The role of heterochromatin protein 1A(HP1a) in the repression of telomeric retroelements in the drosophila female germline

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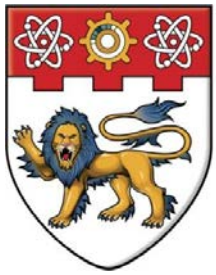

NANYANG TECHNOLOGICAL UNIVERSITY

THE ROLE OF HETEROCHROMATIN PROTEIN 1A(HP1a) IN THE REPRESSION OF TELOMERIC RETROELEMENTS IN THE *Drosophila* FEMALE GERMLINE

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2014

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Declaration

All experiments were performed by me aside from the bioinformatics analysis of the piRNA sequencing data. However the figures from the analysis were generated by me. Immunohistochemistry performed after the first revision of the thesis were done by Yee Wei (Ryan) Teo on stains generated by me.

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Abbreviations

***Drosophila* genes and proteins**

(Nomenclature: Gene names are italized, Dominant mutants begin with a capital while recessive are in small, Proteins begin with a capital letter)

Ago3	Argonaute 3
Armi	Armitage
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3-related protein homolog
Aub	Aubergine
C(3)G	Crossover suppressor on 3 of Gowen
Cav/HOAP	caravaggio
Da	daughterless
E(z)	Enhancer of zeste
Egg/ dSETDB1	eggless
endo-siRNA	endogeneous small interfering ribo nucleic acid
HipHop	HP1-HOAP-interacting protein
HP1a/ Su(var)205	Heterochromatin associated protein 1a/ Suppressor of variegation 205
HP2	Su(var)2-HP2
Mael	Maelstrom
Moi	Modigliani
MRN	marionette
Nos	nanos
Pc	Polycomb
Piwi	P-element induced Wimpy testes
Prod	proliferation disrupter
Rhi	Rhino

Rif1	RAP1 Interacting Factor
SpnE	Spindle E
Tj	trafficjam
Ver	verrocchio
Z4	putzig
γH2Av	Phospho Histone H2A variant

Other abbreviations

ChIP	Chromatin Immuno-precipitation
DAPI	4',6-diamidino-2-phenylindole
Dfs	dominant female sterile
DNA	Deoxyribonucleic acid
DSB	Double stranded breaks
GAL4-UAS	GAL4 upstream activator sequence
GFP	Green fluorescent protein
H3K9me	Histone 3 Lysine 9 methylation
H3K9me2/3	Histone 3 Lysine 9 di/trimethylation
HMT	Histone methyltransferase
HTT	*HeT-A, TART, TAHRE*
LINE	Long Interspersed Nuclear element
LTR	Long Terminal Repeat
miRNA	micro RNA
mRNA	messenger RNA
MTOC	Microtubule organizing centre
nt	nucleotide
ORF	Open Reading Frame
PB(S/X)	Phosphate Buffer(Saline/ Triton X)
PEV	Position Effect Variegation

PH3	phosphorylated Histone 3
piRNA	piwi interacting RNA
qRT-PCR	quantitative Reverse Transcriptase Polymerase Chain Reaction
rasi-RNA	repeat associated small interfering RNA
RNA	Ribonucleic acid
RNA PolII	RNA polymerase II
$RNAi^{HP1a2/3Chr}$	TrIP lines with hairpin RNA construct on the 2^{nd} and 3^{rd} Chromosome respectively
RP	Ribosomal protein
RT	Reverse Transcriptase
siRNA	small interfering RNA
TAS	Telomere associated sequence
TGS	Transcriptional Gene Silencing
TSE	Telomeric Silencing Effect
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Abstract

Transposable elements can cause deleterious mutations to the genome and thus organisms evolved a robust mechanism to control transposition events. The *Drosophila* germline has a specialized small RNA silencing pathway called Piwi interacting RNA pathway (piRNA pathway) for silencing transposable elements (TEs)(Aravin et al. 2007). Heterochomatin Protein 1a (HP1a) is an important component necessary for heterochromatin maintenance. It has been shown that HP1a interacts with Piwi, a central member of the piRNA pathway for TE silencing function in somatic cells (Brower-Toland et al. 2007; Pal-Bhadra et al. 2004). I wanted to study the specific role of HP1a in Drosophila germline using RNAi and clonal analysis, to investigate if HP1a also has a piRNA related function in the germline.

Knockdown of HP1a in the female germline cells led to de-repression of telomeric TEs, *HeT-A, TART* and *TAHRE,* while there was a minimal effect on other TEs. By contrast, upon HP1a loss in the gonadal somatic cells, expression levels of the telomeric TEs remained unaffected, indicating that HP1a exclusively functions in germline for suppression of telomeric TEs. Deep sequencing analysis of small RNA library from HP1a knockdown ovaries showed severe reduction in piRNAs mapping to the telomeric TEs, without any significant decrease in overall transposons mapping piRNA levels, corroborating the TE de-repression pattern. I also observed a reduction in piRNA originating from piRNA clusters having *HetA* copies (Brennecke et al. 2007). HP1a absence led to a significant reduction of heterochromatic signature; tri-methylation at 9^{th} lysine of Histone H3 at telomeric reteroelements. ChIP-seq for HP1a occupancy on the germline chromatin showed enrichment at the transposon loci at pericentromeric and telomeric heterochromatin region; where major piRNA clusters are located.

HP1a is known to be required for telomere capping in *Drosophila.* Telomere maintenance in fruitfly is based on controlled transposition of the telomeric TEs, *HeT-A, TART* and *TAHRE*. In the germline the piRNA pathway has been suggested to be required for telomere replication (Savitsky et al. 2002; Savitsky et al. 2006). I wanted to ask if HP1a was working in the piRNA pathway to regulate telomeric TEs in the ovaries.

Since the piRNAs against other TEs remained unaffected and perinuclear (nuage) localization of downstream components of the piRNA machinery was not perturbed, I hypothesized HP1a probably functions in primary piRNA cluster transcription from telomeric clusters in the female germline. To address if selective cluster transcription occurs for telomeric transposons analysis of data from deep sequencing and qRT- PCR using cluster specific primers was performed.

The novelty of these findings is in the specific role of HP1a in conjunction with the piRNA pathway in repressing a subset of retroelements. The lines of evidence suggest that HP1a cross talks with the piRNA pathway in *Drosophila* female germline specifically for telomere maintenance.

1. Introduction

Repetitive DNA sequences that can change position within the genome are called transposons (McClintock, 1950; McClinock, 1951). These transposons occupy a significant percentage of the genome (nearly 50% of the human genome Lander et al. 2001; Konkel and Batzer 2010). The mobile genetic parasitic elements are usually maintained as facultative heterochromatin in the pericentromeric or telomeric regions of chromosomes. Heterochromatinization helps organisms repress the expression of transposons and hence prevent their genome from deleterious effects of transposon mobilization (Grewal and Jia 2007).

The germline of an organism is essential for continuity of sexually reproducing multicellular animals. Thus, all animals deploy specialized mechanisms to maintain germline genome integrity especially to control transposons. There is a well conserved small RNA based defence mechanism that represses transposon mobilization; namely the piRNA pathway in both somatic and germline cells but the pathway is more intricate in the germline (Aravin et al. 2008; Malone and Hannon 2009).

Thus, animals have two main methods to combat transposons: heteochromatinization and RNAi based defence. Recent evidence from *Drosophila* suggests that there may be a connection between the piRNA pathway and establishment of heterochromatin to silence transposons. Two candidate proteins for this hypothesis are Piwi, - the only nuclear Argonaute family protein known to associate with piRNAs and HP1a is part of all facultative heterochromatin (Sentmanat et al. 2013). Most of the focus so far has been on Piwi so I focused on the role of Heterochromatin associated protein (HP1a) in transposon silencing using the *Drosophila* germline as a model.

1.1 *Drosophila* female germline

Drosophila has been used as a model organism for genetics since T.H Morgan began his experiments in 1910. The century long study on it has enabled genetic tools to be widely available making it an ideal choice for geneticists. Also it is inexpensive to maintain and has a short generation time of 10 days.

The *Drosophila* germline is well characterized and the tissue morphology allows distinction between different cell types (Spradling, 1993). The female germline consists of a pair of ovaries each housing 15-16 ovarioles. Ovarioles begin with a germarium hosting 2-3 germline stem cells which divide asymmetrically; one continues to differentiate progressively to form the cyst and then egg chambers and finally the egg. The cystocytes remain connected to one another by cytoplasmic bridges or ring canals until the 16 cell stage. 15 of these serve as the nurse cells that nourish the oocyte. All the germline nurse cell nuclei are polyploid making visualization of the nuclei easy. The germline cells are surrounded by a layer of somatic follicle cells. This makes comparative studies between germline and somatic cells feasible within the same tissue (Refer to Figure 1.1).

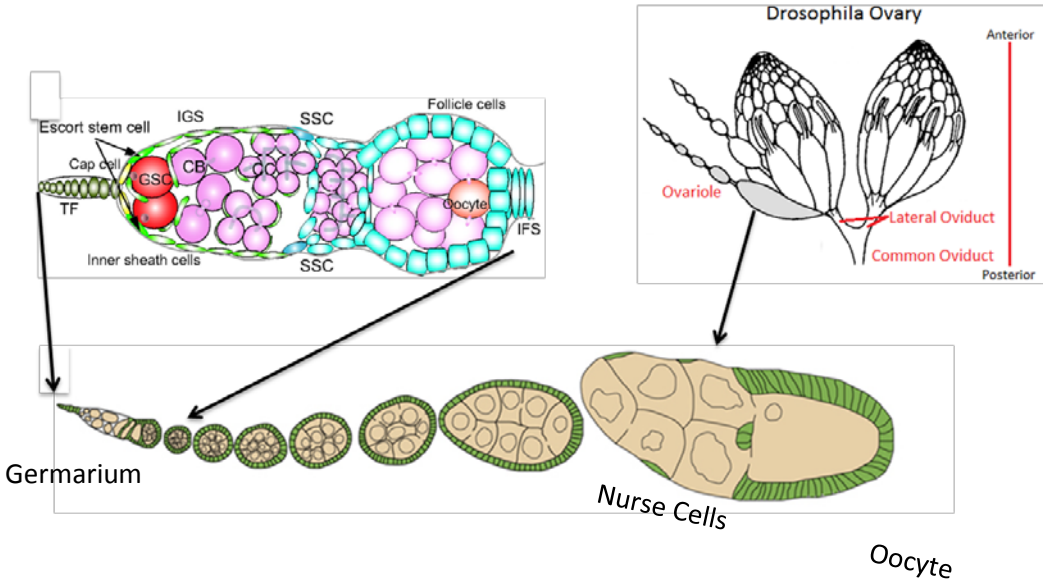


Figure 1.1 : **The *Drosophila* female germline.**

The *Drosophila* female germline contains a pair of ovaries made up of 16-18 ovarioles. Wild-type germarium. GSCs are located adjacent to the terminal filament (TF) and cap cells, and they divide to produce another GSC and a cystoblast (CB). The CB will subsequently divide synchronously four times to produce a cyst of 16 cystocytes (CC), one of which will differentiate into the oocyte. A number of inner gonial sheaths (IGS) line the outside of the germarium in the region where the initial four divisions of the CB occur. The transitory escort cells derive from IGSs adjacent to the cap cells. The SSCs are located posterior to the IGSs and are responsible for producing the follicular epithelium. Each chamber is separated by interfollicular stalks (IFS), which are derived from SSCs. The GSC differentiates to give rise to egg chambers which have a layer of somatic follicle cells (shown in green) surrounding the (beige) germline cells. The germline cells consist of 15 nurse cells and 1 oocyte.Adapted from Handler et al. 2011 and Niki Y et al. 2006.

1.2 Transposons in the *Drosophila melanogaster* genome

1.2.1Transposons

In 1951 Barbara McClintock first proposed the existence of transposons (McClintock 1950; McClintock 1951) . Transposons are mobile genetic elements consisting of repeat sequences and often a sequence coding for a transposase to enable hopping. They can be classified into two different categories based on the molecular intermediates during transposition: DNA transposons and retrotransposons. DNA transposons mobilize via a cut and paste mechanism, while retroelements employ a copy and paste mechanism via an RNA intermediate. The retroelements can be further classified into Long terminal Repeats (LTR) and non-LTR categories (Capy 2005). Transposons occupy as much as 50% of the human genome and 15-20% of the *Drosophila* genome (Hoskins et al. 2002; Kaminker et al. 2002). A question that haunts biologists is why such potentially mutagenic elements have been tolerated through evolution. Current hypothesis proposes that transposons play an important role in evolution but there is weak evidence for this premise (Hurst and Werren 2001 and references therein).

Drosophila melanogaster contains 96 transposon families of which the LTR are most abundant (Appendix 1). Much of the heterochromatin remains un- sequenced or unannotated in *Drosophila* so the transposon insertions are not well accounted for. However, there have been reports on euchromatic insertions of transposons which gives an idea of their genome occupancy (Kaminker et al. 2002). Of course copy number and insertional variations occur among different stocks but massive changes would lead to genome instability and probably would not be tolerated.

1.2.2 Detrimental effects of transposon mobilization

Uncontrolled transposition leads to severe genomic instability (Orgel and Crick 1980). In order to avoid such circumstances animals have evolved a conserved small RNA based defence mechanism called the piRNA pathway (Vagin et al. 2006). In the somatic cells a combined action by endo-siRNA and piRNA fight against transposons whereas in the germline the piRNA pathway is more significant and complex

(Rehwinkel et al. 2006; Czech et al. 2008). In the absence of the piRNA pathway most prominent phenotype is female sterility and male sterility in flies and mice, respectively (Aravin et al. 2008; Brennecke et al. 2008).

The phenotypes associated with uncontrolled transposition events can be studied in mutants of the piRNA pathway where the defence mechanism against transposons is disrupted. Since the *Drosophila* female germline is my model system I would like to enumerate the defects that are observed upon transposition events in piRNA pathway mutants in the fly ovaries (reviewed in Khurana and Theurkauf 2010).

Aberrations in the *Drosophila* ovaries in piRNA pathway mutants:

1) **DNA damage** manifested as **double stranded breaks** (DSB) at region 2a- the 16 cell cyst stage (Figure 1.2 B). However, there has been no evidence which shows a direct link between transposition events and DSB formation.
2) **Axis specification and MTOC assembly defects** seen as Gurken and Oskar (two of the many asymmetrically distributed components required for appropriate germline development) mislocalization (Figure 1.2 A). These defects probably arise as secondary effects due to DNA damage checkpoint activation as a result of DSB formation. This conclusion is based on the fact that a DNA damage checkpoint and piRNA pathway double mutant rescues axis specification and MTOC defects (Klattenhoff et al. 2007).
3) **Disruption of the nuage**- a gemline specific electron dense perinuclear organelle. The nuage is the site for piRNA amplification (ping-pong cycle) in the germline that helps maintain significantly higher pools of piRNA to combat transposons in the germline. Most piRNA pathway components localize to this organelle most probably in a hierarchical manner and the loss of one can affect recruitment of downstream components resulting in mislocalization of the nuage proteins as seen in the Figure 1.2 C (Pek et al. 2012).
4) **Reduction of piRNAs** as determined through deep sequencing of several piRNA pathway mutants (Refer to Table 1.1; Malone et al. 2009). Different mutants have distinct profiles and magnitudes of piRNA loss.

#piRNA_cluster	chr	Aub -/-	Armi -/-	Flam -/-	Krimp -/-	Piwi -/-	SpnE -/-	Squ -/-	Vasa -/-	Zuc -/-
42AB	arm_2R	3.8	31.6	1.9	19.3	5.7	66.8	1.9	6.2	4.2
piRNA_cluster_02	arm_X	2.6	19.6	3.0	3.4	6.0	28.8	2.2	3.0	5.8
piRNA_cluster_05	arm_2L	2.9	12.9	1.1	17.6	17.8	32.9	3.6	3.5	2.8
piRNA_cluster_06	arm_3L	5.3	47.7	1.7	22.8	9.0	69.0	2.4	5.0	6.0
flamenco	arm_X	1.4	0.8	33.2	0.4	39.8	0.8	0.9	0.8	4.6
piRNA_cluster_08	arm_X	7.8	32.8	1.9	15.9	1.6	37.9	3.6	6.2	5.4
piRNA_cluster_10	3LHet	4.5	5.2	2.2	5.1	4.9	10.4	2.4		
piRNA_cluster_11	3LHet	3.5	16.8	1.6	14.3	5.4	38.9	2.7		
piRNA_cluster_12	3LHet	2.8	23.9	1.5	13.1	5.8	33.6	2.9	4.2	3.9
piRNA_cluster_13	3RHet	4.7	20.8	1.7	16.0	5.4	29.7	3.1	3.7	4.4
piRNA_cluster_14	ArmU	1.6	1.3	1.7	1.3	2.2	1.2	0.8	1.5	3.3
piRNA_cluster_15	ArmU	3.0	3.2	1.5	4.8	0.9	6.3	1.8	1.9	4.4
piRNA_cluster_16	arm_3R	2.2	4.2	0.9	0.5	0.9	1.6	1.0	0.6	5.0
piRNA_cluster_17	3LHet	4.1	14.6	1.6	8.3	3.4	22.9	3.1	6.8	3.6
piRNA_cluster_18	2RHet	1.6	1.6	0.9	2.2	2.2	6.1	2.9	1.3	2.7

Table 1.1 **piRNA profiles of sequenced small RNAs in piRNA pathway mutants** (adapted from Malone et al. 2009). The table shows the fold change of piRNAs in comparison to the heterozygotes of each genotype. This table has been shown to give the reader an idea of the fold up-regulation of transposons in piRNA pathway mutants.

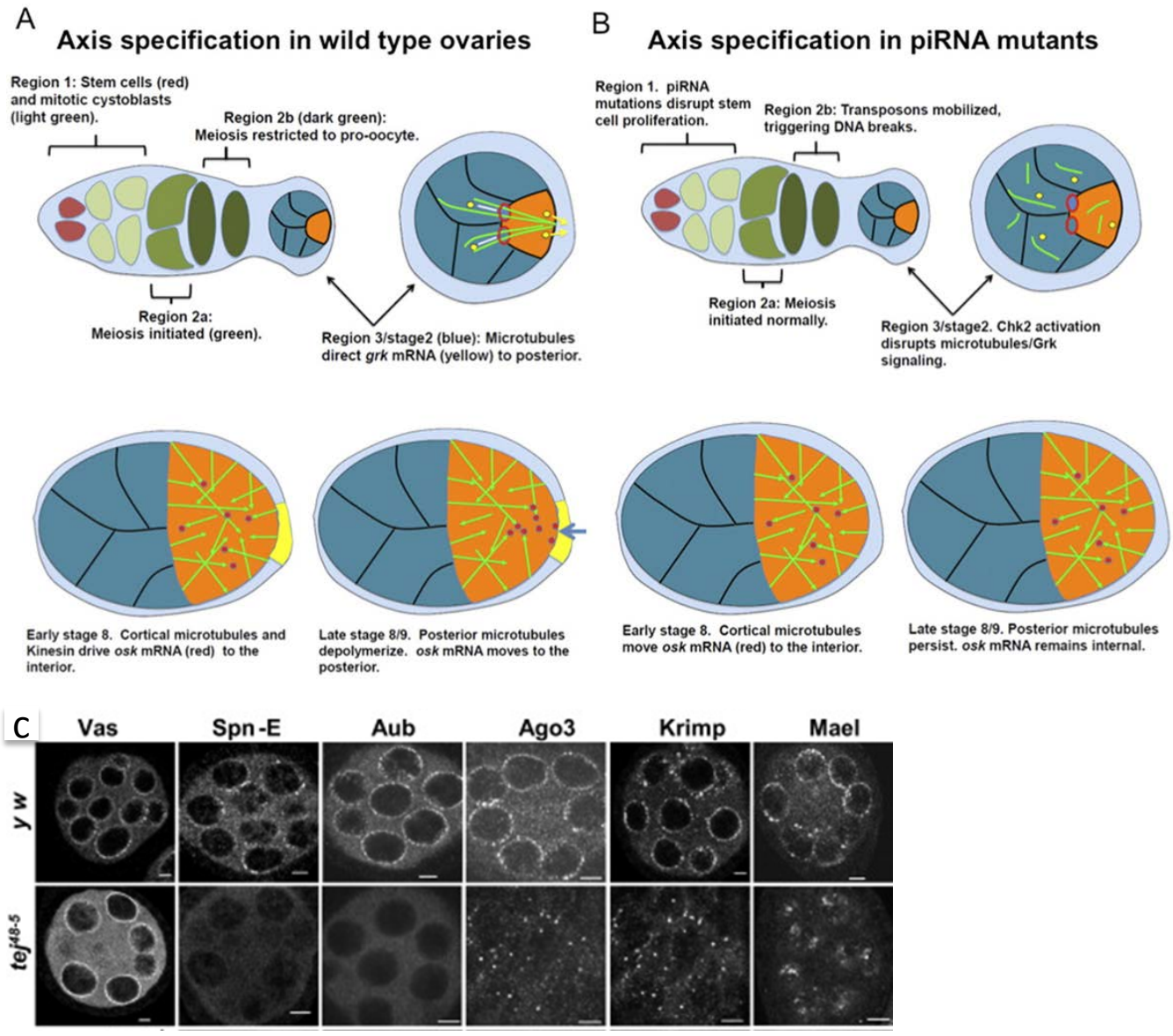


Figure 1.2 **Defects in the *Drosophila* female germline due to transposon mobilization**

(A,B) The axis specification in the germarium and MTOC organization in the oocyte is impaired piRNA pathway mutants (Adapted from Khurana and Theurkauf 2010).

(C) shows the nuage component (Vasa, Spindle E, Aubergine, Argonaute 3, Krimper and Maelstrom) mislocalization in piRNA pathway mutant tejas (adapted from Patil and Kai 2010).

1.2.3 Transposons - a necessary evil

Transposons are not always detrimental. A recent report discusses the role of transposons in long term memory formation in the *Drosophila* αβ neurons where transposons are allowed to be active (Perrat et al. 2013). Phenomena like Position Effect Variegation (PEV), Xist (X chromosome inactivation for dosage compensation in females), chromatin insulators for certain genes, paramutation (a phenomenon where an allele can induce heritable changes in the other allele without DNA sequence changes), epialle formation, parent of origin effect in imprinting and V(d)J recombination for antibody variability also depend on transposons (reviewed in Slotkin and Martienssen 2007). Other than that transposons aid heterochromatin formation which is essential for centromere and telomere formation in most organisms (Pidoux and Allshire 2005). Centromeres are essential during replication for chromosome segregation. Telomeres prevent chromosome shortening and fusions and have also been implicated in cancers (Autexier and Greider 1996). The role of transposons in telomere establishment is most well characterized in flies. My study on HP1a led me to look into the mechanisms of telomeric transposon regulation so I will focus on elaborating the background on *Drosophila* telomeres in a later section.

1.3 The piRNA pathway – defense mechanism against uncontrolled transposition

Transposons are highly active in the germline genome. The organisms have thus evolved a more intricate pathway to ensure a larger population of piRNAs to counter their effect in this tissue. Much of the knowledge on the piRNA pathway comes from studies on model organisms *Drosophila melanogaster, C.elegans*, or mouse. Each has some unique features but most of the machinery is highly conserved (Girard et al. 2006).

The existence of piRNAs was first suggested in 1990 by Livak, although there was no molecular evidence (Livak 1990). A decade later, several groups concomitantly reported the first molecular evidence for piRNAs (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Watanabe et al. 2006). Initially they were called rasi- RNAs (repeat associated small interfering RNAs) owing to their complementarity to the repeat rich regions on the genome (Aravin et al. 2001; Aravin et al. 2003). In the two following sections I will summarize the piRNA pathway as it stands today and also discuss the questions that remain to be addressed.

1.3.1Known features of the piRNA pathway

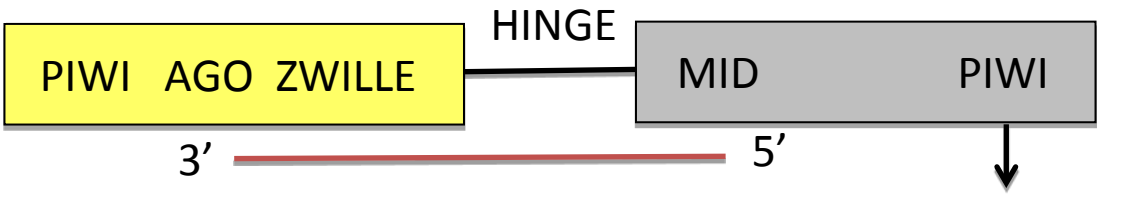
piRNAs are 26-30 nucleotide RNA molecules that differ from other small RNAs in their Dicer independent processing (Vagin et al. 2006). They are loaded onto Piwi family of proteins to silence transposons post transcriptionally via complementary base pairing and RNase H activity on transposon transcripts, or transcriptionally through chromatic modifications and sometimes through translational inhibition (reviewed in Luteijn and Ketting 2013). Recently non transposon related function for piRNAs in relation to memory formation and early development have been reported (Lee et al. 2011; Rajasethupathy et al. 2012).

1.3.1.1 Characteristics of piRNAs :

1) They are longer than most small RNAs known so far; **26-30 nucleotides** while miRNA and endo-siRNA are 21-23nt long on an average.

2) No **secondary structure** has been identified yet
3) They have a **5' U bias**
4) Their **3' end has a 2'O-methylation** which is conserved also in plants and probably ensures stability of the molecule.
5) The biogenesis involves a dicer independent process. piRNA **precursors are single stranded** and their generation is still not well understood.
6) They are loaded onto the highly conserved **Piwi family proteins** that have two major domains connected by a hinge region (Figure1.3). *Drosophila* has 3 Piwi family proteins. Piwi is present predominantly in somatic and germline nuclei in the gonad. Two others, Ago3 and Aub are germline specific and localize to the cytoplasm and perinuclear nuage. Mice have 3 variants Miwi, Mili and Miwi2 and humans have 4 Hiwi, Hili, Hiwi2 and Hiwi3 while *C. elegans* has 3 Piwi family proteins (Hock and Meister, 2008).

A



B

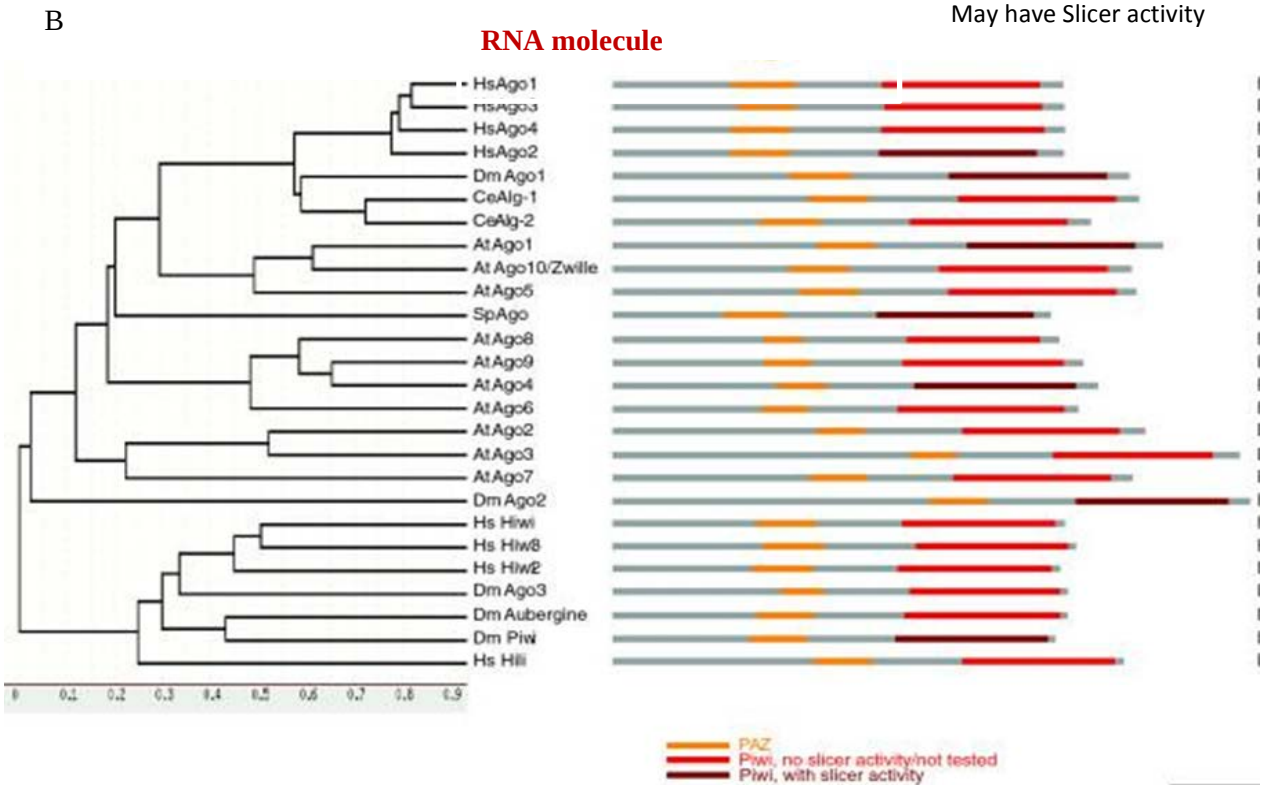


Figure 1.3 **Domains architecture and conservation of Piwi family proteins**

A. Piwi proteins have 2 domains connected by a hinge region. The N-terminal domain contains PIWI, ARGONAUTE (AGO) and ZWILLE domains and the C terminal has a MID and PIWI domain. The N terminal domain is essential for piRNA loading onto PIWI proteins. The 3'end of the piRNA molecule (depicted in red) binds the AGO domain. This bends the molecule and allows the 5' end of the RNA to bind the MID domain. The PIWI domain has RNAseH activity and is believed to cleave target RNA molecules by endonuclease activity (based on *in vitro* data).

B High conservation of Piwi family proteins across animal species (adapted from Hock and Meister 2008).

7) In all organisms they function **predominantly in the germline**.
8) They exhibit **poor conservation** in closely related species unlike miRNA but are **highly syntenic** (Farazi et al. 2008).

1.3.1.2 The biogenesis and functions of piRNAs

In this section I will summarize the steps for two different piRNA biogenesis pathways in the *Drosophila* follicle cells/ Ovarian Somatic cell lines and germline cells respectively. Mechanisms and necessary factors are tabulated below.

STEP 1: Production of long piRNA precursor transcripts

Firstly precursor transcripts, some upto 100 kilobases, arise from specific genomic loci called clusters that house multiple full-length or partial copies of repeat elements.

	CLUSTERS*	TRANSCRIPTION	FACTORS
SOMATIC (ovarian follicle cells or OSS)	Flamenco X (20A-B) which gives rise to most of the transposon matching piRNAs mainly against the gypsy family of retrotransposons. The Traffic jam locus is responsible for Piwi expression regulation	Unidirectional transcription	Factors involved in transcription are not well known. Piwi and HP1a might play an epigenetic role.
GERMLINE (ovarian germline)	42AB (largest)(2R) X-TAS (1A), 3R(100F), 4(102E) (sub-telomeric/telomeric) 2L(38C) 3L(80E-F) Some unmapped loci	Bidirectional transcription	Epigenetic marks by germline specific variant HP1d (Rhino) and Cutoff play a role in transcription.[1]

*(Brennecke et al. 2007)[1](Klattenhoff et al. 2009)

STEP 2: Export of the precursor cluster transcripts from the nucleus to the cytoplasm for further processing.

	MECHANISM	SITE	FACTORS
SOMATIC(ovarian follicle cells or OSS)	Mechanism and factors of transport are unknown protein	Cytoplasmic organelle Yb body(associated with mitochondria) is the processing body	Other components in the pathway: Tudor domain protein: Vreteno Helicase: Armitage Yb[1]
GERMLINE (ovarian germline)	UAP56, the nuclear channel is involved in transport of the piRNA to the cytoplasmic/perinuclear component Vasa *	The electron dense perinuclear organelle nuage is the cytoplasmic processing body that amplifies the germline pool of piRNAs via a ping pong cycle(figure1.4)[2]	Other components in the pathway: Post-translational modifications of Piwi proteins includes SDMA which can be recognized by tudors Tejas, Krimper, SPnE Helicase: Vasa, SpnE Piwi: Aub, Ago3 HMG: Mael Cutoff[3]

*(Zhang et al. 2012)[1](Saito et al. 2010)[2]Nuage components reviewed in (Pek et al. 2012)[3](Kirino et al. 2009)

STEP 3: 5' end generation with a U bias and loading onto Piwis

	FACTORS	SEQUENCE	LOADING:
SOMATIC	No in-vivo evidence. In vitro candidate Zucchini.* (*C. elegans* has no homolog). Mouse mutant of Zucchini homologue has lower number of processed piRNAs.	Unknown how U bias is generated	Loading onto Piwi occurs through chaperone Hsp90 homologue (Hsp83) and co-chaperone Shutdown. Chaperone Hsp 83 and co-chaperone Shutdown are required in the germline for loading onto Aub and Ago3.[2]
GERMLINE	Aub (binds antisense piRNAs) and Ago3 (binds sense piRNAs) work in an amplification loop called ping pong cycle to generate 5' ends. (Figure1.4)[1]	Sequence complementarity leads to cleavage site determination	

*(Nishimasu et al. 2012) [1](Aravin et al. 2007) [1](Gunawardane et al. 2007) [2](Ipsaro et al. 2012; Olivieri et al. 2012; Preall et al. 2012; Izumi et al. 2013)

STEP 4: 3' end generation and 2'O methylation. Somatic and germline pathways probably converge at this juncture.

The factor that generates the 3' cleavage is yet to be identified. Size bias variability among different Piwi family members is probably owing to the protein occupancy on RNA molecule. Piwi bound piRNAs are on average around 26-nt long, Ago3-bound ones are 24-nt long, while Aub-bound ones are 25-nt (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Watanabe et al. 2006). This bias probably arises because of the size of the proteins themselves i.e., the protein occupancy on the RNA molecule. This hypothesis comes from *in vitro* studies (Kawaoka et al. 2011).

Methylase Hen1 is involved in the 2' O methylation and this methylase is well conserved (Saito et al. 2007 ; Horwich et al. 2007).

STEP5: Silencing targets

STEP 5a: Post-transcriptional silencing by germline piRNA

In this process the Piwi-piRNA complex recognizes the target mRNA via sequence complementarity and the transcript- piRNA duplex is destroyed most probably by slicer activity of Piwi family proteins (Gunawardane et al. 2007). Perfect sequence complementarity between mRNA and piRNA is not necessary at least in case of *C. elegans* (Bagijn et al. 2012) .

STEP 5b: Transcriptional silencing by Piwi- piRNA complex in soma and germline

Piwi proteins enter the nucleus after piRNA loading (Ishizu et al. 2011). This implies that they have a role in transcriptional gene silencing (TGS). TGS has been modelled along *S. pombe* where the RNA-protein complex recognizes the nascent pericentromeric transcripts. This is followed by the recruitment of the Histone Methytransferase (HMT) for H3K9 modification and the HP1 homologue (Swi6) is recruited. Swi6 can then compact DNA and establish heterochromatin and also spread heterochromatin marks through dimerization (Nakayama et al. 2001; Buhler and Gasser 2009). In flies, loss of Piwi both in the germline and soma led to H3K9me3 loss (this data is controversial as several reports provide contradictory evidence) and RNA polII occupancy increase at the silenced loci. PolII increase correlated with nascent transcript increase (Wang and Elgin 2011; Le Thomas et al. 2013; Rozhkov et al. 2013). Studies on Ovarian Somatic cells (OSS) showed that even when H3K9me3 levels are normal, the absence of HMG protein Mael leads to failure of TGS suggesting a H3K9me3 independent mechanism or other downstream components for TGS (Sienski et al. 2012).

Evidence for epigenetic inheritance through generations has only been noted in *C. elegans* where TGS can be maintained independent of Piwi (Luteijn et al. 2012). Such a mechanism has not been observed in flies yet.

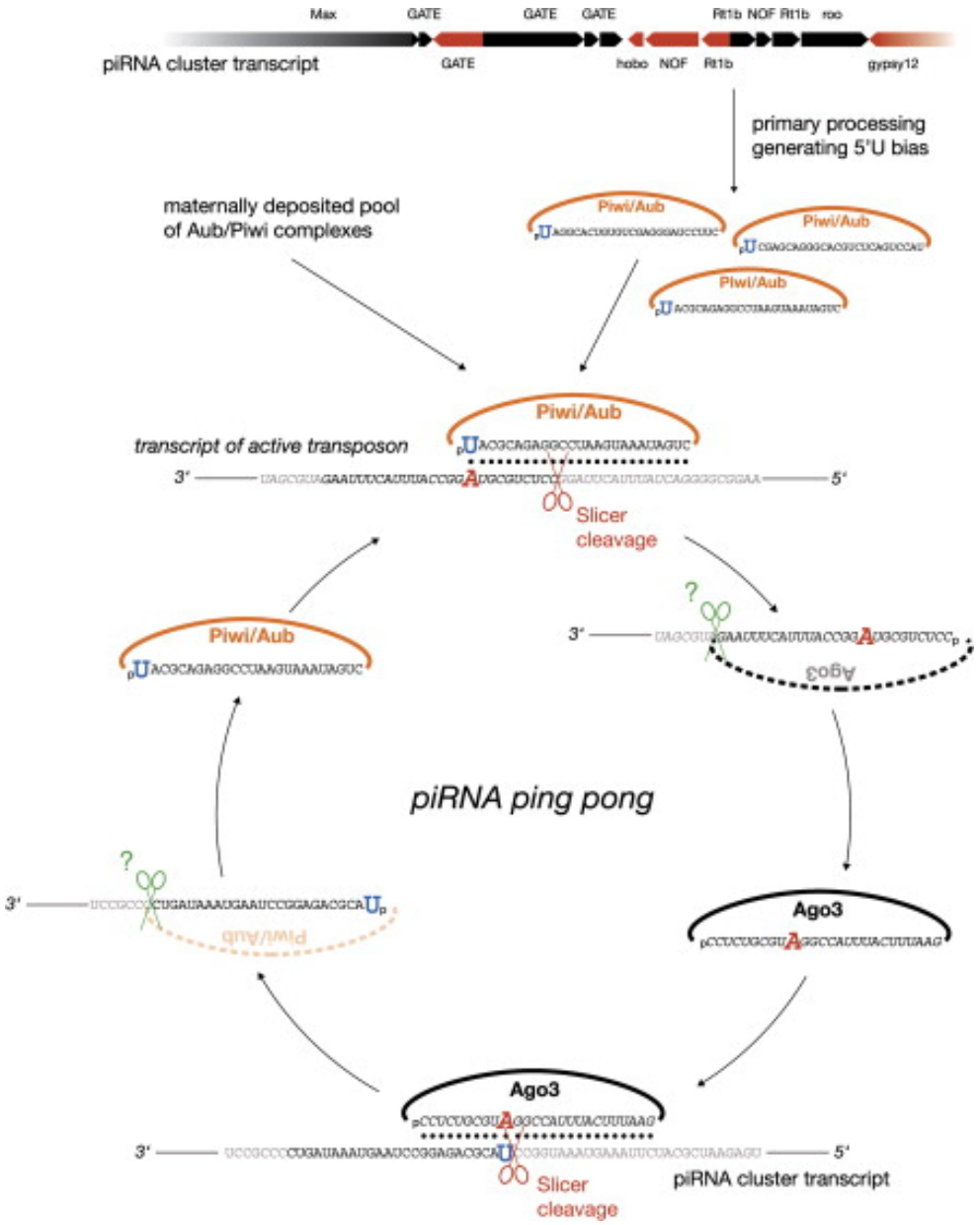


Figure 1.4 **Biogenesis of piRNAs in the *Drosophila* female germline**

The primary piRNAs processed from cluster transcripts or obtained through maternal deposition are usually in antisense orientation to the transposons and can be bound by Piwi and Aubergine (Aub). This Piwi/Aub piRNA complex recognizes nascent transposon transcripts through sequence complementarity and through slicer activity specifically chops the transposon transcript at a particular base. This newly sliced sense transcript can then be bound by Ago3 and target new transcripts and cleave them to generate an antisense template for Aub/Piwi to bind (Ping -pong amplification loop). Aub and Ago3 bound transcripts show a 10 nucleotide overlap supporting the model. The Ping- pong cycle is unique to the ovarian

germline to generate a large pool of piRNAs to combat active transposons (Adapted from Brennecke et al. 2007).

1.3.2Open questions in the piRNA field

1) What are the precursors for piRNAs?

The first major question is where and how these small RNAs transcribe from the genome. In the fly, specific loci on chromosomes termed piRNA clusters have been identified as regions that give rise to the precursor transcripts that are later processed to piRNAs but the mechanism remains uncharacterized (Brennecke et al. 2007). Till date, transcription of the *C. elegans* 21U RNAs (the piRNAs) is best understood. *C. elegans* have individual and separate piRNA genes characterised by a 40 base pair motif upstream of each piRNA sequence. Forkhead family of transcription factors recognize this motif and begin transcription at a particular base and all *C.elegans* piRNAs are 26 nucleotides long. It is not known how transcription is terminated precisely at the 26^{th} base (reviewed in Luteijn and Ketting 2013). In mice A-MYB transcription factors have been associated with piRNA precursor transcription but the details remain elusive. In flies there is no fixed length for the piRNAs and precursors as long as 100 kilobases have been observed (Li et al. 2013).

2) How are these precursors targeted for piRNA processing and transported out of the nucleus?

In flies, UAP56 may be involved in nuclear to cytoplasmic transport. This has been based on Vasa-bound primary transcript reduction in UAP56 mutant (Zhang et al. 2012).

3) How does the cell know that they need to process a particular transcript for piRNA production?

One hypothesis is the epigenetic marks laid at the genomic loci may serve as a guide for other proteins to associate and prime the transcript for subsequent piRNA processing. In support of this theory, in the *Drosophila* female germline a HP1 variant HP1d/ Rhino has been shown to be associated with dual strand clusters (Klattenhoff et al. 2009). However, there have been no direct experiments to confirm this.

4) **5’ end generation**

In the *Drosophila* germline 5’ end generation occurs via Piwi family protein induced cleavage however in the somatic cells the Piwi variant is absent which leaves an open question for a candidate. In vitro data suggests this role may be achieved by Zucchini (Nishimasu et al. 2012). However, there is no clear *in vivo* evidence in support of it.

5) **Loading of the RNAs onto Piwi has only been studied in vitro and conserved** Kawaoka et al. 2011 showed that Hsp90 homologue Shutdown is suggested to be a chaperone for the process of loading however, *in-vivo* evidence is lacking (Olivieri et al. 2012).

6) **3’ end generation**

It is unknown which enzyme is responsible for 3’ end is generation. Most organisms studied aside from *C. elegans* seem to have a variable length of piRNA molecules.

The conserved enzyme, Hen1 is responsible for 3’ OH methylation of piRNA molecules however Hen1 mutants show little or no visible phenotypes (Saito et al. 2007; Kamminga et al. 2010). The methylation probably serves to stabilize the piRNA molecule and further study is needed to confirm this.

7) **An apparent paradox stems from the fact that a piRNA producing locus and its target are marked by the same heterochromatin marks**. (Figure1.5) Recent studies have shown that a usually transcribed locus can be silenced, in trans and through generations in the presence of homologous piRNAs against that locus (de Vanssay et al. 2012). The concept of master loci which serve as the source of piRNAs has been challenged in a recent study where euchromatic transposon insertions converted the euchromatic locus as a piRNA or endo-siRNA generating locus (Brennecke et al. 2007; Shpiz et al. 2014).

This paradox and the epigenetic mechanisms involved in the piRNA pathway were the major questions I wanted to address though this study and I chose to focus on *HP1a* since it is a highly conserved component whose function remains elusive. This study led me to the unique nature of *Drosophila* telomere maintenance and the role of

piRNA in the process which is why I want to elaborate on telomeres in flies in the next section.

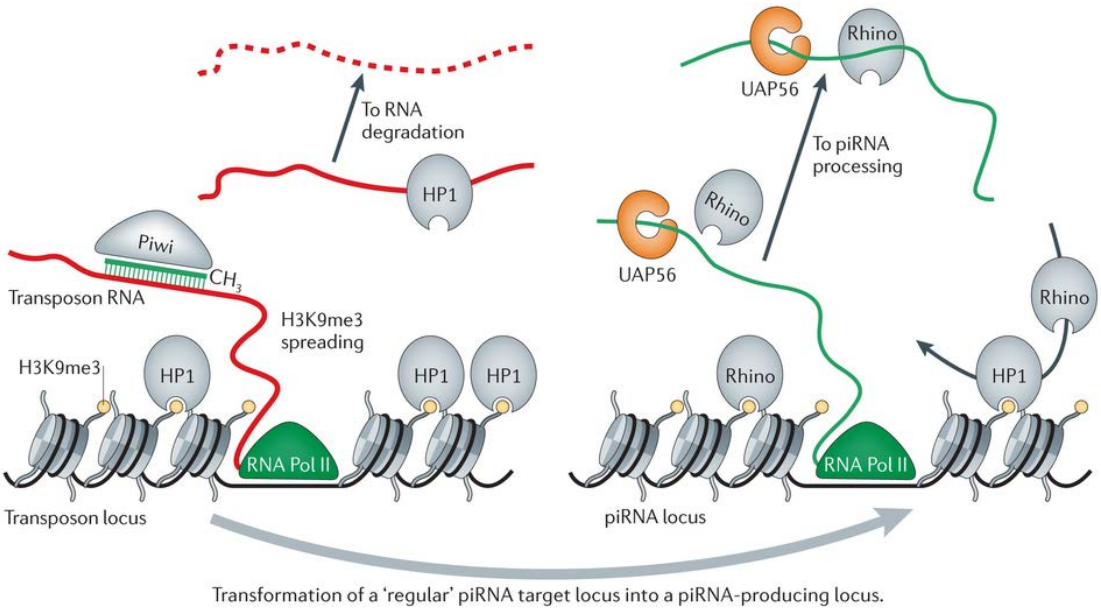


Figure 1.5 **Paradox: piRNAs silence the locus from which they are transcribed**

Current hypothesis states that piRNAs can ensure cluster transcription for their own precursors yet silence the transposon mRNA transcription from the very same locus by an epigenetic process. Variants of HP1 like Rhino are known to associate with clusters to ensure transcription for piRNA precursors and maybe the presence of this variant primes the transcript to be targeted for piRNA processing. However this model needs experimental confirmation. Adapted from (Luteijn and Ketting 2013).

1.4 piRNA pathway in telomeres

1.4.1 ***Drosophila*** telomeres

Chromosome ends are in jeopardy of gene erosion due to under-replication or being recognized by the damage check point and hence fusions (Biessmann et al. 2005). Hence, organisms have various mechanisms to ensure the ends of chromosomes are protected. Most eukaryotes have telomerases which are RNA enzymes that add stretches of repetitive DNA sequences to chromosome ends (Greider 1996; Shpiz 2012).

A unique feature in *Drosophila* is that it lacks a telomerase. Telomere maintenance depends on controlled transposition of three 3 non LTR/ LINE (Long Interspersed Nucleotide elements) retrotransposons *HeT-A*, *TART* and *TAHRE* (Biessmann et al. 1990a; Biessmann et al. 1990b; Levis et al. 1993; Abad et al. 2004b; Pardue et al. 2005). These retroelements rarely occupy the euchromatic region (refer table: highlighted in appendix 7.1; (Kaminker et al. 2002). *HeT-A* is the most abundant telomeric transposon followed by *TART* and *TAHRE* respectively. There are just three copies of *HeT-A* are present on the third chromosome. However all three non LTR retrotransposons are clustered in the heterochromatic telomeres (Figure 1.6).

A

B

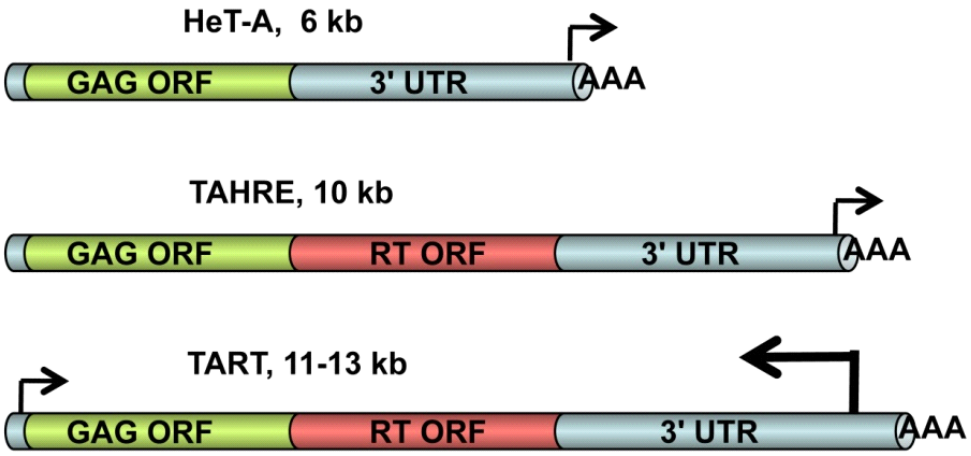


Figure 1.6 ***Drosophila*** **Telomeres**

A. Basic structure and domains of the telomere (from left to right). The cap (black bullet) marks the end of the telomere. This is followed by the Telomere Associated Sequence (blue rectangles TAS) and then the retrotransposon array (red arrows) of *HeT-A*, *TART* and *TAHRE* arranged with their 5' ends facing the telomere and 3' towards centromere. Adapted from Sphiz et al, 2014.
B. The retrotransposons *HeT-A*, *TART* and *TAHRE*. *HeT-A* is 6 kb long and the most abundant element. It is the most important element in telomere maintenance. However it lacks an Reverse Transcriptase(RT) Open Reading Frame (ORF). *TART* RT ORF provides *HeT-A* Gag reverse transcriptase in trans during transposition. *HeT-A* and *TAHRE* share high sequence similarity (adapted from Mason et al, 2011).

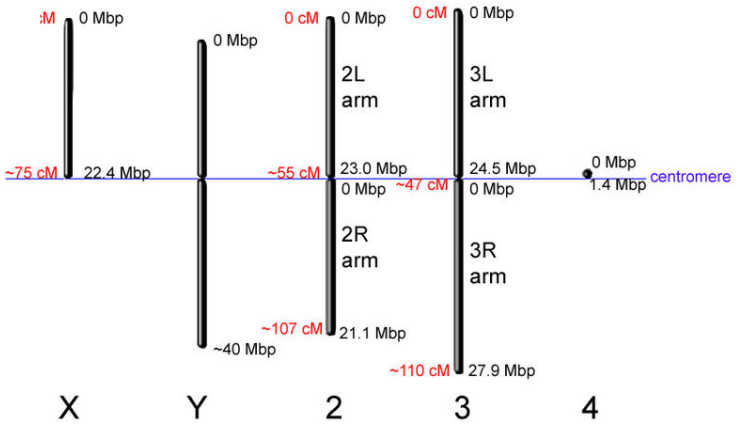
1.4.2 Structure of the ***Drosophila*** telomere:

The *Drosophila* telomere consists of three zones which are distinct in their chromatin configuration (Andreyeva et al. 2005) and references therein (Figure 1.7). Only telomeres on the X chromosome and 4th chromosome have been characterized to a certain extent, the rest remain poorly annotated.

REGIONS OF TELOMERE	**PROTEINS ASSOCIATED**	**FUNCTION**	**TRANSCRIPTIONAL STATE/CHROMATIN STATE**
CAP (end of the chromosome)	SUUR,HP2, Su(var)3-7, HOAP, HipHop, HP1a etc	Prevent telomeric fusion	H3K27me3 transcriptionally silenced.
	ATM, ATR, MRN	DNA damage checkpoint	
TAS/ telomere associated sequence. (Variable length: 2L and 3L are around 460bp repeats whereas X, 2R and 3R are more complex 0.9-1.8kb.)	Polycomb group proteins E(z) and Pc	Implied in TSE (Telomeric Silencing Effect)	H3K27me transcriptionally silenced
HTT array (viariable length of . a head to tail repeat region of the three retrotransposons *HeT-A,TART TAHRE* array;	HP1a, unknown components	Transcription of retrolements to maintain telomere length.	Euchromatic yet has heterochromatin associated proteins

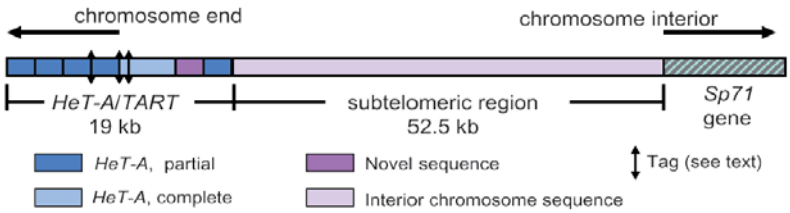
HeT-A being the major component. The 3’ ends of the transposons face the centromere and the 5’ is towards the cap region. Only X and 4th chromosome have somewhat characterized telomeres (George and Pardue 2003).			

A



	X	2L	2R	3L	3R	4
HTT ARRAY	9 *HeT-A* 2*TART*A 1*TART*B		1*TART* A	1*HeT-A* 2*TART*A	1*TART*B	15 *HeT-A* 6*TART*A 1*TART*B 1*TART*C 1*TAHRE*
TELOMERE	147KB	0-50KB	90KB	26KB	43KB	
TAS	COMPLEX 0.9-1.8KB REPEATS	460BP REPEATS	COMPLEX 0.9-1.8KB REPEATS	460BP REPEATS	COMPLEX 0.9-1.8KB REPEATS	

B



C

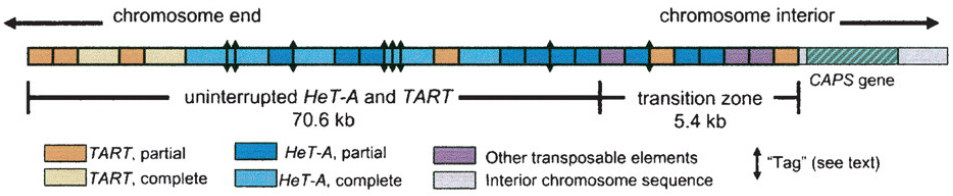


Figure 1.7 ***Drosophila*** **telomeric subdomains**

A. Drosphila chromosomes and a summary of the approximate length occupied by telomeres and TAS.(Adapted from Carvalo 2002 ; Kaminker et al. 2002; Abad et al. 2004a).
B. Characterized telomere on the X chromosome adapted from George and Pardue 2003
C. Characterized telomere on the 4th chromosome adapted from George and Pardue 2003

Rest of the telomeres remain to be characterized owing to technical challenges in sequencing repeat regions.

1.4.3 Mechanisms regulating retrotransposon expression at telomeres

End under-replication in *Drosophila* causes 75bp loss/generation; whereas each retrotransposition adds 6-12kb (Biessmann and Mason 1988; Levis 1989; Biessmann et al. 1990a). Two significant points for the success of this mechanism lie in

a) control of transposon transcription and
b) targeting transposition specifically to the broken or damaged chromosome ends.

HeT-A is the most abundant element and most transposition events to broken chromosome ends are related to *HeT-A* (Savitsky et al. 2006). Expression systems using S2 cells transfecting gag regions of the 3 transposons have also highlighted *HeT-A* as the first to target telomeres. *HeT-A* lacks a reverse transcriptase (RT) region and subcellular localization of *TART* and *HeT-A* suggest that *TART* may provide the polymerase in trans to allow *HeT-A* attachment to chromosome ends. *TAHRE* is mostly excluded from the nucleus in this expression system hence its role in telomere maintenance remains to be studied (Rashkova et al. 2003).

Transcription of the HTT array has been puzzling as this region is bound by the hererochromatin component HP1a and yet retains euchromatic nature. The evidence for the role of small RNAs especially piRNAs, in this process is strong especially in the *Drosophila* female germline evident from the fact that piRNA pathway component mutants express higher levels of telomeric retroelement transcripts. *HeT-A* being the most abundant and important component several studies tried to characterize the transcription of the *HeT-A* element. The *HeT-A* promoter region was found at its 3'UTR region and each *HeT-A* 3'UTR serves as a promoter for the adjacent element (George and Pardue 2003). Antisense transcription is also significant for both *HeT-A* and *TART* loci (Danilevskaya et al. 1999). In fact *TART* antisense transcripts are 10 times more abundant than the sense transcripts. Shpiz et al. 2009, reported nuclear bi-directional transcripts arising from both *TART* and *HeT-A* and the ratio of sense:antisense transcript increased in some piRNA pathway mutants. However, the same piRNA pathway mutants do not affect male telomeric retrotransposition suggesting different tissues may employ varying strategies to maintain telomere length.

1.4.3.1 Somatic regulation of telomere length

In somatic tissues, components like HP1a and PROD (proliferation disrupter) are negative regulators of *HeT-A* transcription (Torok et al. 2007; Silva-Sousa et al. 2012). JIL1 a histone Serine10 kinase has been reported to be involved in transcriptional activation of *HeT-A* elements by establishing open chromatin conformation at the promoters whereas Z4 (Putzig) seems to create a boundary between HP1a and J1L1 (Figure 1.8). However, JIL1 expression does not seem very abundant in the fly germline, implying a different mechanism of telomere maintenance in this tissue.

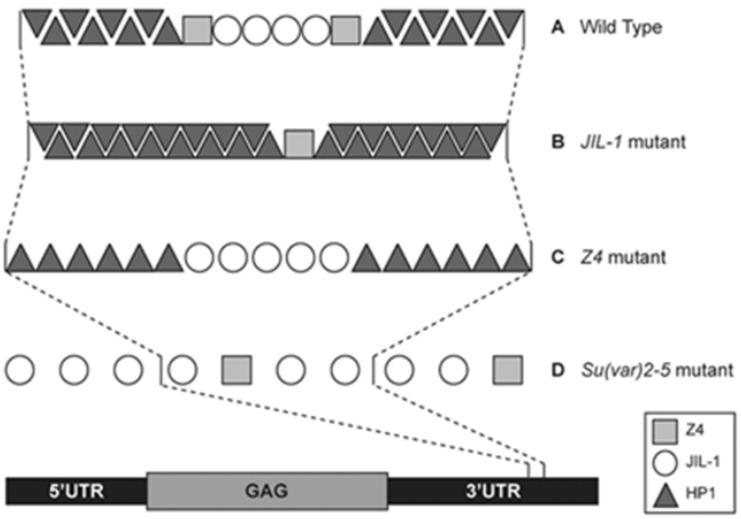


Figure 1.8 **Regulation of *HeT-A* transcription through promoter regulation in somatic tissues of *Drosophila*** The *HeT-A* promoter lies in the 3'UTR region and each *HET-A* element in responsible for the activation of the adjacent *HeT-A* element.

A. In a wild type context, the balance of JIL-1, HP1a and Z4 at the promoters regulates *HeT-A* transcription.
B. In a JIL-1 mutant HP1a occupies the entire promoter region hence silencing the locus completely.
C. In a Z4 mutant the boundary between JIL-1 mediated transcription activation and HP1a mediated silencing is lost and JIL-1 can spread further to activate more *HeT-A* transcripts.

D. In an HP1 mutant context [*Su(var)2-5* mutant], JIL-1 spreads on the entire region and there is no heterochromatinization. As a result the *HeT-A* locus is highly active. Adapted from Silva-Sousa et al. 2012.

1.4.3.2 Germline regulation of telomere length

Two groups have drawn attention to piRNA-mediated telomere maintenance in fly ovaries – the Theurkauf group and Gvozdev and Kalmykova groups in a series of papers reviewed in (Khurana et al. 2010 ; Sphiz et al, 2014).

Most piRNA pathway component mutants show increased transcription of telomeric retroelements in the *Drosophila* female germline. However, only a few manifest telomeric defects or fusions (Khurana et al. 2010). *armitage* and *aubergine* mutants show damaged telomeres whereas *argonaute3* and *rhino* mutants have normal telomeres. s*pnE* mutants have severe *HeT-A* upregulation and transpositions to telomeric regions. However these piRNA pathway mutants do not show increased telomere length like HP1a heterozygous mutants. To check if piRNAs were required for *HTT* silencing, Khurana et al studied piRNAs arising from the 4th chromosome that houses a telomeric cluster with mostly HTT and found a unique population of 19-22nt small RNAs. These piRNAs were reduced in *aub* and *armi* mutants but remained unchanged in *ago3* and *rhi* mutants suggesting there may be a subset of piRNA pathway components dedicated to telomere maintenance.

Dr. Kalmykova and co-workers looked at retroelement transcripts visualized by in-situ hybridization in the ovaries. While *TART* seemed to accumulate in the nurse cell nuclei, *HeT-A* and *TAHRE* target the oocyte. They also showed that *HeT-A* and *TART* loci are bi-directionally transcribed and in some piRNA pathway mutants namely, *spnE* and *aub* the transposon transcripts accumulate in the ovaries (Shpiz et al. 2009; Shpiz et al. 2011). An assay to determine telomeric transposition frequency in piRNA pathway mutants showed increased transposition in *aub* and *spnE* mutants. This did not lead to increased telomere length as is the case for *Su(var)205* mutants suggesting the transposition events were still targeted to eroded chromosomes (Perrini et al. 2004).

To determine whether piRNA mediated silencing of telomeric *HTT* was transcriptional or post-transcriptional, nuclear run on assays were performed at the

HeT-A promoter region in piRNA pathway mutants (Shpiz et al. 2011; Shpiz and Kalmykova 2014). Transcription rates increased significantly and open chromatin marks associated with RNA PolII transcription were also found. Phenomena like TSE have also been shown to be piRNA dependent and H3K9 methylase dSETDB1 has been shown to play a role in *HeT-A* suppression. Thus, the piRNA mediated silencing at telomeres may involve transcriptional silencing.

The chromatin state of the telomeric region is unique as it needs to allow transcription (probably antisense for piRNA production and sense for telomere length maintenance) and yet the transposons - retroelements *HeT-A*, *TART* and *TAHRE* are also targets to be silenced. Through this study I found that HP1a plays a critical role in this process of maintaining the balance in the germline and that it works in conjunction with the telomeric piRNA pathway. Before discussing the details of my results, I would like to review the current knowledge in the field regarding mechanisms by which epigenetic components and piRNA pathway components can work hand in hand for transcriptional gene silencing (TGS).

1.5 Crosstalk between epigenetic components and the piRNA pathway/ small RNA pathways in the germline

In the *Drosophila* germline, the clusters from which piRNAs arise are marked by repressive chromatin marks like H3K9me2/3.Yet, precursor transcripts arise from therein. Though piRNAs are complementary to this region but surprisingly these loci are not transcriptionally silenced by Piwi-piRNA complex. This apparent paradox is still not clearly understood but recent research has led to some insight. Evidence of small RNA guided heterochromatin formation in *S. pombe, A. thaliana* and in somatic cells of *Drosophila* have shown that different strategies are employed to silence a gene locus (Figure 1.9 ,reviewed in Castel and Martienssen 2013). However, in the germline the fight against transposons is vital for the continuity of the organism. So, in early stages of development the offspring has to be primed to recognize transposons as a target to be destroyed. This is achieved through RNAi mediated nuclear silencing through the establishment of epigenetic marks.

In the *Arabidopsis* germline, companion cells allow expression of transposons that generate 21 nt small RNA that can then trans-silence the active transposons in the sperm. In females, de-methylation of Cytosine is followed by 24 nucleotide siRNA production. These RNAs along with Ago9 silence transposons in the egg cell. In mice, there is a phase during early development when transposons are allowed to be active and these loci generate the piRNAs that can then establish a DNA methylation pattern to silence future transposons (Figure 1.9 a and b).

In *Drosophila* somatic follicle cells the flamenco locus housing *Gypsy* family transposons are active. They can produce viral particles and attack the germline. piRNA pathway kicks in to prevent germline attack. Also in the nurse cell Ping-pong helps post-transcriptional silencing. However transcriptional silencing mediated by Piwi and Hetechromatin Protein 1a (HP1a) also silences transposons at euchromatin. To distinguish between a piRNA cluster vs an active euchromatic transposon a variant of HP1, Rhino (HP1d) is used to mark the clusters. Presumably the presence of Rhino can prime the site as a piRNA generating locus and hence it is not a target to be silenced. Also Rhino has been shown as a rapidly evolving protein corroborating host-pathogen co-evolution like mechanism to battle transposons (Klattenhoff et al. 2009).

However this hypothesis has not been verified and remains an interesting point of study as mentioned earlier (Figure 1.9 c).

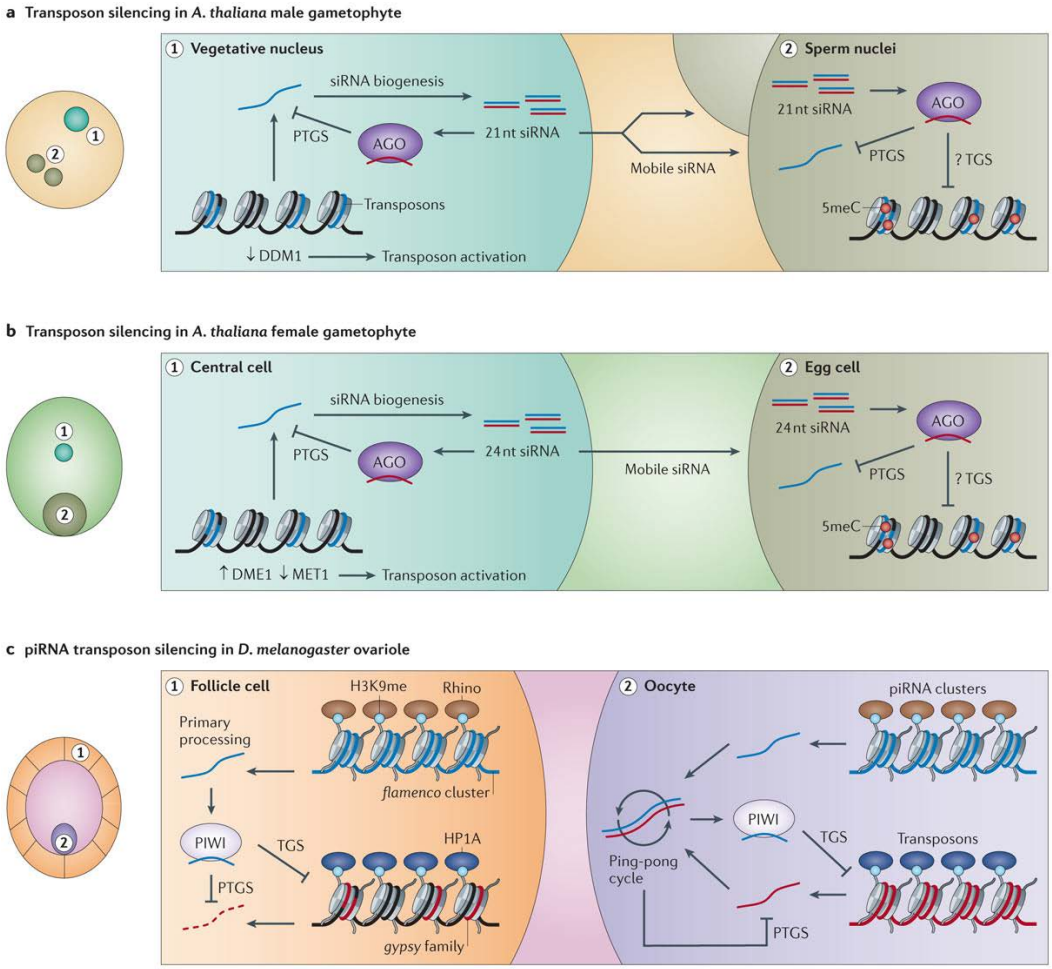


Figure 1.9 **Crosstalk between epigenetic components and the piRNA pathway in the germline in *A. thaliana* male and female gametophyte and *Drosophila* ovariole.** Adapted from Castel and Martienssen 2013.

1.5.1 Epigenetic regulation by piRNAs in the ***Drosophila*** female germline

Nuclear silencing in *Drosophila* is achieved in a manner probably similar to S. pombe where small RNA-protein (here Piwi-piRNA) complexes recognize the nascent transcripts via base complementarity and then recruit methyl-transferases that deposit H3K9me repressive marks, which in turn bring HP1a or Swi6 which is capable of spreading the suppression to neighboring genes.

In *Drosophila*, Piwi is the major candidate for small RNA mediated chromatin remodelling and is the only nuclear Argonaute family protein. Position effect variegation or PEV is a prominent phenotype in flies (Schotta et al. 2003). This refers to silencing of genes in close proximity to heterochromatic loci a fact that can be modelled along S. pombe where Swi6 (HP1a homologue) has been shown capable of spreading repressive marks. Screens for suppression of PEV phenotype identified several piRNA pathway components in addition to expected heterochromatin associated genes. This triggered a plethora of studies to establish the mechanism of transcriptional gene silencing mediated by epigenetic modifications set by Piwi-piRNA complex. In the following segment I will summarise these studies and also highlight the lacunae in the studies. Most of the early studies were based on the variegation phenotypes detected through eye color when a white transgene was inserted at or near a heterochromatic/telomeric locus.

One of the first studies came from Elgin and coworkers where they first showed that heterochromatic silencing or PEV was dependent on several piRNA pathway components namely Piwi, Aub and SpnE, and the effect of spnE was most pronounced (Pal-Bhadra et al. 2004). Three years later work by Lin and coworkers established a link between nuclear Argonaute protein Piwi and non-histone heterochromatin binding protein HP1a. His group identified the domains on both proteins through yeast two hybrid assays that enable binding; probably an RNA dependent association. A mutant of Piwi that failed to bind HP1a in-vitro also failed to silence a PEV reporter transgene located at the heterochromatic region (Brower-Toland et al. 2007). Up until this point there was no evidence of small RNAs mediating a direct role in PEV, although the role of RNAs was implied from the fact that RNase treatment inhibited Piwi association in the somatic tissues. In 2010, Ronserray's group for the first time showed the presence of small RNAs- piRNAs as a requirement for trans-silencing effect seen in the *Drosophila* germline (Todeschini et al. 2010). In their study a heterochromatic insertion (in the telomere associated sequence) of a transposon (P element) could silence a euchromatic insertion of the same P element. They showed that this effect was due to piRNAs arising from the Telomere associated sequences (TAS) that could then silence the euchromatic insertion in an HP1a dependent manner. The apparent paradox of heterochromatic transcription as a pre-requisite for epigenetic silencing is discussed here. A further study in 2011 by Dr. Ruth Lehmann's group then found that

heterochromatin formation was essential for piRNA cluster transcription (Rangan et al. 2011). They used the histone methyltransferase mutant Eggless/ SetDB1 mutant and showed that they phenocopy transposon upregulation like in a piRNA pathway mutant. Cluster transcription from transposon loci was also nearly abolished in the *Eggless* mutant. The hypothesis until this point for somatic PEV was that Piwi- piRNA complex interacts with chromatin modellers HMT and HP1a to establish silencing. Incidentally two papers one from Haifan Lin and another from Elissa Lei, observed HP1a recruitment uncorrelated or independent of Piwi (Lin and Yin 2008; Figueiredo et al. 2012). The study by Dr. Lin on telomere associated sequence on 3R TAS suggested that Piwi and HP1a are antagonistic in nature. Furthermore, the Lei group showed that HP1a recruitment did not require a small RNA pathway (piRNA/ endo-siRNA) however changes in piRNA production did affect global distribution of HP1a. HP1a could spread over longer stretches suggesting the Piwi-piRNA complex might serve a restrictive role on HP1a mediated heterochromatinization, maybe to enable cluster transcription.

The only group that showed a direct evidence for Piwi occupancy on chromatin through a Myc tagged-Piwi ChIP and showed that Piwi bound to 2 transposon loci was Dr. Lin. Subsequently, no group has been able to show direct association of Piwi to chromatin other that by colocalization studies with RNA-PolII through immunohistochemistry on polytene chromosomes (Le Thomas et al. 2013). Indirect evidence of Piwi affecting chromatin has been obtained through H3K9me modification alterations upon Piwi knockdown. The fact that Piwi is multifunctional and required both in the germline and somatic follicle cells has complicated the study. To simplify matters, Elgin's group used a tissue specific knockdown approach to study the effect of Piwi on transcriptional gene silencing in the female germline (Wang and Elgin 2011). Through this study they showed that Piwi knockdown affected only a subset of transposons and did not affect piRNA production for *HeT-A, Roo* and *AtCh X.* Also, they showed a decrease in H3K9me2 upon Piwi loss at those transposon loci suggesting an epigenetic role of Piwi. To connect HP1a into the picture they did a germline knockdown for HP1a whose loss of function is lethal. The subset of transposons derepressed upon Piwi knockdown matched those of HP1a knockdown implying they may work in the same pathway. Contradictorily, the Piwi mutant that was incapable of binding to HP1a could still rescue the Piwi null, signifying that HP1a

association was dispensable for Piwi mediated silencing. Two studies in 2013, using the tissue specific knockdown of Piwi in the germline and somatic gonadal cells of the ovary made use of the new techniques like RNA-seq, GRO-seq, small RNA seq and ChIP seq to address the more global genomic changes associated with the loss of Piwi (Le Thomas et al. 2013; Rozhkov et al. 2013). The aim of these studies was to determine whether the main mode of silencing by Piwi was transcriptional or post-transcriptional. Somatic knockdown studies pointed to a transcriptional silencing role of Piwi but the germline seemed more complex. Their data also shows that Piwi loss leads to loss of piRNA refuting previous data (Wang and Elgin 2011). Recently, a CHHC zinc finger protein Gtsf1 has been shown to be important for Piwi mediated TGS (Donertas et al. 2013; Ohtani et al., 2013 ; Muerdter et al., 2013).

The germline link between Piwi and HP1a remains weak. The Piwi mutant that was incapable of binding to HP1a could still rescue the Piwi null signifying that HP1a association was dispensable for Piwi mediated silencing. The Piwi mutant lacking the nuclear localization signal, Piwi also did not affect HP1a and H3K9me3 localization on all the transposon families de-repressed. The only affected transposon was *HeT-A* suggesting another player with Piwi that mediates silencing. Maelstrom could be one of them (Sienski et al., 2012). Mael loss and Piwi loss in soma lead to nearly overlapping transposon de-repression however H3K9me3 is not lost from these loci in Mael unlike in Piwi kockdown/ loss (Sienski et al. 2012).

The contradictory evidence may be because different loci on the genome are different. Piwi seems to mediate silencing and heterochromatin establishment at euchromatic loci (Sienski et al. 2012). However the 3R TAS seems to require Piwi for active transcription (Lin and Yin 2008). A recent study by Dr. Elgin's group showed that maternal Piwi was required for heterochromatin establishment in embryo (Gu and Elgin 2013) and further studies need to be done in order to find out if this process requires piRNAs. The multi-functional role of Piwi complicates mechanistic studies seeking its role in TGS, especially in the germline. However, all studies have pointed out the importance of HP1a and HMT namely, Eggless in the process. In an attempt to understand TGS in the *Drosophila* germline, I focussed on HP1a.

1.6 HP1a

HP1a was first discovered in *Drosophila* as a tightly DNA bound nuclear protein in 1986 (James and Elgin 1986). It is a 206 amino acid protein which contains a chromo domain and a shadow domain connected by a hinge region. The chromo domain recognizes and binds histone modifications like H3K9me and the shadow domain is involved in protein interactions including dimerization with another HP1a molecule. The hinge domain has nucleic acid binding capacity especially to RNA in mammals (Muchardt et al. 2002). Most of the studies on HP1a DNA binding comes from in vitro mobility shift assays and in vivo evidence remains weak particularly with respect to the hinge domain (Figure 1.10).

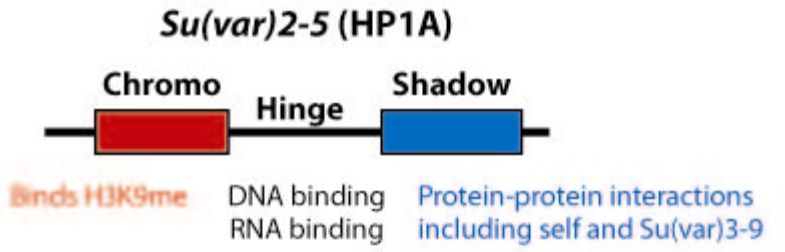


Figure 1.10 **Structure and Domains of HP1a** Adapted from Vermaak and Malik 2009.

Following the characterization of HP1a it was found to be a highly conserved eukaryotic protein (Figure 1.11 ; Lomberk et al. 2006). There are multiple isoforms of this protein in most species showing tissue specific expression patterns (Table 1.2). In *Drosophila* melanogaster there are 5 isoforms tabulated in table (reviewed in Vermaak and Malik 2009). HP1e is the least conserved even among the *Drosophilids* (Figure 1.11).

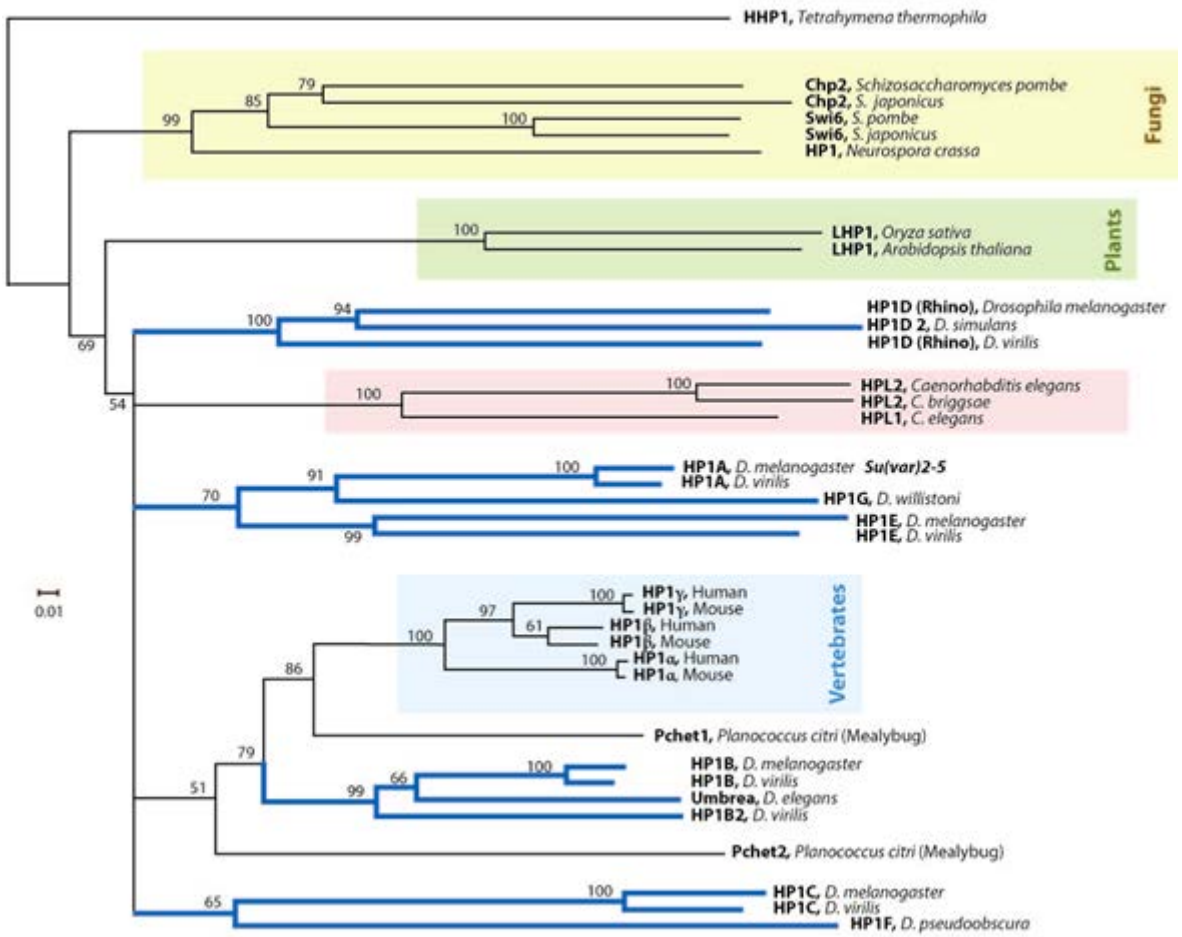


Figure 1.11 **Evolutionary tree for HP1 family proteins depicts high conservation.** Adapted from Vermaak and Malik 2009.

NAME	TISSUES EXPRESSED IN	CHROMATIN STRUCTURE& MODIFICATION
HP1a	multiple	Heterochromatin,H3K9me
HP1b	multiple	Euchromatin& heterochromatin
HP1c	multiple	Euchromatin
HP1d	Female(Rhino)	Heterochromatin,no H3K9me
HP1e	Male	Work in progress by Dr. Mallick and group.

Table 1.2 **HP1 variants in *Drosophila* melanogaster**

1.6.1 HP1a occupancy on the ***Drosophila*** genome

HP1a is predominantly bound to the pericentromeric and telomeric heterochromatin (Vermaak and Malik 2009). However through ChIP studies on different tissues its occupancy appeared more widespread corroborating its multifunctionality (Fanti and Pimpinelli 2008). Two groups studied the localization of HP1a in the subdomains of the telomere. Though ChIP studies on elongated telomeres in *tel* mutants in and Giano stocks in, it was established that HP1a occupies not only the cap region but also spreads onto the TAS and HTT array in a sequence independent manner (Frydrychova et al. 2008 ; Gao et al. 2010). These studies were performed on somatic larval tissues. HP1a occupancy was studied both in the soma and germline by ChIP-qPCR with special focus on the piRNA clusters and transposons (Moshkovich and Lei 2010). HP1a showed significant enrichment at the clusters and transposons. *TART* element showed higher HP1a levels as compared to 1360 element both in the germline and soma. Two groups further showed that HP1a also binds RNA transcripts and gene bodies on euchromatic regions on the fourth chromosome to activate certain genes rather than silence them (Piacentini et al. 2009; Figueiredo et al. 2012).

1.6.2 HP1a is a multifunctional protein.

The multiple functions of HP1a have been summarized as follows:

a) Gene silencing: It plays a role in silencing chromatin via recognition of histone modification and chromatin fibre condensation that enables heterochromatin formation (Figure 1.12). It may also play a role in transcript degradation utilizing its hinge region. In S. pombe, Swi6 (HP1a homologue) is likely to bind heterochromatic reporter genes and target them to the nuclear exosome for degradation (Keller et al. 2012).

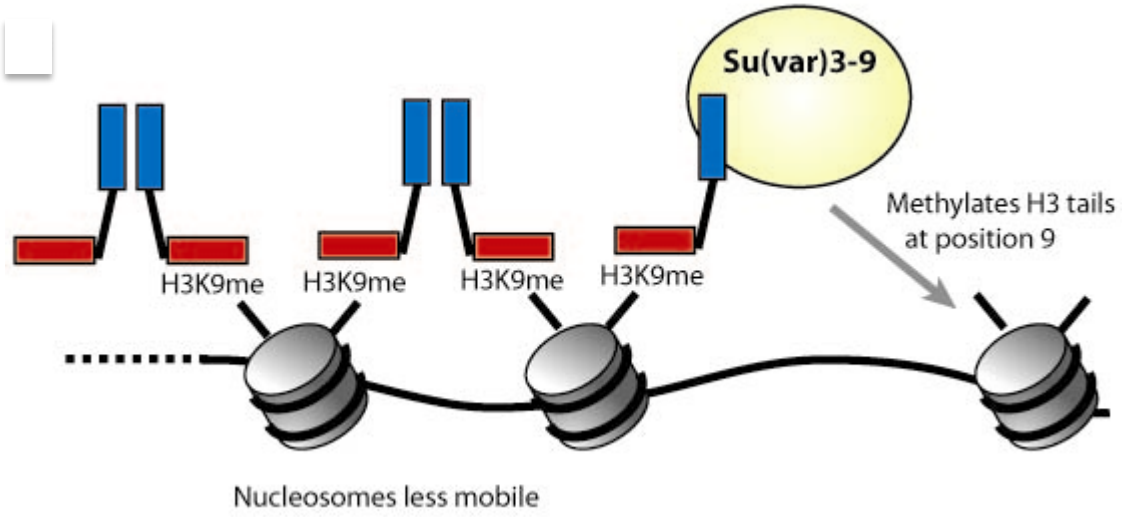


Figure 1.12 **Mechanism of HP1a mediated heterochromatinization**

HP1a chromo domains associate with H3K9me modification on nucleosomes and the shadow domains can dimerize and recruit another HP1a molecule to the adjacent locus. The shadow domain can also interact with the Histone Methyl Transferase (Su(var)3-9) to methylate H3 at Lysine 9 and this in turn can be bound by HP1a chromo domain to spread the heterochromatin mark. Adapted from Vermaak and Malik 2009.

b) Gene activation: HP1a can also recognize other positive histone marks (Fanti and Pimpinelli 2008). Some genes, for example, genes on the fourth chromosome in *Drosophila* need a heterochromatic environment for expression (Lu et al. 2000). Loss of HP1a from these loci reduced transcription of these genes. Also piRNA precursor transcription needs heterochromatin formation (Rangan et al. 2011).

c) Transcript processing: HP1a has also been seen to associate with chromosome puffs and hnRNPs suggesting a function in transcript processing. In mammals recruitment of splicing factors is aberrant in absence of HP1a (Piacentini et al. 2009; Smallwood et al. 2012).

Hence the role of HP1a is contradictory and context dependent and probably depends on the associated factors and chromatin environment. One possible way that a protein can achieve such a multifaceted role is via post-translational modifications. Phosphorylation at multiple sites, ubiquitination and sumoylation of HP1a have also been observed (reviewed in Eissenberg and Elgin 2014).

2. Materials and Methods

2.1 Fly genetics

2.1.1 Fly husbandry

Drosophila melanogaster used in this study were grown on standard cornmeal agar medium at 25ºC. Sco/CyO and TM3/TM6 was used as second and third chromosome balancers respectively. The other strains used in the study are tabulated in table 2.1. For egg laying assays 2 females and 2 males were added to vials and number of eggs layed was counted everyday. Hatch rates were calculated every alternate day.

Table2.1: Drosophila melanogaster strains used in this study

Fly Stock	Source (Bloomington ID/ Laboratory)
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00278}attP2	33400 ($RNAi^{HP1a3Chr}$)
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00531}attP40	36792($RNAi^{HP1a2Chr}$)
w^{-}; *ngt*40; *nanos* GAL4::VP16	Laboratory stocks
TJ GAL4/ CyO	Laboratory stocks
In(1)wm4h;Su(var)2055/In(2L)Cy, In(2R)Cy, Cy1	6234
w[1118];P{w[+mC]=NM}31E P{ry[+t7.2]=neoFRT}40A	1835
P{ry[+t7.2]=hsFLP}12, y[1] w[*]; sna[Sco]/CyO	1929
P{ovoD1-18}2La P{ovoD1-18}2Lb P{neoFRT}40A/Dp(?;2)bwD, S1 wgSp-1 Ms(2)M1 bwD/CyO	2121
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM20-EGFP.shRNA.1}attP2	41556
y[1] w[*]; P{w[+mC]=Ubi-GFP.D}33	5189

P{w[+mC]=Ubi-GFP.D}38 P{ry[+t7.2]=neoFRT}40A/CyO	

2.1.2 Tissue specific knockdown using the GAL4-UAS system (Fischer et al., 1988)

Female driver lines were crossed with male TRiP lines for all the experiments. Further experiments were carried out in 2-3 day old female progeny in most cases. Siblings from the *nos*GAL4>RNAi^{HP1a2chr} were used as controls.

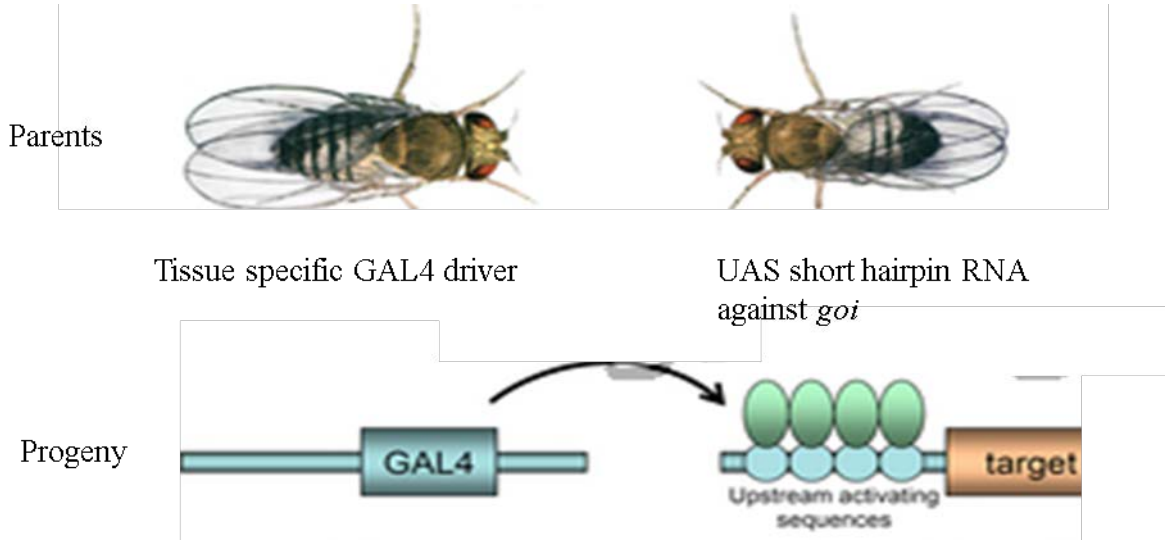


Figure 2.1 Tissue specific knockdown of HP1a using the GAL4-UAS system

Female driver lines were crossed with males bearing a short hairpin RNA against the *goi* (gene of interest, HP1a). The progeny from the cross expressed RNAi in the tissue of interest as a result of which knockdown of the gene was obtained.

2.1.3 Germline Clonal Analysis based on DFS strategy (Chou and Perrimon 1996) and Mitotic clonal analysis.

Cross scheme for germline clonal analysis:

Su(var)205^{05} X w[1118];P{w[+mC]=NM}31E P{ry[+t7.2]=neoFRT}40A

ln(2R) CyO

↓

Pick straight winged female flies ie,

$$\frac{\text{Su(var)}205^{05}}{\text{w[1118];P\{w[+mC]=NM\}31E P\{ry[+t7.2]=neoFRT\}40A}} \quad X \quad \frac{\text{Sco}}{\text{CyO}}$$

Recombination should happen here ↓

Choose >50 CyO progeny and set up individual crosses to Sco /CyO

Set up sibling crosses among CyO progeny from the above cross and selection was based in homozygous lethality for Su(var) 205^{05} allele.

Secondly check for the presence of FRT sequence by PCR among flies selected in the above step.

Stock Su(var)205^{05} FRT 40A/CyO virgins should then be crossed with males containing Heat shock flippase and OvoD1 mutation as follows

$$\frac{\text{Su(var)}205^{05}\text{ FRT 40A}}{\text{CyO}} \quad X \quad \frac{\text{y,w P[ry+;FLP]}^{12}}{\text{CyO}} \; ; \; \frac{\text{P[mini w}^{+}\text{; ovo }^{D1}\text{]FRT 40A}}{\text{CyO}}$$

↓

Heat shock third instar larvae and select straight winged progeny for germline clonal analysis

<u>Cross scheme for mitotic clonal analysis:</u>

$$\frac{\text{Su(var)}205^{05}\text{ FRT 40A}}{\text{CyO}} \quad X \quad \frac{\text{y[1] w[*] P[FLP]}}{} \; ; \; \frac{\text{33 P\{w[+mC]=Ubi-GFP.D\}38 P\{ry[+t7.2]=neoFRT\}40A}}{\text{CyO}}$$

4 Heat shocks ↓

The female progeny were immunostained for GFP, HP1a and *Het-A.*

2.2 Immunohistochemistry and Microscopy

2.2.1. Antibody Staining

Ovaries or brains were dissected in Graces cell culture media (Life Technologies) and teased apart a bit, then fixed for 5 min in Grace's with formaldehyde (2:1) followed by rinses first in PBS and then in PBX [PBS and 0.2% Triton X100 (Sigma)] 2-3 times and washes 2-3 x 10 min in PBX with rocking on a nutator. Then, pre-absorbed for 30 min or longer with PBX-NGS (5%) followed by a rinse once with PBX. They were then incubated for 4 hours at room temperature or overnight at 4°C in primary antibody diluted in PBX-BSA (0.5%) followed by removal of the primary antibody and rinses with PBX and washed for 1 hr with rocking on the rotator with several changes of PBX. Working concentrations for antibody dilutions are listed in Table 2.2. Next, they were incubated for >2 hours at room temperature or overnight at 4°C in secondary antibody diluted in PBX-BSA (0.5%). Secondary antibodies used in the study were Alexa Flour-488,-555, or -633 conjugated goat anti-rabbit, anti-mouse, anti-guinea pig IgG (1:500, Molecular Probes).Rinses and washes with PBX for 1hr followed. Then samples were stained with DAPI (1:500 for 5 minutes) and rinsed with PBX. A rinse with PBS next ensured removal of TritonX100 and 1 drop of vectashield (Vector Labs) was added to the sample. Samples were mounted on a slide glass and teased apart completely. Ovarioles were picked up carefully, and place onto 10ul of vectashield spread-slide glass. Cover glass was placed gently with microneedle.

Table2.2: Antibodies used in this study

Antibody	Dilution	Source
Anti α-Spectrin mouse monoclonal	1:2500	Hybridoma bank, Iowa
Anti HP1a [mouse monoclonal(C1A9)	1:50 1:200	Hybridoma bank, Iowa Covance (Product # PRB-

and rabbit polycolonal]		293C)
anti Krimp (guinea pig polyclonal)	1:2500	Lim and Kai, 2007
anti Aub [mouse monoclonal (clone 4D10)]	1:1000	Gift from Dr. Siomi MC (Gunawardane et. al.,2007)
Anti-Vasa (guinea pig polyclonal)	1:2500	Patil and Kai,2010
Anti-Tejas (rabbit polyclonal)	1:500	Patil and Kai,2010
Anti-Ago3 (rabbit polyclonal)	1:500	Gift from Dr Dahua Chen
Anti-SpnE (rabbit polyclonal)	1:200	Gift from Dr Siomi (Nishida et al, 2009)
Anti Gurken [mouse monoclonal (clone 1D12)]	1:10	Hybridoma Bank, Iowa
Anti-C(3)G (mouse monoclonal)	1:500	Gift from Dr Hawley (Page and Hawley, 2001)
Anti H2Av (rabbit polyclonal)	1:100	Abnova(Product #PAB9920)
Anti- *TART-ORF1* (rat polyclonal)	1:200	Maxwell et al., 2008
Anti *HeT-A* (rabbit polyclonal and guinea pig polyclonal)	1:1000	Guinue pig polyclonal was a gift from Dr Rong (Zhang et al. 2014a) rabbit polyclonal was generated in this study
Anti HipHop (rabbit polyclonal)	1:500	Gift from Dr Rong (Gao et al. 2010)
Anti *I-element*, (rabbit polyclonal)	1:1000	Gift from Dr Finnegan (Brennecke et al. 2008)

2.2.2 TUNEL assay to determine Apoptosis

The TUNEL assay was performed using an ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Millipore) in accordance with the manufacturer's protocol.

2.2.3 Microscopy

Images were taken with a 3-Photomultiplier Tube detector using a 40X or 100X 1.3 NA Plan-Apochromat oil objective by upright confocal microsocope (EXCITER LSM510, Carl Zeiss Inc.). For general ovarian morphology 10X Widefild Metamorph, Carl Zeiss Inc. was used. Image J, LSM software and Adobe Photoshop CS3 software were used for image processing.

2.2.4 Image processing using IMARIS (Bitplane) software for HipHop foci analysis

Using the Cell plugin on Imaris, nuclei were defined as 4µm spheres and the HipHop foci were defined as 0.5 µm foci. Background subtraction was set to the automatic thresholds set by the program. Average values of number of vesicles (HipHop foci) per cell (nuclei) were used for the data analysis. Meredith Calvert and Steve Chai very kindly shared their expertise and knowledge on how to use this software with me.

2.3Molecular Biology

2.3.1 Recombinant DNA methods

2.3.1.1 Bacterial Strains and culture conditions

For all the recombinant DNA protocols the *Escherichia coli* strains used in the study were either XL1 blue or DH5α. For pENTR-D-TOPO cloning the TOP10 *E. coli*

(Invitrogen) was used. For the recombinant protein expression purpose BL21(DE3) *E. coli* was used. All the bacterial strains were grown on Lurial-Bertani {LB; 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract. 1% (w/v) sodium chloride (NaCl) broth or agar medium at 37^{o}C. For the drug resistance selection purposes the media or the agar plates were supplemented with the antibiotics like ampicillin (100µg/ml) or kanamycin (50µg/ml).

2.3.1.2 Bacterial transformation

For heat-shock transformation, competent cells were mixed with an appropriate volume of DNA and incubated on ice for 30-40 min. The DNA/cell mixture was then heat-shocked at 42°C for 60 sec and immediately placed on ice. For electroporation, competent cells were mixed with an appropriate volume of DNA and transferred to a pre-chilled electro-cuvette on ice. The DNA/cell mix was pulsed once at 2.5 kV and 25 µF, and immediately placed on ice. Following either transformation, 1 ml of LB was added to each mixture and mixed gently before incubating the culture at 37°C, with shaking at 250 rpm for 1 hour for recovery. The cells were then plated on LB agar supplemented with the respective antibiotics, depending on the plasmids.

2.3.1.3 Plasmid DNA preparation (mini prep)

A 3ml LB culture of the plasmid containing cells, supplemented with required antibiotics were shaken vigorously overnight at 37^{o}C. Following morning the cells were collected at 5000rpm for 5min. Plasmid mini preps were carried out using Geneaid High Speed Mini Kit (Geneaid) in accordance with manufacturer's instructions.

2.3.1.4Gateway® cloning strategies

Gateway® cloning is two step protocol. First step is the directional TOPO cloning of gene of interest into pENTR™/D-TOPO® (Invitrogen) and the 2nd being

the site-specific recombination step to swap the gene of interest from pENTR™/D-TOPO® into the destination vectors (The Drosophila Gateway™ Vector Collection).

For TOPO cloning, PCR product with a flanking CACC sequence at the 5'-end and a blunt 3'- end was mixed with pENTR™/D-TOPO® in a molar ratio of 2:1, and incubated at room temperature for 30 minutes. Half the ligation mixture was then transformed into OneShot® TOP10 chemically competent E. coli (Invitrogen) in accordance to the manufacturer's instructions.

For site-specific recombination, equal volumes of pENTR™/D-TOPO® harbouring the gene-of-interest or DNA fragment and the Gateway® destination vectors were mixed, in the presence of LR Clonase™ II enzyme mix (Invitrogen). The DNA/enzyme mix was incubated at 25°C for 2 hr to promote recombination. The reaction was subsequently terminated by adding Proteinase K and incubating at 37°C for 20 min. One-half of the reaction mix was used for transformation.

2.3.2RNA isolation and DNAse treatment

Drosophila ovaries were dissected in cold grace's medium supplemented with BSA (0.5%) and transferred to TRIzol reagent (Invitrogen) on ice. Total RNA was extracted from the tissue according to the manufacture's protocol. DNAse treatment was performed using Ambion (name kit) according to protocol provided. Extracted total RNA was stored at -80ºC.

2.3.3 quantitative PCR

1µg of DNAse treated RNA was reverse-transcribed using oligo(dT)20 and Superscript III reverse transcriptase (Invitrogen). 1µl of newly synthesized cDNA was subject to PCR amplification using gene specific primers using the SYBR green master mix (Life Technologies). Primers used in this study are listed in table 2.3.

Table 2.3 Primers used in this study

Primer name	Sequence

Actin5C F Actin5C R	TGCCCATCTACGAGGGTTAT AGTACTTGCGCTCTGGCGG
Bari F Bari R	TTGAAAACGTTTGGGCTTTT TAACACCACCTTTGGCATCA (Wang and Elgin 2011)
BURDOCK F BURDOCK R	ATTAGAAGCGTCGGTCATCG GGGCGCCAATTATCATTTTA (Wang and Elgin 2011)
CLUSTER 1A F CLUSTER 1A R	CGTCCCAGCCTACCTAGTCA ACTTCCCGGTGAAGACTCCT
CLUSTER 1C F CLUSTER 1C R	GTGGAGTTTGGTGCAGAAGC AGCCGTGCTTTATGCTTTAC
HetA antigen F Het A antigen R	CACC CCCTACTGGAAAAGCTGAAC CTACAGGGCATCCTTTGTACGCGCT
HeT-A F *HeT-A* R	TCATTGACGATACCAGCGCATC TCCGGGTGCGTTTAGGTGAG
HP1a Seq 1F HP1a Seq 1R HP1a Seq 2F HP1a Seq 2R	AGGCTCCAGCAGAATAAAGG AAATATTTTCTGTACACAAATTGGTA TAGGTACACTCAATTCCATAGCTTA CGCACACTGTTCGGCCG
I ele F I ele R	GACCAAATAAAAATAATACGACTTC AACTAATTGCTGGCTTGTTATG
Roo F Roo R	AATAAAATTGAATTTTTATGGCATAAAATA GGTAACTCCTCCGCCTTAAC
RP49 F RP49 R	CCGCTTCAAGGGACAGTATCTG ATCTCGCCGCAGTAAACGC
TAHRE F TAHRE R	CTGTTGCACAAAGCCAAGAA GTTGGTAATGTTCGCGTCCT
TART F TART R	TTCTATCAACAGGCTGTCCACAGGTT CCTTCGTAGTCGGGTAGGATTATTCGT
ZAM F ZAM R	ACTTGACCTGGATACACTCACAAC GAGTATTACGGCGACTAGGGATAC

2.3.4 Polymerase Chain Reaction

Approximately 50ng DNA template, 200µM deoxynucloetide triphosphate(dNTP), 500 µM of each primer (forward and reverse), and 0.4 units of Taq polymerase (Roche) were subject to the following cycling conditions: 1 cycle of 94ºC for 30 seconds, 30 cycles of 94ºC for 2 minutes/ 55-62ºC for 45 seconds/68ºC for 1 minute per kb, 68ºC for 10 minutes and the final holding temperature at 16ºC.

2.3.5Sequencing

Sequencing reactions were carried out using BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) with 50-100ng of template DNA and 100µM primers.

2.3.6 DNA preparation from Single fly

Genomic DNA was prepared from a single fly by mashing it in 50µl squishing buffer [10mM Tris-Cl pH 8, 1mM EDTA, 25mM NaCl and 200 µg/ml freshly diluted Proteinase K(Sigma)] with a pipette tip (Gloor and Engles, 1992) and incubated at 37ºC for 30 minutes. Proteinase K was then heat inactivated at 95ºC for 10 minutes and the genomic DNA could be stored at 4ºC or -20ºC for future use. 1.5 µl of this preparation was used per PCR reaction.

2.3.7 DNA preparation from ovaries for genomic copy number analysis

50-100 pairs of ovaries were thoroughly ground with a micropestle in 250ul of Buffer A (10mM Tris-Cl pH 7.5, 60 mM NaCl, 10mM EDTA, 150uM spermidine, 150uM spermine, 200ug Proteinase K/ml). 250ul of Buffer B (200mM Tris-Cl pH 7.5, 30mM EDTA, 2% SDS, 200ug/ml Proteinase K) was then added, mixed gently and incubated at 45ºC for 3 hours. Equal volume(500ul) of Phenol-Chloroform-Isoamyl alcohol was added following the incubation and centrifuged at 12K rpm for 10 minutes. The aqueous phase was collected and an equal volume of Isopropanol and 1/25th volume 3(M) NaOAc were added. Overnight precipitation was followed by high speed

centrifugation at 14000rpm for 30 minutes and ethanol washes. The pellet was dissolved in Tris-Cl, pH 8 and the sample was treated with RNaseA(100µg/µl) for 1 hour at 37ºC to remove RNA (ovaries have very high RNA content). Phenol-Chloroform extraction was repeated.

2.4 Biochemistry

2.4.1 Chromatin Immuno-precipitation

Approximately 240 ovaries were dissected in cold PBS with protease inhibitor. The ovaries were crosslinked by adding formaldehyde to 1.8% (final concentration) and rocking on a nutator for 10min at room temperature. To terminate cross-linking glycine was added to final concentration of 0.125M followed by centrifugation at 2,000 rpm for 5min at 4ºC. Nuclei were isolated by the use of a dounce homogenizer followed by sonication using a bioruptor (10 pulse 30 seconds each). ChIP was performed using the ChIP assay kit (Millipore) according to the manufacturer's protocol with minor modifications. Antibodies used were α HP1a (rabbit polyclonal Covance) and αH3K9me3 (Abcam) and respective IgG controls. DNA was obtained by conventional Phenol Chloroform Isoamyl alcohol extraction followed by Ethanol and sodium acetate precipitation.

2.4.2 Immunoprecipitation of protein RNA complexes

Approximately 300 pairs of ovaries were homogenized in 0.05% NP-40 HEPES IP buffer [30 mM HEPES-KOH (pH 7.3), 2 mM magnesium acetate, 150 mM potassium acetate, 5 mM dithiothreitol, 0.1% NP40] supplemented with an EDTA-free protease inhibitor cocktail tablet (Roche). The homogenate was centrifuged to pellet the debris, and the supernatant was pre-absorbed twice with equilibrated Protein G plus/Protein A agarose suspension beads (Calbiochem). Antibodies were coupled to the equilibrated beads for 3 h. The antibody-bead complexes were incubated with pre-absorbed extract overnight. After three washes 10 minutes each in the wash buffer (used in (Piacentini et al. 2009), HP1a-bound RNAs, RNAs associated with HP1a were obtained through Trizol extraction.

2.4.3 Protein extraction

Protein extracts were obtained by homogenizing ovaries on ice in 2× sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 200 mM dithiothreitol, 300 mM Tris–HCl (pH 6.8), 20% (v/v) glycerol, 0.04% (w/v) bromophenol blue] and denatured by boiling for 5 min. Following the boiling the debris were separated by centrifuging the samples at 10000 rpm for 10 min and stored at -20^{0}C.

2.4.4 PAGE separation of proteins and western blotting

Proteins were separated on 8-12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) [8-12% (v/v) acrylamide/bis-acrylamide (29:1, Bio-Rad), 375 mM Tris-HCl pH 8.8 (for separating gel) or pH6.8 (for stacking gel), 0.1% (v/v) SDS, 0.1% (w/v) ammonium persulphate (APS), 0.4% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED)] using a Bio-Rad gel electrophoresis system at 80 volts (V) till the samples pass through the stacking gel and at 100-120V till the dye front reaches the end. For the direct visualization of the proteins the gels were stained with coomassie brilliant blue staining buffer [0.0025% (w/v) coomassie brilliant blue R250 in 1 part glacial acetic acid: 9 parts of 50% (v/v) methanol]. Excessive stain was removed from the gel by destaining the gels in destaining solution [40% methanol 10% acetic acid] till clear bands appeared. For western blotting the proteins were transferred by electrophoretic blotting onto nitrocellulose or polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in the transfer buffer [3.03 g/L Tris-base, 14.4 g/L glycine, 20% (v/v) methanol] at 100 V for 60 min.

2.4.5 Immunological detection of proteins

Following the transfer the membranes were blocked using blocking solution [PBST (PBS+0.05% Tween 20) supplemented with 5% skimmed milk and 3-5% BSA] for at least 30 min. Membranes were rinsed once with PBST and incubated with primary antibody diluted in PBST with 3-5% BSA overnight at 4^{0}C or at room temperature for 2hrs. Excess primary antibody was washed with PBST for 1hr with change of buffer every 10min and incubated in secondary antibody tagged to HRP diluted in PBST+3-5% BSA for 45min followed by 6 washes with change of buffer

every 10mins with PBST. Detection was performed with SuperSignal® West Pico Chemiluminescence substrate (Thermo Scientific) and the signals were visualized on Kodak BioMax MS film. The following antisera and primary antibodies were used: Anti HP1a C1A9 (1:200; Hybridoma bank) and mouse anti-α-Tubulin DM1A (1:1000; Santa Cruz Biotechnology). Anti-mouse (1:5000; Bio-Rad) HRP-conjugated immunoglobulin was used as secondary antibody.

The bound antibodies from the membranes were stripped by incubating the membranes in 0.1M Glycine pH2.5 at 55^0C for 30-45min and incubating at room temperature for another 1hr with shaking. The stripped membranes were incubated with the next antibody after several washes with PBST. This procedure was performed for loading controls.

2.4.6 Antibody generation

For generation of antibodies against *HeT-A*-GAG region, a 201 amino acid fragment was amplified from cDNA of piRNA pathway mutant *krimper* using the primer pair *HeT-A* antigen F and R. This fragment was cloned into pDEST17 Gateway cloning system according to the manufacturer's protocol. The 6-His tagged fusion proteins were finally used to immunize rabbits and guinea pigs. Antigens were mixed with complete or incomplete Freund's adjuvant (Pierce) and vortexed vigorously for 60 min at 4°C to form emulsions. 0.25 mg for rabbits and guinea pigs were administered intra-peritoneal and/or intramuscularly per injection for up to six rounds of immunization at the TLL Animal Facility. The animals were bled in accordance to standard animal handling procedures. The bleeds were first left at room temperature for 30 min to deactivate the complement and subsequently overnight at 4°C to allow clotting. To separate the serum, the bleeds were centrifuged at maximum speed for 60 min at 4°C. Glycerol and sodium azide were added to final concentrations of 50% (v/v) and 0.1% (w/v), respectively and the anti-serum was kept at -20°C or -80°C.

2.5 Bioinformatics

The bioinformatics analysis was conducted by Dr. Amit Anand in Kai laboratory.

2.5.1 Small RNA sequencing analysis

RNAs were extracted from hand-dissected ovaries of *HP1a* knockdown ovaries and the control. Small RNAs ranging from 18- to 30-nt in length were isolated by PAGE fractionation and were used for library generation for deep sequencing. Deep sequencing was performed on HiSeq2000 at University of Utah (Microarray and Genomic Analysis Core Facility).

The three libraries were normalized with miRNAs. The libraries were mapped to *Drosophila* genome (Rel 5, excluding Uextra). To analyze piRNA matching to clusters, only 23-29-nt reads that uniquely mapped to genome were considered (cluster information was taken from (Brennecke et al. 2007). Reads were mapped to the genome and annotated transcripts using Bowtie (Langmead et al. 2009) allowing zero mismatches. The alignment of reads to transposon sequences was performed with Bowtie, allowing two mismatches. Samtools and BEDTools were employed during the analysis of the alignments (Li et al. 2009) .

The piRNAs mapping to the clusters were counted in 10-nt windows for plotting. To analyze ping-pong generated piRNAs, sense-antisense piRNA pairs having overlap between 2-26-nt were counted by in-house programs. The ping-pong ratios were calculated by dividing the numbers of reads containing 10-nt overlap with sum of reads containing any overlap between 2-26-nt.

2.5.3 ChIP-Sequencing analysis

The DNA recovered after Chromatin immune precipitation from ovarian lysate with HP1 polycloncal antibody (2µg of antibody; Covance, Product # PRB-293C) and IgG (Santa Cruz sc-2027) were used for library preparation. Library preparation and sequencing (single end, 50 nt) was performed at Macrogen Inc (Seoul, South Korea) on Illumina Hiseq 2000.

The reads were aligned to *Drosophila* genome (r 5.25, flybase) without any mismatches using bowtie short read aligner (Langmead et al. 2009) (v. 0.12.7). Only 35% of total reads in HP1a library was mapped to genome. This is most likely due to absence of sequence information on telomeric regions in the current genome release. Reads were also aligned to the canonical transposon sequences (Repbase) with two mismatches. The genomic loactions of all full-length and partial transposon copies in genome was determined using repeat masker and also from flybase. Alignment from specific regions was fetched using samtools. Peak calling was performed using MACS2 software (Zhang et al 2008). IgG was used as control. We used parameters that scaled the peak size in the IgG library since it had less number of reads. The scaling up peaks in IgG library risked increase in false positive peaks for IgG library and this could have led to a conserved number of HP1 associated peaks. However, this parameter would likely curtail false positive peaks associated with HP1.

3. Results

3.1 Knockdown of *HP1a* in the *Drosophila melanogaster* female germline leads to sterility

3.1.1 Tissue specific knockdown approach to study the role of *HP1a* in the *Drosophila* germline

Drosophila melanogaster Heterochromatin Protein 1 a (HP1a) is essential and multifunctional. Since the loss-of-function of HP1a results in lethality, to study its role specifically in the germline, a tissue specific knockdown approach had to be adopted. The *Drosophila* female germline consists of germline cells surrounded by a layer of somatic follicle cells (Figure 3.1.1; Handler et al. 2011). The male germline somatic cells are depicted in blue while the germline in yellow Figure 3.1.2 (Matunis et al. 2012). To knockdown HP1a in the germline cells, GAL4-UAS system was used (Duffy 2002).

Driver lines *nanos*GAL4 and *trafficjam*GAL4 worked efficiently to knockdown HP1a in the female germline (Figure 3.2 A-C) and somatic cells, respectively as seen in (Figure 3.2 D). A combination of *nanos* and *daughterless* GAL4 lines worked best for male germline knockdown (Figure 3.2 E and F). For the knockdown two short hairpin RNAi lines which were generated against *HP1a* gene, encoded by *Su(var)205*, by the TRiP consortium were used (Ni et al. 2011). These lines are available from the Bloomington Stock Center. One of the lines used a valium vector 20 and this was inserted on the 3rd chromosome and the other used a valium 22 vector which had the small hairpin RNAi inserted onto the 2nd chromosome. The two lines will be referred as RNAi^{HP1a2chr} and RNAi^{HP1a3chr}. Of the two lines, knockdown efficiency, visualized by loss of HP1a by immuno-histochemistry, the insertion line on the 3rd chromosome i.e., RNAi^{HP1a3chr} appeared more efficient than RNAi^{HP1a2chr} (Figure 3.3).

Figure 3.2 A shows a wild type ovariole immunostained with anti HP1a, anti-Vasa (a germline marker) and DAPI to stain nuclei. HP1a colocalizes with DAPI, signifying it is a nuclear protein in both soma and germline. In Figure 3.2B and C a

specific loss of HP1a from the germline (Vasa positive cells) is observed, reflecting the specificity of the *nanos*GAL4 driver for the germline. Similarly HP1a was selectively significantly reduced from the somatic follicle cells as seen in Figure 3.2 D upon the use of the *trafficjam*GAL4 driver.

In the male germline, HP1a occupies the nucleus just as in females (Figure 3.2E). I achieved a germline specific knockdown as seen in Figure 3.2F. The germline cells marked by Krimper (since this antibody worked best in this tissue), showed a complete loss of HP1a.

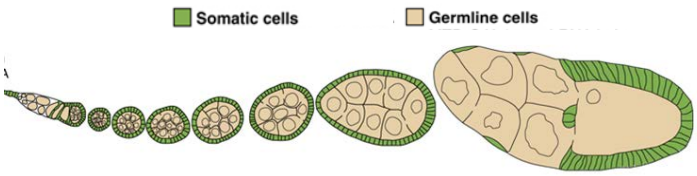


Figure 3.1.1 **Schematic representation of the *Drosophila* ovariole.** The ovariole consists of germline cells depicted in beige and a layer of somatic follicle cells shown in green. Adapted from (Malone et al. 2009)

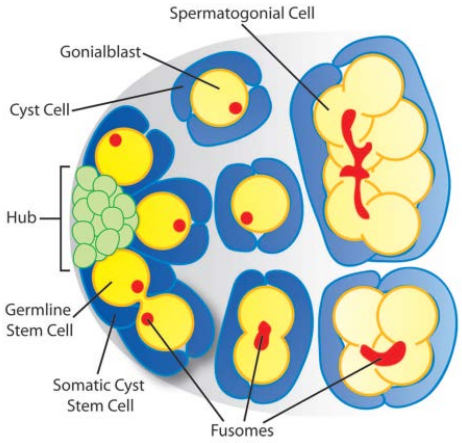
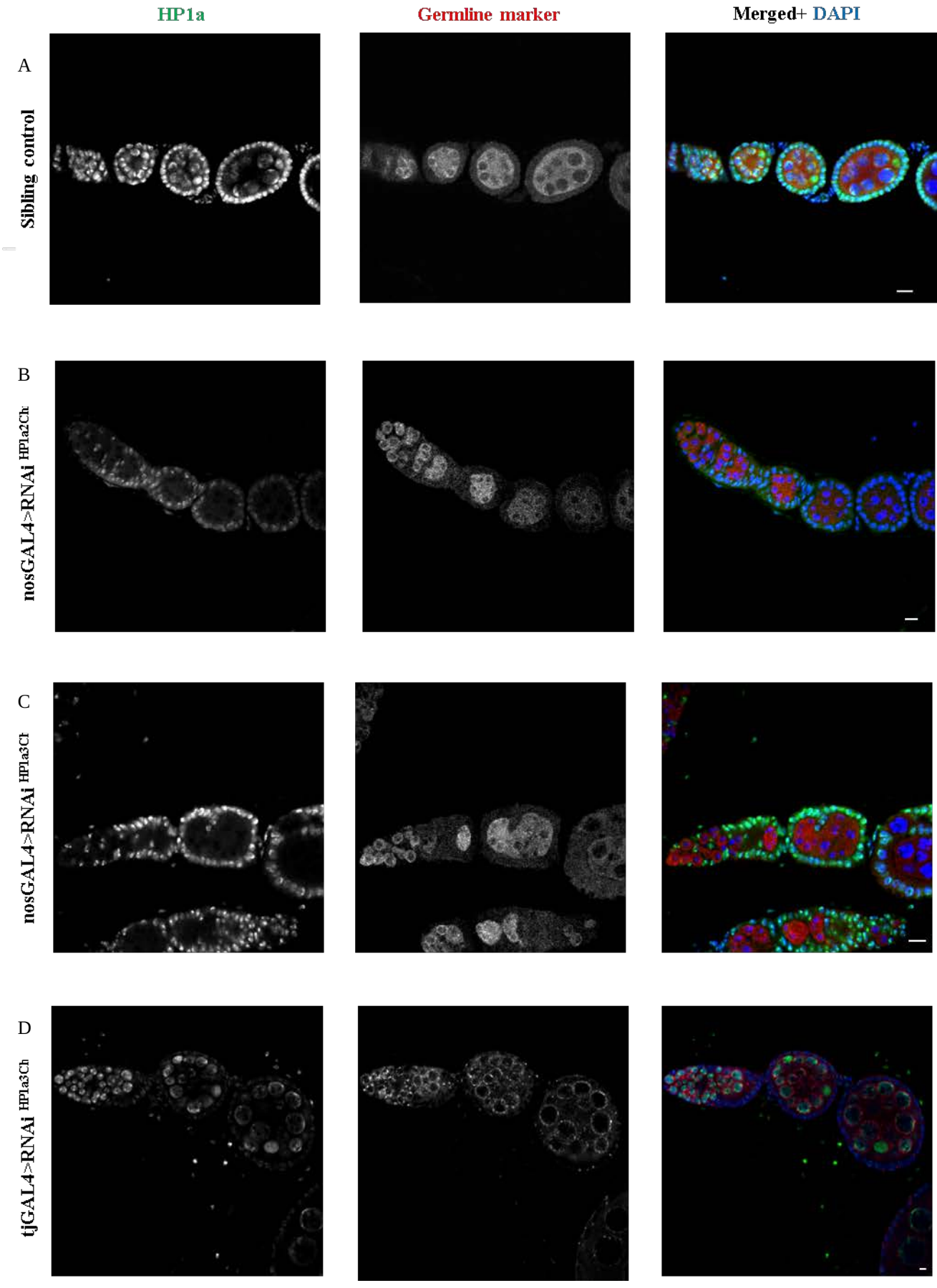


Figure 3.1.2 **Schematic representation of the *Drosophila* testes.** The hub gives rise to both somatic cyst stem cells and germline stem cells which differentiate progressively. The germline is depicted in yellow and the somatic lineage in blue. Adapted from Matunis et al. 2012.


HP1a
Germline marker
Merged+ DAPI
A
Sibling control
B
nosGAL4>RNAi HP1a2Ch
C
nosGAL4>RNAi HP1a3Ch
D
tjGAL4>RNAi HP1a3Ch

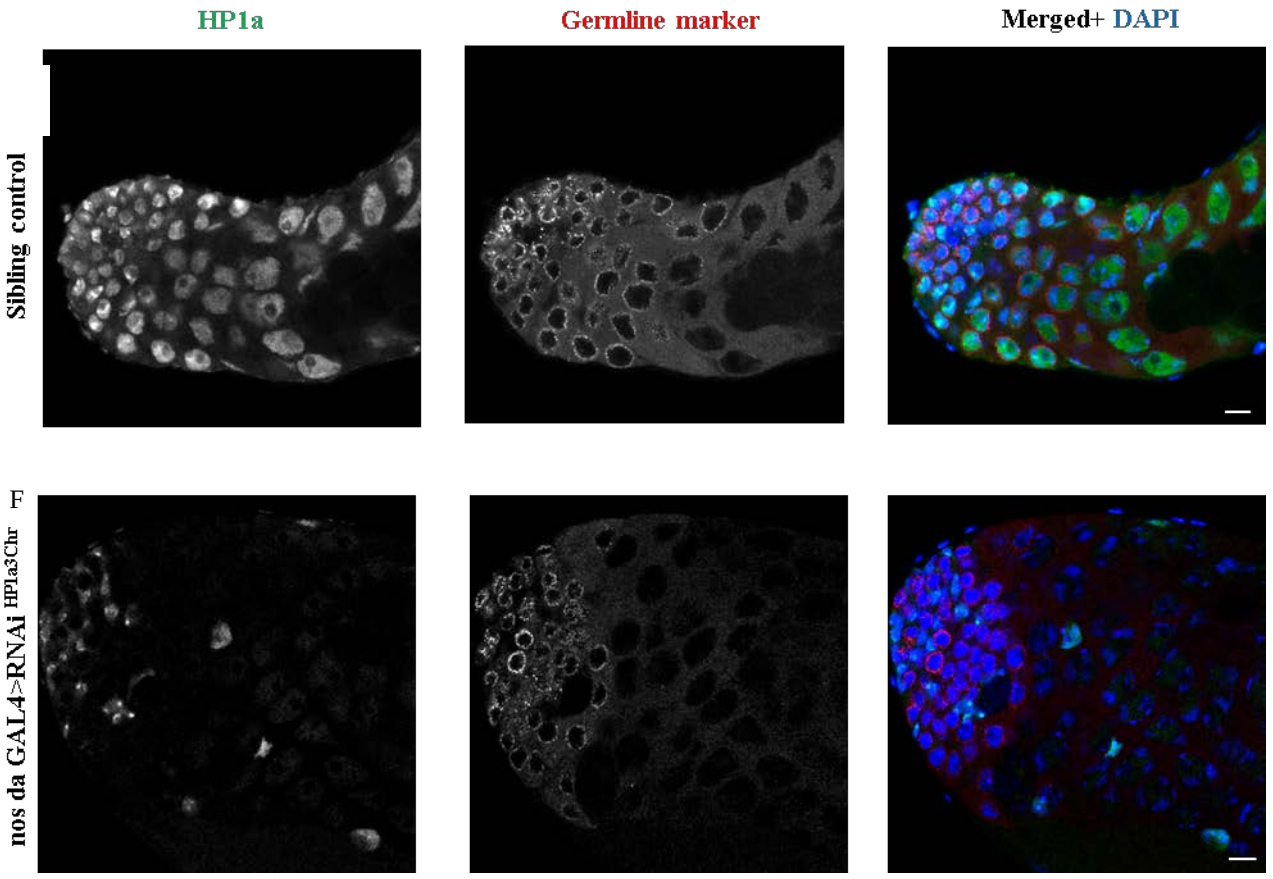


Figure 3.2 **Robust tissue -specific knockdown of HP1a in the Drosophila germline**

A. Control for female germline: Immunostaining with anti-HP1a (green) antibody for wild type ovaries showed both somatic and germline nuclear staining with stronger HP1a in the oocyte nucleus (DAPI) probably due to compaction of the oocyte nucleus. Germline marker Vasa(red) stains the perinuclear region and cytoplasm.

B. Female germline knockdown using *nanos* GAL4 driver and HP1a RNAi line on the 2nd chromosome: The germline nurse cell and oocyte nuclei are completely devoid of HP1a whereas the somatic follicle cells retain strong nuclear signal suggesting the specificity of the knockdown.

C. Female germline knockdown using *nanos* GAL4 driver and HP1a RNAi line on the 3rd chromosome: The germline nurse cell and oocyte nuclei are completely devoid of HP1a whereas the somatic follicle cells retain strong nuclear signal suggesting the specificity of the knockdown. This RNAi line shows more robust knockdown of HP1a.

D. Female ovarian somatic knockdown using *trafficjam* GAL4 driver: HP1a is significantly reduced specifically from follicle cell nuclei while it is retained in the germline nuclei.

E. Control for male germline: HP1a (green) shows somatic and gemline nuclear localization. Krimper (red) marks the germline and DAPI (blue) the nuclei.

F. Male germline knockdown using *nanos* and *daughterless* GAL4 driver and HP1a RNAi line on the 3rd chromosome: Nuclear HP1a is specifically lost from the germline cells (Krimper positive). The RNAi line on the 2nd chromosome showed less robust knockdown (data shown later). Scale bars are 10 µm.

3.1.2 Loss of HP1a in female germline somatic follicle cells shows no significant phenotype

A significant reduction of HP1a from the somatic follicle cells of the ovaries did not show any phenotypic defects in morphology or fertility rates when compared to sibling controls from the same cross (Data not shown).

3.1.3 Loss of HP1a in the male germline leads to partial sterility

Efficient knockdown in the male germline could be obtained using the combined drivers *nanos* and *daughterless* and the RNAi^{HP1a3chr}. Figure 3.3 shows the efficiency of the knockdown procedure. Males from the cross did not show any consistent and/or obvious phenotype although preliminary experiments like fertility assay suggested the males may show semi sterility. It is likely, that residual amounts of HP1a, remained in the tissue (Figure 3.3), owing to which there was no consistent phenotype. Since female germline knockdown showed complete sterility I decided to focus my study in females. Further study on the role of HP1a in the male germline was beyond the scope of this study.

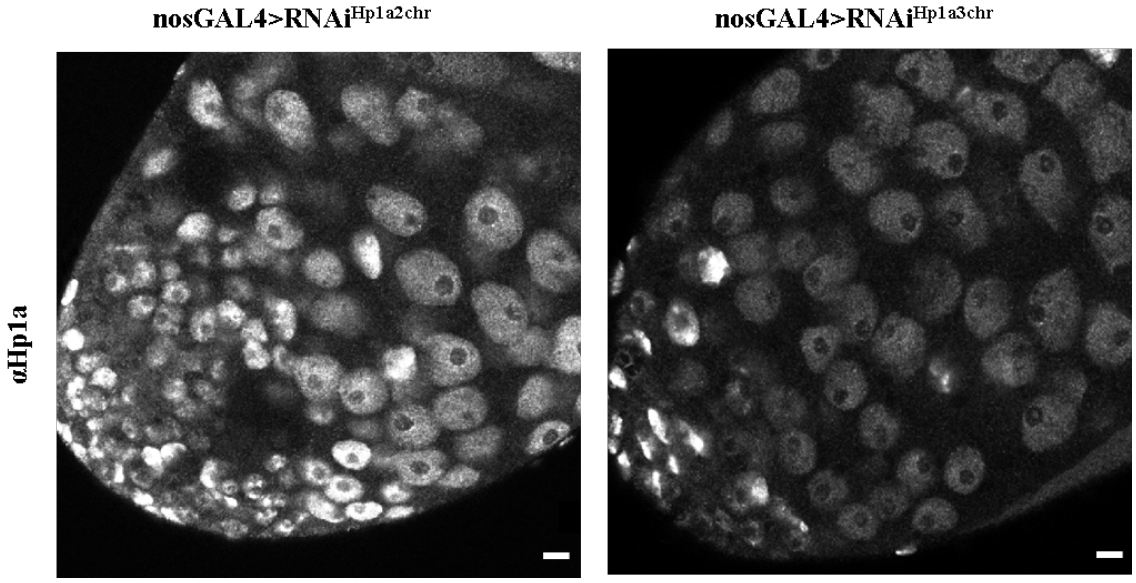


Figure3.3 **RNAi ^{HP1a3chr} showed more efficient knockdown.**

Immunostaining using antibodies specific to HP1a, show more efficient loss of HP1a from the tissue of interest (testes) in nosGAL4>RNAi ^{HP1a3Chr} as compared to nosGAL4>RNAi ^{HP1a2Chr}.

Scale bars are 10 µm

3.1.4 Loss of HP1a in the female germline leads to sterility and some defects in germline development

3.1.4.1 Knockdown of HP1a in the germline of ovaries severely reduced egg laying and hatching

Females with germline loss of HP1a using both RNAi lines were sterile. Knockdown fly ovaries were also smaller in size as compared to wild type and egg chambers later than stage 10 were not observed in 2-3 day old flies. There was a difference in severity of phenotype between the 2 RNAi lines: ovarian size of *nos*GAL4>RNAi^{HP1a3Chr} was smaller than that of *nos*GAL4>RNAi^{HP1a2Chr}. This difference can be due to the disparate efficiency of knockdown as apparent in Figure 3.3. The defects arising from tissue specific knockdown of HP1a in the *Drosophila* germline have been tabulated in Table 3.1.

Under similar conditions, *nos*GAL4>RNAi^{HP1a2Chr} laid just 2-5 eggs/fly/day as compared to sibling controls which layed approximately 30 eggs/fly/day as monitored by the egg laying assay (Figure 3.4). Eggs laid showed some morphological defects (data not shown). *nos*GAl4>RNAi^{HP1a3Chr} did not lay any eggs.

Tissue	Driver	Effect on gonads
Ovarian somatic cells	*tj*-GAL4	None
Female germline	*nos*-GAL4 (both RNAi lines)	**Sterile** Small, egg chambers arrest after st. 10
Testes	*nos*-GAL4 *nos*-GAL4; *da*-GAL4 (RNAi line on 3rd chr)	None Semi-sterile

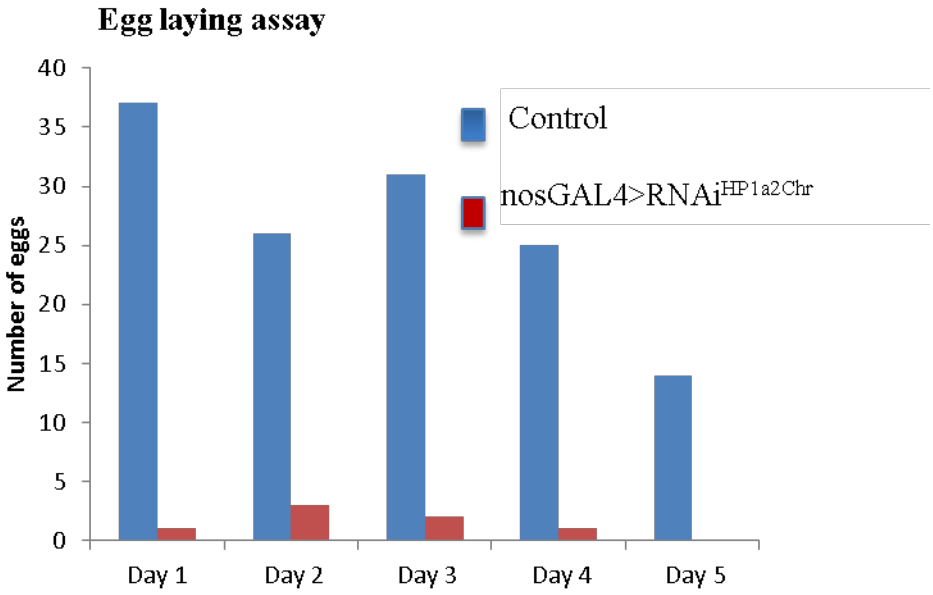


Table 3.1 **Effect of tissue specific knockdown of HP1a in the Drosophila germline**

Figure 3.4 **Egg laying assay .** Graph showing a comparison between eggs layed by control and *nos*GAL4>RNAi^{HP1a2Chr} female germline knockdown flies. There is a significant reduction in the number of eggs layed by the knockdown flies and the eggs do not hatch. The 3rd chromosome RNAi line does not lay eggs at all.

3.1.4.2 Early germline development, oocyte determination are slightly affected while axis specification is not highly perturbed upon HP1a knockdown

To determine the cause of sterility I examined ovarian development, especially oogenesis, upon knockdown of *HP1a* by immunostaining using specific markers for germline development. The germarium consists of germline stem cells that divide asymmetrically to 2 daughter cells, one of which, namely, the cystoblast differentiates. The cystoblast undergoes 4 rounds of incomplete mitosis as a result of which the daughter cells remain connected to one another by a cytoplasmic bridge structure called the fusome. The fusome is a germline specific organelle and α- Spectrin is a component of this structure (Lin et al. 1994; McKearin and Ohlstein 1995). Approximate zones for germline stem cells (GSC) and their progressive differentiation was scored by using Bag of marbles (Bam), the marker for early differentiating cystoblasts (McKearin and Spradling 1990), fusomes were marked by α-spectrin (de Cuevas et al. 1996). The Bam positive cell zones and branched fusome morphology were not altered significantly between sibling control and the germline knockdown of HP1a in young flies suggesting that cystoblast differentiation is unaffected upon *HP1a* germline knockdown. However aged females from *nos*GAL4>RNAi ^{HP1a3chr} showed loss of Bam staining but this could be a secondary effect due to overall degeneration of the ovaries (Figure 3.5 A and B).

Another important feature is oocyte determination. Two pro-oocytes exist at region 2 of the germarium but by stage 3 one is selected for oocyte fate while the other exits meiosis to join the other 14 nurse cells. The oocyte forms a compacted karyosome and using a synaptonemal complex marker, C(3)G. I checked if there were defects at the step of oocyte formation (Page and Hawley 2001). The knockdown of HP1a using *nos*GAL4>RNAi ^{HP1a3chr} but not *nos*GAL4>RNAi ^{HP1a2chr} caused a slight delay in oocyte fate determination apparent from the de-condensed DAPI staining which is a common feature of many piRNA pathway component mutants (Figure 3.5 C).

Oocytes also have polar deposition of certain components required for axis specification. By using Gurken; one of the asymmetrically deposited components, as a marker, I found that female germline knockdown of HP1a did not affect axis specification (Chen et al. 2007). Both control and the knockdowns show the crescent

shaped deposition of Gurken in the oocyte nucleus stage 8 onwards (Figure 3.5 D).

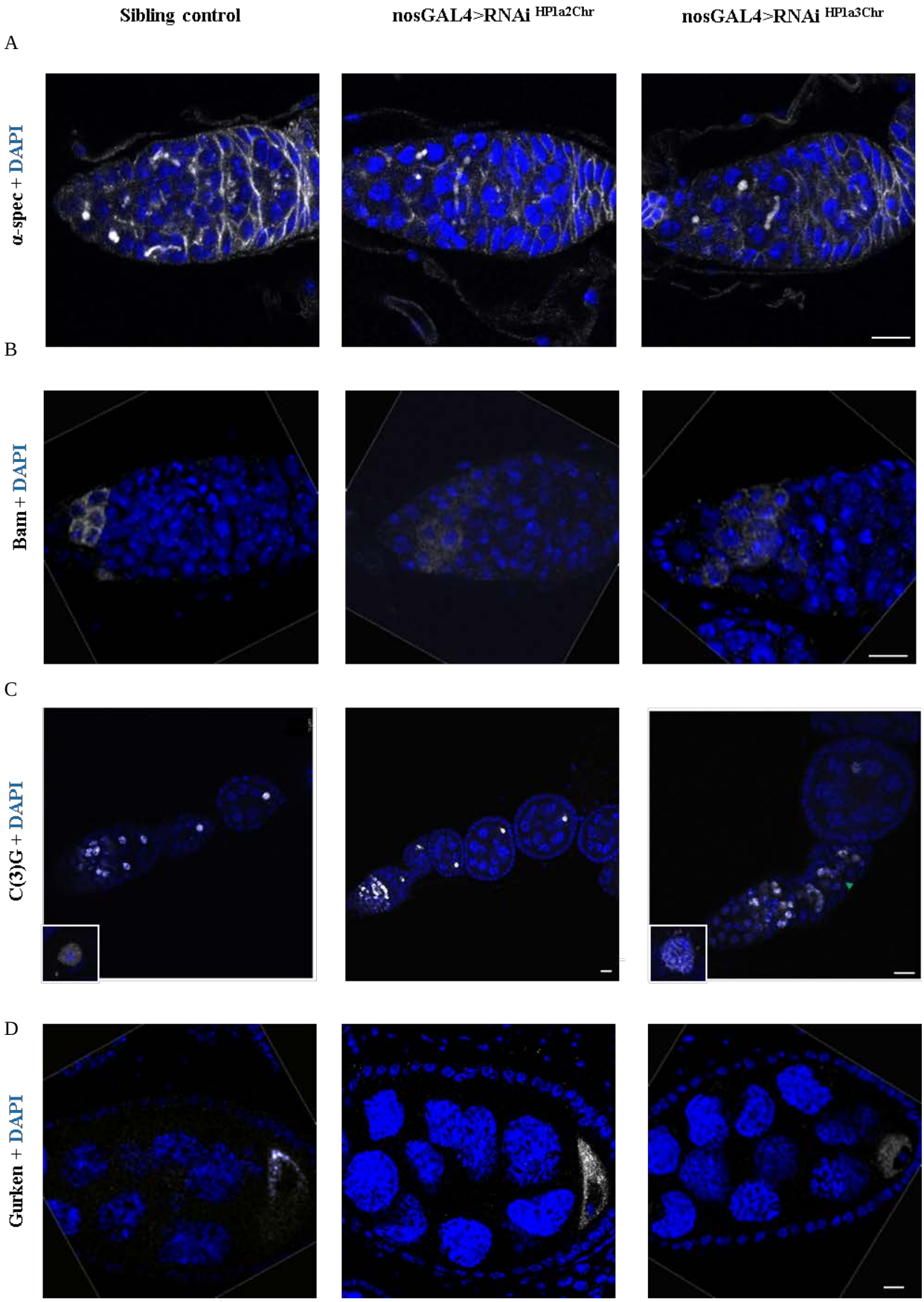


Figure 3.5 **Early germline development and axis specification are not highly perturbed upon HP1a knockdown while oocyte determination is slightly delayed as determined by immunostaining.**

A Germarium showing α-Spectrin staining to mark fusomes in control and knockdown ovaries. The branching of fusomes, signifying connected cysts, is not affected upon loss of HP1a from the ovarian germline.

B. Bam staining was affected slightly in aged *nos*GAL4>RNAi^{HP1a3Chr}, however in young flies there was minimal difference in expression levels between control and knockdown germarium with respect to the Bam positive zone.

C. Oocyte determination is slightly delayed (arrowhead points to the region) in *nos*GAL4>RNAi ^{HP1a3Chr} as evident from the C(3)G staining in the knockdown ovaries as compared to the control. The zoomed in panel in control shows the compaction of nuclei(DAPI staining) as compared to the uncompacted DAPI in the knockdown panel for an egg chamber at the same stage of development.

D. Axis specification and polarization appeared unperturbed as Gurken staining is identical both in the control and knockdown oocytes. *nos*GAL4>RNAi^{HP1a3Chr} showed slight perturbation in expression levels.

Scale bars are 10 μm.

3.1.4.3 An age dependent deterioration was observed on ovarian morphology upon HP1a germline knockdown

Even though there were no severe defects in the germline developmental events, the females were sterile and oogenesis was arrested at stage 8-10 of development. Also, there was an age dependent deterioration of the ovaries. By 8-10 days the ovaries showed severe degeneration, though they never became agametic. Most ovaries lost later stage egg chambers (Figure 3.6).

Older ovaries showed degenerated egg chambers and to check if cell death was a result of apoptosis I performed the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the Apoptag kit provided by Millipore on 5 day old ovaries obtained from both control and germline knockdown flies. *nos*GAL4>RNAi $^{HP1a\ 2chr}$ showed apoptosis from stage 8 onwards as shown in the Figure 3.7. *Nos*GAL4> RNAi $^{HP1a\ 3chr}$ however, showed a much stronger phenotype where the entire ovary including somatic cells (HP1a positive cells in panel C) showed apoptosis. To avoid indirect effects due to age dependent deterioration of the ovaries upon HP1a

germline knockdown, all further experiments were carried out in 2-3 day old flies when the effect on general ovarian morphology was minimal.

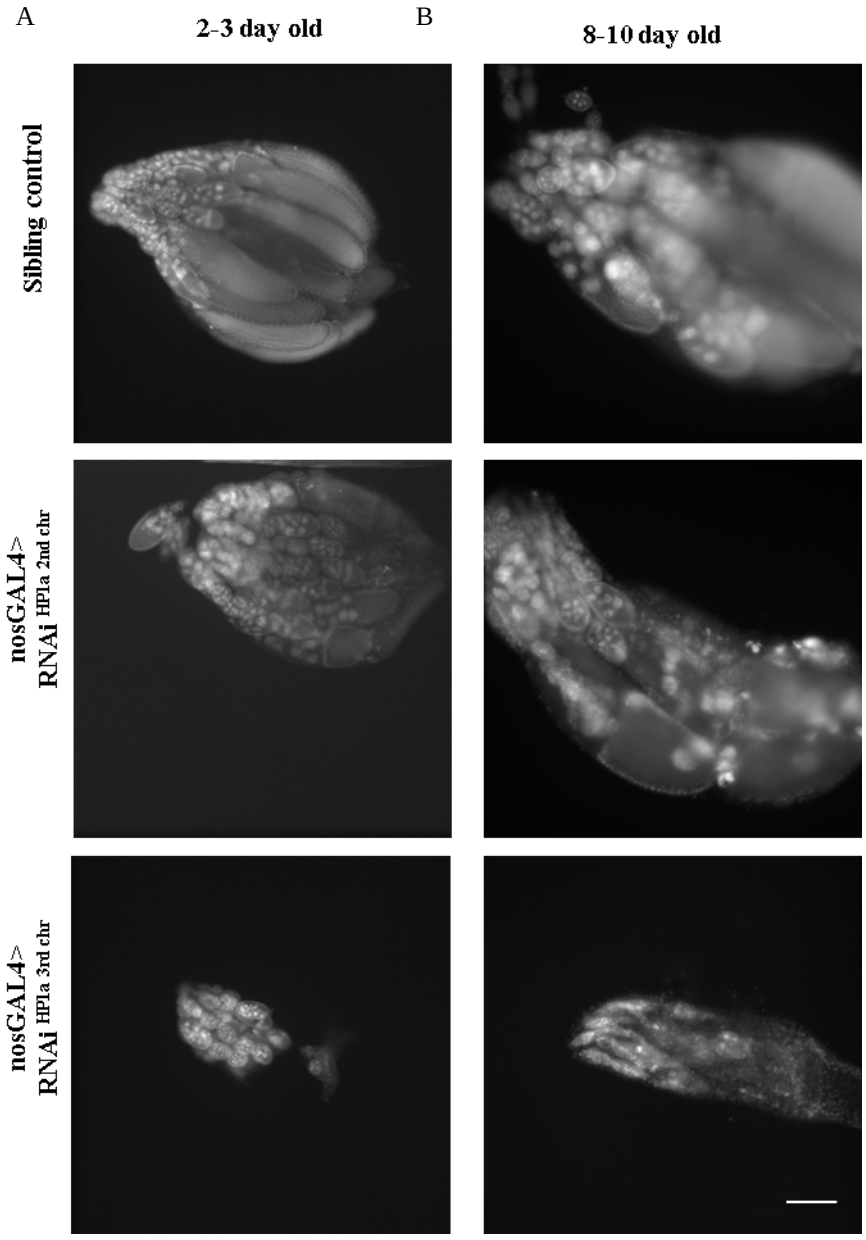


Figure 3.6 **An age dependent deterioration was observed on ovarian morphology upon HP1a germline knockdown**

A. Whole mount images of DAPI stained ovaries from 2-3 day old control and knockdown flies. The knockdown ovaries from *nos*GAL4>RNAi ^{HP1a3Chr} were smaller than control and *nos*GAL4>RNAi ^{HP1a2Chr} but there were no degenerating egg chambers. Knockdown ovaries did not bear many late stage egg chambers.

B. Whole mount images of DAPI stained ovaries from 8-10 day old control and knockdown flies. Severe age- dependent degeneration was observed upon knockdown of HP1a from the germline. These images were taken using a 10x lens on the Zeiss Widefield Microscope.
Scale bars are 10 μm.

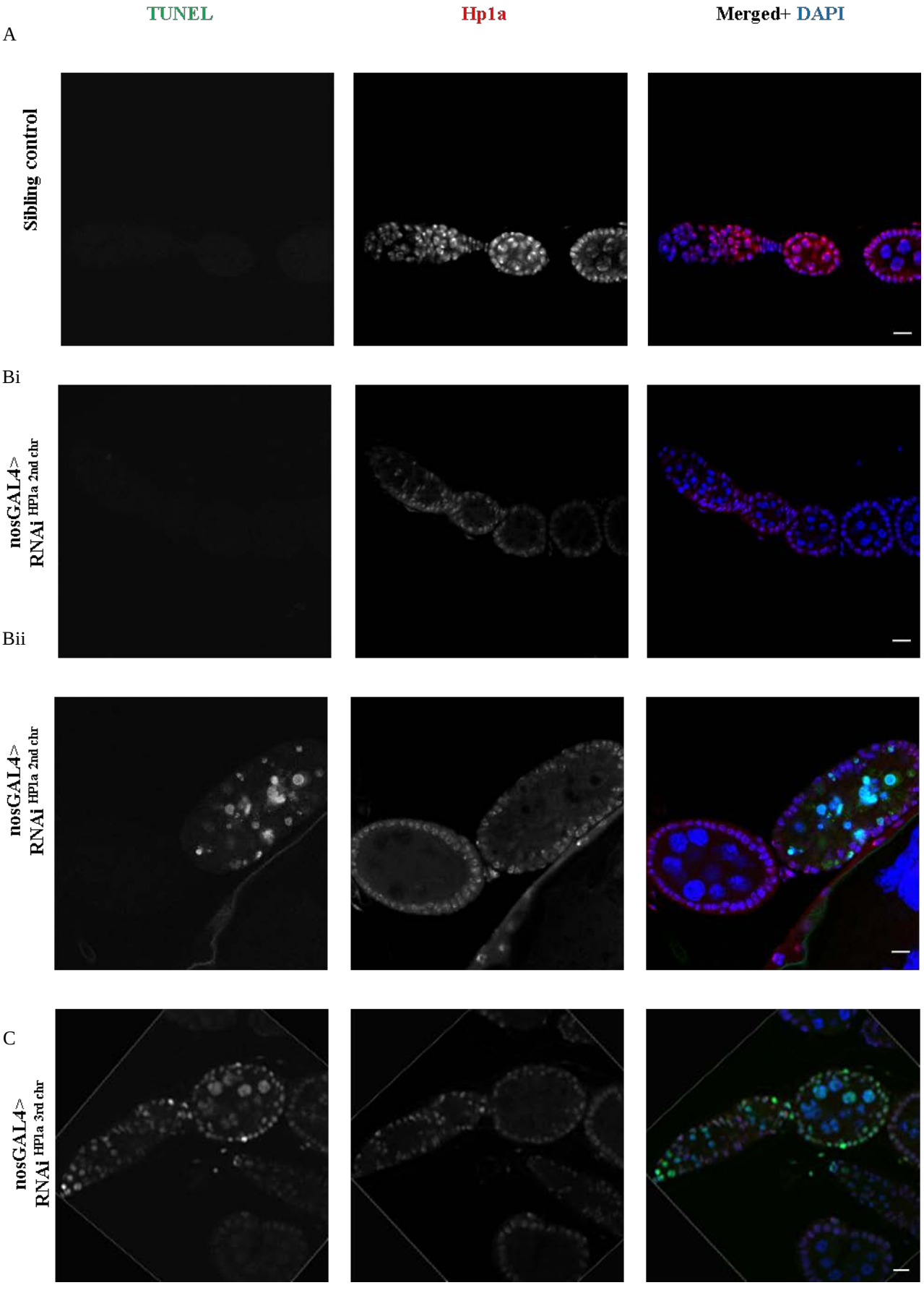

TUNEL
Hp1a
Merged+ DAPI
A
Sibling control
Bi
nosGAL4>
RNAi HP1a 2nd chr
Bii
nosGAL4>
RNAi HP1a 2nd chr
C
nosGAL4>
RNAi HP1a 3rd chr

Figure 3.7 **Apoptosis is the cause of degeneration in older ovaries**

A Staining for TUNEL positive cells was performed to track apoptosis. Control 5- day old ovaries show no apoptosis (no TUNEL positive egg chambers).

B *nos*GAL4> RNAi $^{HP1a\ 2chr}$ show TUNEL positive staining in egg chambers older than stage 8 (ii). There are no TUNEL positive cells in the germarium (i).

C. *nos*GAL4> RNAi $^{HP1a\ 3chr}$ showed high apoptosis in 5-day old ovaries. Apoptosis was also apparent in tissues where HP1a was not knocked down.

Scale bars are 10 µm.

3.1.4.4 Loss of HP1a leads to an accumulation of double stranded breaks in the germline nurse cell nuclei

Early germline development was not significantly perturbed upon knockdown of HP1a. However, sterility and degenerating late stage egg chambers remained obvious phenotypes. Sterility can arise due to many causes (Schupbach and Wieschaus 1991), however, given the known functions of HP1a I focused on the following hypotheses.

HP1a is known to bind to DNA and also maintain stable telomeres (Fanti and Pimpinelli 2008). Also, in the somatic cells HP1a is known to play a role in the piRNA pathway to silence transposable elements (Todeschini et al. 2010). Experiments with germline knockdown of HP1a by (Wang and Elgin 2011) showed up- regulation of certain transposable elements. Given these known functions of HP1a, sterility could arise due to DNA damage that could be attributed to:

i) chromosomal instability rendered by loss of HP1a from the heterochromatic telomere,or

ii) due to double strand breaks arising due to transposition events, or

iii) a combination of both the above factors

Thus, to account for the sterility phenotype I checked for DNA damage or perturbation to genomic integrity in the female germline. Double strand breaks are common upon loss of genome integrity and phosphorylated H2Av (γH2Av) which recognizes the site of double stranded DNA breaks can be used as a marker for DNA damage (Modesti and Kanaar 2001).

In the control ovary double strand breaks can usually be observed in region 2b of the germarium where meiosis occurs (not visualized in the Figure). However, additional DNA breaks were observed in the nurse cell nuclei and beyond region 2b of the germarium upon HP1a knockdown in the ovarian germline akin to several piRNA pathway mutants (Figure 3.8). However no double stranded breaks were observed in the oocyte as is the case for several sterile piRNA pathway mutants.

In view of the DNA damage results I wanted to address whether the cause was

i) loss of chromosomal integrity especially at telomeres or

ii) transposon mobilization upon depletion of HP1a in the female germline, as shown previously by Wang et. al, 2011.

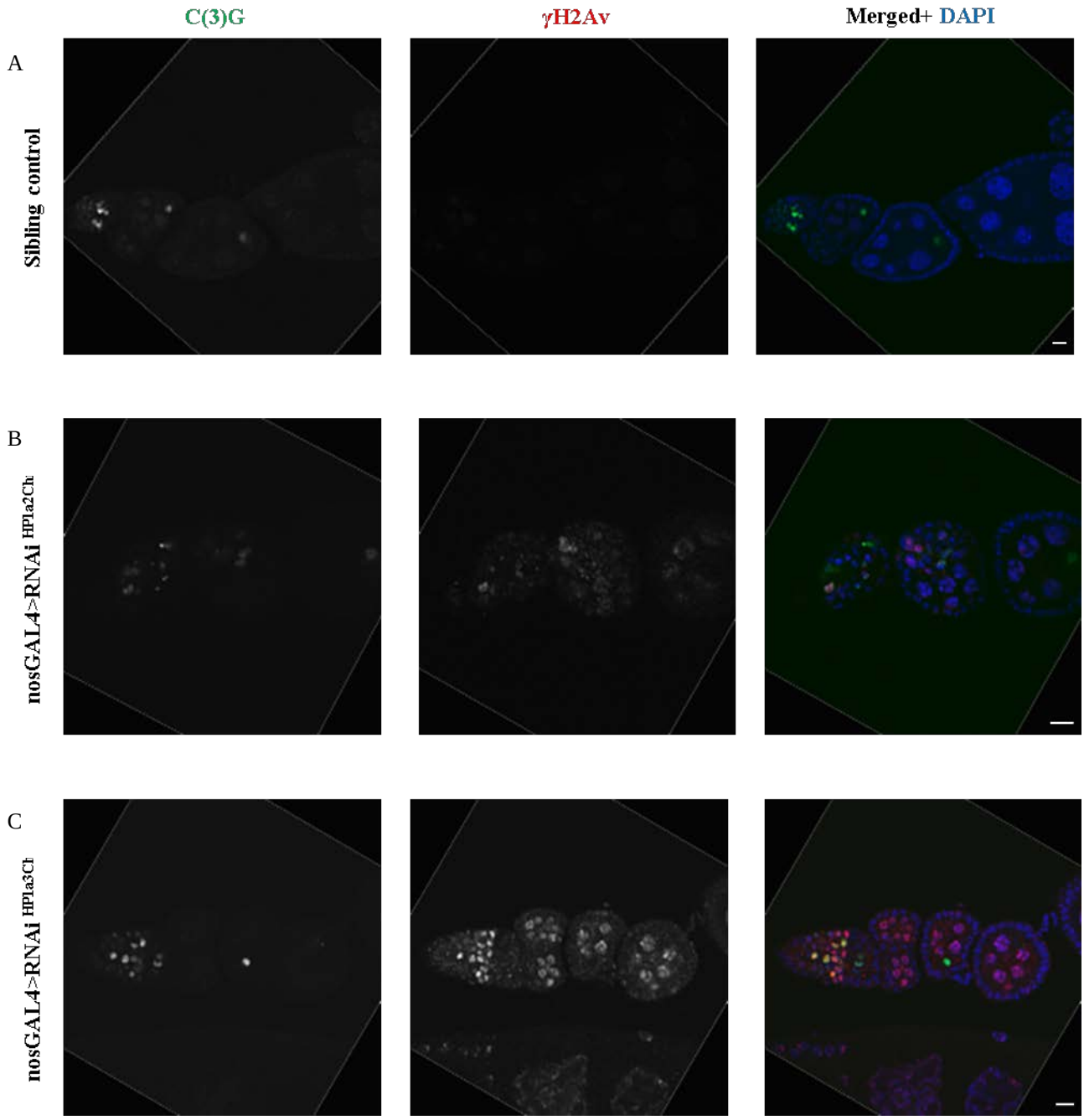


Figure 3.8 **Severe DNA damage occurs upon *nos*GAL4> RNAi $^{HP1a3rd\ chr}$ knockdown.**

Double strand breaks as visualized by γH2Av (red) staining are observed in the germarium and even nurse cells but not the oocyte (marked by C3G (green) in the knockdown ovaries (B and C) as compared to the control (A).
Scale bars are 10 µm.

3.2 The loss of HP1a does not compromise the integrity of telomeres but may lead to slight telomere clustering defects in the ovaries

3.2.1 Lagging chromosomes were not seen in the germarium of *HP1a* knockdown ovaries

Previous reports by (Khurana et al. 2010) have shown the presence of lagging chromosomes owing to telomeric defects and I tried to visualize such defects in the mitotic germline cells in the germarium of *HP1a* knockdown ovaries.

To visualize mitotic cells in the germarium I used PH3 as a marker. Additionally I had a germline marker, Vasa to mark the germline cells. DAPI was used to visualize DNA. I looked for mitotic germline cells i.e., both Vasa and PH3 positive, preferably between late metaphase and anaphase. There are indeed very few such cells seen in each ovary so I had a relatively small number of samples- 10 cells from several knockdown ovaries. Lagging chromosomes are supposed to be seen as DAPI dense regions in the metaphase plate region when the chromosomes have receded to the poles during anaphase. No such DAPI pockets were seen upon HP1a knockdown, for the sample size studied (Figure 3.9 A).

3.2.2 HipHop foci decreased in number upon HP1a knockdown implying defects in telomere clustering

To analyse if the telomeres were affected upon HP1a knockdown I used an approach similar to the one reported in Wesolowska et al. 2013; where the authors scored telomeric clustering using EGFP-HOAP as a reporter. Instead of HOAP, which occupies telomere caps, and I used Hip-Hop which is an interacting partner of HOAP to label telomeres(Gao et al. 2010). In the wild type context the 16 telomeres in *Drosophila melanogaster* cluster to form 4-6 foci per nuclei. I reasoned that if telomere integrity was lost upon loss of HP1a, there may be defects in clustering.

30 images each for sibling controls, *nos*GAL4>RNAi^{HP1a2chr} and *nos*GAL4>RNAi^{HP1a3chr} germarium co-stained for HipHop; the telomere marker and DAPI the nuclear marker, were taken. These images were processed by the Imaris software to determine the number of Hip-Hop foci per nucleus. As seen from the graph

in Figure 3.9B the sibling controls had 4.5foci/nucleus on average. *nos*GAL4>RNAi^{HP1a2chr} showed a decrease in the number of foci 2.7foci/nucleus whereas *nos*GAL4>RNAi^{HP1a3chr} showed no considerable defects. The slight reduction may imply some telomeric fusion but on the whole it does not seem that HP1a knockdown in the germline disrupts telomere integrity.

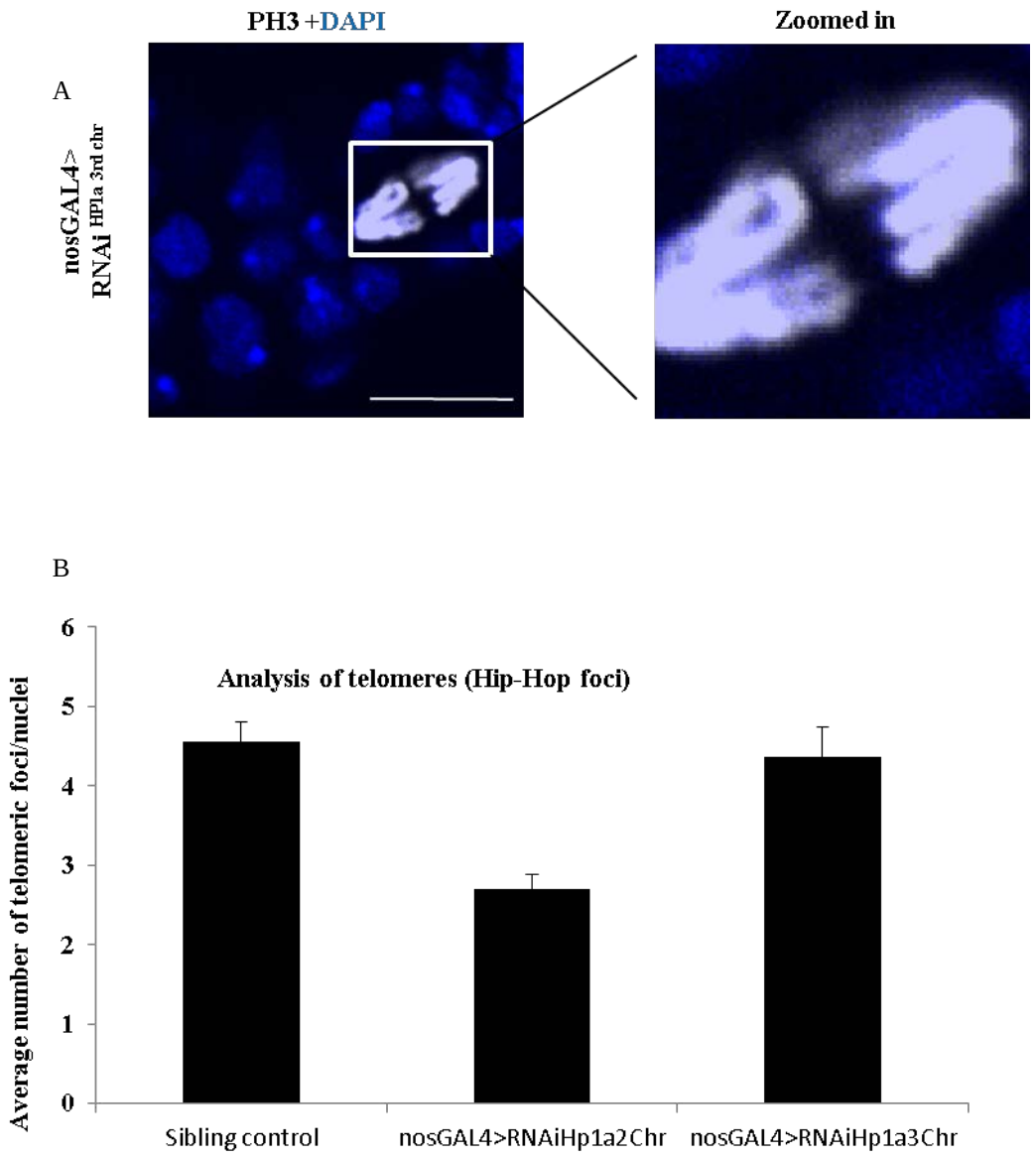


Figure 3.9 **Effect of germline HP1a knockdown on telomeres**

A. Confocal section of a germarium with a mitotic germline cell in late anaphase. There were no DAPI dense regions between the 2 spindles as they retreat to the poles signifying absence of lagging chromosomes. Scale bars are 10 µm.
B. Telomere clustering as analysed by number of Hip-Hop foci/nuclei may be slightly affected upon germline knockdown of HP1a. For the analysis 30 germarium for each sample were analysed using the 3D image analysis Cell plugin available from Imaris.

3.3 A subset of transposons, especially, the telomeric retroelements are de-repressed upon loss of *HP1a* from the *Drosophila* female germline

3.3.1.1 Telomeric retroelements *HeT-A, TART* and *TAHRE* showed most significant upregulation upon female germline knockdown of *HP1a*

To address whether double stranded breaks were as a result of transposition events, qRT-PCR was performed on RNA samples isolated from HP1a knockdown ovaries to check for transposon mRNA expression levels. Telomeric *HeT-A, TART*, and *TAHRE* showed significant up-regulation (>5 times) upon loss of HP1a while other transposable elements like *I element, Bari, Burdock* did not show major changes in expression. R2 (Kraminker et. Al.,2002) showed a significant upregulation too but this is not unexpected given that the genomic location of R2 is mostly subtelomeric This suggests that HP1a is required for silencing a subset of transposons in the ovarian germline (Figure 3.10 A).

3.3.1.2 Loss of HP1a from the somatic follicle cells of the ovary did not lead to upregulation of telomeric retroelements

HP1a is ubiquitously expressed and occupies telomeres in both the somatic and germline tissues of Drosophila. To determine if the role of HP1a in silencing the telomeric subset of transposons was germline specific I checked transposon expression levels upon *tj*GAL4>RNAi$^{HP1a2\&3chr}$. There was no upregulation in *HeT-A, TART* or *TAHRE* expression levels upon loss of HP1a from the somatic follicle cells of the ovaries (Figure 3.10 B).

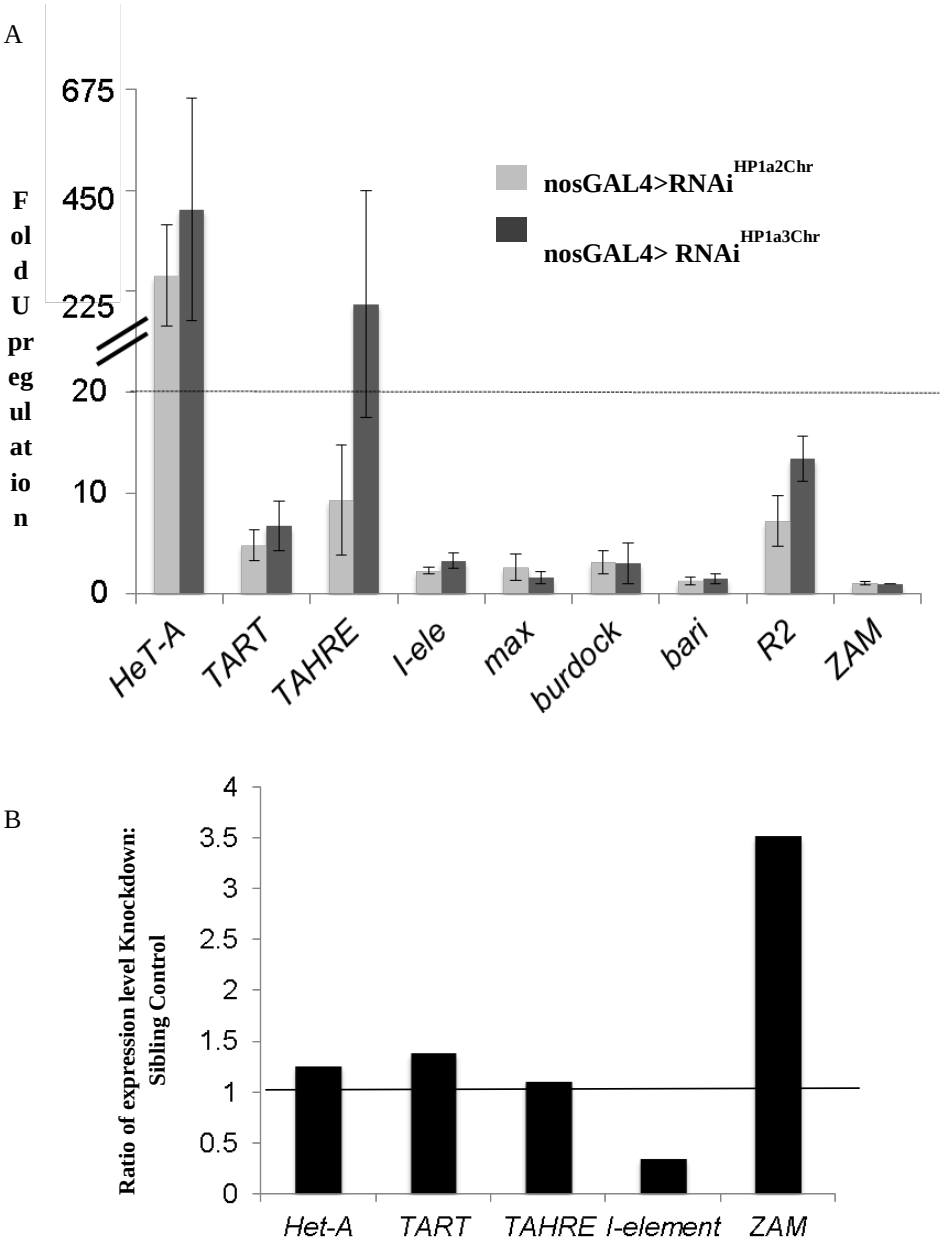


Figure 3.10 ***nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$ knockdown selectively derepresses telomeric retroelements and has little or no effect on other transposons.**

A qRT-PCR results on RNA extracted from control and *nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$

Results show fold upregulation with respect to sibling control. Telomeric elements *HeT-A*, *TART* and *TAHRE* show most significant upregulation. A break in the graph had to be included to accommodate *Het-A* levels. The levels of upregulation were more significant using the RNAi^{HP1a3Chr} line.

B qRT-PCR results on RNA extracted from sibling control and *tj*GAL4> RNAi $^{HP1a3rd\ chr}$

Results show ratio between knockdown and control. There are no significant changes in transposon expression levels especially telomeric retroelements, unlike upon germline knockdown.

3.3.2 *HeT-A* and *TART* showed strong oocyte nuclear accumulation

To confirm the qRT-PCR results and determine the localization of the retroelements in the ovaries upon HP1a loss, I immunostained for retroelements *HeT-A, TART* (upregulated) and *I element* (unaffected) using specific antibodies. *HeT-A* and *TART* showed strong oocyte nuclear accumulation. I element was not seen to be up-regulated correlating with the q-RT PCR results (Figure 3.11A and B). To ascertain that the I element antibody was functional I used another piRNA pathway mutant, *krimp,* known to de-repress I element (Lim and Kai 2007) and found strong oocyte nuclear accumulation in keeping with previous reports (Figure 3.10C; Van De Bor et al. 2005).

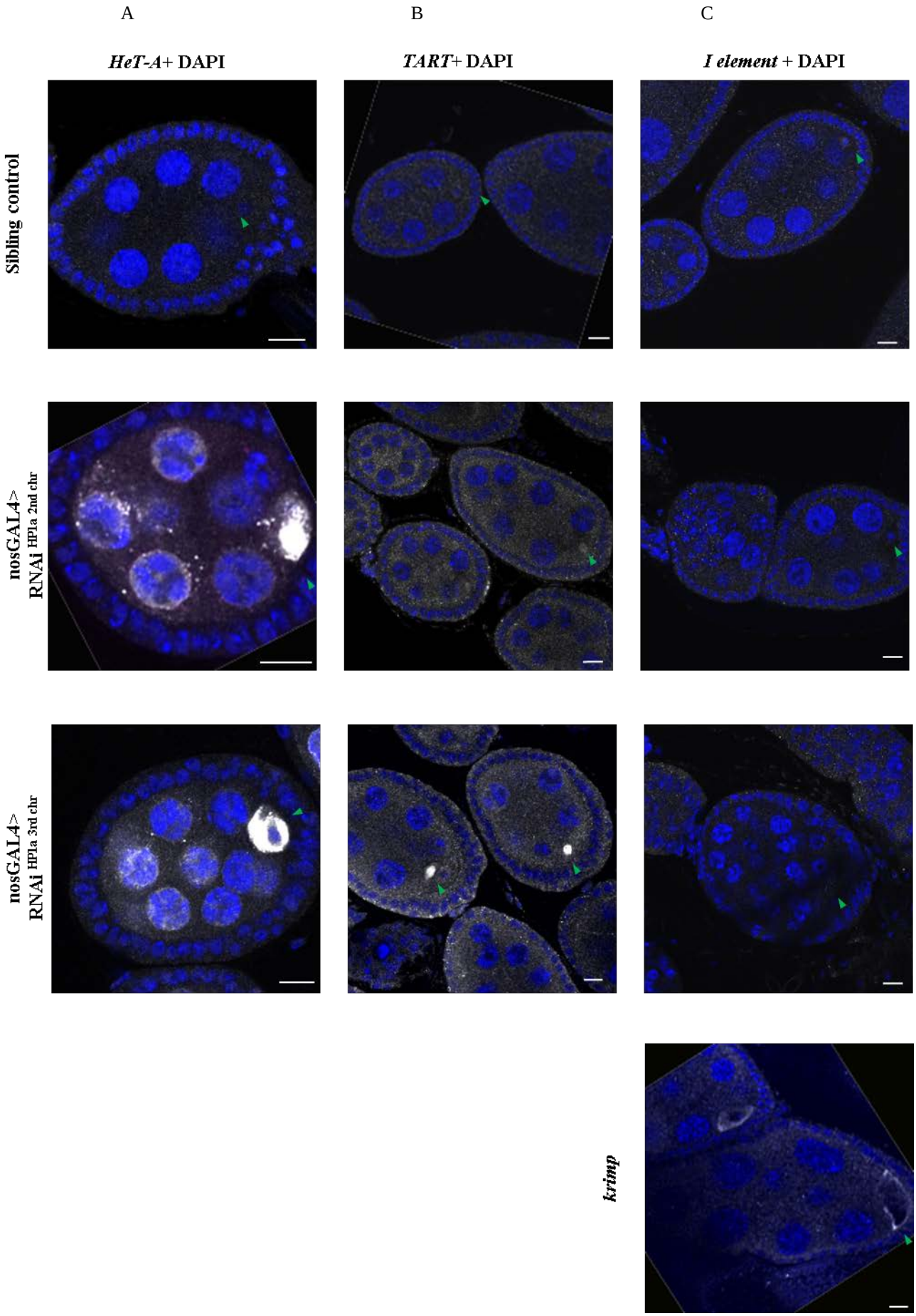

A
B
C
HeT-A+ DAPI
TART+ DAPI
I element + DAPI
Sibling control
nosGAL4> RNAi HP1a 2nd chr
nosGAL4> RNAi HP1a 3rd chr
krimp

Figure 3.11 **Telomeric retroelements *HeT-A* and *TART* protein accumulate in the oocyte nuclei upon *nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$ knockdown but I element does not.**

A. anti *HeT-A* staining showed strong oocyte (marked by green arrow heads in all panels) and nurse cell nuclear accumulation of *HeT-A* in the knockdown ovaries and a complete absence from the control.

B. Telomeric retroelement *TART* showed *HeT-A* like oocyte nuclear accumulation in the knockdown ovaries especially in *nos*GAL4>RNAi ^{HP1a3chr} in contrast to complete absence in the control.

C. I element accumulation was not seen upon knockdown of HP1a in the germline corroborating the qPCR data. By contrast, piRNA pathway mutant *krimp* shows oocyte accumulation of I element, confirming that the antibody was functional.

Scale bars are 10 μm.

3.3.3 Genomic copy number of telomeric *HeT-A* increases upon HP1a knockdown suggesting increased transposition events

To assess if transposition events were really occurring I isolated genomic DNA from HP1a knockdown flies of varying ages and performed qPCR for telomeric *HeT-A* copy numbers (Figure 3.12). A >5 fold increase in *HeT-A* was observed suggesting increased transposition events whereas I element copy number remained unchanged. This corroborated the qRT-PCR results.

HP1a is known to be present at telomeres (Frydrychova et al. 2008). The above mentioned results revealed that the most affected transposons in HP1a germline knockdown: *HeT-A, TART*, and *TAHRE* are those present at the telomere. Perrini et al., 2004, characterized different alleles of HP1a and suggested that HP1a plays two distinct roles in telomeric retroelement transposition and telomere capping and stability. I wanted to address the transposon related functions of HP1a in the germline especially since my results suggested that the telomere silencing function was more severely affected upon female germline specific loss of HP1a.

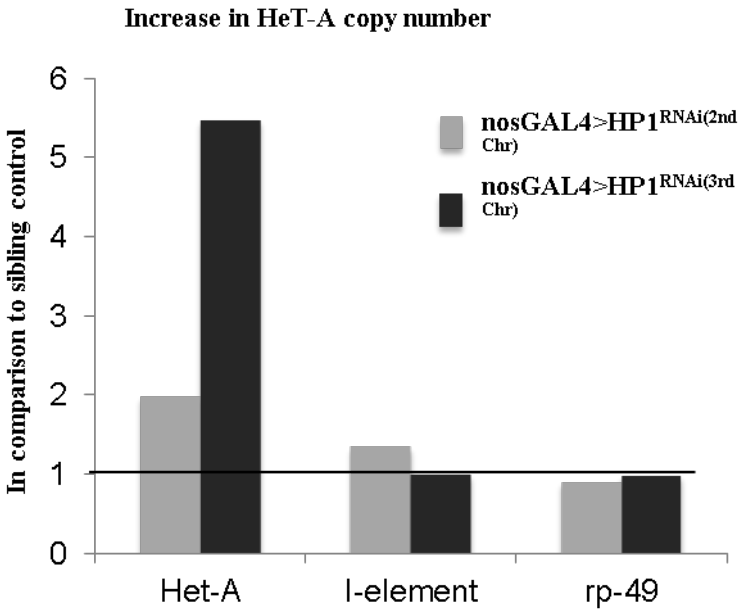


Figure 3.12 **Genomic copy number for telomeric retroelements increased upon *nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$ knockdown in the germline.**
qRT-PCR on genomic DNA isolated from aged *nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$ flies showed increased telomeric *HeT-A* copy number but not I element transposon or euchromatic coding gene RP-49.

3.4 Clonal analysis using *dfs* strategy to corroborate HP1a knockdown studies

Off target effects of RNAi lines and inefficient knockdown are long standing concerns for knockdown studies. Although the use of short hairpin dsRNA targets and not long dsRNA targets reduces the risk of off target effects further confirmation was necessary (Kulkarni et al., 2006). To ensure that the phenotype I saw upon female germline knockdown of HP1a was solely due to the loss of HP1a I used the *dfs* (dominant female sterile) clonal analysis strategy to generate germline clones for *HP1a* (Chou and Perrimon 1996). Germline clones for *HP1a* using the dfs strategy was technically challenging to obtain. I could not generate sufficient numbers of flies for further experiments like qRT-PCR to check for transposon upregulation. Thus a mitotic clonal analysis was performed instead (Xu and Rubin 1993). A fly line bearing a homologous FRT, ubiGFP and containing a heat shock flippase was crossed to the *Su(var)205*^{05} FRT/ CyO. The progeny were heat shocked four times, twice at the larval stage and twice at pupae. The adult females were then scored for mitotic clones. As seen in Figure 3.13, the GFP negative egg chambers were devoid of HP1a and this confirms the FRT lines and recombination events. Only the clones showed massive upregulation of *Het-A;* whereas no upregulation was observed in the adjacent egg chambers bearing functional copies of *HP1a*. These results conclusively show that the reason for telomeric retroelement upregulation is solely due to loss of function of HP1a and not an off target effect of RNAi in general.

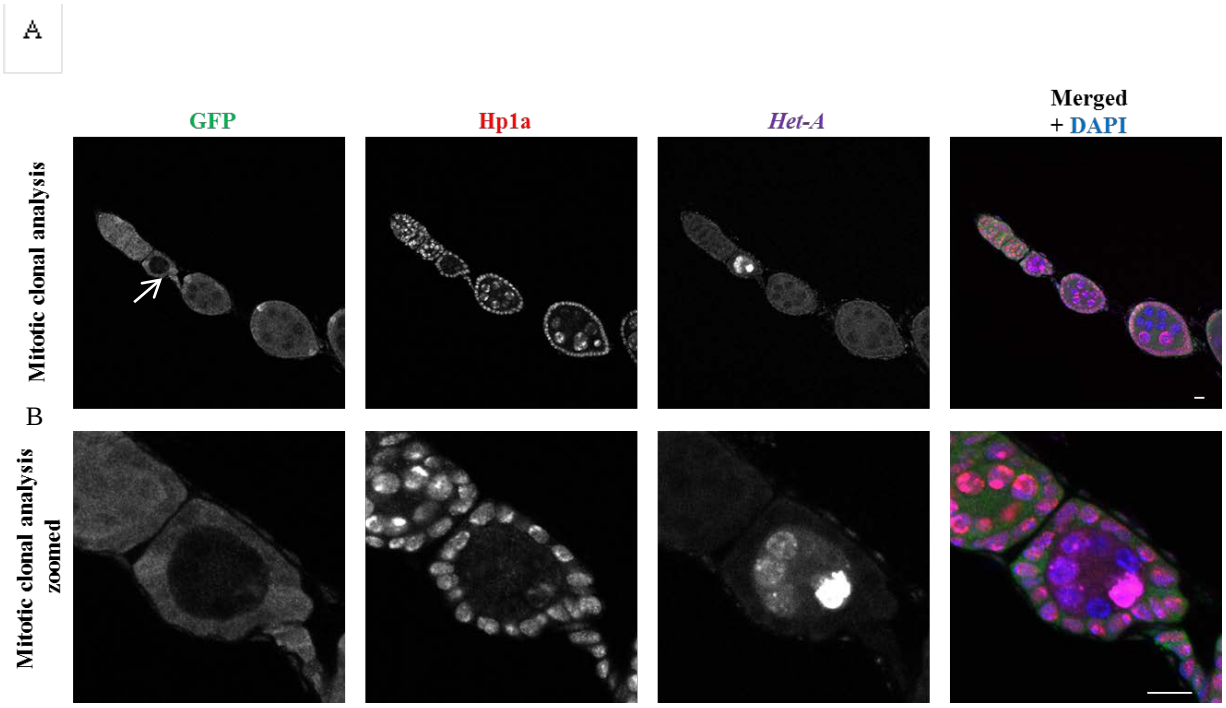


Figure 3.13 **Mitotic Clones for *Su(var)205*^{05} in the Drosophila ovary.**
A fly line bearing a homologous FRT, ubiGFP and containing a heat shock flippase was crossed to the Su(var)20505 FRT/ CyO and the progeny wereheat shocked. GFP negative clones were scored for the upregulation of telomeric retrolement *Het- A.* In panel A the arrow points to a GFP negative egg chamber representling a stable mitotic clone. The egg chamber was deviod of HP1astaining confirming the HP1a mutant clone. Temoleric retrolement *Het- A* was upregulated selectively upon loss of HP1a. Panel B shows a zoomed view of the mitotic clone egg chamber.
Scale bars are 10 µm.

3.5 HP1a is required for piRNA production against telomeric HTT elements

Massive, but selective transposon families showed up-regulation and mobilization upon *HP1a* knockdown in the *Drosophila* female germline. The mechanism of suppression of transposons in the germline involves the piRNA pathway. To ascertain if HP1a was working hand in hand with the piRNA pathway and if so, at what step of the pathway, deep sequencing analysis for piRNAs from the knockdown ovaries was performed. To make sure that the phenotypes were not a result of RNAi in general I used *nos*GAL4>RNAi^{GFP} in addition to the sibling controls. Sibling controls and RNAi^{GFP} showed similar profile hence for simplicity RNAi^{GFP} will be used as a control for the thesis.

I hypothesized that if HP1a is solely involved in Transcriptional Gene Silencing (TGS) there should be no significant change in piRNAs between control and germline knockdown ovaries. However, if it is involved in more upstream processes of piRNA production one would expect to see perturbation in piRNA levels. Also, I wanted to see if the selective effect on the telomeric subset of transposons could be explained from the piRNA profile.

The sequencing was performed on the Hiseq2500 platform for the 4 piRNA libraries. The libraries were normalized using mature miRNAs since miRNA numbers were comparable across all the libraries (Table 3.2). Many non-coding RNAs lie in the heterochromatic regions so it was not surprising that they exhibited variability across the libraries. Endogenous siRNAs (esiRNAs) could not be used to normalize the libraries because they were too few in number. All further analysis was performed on normalized reads (Figure 3.13).

Genotype / Number	*nos*GAL4>RNAi GFP	*nos*GAL4>RNAi HP1a2chr	*nos*GAL4>RNAi HP1a3chr
Total reads	64626724	36867561	44349749
Mature miRNA	151661	141593	100696
Precursor miRNA	158725	151661	106426
esiRNA	182	598	292
Non codingRNA	8791	26815	187580

Table 3.2 **Normalization of piRNA libraries**

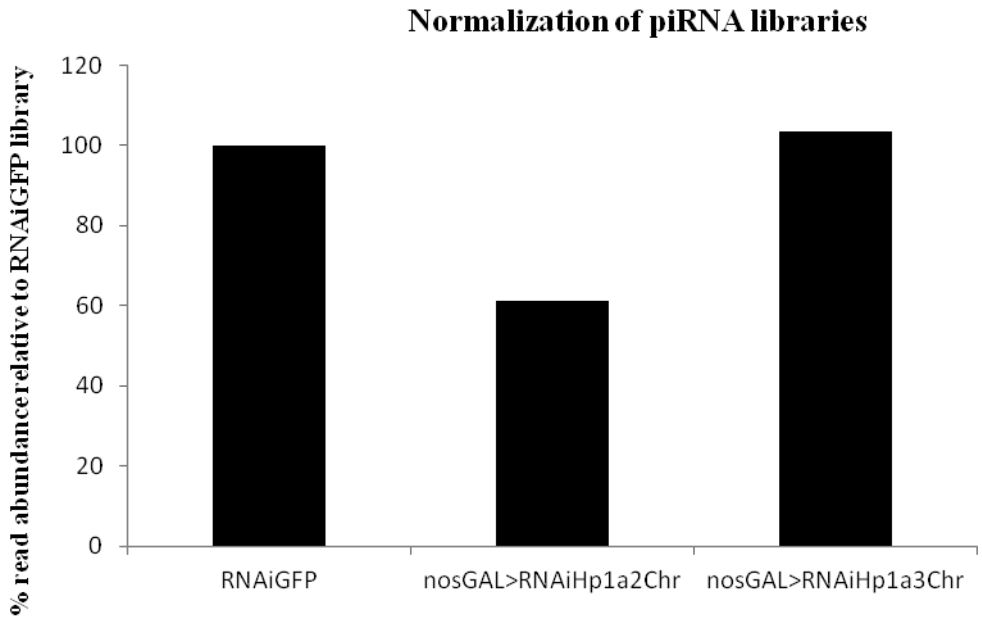


Figure 3.14 **Normalization of the sequence data obtained from the three ovarian piRNA libraries**

The raw data was normalized using mature microRNAs the reads of which were most comparable across all three libraries sequenced for small RNAs (piRNAs) on the HiSeq2500 platform.

3.5.1Most germline piRNAs were unaffected upon germline specific HP1a knockdown

Firstly, I looked at the size profile of small RNAs sequenced across all the libraries. Both knockdown libraries showed a slight decrease in the range of 23-29 nucleotides, i.e., the range for piRNAs (Figure 3.15). Thus HP1a loss most likely affected piRNA production. To ascertain which piRNAs were responsible for this drop, the piRNAs were aligned against the genome and transposons. Most piRNAs showed little or no effect upon loss of HP1a from the germline, as evident from Figure 3.16 (gray data points that lie within the 2 fold up or down regulation trendlines).

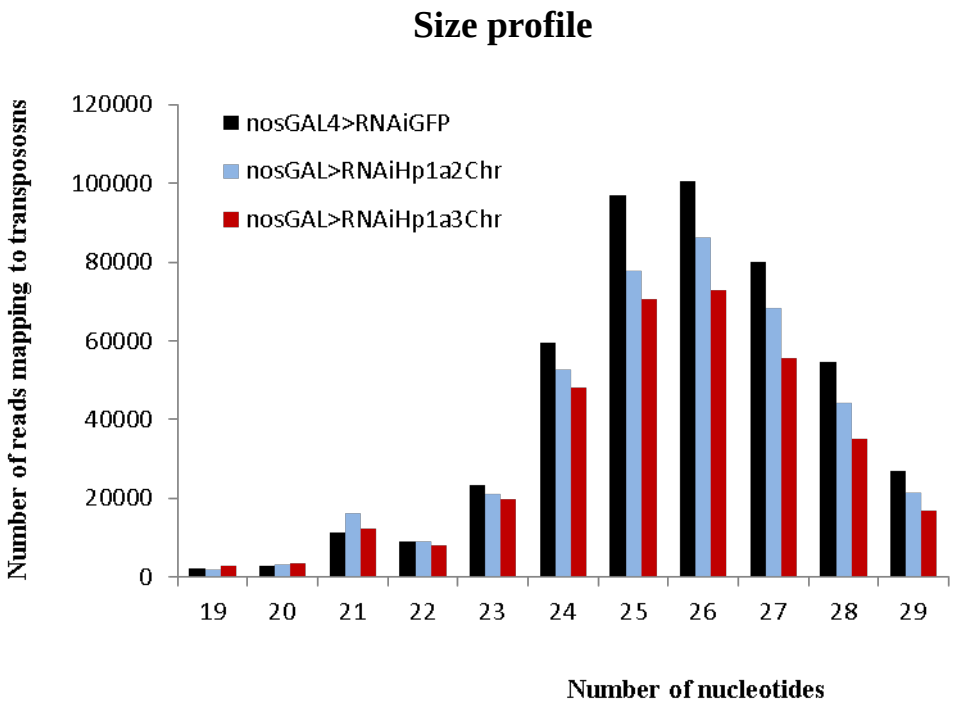
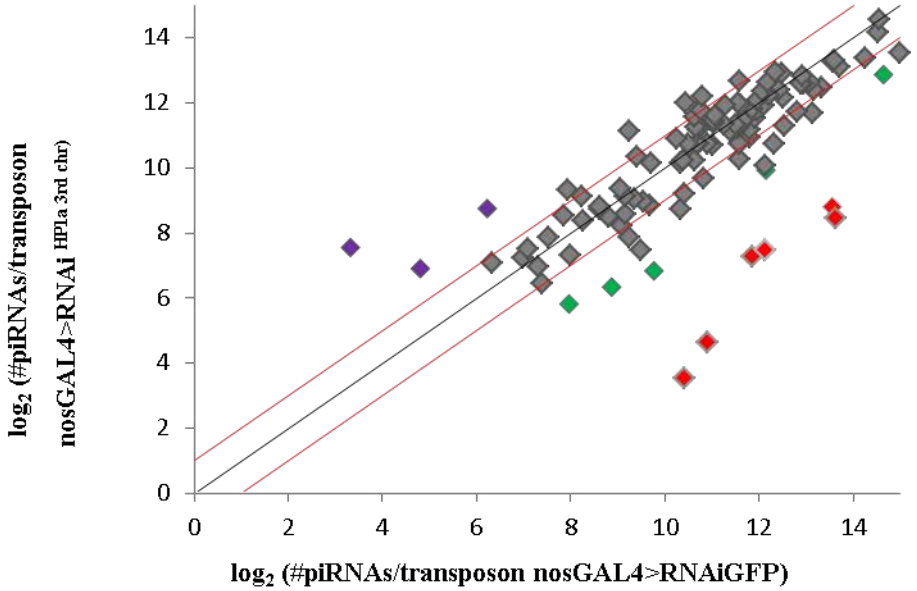
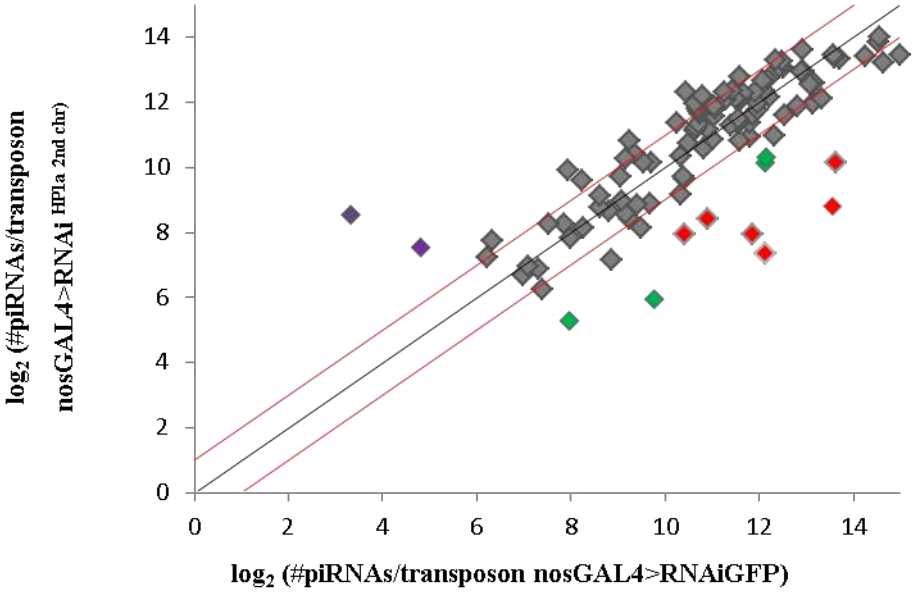


Figure 3.15 **Size profile for normalized transposon mapping small RNAs for the three ovarian libraries sequenced** piRNAs lie in the size range of 23-29 nucleotides. The general size distribution for both control (nosGAL4>RNAi^{GFP}) and knockdown libraries (nosGAL4>RNAi$^{HP1a2/3chr}$) is identical however the numbers of transposon mapping piRNAs seemed reduced in the knockdown samples.

A



B



◆ **Most reduction in piRNAs**

◆ **Moderate reduction in piRNAs**

◆ **Increase in piRNAs**

2 fold trendlines

y=x trendline

Figure 3.16 **Transposon mapping piRNA profile upon germline knockdown of HP1a**

A&B Graphs showing a comparison between numbers of piRNA against all germline transposons in nosGAL4>RNAi^{HP1a2chr} and the control and nosGAL4>RNAi^{HP1a3chr} respectively. A >2 fold up or down regulation was considered significant. Red points mark piRNAs against *HTT* elements which showed most reduction in the knockdown library. Green points mark elements that showed a moderate reduction in piRNAs and the purple points mark elements against which there were increased numbers of piRNAs upon knockdown of HP1a in the germline. In most cases the same piRNAs were affected in both lines used.

3.5.2 Only piRNAs mapping to telomeric *HTT* transposons showed massive reduction upon loss of HP1a from the germline

Only a of subset transposon mapping piRNAs showed significant reduction in the knockdown libraries. piRNAs against the telomeric retroelements *HeT-A*, *TART* and *TAHRE* were 5-30 fold reduced in *nos* GAL4>RNAi^{HP1a2chr} and 30-120 fold reduced in *nos* GAL4>RNAi^{HP1a3chr} (data points marked in red, on Figure 3.15 A and B). This was in keeping with the transposon up-regulation profile observed by qPCR. A few other transposons namely; *Looper1*, *NOF*, *Roo, BS and Rt1c* showed a moderate reduction in numbers of piRNAs in the range of 2-5 fold in the knockdown libraries (marked in green in Figure 3.14). piRNAs against *Gypsy, Tirant* and *Frogger* showed >2 fold increase (marked in purple in Figure 3.16).

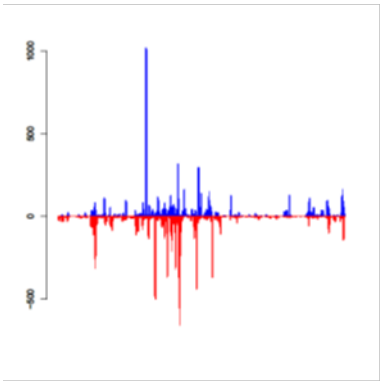
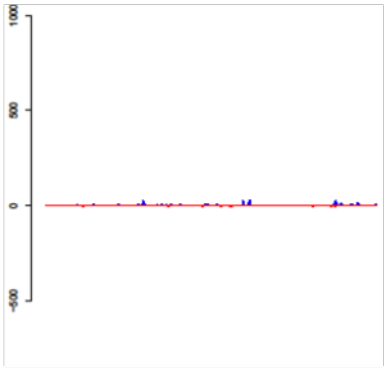
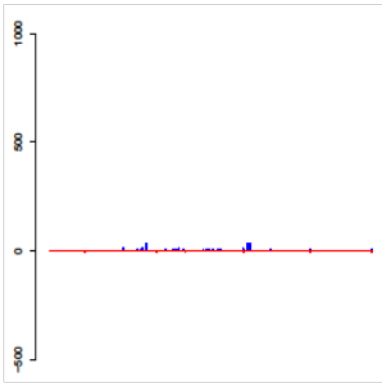
To look into the specificity towards certain piRNAs I further checked all individual germline piRNAs and their sense:anti-sense distribution. A few examples of each category are provided in Figure 3.17. Nearly all piRNAs against the *HTT* (A) elements were lost, piRNAs against *Roo* (which occupies subtelomeric regions on the genome) (B) were lost to a lesser extent while piRNAs against *I element* and *Max* remained unperturbed (C). There was a perfect correlation between the loss of piRNAs and upregulation of retrotransposon transcripts from sequencing and qPCR data.

nosGAL4>RNAiGFP

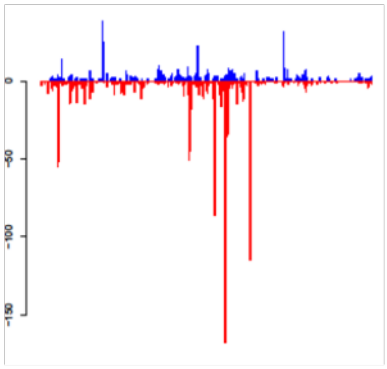
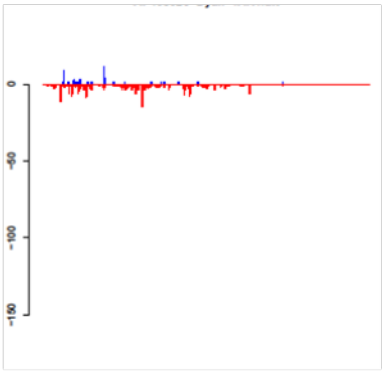
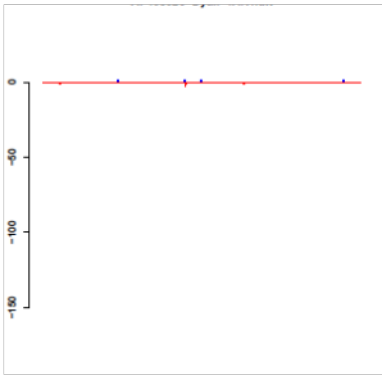
nosGAL4>
RNAi HP1a 2nd chr

nosGAL4>
RNAi HP1a 3RD chr

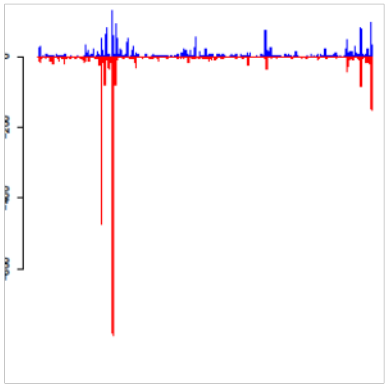
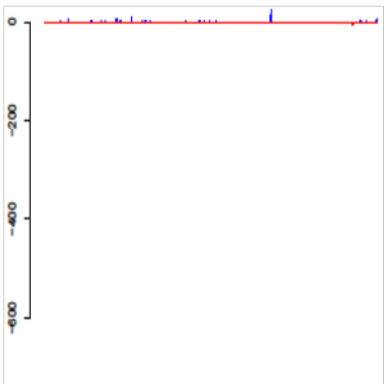
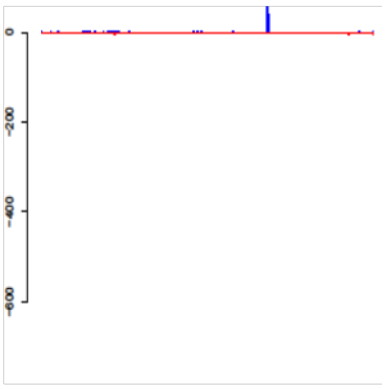
HeT-A

TART

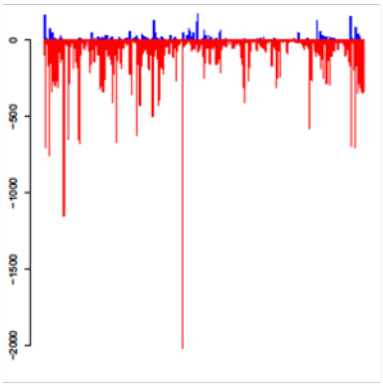
TAHRE

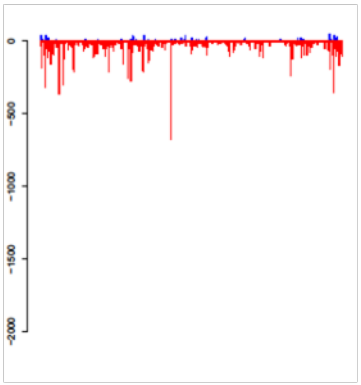




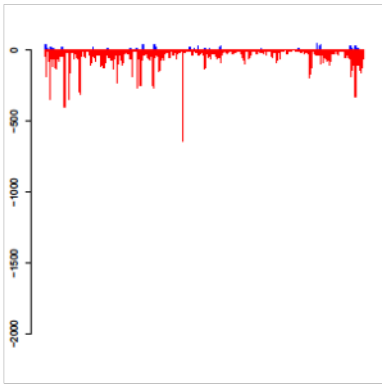
nosGAL4> RNAi HP1a 2nd chr

nosGAL4> RNAi HP1a 3rd chr

Roo

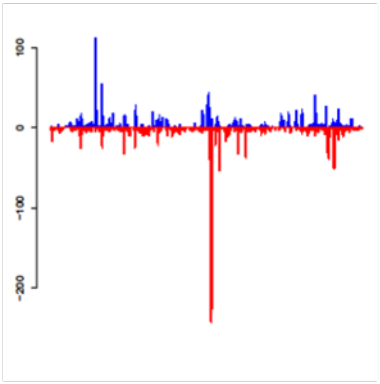


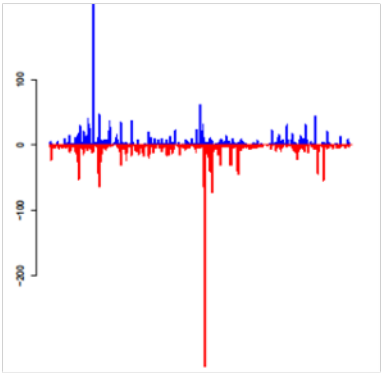


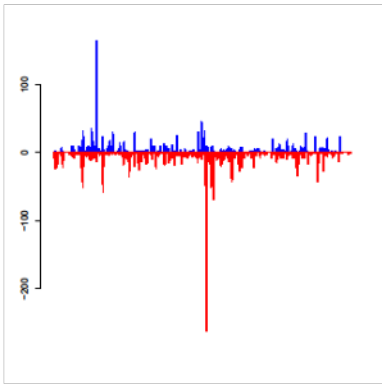


C. Unaffected

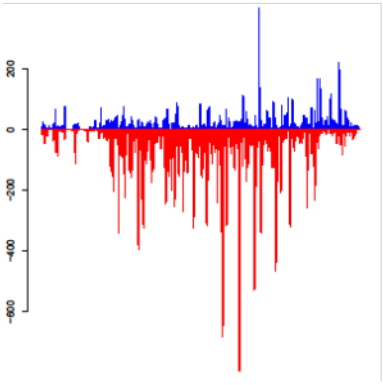
I-element

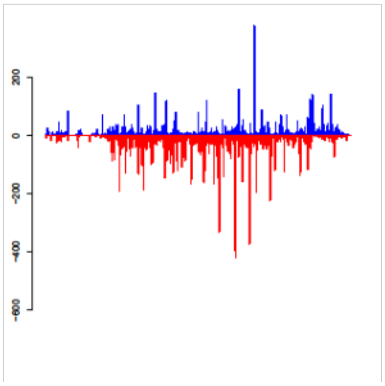






Max





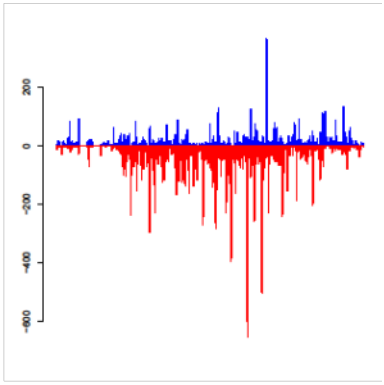


Figure 3.17 **Specific reduction in *HTT* mapping piRNAs is observed in the knockdown libraries.**

A. Most affected- piRNAs against the teloemeric retroelements were abolished upon knockdown of HP1a.

B. Moderately affected- *Roo* piRNAs were moderately reduced upon loss of HP1a from the ovarian germline.

C.Unaffected- piRNAs against *I element* and *Max* show similar profiles in control and knockdown libraries.

3.5.3 Cluster transcripts arising from clusters housing telomeric retroelements showed a severe drop

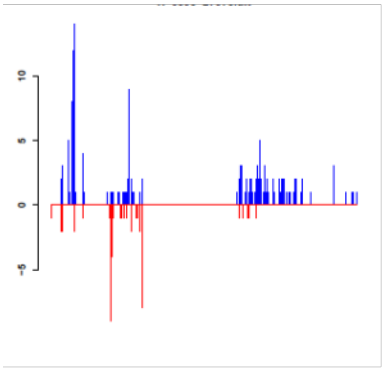
Thus far is it evident that HP1a plays an important role in the production of piRNAs against telomeric retroelements in the female germline. One of the possible ways to attain this specificity can be achieved if HP1a promotes cluster transcription from a subset of piRNA clusters, ie, telomeric piRNA clusters. This hypothesis is based on the function of its homolog *Rhino* which is known to be required for dual strand cluster transcription in the *Drosophila* female germline (Klattenhoff et al. 2009).Using the piRNA cluster information provided by Brennecke et al. 2007 (Appendix 7.3) I mapped the piRNAs from sequenced libraries to the annotated clusters. piRNAs from the telomeric clusters of Chromosome 4 (chromosome band 102 E), X-TAS(1A) and 3R (100F) were severely reduced as expected (Figure 3.18 A). However, a strong reduction was also observed from the largest germline piRNA cluster 42 AB on 2R (Figure 3.17 B). Some other germline piRNA clusters like the one on 2L and 38C were also affected but the somatic piRNA cluster *flamenco* was not affected at all. Thus, germline loss of HP1a affects germline cluster transcritption.

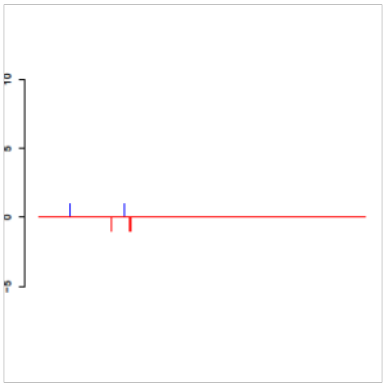
nosGAL4>RNAiGFP

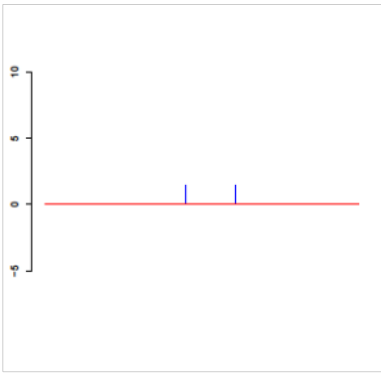
X-TAS (1A)

nosGAL4>
RNAi HP1a 2nd chr

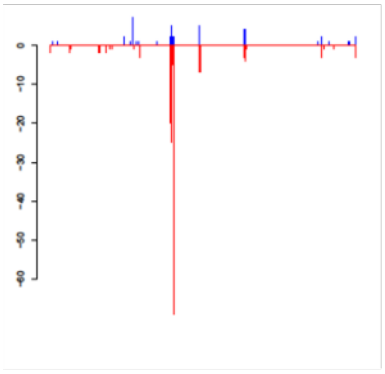
nosGAL4>
RNAi HP1a 3Rd chr

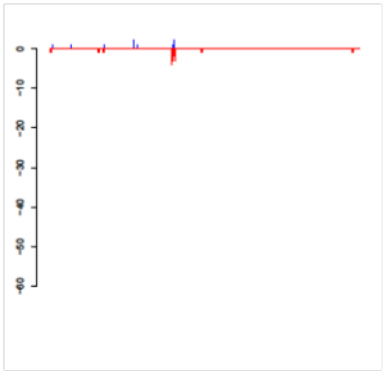


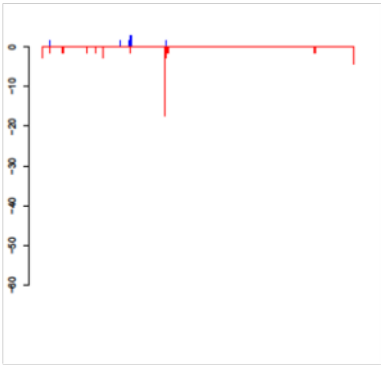




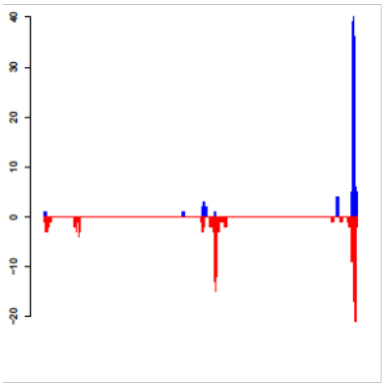
102 E (4)

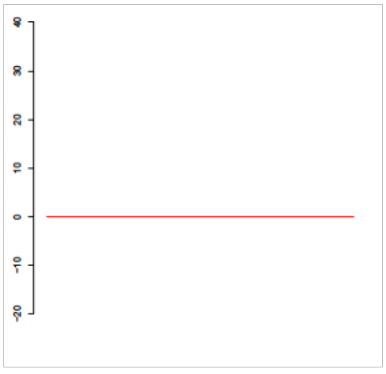


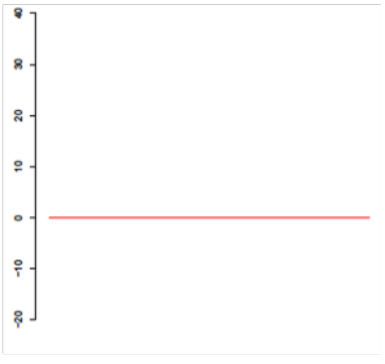




100F(3R)





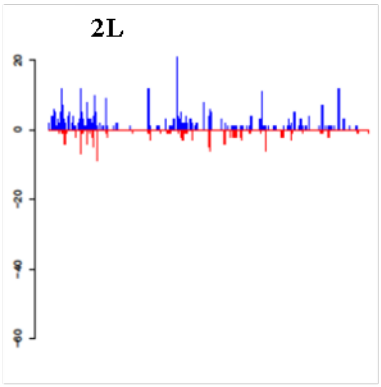


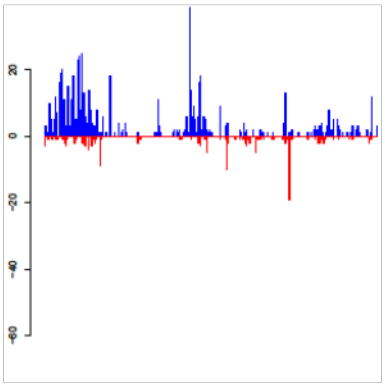
nosGAL4>RNAiGFP

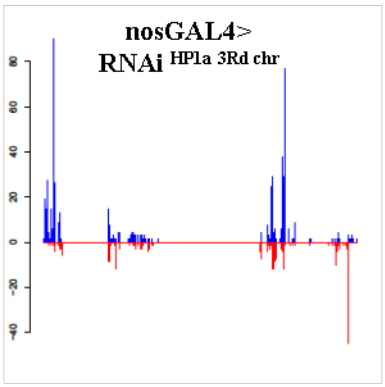
nosGAL4>
RNAi HP1a 2nd chr

nosGAL4>
RNAi HP1a 3Rd chr

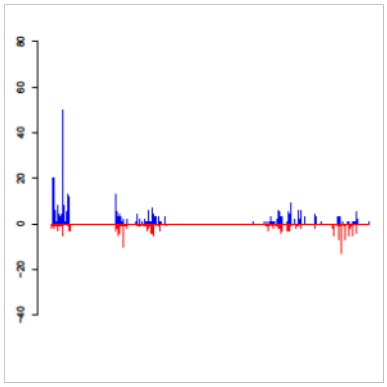
2L

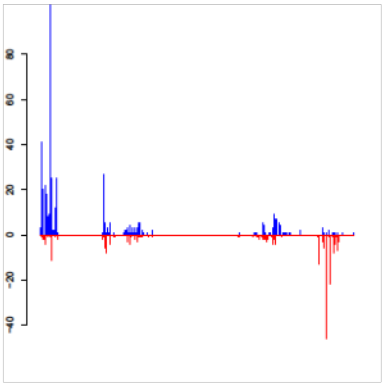


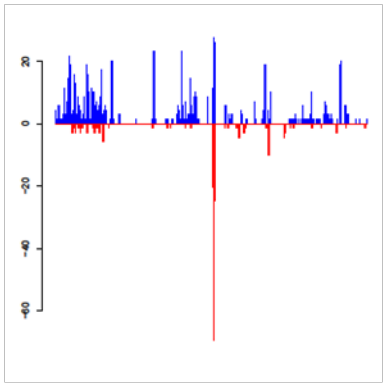




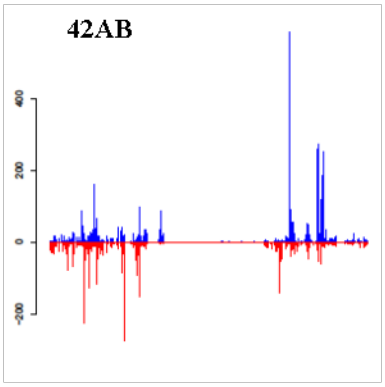
38C

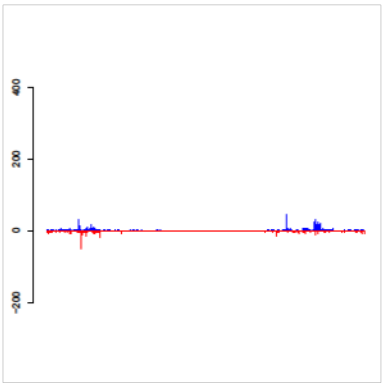


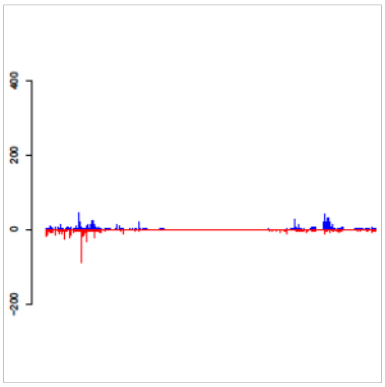




42AB

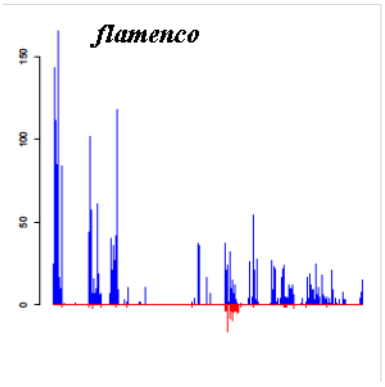


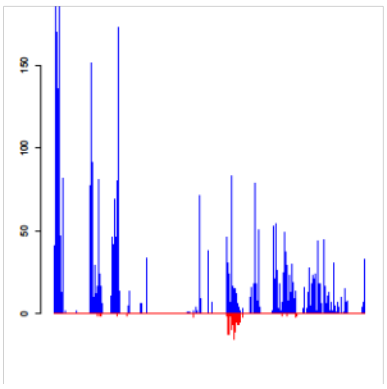




C.Somatic cluster

flamenco





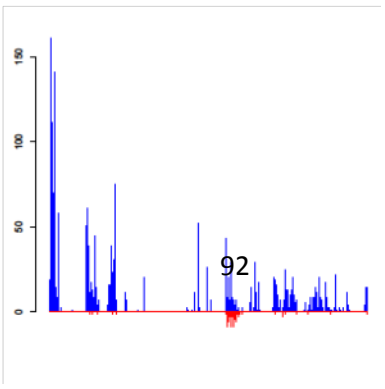


Figure 3.18 **Cluster transcription from telomeric and some other germline clusters is affected upon germline knockdown of HP1a**.

A. Telomeric cluster transcription is abolished as is evident from the barplots.

B. Other germline cluster transcription is also affected upon loss of HP1a, with a significant loss from the most important germline cluster on 2R (42AB).

C. Somatic piRNA cluster transcription is not affected upon loss of HP1a from the germline as is evident from the unidirectional *flamenco* cluster mapping piRNA data.

3.6 Global changes in the germline transcriptome and genome upon loss of HP1a from the ovaries

HP1a is a multifunctional protein and hence its loss could lead to global changes in the transcriptome and the phenotypes I observe may arise as secondary effects. Although the selective loss of piRNAs implies a very specific function for HP1a in the female germline, I performed ChIP- sequencing and RNA sequencing to validate the observations. The RNA sequencing results will be analysed to determine if there are global changes to the transciptome upon knockdown of HP1a in the ovarian germline.

3.6.1 HP1a occupies transposon loci in the female germline

In the germline, HP1a mostly (85.7%) occupies the transposon loci as seen by ChIP-Sequencing data in my hands (Figure 3.19A). This has also been shown by ChIP-qPCR by (Moshkovich and Lei 2010). I planned to analyse if HP1a was more abundant at the telomeric retroelements as compared to the ones that were unaffected by its loss in the germline. A major difficulty in analysing ChIP sequencing data stemmed from the poor annotation of *Drosophila* telomeres (Kaminker et al. 2002; Abad et al. 2004a). Thus I could not determine HP1a distribution on individual transposons from the sequence data however ChIP-qPCR showed that HP1a occupied the telomeric retrotransposons more robustly than the others used in the experiment (Figure 3.19B).

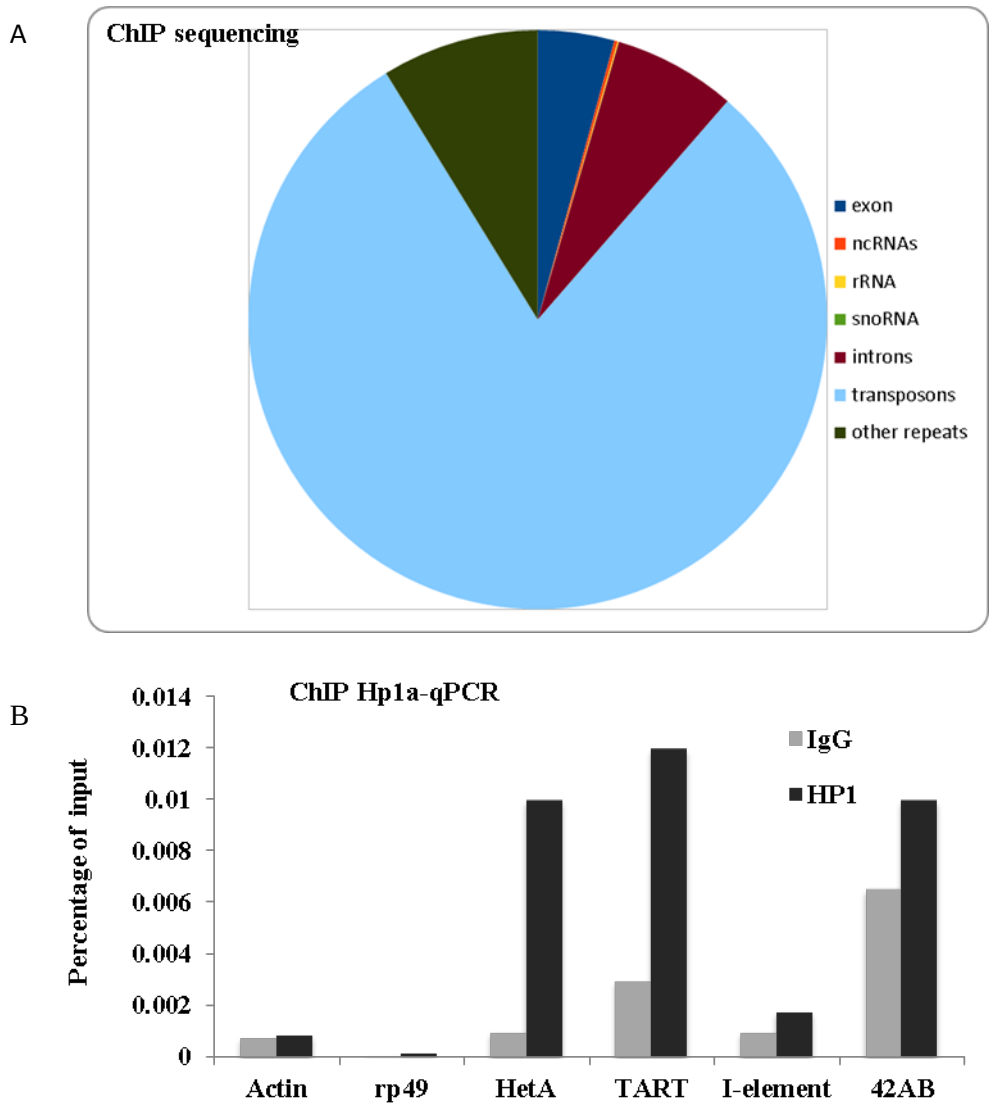


Figure 3.19 **HP1a is most abundant at transposon loci in the Drosophila female germline**

A. ChIP- sequencing from wild type *Drosophila* ovaries showed that 85.7% of HP1a resides at transposon loci in this tissue. Further analysis to determine whether HP1a occupied specific transposon loci could not be performed owing to poor annotation of heterochromatin, especially telomeres *in Drosophila*.

B. ChIP-qPCR for HP1a showed that it preferentially binds telomeric retrotransposon loci (*HeT-A and TART*) compared to other transposon *I element* or euchromatic loci (Actin and RP-49). HP1a also showed enrichment at the largest germline piRNA cluster 42AB. IgG pull down was used as a control for this experiment.

3.7 HP1a is part of the piRNA machinery that regulates telomeric cluster transcription

3.7.1 Loss of HP1a does not disrupt the germline piRNA pathway machinery at the nuage

Loss of HP1a did not cause a general loss of piRNAs suggesting that probably the machinery producing piRNAs in the germline was not affected. The major source of piRNAs comes from the Ping-pong cycle in the germline. The nuage is the processing body where Ping-pong amplification occurs and most mutants with severe loss of piRNAs depict defects in the assembly of the nuage. Nuage components are suggested to be recruited in a hierarchical manner to the perinuclear regions and loss of an upsteam component causes delocalization of the downstream proteins from the nuage (Summarized in Appendix 7.2; Pek et al. 2012). Upon loss of HP1a most nuage components retained perinuclear localization as determined by immunostaining, however Ago3, Krimper and Tejas seemed to form bigger puncta (Figure 3.20). This suggests that HP1a probably does not play a very significant role in the recruitment of amplification cycle/ Ping-pong cycle components to the nuage for piRNA production.

Given the known functions of HP1a in conjunction with Piwi in the soma I also checked if nuclear localization of Piwi was affected upon germline knockdown of *HP1a*. Immunostaining results showed that there were no defects in Piwi localization.

Furthermore the Ping-pong score for individual germline piRNA was calculated, using the deep sequencing libraries. One of the characteristics of Ping-Pong pairs of piRNAs is a 10 nucleotide overlap. The Ping-pong score for each transposon was calculated as a ratio of piRNA pairs with 10 nucleotide overlap versus the total number of piRNAs against the respective transposon. Although overall sense piRNAs seemed to increase upon knockdown of HP1a in the ovaries (Figure 3.21A), Ping-Pong score was not significantly affected for most transposons. For the piRNAs that do show a change in Ping-pong score, the reason is that piRNAs against those transposons were completely abolished upon loss of HP1a (eg: those against *HTT* as marked on the scatter plot Figure 3.21B). Since most Ping-pong piRNAs were unaffected it implies that the loss of Ping-Pong piRNAs for the small subset was probably not owing to defects in the amplification machinery.

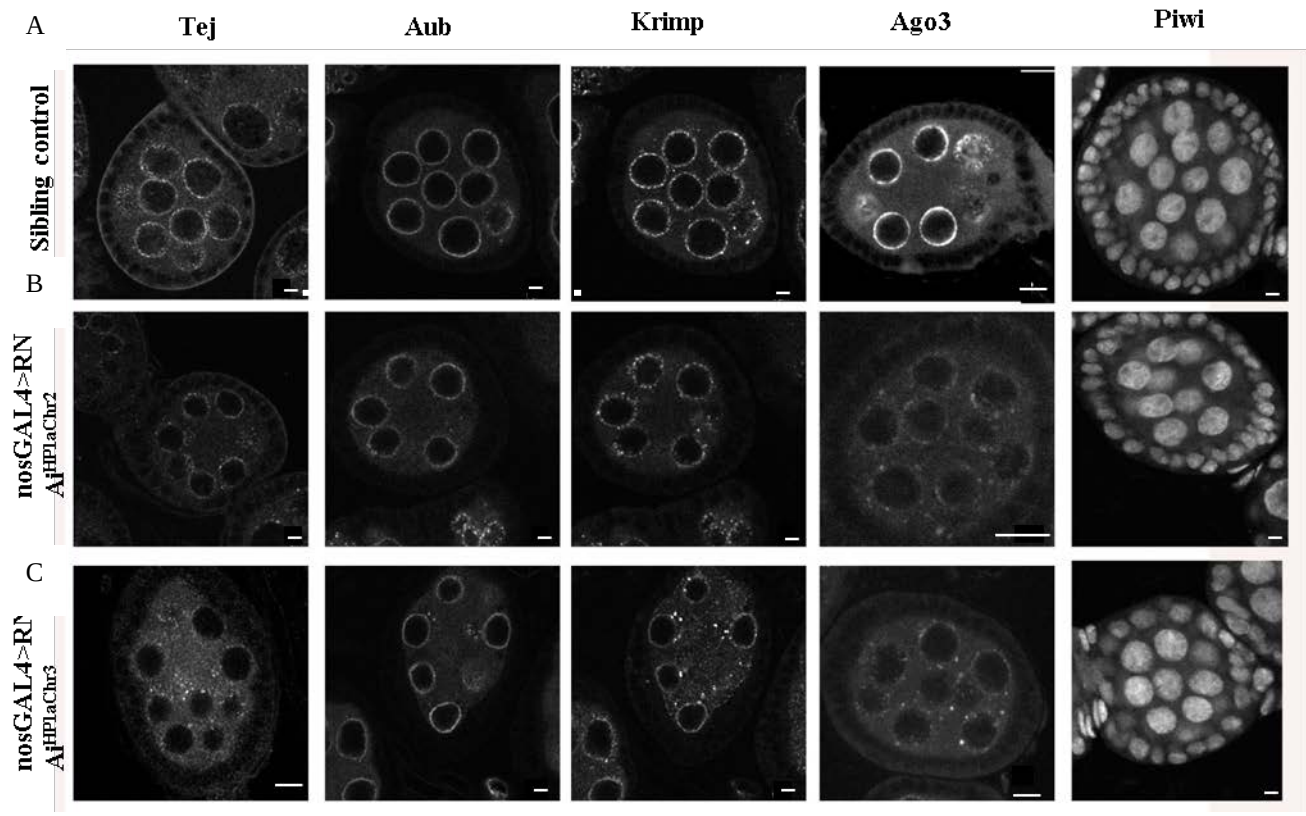
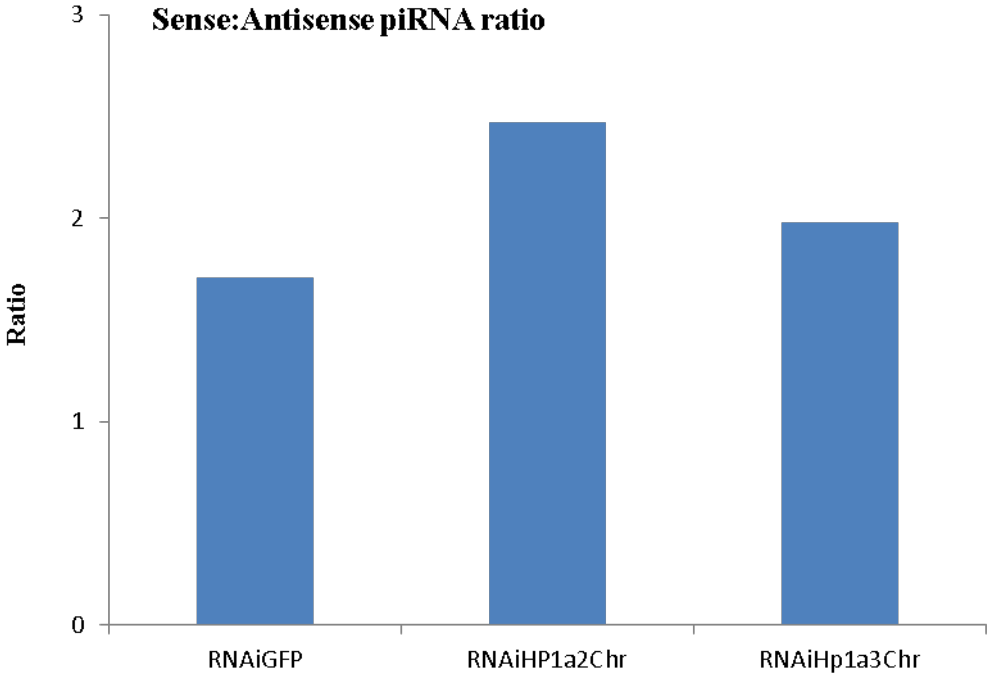


Figure 3.20 **The piRNA processing machinery at the nuage is not grossly affected upon *nos*GAL4> RNAi $^{HP1a2nd/3rd\ chr}$ knockdown.**

A. Immunostaining images for sibling control egg chambers showing nuage components Tejas, Aub, Krimp, Ago3 and nuclear Piwi
B. Immunostaining images for *nos*GAL4>RNAi^{HP1a2Chr} egg chambers showing nuage components Tejas, Aub, Krimp, Ago3 and nuclear Piwi. The components show no disruption in localization as compared to control
C. Immunostaining images for *nos*GAL4>RNAi^{HP1a3Chr} egg chambers showing nuage components Tejas, Aub, Krimp, Ago3 and nuclear Piwi nuage components. The nuage components Krimper, Ago3 and Tejas show more punctate foci.
Scale bars are 10 µm.

A


Sense:Antisense piRNA ratio
Ratio
3
2
1
0
RNAiGFP
RNAiHP1a2Chr
RNAiHp1a3Chr

B

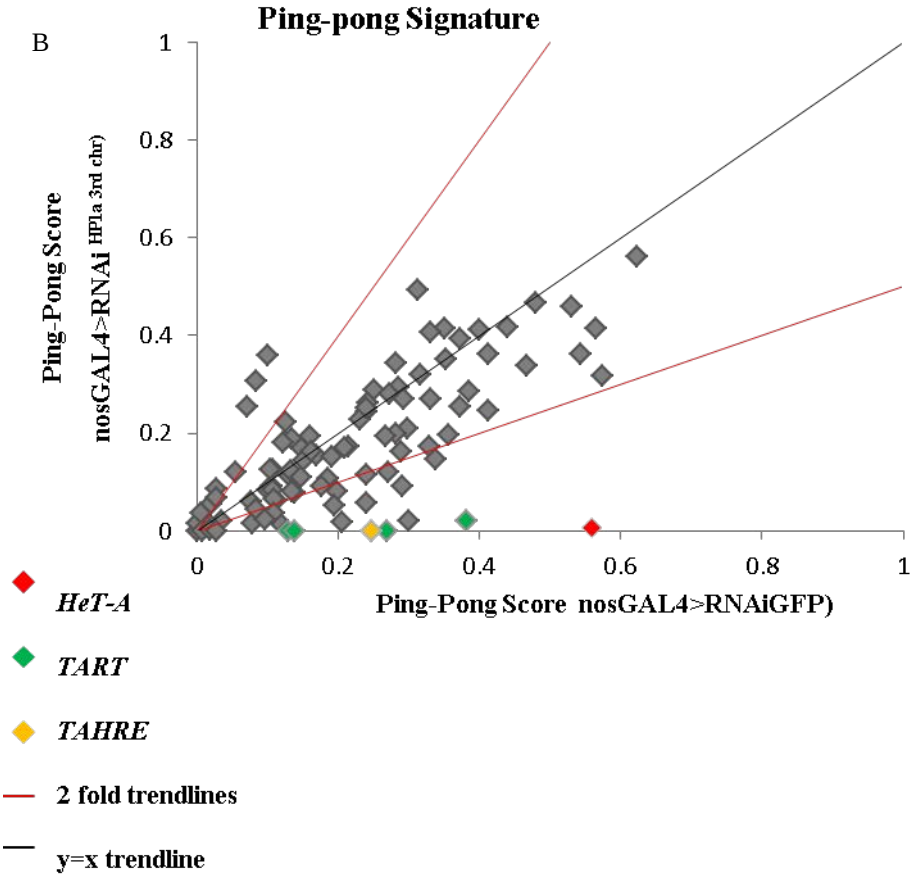

Ping-pong Signature
Ping-Pong Score nosGAL4>RNAi HP1a 3rd chr)
Ping-Pong Score nosGAL4>RNAiGFP)
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
HeT-A
TART
TAHRE
2 fold trendlines
y=x trendline

Figure 3.21 **Ping-pong ratio for most piRNAs remain unaffected upon loss of HP1a from the ovarian germline**.

A. Bar graph showing the ratio of sense versus antisense piRNAs in the piRNA libraries. There is a slight increase in sense piRNAs upon loss of HP1a.

B. Scatter plot showing the Ping-Pong score for piRNAs against all transposons. Most Ping - pong piRNAs are unaffected (gray data points lie within the 2 fold trendlines). Telomeric piRNAs are lost from the knockdown library entirely. *nos*GAL4>RNAi^{HP1a2Chr} showed a similar profile (data not shown).

3.7.2 Cluster transcription decreases upon HP1a knockdown

Most transposons are unaffected upon germline knockdown of HP1a and downstream piRNA processing seems unperturbed. Also HP1a occupies the heterochromatic telomeric regions housing HTT. This suggests HP1a may be required for primary piRNA production for telomeric retroelements. Zhang et al. 2012, used Vasa bound RNA as a means to determine cluster transcripts. This is based on the notion that Vasa is on the top of the cytoplasmic nuage hierarchy and receives the transcripts deemed for piRNA production from the upstream nuclear processing machinery. I isolated Vasa bound RNA from controls and HP1a knockdown ovaries and checked for cluster transcripts arising from the 42AB cluster (most germline piRNAs map to this cluster) by qPCR and observed a drop in cluster bound piRNAs in the knockdowns as seen in Figure 3.22. However piRNA sequencing results show that most piRNAs can be processed normally upon loss of HP1a hence it was essential to determine the specific clusters or loci on the genome that were affected upon HP1a knockdown in the germline. One difficulty to test this hypothesis stems from the poor annotation of heterochromatin as a result of which I could not map the cluster transcripts arising from telomeric heterochromatin regions. To circumvent this problem, total RNA from ovaries of HP1a knockdown flies and the corresponding sibling control were sequenced. The data analysis is pending and I hope to identify and annotate the clusters dependent on HP1a for primary piRNA transcription through this study. A recent study shows that HP1a binds RNAs arising from repeat regions corroborating my hypothesis (Alekseyenko et al. 2014).

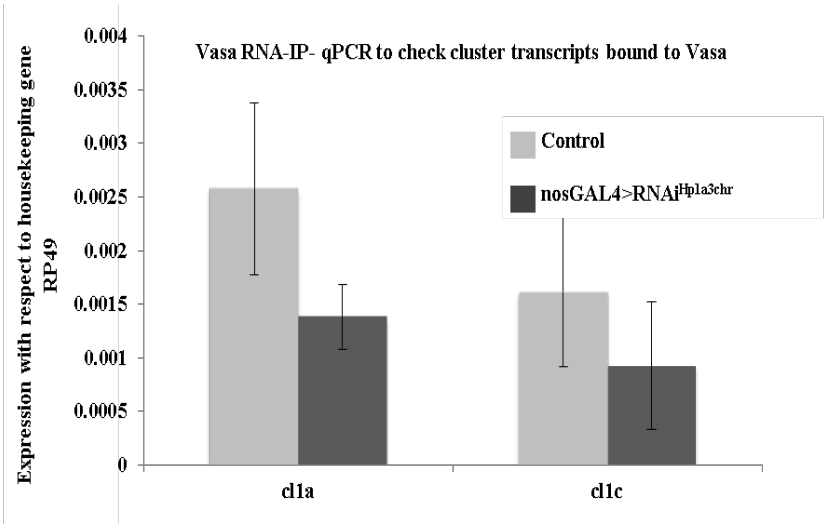


Figure 3.22 **Vasa bound piRNA cluster transcripts showed a reduction upon HP1a knockdown in the ovarian germline.**

RNA is bound to Vasa were isolated by an RNA-IP experiment and a qRT-PCR was performed to determine amount of cluster transcripts bound. A drop in Vasa bound cluster transcripts arising from the 42AB cluster was observed upon *HP1a* germline knockdown. The data was normalized with respect to the expression of the housekeeping gene Ribosomal Protein49 (RP49).

3.8 HP1a is required to enforce heterochromatinization at telomeric *HTT* array

3.8.1H3K9me3 marks specifically on telomeric retroelements are reduced upon loss of *HP1a*

Heterochromatin establishment by HP1a has been associated with the establishment of histone methylation marks, H3K9me2/3 (Piacentini et al. 2009; Wang and Elgin 2011; Figueiredo et al. 2012; Sienski et al. 2012; Gu and Elgin 2013; Le Thomas et al. 2013; Muerdter et al. 2013; Eissenberg and Elgin 2014; Klenov et al. 2014). To determine if the mechanism by which *HP1a* regulates telomeric transposons involves histone methylation I performed ChIP-qPCR for heterochromatin marks on ovarian lysate obtained from HP1a knockdown ovaries and sibling controls. H3K9me3 levels were significantly reduced at the telomeric transposon *HeT-A, TART* and *TAHRE* loci upon germline knockdown of HP1a while it remained unperturbed at

the transposons which showed no up-regulation upon HP1a loss (Figure 3.23). The use of entire ovaries, which includes somatic follicle cells and other somatic cell types, may undermine the effects somewhat.

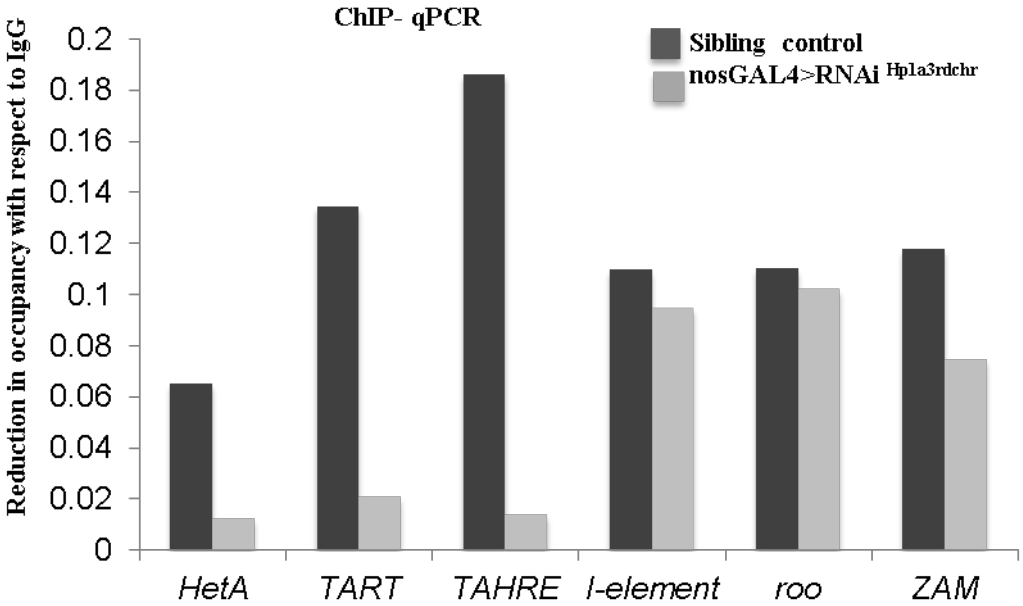


Figure 3.23 **H3K9me3 marks are lost specifically on telomeric *HTT* upon loss of HP1a from the ovarian germline**

ChIP-qPCR on HP1a knockdown ovaries using anti H3K9me3 showed a sharp reduction of histone tri methylation at Lysine 9 from the telomeric transposons but showed no change at transposons that were not affected by the loss of HP1a.

4. Discussions

4.1 HP1a is essential for the female germline

This study demonstrated that the loss of HP1a from the ovarian germline caused complete sterility and enhanced telomeric retroelement expression. The transposon upregulation correlated with a complete loss of piRNAs against the telomeric transposons *HeT-A, TART and TAHRE (HTT).* The piRNA production machinery including the amplification loop at the nuage (a germline specific loop) remained functional, as most non *HTT* piRNAs were unaffected upon germline loss of HP1a. HP1a is enriched on transposon loci, particularly telomeric *HTT* in the female germline genome. HP1a is involved in the recruitment of H3K9me3 to *HTT* elements. My study proposes that HP1a is most probably required for precursor piRNA transcription especially from telomeric piRNA clusters, annotated on X, 4th chromosome and on 3R.

4.1.1 HP1a is not essential in somatic follicle cells of the ovary

The knockdown using female germline specific driver *nanos* GAL4 caused a sterile phenotype while the knockdown in somatic follicle cells did not. This suggests that HP1a may not have an essential function in the ovarian soma or there may be redundant pathways in this tissue especially with regard to maintenance of telomere homeostasis. The telomeric transposon families are not upregulated in piRNA pathway mutants in the ovarian somatic cells and also HP1a knockdown in this tissue did not increase *HTT* expression as observed via qRT-PCR (Figure 3.10B). Telomere maintenance has been attributed to components *Z4* and *Jil1* in somatic tissues as discussed in the introduction for this thesis (Silva-Sousa et al. 2012). *Jil1* expression is not too high in the female germline of *Drosophila* (Chintapalli et al., 2010), thus, HP1a probably has a more prominent role for telomere maintenance in this tissue.

4.1.2 Role of *HP1a* in the male germline

Male germline knockdown could only be achieved by a combination of GAL4 drivers *nanos* and *daughterless.* Although female germline knockdown of HP1a

resulted in complete sterility, fertility assays on male suggest that loss of HP1a has a slight effect on male fertility. However, in my PhD studies I did not do further experiments to characterize the weak effect on male fertility. There is a possibility that complete knockdown could not be achieved in the testes (Figure 3.3).

Telomeric transposition was the focus of my studies and in males telomere maintenance probably does not rely on the piRNA machinery, since piRNA pathway mutant males do not show upregulation of *HTT*, in contrast to females (Shpiz et al. 2009). Using the tools generated in this study, it will be interesting to study the mechanism of telomere length maintenance in males especially with regard to *HeT- A*, since the vast majority of the Y chromosome contains *HeT-A* repeats.

4.2 HP1a as a member of the piRNA pathway

4.2.1 The physiological relevance of TSE and PEV (Appendix 7.4 and 7.5)

The suggestion that HP1a is a player in the piRNA pathway came from two sets of observations:

i) The phenomenon of Telomere Silencing Effect (TSE) and Hybrid dysgensesis, and
ii) Position effect variegation (PEV) and Transcriptional gene silencing (TGS) in conjunction with Piwi (especially in somatic tissues)

Incidentally, most hybrid dysgenesis studies used *P element* insertions at telomeric/ TAS loci. TSE is a phenomenon where the P elements located at telomeres could silence euchromatic insertions of the same P element in trans. Work by the Ronserray group showed the importance of HP1a in TSE (Todeschini et al. 2010). TSE was later shown to be dependent on another piRNA pathway component, Aub and a maternally inherited trait (Haley et al. 2005). Furthermore, piRNAs were deemed responsible for this effect. Todeschini et al showed that in absence of *Su(var)205*, the piRNA population was lost, leading to loss of TSE (Todeschini et al. 2010).

My results imply that HP1a is involved in precursor piRNA transcription from telomeric loci and explains its role in TSE via the piRNA pathway. Additionally,

this study shows the physiological relevance of this function of HP1a at telomeres to maintain telomere length in *Drosophila*.

HP1a and Piwi have been implied in transcriptional gene silencing in somatic tissues (Pal-Bhadra et al. 2004; Brower-Toland et al. 2007). In the germline Wang and Elgin, 2011, showed that same subset of transposons were upregulated upon female germline specific knockdown of Piwi and HP1a. The fact that HP1a affects only a subset of transposons was corroborated by my results. However, in my hands, the same set of transposons was not upregulated: results for transposon *Bari* were inconsistent- it showed no significant change for me. This may be due to potential differences between strain backgrounds or off target effects due to the use of the long ds RNAi construct in their study (Ni et al. 2011).

Elgin's group has reported that *piwi* mutants in which HP1a interacting domain was disrupted and failed to show transposon upregulation in the germline Wang and Elgin, 2011, suggesting that Piwi and HP1a may work at different steps in the female germline piRNA pathway. Recent results in Klenov et al. also suggests that transcriptional silencing by Piwi is not necessarily coupled to H3K9me3 and HP1a marks (Klenov et al. 2014). Thus far, HP1a has been implied in TGS, at the final step of silencing and heterochromatin formation. Although heretrochromatin formation for piRNA cluster transcription is known to be essential (Rangan et al. 2011), HP1a's involvement has not been shown directly. HP1d/ Rhino was the first protein of the HP1 family to be essential for dual strand piRNA cluster transcription (Klattenhoff et al. 2009) . Results from my RNA sequencing will allow me to find out if HP1a is involved in telomeric cluster transcription.

4.2.2 Phenotypes of typical piRNA pathway mutants and a comparative study with HP1a germline knockdown

piRNA pathway mutants show a vast repertoire of phenotypes in the female germline. However there is variability among different mutants and it is unclear if the piRNA pathway components may have other piRNA independent functions. Among the phenotypes, female sterility, delay in oocyte fate determination, age dependent deterioration of ovaries due to arrest in egg chamber development and apoptosis are

some of the characteristics germline knockdown of HP1a shared with other piRNA pathway mutants. However the HP1a germline knockdown did not result in the typical defects in oogenesis like axis specification. This exception can be seen in other mutants like *tejas*, which does not show axis specification defects (Patil and Kai 2010). Hence the repertoire of phenotypes associated with piRNA pathway mutants can be variable and distinct.

Most piRNA pathway mutants exhibit extensive DNA damage owing to transposon mobilization, and accumulation of double strand DNA breaks (DSBs) In most cases, the oocyte is the target for most transposons and shows maximum DNA damage and possibly why most piRNA pathway mutants are sterile. In the HP1a knockdown females DSB were apparent only in the germarium (extended beyond the meiotic zone) and also in the nurse cells (Figure 3.8). However, antibodies against telomeric retroelements showed strong oocyte signal (Figure 3.11), indicating a massive accumulation of *HeT-A* and *TART* transposons in the oocyte. Contradictorily, DNA breaks were not discernable in the oocyte. One plausible explanation is that the *HTT* retroelements are targeted predominantly to the telomeres/chromosome ends for insertion. If most insertions are targeted to chromosome ends it may explain why one does not observe massive DSB. This implies that loss of HP1a does not disrupt the machinery that directs the *HTT* elements to the telomeres. Indeed, heterozygous mutants of *Su(var)205,* encoding HP1a exhibit elongated telomeres supporting the above hypothesis (Perrini et al., 2004). Further evidence by Savitsky et al. 2002,where loss of HP1a did not lead to loss of telomere targeting capability of *HeT-A, TART* and *TAHRE* and furthermore *HeT-A* elements could still be targeted to broken chromosome ends in a mutant of HP1a, corroborating my hypothesis.Experiments using qRT-PCR on genomic DNA isolated from germline knockdown of HP1a showed an increased copy number for *HeT-A* (Figure 3.12). An inverse PCR or TAU PCR or Next-generation sequencing can be performed to check for the genomic location for these insertions to verify that transposon insertions are predominantly telomeric / at chromosome ends.

Rhino, a HP1 homologue, known to be required for dual strand piRNA cluster transcription in the germline, mutants show a similar DSB profile as HP1a knockdown females. Since both Rhino and HP1a seem to be involved in cluster transcription the similarity in phenotype is noteworthy.

Another technical flaw with the scoring of double strand breaks using the antibody for phospho-histone2a variant was that the only available antibody showed significant variability between samples even when proper controls were used each time.

4.2.3 Phosphorylation of HP1a in piRNA pathway mutants

HP1a is suggested to directly bind telomeric DNA(Figueiredo et al. 2012). The phosphorylation status of HP1a has been correlated with its DNA binding ability (Eissenberg et al. 1994;Badugu et al. 2005). Furthermore, phosphorylation on different sites of HP1a by Casein Kinase II may be essential for heterochromatinization and PEV silencing (Zhao and Eissenberg 1999; Zhao et al. 2001). There are two phospho-isoforms of HP1a: a hyperphosphorylated and a hypophosphorylated form and the functions of both uncharacterized. Preliminary results in the Kai laboratory show that some piRNA pathway mutants namely, *kumo, spnE and mael* but not *tejas* increased phosphorylation of HP1a in the ovaries. This suggests that some piRNA pathway components could modulate HP1a binding to DNA and hence affect heterochromatinization and this is an interesting direction to pursue in the future, for a detailed mechanistic insight into the molecular role of phosphorylation. Mass spectrometric analysis can be used to determine phosphorylation sites on HP1a and a kinase mutant screen will enable identification of the kinase responsible for HP1a phosphorylation in the female germline.

4.3 Production of the telomeric subset of piRNAs depends on HP1a in the female germline.

4.3.1 Compartmentalization of the piRNA pathway to tailor specific transposons

Several studies have directly or indirectly implied that a subset of piRNA pathway components may be required for telomere maintenance in the female germline (Savitsky et al. 2002; Savitsky et al. 2006; Khurana et al. 2010; Czech et al. 2013). Aub and Armi were reported to be involved in telomere maintenance by (Khurana et al. 2010). Another component of the piRNA pathway required for

telomere homeostasis in the female germline is SpnE (Savitsky et al. 2006). In the *spnE* mutant *HeT-A* but not *I element* showed a severe upregulation by RT-PCR suggesting SpnE's specific role for the telomeric elements. Additionally *spnE* mutants showed elongated telomeres like the HP1a mutant alleles. An RNAi screen also showed that telomeric retroelements *TAHRE* and *HeT-A* showed very high correlation for the factors required for their silencing while *HeT-A* and *Blood* had fewer common components, suggesting that different transposons rely on different piRNA pathway components for their silencing while some families, like the telomeric elements have a similar subset of components for their regulation (Czech et al. 2013).

Most piRNA pathway mutants cause *HeT-A* de-repression in the female germline yet they do not all disrupt telomeres. The Drosophila telomere has 3 domains and its homeostasis involves transposon regulation at the *HTT* domain and the capping function at the extreme end of the chromosome (Andreyeva et al. 2005). In the study by Khurana et al. they showed that a*ub* and a*rmi* but not a*go3* and r*hi* were required for telomere stability. *Aub* and a*rmi* were implied in the capping function of telomeres.

Additionally, mutants of *aub* and *armi* caused loss of 19-22nt piRNAs from a piRNA cluster on the fourth chromosome. However, I find that small RNA sequencing data from HP1a knockdown ovaries did not show any such piRNA loss. One possible explanation is that the capping function and transposon regulation are not correlated, although both require piRNA pathway components. This is supported by a study where telomere capping and transposon related functions could be segregated for HP1a by the use of an allele *Su(var)205*^{02}. This allele caused massive *HTT* upregulation but this chromodomain mutant of HP1a could still localize to and protect telomere caps (Perrini et al. 2004). Also telomere elongation is not observed for other telomere capping protein mutants *cav, moi* or *ver*, suggesting that capping and telomere length regulation are distinct even though there may be some overlapping components (Raffa et al. 2011).

4.3.2 Cluster transcription

After the RNA-sequencing data analysis from HP1a knockdown ovaries I hope to be able to reach a conclusive insight into the role of HP1a in cluster transcription. Current piRNA sequence data suggests HP1a loss leads to disruption of cluster transcription from several germline piRNA clusters (Figure 3.18). Telomeric cluster

annotation is incomplete because of inherent technical challenges associated with assembling repeat regions. This is one of the major challenges of the study on HP1a's role in precursor transcription from telomeric clusters.

Also it will be interesting to see if HP1a and HP1d (Rhino) work in conjunction with one another in ovaries, especially now that dual strand cluster transcription is better understood (Mohn et al. 2014; Zhang et al. 2014b).

4.4 Mechanism of telomere length maintenance in the Drosophila female germline.

4.4.1 H3K9me3 modification is enforced by HP1a to induce heterochromatinization at telomeres

Heterochromatin formation has been deemed essential for piRNA cluster transcription (Rangan et al. 2011). Egg/ SetDB1 the histone methyl transferase mutant showed significant loss of piRNAs. The *eggless* mutant also shows a female sterile phenotype. However, a recent paper dissected its role in the female germline and showed that it is required for germline stem cell differentiation, progression of oogenesis beyond stage 5 and also in somatic follicle cells to maintain GSCs (Clough et al. 2014). The phenotypes of HP1a knockdown do not overlap with the *eggless* mutants, suggesting that they may work in distinct pathways. However, the longstanding model for heterochromatin establishment involves HP1a and H3K9me2/3 (Vermaak and Malik 2009). However, HP1a can directly bind DNA, especially telomeric DNA and has been shown to be recruited to the fourth chromosome of Drosophila independent of H3K9me3 (Figueiredo et al. 2012). My data suggests that transposon regulation at ovarian germline telomeres by HP1a is mediated by H3K9me3 modification. Recent data by Klenov et al. 2014, supports this hypothesis. They showed a high correlation between HP1a and H3K9me3 on *HeT-A*, *TAHRE* and *TART* (although controversial) in the ovaries. The current understanding points at a H3K9me2/3 independent and dependent role of HP1a in the ovarian germline and my study shows that telomeric length maintenance via *HTT* transposition relies on HP1a's recruitment of H3K9me3.

4.4.2 RNAi as a conserved mechanism for telomere maintenance

Although *Drosophila* telomeres are unique and from several studies including mine, it appears that different tissues adopt different mechanisms to maintain telomere length.

A recent study on embryonic stem cells identified *Rif1* as a telomeric factor that regulates telomere length by maintaining heterochromatic silencing though H3K9me3 modifications (Dan et al. 2014). Thus telomere length homeostasis via heterochromatin modifications is not unique to Drosophila telomeres.

4.5 Perspective

The role of piRNAs has been implicated in telomere length homeostasis in Drosophila ovaries (Shpiz et al., 2014).

HP1a has been linked to the piRNA pathway in the Drosophila germline (Wang et al, 2011; Brower-Toland et al. 2007; Pal-Bhadra et al. 2004). However, through this study I showed that the role of HP1a in the fly female germline piRNA pathway is exclusive to the telomeric retroelements. Futhermore, I show evidence that telomeric HP1a is required for piRNA precursor transcription contrary to the hypothesis of its involvement in transcriptional gene silencing along with Piwi (Brower-Toland et al. 2007).

5. Future Directions

5.1 Is HP1a required for Cluster transcription of piRNAs?

HP1a is a member of the Drosophila ovarian piRNA pathway. Data presented so far implies its role in the upstream processing of telomeric retroelement piRNAs. qRT-PCR data on cluster transcripts suggests that HP1a may be involved in cluster transcription of piRNAs (Figure 3.22). In order to verify this hypothesis, RNA sequencing for RNA isolated from HP1a germline knockdown ovaries (with appropriate control) was performed in the lab. Both mRNA expression levels for transposable elements and piRNA precursor transcript levels will be examined. The aim is to determine if a decrease in between piRNA precursor levels can be correlated to increased mRNA transcripts for the affected TEs. Previously Kalmykova group reported changes in sense: antisense transcript ratios from the telomeric retroelement loci (Shpiz et al 2009). We plan to check if HP1a depletion leads to a shift in the ratio. Also since other germline clusters like 42AB were affected in the HP1a knockdown fly ovaries, we would like to address if piRNA precursors mapping to these clusters are reduced too. Potential problems for precursor annotation especially for the telomeric clusters will remain a challenge.

In the event that there are no significant changes in the levels of precursor piRNAs, it is likely that HP1a has a role in precursor piRNA transport to the nuage. It is known that HP1a has a high affinity to bind RNA (Keller et. al. 2012). It is thus likely that HP1a binds the precursor piRNA to aid transport to the perinuclear foci for further processing of piRNAs. HP1a RNA-IP followed by sequencing can help determine if HP1a directly binds piRNA precursors. This experiment has been performed by me though the sequencing remains to be done.

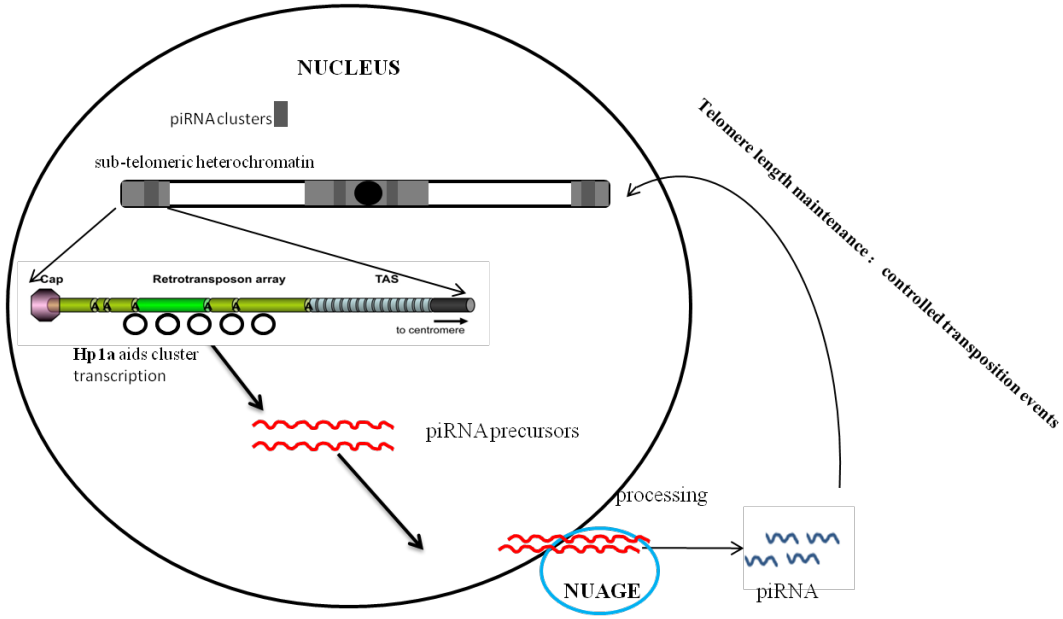
Given the multifunctional role of HP1a, the RNA sequencing data will also be analysed to check if there are any global changes to the germline transcriptome upon loss of HP1a.

5.2 Is the role of HP1a telomeric chromatin context dependent?

Trans-silencing of euchromatic insertions when a copy of the same transgene was also inserted into the telomeric heterochromatin (potentially piRNA producing loci) was shown to be dependent on HP1a and its ability to generate piRNAs (Todeschini et al. 2010, Appendix 7.4). To examine if HP1a's role in cluster transcription is telomeric chromatin context dependent,. *HeT-A* promoter Lac-Z transgenes can be used in the study (Shpiz et al. 2011). Shpiz et al showed that a euchromatic *HeT-A* promoter-Lac-Z transgene is silenced in the wild type ovary but upregulated in piRNA pathway mutant, suggesting suppression of the *HeT-A* promoter is most likely piRNA-pathway dependent (Shpiz et al. 2011). To see if HP1a loss in germline could lead to a similar loss of euchromatic *HeT-A* silencing the transgenic lines are combined with the HP1a RNAi lines, and Lac-Z expression will be examined by immunostaining and qRT-PCR upon knockdown of HP1a from the germline. This experiment will enable us to determine the importance of chromatin configuration in HP1a's role in telomeric retroelement cluster transcription.

6. Conclusion

The aim of this study was to determine the role of epigenetic factor Heterochromatin protein 1a (HP1a) in the female germline piRNA pathway. I found that *HP1a* is essential for controlling transposition of telomeric retroelements *HeT-A*, *TART* and *TAHRE* which is the sole mechanism of telomere length control in *Drosophila*. I propose that *HP1a* is required for precursor transcription of piRNAs from the telomeric clusters in the ovarian germline. The model shown below summarizes the findings of my PhD studies.



Model for proposed function of HP1a in the repression of telomeric retroelements to maintain telomere length in the *Drosophila* female germline.

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8. Appendix

8.1 Genome wide euchromatic distribution of transposons in *Drosophila melanogaster* (Adapted from Kaminker et. al 2002)

Transposon Class	Transposon Family	*Drosophila* Chromosome						Total	Number full length
		X	2L	2R	3L	3R	4		
LTR	*17.6*	0	3	5	2	0	12	7	5
	1731	0	0	0	1	0	2	1	1
	297	12	6	7	10	0	57	18	39
	3S18	0	1	1	0	0	6	4	2
	412	0	7	11	5	0	31	24	7
	accord	0	1	0	0	0	1	0	1
	aurora	0	2	1	0	0	3	1	2
	blastopia	2	7	1	2	0	17	13	4
	blood	11	2	3	5	0	22	22	0
	Burdock	4	4	0	3	0	13	7	6
	Circe	0	2	0	0	0	2	0	2
	copia	13	4	5	4	0	30	26	4
	diver	1	3	1	3	0	9	9	0
	diver2	4	3	2	0	0	9	0	9
	Dm88	0	2	0	30	0	32	0	32
	frogger	1	0	0	0	0	1	1	0

	GATE	0	16	0	0	3	20	0	20
	gtwin	2	0	2	0	0	6	2	4
	gypsy	1	1	0	0	0	2	1	1
	gypsy2	0	0	2	0	0	3	1	2
	gypsy3	0	2	0	0	0	2	1	1
	gypsy4	1	0	0	1	0	2	1	1
	gypsy5	0	0	1	0	0	2	1	1
	gypsy6	1	0	0	0	0	1	0	1
	HMS-Beagle	5	1	0	3	0	13	9	4
	Idefix	2	0	3	1	0	7	2	5
	invader1	0	4	3	18	1	26	1	25
	invader2	4	3	2	0	0	10	3	7
	invader3	5	2	2	5	0	16	3	13
	invader4	4	2	1	1	1	9	2	7
	invader5	4	0	1	0	0	6	0	6
	McClintock	0	0	1	0	1	2	2	0
	mdg1	2	9	6	3	0	25	13	12
	mdg3	5	2	2	4	0	16	8	8
	micropia	0	0	0	4	0	5	2	3
	opus	6	6	6	3	0	24	16	8
	qbert	0	1	0	0	0	1	1	0

	Quasimodo	7	0	4	1	0	14	5	9
	roo	22	31	31	27	0	146	58	88
	rooA	0	0	1	1	2	5	0	5
	rover	0	1	0	2	0	6	3	3
	springer	1	4	1	3	0	11	5	6
	Stalker	1	0	5	3	0	12	3	9
	Stalker2	0	5	2	1	1	13	4	9
	Stalker4	0	0	0	1	0	2	2	0
	Tabor	2	0	0	0	0	3	2	1
	Tirant	3	3	4	5	1	20	15	5
	Transpac	1	0	0	2	0	5	5	0
	ZAM	0	0	0	0	0	0	0	0
LINE-like	*baggins*	2	10	0	0	1	14	0	14
	BS	6	6	7	8	0	29	6	23
	Cr1a	5	17	21	2	10	56	1	55
	Doc	16	5	19	10	0	55	30	25
	Doc2	0	1	0	0	0	1	0	1
	Doc3	1	6	1	0	1	9	0	9
	F	7	10	10	11	2	42	16	26
	G	2	0	0	0	0	3	0	3
	G2	9	1	2	1	0	14	2	12

	G3	1	2	0	0	0	4	0	4
	G4	5	0	2	3	1	11	0	11
	G5	5	0	1	2	1	9	0	9
	G6	1	0	1	0	0	3	1	2
	Helena	1	1	1	2	0	7	0	7
	HeT-A	0	0	0	0	3	3	0	3
	I	5	6	3	5	2	28	8	20
	Ivk	3	0	1	0	0	7	2	5
	jockey	9	15	13	14	2	69	12	57
	jockey2	2	0	0	1	3	10	0	10
	Juan	1	1	1	2	0	9	6	3
	R1	1	2	2	0	3	10	2	8
	R2	0	0	0	0	0	0	0	0
	Rt1a	0	6	5	2	0	13	5	8
	Rt1b	6	14	7	5	1	37	5	32
	Rt1c	2	2	1	0	1	17	1	16
	TART	0	0	0	0	1	1	0	1
	X	8	4	8	2	0	25	6	19
TIR	*1360*	18	16	20	11	30	105	10	95
	Bari1	1	1	0	2	1	5	5	0
	Bari2	2	0	0	1	1	4	1	3

	HB	7	6	3	7	4	32	5	27
	H	11	2	1	5	0	24	1	23
	hopper	2	3	2	3	0	15	11	4
	hopper2	3	0	2	0	0	5	1	4
	looper1	0	0	0	2	0	3	0	3
	mariner2	5	2	2	0	3	17	4	13
	NOF	0	2	2	1	0	7	0	7
	P	0	0	0	0	0	0	0	0
	pogo	12	5	7	11	0	44	5	39
	S	4	11	11	12	4	51	14	37
	S2	3	3	3	2	1	13	0	13
	Tc1	1	4	1	2	11	21	1	20
	transib1	0	2	0	0	0	2	0	2
	transib2	5	2	2	0	0	12	0	12
	transib3	1	1	1	1	3	7	0	7
	transib4	1	2	0	0	0	5	1	4
FB	*FB*	4	2	8	4	2	32	13	19

8.2 Drosophila nuage components and their functions (adapted from Jun Wei Pek)

Protein	Domain composition	Functions	References
Argonaute3 (Ago3)	PAZ domain PIWI domain D-D-H motif	piRNA biogenesis, transposon repression, piRISC component, exhibit slicer activity in vitro	Brennecke et al., 2007; Gunawardane et al., 2007.
Aubergine (Aub)	PAZ domain PIWI domain D-D-H motif	piRNA biogenesis, transposon repression, oocyte polarity, chromosome condensation	Vagin et al., 2006; Harris and Macdonald, 2001; Pek and Kai, 2011.
Cutoff (Cuff)	Putative exoribonuclease	Transposon silencing, oocyte polarity	Chen et al., 2007.
Krimper (Krimp)	Tudor domain	piRNA biogenesis, transposon silencing, oocyte polarity	Lim and Kai, 2007.

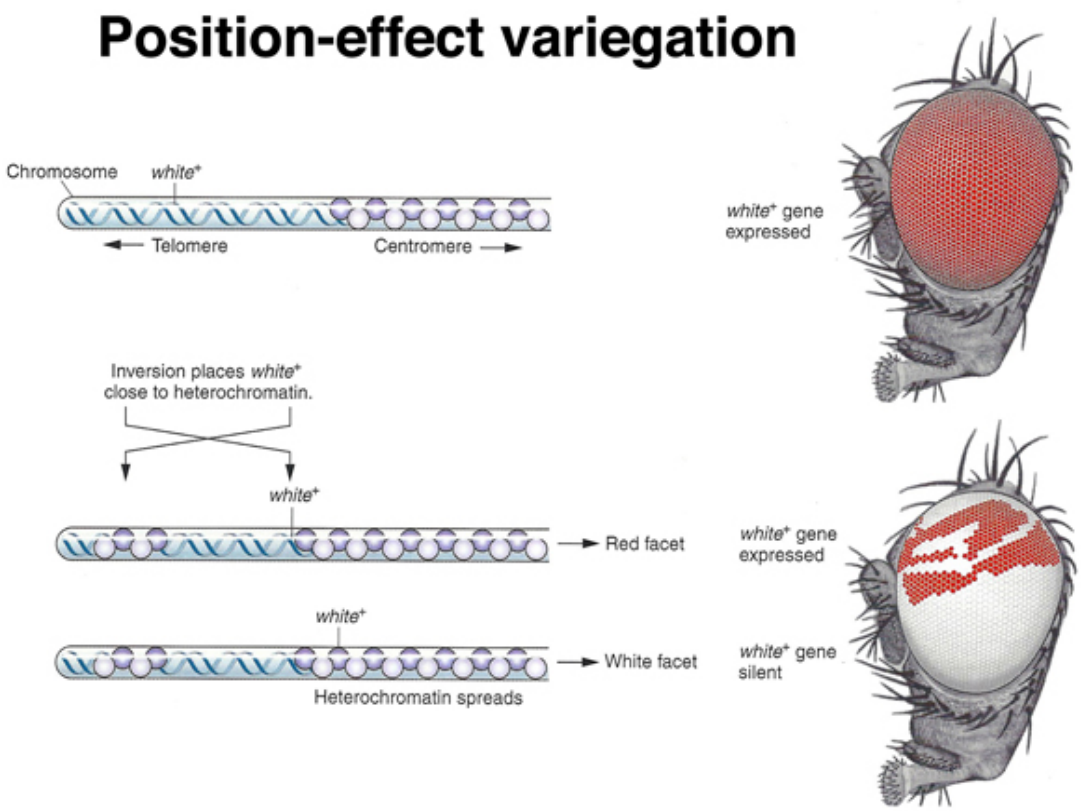
Maelstrom (Mael)	HMG box	piRNA biogenesis, transposon silencing, oocyte polarity, GSC differentiation	Findley et al., 2003; Lim and Kai, 2007; Pek et al., 2009.
Vasa (Vas)	RNA helicase DEAD box	piRNA biogenesis, transposon silencing, oocyte polarity, chromosome condensation	Lim and Kai, 2007; Pek and Kai, 2011; Styhler et al., 1998.
Spindle-E (Spn-E)	Tudor domain DEXH box	piRNA biogenesis, transposon silencing, oocyte polarity, chromosome condensation	Pek and Kai, 2011; Vagin et al., 2006; Gillespie and Berg, 1995.
Tudor	Tudor domain	piRNA biogenesis, transposon silencing, regulates RISC assembly	Nishida et al., 2009.
Tejas	Tudor domain Tejas domain	piRNA biogenesis, transposon silencing	Patil and Kai, 2010.

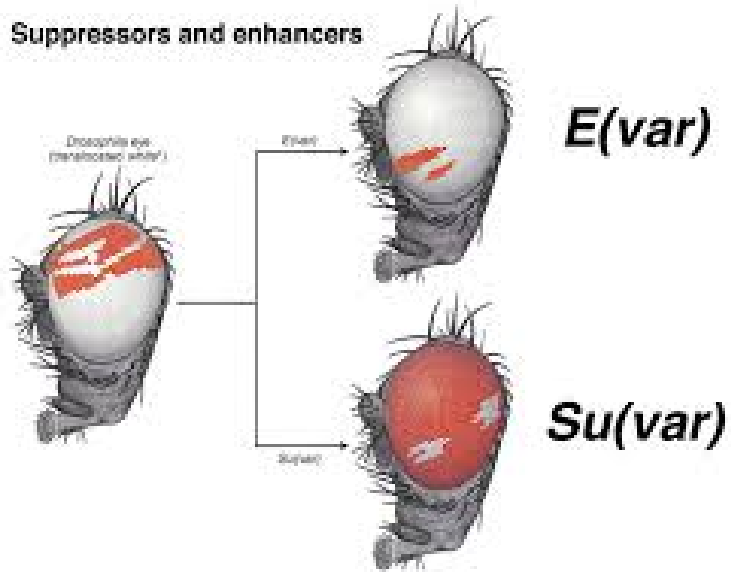
8.3 piRNA cluster annotation (adapted from Brennecke. et al 2007) Telomeric clusters are highlighted in gray.

cluster ID	chromosome	chromosomal band	start	stop	length [kb]	Transposon content (+/- in %)	cluster unique piRNAs	number of potential piRNAs derived from this cluster and fraction of all piRNAs	piRNA strand distribution (+/- strand in %)
1	2R	42AB	2144349	2386719	242	38/32	1686	15102/30.1%	49/51
2	X	20A	21392175	21431907	40	0/78	986	8621/17.2%	100/0
3	4	102E	1258473	1348320	90	6/83	684	2519/5.0%	23/77
4	X-TAS	1A	-	-	7	0/3	484	1306/2.6%	44/56
5	2L	38C	20148259	20227581	79	23/64	482	1851/3.7%	54/46
6	3L	80E-F	23273964	23314199	40	29/37	228	1455/2.9%	64/36
7	U	-	4015849	4029971	14	67/0	176	317/0.6%	62/38
8	X	20A-B	21505666	21684449	179	12/75	170	6649/13.2%	99/1
9	X	20B	21759393	21844063	85	23/55	155	2187/4.4%	63/37
10	U	-	5766708	5772171	5	100/0	133	281/0.6%	54/46
11	3R	100F	27895169	27905030	10	11/4	107	932/1.9%	0/100
12	3LHet	-	1402377	1557939	156	28/39	102	4789/9.5%	51/49
13	3LHet	-	2011004	2180268	169	35/37	86	7062/14.1%	31/69
14	U	-	7542733	7545114	2	100/0	84	149/0.3%	59/41
15	3LHet	-	238123	332969	95	27/46	71	4266/8.5%	43/57

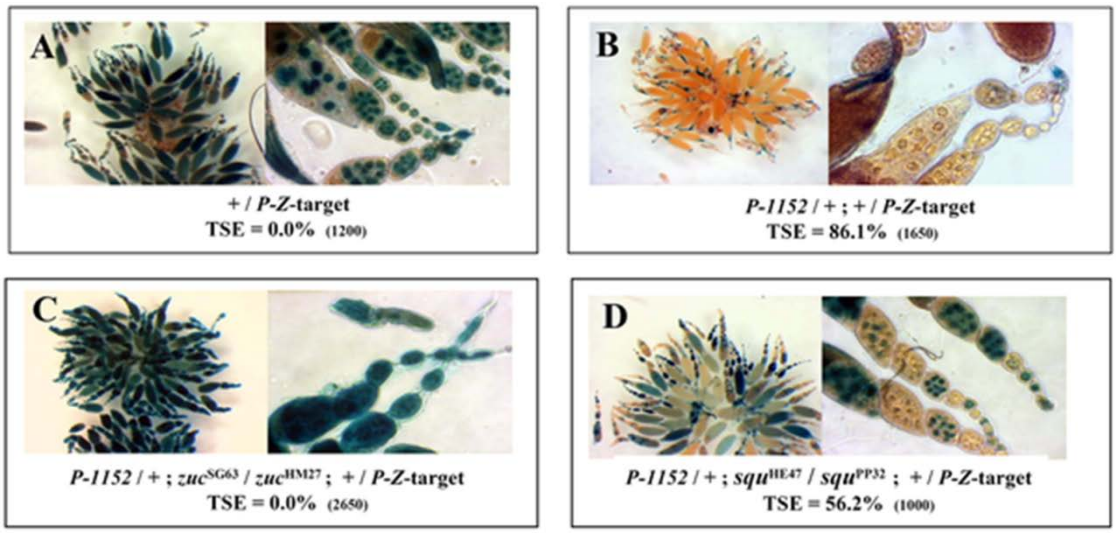
Table S1. Top piRNA Clusters in the Drosophila melanogaster Genome

8.4 Synopsis of Position Effect Variegation (PEV) (Adapted fromhttp://www.discoveryandinnovation.com/BIOL202/notes/lecture18.html)





8.5 Synopsis of Telomeric Position Effect (TPE) (adapted from Todeschini et al ; 2010)



A. Expression of the euchromatic Lac-Z construct in wild type fly ovaries is not supressed.

B. Expression of the euchromatic Lac-Z construct is suppressed when another copy of the Lac-Z insertion is in telomeric locus

C and D. Show the release of suppressive effect on Lac-Z expression in B in a piRNA pathway mutants *zucchini* and *squash*.

9. Author's publications

From January 2012- August 2014 in Dr. Toshie Kai's Laboratory I have contributed to:

1) The role of Heterochromatin Protein 1a (HP1a) in the repression of telomeric retroelements in the Drosophila female germline.
Amit Anand*, Alisha Chakrabarti *, Teo Yee Wei (Ryan) and Toshie Kai.
(* authors contributed equally to the work)
Manuscript in preparation.

2) The Tudor domain Tapas, a homolog of the vertebrate Tdrd7, functions in piRNA pathway to regulate retrotransposons in the germline of *Drosophila melanogaster*.
Veena S. Patil *, Amit Anand*, Alisha Chakrabarti and Toshie Kai.
(* authors contributed equally to the work)
BMC Biology 2014, 12:61.

10. Posters, awards, invited talks

1) TLL annual symposiums (2011) at Temasek Lifesciences Laboratory, Singapore (Poster).