THE HYBRID INCOMPATIBILITY GENE LETHAL HYBRID RESCUE REPRESSES REPETITIVE DNA

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Heterochromatin keeps in check selfish elements such as transposable elements (TEs) and satellite DNAs, which can wreak havoc on a genome by mobilizing and increasing their copy number, leading to genomic instability and sterility. Heterochromatin proteins (HPs) that mediate repression of selfish DNA may therefore be in an eternal arms race with selfish DNA. This arms race might explain the extensive sequence divergence discovered in some HPs which cause post-zygotic reproductive isolation. However, evidence for this model is limited. For my thesis work, I studied *Lhr*, a strong candidate gene, to test this model. *Lhr* encodes a rapidly evolving, HP1a interacting, HP that causes lethality in hybrids between *D. melanogaster* and *D. simulans*. To determine *Lhr's* normal function we knocked-out *Lhr* via homologous recombination in *D. melanogaster.* I discovered that *Lhr* mutant females have reduced fertility. Using mRNA-Seq, I found that *Lhr* regulates the steady state levels of many different satellite and TE transcripts. ChIP data argue that this increase is due to a defective post-transcriptional pathway. However, my analysis of small RNA-Seq data shows that small RNAs targeting most misregulated transposable elements are not affected and suggests instead that *Lhr* functions independently or downstream of the small RNA pathway. To address the effects of extensive sequence divergence of *Lhr* between *D. melanogaster* and *D. simulans,* I performed a RNA-Seq comparison of wild-type and *Lhr* mutant *D. simulans* lines. I discovered that loss of *Lhr*

upregulates different transposable elements in *D. melanogaster* and *D. simulans*. Further, comparing the two species, I made the striking observation that localization of Lhr protein has expanded in *D. melanogaster* to encompass two satellites which account for nearly 6% of the *D. melanogaster* genome, but only 0.7% in the inferred ancestor of *D. melanogaster* and *D. simulans.* Finally, I found that *Lhr* is required for expression of heterochromatic genes, suggesting that it helps the host genes in *D. melanogaster* to adapt to the greatly expanded heterochromatic content of this species. My studies uncover an important component of the machinery that an organism uses to repress TEs and satellites, and to adapt to changes in selfish DNA. My work further demonstrates that each *Lhr* ortholog has adapted to repress different selfish elements in each species and provides support for the arms race model.

BIOGRAPHICAL SKETCH

Satyaki P Rajavasireddy grew up in the city of Hyderabad, India. He studied at the Hyderabad Public School for twelve very painful years, where much pain was inflicted by bullies and the P.T. teacher. After accidentally choosing to pursue biology in high school and liking it very much, he went on to obtain a Bachelor's Degree in Biochemistry and Chemistry from Aurora's Degree College (Osmania University) in 2004. These were wonderful years which allowed much freedom to pursue joyous reading beyond the limits of the very stale syllabi of Indian academics. This was followed by a three year Master's degree at the Tata Institute of Fundamental Research where, free of the tyranny of formal exams, he studied the development of the mouse forebrain and experienced the vagaries and joys of research. In 2007, he moved to Ithaca to pursue a PhD at Cornell in the field of Biochemistry, Molecular and Cellular Biology. He joined the Barbash Lab which, along with the Department of Molecular Biology and Genetics, provided a very nourishing environment that helped him expand his perspective of biology. Satyaki will be taking with him, this perspective, ideas, memories and experiences to a post-doctoral fellowship in the lab of Dr. Mary Gehring at the Whitehead Institute in Boston.

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The lab was a lively place to be in, full of bright and caring people. The many friends I made here will ensure that I will always be tethered to the Barbash Lab. Patrick Ferree was the elder brother figure in the lab. He taught me much about immunos, FISH, centromeres and satellites and has been a great companion through the years. He was, is and will remain a great friend. Heather Flores was my

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LIST OF ABBREVIATIONS

Ago3: Argonaute 3
Aub: *Aubergine*
BESS: domain found in BEAF32, Stonewall, Suvar 3-7
CenH3: Centromeric Histone 3
CID: Centromere Identifier
CPC: Chromosome Passenger Complex
F.E.T: Fisher's Exact Test
GFP: Green Flourescent Protein
H3K9Me2: Histone 3 Lysine 9 Dimethylation
HI: Hybrid Incompatibility
Hip: HP1 interacting protein; synonymous with *Hp4*
Hmr: *Hybrid Male Rescue*
Hmr^{3}: A mutant allele of *Hmr*
Hp1a: *Heterochromatin protein 1a*
Hp1c: *Heterochromatin protein 1c*
Hp3: *Heterochromatin protein 3*; synonymous with *Lhr*
HP4: *Heterochromatin protein 4*
Lhr: *Lethal hybrid rescue*
Lhr^{1}: strong loss of function allele of *Lhr* in *D. simulans*
Lhr^{2}: hypo-morphic allele of *Lhr* in *D. simulans*
Lhr^{KO}: *Lhr* knockout in *D. melanogaster*
$Lhr^{KO/+}$: Heterozygous control for *Lhr* knockout in *D. melanogaster*
Lhr^{+}: Wild-type control for *Lhr* knockout in *D. melanogaster*
LTR: Long terminal repeat
MADF: Myb/Adf-1 like
Mel: *Drosophila melanogaster*
mel-Hmr-FLAG: FLAG tagged Hmr mel transgene
mel-Hmr-HA: HA tagged Hmr mel transgene
piRNA: piwi associated RNA
piwi: *P- element associated wimpy testes*
qpcr: quantitative polymerase chain reaction
qRT-PCR: quantitative real time polymerase chain reaction
rDNA: ribosomal DNA
rnaseq: RNA sequencing
sim: *Drosophila simulans*
siRNA: small interfering RNA
Spn-E: Spindle-E
Su(var)205: *Suppressor of variegation 205*
Su(Var)3-9: *Suppressor of variegation 3-9*
TAS: Telomere associated satellites
TE: Transposable element

Chapter 1
Introduction

Transposable elements: Invasion, conflict, armistice, disarmament and domestication.

Invasion

Transposable elements are horizontally transferred into new hosts by vectors such as viruses, bacterium or blood feeding parasites or through the TE itself producing virus like particles[1]. They can also spread through gene flow between or within species [2–5]. Once they invade a new species, they proliferate via either RNA or DNA intermediates. Sometimes, the same TE family can invade the same species multiple times [6]. The TE family's success in a new species, upon horizontal transfer, is dependent on its ability to interact with host machinery[7] and its post transfer dynamics. TEs that proliferate slowly upon infection are thought to be quickly lost from the population while those proliferating too rapidly are thought to lead to sterility (see below). Instead, modelling suggests that TE proliferate rapidly upon infection but slow down proliferation soon after [8]. Invading TE families are also known to reactivate and proliferate after dormant phases [9].

Conflict

This rapid proliferation of TEs can have many negative effects on fitness of the host. The most immediate effect is genomic instability. The increased activity from TE enzymes such as endonucleases as well as increases in transposition lead to increased double stranded breaks. In *Drosophila,* such activity has been linked to female sterility [4,5]. The presence of

increased number of TE copies also leads to increased ectopic recombination [10]. Thus, TE invasions can have serious effects on the host and many eukaryotes have evolved machinery to combat TEs. At the center of this machinery in most eukaryotes is a small RNA based TE recognition machinery that can adaptively respond to invasions by new TEs. In *Drosophila,* such a defense is chiefly mounted by the piRNA pathway. The piRNA pathway begins at the piRNA cluster- a library of fragments from extinct and extant TEs [11]. piRNA clusters are several KB long and attract invading TEs. These piRNA clusters are heterochromatic, a state which may help repress remobilization of these captured TEs[4]. Within these piRNA clusters, over evolutionary time, the TEs become truncated and often leave behind only small fragments. Transcription of these piRNA clusters produce long mRNAs called primary piRNAs that are transported to a peri-nuclear compartment called the nuage. Here, two PIWI protein *Ago3* and *Aub,* aided by a number of other accessory proteins, convert the primary piRNAs into 23-30 nucleotide long piRNA. These piRNA acting like seeds then detect and guide Ago3*,* Aub and a number of nucleases to TE transcripts in the cytoplasm. The activity of these nucleases produces secondary piRNAs which serve to amplify the number of piRNAs targeting active TEs and also to recognize TEs sequences beyond the fragment in the piRNA clusters. Once recognized, these TEs are degraded. The complete mechanism underlying this degradation remains unclear but the decapping and mRNA degrading enzyme rich “pi-bodies” have been implicated [12,13]. These secondary piRNAs are also recruited by PIWI which enters the nucleus and uses the piRNA to recognize a TE. It remains unclear if PIWI uses the piRNA to recognize a DNA sequence or the RNA being freshly produced at a TE insertion. Upon binding, PIWI mediates recruitment of the heterochromatin machinery, including HP1a and H3K9 methylases. These proteins are thought to help repress TEs [14]. The specific contribution of each component of the piRNA pathway and heterochromatin machinery to final repression seems to vary from one TE family to another[13].

It has been suggested that the heavy host repression of TEs selects for variants that can escape it and proliferate. These TE variants then proliferate and select for variants of host proteins that can restore repression to the newly escaped TEs[15]. Two observations are consistent with this model. Proteins in the piRNA pathway show increased codon bias, arguing that the host optimizes the production of piRNA proteins in order to combat TEs[16]. Second, the signatures of adaptive evolution seen in many piRNA pathway and heterochromatin proteins are also consistent with such an "arms race"[17]. However, this model still lacks biochemical or molecular evidence. Barring some heterochromatin proteins, most TE repressors make no sequence specific contacts. While some viruses make siRNA inhibitor proteins, no TE encoded piRNA inhibitor has yet been found. Additionally, the discovery of piRNAs against arboviruses in mosquitoes has led to speculation that the rapidly evolving piRNA proteins, like their siRNA counter parts are also in conflict with viruses rather than TEs[18]. However, molecular studies of host-TE interactions are in their infancy and it is entirely plausible that a TE inhibitor of the piRNA pathway will be found.

Armistice

While the host can often vanquish the active TE without fitness costs to the host itself, the loss of the host guarantees the destruction of the TEs. TEs avoid this outcome via a multitude of strategies. Some TEs use an auto-regulation mechanism that limit their copy number in the genome[19]. Other TEs hide in niches where they may make copies without seriously compromising host fitness. Some examples of this include the *R1* and *R2* retro-transposons in *Drosophila* which preferentially insert into the ribosomal DNA locus[20]and the *HeT-A* and *TART* elements which insert only at the telomeres of *Drosophila.*([21], see below). Insertion into such niches is so successful that these elements have been vertically transmitted for millions of years and can be found through many *Drosophilids.* This doesn't mean that these

elements are no longer in conflict with the host, but possibly represent only a curtailment of conflict. Using the conflict analogy, this next step in TE-host interactions can be described as an armistice, where hostilities between the host and the TE have not ended in a permanent peace but are curtailed.

Disarmament and Domestication.

The piRNA pathway and heterochromatin proteins help repress TEs. Most TE insertions once repressed slowly degrade, either through the accumulation of mutations or deletions, losing their ability to copy themselves. However, some TEs are domesticated, providing genetic material for the evolution of completely new host components. Recombination Signal Sequences (RSS) and RAG-1 recombinases that help generate antibody diversity in jawed vertebrates were originally components of the *Transib* family of TEs [22].Remnants of TEs also regulate gene expression- acting as promoters, differentially methylated regions that control imprinting and sources of small RNA [23–25]. They provide starting material for completely novel genes, such as *Iris* in *Drosophila*[26] and new protein domains such as MADF sourced from the *PIF* family of TEs [27] and the CENPB sourced from the *Pogo* TE family[28].

An extreme case, where not individual insertions but whole families are domesticated, is associated with the telomeric retrotransposons in *Drosophila.* In what is also an interesting example of how versatile biological systems can be, the seemingly fundamental and well conserved reverse transcriptase system set around telomerase has been replaced by a reverse transcriptase system sourced from retrotransposons. In *D. melanogaster*, three related TE families- *HeT-A,TART* and *TAHRE* which insert only at the telomeres, have completely replaced the telomerase based telomeres; their addition to the chromosomal ends being regulated by host machinery[29]. This extreme domestication event may have occurred in multiple steps. In the first step, these TEs would have used the telomere as a niche, inserting into the normal

telomerase extended repeats. In fact, SART1 and TRAS1 TEs of *B. mori* and the SARTTc TE family of *T.castaneum*, which may be related to the *Drosophilid* telomeric TEs, insert specifically into the more canonical telomeres of their hosts [30,31]. In *Drosophilids,* over time, the telomeric TEs would have started to communicate with cellular cues such as chromatin states that regulate telomeric regions and would have eventually replaced the telomerase system.

Satellite DNA: Development, function and adaptation.

Development of satellite DNA: Formation and Growth

Satellites are tandem repeats which usually have no protein coding potential. In this dissertation, I use satellites to describe all heterochromatic tandem repeats excluding rDNA. Satellite DNA comes in two flavours- simple repeats and complex repeats. Simple repeats consist of monomers ranging from 5-12 bp. Longer monomers, up to 359bp and even longer are also found [32,33]. In some cases, the genesis of the satellite sequence can be linked to the rearrangement and/or amplification of simple sequence motifs and transposable element fragments [34–36]. Independent of the origin of the satellite DNA's sequence, they often seem to share structural characteristics. For example, the *Drosophila* Y chromosome's centromeric satellite, *18HT*, derived from telomeric TEs has a purine rich strand that can form non-Watson-Crick GA base pairs[37]. Interestingly, chromosome 3's centromeric satellite *Dodeca* is also capable of forming such structures[37,38].Satellite monomers, once created can either increase or decrease their copy number in an individual through a multitude of mutational mechanisms including unequal crossover[39] and replication slippage[40].Such a process would produce large intra and inter-specific variations. Bosco et al cytologically examined differences in total heterochromatin content and found that there was far lesser intraspecific variation than there

was interspecific variation. They speculated that this pattern supported the existence of a mechanism by which the satellites affected meiosis to drive themselves to fixation (see below) or that there were “other species specific selection pressure” which enabled the fixation of specific satellite DNA families within species [41]. The identity of this proposed mechanism is one of the great mysteries of the field and maybe associated with putative functions of satellite DNA and the regulators of satellite DNA and will be discussed in more detail below.

Satellites: Functions and Effects.

Satellite DNAs are often found organized in blocks, hundreds of KB long. They are not rich in translatable information, like protein coding genes, and have thus been relegated to junk in popular discourse. However, such a designation misses the versatility of DNA. In satellites, DNA plays a structural role more akin to other cellular macro-molecules; acting as a scaffold upon which proteins necessary for other functions assemble. Far from being junk, satellite DNA and attendant proteins play important roles in the cell.

The most important role is at the centromeres, mediating chromosome segregation. Here, satellite DNA complexes with CenH3 and other centromeric proteins to form a docking point for the kinetochore that connects the chromosomes to the microtubules which generate the force necessary for chromosome segregation. Centromeric satellites are species specific in length and sequence. In *S. cerevisiae,* the monomer is 125bp long and forms small “point centromeres”[42]. In *H. sapiens,* the monomer is 171bp long. Unlike budding yeast, D*. melanogaster* has “regional” centromeres[42].Deletion analysis followed by sequencing showed that the minimal X chromosome centromere was several tens of KB long and included a mish-mash of several satellites[43]. While the centromeres of other *D. melanogaster* chromosomes have not been similarly mapped, they chromosomes completely lack several of the satellites described for the X chromosome’s centromere[32]. Interestingly, while the centromeric

sequences as well as CenH3 sequences differ between species, CenH3 orthologs from *H. sapiens*, *C. elegans* and *S. pombe* all localize to the pericentric regions of *D. melanogaster* S2 cell chromosomes[44]. This result has been interpreted to suggest that CenH3 needs some association with heterochromatin but has no sequence specificity. This model is also supported by human neo-centromeres which lack similarity to the alpha satellite monomer, beyond having an AT rich sequence, but often show some heterochromatic features [45]. Thus, while an accumulating amount of evidence argues that centromeres are epigenetically determined, it remains unclear if the extensive correlation between satellites and centromeres suggests that satellites are better at creating the epigenetic state or if the satellites create a higher order structure that is necessary for segregation.

The interactions between heterochromatin and centromeres can be understood from observing the over expression of CID in *Drosophila* S2 cells [46].The over expression of CID leads to the formation of “CID islands” which can act as neo-centromeres capable of recruiting kinetochores and microtubules. These neo-centromeric CID islands are most enriched close to the telomeres and enriched to a lesser amount in the pericentric regions, both areas rich in heterochromatin. However, the CID islands seemed to abut but not overlap with the HP1 rich heterochromatic regions. In fact, they formed in euchromatin-heterochromatin boundary areas which had neither the active H4 acetylation nor the inactive H3K9 methylation modifications. This dependence of centromere localization on heterochromatin was further supported by the recruitment of CID to the neighbourhood of LacO repeats to which HP1-LacI was targeted. The importance of the balance between euchromatin and heterochromatin is also made clear by another elegant experiment that utilized human artificial chromosomes [47]. In this experiment, targeting of chromatin state changing transcriptional activators or repressors to the centromere of the artificial chromosome affected its segregation. Olszak et al., 2011 suggest that the non-

heterochromatic and non-euchromatic state of centromeric domains allows the accumulation of CID at these sites.

Despite the requirement for this inert state, transcripts from several centromeric satellites can be detected at low levels. Such satellite transcripts accumulate in murine cells [48],humans[49] and maize [50]. These transcripts are thought to have functional roles, interacting with CenH3 in maize[50] and mediating chromosome passenger complex function and protein assembly in human cells[49,51]. Additionally, the levels of these centromeric RNAs seems to be important, with satellite transcription over expression leading to mis-localization of CPC components[48]. While evidence for such roles is accumulating, the roles of satellite transcripts seems to be species specific[51]. The production, regulation and function of satellite transcripts have not been well studied in *Drosophila.* The 1.688/359 satellite block is known to be transcribed by RNAP2; the mRNA being processed into small RNA by the piRNA pathway[52]. mRNA as well as small RNA against many satellites such as GAGAA, and Responder can also be observed in many RNA-Seq and small RNA-Seq datasets (Saito et al., 2006). It is plausible that satellites producing small RNA help create a heterochromatic zone via the PIWI mediated recruitment of HP1a. In contrast, satellites such as AACAC which produce transcripts but no small RNA may form a euchromatic zone[supplementary materials 13,54]. The euchromatic and heterochromatic zone could then create a euchromatin-heterochromatin transition zone which is competent to become a centromere.

Satellites play other roles. For example, they act as repositories for proteins and may affect development. The ability of satellites to affect genes in their neighborhood has been more famously observed in position-effect variegation assays. However, satellites are capable of more sophistication and can also affect gene expression in trans. It has been suggested that satellites can act as sinks, soaking up DNA binding factors and affecting gene expression

genome-wide [55]. In one example in *Drosophila,* the GAGAA satellite acts as a repository for the GAGA factor, a transcription factor that regulates *Ultrabithorax (Ubx).* In one incredible case, probably mediated by GAGA factor recruitment, a polyamide that specifically binds to and opens the GAGAA satellite was able to enhance the wing-haltere transformation phenotype of *Ubx* heterozygotes with an extra dose of GAGAA satellites [56].

Thus, satellites have the all-important function of assisting in chromosome segregation and can have effects on gene expression, genome-wide. It is therefore critical that satellites remain well regulated.

Satellites and transposable elements in the *D. melanogaster- D. simulans* system

The *D. melanogaster- D. simulans* siblings are an excellent system to study satellites and transposable elements. They allow us to draw on knowledge and tools that were generated over a century. They also have strikingly different genomes. In *D. simulans,* TE derived sequences account for 5% of the genome. They are mostly fragmented, though there are some active. In *D. melanogaster,* 15% of the genome comes from TE derived sequences and there are several actively transposing TEs [57,58]. Explanations for these differences range from *D. melanogaster* encountering new TEs as it spread to newer habitats to the smaller population size of *D. melanogaster* preventing efficient selection against TEs. However, atleast two observations suggest that *D. simulans* may be mechanistically more adept at repressing TEs. First, reporters linked to the *Copia* TE were repressed more efficiently in *D. simulans* than in *D. melanogaster.* Additionally, comparisons of the piRNA pathway in *D. melanogaster* and *D. simulans* suggest that this pathway maybe more stringent in the latter species.

The explosive proliferation of TEs in *D. melanogaster* is thought to have had other secondary effects. It has been speculated that the TE proliferation may have directly lead to the increase in satellite DNA content as well. According to this hypothesis, the hyper-proliferating TEs would have inserted into centromeric satellites and upset the delicate balance of chromatin states essential for centromeric function [42]. Consistent with this hypothesis, full length copies of several TEs have been found embedded in *D. melanogaster* centromeric satellites[43]. It is speculated that centromeres so disrupted would have led to the evolution of new centromeric satellites, thus converting the old centromeric satellites to peri-centromeric satellites[42].

It has been much speculated that some of these centromeric satellites help increase their frequency in the population by driving the transmission of chromosomes they are a part of during assymetric meiosis such as that in females. In this model, the variant alleles of host centromeric and heterochromatin proteins that would combat this drive would be selected [42]. Indeed, segregation distortion loci can be centromeric, such as in monkeyflowers[59]. However, to date, such phenomena have not been found in *D. melanogaster* and it is plausible that heterochromatin proteins are rapidly evolving to help the host adapt to satellites which are evolving due to mutational causes.

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CHAPTER 2

The *Hmr* and *Lhr* hybrid incompatibility genes suppress a broad range of heterochromatic repeats

NOTE: This paper is largely the result of work done by me. The experiments were planned by Dr.Dan Barbash and me. The following figures are those with contributions from other authors*. Fig 2.3D and Fig 10 were done in collaboration with Kevin Wei who generated this data and is using it to write a separate paper. Figures 4B and 6C were generated by Tawny Cuykendall using analytical pipelines developed by me. Other authors independently generated Fig 2.1 (except C and G which are my contribution), 2.2 B and C, Fig2.3, Fig 2.3B and Fig 2.9.*

Introduction

As populations diverge, their ability to reproduce with each other diminishes. Hybrid incompatibility (HI), the reduced viability and fertility of interspecific hybrids, is a major cause of reproductive isolation between nascent species and thus an important contributor to speciation. Many of the genes causing HI show evidence of adaptive evolution, typically manifest as excessive numbers of amino-acid-changing mutations compared to neutral expectations [1,2]. These data do not, however, imply that natural selection acts directly on HI phenotypes. Rather, the prevailing model of HI formulated by Dobzhansky and Muller (D-M) emphasizes that incompatibilities evolve in two distinct steps. First, two or more loci diverge independently in two nascent species. Then, if these species later interbreed, these diverged genes may interact to cause deleterious HI phenotypes. The key insight of the D-M model is that hybrid lethality and sterility evolve as byproducts of intraspecific divergence [1].

Adaptive evolution therefore does ultimately lead to HI, but if we wish to identify the evolutionary forces that drive the divergence of HI genes, then we need to understand the function of these genes within species. The mechanisms by which HI genes cause sterility or lethality are important but separate issues. In fact, it remains uncertain whether the wild type functions of HI genes are generally predictive of the deleterious phenotypes that they cause within hybrids.

Pinpointing the function of HI genes and the causes of their adaptive evolution is a challenging goal. For example, the *Hybrid male rescue* (*Hmr*) gene causes large reductions in hybrid fitness [3]. Loss-of-function mutations in *D. melanogaster*, however, have only moderate effects on fertility and provide few insights into mechanistic underpinnings [4]. The nucleoporins provide an intriguing counterexample. Several have been implicated in hybrid lethality and found to evolve under adaptive evolution [5]. Mutations in nucleoporin subunits are lethal in *D.*

melanogaster, but the genes have many pleiotropic functions and the challenge is to pinpoint which one(s) are driving evolutionary divergence.

Here we investigate two hybrid lethality genes, *Lethal hybrid rescue* (*Lhr*) and *Hmr*, which interact to cause F1 hybrid male lethality between *D. melanogaster* and *D. simulans* [6]. Both genes show extensive divergence in their coding sequences that is consistent with positive selection [6,7]. For *Hmr* this sequence divergence appears to be required for hybrid lethality because the *D. melanogaster* ortholog of *Hmr* causes hybrid lethality but the *D. simulans* ortholog does not [7]. For *Lhr*, however, both orthologs have hybrid lethal activity, with *D. simulans Lhr* having greater activity due to its higher expression level in hybrids [8]. That study left open the possibility that *Lhr* coding sequence divergence makes some contribution to hybrid lethality. Furthermore we found that *Lhr* from the more diverged species *D. virilis* has no hybrid lethal activity, suggesting that more extensive coding sequence divergence does have substantial functional consequences [9].

These previous studies leave unanswered the fundamental question of what evolutionary force is driving adaptive sequence change, and necessitate a detailed understanding of *Hmr* and *Lhr* function within each of the hybridizing species. Loss of function alleles of *Hmr* and *Lhr* are strong suppressors of hybrid lethality, but are largely viable within *D. melanogaster* and *D. simulans*, respectively [10,11].

Lhr (also known as HP3) protein localizes to heterochromatin [6,12]. Several other Drosophila HIs also involve heterochromatin or heterochromatin proteins, which is intriguing because genome size varies widely among Drosophila, largely as a consequence of variation in repetitive DNAs that make up the heterochromatin [13,14]. Heterochromatin may have a much wider role in incompatibility because repetitive DNA variation is the major cause of the ~1000-fold variation in genome size among multi-cellular eukaryotes [15]. These DNAs can increase in

copy number by general host processes such as unequal crossing over and duplication [16]. Alternatively, they may increase copy number by selfish properties such as transposition for TEs [17] and meiotic drive for satellite DNAs [18]. In either case, over-proliferation can be deleterious to their host species by causing genome instability, leading to the evolution of host defense mechanisms [19]. For example, one major mechanism is the piRNA pathway, where small (23-30 nt) RNAs derived from TE sequences are used to silence TE activity [20]. There are also hints that the piRNA pathway may regulate satellite DNAs [21]. Interestingly, piRNA regulatory genes often show signatures of adaptive evolution among Drosophila species [22].

Genetic conflicts with selfish DNAs have been proposed as an important driver of HI [1,2,23], but little is known about what specific sequences are interacting with HI genes. *D. simulans* and *D. melanogaster* have great potential for addressing this question because they differ substantially from each other in genome size [14], satellite DNA content [13,14], and in both the types and number of TEs that they harbor [24]. Here we report that *Hmr* and *Lhr* are required to repress transcription from both TEs and satellite DNAs. *Hmr* and *Lhr* also regulate telomeres, a third specialized type of heterochromatic sequence that serves to protect the ends of linear chromosomes [25] and is composed of rapidly evolving DNA and proteins [26-28]. Telomere variation can affect host fitness and genome stability, and has been proposed as another potential source of meiotic drive [27,29]. We used a *D. simulans* mutation in *Lhr*, comparative cytology, and interspecific complementation with *Hmr* transgenes to identify classes of TEs and satellites that are regulated differentially between the species. We conclude that *Hmr* and *Lhr* provide an adaptive defense against multiple classes of repetitive DNA sequences that change rapidly in evolutionary time, can reduce host fitness, and have high potential to provoke genetic conflict.

Results

Lhr and Hmr form a complex with HP1a

Lhr protein localizes to a subdomain of pericentric heterochromatin in early embryos [8]. To explore possible similarities with Hmr, we examined the localization of Hmr with a 3X-HA epitope-tagged *Hmr* transgene (see Materials and Methods). mel-Hmr-HA colocalizes with HP1a and H3K9me2 at heterochromatin in nuclear cycle 14 embryos (Figure 2.1A). We then used Immuno-FISH to determine its localization relative to specific heterochromatic satellite DNA sequences. mel-Hmr-HA does not overlap with the X-linked 359-bp satellite but colocalizes with dodeca, a GC-rich pericentromeric satellite on chromosome 3. This pattern mimics that seen previously with Lhr [8]. Additionally, mel-Hmr-HA colocalizes with GA-rich repeats and the 2L3L satellite in embryos (Figure 2.1B). Colocalization between mel-Hmr-HA with both dodeca and GA-rich repeats is also observed in ovarian nurse cells from *Hmr^{3}; mel-Hmr-HA* females, indicating that localization is not a consequence of overexpression (Figures 2.2B, C). Unlike Lhr [8], mel-Hmr-HA localizes to the nucleolus in early embryos (Figure 2.1C), suggesting that Hmr may have some functions distinct from Lhr.

The largely similar localization patterns of Hmr and Lhr raise the possibility that they physically interact. We performed co-immunoprecipitation (co-IP) studies from embryo extracts and found that mel-Lhr-HA and mel-Hmr-FLAG co-IP (Figure 2.1D). *mel-Lhr-HA* was previously shown to express at wild type levels [8], and *mel-Hmr-FLAG* is expressed significantly lower than wild type levels (Figure 2.3), demonstrating that these results are not due to overexpression. Lhr was previously shown to bind to, co-localize with, and be dependent on HP1a for correct heterochromatic localization [6,9,12,30]. We therefore tested if HP1a also associates with Hmr. IPs with HP1a pulled down mel-Lhr-HA and mel-Hmr-FLAG, but the reciprocal IPs failed to pull down detectable HP1a (Figure 2.1E).

Yeast two-hybrid assays show that *Hmr* and *Lhr* from *D. melanogaster* interact, suggesting that the co-IP reflects a direct interaction between the proteins (Figure 2.1F). This interaction is likely mediated via the BESS domains within Lhr and Hmr [6], a 40 amino-acid motif found in 19 proteins in *D. melanogaster* that has been implicated in protein-protein interactions and homo-oligomerization [31]. We also found that the *D. simulans* orthologs interact, as do the heterospecific combinations; the strength of interactions varied widely but exploring the potential significance of this result will require a more quantitative assay.

We next examined protein localization in mutant backgrounds to test the potential mutual dependence of Lhr and Hmr for their localization to heterochromatin. We made a *D. melanogaster Lhr* mutation by recombining a *mini-white* gene into the *Lhr* locus to create the Lhr^{KO} allele (Figure 2.4A). In Lhr^{KO}, transcription from *Lhr* but not flanking genes is greatly reduced, and no Lhr protein is detectable (Figure 2.4B, C). These results demonstrate that Lhr^{KO} is a strong loss of function allele, which we confirmed in hybrid rescue, crosses (see Materials and Methods).

Lhr-HA levels are greatly reduced in Hmr^{3} mutant embryos but when examined at high gain a small amount of Lhr-HA is detectable in heterochromatin (Figure 2.1G). This result suggests that Hmr is not absolutely required to localize Lhr to heterochromatin, though it remains possible that some Hmr protein is made in the Hmr^{3} mutant. In a reciprocal experiment, Hmr-HA localization appears normal in Lhr^{KO} (Figure 2.1H). In combination with previous results, our data suggest that Lhr localization to heterochromatin depends on HP1a, and that Hmr stabilizes Lhr.

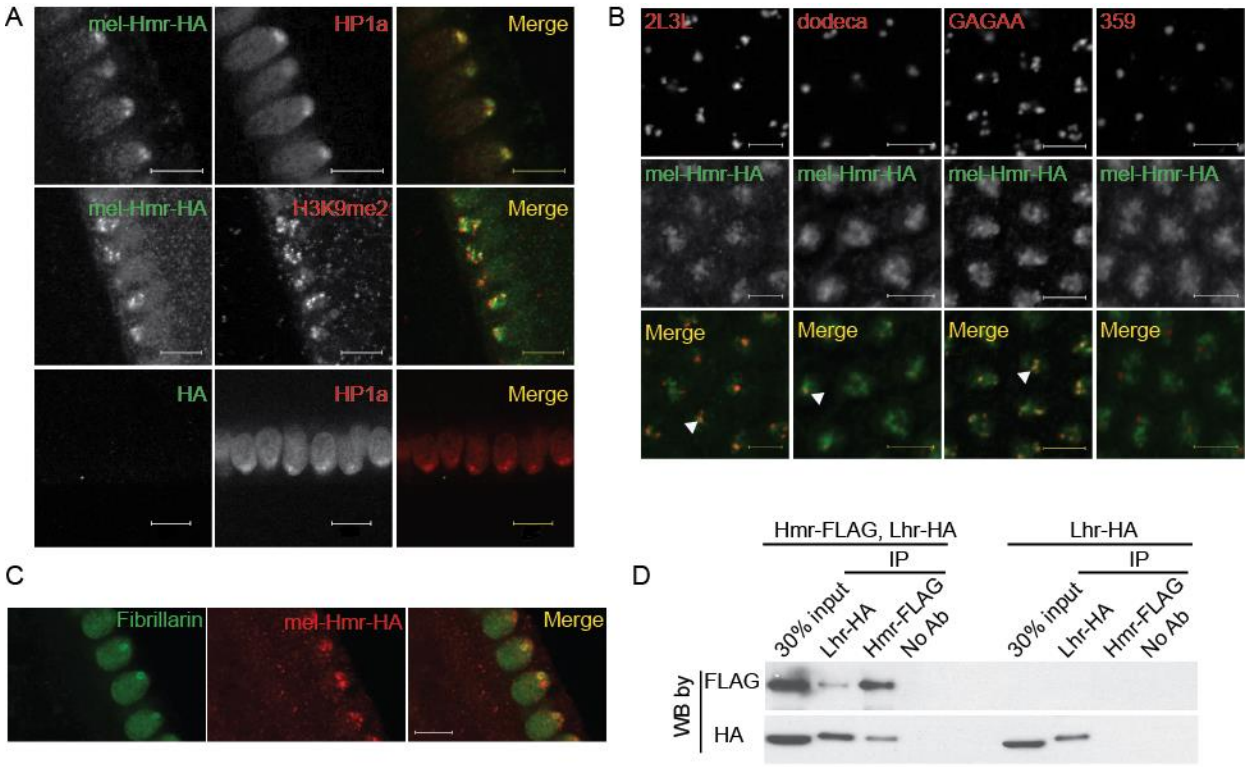


Figure 2.1 Hmr forms a complex with Lhr and HP1a and is required to stabilize Lhr. **(A)** mel-Hmr-HA (green) colocalizes with HP1a (top) and H3k9me2 (middle; both red) in nuclear cycle 14 embryos. The HP1a costain is in a *mel-Hmr-HA* background, while the H3k9me2 costain is in a *Hmr*^{3}*; mel-Hmr-HA* background. A negative control shows no HA signal in *w*^{1118} embryos lacking the *mel-Hmr-HA* transgene (bottom). Scale bars represent 10μm. **(B)** Arrowheads show where mel-Hmr-HA (green) colocalizes with 2L3L, dodeca and GA-rich satellites but not with the 359 bp repeat satellite in *mel-Hmr-HA* (all FISH probes red). Scale bars represent 5μm. **(C)** mel-Hmr-HA (red) colocalizes with the nucleolar marker Fibrillarin (green) in *mel-Hmr-HA* early embryos. Scale bars represent 10μm. **(D)** mel-Lhr-HA and mel-Hmr-FLAG co-immunoprecipitate from *D. melanogaster* embryo extracts derived from flies expressing both transgenes (left 4 lanes) but not from flies expressing only Lhr-HA (right 4 lanes). Extracts were IP'd with the indicated antibodies, and then probed by Western Blots (WB) with the same or different antibodies.

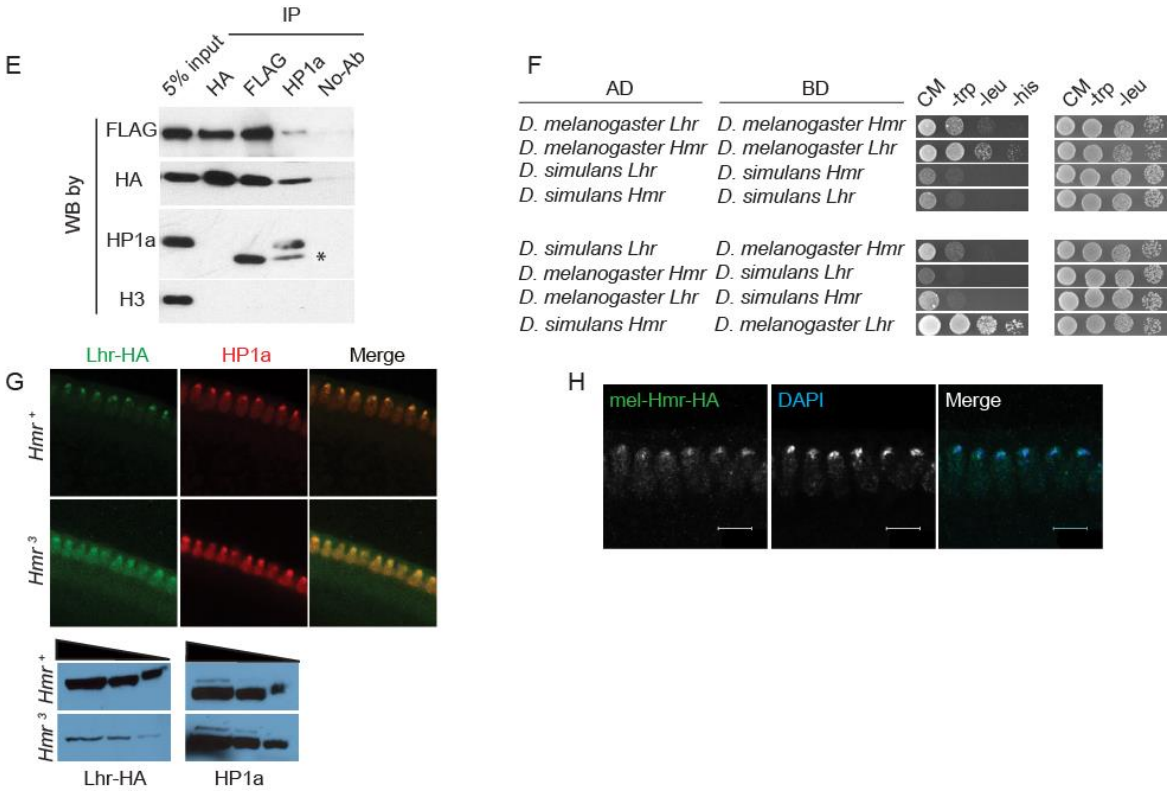


Figure 2.1 contd. Hmr forms a complex with Lhr and HP1a and is required to stabilize Lhr. **(E)** Lhr-HA, Hmr-FLAG and HP1a co-immunoprecipitation from embryo extracts. Specificity is indicated by lack of immuno-precipitation of histone H3. Asterisk indicates the antibody light chain. **(F)** Lhr and Hmr interact in a yeast-two hybrid assay. Interactions were detected by growth on complete media (CM) lacking histidine (his); growth controls were performed on CM lacking tryptophan (trp) and leucine (leu). The top 4 panels test for interactions between orthologs from the same species; the bottom 4 between heterospecific orthologs. AD, activation domain; BD, DNA binding domain. **(G)** Lhr-HA is detectable in *Hmr*^{3} and localizes to heterochromatin, as indicated by co-localization with HP1a. Note that a higher gain was used in the *Hmr*^{3} panels compared to the *Hmr*$^{+}$ panels in order to detect Lhr-HA, and is reflected in the higher background. Western blots confirm that Lhr-HA levels are reduced in *Hmr*^{3}. HP1a is used as a loading control. **(H)** Hmr-HA maintains its localization to DAPI-dense heterochromatin in *Lhr*^{KO}*; Hmr-HA* embryos. Scale bars represent 10μm.

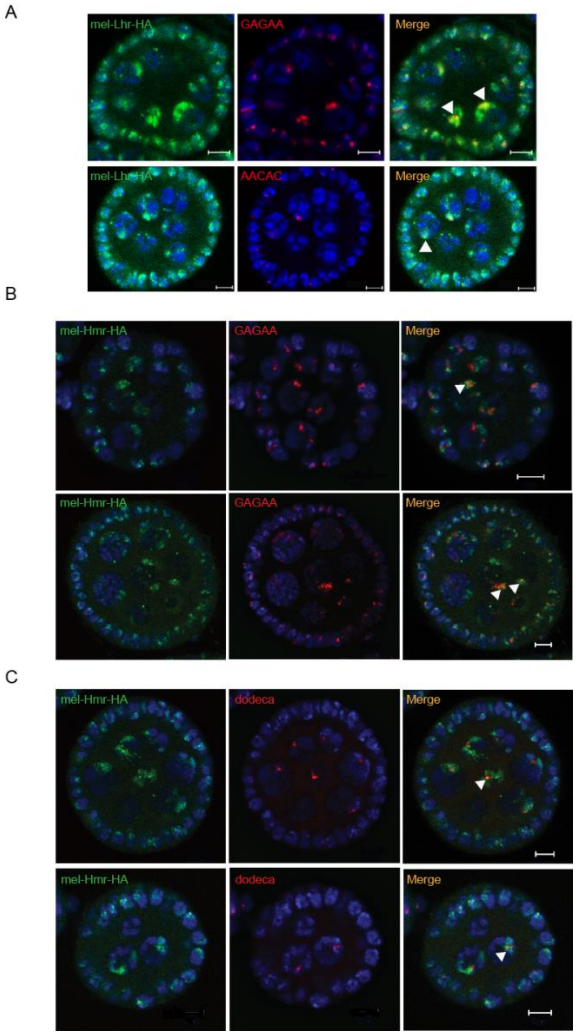


Figure 2.2 Lhr and Hmr colocalize with specific satellite sequences in ovaries.
Nurse cell nuclei (blue) are stained with DAPI in all panels. Scale bars represent 5μm. **(A)** mel-Lhr-HA (green) colocalizes with GAGAA(red, top panel) and AACAC (red, bottom panel) in the nurse cells of Lhr^{KO}/+ ; *LhrHA*/+ ovaries. Arrows point to overlaps between bright FISH and HA-staining foci. **(B)** mel-Hmr-HA (green) colocalizes with GAGAA (red) and **(C)** dodeca (red) in nurse cells of Hmr^{3} ; *mel-Hmr-HA/mel-Hmr-HA* ovaries in a subset of nuclei. Arrows point to overlaps between FISH signals and the brightly staining foci of mel-Hmr-HA. Two different egg chambers are shown for both dodeca and GAGAA.

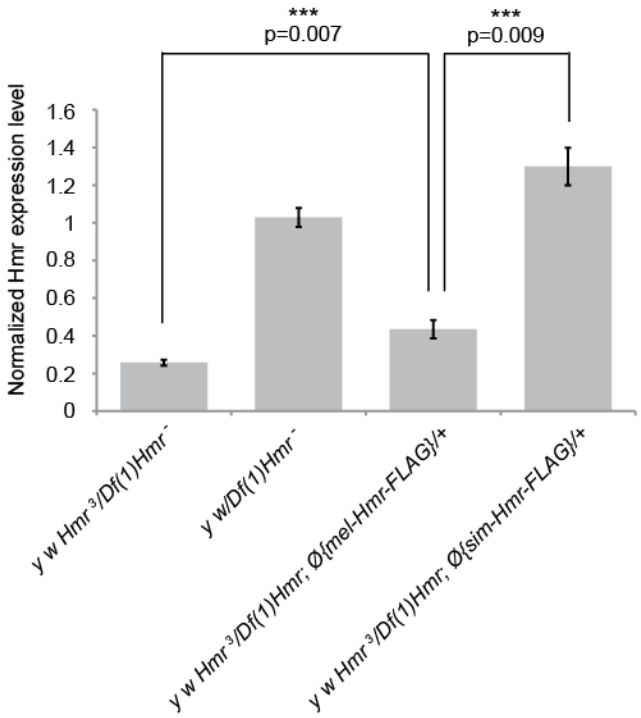


Figure 2.3 qRT-PCR analysis of *Hmr-FLAG* transgenes. *Hmr* transcript levels in transgenic lines were compared to the host strain (*yw Hmr^{3}/Df(1)Hmr-)* and also to heterozygotes (*yw/Df(1)Hmr*). The transgenes are heterozygous, therefore both the transgenic lines and *Hmr$^{+/-}$* carry one copy of *Hmr^{+}*. RNA was isolated from ovaries and *Hmr* expression levels were normalized relative to *RpL32*. Error bars represent standard error within 3 biological replicates. The difference in the expression level of *mel-Hmr-FLAG* and *sim-Hmr-FLAG* is significant (*p*=0.009, two-tailed *t*-test with equal variance). Additionally, the expression of *mel-Hmr-FLAG* is significantly different than an endogenous copy of *Hmr* (*p*=0.007, two-tailed *t*-test with equal variance).

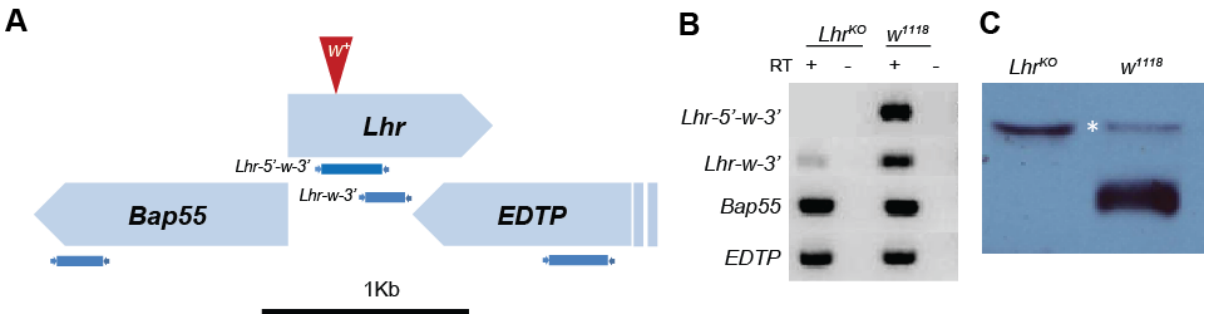


Figure 2.4 The *D. melanogaster Lhr^{KO}* allele generated by homologous recombination (A) *Lhr* and flanking genes are shown, the red triangle labeled w^+ indicates the site of the insertion in the *Lhr^{KO}* allele, which is predicted to be ~4.7kb based on the structure of the targeting vector. Products used in RT-PCR reactions in **(B)** are shown below the genes. *EDTP* gene is partial; *w*+ insertion not to scale. **(B)** RT-PCR from adult females shows no *Lhr* transcript spanning the w^+ insertion (*Lhr-5'-w-3'*) in *Lhr^{KO}*. A highly reduced amount of *Lhr* transcript is detected 3' to the *w*+ insertion (*Lhr-w-3'*). The flanking genes *Bap55* and *EDTP* are not affected. w^{1118} was used as a *Lhr*$^+$ control. +, - indicates presence or absence of reverse transcriptase (RT). **(C)** Western analysis shows that *Lhr^{KO}* produces no protein. A non-specific band indicated by the asterisk is used a loading control.

Lhr is required for female fertility

Lhr^{KO} flies are almost fully viable (22.25% compared to the expected 25% in crosses between heterozygotes at 27°; $p<0.05$ by Chi-squared; N=2813 total flies scored). However, comparison of Lhr^{KO} with a background-matched Lhr^{+} control (see Materials and Methods) showed that Lhr^{KO} females have substantially lower fertility, particularly at higher temperatures. One to five day old Lhr^{KO} females display only a fraction of the fertility of Lhr^{KO}/+ and later become sterile (Figure 2.5A). We confirmed this in a different Lhr^{-} background where a similar reduction in fertility occurs at later ages (Figure 2.5B). In a separate experiment we found that the hatch rate of the eggs laid by Lhr^{KO}/Lhr^{KO} mothers is low and declines with increasing maternal age (Table 2.1). This Lhr^{KO} female fertility phenotype is strikingly similar to that of *Hmr* mutants [4], suggesting that *Hmr* and *Lhr* may function in a common regulatory pathway.

Lhr and *Hmr* are required to repress transposable elements

We performed an RNA-Seq comparison of ovaries from Lhr^{KO} and Lhr^{+} to investigate the cause of this fertility reduction and discovered a widespread increase in transposable element (TE) transcripts. Using two different TE mapping methods (see Materials and Methods) we found that transcripts from 99 families were at least 2-fold upregulated, with 38 elements being at least 10 fold upregulated (Figure 2.6A). Mis-regulated TEs include elements with germline expression such as the telomeric non-LTR retrotransposons *HeT-A* (350.7 fold) and *TART* (51.76 fold), the LTR retrotransposon *copia* (19.8 fold), and the DNA transposon *bari-1* (44.7 fold). TEs expressed only in the somatic follicle cells, such as *Gypsy* (3.8 fold) and *Zam* (7 fold) were also upregulated. In addition, qRT-PCR in two different genetic backgrounds confirmed the massive increase in *HeT-A* transcript levels (185-846-fold; Figure 2.7). These results demonstrate that the telomeric TEs are especially sensitive to *Lhr* regulation.

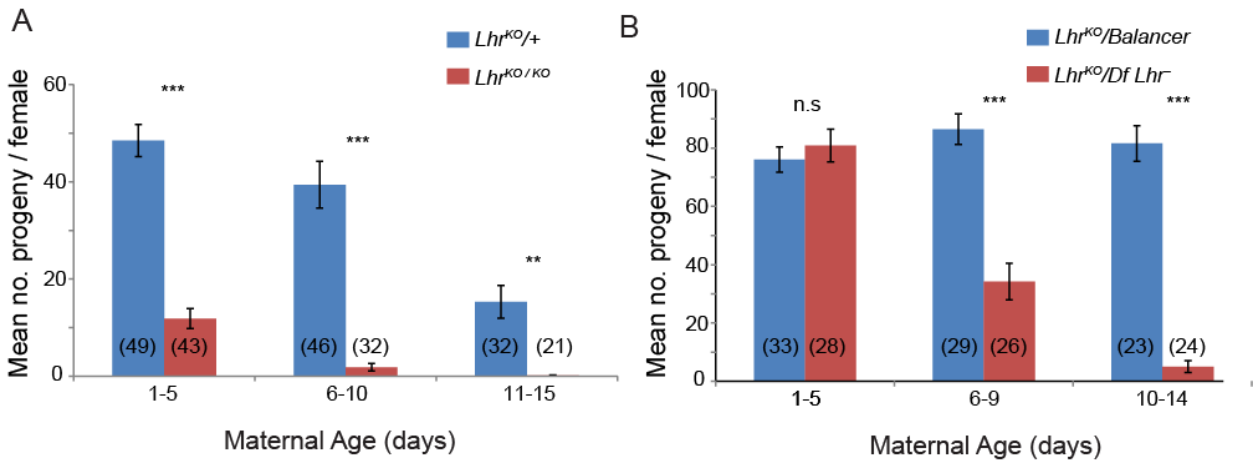


Figure 2.5 *Lhr* mutant females have reduced fertility Total adult progeny from single $Lhr^{KO}/^{KO}$ **(A)** or $Lhr^{KO}/Df(2R)BSC44$, Lhr^{-} **(B)** females were compared at 27° to heterozygous female siblings ($Lhr^{KO}/+$ for **(A)**; $Lhr^{KO}/SM6a$ for **(B)**). The difference between the fertility of genotypes was tested by a two-tailed *t*-test. n.s= not significant, **p<0.01,***p<0.001. The number of individuals tested for each experiment is shown at the bottom of the bars. The error bars represent S.E.M. Crosses were performed at 27°.

Maternal Age	Hatch rate *Lhr*^{KO}/+	Hatch rate *Lhr*^{KO}/*Lhr*^{KO}
2-3 days	64.70	17.39
5-6 days	51.90	8.13
10-11 days	54.64	2.78

Table 2.1 Eggs laid by *Lhr*^{KO} mothers have a reduced hatch rate. Hatching of eggs laid by *Lhr*^{KO}/+ or homozygous *Lhr*^{KO} mothers crossed to wild-type fathers was followed for 36 hrs after egg lay. For *Lhr*^{KO}/+, 34 eggs from days 2-3, 289 from days 5-6 and 668 eggs from days 10-11 were counted. For *Lhr*^{KO}, 46 eggs from days 2-3, 209 from days 5-6 and 287 eggs from days 10-11 were counted. The significance of the difference in the hatch rates of the eggs laid by *Lhr*^{KO} and *Lhr*^{KO}/+ mothers was calculated by one tailed F.E.T., and was significant at all time points ($p<10^{-4}$).

We also performed RNA-Seq analysis of an *Hmr* mutant (*Df(1)Hmr⁻/Hmr³*, abbreviated below as *Hmr⁻*). We compared it to a heterozygous control (*Df(1)Hmr⁻/y w Hmr⁺*, abbreviated below as *Hmr⁻/Hmr⁺*) because it closely matches the genetic background of the mutant genotype, and also serves as a control for *Hmr* transgenic genotypes that are described below. We found that 55 different TE families are upregulated at least 2 fold in *Hmr* mutants, with 14 being upregulated at least 10 fold (Figure 2.6B). Notably, the telomeric retrotransposons *HeT-A* and *TART* are again among the most highly upregulated. Strikingly, the TEs affected by *Hmr* are largely a subset of *Lhr*-regulated TEs, suggesting that they act together to regulate multiple TE families (Figure 2.6C). The smaller number of mis-regulated families in *Hmr⁻* likely reflects the fact that we are comparing *Hmr⁻* mutants to heterozygotes, but *Lhr* mutants to wild type.

Since some germline TE repressor genes also regulate somatic TE expression [32], we performed RNA-Seq to compare TE expression between 72-76 hour-old *Df(1)Hmr⁻/Y* and *Hmr⁺/Y D. melanogaster* male larvae. This also served as a control for experiments described below to address whether TE mis-expression may be contributing to hybrid lethality. We found that 31 TEs exhibit a statistically significant ≥2 fold upregulation (Figure 2.6D), but there are two striking differences compared to *Hmr* mutant ovaries. First, different TEs are affected, with the telomeric retrotransposons in particular not upregulated in the larvae. Second, the magnitude of TE derepression is lower in larvae.

Lhr and *Hmr* affect expression of heterochromatic genes

We next examined potential effects on protein-coding genes. Remarkably few genes (11 in *Hmr⁻*; 0 in *Lhr^KO*) show a statistically significant misregulation in either *Lhr* or *Hmr* mutants (FDR 0.05). However, a comparison of fold change in the expression of all heterochromatic versus all euchromatic genes found that heterochromatic genes are down-regulated to a greater extent for both mutants, although the effect is stronger in *Lhr^KO* (Figure 2.8). Lhr preferentially associates

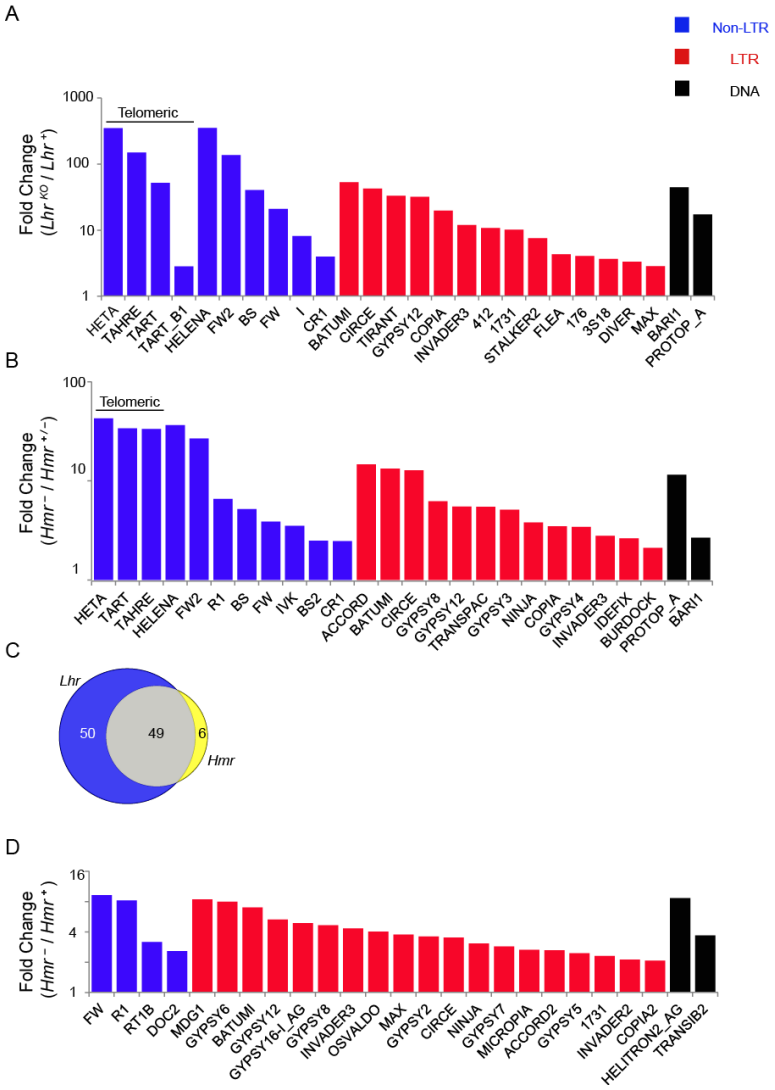


Figure 2.6 TE misregulation in *Lhr* and *Hmr* mutants. **(A** and **B)** Analysis of Lhr^{KO} **(A)** and Hmr^{-} **(B)** ovaries. Reads with zero mismatches were mapped separately to the individual-insertion or consensus-sequence TE databases. A subset of TEs that are significantly different between genotypes are shown and include those with the 25 lowest p-values obtained from individual-insertion mapping analysis, but excluding all *centroid* repeats [96]. Additionally shown are *TAHRE*, which is only found in the consensus-sequence database, as well as *TARTB1* for Lhr^{KO}, which is significant but not among the 25 top hits in the Lhr^{KO} individual-insertion analysis. **(C)** 49 TEs are upregulated at least 2 fold in both Lhr^{KO} and Hmr^{-}. TE families include those resulting from mapping reads to the insertion database, as well as families found only when reads were mapped to the consensus database. **(D)** Reads from *Hmr* mutant or wildtype male larvae with up to three mismatches were mapped to the individual-insertion or consensus-sequence TE databases. All TE families, excluding *centroids*, that were significantly upregulated in the insertion sequence based analysis are shown here. Note the different Y-axis scales in **A**, **B** and **D**. Classification of DNA, LTR and non-LTR elements is from reference [97].

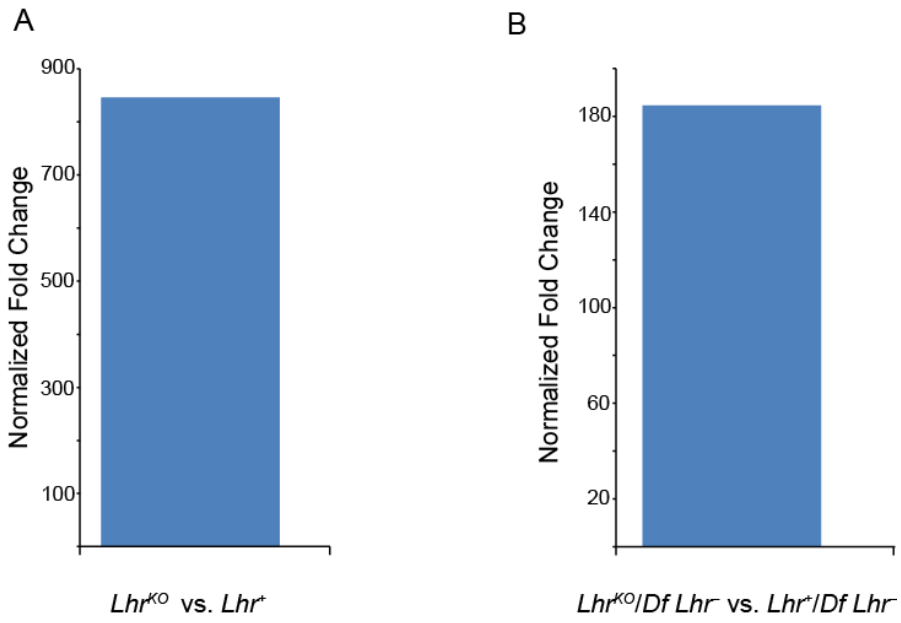


Figure 2.7 qRT-PCR analysis shows elevated *HeT-A* levels in *Lhr* mutants. qPCR was used to estimate the transcript levels of *HeT-A* relative to the gene *RpL32* in poly-A primed cDNA samples obtained from ovarian RNA from two different Lhr^{-} backgrounds and matching controls. **(A)** Ratio of *HeT-A*/*RpL32* in Lhr^{KO} vs. Lhr^{+}, showing mean from 3 biological replicates. Significance of fold change was calculated using Welch's one-tailed *t*-test; $p < 0.05$. **(B)** Ratio of *HeT-A*/*RpL32* in Lhr^{KO}/*Df(2R)BSC44* vs. Lhr^{+}/*Df(2R)BSC44*, showing mean from 4 biological replicates. Significance of fold change was calculated using the one tailed Wilcoxon rank sum test; $p < 0.05$.

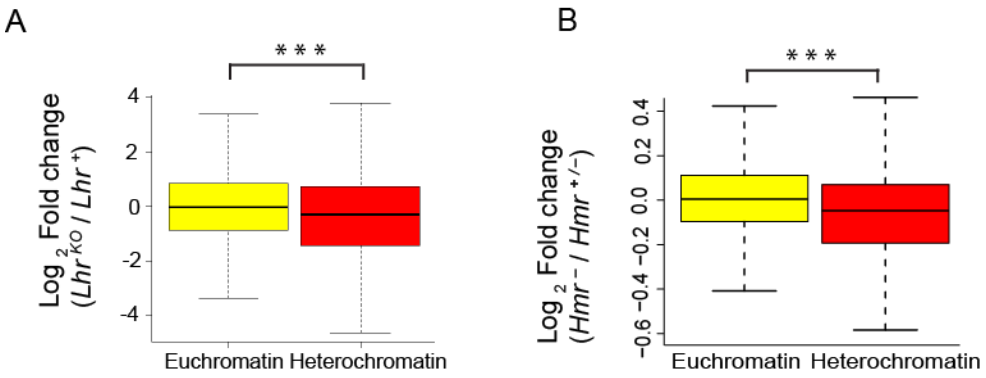


Figure 2.8 Reduced expression of heterochromatic genes in *Lhr* and *Hmr* mutants. Loss of *Lhr* **(A)** and *Hmr* **(B)** leads to a statistically significant reduction in the expression of heterochromatic genes. Significance of difference was calculated using the Wilcoxon rank sum test with continuity correction (for **(A)** p = 3.549e-05, for **(B)** p =1.461e-09). Box plots show $\log_2$ fold change of 7838 euchromatic and 370 heterochromatic genes for **(A)** and 7451 euchromatic and 344 heterochromatic genes for **(B)**. The definition of the euchromatin-heterochromatin boundary for all chromosomes comes from experiments done in S2 tissue culture cells, except for 3R, which comes from the cytogenomic border [98].

with heterochromatic genes in an embryonic cell culture line [12]; our results suggest that Lhr and Hmr have a small positive effect on expression of some heterochromatic genes.

Lhr **and** ***Hmr*** **mutants have long telomeres**

Drosophilidae have lost the telomerase-based mechanism of telomere elongation and instead use the regulated transposition of the *HeT-A*, *TART* and *TAHRE* retrotransposons [33]. Strikingly, these were among the 3 most strongly affected TEs in Lhr^{KO} and Hmr^{-} ovaries (Figure 2.6). We therefore investigated in more detail the localization of Lhr and Hmr proteins to the telomere [6]. Cytological markers on polytene chromosomes have been used to describe three distinct regions in the telomere, with HP1a localizing exclusively to the “cap”, a proteinacous structure at the most distal end of telomeres [25,28].

mel-Lhr-HA and mel-Hmr-HA overlap with HP1a, showing that Lhr and Hmr localize to the cap but not to more proximal regions (Figure2.9A, B). Localization is not due to the doubling of the dosage of these proteins in the transgenic lines because it also occurs in the Hmr^{3}*; Hmr-HA/Hmr-HA* and Lhr^{KO}*/+; Lhr-HA/+* genotypes (Figure 2.10). The localization of Lhr and Hmr to the cap, the primacy of the cap in the regulation of telomeric length, and the increase in the transcript levels of telomeric retro-transposons in *Lhr* and *Hmr* mutants led us to ask if these mutations cause long telomeres. We quantitated *HeT-A* DNA copy number by qPCR in Lhr^{KO} flies maintained at 27°C separately from its matched wild-type control strain for ~40 generations. We found that *HeT-A* copy number increased approximately 6 fold in Lhr^{KO}

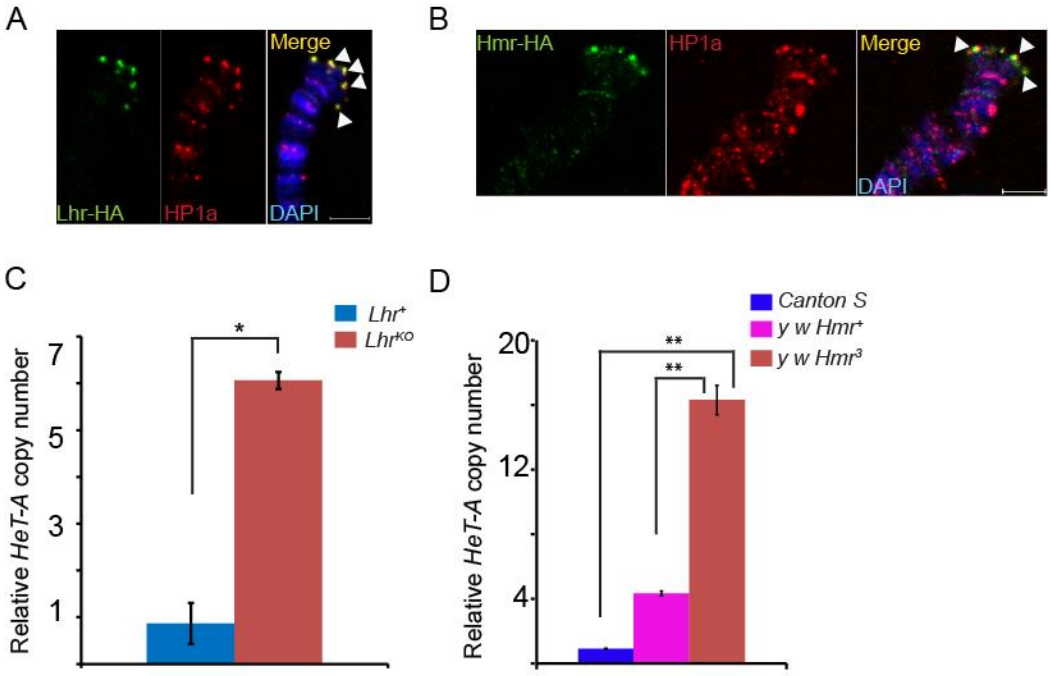


Figure 2.9 Lhr and Hmr are telomere cap proteins required for regulating telomere length. Lhr-HA **(A)** and Hmr-HA **(B)** localize to telomeres. Co-immunostaining with anti-HA and anti-HP1a shows that both proteins colocalize at the cap (arrowheads). The merged images include DAPI to stain DNA, shown in blue. *Lhr*^{KO} **(C)** and *Hmr*^{3} **(D)** have increased *HeT-A* copy number. qPCR was used to estimate the abundance of *HeT-A* and *rp49* from *Lhr*^{KO}, *Lhr*$^{+}$*, y w Hmr*^{3}, a matched *y w Hmr*$^{+}$ control, and the wild-type Canton S strain. Genomic DNA was isolated from carcasses of females whose ovaries were removed in order to minimize the amount of polytenized DNA present. Relative *Het-A* copy number is the ratio of *Het-A/rp49.* The error bars represent S.E.M for three replicates. The significance of the differences between the genotypes was calculated using two tailed *t*-test; *= p<0.05; ** = p< 0.01. Scale bars = 5µm.

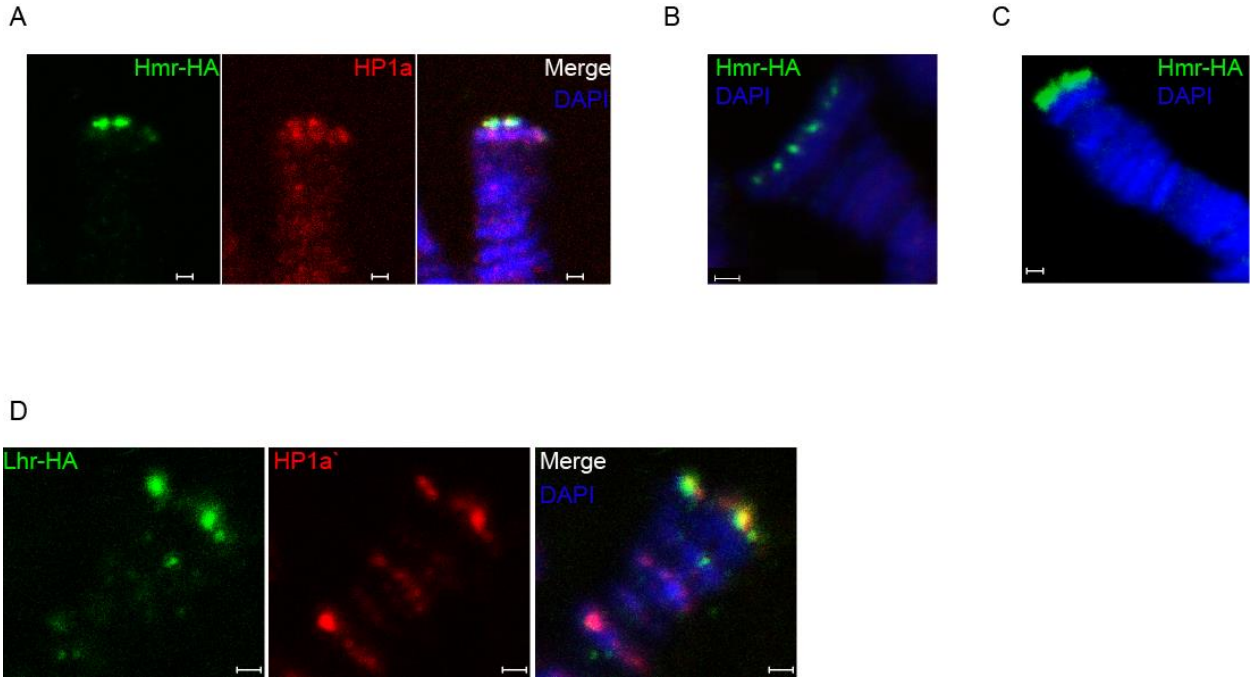


Figure 2.10 Localization of Hmr-HA and Lhr-HA to the telomeres is independent of dosage of endogenous copies. mel-Hmr-HA (green) in *Hmr*^{3}; *Hmr-HA* **(A-C)** and mel-Lhr-HA (green) in *Lhr*$^{KO/}$+; *Lhr-HA/*+ **(D)** colocalize with HP1a (red) at the telomere cap on polytene chromosomes. mel-Hmr-HA shows a range of distributions at the telomere, including punctate **(B)** and continuous across the chromosome terminus **(C)**. Scale bar is 1µm.

(Figure 2.9C). We also examined *HeT-A* DNA copy number in an Hmr^{3} mutant stock, and found ~4-16 fold higher abundance than in the Hmr^{+} stocks *y w* and Canton-S (Figure 2.9D).

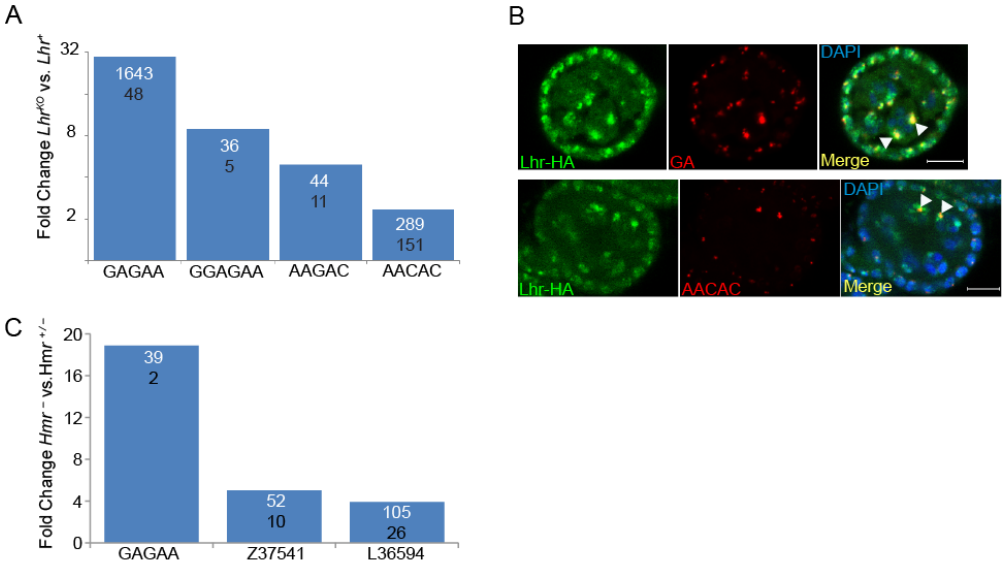
Satellite DNA transcripts are upregulated in *Lhr* and *Hmr* mutants.

Hmr and Lhr both localize to pericentric heterochromatin, which is largely composed of TEs and satellite DNAs. The potential effects of heterochromatin proteins on the levels of transcripts from satellites have not been widely explored. We therefore used our RNA-Seq data to examine transcript levels from 143 repeats in a repeat-sequence database (see Materials and Methods). Transcripts from most repeats are found at low abundance in Lhr^{+} with only 17 producing more than 10 reads. Four different satellite classes are significantly higher in Lhr^{KO} versus Lhr^{+} ovaries, including three that collectively make up more than 8% of the *D. melanogaster* genome [13]: AAGAC, AACAC, and the GA-rich satellites (Figure 2.11a). The GAGAA satellite showed the strongest effect, with an approximately 30-fold increase.

These results raise the question of whether transcriptional regulation of specific satellite DNAs reflects a direct association with Lhr. Lhr was not previously tested for association with either GA-rich satellites, which are found on all chromosomes in *D. melanogaster* [34], or with the AACAC satellite found on chromosomes 2 and Y [35]. We found that Lhr-HA colocalizes extensively with the GA-rich and AACAC satellites in the nurse cell nuclei of early stage egg chambers (Figure 2.11B, 2.2A).

In our *Hmr* RNA-Seq data the number of reads mapping to each repeat family was generally very small, but 3 satellite families are significantly derepressed by at least 4 fold in Hmr^{-} (Figure 2.11C), including GAGAA, which has a 19 fold increase in expression. This finding is consistent with the localization of mel-Hmr-HA to GA-rich satellites above (Figure 2.1B).

Figure 2.11 *Lhr* and *Hmr* repress satellite DNA transcription (A) Fold increase in satellite transcripts of Lhr^{KO} versus Lhr^{+}. Numbers within the bars show normalized reads mapping to each satellite from Lhr^{KO} (black) and from Lhr^{+} (white). All differences have $p<0.01$ by F.E.T. test. **(B)** Lhr-HA (green) colocalizes with GA-rich and AACAC satellites (red) in ovarian nurse cell nuclei (arrowheads) DAPI is shown in the merged images in blue. Scale bar = 10µm. **(C)** Fold increase in satellite transcripts in Hmr^{-} versus $Hmr^{+/-}$. Numbers within the bars show normalized reads mapping to each satellite, the numerator from Hmr^{-} and the denominator from $Hmr^{+/-}$. All differences have $p<0.001$ by F.E.T. test.



Additionally, the satellite Z37541, which binds nuclear lamins, is upregulated 5 fold in *Hmr*$^{-}$ *[36]*.

Although Lhr-HA localizes to the dodeca satellite [8]; we detected very few reads in either our *Lhr*$^{+}$ or *Lhr*^{KO} samples; likewise we did not find upregulation of dodeca in our *Hmr* RNA-Seq data. We conclude that Hmr and Lhr proteins are required to regulate transcript levels of a subset of satellites to which they localize.

siRNA and piRNA patterns are largely normal in *Lhr*^{KO}

The wide spectrum of TEs derepressed in *Lhr* and *Hmr* mutants is similar to mutations in piRNA regulatory genes such as *Ago3* and *aub* that post-transcriptionally regulate TEs via small-RNA-mediated silencing [37,38]. We therefore investigated a range of phenotypes that are associated with defects in the piRNA pathway. *Ago3* and *aub* mutants disrupt Vasa localization to the peri-nuclear small-RNA processing center, the nuage, and exhibit drastic reductions in the piRNA fraction (23-30nt) [38,39]. We found, however, that Vasa localizes normally in *Lhr*^{KO} (Figure 2.12A). We then sequenced the small RNA pool in *Lhr*^{KO} and found that the piRNA level is broadly comparable to *Lhr*$^{+}$ with only a minor reduction in longer piRNAs (Figure 2.12B). This pattern contrasts with mutants such as *aub* and *spn-E* that show a severe loss of piRNAs [39]. We looked more closely for TE-specific defects and found that piRNAs mapping to most individual TE families are comparable between *Lhr*$^{+}$ and *Lhr*^{KO} (Figure 2.12C). We also examined “ping-pong” processing, which produces piRNAs from opposing strands with a characteristic 10 nucleotide overlap [38,39]. Most TEs derepressed in *Lhr*^{KO}, including *HeT-A*, have ping-pong scores that are comparable to wild-type (Figure 2.12D).

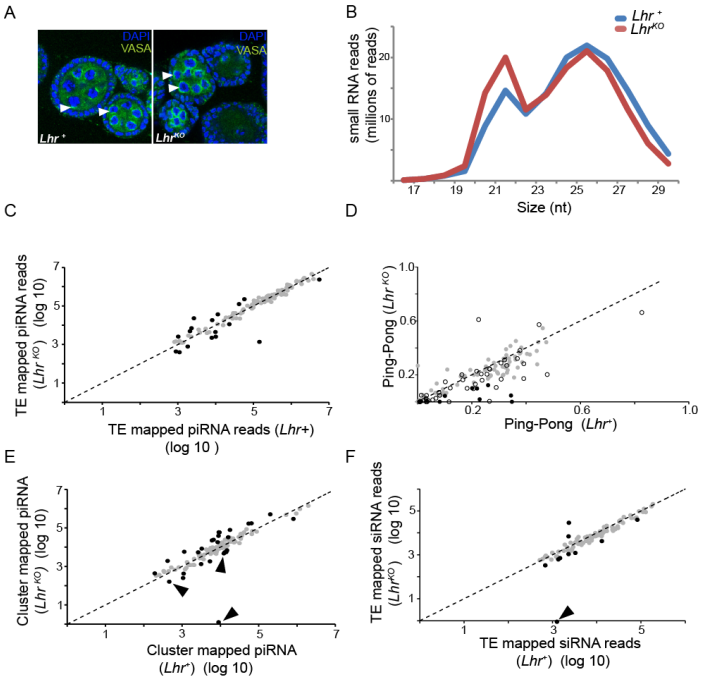


Figure 2.12 Small RNA patterns are largely unaffected in *Lhr*^{KO} (A) VASA (green) marks the peri-nuclear nuage (white arrowheads) and shows no difference in localization between *Lhr*$^{+}$ and *Lhr*^{KO} ovaries. **(B)** siRNA (17-22 nt) without mismatches and piRNA (23-30 nt) with up to one mismatch were mapped to a reference sequence set containing the *D. melanogaster* r5.68 genome, *D. melanogaster* sequences from Repbase and the repeat-sequence database. The number of mapped *Lhr*^{KO} reads was normalized to the total number of mapped *Lhr*$^{+}$ reads. **(C)** Filtered piRNA reads were mapped uniquely to the Repbase TE consensus sequences with one allowed mismatch. 121 TE families producing >=1000 reads summed over both genotypes are shown. Black circles represent TE families whose fold change between *Lhr*^{KO} and *Lhr*$^{+}$ is greater than 2 fold ($p < 0.001$). **(D)** Ping-pong scores of TE families in *Lhr*^{KO} and *Lhr*$^{+}$. Among TEs whose mRNA levels are significantly increased in *Lhr*^{KO} (Figure 3), those with >2-fold or <2-fold changes in ping-pong score are represented by black circles and gray circles, respectively. TEs whose mRNA levels are not significantly increased in *Lhr*^{KO} are represented by open circles. **(E)** Plot shows the number of unique piRNAs mapped to piRNA clusters, with one allowed mismatch and normalized between genotypes. piRNA clusters with >=500 reads summed over both genotype are shown. Black arrowheads point to sub-telomeric piRNA clusters. Black circles indicate clusters whose fold change between *Lhr*^{KO} and *Lhr*$^{+}$ is greater than 2-fold ($p < 0.001$). **(F)** Unique siRNA (17-22 nt) were mapped as in **(C)**, except no mismatches were allowed. 96 TE families are plotted that have >=1000 reads summed over both genotypes. Black circles represent TEs whose siRNA levels changed by >2 fold. siRNA mapping to the TAS repeat HETRP are almost completely lost (arrow). For **(C, D, F)** significance values were calculated using F.E.T., implemented in DEG-seq.

We searched further for possible defects in piRNA production by examining piRNAs that map to 122 primary-piRNA-generating heterochromatic clusters [40]. piRNAs originating from most of the major clusters are not significantly affected in Lhr^{KO} but 16 and 11 of the 122 clusters are at least two-fold higher or lower, respectively, in Lhr^{KO} (Figure 2.12E). Some of the most strongly affected clusters are associated with telomeres. Cluster 3 consists entirely of telomeric retrotransposons and is upregulated 4.3 fold in Lhr^{KO}. Sub-telomeric cluster 11 shows a complete loss of unique piRNAs, while clusters 33 and 4 are 2.6 and 2.9 fold downregulated, respectively. These 3 clusters consist mainly of HETRP telomere-associated (TAS) repeats and are therefore not expected to contribute to TE repression; their misregulation instead suggests that *Lhr* is required for regulating chromatin states at telomeres.

The siRNA pathway has also been implicated in repressing TEs in the ovary [41-43]. We found that siRNAs mapping to the vast majority of TE families, including those mapping to *HeT-A*, are not significantly different between Lhr^{KO} and Lhr^{+}, suggesting that *Lhr* is not generally required for siRNA biogenesis (Figure 2.12F). Taken together, our results indicate that defects in small RNA synthesis are not the cause of TE derepression in Lhr^{KO}. An intriguing possibility is that Lhr is a piRNA-dependent effector of TE silencing.

Comparing *Lhr* function in *D. simulans* and *D. melanogaster*

We propose that the dynamic sequence turnover of repetitive DNAs is the selective pressure driving the adaptive sequence divergence of *Lhr* and *Hmr*. This hypothesis implies that the localization and/or function of the Lhr protein has changed between species, due to co-evolution with species-specific repetitive DNAs. The Lhr^{1} allele in *D. simulans* [10] presents a rare opportunity to compare the function of a rapidly evolving heterochromatin protein between

sibling species. We performed RNA-Seq from ovaries of *Lhr*^{1} females and a matched *Lhr*$^{+}$ control (see Materials and Methods). We found essentially no *Lhr* transcript reads in the *Lhr*^{1} mutant strain, strongly suggesting that this allele is null.

D. simulans has many of the same satellites as *D. melanogaster* but they are generally of lower abundance [13]. We therefore first examined satellite DNA expression in the *Lhr*^{1} and *Lhr*$^{+}$ (control) RNA-Seq data. Unlike in *D. melanogaster Lhr*^{KO}, we found few satellite reads in either genotype and no significant differences between them. We conclude that *Lhr* has a unique role in *D. melanogaster* to repress satellite DNA transcription. The AACAC satellite that Lhr co-localizes with in *D. melanogaster* (Figure 2.11B) is absent in *D. simulans* [35]. The GAGAA satellite is also drastically different in *D. simulans*, being eight-fold less abundant and found only on the sex chromosomes [13,35]. To determine if this interspecific difference in satellite content reflects divergent localization of Lhr orthologs, we examined *D. simulans* ovaries expressing a previously characterized sim-Lhr-HA transgene [8]. While Lhr-HA is juxtaposed to dodeca in both species, as previously described [8], the strongest foci in *D. simulans* do not overlap with GAGAA (Figure 2.13A). These results demonstrate that Lhr has evolved distinct localization patterns to at least two satellites between *D. melanogaster* and *D. simulans*.

We next examined TE expression and discovered a broad spectrum of TEs derepressed in *D. simulans Lhr*^{1}, with 80 TEs showing a greater than two-fold up-regulation (Figure 2.13B). Upregulated TEs again include the telomeric transposable elements *HeT-A, TART,* and *TAHRE,* other germline elements such as *Nomad,* and somatic TEs such as *Zam* and *Gypsy 5*. 53 transposable elements were commonly mis-regulated in both *D. melanogaster* and *D. simulans,* showing that the function of *Lhr* in repressing TEs is broadly conserved between species (Figure 2.13C). However, the fold increases of most individual TE families are lower than seen in *D. melanogaster Lhr*^{KO}. For example, *HeT-A* is 352 fold upregulated in *Lhr*^{KO} but only

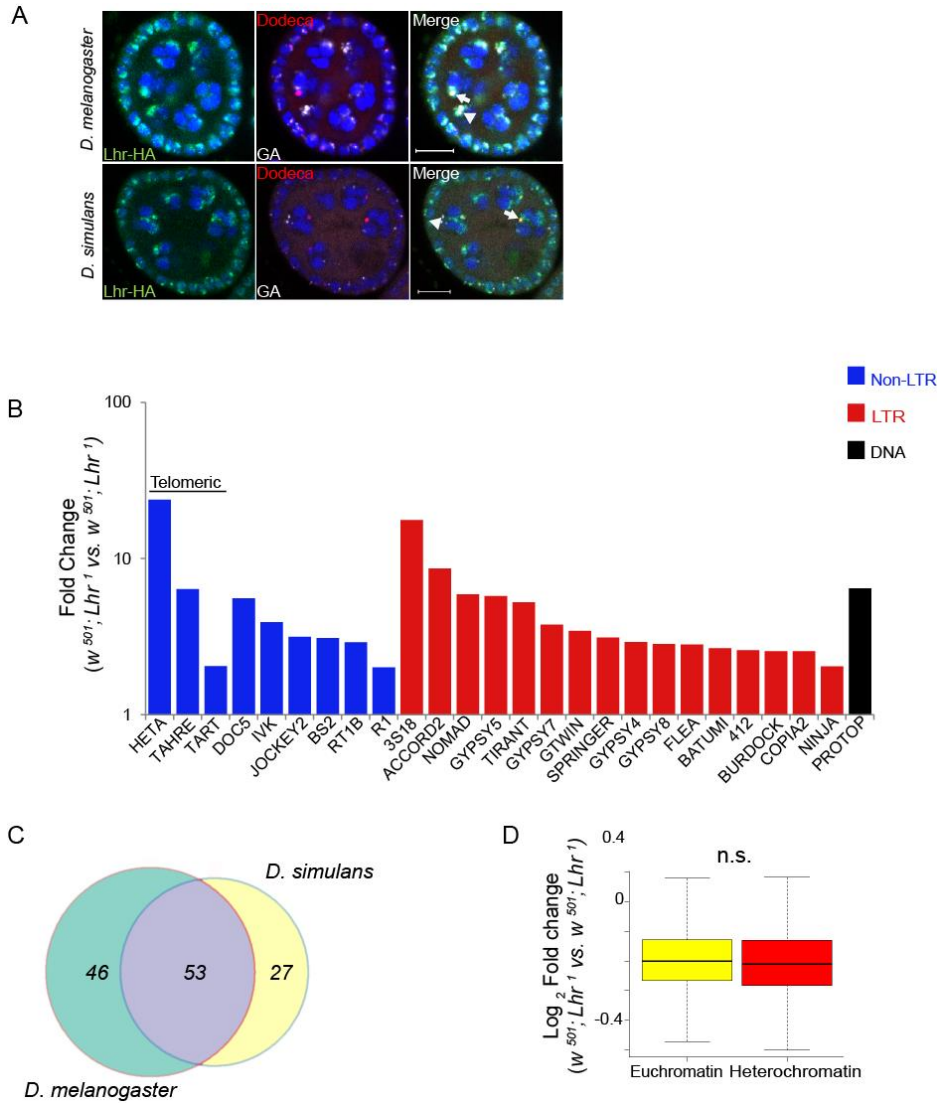


Figure 2.13 Analysis of *Lhr* function in *D. simulans* **(A)** Immuno-FISH experiment shows that the brightest mel-Lhr foci colocalize with dodeca (red, arrow) and GA satellites (white, arrowhead) in *D. melanogaster* (upper panel). The brightest sim-Lhr foci either colocalize or are juxtaposed with dodeca (arrow) but are not associated with GA-rich satellites (arrowhead). All panels contain DAPI shown in blue. Scale bar = 10μm. **(B)** Fold changes in TE expression between w^{501}; Lhr^{1} and w^{501}; Lhr^{+} were calculated for uniquely mapping reads with zero mismatches to the individual-insertion database and with three mismatches to the consensus-sequence database. Three mismatches are required to account for the divergence of TE insertions in *D. simulans* from the consensus sequences, which are largely defined from *D. melanogaster* TEs. The 25 most significantly derepressed TE families in the individual-insertion sequence based analysis are shown here (excluding *centroids*), as well as *TAHRE*, which is found only in the consensus-sequence database. Classification of DNA, LTR and non-LTR elements is from reference [97]. **(C)** Comparison of TE misregulation between *D. melanogaster* and *D. simulans Lhr* mutations.

23.8 fold upregulated in *Lhr*^{1}. We further discovered that *Lhr* loss in *D. simulans* does not significantly affect the expression of heterochromatic genes (Figure 2.13D), in contrast with our similar analysis of *Lhr*^{KO} in *D. melanogaster* (Figure 2.8A). This result suggests that pericentric genes in *D. melanogaster* are more sensitive to changes in heterochromatin state than in *D. simulans.*

Overall, our results demonstrate that *Lhr* function correlates with the increased repeat content and larger amount of heterochromatin found in *D. melanogaster*.

Comparison of *Hmr* ortholog function

To examine the functional consequences of *Hmr* divergence, we took an alternative approach of transforming *sim-Hmr* transgenes into *D. melanogaster*. We found that sim-Hmr-HA, like mel-Hmr-HA, localizes to heterochromatin in *D. melanogaster* (Figure 2.14A). To examine potential differences in TE and satellite regulation, we used parallel *mel-Hmr-FLAG* and *sim-Hmr-FLAG* transgenes, crossed them into an *Hmr*$^{-}$ background (*Df(1)Hmr*$^{-}$*/Hmr*^{3}), and performed RNA-Seq on ovarian mRNA. Our expectation was that divergence of *Hmr* between the orthologs might manifest as the failure of *sim-Hmr-FLAG* to complement the derepression of TEs in *Hmr*$^{-}$As a control for the function of the transgenes, we compared the heterozygous wild type *Hmr*$^{-}$/*Hmr*$^{+}$ to *Hmr*$^{-}$*; ø{mel-Hmr-FLAG}/*+, as each genotype has one wild type copy of

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The diagram includes all TE families that were upregulated at least two fold, including those in individual-insertion database analysis as well as those that are only represented in the consensus-sequence database analysis. (D) Comparison of euchromatic and heterochromatic gene expression in *D. simulans w*^{501}*; Lhr*^{1}, as described in Figure 2.4. The euchromatin-heterochromatin border has not been experimentally determined in *D. simulans* and was defined from *D. melanogaster,* Analysis includes 7479 euchromatic and 350 heterochromatic genes (p = 0.12, Wilcoxon rank sum test with continuity correction).

Hmr^{+}*.* The majority of the upregulated TEs in *Hmr*$^{-}$ (Figure 2.14B) are suppressed by the *mel-Hmr-FLAG* transgene; however, 9 out of 182 families ranged from 2 to 9 times more highly expressed in *Hmr*$^{-}$*; ø{mel-Hmr-FLAG}/*+ than *Hmr*$^{-}$/*Hmr*$^{+}$ (Figure 2.14B). This result suggests that *mel-Hmr-FLAG* does not fully complement the *Hmr* mutant phenotype, which may reflect its decreased expression compared to a wild type allele (Figure 2.3), though it is also possible that some differences may result from TE polymorphisms that remain between the strains. qRT-PCR also demonstrated that *sim-Hmr-FLAG* expresses in *D. melanogaster* at ~3x the level of *mel-Hmr-FLAG* (Figure 2.3), a difference previously seen with *Lhr* transgenes [8]. Because *Hmr* is a negative regulator of TE expression, we suggest that this expression difference will not bias against our goal of identifying TEs that are not fully repressed by *sim-Hmr-FLAG*.

We did not find any difference in satellite DNA expression; however, we found 11 TE families that are differentially expressed between the transgenic genotypes (Figure 2.14C). Five are more highly expressed in *Hmr*$^{-}$*; ø{mel-Hmr-FLAG}/*+ with fold changes ranging from 2-3, of which 3 are incompletely repressed by *mel-Hmr-FLAG* in the control cross described above (*Transpac*, *Tirant*, and *Batumi*). The differential expression of these 5 families likely reflects the inability of *mel-Hmr-FLAG* to fully complement *Hmr*$^{-}$ and the higher expression level of *sim-Hmr-FLAG.*

More intriguing are 6 TE families that are 2-6x more highly expressed in *Hmr*$^{-}$*; ø{sim-Hmr-FLAG}/*+ than in *Hmr*$^{-}$*; ø{mel-Hmr-FLAG}/*+, implying that *sim-Hmr-FLAG* is unable to fully complement the derepression of these elements. *BS* and *Doc6* (also known as *Juan*) elements are present at a mean frequency of about 0.1 in a population of Portuguese *D. melanogaster* [44] and have low pairwise identity in the reference genome [45], suggesting that they are likely active. The mean population frequencies of 4 of the other families (*BS3*, *Circe*, *Helena*, and *FW2*) are near 1, suggesting that these TEs are fixed and therefore currently inactive in

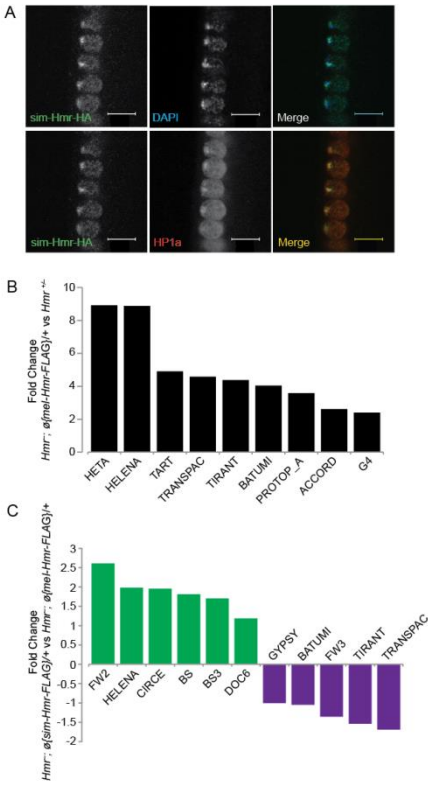

Figure 2.14 *Hmr* orthologs have diverged in their effects on a small subset of TEs. **(A)** sim-Hmr-HA colocalizes with HP1a (red) in nuclear cycle 14 *D. melanogaster Hmr*^{3}; *sim-Hmr-HA* embryos. The *sim-Hmr-HA* transgene was transformed into *D. melanogaster* at the identical *attP2* site used for *mel-Hmr-HA* above (Figure 2.1). DAPI is shown in blue. **(B)** *mel-Hmr-FLAG* does not fully complement TE derepression in *Hmr*$^{-}$. 9 TE families are 2-9x more highly expressed in *Hmr*$^{-}$; *ø{mel-Hmr-FLAG}/+* compared to *Hmr*$^{+/-}$. **(C)** Comparison of TE expression in *Hmr*$^{-}$*; ø{mel-Hmr-FLAG}/+* and *Hmr*$^{-}$*; ø{sim-Hmr-FLAG}/+*. For **B** and **C**, reads were mapped to the individual-insertion database. TEs are considered differentially expressed in the pairwise comparisons if there was at least a 2x fold change and $p<0.001$.

D. melanogaster. *Helena,* though, appears to have been active more recently within *D. simulans [46]*. We suggest that *BS, Doc6* and *Helena* are candidates for future investigation of co-evolution with *Hmr* in either *D. melanogaster* or *D. simulans*.

Transposable elements are upregulated in hybrids

In light of our discovery that *Lhr* and *Hmr* are required for TE repression within *D. melanogaster* and *D. simulans*, we investigated TE activity in lethal (Hmr^+) hybrid male larvae. Because most TEs have different expression levels between *D. melanogaster* and *D. simulans*, we defined mis-regulated TEs as being at least two-fold higher than both parental species, as done in a previous analysis [47]. We found that 42 LTR and non-LTR elements are significantly upregulated in lethal (Hmr^+) hybrid male larvae with 2 others being downregulated (Figure 2.15A).

We next examined whether TE misregulation correlates with hybrid lethality by comparing the lethal Hmr^+ hybrid males to viable Hmr^- hybrid males (Figure 2.15B). The expression of 29 TEs is significantly lower in Hmr^- hybrids. Because *Hmr* functions as a repressor of TEs in *D. melanogaster* male larvae (Figure 2.6C), these differences may reflect a general difference between lethal and viable hybrids rather than the presence or absence of *Hmr* activity. In fact, only 4 of the 29 TEs downregulated in Hmr^- hybrid male larvae are upregulated in Hmr^- *D. melanogaster* male larvae.

In addition, we found modest increases (2-4 fold) in the activity of 5 TE families in living hybrids. None of these are significantly upregulated in Hmr^- *D. melanogaster* male larvae. They

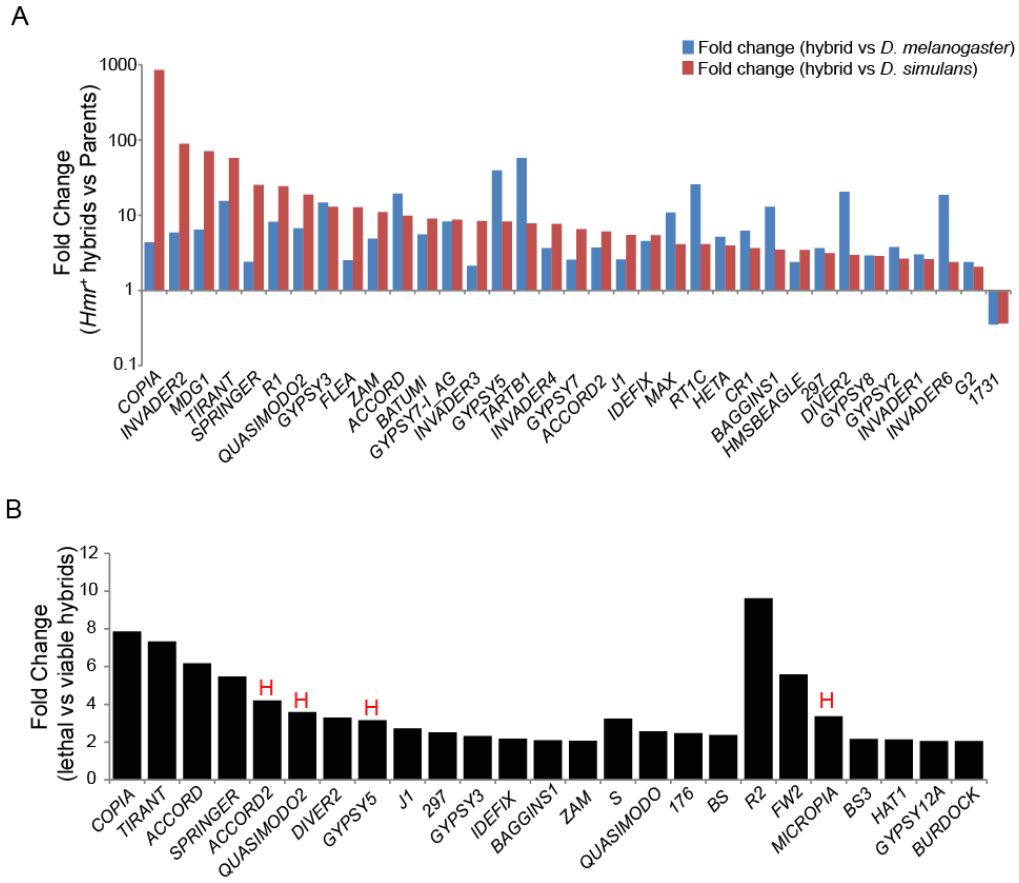

Figure 2.15 TE misregulation in hybrid males (A) Fold change of TEs up- or downregulated ≥2-fold in *Hmr*$^{+}$ hybrid male larvae relative to both *D. melanogaster* and *D. simulans* male larvae. Uncharacterized *centroids* are not shown. **(B)** Fold change of TEs with significantly higher expression in lethal *Hmr*$^{+}$ versus viable *Hmr*$^{–}$ hybrid male larvae. "H" indicates TEs that are significantly upregulated in *Hmr*$^{–}$ *D. melanogaster* male larvae compared to *Hmr*$^{+}$ *D. melanogaster* male larvae from Figure 2.3D. Note the different Y axis scales between panels A and B. TE families include those resulting from mapping reads to the individual-insertion database, as well as families found only when reads were mapped to the consensus-sequence database. Reads unique to each TE class were mapped allowing for up to 3 mismatches.

include *TAHRE* and may reflect higher levels of cell proliferation in viable hybrids. Taken together our results suggest that TE overexpression is unlikely to be causing hybrid lethality.

Discussion

Lhr and Hmr interact with HP1a

We and others previously reported that Lhr (also known as HP3) interacts with HP1a [6,9,12,30]. Here we report that Hmr also interacts with Lhr, and both are present in a complex together with HP1a. Consistent with this interaction, many of the roles we report here for Lhr and Hmr have been described for HP1a, including localizing to heterochromatin, regulating TE and pericentric gene expression, and controlling telomere length [48-50]. However, unlike mutations in *Su(var)205* which enodes HP1a [51], mutations in *Hmr* and *Lhr* are viable. Furthermore, Hmr and Lhr do not localize to the 359 bp satellite which forms a substantial fraction of X-linked pericentric heterochromatin [Figure 1;8]. These findings suggest that Hmr and Lhr are not ubiquitous heterochromatin proteins, leaving open the intriguing question of what guides their localization specificity. The interaction of Hmr and Lhr with HP1a, as well as their effects on TEs in somatic cells, have recently been independently reported [52].

Rapidly evolving heterochromatin proteins and repetitive DNA variation

Several HIs involve heterochromatin proteins or heterochromatic sequences, leading to the suggestion that genetic conflicts between selfish DNAs and host fitness are an important force that is driving the evolution of HI [1,2,23,53].

TE and satellite abundance varies widely among species and is a major contributor to genome-size variation. The evolutionary causes of this variation have been widely debated for many years [54]. When considering genetic conflict theories, it is important to first exclude alternative evolutionary causes of repetitive DNA variation. One explanation is neutrality, with repeat variation governed by mutational processes, in particular the balance between insertions and deletions [55]. Insertion/deletion models are particularly appropriate for inactive and degenerate TEs, and perhaps also for certain classes of satellites that are no longer homogenized by concerted evolution [56].

Selectionist models fit better for active repeats, and must be invoked if the adaptive evolution of heterochromatin proteins is proposed to reflect co-evolution with repetitive DNA. One model is that some repeats are co-opted for host functions. Drosophila's telomeric retrotransposons are a relevant example that is discussed below. We also consider three, non-mutually exclusive selective costs associated with repetitive DNA when discussing the evolution of *Hmr* and *Lhr*

One potential cost arises from the overall load of repetitive DNAs, including increased genome size and instability. A second is direct genetic conflict. We define genetic conflict here to refer to fitness costs imposed by selfish DNAs that have evolved specific mechanisms to increase their transmission [57]. Such conflicts could be caused by highly active individual repeats, for example during hybrid dysgenesis caused by introduction of a TE family into naive strains [58]. Finally, genetic conflicts can have indirect costs, such as pleiotropic fertility defects caused by repeat expansions involved in meiotic drive [59].

Hmr and *Lhr* repress transposable elements

TEs define selfish DNA [54]. They infect most genomes, can self-mobilize and increase their copy number, and destabilize genomes via spontaneous mutations, ectopic recombination, and deleterious increases in genome size [60,61]. Adaptive evolution of TE-defense genes can therefore be readily interpreted as the host species responding to the fitness cost of TEs [19].

Like *Hmr* and *Lhr*, many piRNA pathway genes are also evolving under positive selection [22]. This raises the possibility that *Lhr* and *Hmr* are co-evolving with the piRNA pathway proteins. However, the lack of major perturbations in the piRNA pool in Lhr^{KO} suggests that Lhr and Hmr function downstream or independently of piRNA biogenesis. Piwi, guided by piRNA, has been proposed to recruit repressive heterochromatin components including HP1a and histone methyl transferases to transposable elements [50,62]. One possibility is that Lhr and Hmr function downstream of Hp1a to repress TEs via RNA degradation machinery such as the nuclear exosome [63].

We note that *Ago3* is moderately down-regulated in both Lhr^{KO} (3.4 fold) and Hmr^{-} (~2 fold), likely because the gene is peri-centromeric. Two results demonstrate that this modest reduction in *Ago3* cannot explain the broad effects on TEs in *Hmr* and *Lhr* mutants. First, *Ago3* expression is unaffected in *D. simulans* Lhr^{1}, which also shows widespread TE derepression. Second, *Ago3* mutants have major disturbances to their piRNA pool [38], which we did not observe in Lhr^{KO} (Figure 2.12).

Hmr and *Lhr* regulate telomeres

While TE repression is typically viewed in terms of genetic conflicts, the relationship between *Lhr*, *Hmr* and the telomeric TEs resembles symbiosis. These TEs have been

domesticated by Drosophila species for tens of millions of years to serve a vital host function, and thus are not considered selfish DNA [33,64]. The telomeric TEs were among the most strongly derepressed in *Hmr* and *Lhr* mutants, in some cases more than 100 fold. We also observed increases in *HeT-A* DNA copy number in *Hmr* and *Lhr* stocks. Increased telomeric TE expression does not necessarily increase *HeT-A* DNA copy number and cause longer telomeres, suggesting that multiple factors control telomere length [65]. If so, then *Lhr* and *Hmr* must control multiple processes at the telomere. This is supported by the localization of both proteins to the telomere cap, a protective structure that prevents telomere fusions [28]. The strong reduction in Lhr^{KO} of piRNAs from three TAS-repeat containing sub-telomeric piRNA clusters is particularly intriguing. piRNA production from clusters is dependent on them maintaining a heterochromatic state [66], which could explain why *Lhr* is required for TAS piRNA expression while it acts as a repressor in most other circumstances.

Hmr and *Lhr* regulate species-specific satellite DNAs

We discovered several striking examples that suggest species-specific co-evolution of *Hmr* and *Lhr* with satellite DNAs. We found that *D. melanogaster* Hmr and Lhr proteins localize to and repress transcripts from GA-rich satellites. GA-rich satellites are ~8 fold less abundant in *D. simulans* [13] but are cytologically detectable; nevertheless we find that sim-Lhr does not localize to them. GA-rich satellites also have low abundance in the outgroup species *D. erecta* [13], implying that the differential abundance with *D. simulans* reflects an increase in *D. melanogaster*. Similarly we discovered that mel-Lhr-HA localizes to AACAC in *D. melanogaster*, a repeat that is absent in *D. simulans* [67]. Furthermore, we detected moderate up-regulation of several other satellite transcripts only in *D. melanogaster*. Our results suggest that *Lhr* and *Hmr* may have evolved in *D. melanogaster* to mitigate the deleterious consequences of satellite

expansion, which can include ectopic recombination, increased genome size, and destabilized chromosome segregation [16,68].

Satellite transcripts have been reported from various tissues in wild type *D. melanogaster* [69,70] but little is known about their production. They could be products of either non-specific transcription or read-through from adjacent TEs. Increased levels of satellite transcripts are observed in *D. melanogaster spn-E* mutants, suggesting that RNA interference or piRNA pathways control satellite transcript levels [21].

Is the adaptive evolution of *Hmr* and *Lhr* driven by diverging heterochromatic repeats?

We find that at a broad scale, *Lhr* and *Hmr* from both *D. melanogaster* and *D. simulans* regulate heterochromatic repetitive DNAs but very few genes. This finding is consistent with previous analyses demonstrating that some functions of these genes are conserved between species [4,7-9]. But many of the repeats regulated by *Lhr* and *Hmr* are rapidly evolving, raising the question of whether specific repetitive DNAs are directly driving the adaptive evolution of the *Lhr* and *Hmr* coding sequences between species. A simple prediction is that *D. simulans* orthologs should fail to fully repress such repeats when placed into *D. melanogaster*, a prediction that we tested for *Hmr*.

The *BS* non-LTR retrotransposon is significantly derepressed in *D. melanogaster* Hmr^{-} and Lhr^{KO}, and in *D. simulans* Lhr^{1} mutants. Interestingly, *BS* appears to be transpositionally active in *D. melanogaster* but inactive in *D. simulans* [71]. One interpretation is that *BS* was active in the common ancestor and regulated by *Hmr* and *Lhr*. The genes would continue to co-evolve with *BS* in *D. melanogaster*, making the *sim-Hmr* ortholog less effective at repressing *BS* elements in *D. melanogaster*. In this scenario *Hmr* and *Lhr* are engaged in a recurrent genetic

conflict with *BS* elements that leads to their sequence divergence. Consistent with this prediction we found significantly higher expression in *Hmr⁻; ø{sim-Hmr-FLAG}*/+ compared to *Hmr⁻; ø{mel-Hmr-FLAG}*/+.

Copia shows a different pattern, with ~20-fold up-regulation in Lhr^{KO} but only ~2-fold in Lhr^{1} (and only when mapping to the consensus-sequence database), as well as significant derepression in *Hmr⁻*. *Copia* expression level can be high in *D. melanogaster* but is variable among populations. In contrast, *copia* elements in *D. simulans* typically contain deletions in regulatory elements required for expression, and transcripts are undetectable by Northern blot analysis [72]. These results suggest that *Hmr* and *Lhr* could be *D. melanogaster* host factors that defend against a TE that is currently active within the species. However, *copia* was fully repressed in *Hmr⁻; ø{sim-Hmr-FLAG}*/+, demonstrating that adaptive divergence of *Hmr* by itself does not affect *copia* regulation.

Overall, we found surprisingly few cases of overexpression associated with *Hmr* divergence, including no effects on satellite DNAs (Figure 2.9). We also note that most of the TEs identified other than *BS* and *Doc6* are likely transpositionally inactive in *D. melanogaster* [44], which makes it more challenging to fit a scenario of direct and recurrent evolution between *Hmr* and specific TEs.

We suggest several possible interpretations of these results. One is that *Hmr* and *Lhr* adaptive divergence is in fact driven largely or solely by *BS* and/or *Doc6*, a hypothesis that will require understanding the mechanism by which *Hmr* and *Lhr* affect expression of these TEs. Second is that *Hmr* and *Lhr* may be co-evolving with other genes, and that multiple diverged genes need to be replaced simultaneously in order to detect their effects on other TEs and satellite DNAs. Third is that more sensitive assays are needed, for example monitoring TE transposition rates over multiple generations. A fourth possibility is an alternative to genetic

conflict scenarios that arises from population-genetic models. These models suggest that the fitness costs of individual TE families are likely extremely weak under most circumstances. The adaptive evolution of repressor proteins may therefore reflect the cumulative load of repeats within a genome [22]. This alternative view could be applicable to *Hmr* and *Lhr* since they repress a large number of TEs and satellites. Finally, *Hmr* and *Lhr* may have additional unidentified phenotypes that are also the targets of adaptive evolution.

Repeat load, adaptation and hybrid incompatibilities

D. simulans has a smaller genome with ~4-fold less satellite DNA [13,14] and significantly fewer TEs [24,73] compared to *D. melanogaster*. This large difference in repeat content between *D. melanogaster* and *D. simulans* may have wider consequences. We found reduced expression from pericentric heterochromatin genes in *Hmr* and *Lhr* mutants in *D. melanogaster*. This reduction may reflect the fact that pericentric genes have evolved to use heterochromatin proteins such as Lhr and Hmr to maintain gene expression in a repeat-rich environment [74]. Pericentric genes in species with fewer repeats would presumably not require these proteins. Consistent with this model, we found that *Lhr* loss in *D. simulans* has a negligible impact on pericentric gene expression. This finding suggests that *Lhr* and *Hmr* have an adaptive role in blocking effects on gene expression arising from increasing repetitive DNA copy number.

If each genome is uniquely adapted to its repetitive DNA content, then the shock of hybridization may lead to misregulation of TEs and satellites. TEs are activated in various animal and plant hybrids but the consequences, if any, for hybrid fitness are largely unclear [75]. We found substantial TE misregulation in hybrid male larvae (Figure 2.15A). Since these hybrids are agametic [76], this TE expression comes from somatic tissues. The fitness cost of

this upregulation is unclear as somatic TE overexpression is not necessarily lethal within *D. melanogaster* [77,78]. Comparison of lethal *Hmr*$^{+}$ and viable *Hmr*$^{–}$ hybrid males demonstrates that lethal hybrids have more TE expression (Figure 2.15B) than the viable hybrids, which in turn have more TE expression than either of its parents. However, this TE misregulation seems unconnected with *Hmr* as the TEs differentially expressed between *Hmr*$^{+}$ and *Hmr*$^{–}$ hybrid male larvae are largely distinct from those between *Hmr*$^{+}$ and *Hmr*$^{–}$ *D. melanogaster* male larvae. Further, while *Hmr*$^{–}$ causes rampant TE over-expression within *D. melanogaster*, it is associated with reduced TE levels in hybrids. These observations argue that the TE derepression in hybrids is unrelated to the pure species function of *Hmr*. This finding is consistent with previous genetic studies that demonstrate that the wild type *Hmr*$^{+}$ allele causes hybrid lethality and thus behaves as a gain-of-function allele in hybrids [79,80]. More generally it underscores the unique nature of the hybrid genetic background [1]. Somatic TE overexpression may result from breakdown in the siRNA or piRNA pathways due to incompatibilities among multiple rapidly evolving TE regulators.

One clear example is known where a species-specific difference in a satellite DNA causes incompatibility between Drosophila species [81]. But the toll caused by heterochromatic differences may more commonly be indirect, as heterochromatin proteins diverge in response to changes in heterochromatic DNA repeats. Recent work suggests that hybrid female sterility may be caused by incompatibilities among rapidly evolving piRNA proteins rather than by species-specific differences in TEs [47]. We suggest that the role of *Hmr* and *Lhr* in regulating the activity of three highly dynamic classes of heterochromatin has led to their recurrent adaptive evolution, and secondarily, to their involvement in interspecific hybrid lethality.

Materials and methods

Construction of the *Lhr^{KO}* mutant

We used the pW25 donor vector and ends-out homologous recombination method to make an *Lhr* mutant allele [82]. The donor vector was designed to recombine a *w*$^{+}$ marker into *Lhr* and simultaneously remove 26 bp of the coding region. iProof (Biorad) was used to PCR amplify two genomic fragments from *y; cn bw sp* (*D. melanogaster*) genomic DNA. The 3768bp *Lhr* upstream fragment, including 128bp of the coding region of *Lhr,* was amplified with primers LUF-Fwd: 5'- ttggcgcgccAACAGGGTCGGCTGTCACATTT and LUF-Rev: 5'- ttggcgcgccGCGAGCATCTCCATGAGCAG (Tm=63°C) and cloned into the *Asc*I site of pW25 using the underlined sequences. The 3935bp *Lhr* downstream fragment that includes 806bp of the *Lhr* coding region was amplified with primers LDF-Fwd: 5'- AAGCGGCCGCAGGTGGAGCCCAAAATGGACG and LDF-Rev: 5'- AAGCGGCCGCCACACATTGCGAATGCA G AAA (Tm=65°C) and cloned into the *Not*I site using the underlined sequences. Restriction digestion was used to pick a clone in which the 2 inserts and the *mini-white* gene were in the same orientation.

The construct was injected into a strain of *w^{1118}* (Genetic Services) and a transgenic line, *P{w*$^{+}$*, Lhr-KO}5-1*, with a lethal insertion on the X chromosome was obtained. *P{w*$^{+}$*, Lhr-KO}5-1/FM6* females were crossed to *y w; P{ry*$^{+}$*, hs-flpase}, P{v*$^{+}$ *hs-I-Sce} /TM6, Ubx* males. Two to three day-old larvae were heat shocked and *P{w*$^{+}$*, Lhr-KO}5-1/y w P{ry*$^{+}$*, hs-flpase}, P{v*$^{+}$ *hs-I-Sce}*/+ female progeny were crossed to *w^{1118}* males. Rare *w* $^{+}$ sons were screened for homologous recombination events by PCR. Primer pairs *Lhr-f1* 5'-TTCGCACGTTGTGTTCAAGTAA-3', / Lhr-r1 5'-GTAGCTTTCTCTTGGCGCTCTT-3' and Lhr-f2 5'- AACGTGCTCGTAGCTTTGGT-3'/, Lhr-r2 5'-TCGCGAAAATACTTCCGTCT-3' (Tm=58°C) produce no amplicons in the presence of the *white* insertion. Attempts to remove the *w*$^{+}$ marker

by *Cre* recombination were unsuccessful and the w^+-disrupted *Lhr* locus was designated as Lhr^{KO}.

To test the genetic effects of this mutation, we took advantage of a recent observation that a deficiency chromosome which deletes *D. melanogaster Lhr* can weakly rescue *D. melanogaster-D. mauritiana* hybrid males to the pharate adult stage [8]. When we crossed Lhr^{KO} homozygous females to *D. mauritiana* males at 18°, we obtained 10.6% rescue of live males (17 males and 161 females). The stronger rescue observed here may be due to the fact that the mothers of the cross were homozygous for the Lhr^{KO} allele, since *Lhr* likely has strong maternal expression based on its high protein abundance in early embryos [8].

Hmr transgenes

A *D. melanogaster Hmr-FLAG* transgene was made by inserting a 3X FLAG tag sequence [83] immediately upstream of the stop codon of *Hmr* using fusion PCR into plasmid p72, which is a pCaSpeR2 vector containing a ~9.7kb fragment of the *Hmr* region [3]. Two *Hmr* fragments (L-arm and R-arm) were amplified from p72 with iProof polymerase by using primer pairs 739/738 and 736/740, respectively. The primers 738 and 736 contain sequence encoding the FLAG tag and partially overlap to allow fusion in the subsequent stage. The primers 739 and 740 were combined with L-arm and R-arm products to produce a fused partial fragment of *Hmr* containing the 3X FLAG sequence. This fragment was cloned into the pCR-BluntII-Topo vector (Invitrogen) and sequenced completely between the *Avr*II and *Kpn*I restriction sites. The *Avr*II/*Kpn*I fragment was then cloned into the corresponding sites of the p72 plasmid. A 300 bp fragment containing the *attB* site was then PCR amplified from plasmid *pTA-attB* (gift from Dr. Michele Calos) using primers 502 and 503 and cloned into the *Not*I site. This fragment was

digested with *Not*I (on the ends of 502 and 503), gel purified, and inserted into the *Not*I site of the plasmid containing *Hmr-FLAG*. We refer to this transgene as *mel-Hmr-FLAG*.

A *D. melanogaster Hmr-HA* transgene was made by inserting a 3XHA epitope tag between codons 466 and 467 of *Hmr*. Primers 215/1246 and 1247/495 were used to amplify 573 and 316 bp fragments, respectively. Primers 1246 and 1247 overlap and encode the HA tag. Fusion PCR containing these 2 products and primers 215/495 was performed. The PCR product was cloned into pCR-Blunt II-TOPO, and the insert was checked by sequencing. The insert was then cloned using *Spe*I and *BsiW*I back into a modified p72 containing an *attB* site inserted into the *Not*I site. The orientation and presence of the HA tag were checked by double digests and PCR. We refer to this transgene as *mel-Hmr-HA*.

A *D. simulans Hmr-FLAG* transgene was made by inserting the 3X FLAG tag sequence upstream of the stop codon in p89, a pBluescript II KS(+) plasmid containing the *D. simulans Hmr* insert that was used for the p92 transformation construct in [7]. Primers 751/753 and 750/752 were used to amplify 1.3kb and 1.8kb fragments of the insert, respectively, which were then joined by fusion PCR using primers 750/751. The fusion PCR product was cloned into pCR-Blunt II-TOPO and confirmed by sequencing. The insert was designed to have an *Hpa*I site near one end and a *Not*I site near the other. The *Not*I site was destroyed during cloning; however, the pCR-Blunt II-TOPO vector contains a *Not*I site within 40bp of the destroyed sequence. The insert was then cloned back into p89 using *Hpa*I and *Not*I. The orientation of the insert, as well as the addition of the FLAG tag, was checked by double digest with *Cla*I and *Hpa*I. The *D. simulans Hmr-FLAG* insert was then removed as a *Sac*II fragment. Klenow enzyme was used to fill-in the ends to allow cloning into the *Stu*I site of pCaSpeR2 containing an *attB* site inserted at its *Not*I site. We refer to this transgene as *sim-Hmr-FLAG.*

The *D. simulans Hmr-HA* transgene was made from plasmid p89 by inserting the HA tag at the region orthologous to *mel-Hmr-HA* [7]. Primers 135/1365 and 1247/1364 were used to amplify 861 bp and 827 bp fragments, respectively, from the p89 template, and were fused together using primers 1364/135. The fusion PCR product was then cloned into pCR-Blunt II-TOPO and the entire insert was checked by sequencing. The insert was then cloned back into p89 using *Spe*I and *Blp*I. Blunt end ligation, used for *sim-Hmr-FLAG* above, proved inefficient for transferring the insert into the transformation vector. Therefore an *Xba*I site was added to the 3' end of *Hmr*-HA by amplifying the entire insert using primers 1402/1403. The PCR product was then gel purified and cloned back into pCR-Blunt II-TOPO. The polylinker contains an *Xba*I site 5' to the insert, allowing us to clone the entire insert into the *Xba*I site of pCaSpeR2 containing an *attB* site inserted at its *Not*I site. We refer to this transgene as *sim-Hmr-HA*.

Oligonucleotides for *Hmr* transgenes (all written 5'-3'). 739: AGCCAAATTGCCGACAGTAGCCAAG; 738: ATCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCAGGCGGTGGCGGATTGACCTTG; 736: GACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTAGCTCTCGAAACTTTTGGCACACGTAG; 740: TTGTACTGCCATTAGGTATAGCTAACCATCC; 502: AAACCCGCGGCCGCATGCCCGCCGTGACCGTC; 503: AAACCCGCGGCCGCGATGTAGGTCACGGTCTCG; 152: TCTTCTTAGACTGCGGGTTG; 215: CAGCGCATGCGCGGCACCGTAT; 1246: ATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTACATTGCACTGTTGGTCATGCTCGT; 1247: TCCCTATGACGTCCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGATTAC;GCTAGCACTGCCACAAGCATTGG; 495: GACACGCCCGTTCCCATAGT; 751: ACAGCGATTTGCGCAAGCCG; 753:

TCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCAGGCGGTGGCGGATTTG CCTTCTTGGCGTATTTAGA; 750: GTGAATTGTAATACGACTCACTATAGGGCG; 752: GACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTAGCTCTCGA ATCATTGGCACACG; 135: GAGGAGGACCCCACCTATAACTAC; 1365: ATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTATGCACT GTTAGAAATGCTTGTGCTG; 1364: GCTGGCAATTTGGACTTTGT; 1402: GCGGGCGGTCATTATTAA; 1403: TATCTAGAGCGGCCGCGAGCTCTAATA.

Transgenic Fly Lines

φC31-mediated transgenesis was performed by Genetic Services using the *P{CaryP}attP2* integration site at cytological position 68A4 [84]. Site specificity of integration was checked by PCR assays described in references [8,85]. *D. melanogaster* transformants were crossed to a *y w* strain. Wild type activity of the *Hmr-HA* transgene was tested for complementation of an *Hmr* rescue mutation in hybrids as done previously for Hmr^{+} transgenes [3,7]. Here we crossed *Df(1)*Hmr^{-}*/FM6; ø{mel-Hmr-HA}* females to *D. simulans* w^{501} males. We recovered 193 w^{501}*/Y;* +/+ hybrid males but only 1 w^{501}*/Y; ø{mel-Hmr-HA}*/+ hybrid male, demonstrating that the transgene is Hmr^{+}.

Drosophila strains

Lhr^{KO} was outcrossed to w^{1118} for six generations. Sibling crosses were then used to generate a homozygous w^{1118}; Lhr^{KO}/Lhr^{KO} (abbreviated as Lhr^{KO}), a heterozygous Lhr^{KO}/+, and a wildtype w^{1118}; Lhr^{+}/Lhr^{+} line (abbreviated as Lhr^{+}). All experiments with *Lhr* in this paper use these matched mutant and sibling controls unless otherwise specified. The *D. simulans* Lhr^{1}

allele is caused by an insertion in the 5' UTR and appears to make no transcript by RT-PCR [6]. *Lhr*^{1} was outcrossed to the inbred wild-type line *w*^{501} for 3 generations to generate the stock *w*^{501}*; Lhr*^{1} (abbreviated as *Lhr*^{1}) and *w*^{501}*, Lhr*$^{+}$ (abbreviated as *Lhr*$^{+}$). *Lhr-HA* transgenes were described previously [8]. *y w* F10 was created by single-pair matings between siblings for 10 generations.

We refer to the *P{EPgy2}Hmr*^{3} allele that is marked with *y*$^{+}$ and *w*$^{+}$ described in [4] as *Hmr*^{3}. *Df(1)Hmr*$^{-}$*, y w v*, abbreviated as *Df(1)Hmr*$^{-}$, is described in [86]. In order to match backgrounds for the *Hmr* RNA-Seq experiments, the *Hmr*^{3} stock and the transgenic lines (*mel-Hmr-FLAG* and *sim-Hmr-FLAG*) were outcrossed to *y w* F10 for 6 generations and then made homozygous.

Fertility assays

Individual 1-2 day old virgin *Lhr*^{KO} and *Lhr*^{KO}/+ sibling females, obtained from crosses of *Lhr*^{KO}/+ at 27°C, were crossed to two *w*^{1118} males. Flies were transferred to a fresh vial every 5 days for 15 days. Vials in which either the female or both males were missing or dead were not scored or transferred. Total progeny from each remaining vial were counted. To create the heteroallelic siblings *Lhr*^{KO}*/Df(2R)BSC44* and *Lhr*^{KO}*/SM6a*, *Lhr*^{KO}*/Lhr*^{KO} were crossed to the *Lhr*$^{-}$ deletion stock *Df(2R)BSC44/SM6a* [6]. The fertility assay was carried out as above except vials were flipped every 4-5 days.

Hatch rate assays

Lhr^{KO}/+ or *Lhr*^{KO}/*Lhr*^{KO} females were crossed to *w*^{1118} males at 27°C. Egg lays were carried out on grape juice/agar plates for 3 hour periods at either 2-3 days, 5-6 days or 10-11 days after eclosion of the female parents. The plates were maintained at 27°C and monitored over the next 24-36 hours for hatched eggs.

Crosses for generating *Hmr* genotypes for RNA-Seq of ovarian mRNA

y w Hmr^{3}*;* +/+ females were crossed to *y w; ø{mel-Hmr-FLAG}/ø{mel-Hmr-FLAG}* males. F1 males were crossed to *Df(1)Hmr*$^{-}$*/FM6*; +/+ females to generate both *y w Hmr*^{3}*/Df(1)Hmr*$^{-}$; *ø{mel-Hmr-FLAG}/+* and *y w Hmr*^{3}*/Df(1)Hmr*$^{-}$; +/+. Similarly, *y w Hmr*^{3}*;* +/+ females were crossed to *y w*; *ø{sim-Hmr-FLAG}/ø{sim-Hmr-FLAG}* males. F1 males were crossed to *Df(1)Hmr*$^{-}$*/FM6*; +/+ females to generate *y w Hmr*^{3}*/Df(1)Hmr*$^{-}$; *ø{sim-Hmr-FLAG}/+.* Lastly, *y w*; +/+ females were crossed to *y w*; *ø{mel-Hmr-FLAG}/ø{mel-Hmr-FLAG}* males. F1 males were crossed to *Df(1)Hmr*$^{-}$*/FM6*; +/+ females to generate the heterozygous wildtype control, *y w*/*Df(1)Hmr*$^{-}$; +/+. These crosses were done at 27ºC and in triplicate to generate 3 biological replicates.

Crosses for generating pure-species and hybrid samples for RNA-Seq of larvae

The *Df(1)Hmr* $^{-}$*, y w v/FM7i, P{w+ mC =ActGFP }JMR* stock (abbreviated as *Df(1)Hmr*$^{-}$ */FM7i, GFP*) was described previously [86]. A stock with the matching *Hmr*$^{+}$ genotype*, y w v*/*FM7i, P{w+ mC =ActGFP}JMR* (abbreviated as *Hmr*$^{+}$*/FM7i, GFP*) was created by crossing *y w v/Y* males with *Df(1)Hmr*$^{-}$*/FM7i, GFP* females. *FM7i, GFP /Y* males from this *Hmr*$^{+}$ stock were

then crossed to *Df(1)Hmr⁻/FM7i, GFP* females for 10 generations in order to make the autosomal backgrounds comparable between the two stocks.

To generate hybrids, *Df(1)Hmr⁻/FM7i, GFP* or *Hmr⁺/FM7i, GFP* were crossed to *v/Y D. simulans* males. For each cross, 6 replicates were made each containing 25 0-12 hour-old virgin females and 50 4-6 day-old virgin males. Hybrid larval sons not carrying the balancer were selected by their *y⁻* mouth hook and *GFP⁻* body phenotypes. Additionally, some crosses were allowed to develop to ensure that only *Df(1)Hmr⁻* crosses produced hybrid sons. To generate *D. melanogaster* samples, 3 replicates of 10 *Df(1)Hmr⁻/FM7i, GFP* or *Hmr⁺/FM7i, GFP* virgin females were crossed to 15 *FM7i, GFP/Y* males. Larval sons not carrying the balancer were selected by *y⁻* and *GFP⁻* phenotypes. To generate *D. simulans* samples, 3 replicates of 10 *y w D. simulans* virgin females were crossed to 15 *v/Y D. simulans* males. Larval sons were selected by *y⁻*.

Preparation of protein lysates for semi-quantitative Western blots.

50 mg of 1-17 hr embryo collections were dounced 30 times with a tight pestle in 500ul buffer A1 (15mM HEPES, pH=7.5; 15mM NaCl; 60mM 1M KCl; 4mM $MgCl_2$; 0.5% TritonX-100; 0.5mM DDT) and then centrifuged for 5 minutes at 4°C. The pellet was washed with 500µl buffer A1 and centrifuged. This process was repeated another two times. The pellet was lysed by douncing in 200 µl SDS lysis buffer (500µl 10% SDS, 200µl 1M Tris, pH=8.0, 40µl 0.5M EDTA, 100µl 100X protease inhibitor, 10µl 0.5M EGTA, 50µl 100mM PMSF, 9.1ml water). The lysate was allowed to rotate at 4°C for 20 minutes and then centrifuged. The supernatant was removed, quantitated using the Bradford assay and was run on an SDS-PAGE gel.

Anti-Lhr antibodies and Western blots

An Lhr cDNA was cloned into pDEST17 (Invitrogen). The expressed protein from *E. coli* was purified using Ni-Ag beads under denaturing conditions (8M urea), dialyzed down to 2M urea and injected into rabbits (Cocalico). The antisera was then purified by coupling purified His-Lhr to CnBr-activated Sepharose beads in the presence of 1% Triton-X and removing urea by dialysis. Antisera was eluted in 0.2 M glycine, pH2.8 and then neutralized with 1M Tris, pH8.5. The antibody failed to detect Lhr in immunofluorescent experiments but was used for Western blots in Figure 2.S3 at 1:4000 in 5% milk-TBST and HRP conjugated anti-rabbit secondary antibody at 1:2000 dilution. HA-tagged Lhr was detected with 1:1000 dilution of rat anti-HA (Roche, 3F10) and HP1a was detected with a 1:700 dilution of mouse monoclonal supernatant (C1A9, DSHB).

Co-immunoprecipitation

0~16 hour-old embryos were collected, dechorionated and snap frozen in liquid nitrogen. Embryos were then resuspended to 10x embryo volume of Buffer A (10mM Tris-Cl pH 8.0, 300mM sucrose, 3mM $CaCl_2$, 2mM Mg $acetate_2$, 0.1% Triton X-100, 0.5mM DTT, 0.5mM PMSF) and homogenized with a dounce homogenizer. The homogenized lysate was centrifuged at 700g for 10 minutes at 4° to pellet the nuclei. The supernatant was removed, the pelleted washed once in Buffer A, the nuclei centrifuged again and then resuspended in 1x embryo volume of Buffer MN (15mM Tris-Cl pH7.4, 250mM sucrose, 60mM KCl, 1.0mM $CaCl_2$, 0.5mM DTT, 1x protease inhibitor cocktail). The nuclear lysate was sonicated briefly, micrococcal nuclease added to a concentration of 500 units/ml, and the chromatin digested for 1 hour at 4° with gentle agitation. EDTA and Triton X-100 were then added to a concentration of

5mM and 0.1% respectively, to inactivate nuclease activity and solubilize the proteins, followed by incubation at 4° for 1 hour. After a second brief sonication, the digest was centrifuged at 12,000g for 10 min at 4° and the supernatant was collected. 50µl of the chromatin digest was diluted in IP Wash Buffer (50mM Tris-Cl pH7.4, 100mM NaCl, 0.1% Triton X-100) with 1x protease inhibitor cocktail to a final volume of 125µl per co-immunoprecipitation mixture. 15 µl of protein G-conjugated magnetic beads and 2-5µl of antibody were added followed by incubation for 4 hours at 4° with gentle agitation. The beads were washed 3 times in IP Wash Buffer. The immunoprecipitated proteins were then eluted by boiling the beads in 1x Laemmli sample buffer for 5 minutes and analyzed by immunoblotting.

RT-PCR and qRT-PCR assays

RNA extraction, cDNA synthesis and qRT-PCR assays were performed as in reference [8], using 2-5µg of RNA. qRT-PCR experiments included three technical replicates of three separate biological replicates. Primers included: Lhr-f1 5'caccATGAGTACCGACAGCGCCGAGGAA, Lhr-r1 5' ACACTTGGTTTTCGGCACATC CGC, Lhr-f2 5' GTAGCTTTCTCTTGGCGCTCTT, Lhr-r2 5' GTAAGTGAACTGAAGCTGC GTTGG, EDTP-F 5'GCTGGCAGGTGG TTACCGACA, EDTP-R 5'CGTGGCCAGGTTCA TGGATGA, Bap55-F 5' CCGAGAGTC TCTTTGACAATGCA, and Bap55-R 5'GCCTCTT CGTACTCCTGCGA. Hmr-f1 5' TAAGTTCGCCTTCCGCACATACC and Hmr-r1 5' GACCAGAAACCTGAGTTGCTCCA. *HeT-A* and *RpL32* (also known as *Rp49*) transcript levels were measured with primers from reference[87].

qPCR of *HeT-A* DNA copy number

The Invitrogen DNEasy kit was used to make genomic DNA from Lhr^{KO} and Lhr^{+} female carcasses that were free of ovarian tissue. Primers Het-s2 and Het-as2 amplify from the coding sequence of *HeT-A* [88]. *HeT-A* copy number was normalized to *RpL32* (also known as *Rp49*) copy number using primers from reference[87].

RNA-Seq samples

For samples from ovaries, flies were kept at 27°C for several generations prior to and during the experiment. Freshly eclosed females were collected and aged 2-3 days and then transferred to fresh food with yeast paste for another 2-3 days. RNA was extracted, from ovaries dissected in chilled 1X PBS, using Trizol. Ovarian mRNA-Seq libraries were constructed at the Epigenomics Core Facility at Weill Cornell Medical College using the poly(A) enrichment method. Libraries were sequenced using the Illumina HiSeq2000 platform to produce 50bp single reads which were then trimmed for quality and filtered to remove rRNA reads. One biological sample each from Lhr^{KO} and Lhr^{+} was duplexed and run in a single lane. 51,193,832 filtered reads were obtained for Lhr^{+} and 41,688,028 reads for Lhr^{KO}. Three biological replicates each of *D. simulans* w^{501} and Lhr^{1} ovarian mRNA libraries were run on a single lane and the number of filtered reads ranged from 36,472,726 to 43,449,879. For experiments with *Hmr*, two biological replicates were included for each genotype and all 8 samples were multiplexed in a single lane. The number of filtered reads for each sample ranged from 23,863,381 to 27,490,644. For larval samples, around 30 larvae were collected for each genotype and flash frozen in liquid N_2. RNA was extracted from 2 biological replicates of each genotype using Trizol. Larval RNAseq libraries were generated and bar-coded using the TruSeq kit, and run in

one lane of an Illumina HiSeq 2000 100bp yielding 13,707,247 to 20,373,267 filtered reads per sample, except for one library which produced only 7,840,004 reads.

RNA-Seq analysis

Reads mapping to either rRNA or repetitive DNA were filtered out using Bowtie [89] and the filtered reads were mapped to the unmasked *D. melanogaster* genome using Tophat [90]. The BAM file outputs were used by Cuffdiff with the -b option [91]. All *.fasta and *.gtf files were based on the release 5.68 of the *D. melanogaster* genome from ENSEMBL. To find differentially expressed genes in *D. simulans*, we aligned reads to the *D. melanogaster* genome with Tophat, allowing two mis-matches. While this approach could potentially reduce mapping ability for diverged genes, it allowed us to take advantage of the better assembly and annotation of the *D. melanogaster* genome.

To maximize the TEs considered in our analyses, we mapped reads to two different databases using Bowtie. First, reads were uniquely mapped to a database consisting of all the annotated TE insertions in the *D. melanogaster* and *D. simulans* genomes [47]; we refer to this as the individual-insertion database. While this database likely represents most TE families present in our stocks, some TEs may either be absent from the assembled genome or be represented by copies that are sufficiently diverged such that they impact our ability to correctly assess transcript levels. These elements include the telomeric element *TAHRE*, which has only a few insertions in the genome and is known to be absent from the reference genome since only two telomeres are included in the assembly [92]. Therefore we also mapped reads, allowing for either 0 mismatches when aligning reads from *D. melanogaster* or 3 mismatches when aligning reads from *D. simulans* or hybrids, to a database consisting of the consensus sequences of the

annotated TEs and repeats found in Repbase as well as *de novo* predicted TEs generated by piler-DF using the 12 *Drosophila* genomes [47]; we refer to this as the consensus-sequence database. Only reads that mapped uniquely within the same family were included in the subsequent analyses of differential expression. Mismatches allowed for each alignment are mentioned in figure legends. Statistical significance of differential expression among TEs was calculated with F.E.T. in the DEG-seq package[93].

To analyze reads mapping to satellite DNAs, we built a database using a curated file from the Berkeley Drosophila Genome Project (http://www.fruitfly.org/sequence/sequence_db/na_re.dros) which itself was constructed from GenBank sequences. This file includes some mis-annotated TEs and non-satellite sequences. We counted reads that mapped to these repeats without any mismatches and calculated statistical significance of differential expression among satellites with F.E.T. in the DEG-seq package.

Small RNA sequencing and analysis

Libraries were prepared as described but no oxidation was carried out [38]. Briefly, total RNA was extracted from 5-6 day old Lhr^{KO} and Lhr^{+} ovaries using the mirVANA kit (Invitrogen). Total RNA was size fractionated on a 15% Urea-PAGE gel to enrich for 18-29 nt small RNA, excised and eluted and then subjected to 2S rRNA depletion. This small RNA was ligated to a 3' RNA adapter, gel purified, and then ligated to a 5' DNA adapter. The adapter-ligated small RNAs were reverse transcribed and PCR amplified. The amplified PCR products were gel purified, quantified and sequenced in two lanes of a HISeq 2000 machine.

Only reads with a 3' adapter were kept, which was then removed using a custom script [47]. These reads were binned by size as either miRNA/siRNA (17-22 nt) or piRNA (23-30 nt). rRNA, tRNA and snoRNA sequences were filtered from these reads and the remaining reads were further filtered to keep only those reads that mapped to either the unmasked genome,or the satellite DNA database described above, or Repbase consensus sequences [94]. These filtered reads included 89,953,149 piRNA reads and 40,859,119 siRNA reads in Lhr^{KO}, and 120,143,855 piRNA reads and 36,388,192 siRNA reads in Lhr^{+}.

piRNA reads were mapped uniquely to all *D. melanogaster* sequences from Repbase using Bowtie, allowing for one mismatch. Ping-Pong scores were calculated using reads mapped with up to 1 mismatch, as described in reference [47]. For mapping to piRNA clusters, we built an index using sequences extracted from the Release 5 DM3 genome on the UCSC genome database and GenBank with coordinates of individual piRNA clusters obtained from reference [40]. piRNA reads were uniquely mapped to piRNA clusters with zero mismatches and significance for differential expression was calculated using F.E.T implemented in DEG-seq. siRNA reads were mapped uniquely to all *D. melanogaster* sequences from Repbase with Bowtie, without allowing for any mismatches.

Immuno-fluorescence and Immuno-FISH

Immunofluorescence and FISH were performed on embryos and ovaries as described in references [4,81]. Polytene chromosomes were dissected in 0.7% NaCl, squashed, and fixed in 1.8% PFA, 45% acetic acid for 17 minutes. They were then washed in 1% Triton X in PBS for 10 minutes, then washed in 5% milk in PBS for 1 hour, incubated with primary antibody overnight at 4°C, washed in 5% milk in PBS for 10 minutes, incubated with secondary antibody

for 1 hour at room temperature, and then washed for 10 minutes in buffer A (0.15M NaCl, 0.2% NP40 substitute, 0.2%Tween 20) followed by 10 minutes in buffer B (0.20M NaCl, 0.2% NP40 substitute, 0.2%Tween 20).

Rat anti-HA antibody (Roche, 3F10) was used at 1:100, rat anti-Vasa (DSHB) was used at 1:25, Fibrillarin (Abcam, Ab5281) was used at 1:100, anti-HP1a antibody (C1A9, DSHB) was used at 1:100. Alexa fluorophore-conjugated secondary antibodies were used to detect the primary antibody. Fluorescently labeled probes against GA-rich satellites, AACAC, 2L3L, 359 bp and dodeca were obtained from Sigma with sequences described in references [8,81,95]. Imaging was carried out using a Zeiss 710 confocal microscope at Cornell University's Microscopy and Imaging Facility.

Yeast two-hybrid assays.

A full-length coding-sequence plasmid of *D. melanogaster Hmr* was made by correcting 3 frame-shift errors in the RE54143 cDNA [3]. Two errors in exon 5 were replaced by ligating in a ~1.6 kb *Xba*I-*Hind*III fragment from the LD22117 cDNA, followed by replacement of a 2172 bp *Nde*I-*Zra*I fragment from the p83 genomic clone [3]. The coding sequence was then PCRd out and cloned into pENTR/D-TOPO. The *D. simulans Hmr* CDS was PCRd out of cDNA and cloned into pENTR/D-TOPO. The *Lhr* plasmids and yeast two-hybrid destination vectors and assays are described in reference [6].

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Chapter 3

Lhr: Functions and interactions

Introduction

The most well studied heterochromatin proteins are HP1a and the Histone3-Lysine 9 methylases Su(Var)3-9 and SetDB1. Loss of these proteins leads to an increase in transposable element (TE) transcripts in the female germline [13,60]. In addition, loss of the former two leads to increased TEs in larval stages and in carcasses of adults [61]. HP1a and Su(Var)3-9 may also affect larval development and adult fitness through processes unrelated to TE activity. Hp1a mutants die in larval development, at least in part due to gene expression defects [62,63]. Hp1a also plays a role in testes specific gene expression [64] and DNA repair [65,66]. Loss of Su(Var)3-9 leads to increased breakage in repetitive DNA, genomic stability, and reduced life span[67].

Lhr is expressed in every tissue that has been examined. I therefore wanted to ask if Lhr, like its key interactor HP1a, affects TE expression and fitness at other stages. To this end, I examined the expression of transposable elements in testes, early embryos, larvae and the carcasses of adult flies. I also examined the effects of Lhr loss on male fertility and radiation sensitivity. Finally, I tested the ability of known and putative interactors of Lhr to influence Lhr activity.

Results and Discussion

Lhr in the male germline

Immuno-fluorescence shows that Lhr-HA is detectable in the testes (Figure 3.1 A). Additionally, mirroring its localization in other tissues, it colocalizes with HP1a at heterochromatin (Figure 3.1B). To test if the loss of *Lhr* affects male fertility, I compared the fertility of Lhr^{KO} and Lhr^{+} males. In contrast to the severe fertility defect observed in Lhr^{KO} females, Lhr^{KO} males show no noticeable reduction in fertility (Figure 3.1C). I also examined if the loss of Lhr affected the expression of transposable elements in the testes. I found that HeT-A was not upregulated while Copia was upregulated 14.6 fold in Lhr^{KO} relative to the wild-type control (Figure 3.1D). The lack of increased HeT-A transcription is consistent with a lack of HeT-A inactivity in the testes of piRNA pathway mutants [29]. Transposable element up-regulation has been traditionally associated with reduced fertility. However, in *Lhr* mutant testes, this assumption seems to be invalid. One possibility is that the fertility defect is subtle and maybe detected by the more sensitive sperm exhaustion assay. A second possibility is that mutations in genes such as *Piwi* that have been shown to affect TE expression as well as fertility do so through two independent functions.

I speculate that Lhr may have many additional roles beyond regulating transposable elements in the testes. The Y chromosome uses a centromere which consists of fragments of telomeric retrotransposons [35,68,69]. The Y chromosome also consists of GAGAA repeats as well as AACAC repeats. Considering that Lhr affects both the satellites as well as the telomeric retrotransposons in ovaries, it is plausible that Lhr can affect the segregation of the Y chromosome.

A more complete understanding of the functions of Lhr in the testes may be obtained by additional assays including RNA-Seq.

Lhr represses TEs in somatic tissues.

Several families of transposons are active in larval stages and in adult somatic tissues and are further upregulated in mutants of chromatin and siRNA pathway proteins [70]. I therefore tested *Lhr* mutants, to see if *Lhr* is required for repressing TEs outside the adult female germline. I examined embryos which inherit a large number of TE transcripts maternally. Consistent with the high expression of *HeT-A* in ovaries, I found that embryos laid by Lhr^{KO} mothers had higher levels of *HeT-A* transcript (Figure 3.2A). Larval stages have several proliferating tissues and show *HeT-A* expression, plausibly needed for telomere maintenance. I therefore compared Lhr^{KO} and Lhr^{+} larvae and found that *HeT-A* was upregulated 12 fold in the absence of *Lhr* (Figure 3.2B). Examination of female carcasses found that *HeT-A* was increased 31 fold in the absence of Lhr (Figure 3.2C). These results show that Lhr is also required for repression of TEs outside the female germline. Whether the mechanisms are the same through which Lhr regulates TEs inside and outside the female germline remains unclear.

***Lhr* mutants are not sensitized to gamma radiation.**

Double stranded DNA damage in heterochromatic repetitive DNA is repaired in a complex, HP1a mediated process where the damaged DNA is transported out of heterochromatin [65]. Consistent with these observations, larvae heterozygous for Hp1a, when compared with wild-type siblings, were more sensitive to gamma radiation[66]. As HP1a is Lhr's key protein interactor, I wanted to ask if Lhr was involved in this Hp1a function. All larvae, Lhr^{KO} and Lhr^{+}, exposed to 5000 rads of gamma radiation failed to develop to adulthood. When

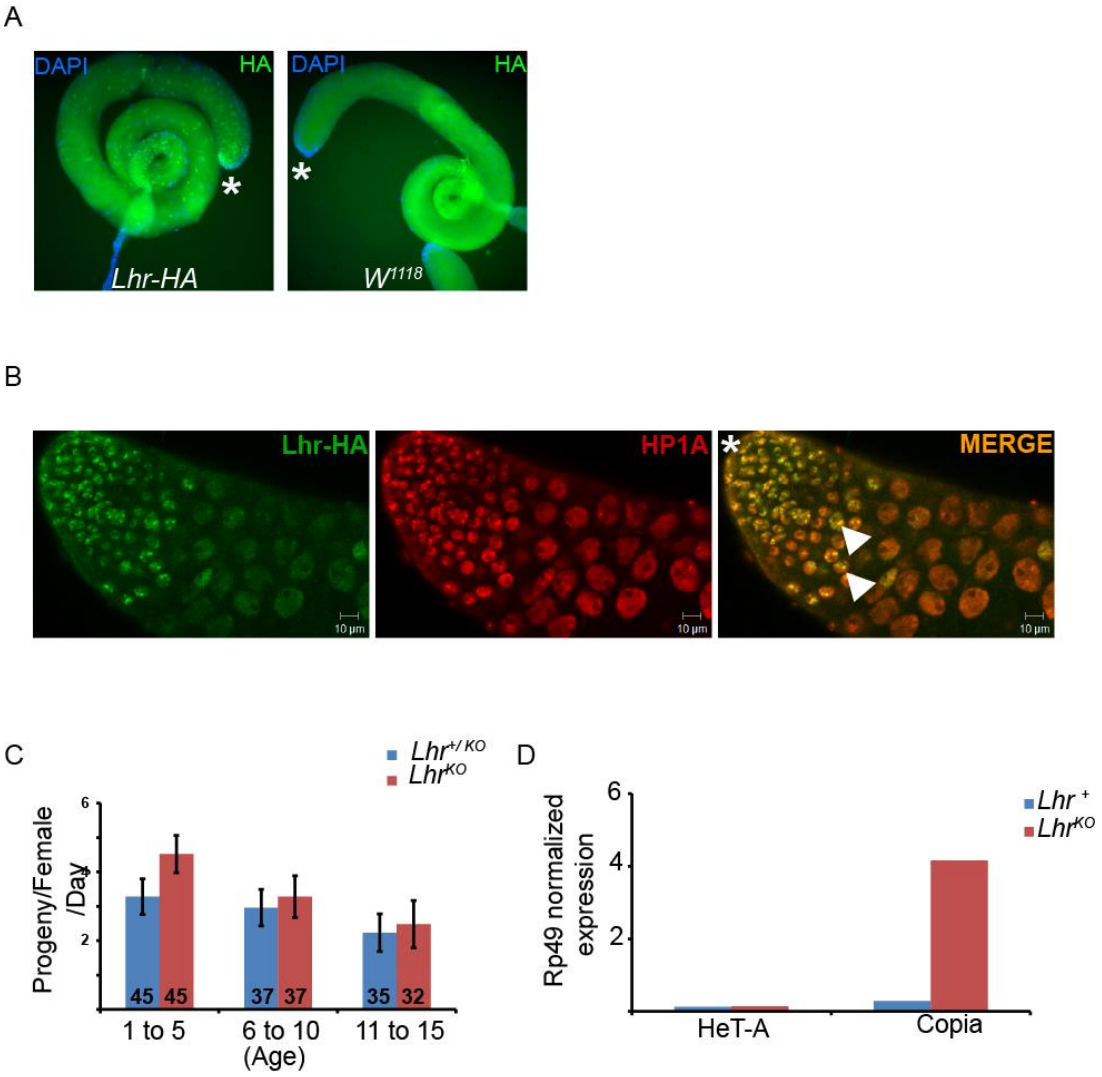

Figure 3.1 Lhr in the testes. (A) Lhr can be seen in the cells of *Lhr-HA* transgene carrying D. melanogaster testes. The testis of a W^{1118}, without a transgene, shows no signal. **(B)** Confocal slice shows Lhr-HA (green) localizing to heterochromatin marked with Hp1a (red). The asterisk in **A** and **B** marks the anterior end of the testes which includes the stem cell hub. Arrowheads show cololcalization of Lhr-HA an HP1a. **(C)** Loss of Lhr does not reduce the fertility of males. Numbers embedded inside bars represents number of males tested at each time point. Two tailed t-test showed that there was no significant difference between the fertility of the genotypes at all three ages. Error bars represent standard error of mean. **(D)** Loss of Lhr increases *Copia* but not *HeT-A* levels in testes. Q-PCR was from only a single biological sample. RT-PCR was carried out by oligo-dT priming.

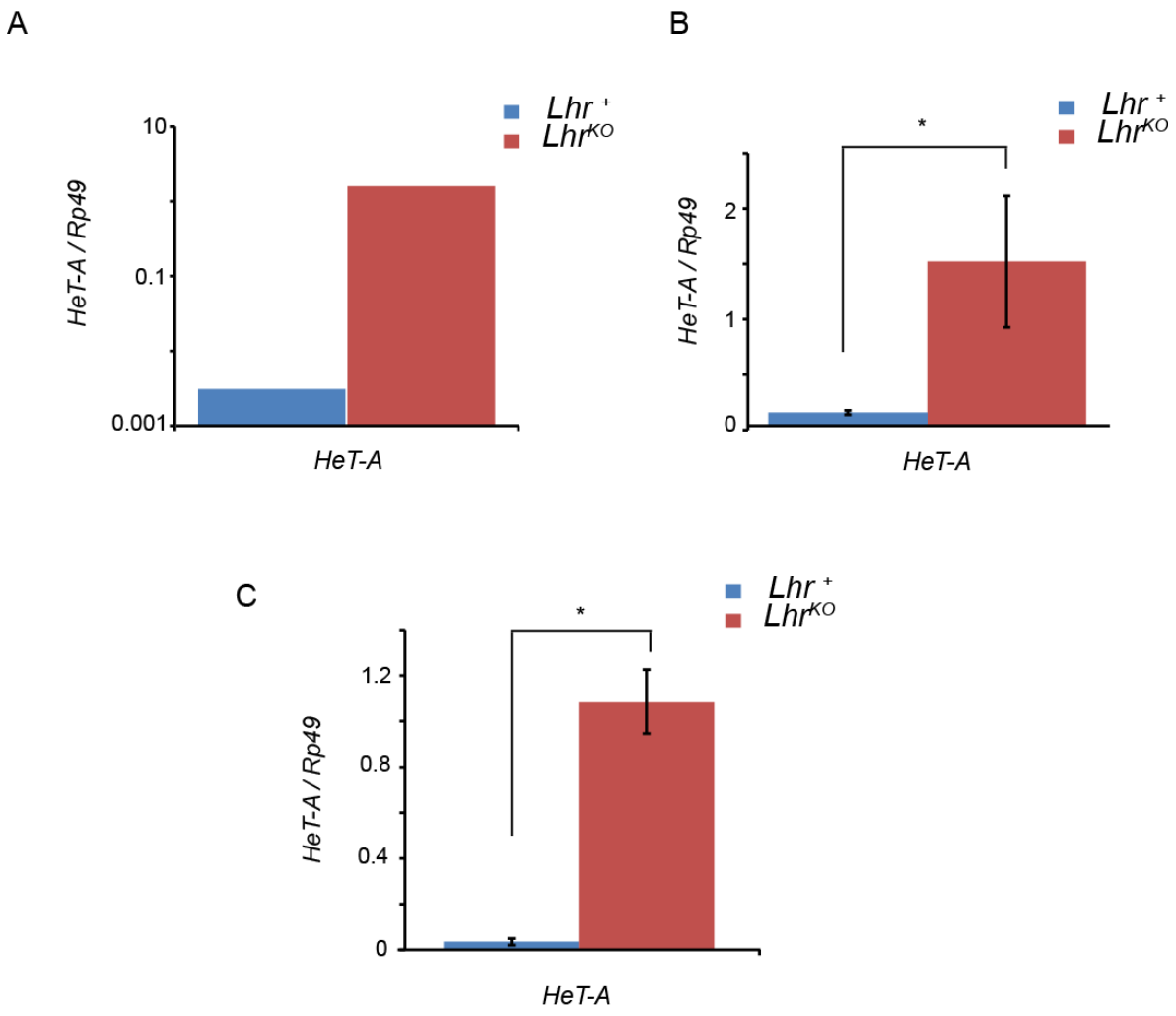

Figure 3.2 *Lhr* regulates transposable element expression in multiple tissues.
qPCR was used to estimate the transcript levels of *HeT-A* relative to *Rp49*. **(A)** Analysis of a single biological sample shows increased *HeT-A* transcripts in 0-3 hour embryos from Lhr^{KO} parents . Ratio of *HeT-A*/*Rp49* in Lhr^{KO} and Lhr^{+} larvae **(B)** and female carcasses **(C)**, showing mean for 3 biological replicates. Error bars represent standard error of mean. Significance of fold change calculated using the one tailed Wilcoxon rank sum test; * $p<0.05$. Samples were collected from organisms grown at 27°C.

exposed to 2000 rads, larvae from both genotypes exhibited melanotic masses, formed melanized pseudo-pupae and showed reduced progression to adulthood. However, in contrast to Hp1a heterozygous larvae which show a 4-5 fold reduction in number of adults reaching adulthood [66], comparable numbers of Lhr^{KO} and Lhr^{+} larvae reached adulthood (Table 3.1).These results suggest that Lhr is not required to repair DNA damage in heterochromatin.

Loss of Lhr derepresses telomere proximal genes.

Lhr localizes to the telomere cap. In Lhr^{KO}, the telomeric retrotransposon *HeT-A* has higher copy number and two sub-telomeric piRNA clusters showed dramatically reduced piRNA output. As previously explained, these observations suggest that the sub-telomeric regions show a general derepression. I therefore asked if sub-telomeric genes are also upregulated in *Lhr* mutants. To do this, I examined genes which are within50 KB of the mapped chromosome termini and found that the loss of *Lhr* in both *D. melanogaster* and *D. simulans* lead to increased expression from telomeric genes (Table 3.2). The expression of telomeric genes is affected by repressive proteins [71] and by telomere length, with longer telomeres linked to higher expression(Golubovsky et al., 2001). The *Lhr* mutant in *D. melanogaster* has long telomeres (Figure 2.9). It is therefore difficult to choose between these two potential causes of increased gene expression in *D. melanogaster*. One way to address the importance of telomere length to sub-telomeric gene expression would be to look at the telomere proximal gene expression of DGRP lines with long and short telomere lengths.

Genotype	Replicate	Larvae irradiated	Larvae reaching adulthood	% reaching adulthood
Lhr^{+}	1	100	33	33
Lhr^{+}	2	325	72	22
Lhr^{KO}	1	100	21	21
Lhr^{KO}	2	295	73	24

Table 3.1 Radiation sensitivity of *Lhr*^{KO}. F.E.T on sum for both replicates of each genotype shows that there is no significant difference in the survival rates between the genotypes after irradiation with 2000 rads. The genotypes used here were *w*^{1118};*Lhr*^{KO} and the matching *W*^{1118} control.

Chr	Gene	Strand	Distance from Chr end	Fold Change (mel)	Fold Change (sim)
2L	CG11023	+	7529	NO TEST	(Not telomeric)
	L(2) gl	-	9839	1.06	**5.31***
	Ir21a	-	21823	NO TEST	**12.97***
	Cda5	-	25402	NO TEST	0.542
2R	CG30428	+	4650	**2.20**	**0.45***
	CG9380	-	3867	2.61	83.47*
3L	Mthl8	+	22428	**4.21***	NO TEST (9.89)
3R	Map205	-	10890	1.23	1.08
	Mod	+	24357	1.79	1.01
	Krz	-	27338	0.84	1.03
X	CG17636	-	20757	**2.86**	**3.41***
	Rhogap1A	+	23836	1.45	1.15
	Tyn/Sp71	+	39034	**2.61**	**2.03***

Table 3.2 Sub-telomeric gene expression in *Lhr* mutants.Gene expression differences for sub-telomeric gene extracted from the Cuffdiff output comparing gene expression between wild-type (Lhr^{+}) and mutant (Lhr^{KO}) *D. melanogaster,* wild-type (w^{501}) and mutant (Lhr^{1}) *D. simulans.* The *D. melanogaster* comparison is between a single sample from each genotype. The *D. simulans* comparison is between three biological replicates from each genotype. **Bold** highlights genes with >/= 2 fold differential expression. " * " indicates statistically significant results." NO TEST " indicates lack of sufficient reads.

Lhr and satellites

Satellite DNAs produce both siRNA and piRNA in the ovary [73]. These small RNAs are transferred into the oocyte and have been suggested to help package the satellite DNA into heterochromatin in the embryo. Different satellite classes have differently composed small RNA pools in wild-type ovaries. I found neither siRNA (18-22nt) or piRNA (23-30nt) from the AACAC satellite which shows abundant numbers of mRNA transcripts, only piRNA from GAGAA, only siRNA from AAGAC, and both piRNA and siRNA from the 359bp satellite, SAT04 and Dodeca (Fig 3.3)

I had earlier described data showing that Lhr is required to repress the production of mRNA transcripts from a subset of satellites that it localizes to, including GAGAA and AACAC. I found that small RNA produced from satellites is also affected in Lhr^{KO} and can be divided into two classes based on the behavior of these satellites in the mutant (Fig 3.3). The first class includes GAGAA, AAGAC and SAT04. In the Lhr^{KO}, GAGAA produces more piRNA, but no siRNA. AAGAC produces more siRNA but no piRNA.SAT04 produces more siRNA as well as piRNA (Fig 3.3). Overall, their behavior can be explained by a model in which the increased production of small RNA in these satellite classes largely reflects the processing of increased amounts of mRNA transcripts in Lhr^{KO}.

The second class, Dodeca and 359, are more intriguing. In the Lhr^{KO}, Dodeca produces two fold more piRNA, but the amount of siRNA is reduced by the same amount (Fig 3.3). Finally, in the case of the 359 satellite, in which case I could detect only a handful of mRNA transcripts, I could find a substantial decrease in the number of small RNA being produced. Neither of these patterns can be explained by the simple model described above. One

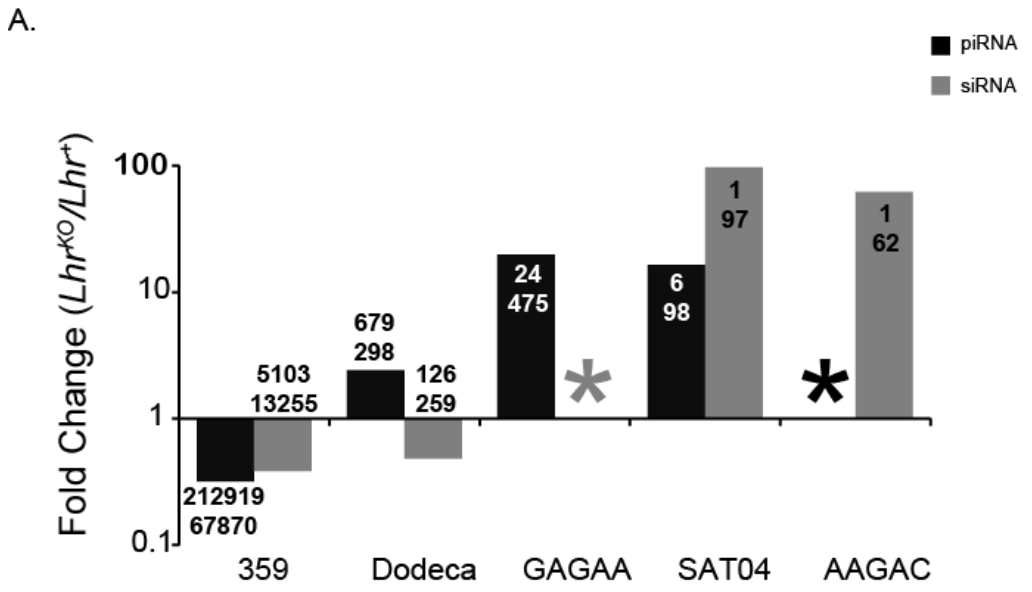

Figure 3.3 Lhr loss affects small RNA from satellites. siRNA and piRNA reads from *Lhr*^{KO} and *Lhr*$^{+}$ were mapped, without allowing any mismatches, to a satellite reference sequence without mismatches. Reference sequences for Dodeca and 359 were obtained from multiple cloned sequences and did not use a consensus sequence. The graph above shows significant normalized fold changes (*p<0.01*). Significance was calculated by F.E.T implemented in DEG-seq. Asterisks (*) represent cases where no reads were recorded. Numbers on bars represent reads mapping to Satellite DNA class in wild-type (bottom) and *Lhr*^{KO} (top).

possibility may be that the location of these satellites within the nucleus is important for repression and that the loss of Lhr changes nuclear organization.

Lhr and Hmr

Lhr and Hmr both show extensive heterochromatic localization and colocalize at the chromocenter of polytene chromosomes (Figure3.4A). In ovaries and embryos, Lhr and Hmr colocalize with the Dodeca satellite (Figure 2.1B,2.2C,2.13A; [74]). However, in salivary gland polytene chromosomes, Hmr appears to surround but not colocalize with the Dodeca satellite (Figure 3.4B). It is unclear if this is a polytene specific pattern.

I have previously shown that Lhr protein levels are drastically reduced in Hmr mutant embryos (Figure 2.1G). To test the effects on increasing Lhr levels in *Hmr* mutants, I compared the fertility of Hmr^3 , Hmr^3;*LhrHA/+* and Hmr^3; *LhrHA/LhrHA* with each other. I found that Hmr^3 mutants complemented with one copy of the transgene had higher fertility than Hmr^3, but it was not significant. Interestingly, the addition of two copies of *Lhr-HA* Hmr^3 mutants significantly enhanced the fertility of 11-16 day old Hmr^3 mutants (Figure 3.4C). This effect of *Lhr* may be mild in part because of the limited increase in Lhr protein levels.

To examine the effects of Hmr loss on Lhr in hybrids, I made cytological observations of Lhr-HA in wild-type hybrid embryos and *Hmr* mutant hybrid embryos. I found that while Lhr can be detected at the chromocenter normally in wild-type hybrids, it cannot be detected in Hmr^3 mutant hybrids (Figure 3.4D).

A preliminary experiment, comparing fertility in Hmr^3; Lhr^{KO}/*Cyo* and Hmr^3;Lhr^{KO}/Lhr^{KO} at 27C found that by day 5, 5 out of 28 (17.85%) of Hmr^3;Lhr^{KO}/*Cyo* and 14 out of 31 (45.16%) of females were completely sterile. This is an intriguing result and raises the possibility that loss of *Lhr* can enhance *Hmr* phenotypes. While fertility assays show some evidence of interaction

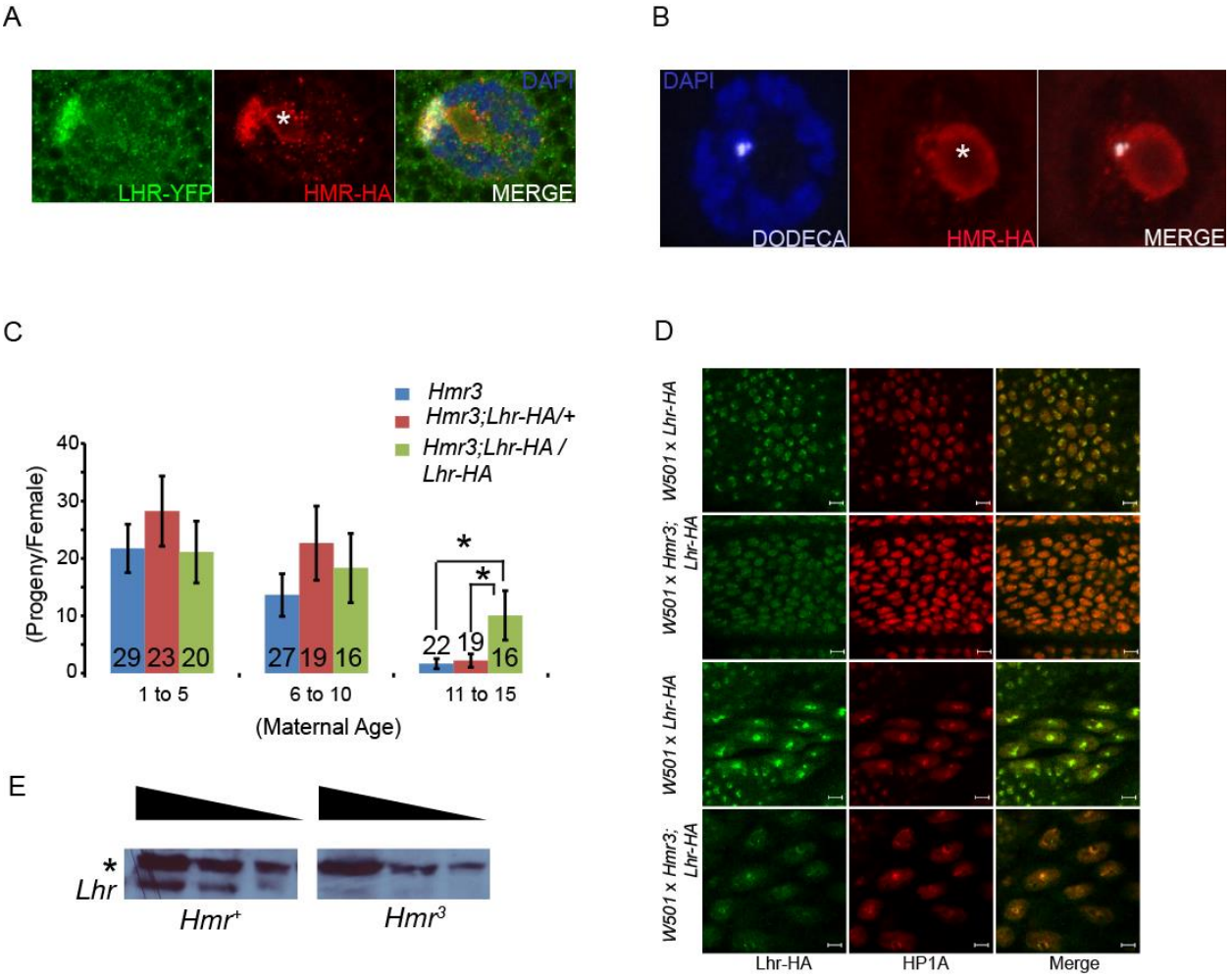

Figure 3.4 Lhr-Hmr interactions in *D. melanogaster*. **(A)**Lhr-YFP (green) colocalizes with HMR-HA(red) at the chromocenter of whole mount salivary gland nuclei. **(B)** Immuno-FISH shows that HMR-HA (red) surrounds but does not completely overlap with the Dodeca satellite(white) in whole mount salivary glands. The asterisk in **A** and **B** marks the nucleolus. **(C)** Assay measuring the female fertility of *Hmr*^{3} mutants, *Hmr*^{3} mutants complemented with a single copy or two copies of the *Lhr-HA* transgene. No significant difference was found in the fertility from days 1 through to 10. However, *Hmr*^{3} mutants alone or mutants complemented with a single transgene show lower fertility than mutants complemented with two copies of the transgene. Significance was calculated using a one tailed T-test. * *p<0.05.* Experiment was carried out at 27˚C. **(D)** Lhr-HA forms bright foci that colocalize with HP1a in wild-type hybrid embryos. In *Hmr*^{3} mutant hybrids, Lhr-HA is vastly reduced, but can be seen to colocalize to the chromocenter. Scale bar is 1µm.All crosses were *D. melanogaster* mothers to *D. simulans* fathers. **(E)** Western blot comparing two fold dilutions of endogenous Lhr in wild-type and *Hmr*^{3} mutant ovaries. The band marked by the asterisk is a non-specific background band used as a loading control.

between Lhr and Hmr, these assays are insensitive and the use of TE expression levels may provide a better way to assay Lhr-Hmr interactions.

Lhr and HP4

HP4, or HP1 Interacting Protein (*Hip*), has two HP1a binding sites and acts as a triplo and haplo suppressor of PEV [75]. HP4 is found in Lhr-containing complexes [76]; its molecular function remains unknown. However, it has been speculated that it stabilizes HP1A dimers and heterochromatin through the use of its two HP1a binding sites [75]. HP4-containing deficiencies enhance male hybrid rescue by the weak Lhr^2 allele [77]. To test this further, I used a P-element excision mutant of HP4, crossed it to Lhr^2 and observed that the loss of *Hp4* enhances male rescue (Table 3.2). However crosses with a large deficiency- *Df(3L)Exel6279*- that also deletes *Hp4* showed a more complicated result (Table 3.3). First, no males were rescued by Lhr^2 alone. This is consistent with previous observations that Lhr^2's ability to rescue male hybrids is often dependent on genetic background. Second, the presence of *Df(3L)Exel6279* increased the number of females that were recovered. In total, these results suggest that *Hp4* can enhance Lhr^2 rescue. This enhancement raises two possibilities. First, it may suggest a direct role for HP4 in hybrid lethality. Alternatively, it may be less direct via affecting HP1a, impact the total amount of Hp1a bound Lhr.

	Tm6			*Df 6279*		
Rep	♀	♂	**% rescue**	♀	♂	**% rescue**
1	80	0	0	130	1	0.008
2	94	0	0	120	0	0
3	85	1[#]	0	120	1[#]	0
4	69	0	0	103	0	0

Table 3.2 Hip41 allele enhances rescue of hybrid males by *Lhr*^{2}. Progeny from crosses of *CyO/Sp; hip*^{41}*/TM6, Tb* females and *Lhr*^{2} males that were maintained at 25°C. *Cy* and *sp* progeny reported together. Replicates 1 to 3 carried out in one batch, replicates 3 to 6 carried out separately. % rescue is (number of males/number of females) x100.

	Tb			*Hip41*		
Rep	♀	♂	**% rescue**	♀	♂	**% rescue**
1	121	32	26	95	137	144
2	83	21	25	108	100	92.5
3	41	3	7.3	47	46	97.8
4	32	0	0	55	41	74
5	77	5	6.4	137	97	70
6	164	2	1.2	262	205	78

Table 3.3 Deficiency covering Hp4 does not enhance rescue of hybrid males by *Lhr*^{2} . Progeny from crosses of *w*^{1118}*; Df(3L)Exel6279, P{XP-U}Exel6279/TM6B, Tb*^{1} females and *Lhr*^{2} males that were maintained at 25C. % rescue is (number of males/number of females)x100. # symbolizes patriclinous males identified by broken cuticles. These males have not been used to calculate rescue.

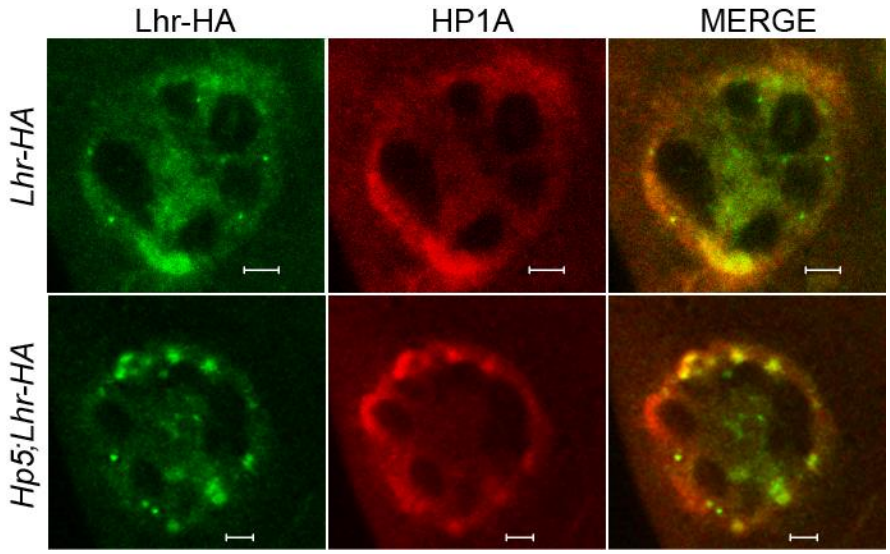

Figure 3.5 Lhr-HA does not mis-localize in the *Hp5* p-element insertion line. A confocal slice of an ovarian nurse cell shows that Lhr-HA (green) continues to localize to heterochromatin, marked by HP1a (red), in the line containing a p-element insertion in the *Hp5* locus. Scale bar represents 5μm.

Lhr and HP5

Hp5 is an HP1A associated heterochromatin protein that immuno-precipitates with Lhr. Previous work from Nicholas Brideau (2010) showed that Lhr-YFP failed to localize to heterochromatin in the salivary glands of Hp5 mutant, made by inserting a p-element into the 5' UTR of *Hp5* (ref). However, my experiments showed that Lhr-HA localized normally to heterochromatin in the ovaries of the same *Hp5* mutant background (Figure 3.5). The cause of this discrepancy remains unclear. To test any potential requirement for Hp5 in the localization of Lhr, I required a Hp5 null mutant. I therefore tried to create a loss of function *Hp5* mutant allele by imprecisely excising the p-element located within *Hp5*. However, after two rounds of screening of progeny of the *Hp5* p-element insertion and transposase, during which I examined 35 excisions, I found no imprecise excisions. Future experiments to remove this p-element should use recombination mutant backgrounds that enhance the rates of imprecise excision. Another possible method to test the relationship between Hp5 and Lhr will be to use an *Hp5* RNAi line.

Lhr and HP1c

HP1c is an HP1a paralog that localizes predominantly to euchromatin and to sub-telomeric regions. HP1c like HP1a, had the chromo-shadow domain, through which HP1a interacted with Lhr. Interestingly, DAM-ID studies, using Lhr-DAM and HP1c-DAM fusions suggested that Lhr and HP1c co-localized at euchromatic loci[78]. This result raised the possibility that Lhr and HP1c physically interacted in vivo. To test the possibility of an Lhr-HP1c interaction, I examined the ability of Lhr-HA to co-immuno-precipitate HP1c from embryonic lysates. I found that while I could confirm the previously reported HP1a-Lhr interaction, I could not find evidence for an interaction between Lhr and HP1c. A IP-Mass Spec study also failed to find HP1c among Lhr interactors [76]. It remains unclear if Lhr physically interacts with HP1c.

The biological relevance of the euchromatic localization of Lhr in the DAM-ID study [78,79] is now unclear, since loss of Lhr does not lead to widespread mis-regulation of these genes.

MATERIALS AND METHODS

RNA preparation, RT-PCR and QPCR: RNA preparation, RT-PCR and QPCR were carried out as described in Satyaki et al with the same set of primers and reagents with the exception of primers for Copia which were ordered using information from Lu et al (2009) ; Copia F: TGCCACTAAGCGTGGTATTG and Copia R: CTCTTGGAGACGCT TTACGG. Embryos for qPCR were collected on grape plates for 3 hours at 27°C. Carcasses were collected from females by removing germline tissue. Testes were collected from 3-5 day old, w1118 background Lhr^{KO} and Lhr^{+} males that were grown at 27°C.

Immuno-fluorescence:

Immunofluorescence on ovaries was carried out as described before. For immuno-fluorescence on testes, testes were dissected from 5-day old males in chilled 1X PBS, fixed for 20 minutes with 4% PFA-PBS,blocked for 2 hours with 3% PBT-BSA, stained with 1:100 anti-HA (3F10) and 1:100 anti-HP1a(C1A9) in 3% PBT-BSA, washed 3X in 0.1% PBS-TritonX.

Fertility Assays

Individual 2-3 day old $Lhr^{KO/+}$ and Lhr^{KO} males obtained from heterozygous parents, maintained at 27C, were mated with two virgin w^{1118} females and the crosses were also kept at 27C. The male was transferred to a new vial with two new female virgins every five days. The females were transferred into fresh vials every five days till no additional progeny were produced. Vials with dead males were not counted. To represent changes in the number of females that can be caused by lethality, the fertility of females is represented as number of progeny/day/female. Female fertility assays were carried out at 27°C as previously described.

Radiation sensitivity assay

Wandering third instar larvae of $w^{1118};Lhr^{KO}$ or $w^{1118};Lhr^{+}$ genotypes were exposed to either 5000 rads or 2000 rads of gamma radiation. After exposure, the larvae were grown at 25C and ecclosing progeny were counted up to 12 days after irradiation.

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CHAPTER 4

Studies of hybrid development

INTRODUCTION

Hybrid male progeny of *D. melanogaster* mothers and *D. simulans* fathers fail to enter the pupal stage and die as larvae [80]. These larvae are abnormal, having under-proliferated brains and lacking imaginal discs and germline tissues[80]. Imaginal discs are essential because they are the progenitors of many adult tissues. The lack of imaginal discs may therefore be a proximal cause for the failure of male hybrids to pupariate. Imaginal discs, when fully grown, signal the larvae to transit to the pupal stage; damaged imaginal discs delay pupariation [81]

It has been argued that imaginal discs and the under-proliferated brains are caused by mitotic defects and this model indeed fits well with observations that the two hybrid incompatibility proteins Lhr and Hmr localize to peri-centromeric or even centromeric heterochromatin[76]. However, this model needs more investigation. A previous study did not find imaginal discs even at embryonic stages [82]. Thus, it's plausible that the lack of imaginal discs and eventual larval lethality is caused by a fate specification defect rather than a mitotic defect. Additionally, though Thomae et al. argue that mitotic defects are the cause of the under-

proliferation, direct evidence that mitotic defects actually occur in hybrids is sparse. They used the mitotic segregation defects in cell lines, from parental species, over expressing Lhr and Hmr to explain hybrid lethality. However, this study does not take into account the heavy over expression of these proteins needed to obtain segregation defects in cell culture and the relatively lower increases in the levels of Lhr and Hmr in hybrid animals. Most importantly, they never show these segregation defects in hybrids; probably stymied by the lack of observable mitotic hybrid tissue.

This study addresses these issues. First, I show that imaginal discs are specified in male hybrids. To address the second problem, I used a Gal4-UAS system to drive the production of LHR^{sim} in the wing imaginal discs of viable male hybrids rescued with the Lhr^{1} mutation. I found that while the wings were formed, without obvious size defects, the hybrid male wings exhibited a cell adhesion defect. This result argues against the model of mitotic defects underlying hybrid lethality and provides a system to supply the mitotic tissue needed for studying hybrids.

Results and discussion

Imaginal discs are specified in hybrid males.

Imaginal discs are specified during early embryogenesis[83]. To test if imaginal discs are missing in hybrid males because they are not specified, previous studies tried to culture cells from hybrid embryos in the embryos of parental species. However, these hybrid cells apparently did not contribute to adult tissues[82]. This experiment, though state of the art for its time, did not provide conclusive answers. To answer this question, I looked at the expression of GFP driven by the promoter of the *Escargot* gene (*esg-Gfp*)- a marker of imaginal disc and histoblast nests[83]. Using a FISH probe against the Y chromosome[84], I was also able to distinguish between male and female hybrid embryos. *Esg-Gfp* can be seen in female hybrids, marking

imaginal discs and histoblast nests (Figure 4.1A). Interestingly, *Escargot* driven GFP can also be seen in male hybrid embryos in structures that appear similar to histoblast nests and imaginal discs (Fig 4.1B,C,D). This led me to conclude that in hybrids, imaginal discs are specified.

Growth defects in male hybrid imaginal discs are cell non-autonomous .

As shown above, imaginal discs are specified in hybrid male embryos (Figure 4.1B,C,D). However, anatomical examination of the male hybrid does not detect the imaginal discs in late larval stages. It has been argued that the disappearance of these imaginal discs is linked to a mitotic defect that prevents the cells from proliferating [76]. One prediction of this model is that the failure of imaginal discs to grow would be a cell autonomous defect. Thus, any imaginal disc cell expressing Lhr^{sim} , Hmr^{mel} and having the hybrid genome must fail to proliferate. To test the cell autonomy of the cell proliferation defect, I made use of the UAS/GAL4 system that allows tissue specific expression. I constructed a *D. melanogaster* line in which *UAS-*Lhr^{sim}*-YFP* is driven in the wing imaginal discs by the wing specific *A9-Gal4* driver. I then crossed *D. melanogaster A9-Gal4;UAS-*Lhr^{sim}*-YFP* with *D.simulans* Lhr^{1} males. In this experiment, the Lhr^{1} allele - a strong loss of function allele- rescues the male hybrid. However, adding back Lhr^{sim} to the wing disc should obliterate the wing if it is a mitotic defect.

The *A9-Gal4* driver is active throughout the wing disc up to the late third instar, at which point its activity narrows to the wing's dorsal pouch and has been used in many studies of wing growth and development [85,86]. Examination of hybrid larvae showed Lhr^{sim}-YFP expression in the wing discs (Figure 4.2A). However, some YFP could also be observed in the salivary glands and fat body of the hybrid larvae (Figure 4.2B,C). This suggested that the *A9-Gal4* driver is leaky and has some activity outside the wing disc. Examination of hybrid progeny showed that

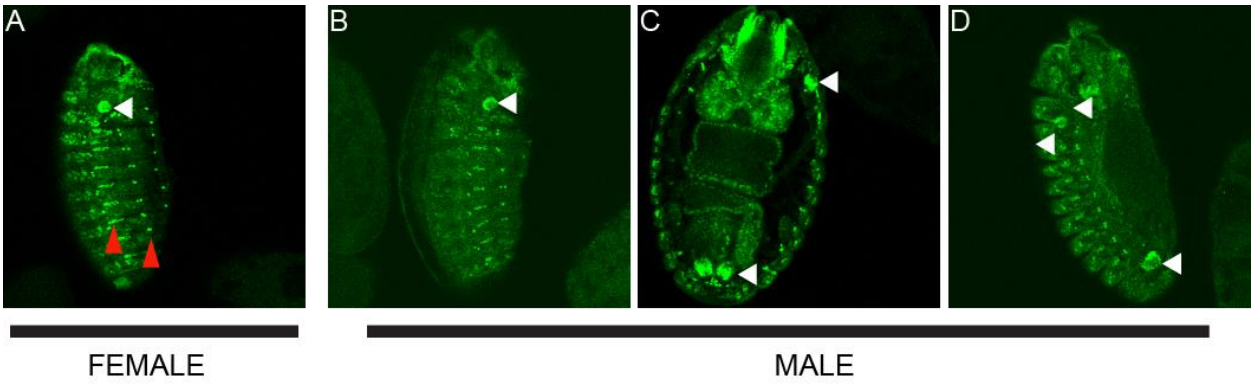

Figure 4.1 Imaginal discs are specified in male hybrid embryos. GFP (green) driven by *Escargot* marks hybrid embryos. White arrows point to some of the imaginal discs while red arrows point to the histoblast nests. To generate these embryos, *Escg-Gfp/Cyo* were crossed to *D. simulans* $w^{501.}$ Embryos were sexed with the Y specific AATAAAC probe and GFP containing embryos were imaged.

females had normal wings (Table 4.1). This is contrary to model put forth by Thomae et al where the over expression of Lhr would cause mitotic defects[76]. Examination of hybrid males showed that they also formed full sized wings. This suggests that expression of Lhr does not cause a mitotic defect. While the wings were full sized, they were also blistered and the dorsal and ventral blades were detached from each other (Fig 4.2G). This is a classic phenotype that is observed in cell adhesion mutants. Interestingly, knockdown of the histone methylase *SetDB1* in wing discs also has a similar phenotype [87]. The drastic reduction in hybrid male viability may be caused by the leaky expression of Lhr^{sim} outside the wing disc (Table 4.1). These preliminary results require further testing. A stronger wing specific Gal4 driver like *Apterous-Gal4* can be used to confirm the result obtained with *A9-Gal4*. Further, to avoid the interference of the cell adhesion defects in judging proliferation, it might be necessary to carry out a similar tissue specific expression in the eye using the *Gmr-Gal4* driver.

The preliminary data described here argues that there are no segregation defects in hybrids. What then is the cause of the imaginal disc growth defect seen in hybrids? The answer might lie in the observation that leaky expression kills male hybrids. I propose that hybrid lethality is actually a cell non-autonomous defect and that this leaky expression, acting in an endocrine tissue such as the fat body, can influence the growth of imaginal discs. The cell non-autonomous effects of the fat body are well known[88]. One excellent example comes from mutants of the *minidiscs* gene which encodes a larval fat body specific amino acid transporter [89]. In *minidiscs* mutants, the fat body fails to sense amino acids and therefore signals a state of starvation to the rest of the imaginal discs, which under-proliferate [88,89]. Future experiments expressing Lhr in the endocrine organs of hybrid males can test this cell non-autonomous model further.

Replicate		Normal wings	Abnormal Wings	Remarks
1	♀	339	0	
	♂	3*	34	*Includes 2 patriclinous ♂
2	♀	209	2	
	♂	1	51*	* includes 3 flies with one abnormal wing.
3	♀	103	0	
	♂	0	15*	*includes 1 fly with serrated wings
4	♀	189	0	
	♂	0	36*	*includes 3 flies with serrated wings

Table 4.1 Male Biased effects of expressing *Lhr ^{sim}* in hybrid wings. Hybrid progeny from room temperature crosses of *A9-Gal4; UAS-Lhr^{sim}-YFP/ UAS-Lhr^{sim}-YFP D. melanogaster* ♀ x *Lhr^{1} D. simulans* ♂, that survived to adulthood were scored for bloated wings. Patriclinous males were scored by the presence of a broken cuticle.

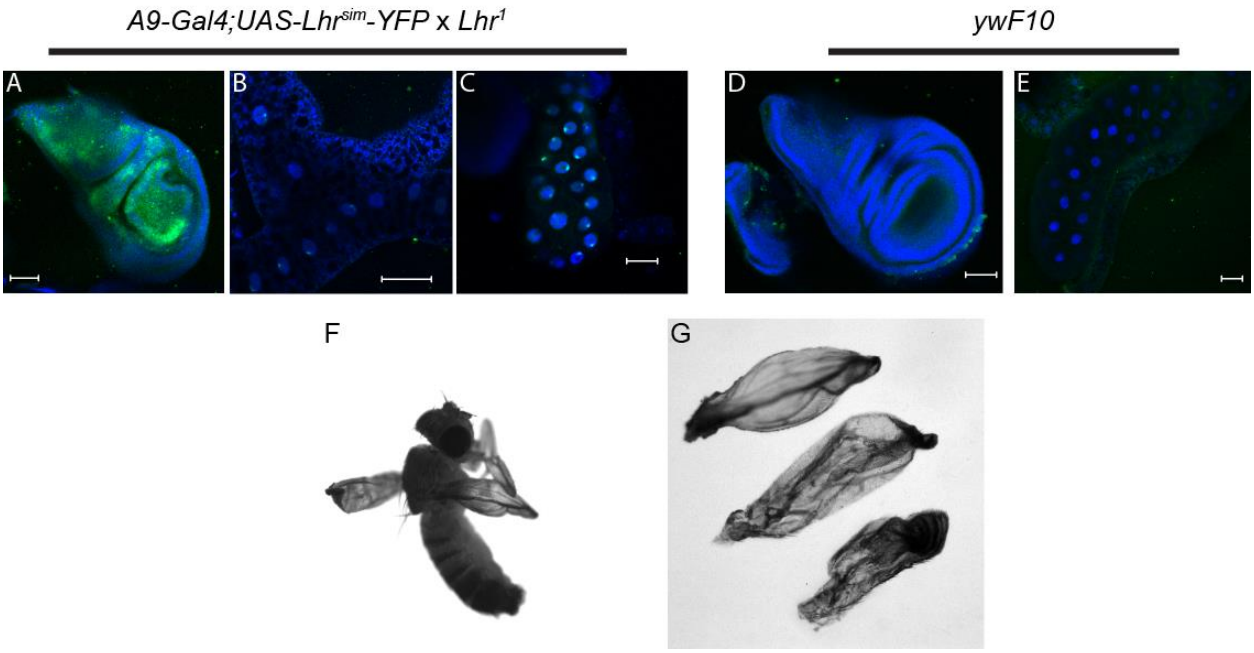

Figure 4.2 Expression of *Lhr^{sim}* in the wing imaginal disc leads to the formation of wing blades with cell adhesion defects. The *A9-Gal4* driver was used to express *Lhr*^{sim}-YFP in the wing disc of *D. melanogaster-Lhr*^{1} *D. simulans* hybrid males. *A9-Gal4* drives expression of *Lhr*^{sim}*-YFP* not only in the wing disc **(A)**, but also in the fat body **(B)** and in the salivary glands **(C)**. Specificity of the anti-GFP antibody can be deduced from the lack of signal in the wing disc **(D)** or in the salivary glands and fat body **(E)** of *y w F10* which carries no *YFP* transgene. Scale bar indicates 50 µm. All crosses were carried out at 25°C.

Materials and Methods

Drosophila strains: The *p1986* line, expressing *escg-GFP*, used to detect the presence of imaginal disc in hybrid males, was obtained from the Fly Trap Consortium [90]. The *UAS-Lhr^{sim}-YFP* transgene was previously described in Brideau & Barbash, 2011. The *A9-Gal4* was obtained from the Bloomington Stock Center (Stock no. 8761)

Immuno-Fluorescence: The Y chromosome specific satellite probe-AATAAAC- used here was previously described in Maheshwari & Barbash, 2012. To detect GFP, the mouse anti-GFP (JL-8, Clontech) was used at 1:100 dilution.

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Chapter 5

The way forward

In this dissertation, I have described strong evidence showing that Lhr is part of the machinery that allows *Drosophila* to adapt to changes in satellite DNA and transposable elements. However, several questions remain unanswered and my work can become foundational to new studies.

They can be divided into the following categories:

1. What biological processes is Lhr involved in?
2. How does Lhr regulate repetitive DNA?
3. Do specific elements cause the rapid sequence divergence of Lhr, and if so, what is the molecular basis of these interactions?
4. How does Lhr cause hybrid lethality?

What biological processes is Lhr involved in?

Role in the male germline

To identify all the selection pressures driving the sequence divergence of Lhr, we need a fuller understanding of Lhr function, beyond its role in the female germline. One critical site of Lhr function is the testes. The male germline is an ecosystem that is distinct from the female germline. The piRNA pathway mainly controls the *stellate* genes in the testes and TE regulation seems secondary, with *Aub* and *Ago3* binding piRNAs derived from a subset of TEs that are expressed in the female germline [92]. Some TEs may be taking advantage of them being the secondary focus of the piRNA pathway in the male germline. For example, *Copia* is known to transpose exclusively in the male germline [93,94]. Because of such differences between the

male and female germlines, there may be TEs uniquely active in the male germline that are driving the sequence divergence of Lhr. I have shown above that Lhr represses *Copia* in the male germline (Fig 3.1D). This raises the possibility that Lhr, mirroring its role in the female germline, represses a broad spectrum of repetitive elements in the male germline. To test this, I suggest carrying out an RNA-Seq experiment comparing Lhr^{KO} and Lhr^{+} testes. In addition to helping understand the forces driving Lhr's sequence divergence, it would shed light on the role of heterochromatin in spermatogenesis. The Y chromosome is littered with transposable elements and satellite DNA and is mostly heterochromatic. Interestingly, the Y chromosome centromeric regions are rich in remnants of telomeric TEs, Dodeca, AACAC and GAGAA satellites [35,95] - all elements regulated by Lhr in the female germline(Fig 2.3A, 2.6A,B). The Y chromosome also includes a small number of genes required for male fertility [96]. It's conceivable that Lhr would be required for the normal expression of these genes, akin to how it regulates heterochromatic gene expression in the ovaries (Fig 2.4). It would be expected that any effects of Lhr on sperm heterochromatin should be manifested by reductions in fertility- something I have not observed in a simple male fertility experiment (Fig3.1C). Interpretation of this result must be tempered by my observation that the Lhr^{KO}'s female germline shows a relatively moderate reduction in fertility (Fig2.2). Therefore, any future endeavor to look for a defect in male fertility must use more sensitive assays like sperm exhaustion assays or test the ability of sperm from Lhr^{KO} to compete with the sperm from Lhr^{+}.

Role outside the germlines

TE repression outside the germline has received little interest primarily because it has been assumed that TEs active outside the germline do not contribute to copy number increases of TEs in the germline. However, a number of TEs including *Gypsy* and *Copia* produce virus-like

particles that move between tissues [97,98]. It is therefore plausible that TEs active in somatic tissues can be transported to the ovaries or the testes by haemolymph.

Loss of Lhr function can have additional roles outside the germline. For example, loss of Lhr severely down regulates the ovarian expression of *Caps*, a gene whose product plays important roles in synaptic transmission [99]. Down-regulation of this gene in the nervous system could lead to behavioural defects.

Does Lhr regulate transposition?

While loss of Lhr increases TE transcript levels, the correlation between TE transcript level and genomic TE copy number is uncertain. It is likely that in some cases, the loss of Lhr, by increasing chromatin accessibility, can lead to increases in TE copy number independent of TE transcript levels. To check for such effects, Lhr^{KO} mutants allowed to accumulate TE copies can be sequenced every few generations. The different effects of Lhr in the male and female germline could be detected by crossing the Lhr^{KO} males and females with Lhr^{KO}/+ and picking up $Lhr^{KO/KO}$ individuals at each generation.

What is the significance of the satellite transcripts?

Transcripts from a number of satellite DNA are increased in Lhr^{KO} ovaries (Figure 2.11A). The importance of this remains unclear, but there are at least three possibilities. One possibility is that the regulation of these transcripts is important for centromeric function. Transcripts from centromeric satellite DNA have been implicated in centromeric function in several species in the plant and animal kingdoms; the over-expression of these satellite transcripts have been linked to centromeric dysfunction [51]. However, the role of these satellite RNAs seems to vary by species and its role in *Drosophila* remains to be studied. Several experiments can be carried out to understand the significance of these satellite transcripts and

the localization of Lhr to satellites. First, the identity of the sequences constituting the centromeres in Drosophila is unclear. It's therefore unclear if Lhr binds centromeric satellites or peri-centromeric satellites. Additionally, it is not clear if Lhr localizes to the centromere. The Lhr-HA transgene used extensively in this dissertation did not localize to the centromere in early embryos [84] but anti-Lhr antibody detected it at the centromeres of tissue culture cells and imaginal disc cells [76]. It is plausible that Lhr is not centromeric in early embryos but becomes centromeric later in development. We can address these issues by carrying out co-immuno-localization of Lhr-HA and the centromeric protein CID at different stages and by carrying out an immuno-FISH examining the co-localization of CID and variouus repeats. The latter study could also be extended to test the oft bandied model that centromeric satellites have changed between *D. melanogaster* and *D. simulans.*

To test the relevance of the over-expression of satellite transcripts, satellite transcripts can be over expressed in a UAS-GAL4 system. However, planning this experiment is hobbled by several unknowns. For example, what should the length of these transcripts be? Do these transcripts act in cis or trans? A second possibility is that while these transcripts may play an important role in centromere function, their over production may lead to the formation of R-loops which interfere with replication and lead to genomic instability. Finally, a third possibility is that these transcripts serve no purpose and the increase in these transcripts is a result of an increase in pervasive transcription, brought about by the loss of the repressive properties of heterochromatin in the Lhr^{KO}.

One way to test the role of Lhr in centromere function would be to measure rates of non-disjunction in meiosis. A limited experiment carried out to examine NDJ rates of the third

Maternal genotype	Approximate number of eggs	# live adults (NDJ rate)
Lhr $^{+}$	4800	2 (0.042%)
Lhr ^{KO}	2400	0 (0)

Table 5.1 No appreciable increase in female NDJ in *Lhr*^{KO}. *Lhr*^{KO} and *Lhr*$^{+}$ females were separately crossed with *w+;C(3) th*^{1}*,sr*^{1} males. Crosses were maintained at 27C. Vials were flipped each day and the number of eggs laid was approximated for each genotype. Both the live adult progeny were males and were marked wth *th*^{1}. Apart from the adult progeny, no larvae or pupae were discovered.

chromosome in Lhr^{KO} found no appreciable increase in NDJ rates (Table 5.1). However, this experiment did not examine enough progeny and additional experiments are needed to ascertain if loss of Lhr affects centromere function at meiosis. One sensitive method that can be used may be to test if loss of Lhr enhances low levels of NDJ in *nod* mutants

How does Lhr regulate repetitive DNA?

I have shown that Lhr regulates transcripts from a wide variety of transposable elements and satellite DNA. Additionally, it also affects heterochromatic genes. These observations suggest that Lhr is a general heterochromatin protein. Lhr is known to interact with the heterochromatin protein Hp1a [79,91,100].I have shown that Lhr doesn't perturb the small RNA biogenesis pathways (Fig 2.7) and that Hp1a is not broadly affected across heterochromatin. Additionally changes in RNAP2 levels may be insufficient to support a transcriptional repression role for Lhr (Shuqing Ji). This suggests that Lhr may be functioning post-transcriptionally. So how then does Lhr regulate the steady-state levels of transcripts from repetitive DNA? There are at least two potential, non-exclusive roles that Lhr might play.

Possible ways Lhr may regulate transcription: First, Lhr may stabilize HP1a binding to chromatin and to other repressor proteins. HP1 proteins dimerize through the chromo-shadow domain and the dimerization interface provides binding sites for proteins [101]. Lhr may either stabilize these HP1a homo-dimers and/or adjacent pairs of dimers. Several observations are consistent with such a model. First, *in vitro* data shows that both the C-terminal and N-terminal halves of Lhr can bind HP1a[77]. This argues that each Lhr molecule can bind two HP1a molecules. Second, such models require that the amounts of Lhr and HP1a maintain precise stoichiometric ratios and deviations from such ratios can lead to derepression of heterochromatin. Consistent with this expectation, high-level over-expression of Lhr can lead to

a derepression of TEs in tissue culture cells [76]. Several experiments will be needed to test this model. First, the model that Lhr binds two molecules of HP1a needs to be more rigorously tested. For example, both C and N terminal Lhr halves should be able to independently localize to heterochromatin *in vivo*. Second, the dose response can be tested further. The over expression of Lhr in the ovary using a UAS-GAL4 system can be used to test the results obtained from cell culture. Such a dose response would also be manifest as a haplo and triplo enhancer of position-effect variegation reporters. A third possible experiment would be to examine the stability of heterochromatin protein complexes in Lhr^{KO}. FRAP can be used to measure the kinetics of the exchange of HP1a or HP1a interactors from heterochromatin in the presence or absence of Lhr.

A second and overlapping mode for Lhr's repressive role might be by post-transcriptional regulation. First, RNAP2 ChIP data shows that loss of Lhr, increases RNAP2 levels by 1.8 fold for *HeT*-A and not at all for *Copia*. This cannot explain the increases in transcript levels(Shuqing Ji). Support also comes from cross referencing proteins known to complex with Lhr and genes whose mutations lead to increases in TE transcripts. A significant part of the overlap between these lists is proteins implicated in RNA metabolism (Table 5.2). To test this model, we will first need to show that post-transcriptional processing is affected. To do this, TE transcripts can be measured in Lhr^{KO} and *Lhr+* ovaries that have been cultured for several hours in the presence of an RNAP2 inhibitor such as alpha-amanitin. Another way to address this issue would be to compare steady state TE transcript levels obtained from RNA-Seq with levels of active transcription at TEs that can be obtained from pro-Seq. If Lhr's affect is indeed post-transcriptional, then Lhr^{KO} would have a large concentration of TE transcripts. Follow up experiments including co-immuno-precipitation and immuno-fluorescence can be used to show a link between Lhr and proteins known to be involved in RNA degradation.

Another unanswered question is the identity of all the factors that determine the localization of Lhr. Lhr localizes to several satellites, chromosome IV and the telomeres. Lhr's broad localization to heterochromatin requires Hp1a[79,91,100]. However, this seems to be only part of the story because it does not overlap completely with Hp1a (Greil et al., 2007, data not shown). It is also unclear if HP1a is necessary or sufficient for Lhr to localize to the telomeric cap. Another potential localization determinant for Lhr is the MADF domain containing Hmr. However, while Hmr is nucleolar in both syncytial embryos as well as larval salivary glands (Fig 2.1C), there is no observable Lhr in the nucleolus [84]. These observations argue that there are determinants beyond HP1a and Hmr that target Lhr localization. Several candidate targeting proteins come from recent studies involving co-immuno-precipitation of Lhr (Table 5.3). Two obstacles had made these experiments difficult to perform in the past. One was the absence of an antibody that could cytologically detect Lhr and the second was the absence of information as to where Lhr precisely localized. A recently described anti-Lhr antibody and my fine mapping of Lhr localization relative to satellites and the telomere cap may go some way in ameliorating this problem[76].

Which specific elements cause the sequence of Lhr to rapidly diverge and what is the physical basis for the interactions?

The argument that adaptive evolution is directly affecting TE and satellite repressors is challenged by the fact that most repressors do not themselves have sequence specificity. We have a number of candidate repetitive elements that may drive the sequence divergence of Lhr, but this same issue of specificity also affects Lhr. There are atleast three potential scenarios through which these elements can act on Lhr. These are:

1. **Direct Conflict:** This model is most directly applicable to Lhr's interactions with transposable elements. Under this model, RNA or proteins encoded by transposable elements such as Gag, reverse transcriptase, or transposase could directly interact with Lhr and inhibit its function or use it to home in on target sites. Testing this model requires the ability to study Lhr's physical interactions. This can be done in one of two ways. First, Lhr can be immuno-precipitated from wild-type ovaries and the pull downs be subjected to mass-spectroscopy or RNA-Seq. Alternatively, Lhr can be used as a bait in a yeast two hybrid experiment that uses transposable element proteins as prey.

2. **Indirect Conflict:** This model works based on the knowledge that Lhr, as a BESS domain containing protein, may act as an adapter that helps HP1a and other proteins increase their protein binding repertoire. Under this model, proteins that interact directly with satellites and are rapidly evolving in response to changes in these selfish entities funnel the effects of adaptive divergence to Lhr. Lhr then acts as a bridge between these rapidly evolving proteins and the more conserved core heterochromatin proteins like HP1a. There are two observations that are consistent with this model. First, Lhr is found in complex with many rapidly evolving proteins such as Hmr, Su(var)3-7, Hp5, Stonewall [76,102]. Second, Lhr's sequence co-varies with these proteins along the *Drosophila* sequence tree (Clark NC, Personal communication). Important to this model, is to understand how Lhr's protein interactors have changed between species, an example of which can be found in Thomae et al [76].

3. **The repeat load model:** This model, explained in chapter 2, argues that Lhr is evolving to overall changes in repeat copy number. How could Lhr's sequence divergence be linked to repeat load? One prediction of this model would be that Lhr protein levels would be higher in *D. melanogaster* that in *D. simulans*. This can be tested by western using an antibody against a

conserved region of Lhr. Alternatively, it can be measured by quantitative mass-spec. A second prediction of this model would be that Lhr is more codon optimized in *D. melanogaster* that in *D. simulans* and this can be tested. Another effect of the sequence divergence in Lhr's sequence may be to increase the affinity of Lhr for interacting partners. This can be a difficult model to test *in vivo* as Lhr may function as part of a large and probably co-evolving complex. In such cases, assays substituting one component of a complex with its ortholog from a sibling species would be hard to interpret. Functional differences caused by changes in amino acids that lead to changes in affinity would be difficult to separate from changes that are caused by differences in unlinked protein interactions. Nevertheless, a naïve expectation is that one dose of Lhr from *D. melanogaster*, where it is adapted to higher TE load, would be more repressive than *D. simulans* Lhr. This model can also be tested by measurement of the association constants of Lhr and its interactors *in vitro*.

How does Lhr cause hybrid lethality?

Our work shows that Lhr gains a function in hybrids that leads to hybrid lethality. However, attempts to understand the molecular nature of this gain of function have been limited by the lack of a suitable tissue for study and by an absence of knowledge of Lhr's interactors in hybrids. I argue in chapter 3 that the growth defects in hybrids may be linked to an endocrine tissue. These endocrine tissues often consist only of a few cells and may be difficult to obtain in amounts sufficient to carry out proper molecular or biochemical analyses. However, any tissue with both parental genomes as well as Lhr and other proteins from both parental species may offer insights into the phenotypes in hybrids. While such studies can be carried out in whole larvae, this approach can introduce post-mitotic and polyploid tissues that are often unaffected by hybrid incompatibility. I propose that sufficient amounts of diploid tissue can be obtained from

Candidate	Functions/Comments	Refs for effects on TEs/small RNA
HP1a	Chromatin state, RNA binding	[13,104]
RpS13	Ribosomal, tissue specific expression	[13,105]
RpL21	Ribosomal and centrosomal	[13,105]
RpL18A	Ribosomal and centrosomal	[13]
Rm62	RNA helicase. Known to cooperate with Su(var) 3-9	[13,106]
RpL14	Ribosomal and centrosomal. Associates with transcription sites.	[13]
Ssrp	Nucleic acid binding	[13]
RpL18	Ribosomal,Mitotic Spindle	[13]
RpL34B	Ribosomal	[13]
RpL4	Ribosomal	
RpS30	Ribosomal,Mitotic spindle	[13,105]
RpL38	Ribosomal	[13]

Table 5.2 Candidate Lhr interactors that process RNA and regulate TEs .Lhr interacts with a number of ribosomal and other RNA binding proteins that have been shown to have roles in screens for effectors of TE repression [13] or in modulators of small RNA [105]. Several of these ribosomal proteins have been shown to have roles outside the ribosome. This list is not exhaustive. Further, the results of the RNAi screen carried out by Czech et al. may have several false negatives.

Candidate	Ref for interactions	Function/Localization/ Phenotypes/Comments
HP1a	Y[1,] M[2,] YC[4] Y[5,] O[6,] YWO[7]	R[12],T[11,12],P[12],Z[12]
Hmr	O[1,] M[2,3,] W[Fig]	T[Fig,2],C[2], Z[Fig]
Hp4	M[2, 3,] O[6]	P[12]
Hp5	M[2,3,] O[6]	C[6]
Hp6/Umbrea	M[2, 3,] O[6],Y[7]	T[11],C[3]
Hp1b	M[2]	over expression artifact ?
Su(var)3-7	M[2]	M[12], T[12]
HP2	M[2,3]	T[11], P[12]
Stonewall	M[8]	T[11,8], P[12]
Su(var)3-9	M[2]	T[11,12], P[12]
SuUR	M[2]	Z[12], P[12]
CTCF	M[2,8]	P[8]
Su(Hw)	M[2,8]	P[8]
Nlp	M[2]	T[12],C[12],P[12]
Msl-1	M[2]	C[12]
Msl-2	M[2]	C[12]
Mle	M[2]	C[12]
Acf-1	M[2]	C[12]
Irbp	M[2]	Z[12]
Pav	M[2]	T[11],Z[12]
Moi	M[2]	Z[12]
Ver	M[2]	Z[12]
Ku80	M[2]	Z[12]

Table 5.3 Candidate interactors that may target Lhr to heterochromatin.

Interaction assay: M- IP/Mass spec, **W**-IP/western blot, **Y**-Y2 H, **C**-Cytological, **O**-Other . **References:** [1] [100], [2][76],[3][102] , [4][77], [5][107], [6] [79],[7][91], [8] personal communication [108] .

F**unctions/Phenotypes/Localization: T –** TE misregulation(ref), **R**-RNA processing, **C**- Centromeric function, **P**-chromatin maintenance, **Z**-Telomere function, **X**-no known function. **References for function etc-** [11][13], [12] (not a result of a screen) .

	+			ϕ *Hmr-HA, w*$^{+}$		
Rep	***f,w***	***w***		***f,w+***	***w+***	
	♀	♀	♂	♀	♀	♂
1	0	0	56	0	0	42
2	0	0	76	0	0	66

Table 5.4 *Hmr* is not the only locus on the *D. melanogaster* X chromosome needed for hybrid lethality. Crosses of *C(1)DX,ywf; ϕ Hmr-HA, w*$^{+}$*/+,* female and *w* ^{501} males were used to generate *D. melanogaster-D. simulans* hybrid males with the *D. simulans* X chromosome. Chi-square test was used to determine deviation from a 1:1 ratio between males with and without the transgene. The difference was not significant.

the imaginal discs of *Lhr*^{1} mutant hybrids, where Lhr can be expressed tissue specifically using the UAS/GAL4 system, as described in Chapter 4. These imaginal discs can either be used directly or be used to create cell lines. A simple assumption would be that such cell lines- with chromosomal complements from both species- are likely to be unstable. However, I argue that it is unclear if they would be considerably more unstable than cell lines from a single species alone, or what the effects of such instability would be. After all, these hybrid imaginal discs are capable of proliferating and differentiating in progeny from crosses of *D. melanogaster* mothers and *D. simulans* fathers and from the reciprocal crosses where the males are viable. Such cell lines or the imaginal discs themselves can provide tissue for immuno-precipitation as well as RNA-seq. I argue that this is better than using whole larvae. Whole larvae are a mix of polyploidy and diploid tissues and it is unclear if ploidy levels in hybrids proportionally represent both parental genomes. Additionally, many larval tissues are post-mitotic and maybe less affected by the genomic shock of hybridization.

The other problem preventing a complete understanding of the hybrid phenotype is what other genes, apart from Lhr and Hmr, are involved in setting up the hybrid incompatibility. Atleast one potentially undiscovered hybrid incompatibility locus is on the X chromosome of *D. melanogaster*. The *D. melanogaster X* harbours at least one known HI locus- *Hmr.* However, I have shown that *D. melanogaster- D.simulans* hybrid males, with the *D. simulans* X chromosome and the *D. melanogaster Hmr* are viable, arguing that there are additional regions on the *D. melanogaster* X chromosome that are necessary for hybrid lethality (Table 5.4). It is plausible that there are no other major effect regions but that the hybrid lethality is the result of a general divergence of the X chromosome. It must be mentioned that its unclear why such effects should also not arise from an equally diverged *D. simulans* X chromosome.

This region can be uncovered either by using traditional EMS mutagenesis coupled to DNA sequencing or by making hybrids with wild-type *D. simulans* fathers and *D. melanogaster* mothers which have a Compound X chromosome, a Y chromosome with an X chromosome duplication and the *Hmr* transgene on the third chromosome.Such a screen would even be able to pick up multiple minor effect loci on the chromosome. While this may seem like a lot of work, a series of such *D. melanogaster* lines can be generated in single generation crosses from a *C(1)Dx ywf, ϕHmr /ϕHmr* parental stock

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