally uninteresting homogeneous repeats, heterochromatin contains many disparate elements of varied functions (e.g., piwi-associated RNA, or piRNA, clusters for genome defense[8]).

Our understanding of heterochromatin function was transformed by a pivotal 1986 publication describing *D. melanogaster*'s Heterochromatin Protein 1 (HP1).[10] Using monoclonal antibodies against a

protein fraction tightly bound to DNA, James and Elgin uncovered a chromosomal protein that localizes primarily to pericentric heterochromatin and was encoded by *Su(var)2-5*,[11] one of the genes previously shown to suppress PEV. Subsequent analysis of HP1 (now called "HP1A") and its numerous interacting partners illuminated an unprecedented number of heterochromatin-dependent essential processes. Centromere maintenance,[12] RNA interference,[13] and telomere protection,[14] for example, all rely on HP1-dependent heterochromatin integrity. This progress highlights the power of heterochromatin-bound proteins as molecular tools to reveal new roles for this ubiquitous but cryptic genome compartment.

More recently, these heterochromatin-bound proteins have been used to reveal the evolutionary forces that may act on the rapidly evolving but unassembled, sometimes undefined heterochromatic sequence to which they bind.[15] For example, the finding that a female germline-restricted HP1 protein, Rhino/HP1D, had evolved under positive selection (faster than expected amino acid divergence) predicted its engagement in a moleculr arms-race with transposable elements.[16] This prediction was supported by the finding that Rhino binds heterochromatin-embedded, rapidly evolving piRNA clusters,[17] which themselves likely evolve under positive selection[18] to "immunize" genomes against new invasions of transposable elements. Thus, rigorous population genetic and molecular evolution analyses on heterochromatin protein "surrogates" could reveal evolutionary dynamics at the repetitive heterochromatin sequence for which such tests are undeveloped.

HP1 Phylogenomics: Expanding the Family Business

We set out to discover new "heterochromatin surrogates" for both functional and evolutionary analysis. We focused our analysis on the gene family founded by *HP1A*.[a] At first glance, the *HP1* gene family seems like a poor target for phylogenomics. For many years, it was con-

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a   We refer to the *Su(var)2-5* gene as *HP1A* here purely for ease of referral and comparison to the other paralogous HP1 genes in *Drosophila*.

sidered small, static, and structurally homogenous.[19] HP1 family members are traditionally defined by a single, common domain structure—a chromodomain, hinge, and chromoshadow domain[20] (**Fig. 1**). Using this definition, *HP1B* and *HP1C* were discovered in the newly sequenced *D. melanogaster* genome in 2000,[21] bringing the family size up to three. Additional HP1s in non-*Drosophila* genomes were also identified, including three in humans (*HP1α, HP1β, HP1γ*),[22-24] which are not orthologous to any of the *Drosophila HP1* genes[25] and instead likely derived from an *HP1B*-like ancestor.[25]

At this time, a family size of three was the maximum number across any eukaryote, including yeast, worms, mouse, and *Arabidopsis*.[19] Furthermore, all three *Drosophila* HP1s and all three mammalian HP1s are highly conserved across broad evolutionary distances.[26] Remarkably, the human HP1α can rescue *D. melanogaster* HP1A-dependent loss of silencing[27] *despite* paralogy. These observations supported the idea that functional and evolutionary stasis is a defining feature of this small family. However, the fortuitous discovery of a new *D. melanogaster HP1* in a female sterility screen,[28] called "*rhino,*" together with its signature of strong positive selection,[16] suggested that HP1s are potentially more numerous and more plastic than originally thought. The subsequent discovery of a fifth *HP1*[16] in *D. melanogaster*, *HP1E*, supported this prediction and motivated our comprehensive phylogenomic analysis.

HP1A through *HP1E* served as entry points into characterizing the *HP1* gene family in the recently sequenced 12 *Drosophila* genomes, which represent over 40 million years of *Drosophila* evolution.[29] Using all five chromodomains and chromoshadow domains as queries, we computationally searched the 12 genomes for significant tBLASTn hits. Our iterative search strategy, in which any significant domain hit becomes a search query itself, returned over 100 hits across this 40 million year snapshot. Using the evolutionary definition of "gene family," we conditioned membership of these hits into the *HP1* family based on phylogenetic relationships. Combined with information about synteny, we identified orthologous,

paralogous, and anciently diverged members relative to known *HP1s*. Orthologs have diverged through speciation events; thus, they are both syntenic (in the same genomic location) and more closely related to each other than to other *HP1* genes. For example, the *HP1A* alleles from all 12 genomes form a single monophyletic (single evolutionary origin) clade. Paralogs represent sister clades; for example, the *HP1G* and *HP1A* gene clades share a common ancestor that is more than 40 million years old. We also identified younger paralogs that cluster *within* older HP1s, like the relatively young, *HP1D/Rhino*-derived *Oxpecker* genes. These represent cases of more recent gene duplication events within pre-existing HP1 clades. Finally, a few paralogs defied groupings within old or new *HP1* gene clades, representing either ancient origins or very rapid divergence (they still share stronger identity to HP1 chromo or shadow domains than to any other *Drosophila* proteins).

We found that *HP1A, HP1B, HP1C* and *HP1D* orthologs occurred in all 12 genomes in the syntenic locations. However, *HP1E* orthologs had clearly degenerated in several species, implicating recurrent *HP1E* gene loss. This seemingly unique species-specificity of *HP1E* proved to be the rule for all new *HP1* paralogs we discovered. Indeed, none of the other *HP1* genes discovered are present in all 12 genomes. Virtually all of these newly identified *HP1s* evolved within the last 40 million years and so appear in only a restricted set of lineages.

Even more unexpectedly, while the canonical HP1 domain structure is defined by the presence of both a chromodomain and a chromoshadow domain (**Fig. 1A**), the majority of new *HP1* family members encoded only one of these domains, having lost or degenerated either the original chromo or chromoshadow domain during or after duplication (**Fig. 1B**). We initially disregarded these "half-*HP1s*" as duplicate genes caught in the act of pseudogenization. However, upon examining the syntenic locations of these half-*HP1s*, we confirmed that many had been retained for many millions of years. Long-term retention is consistent with function, particularly in *Drosophila* where the half-life

of pseudogenes is remarkably short.[30] Further supporting this prediction, we found evidence of transcription for 18 of the 19 genes in our list. Intriguingly, virtually all are transcribed primarily in the male germline. Four genes are encoded in the well-annotated genome of *D. melanogaster*—one chromodomain-only HP1, and three chromoshadow-only HP1s (one of which we already know is essential[31]).

Our data are consistent with a minimum *HP1* gene family size of 26 in the 12 *Drosophila* species sampled. This represents a 4-fold increase in *HP1* gene number. Furthermore, the unprecedented structural diversity of the new *HP1* family members offers a dramatically expanded toolkit for discovering new heterochromatin functions.[b] The pervasive lineage-restriction is consistent with species- or clade-specific adaptations that rely on young *HP1* genes (below), and may offer insights into the evolutionary significance of the rampant between-species divergence observed at the heterochromatin sequence itself. Moreover, the predominance of male germline expression is consistent with currently uncharacterized male-specific chromosome biology driving this lineage-specific adaptation.

A 'Revolving Door' of HP1 Proteins in the *Drosophila* Male Germline

The male germline has recurrently emerged as a venue enriched for signatures of positive selection across many gene classes.[32] These DNA signatures include statistical enrichment for new amino acid changes and retention of gene duplicates, the latter of which results in expansion of gene families. An agnostic analysis of *Drosophila* gene family evolution across the 12 genomes[33] demonstrated that significant family expansions (and contractions) are enriched for male reproduction-related functions. Intriguingly, the abundant male germline-expressed *HP1* paralogs are *not* part of gene family expansion in the strict sense. Despite rampant gene birth and death over the 40 million

Figure 1. (**A**) Canonical HP1 domain structure. The chromodomain ("chromo") recognizes H3K9me, the hinge binds DNA and/or RNA, and the chromoshadow ("shadow") homodimerizes and heterodimerizes. (**B**) Alternative paths underlying half-HP1 birth. Drift or selection drives the degeneration of the chromodomain (in this example) following a full HP1 duplication event. Alternatively, the duplication itself is restricted to a single domain. A 3' retrotransposition bias[43] may underlie the enrichment of chromoshadow domain-only HP1s observed in our data set.[1]
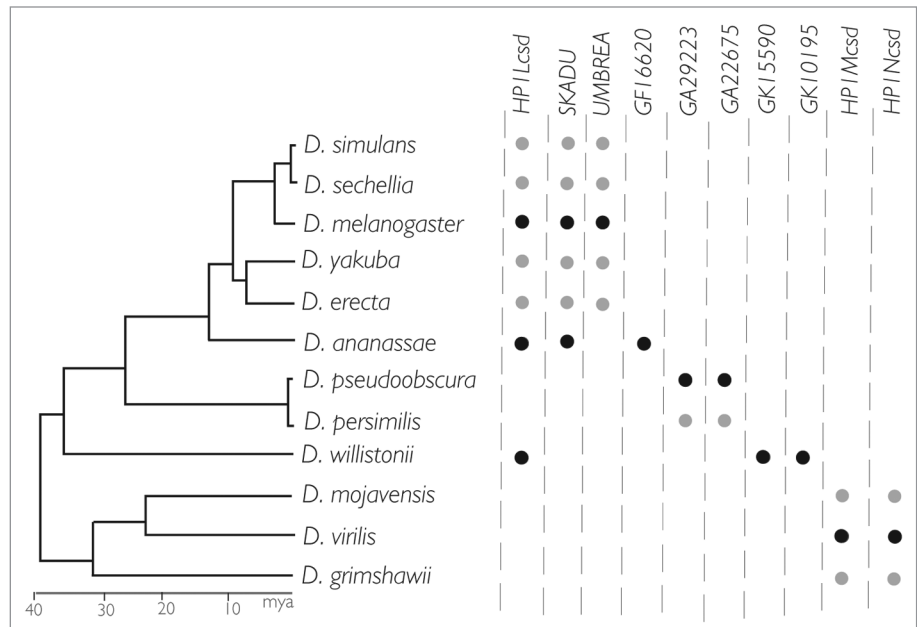
Figure 2. Revolving door dynamics: gene number stasis, recurrent birth, and recurrent death. The 10 chromoshadow-only genes represented are all expressed primarily in testis. Each lineage harbors either two or three HP1s of this domain class, but these genes are rarely shared across distant lineages. ● Expression assayed directly by tissue-restricted RTPCR. ● Expression inferred.

years, the number of *HP1* genes in a given *Drosophila* species varies only modestly (for the chromoshadow-only class, see **Figure 2**). This gene number stasis, despite prolific gene birth, is consistent with functional gene replacements over time. Borrowing a term from Demuth and Hahn,[34] we refer to this pattern as an *HP1* gene family "*revolving door*."

At a genome-wide scale, recurrent gene turnover is consistent with a neutral model of gene family evolution.[35] Averaged across all genes, a steady-state birth/death process (that assumes an equilibrium genome size) readily accounts for gene turnover. Under this model, a gene duplication event generates a daughter copy that is ultimately retained

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b   The *HP1* family designation is an evolutionary classification; significant functional work needs to be done to ascertain whether any or all the new genes indeed encode heterochromatin-binding proteins.
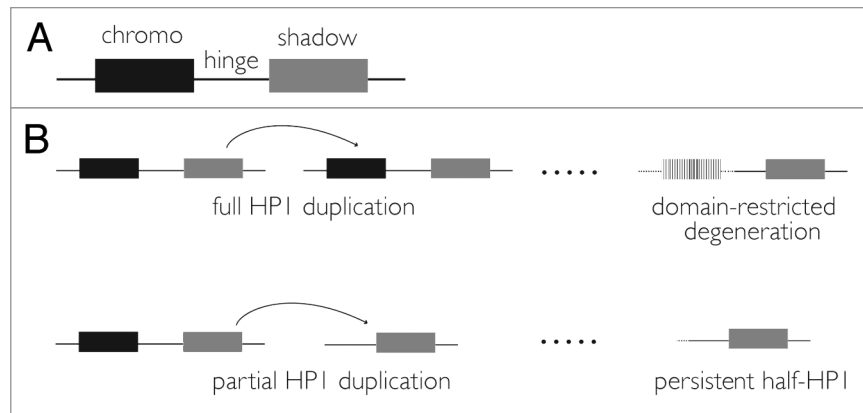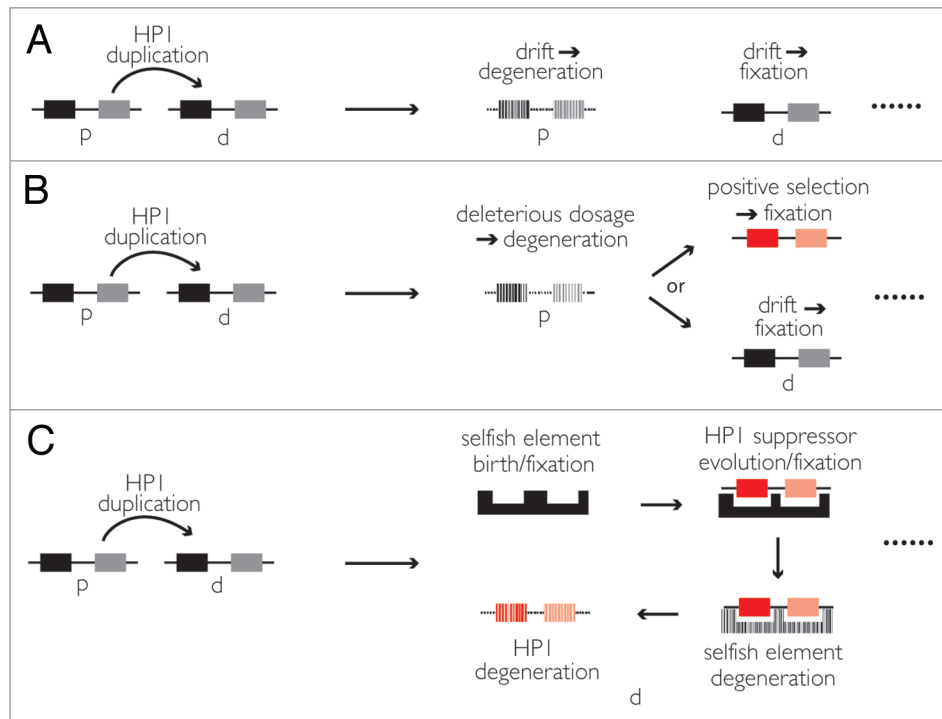
Figure 3. Alternative forces driving gene replacements. (**A**) Birth and then fixation/death under neutral forces. (**B**) Birth and then fixation under neutral forces or positive selection, death driven by negative selection to relieve dosage effects, such as heterochromatin expansion/contraction or positive/negative transcriptional regulation.[44-46] (**C**) Birth and then fixation under positive selection to suppress recurrently evolving selfish elements, death under neutral forces. p, parent gene, d, daughter gene.

while the parent copy accumulates mutations under genetic drift (**Fig. 3A**). At the level of a single gene family with elevated birth/death rates, however, this model is less satisfying; specifically, under neutrality the gene death rate varies independently of gene copy number. Chromatin proteins, and specifically HP1s, however, are typically dosage-sensitive.[36] An extra gene copy can, for example, suppress or enhance heterochromatin spreading along a chromosome.[37] We speculate that gene duplications of some chromatin-protein encoding loci are instantly visible to natural selection. Consequently, an *HP1* gene death rate parameter may vary positively with gene copy number, which at least partially explains the gene family-wide revolving door pattern (**Fig. 3B**). This slight variation on Birchler's "gene balance hypothesis"[38] may also explain our observation that half-*HP1s* evolve exclusively from full *HP1s*. A mutation that breaks a chromodomain or chromoshadow domain in the full HP1's daughter copy instantly

relieves deleterious dosage-effects. Relief from deleterious dosage effects may free up the daughter copy to evolve along its own evolutionary trajectory.

In addition to this negative selection, positive selection may also explain the recurrent gene birth and death across the *HP1* family (**Fig. 3C**). Heterochromatin is riddled with selfish elements.[39,40] These genomic parasites gain a fitness advantage upon self-replication or drive in the germline where they have direct access to the next generation. Germline-restricted *HP1s* like the numerous *Rhino/HP1D*-derived chromodomain-only *Oxpecker* genes may suppress this selfish activity. Once successfully silenced, the selfish element and its suppressor degenerate. Recurrent bouts of selfish element birth and degeneration[41] may explain at least some of this *HP1* turnover in the male germline.

Just like the discovery and study of histone variants have greatly transformed our understanding of chromatin functions and states,[42] analysis of this diverse toolkit

of heterochromatin surrogates promises to reveal both currently unknown cellular roles for heterochromatin as well as the evolutionary forces that act on this still understudied genome compartment. Moreover, this kind of phylogenomic approach is gene family- and taxon-independent. As more and more complete genome sequencing data sets become available, we anticipate many more analyses that overturn false perceptions of stasis at gene families that encode essential proteins.

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