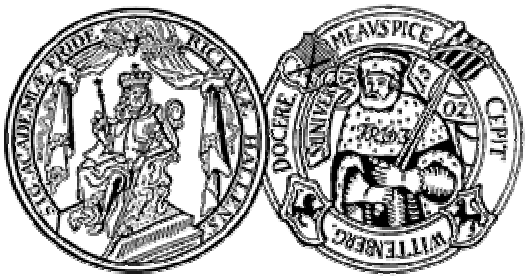
Alternative DNA methylation dependent gene silencing pathways and its affect on genome stability in *Drosophila*



Dissertation

zur Erlangung des akademischen Grades

Doctor rerum naturalium

(Dr. rer. nat)

Vorgelegt der

Naturwissenschaftliche Fakultät I – Biowissenschaften
Martin-Luther-Universität Halle-Wittenberg

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Halle (Saale), 23.09.2008

Tag der mündlichen Prüfung……

For my loving parents (“Aai & Bhau”)

Abbreviations

AEL	After Egg Laying
bp	base pair
BSA	bovine serum albumine
ChIP	Chromatin Immunoprecipitation
DAPI	4', 6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
EDTA	Ethylendiaminetetraaceticacid
EGTA	Ethylenglycol-bis-(β-aminoethylether)N,N,N',N'-tetraaceticacid
E(var)	Enhancer of variegation
FISH	Fluorecent *in situ* hybridization
h	hour
H3K9	Lysine 9 of histone H3
H3K27	Lysine 27 of histone H3
H4K20	Lysine 20 of histone H4
HMTase	Histone methyltransferase
HDAC	Histone deacetylase
kb	kilobases
kDa	kilodalton
LSM	Laser Scanning Microscope
MB	Megabases
MBD	methyl-CpG-binding domain
NP40	Nonident P40
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + 0,2% Tween 20
PCR	polymerase chain reaction
PEV	position-effect variegation
PMSF	Phenylmethanesufonylfluoride

PVDF	Polyvinylidenfluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
SDS	sodiumdodecylsulfate
Su(var)	Suppressor of Variegation
TAS	Telomere associated sequences
TEMED	N,N,N',N'-Tetramethylethylene-diamin
Tris	tris(hydroxymethyl)aminomethane
U	unit
X-Gal	5-Bromo-4-chloro-3-indoxyl-β-D-galactoside

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Abstract

Previous genetic analysis suggested a differential control of gene silencing at different genomic loci in *Drosophila*. In the present work, a series of 67 *white* variegating P element insertions monitoring silenced genomic loci was used to dissect out functional gene silencing pathways not only in the heterochromatin but also within the euchromatic regions in *Drosophila*. To assess the role of DNA methylation in gene silencing processes new mutant alleles for *Dnmt2* were generated. At least five independent gene silencing pathways were identified and a function of DNA methylation in three silencing pathways could be demonstrated. Using newly generated *Dnmt2* null mutations we show that in *Drosophila* significant DNMT2 dependent DNA methylation is only found in early embryos and controls retrotransposon silencing in the somatic tissues. LTR sequences were identified as targets for the DNMT2 mediated DNA methylation. Genetic and molecular analysis demonstrated a sequential pathway for DNMT2/SUV4-20 mediated silencing of retrotransposons. In early embryos DNMT2 binds to LTR sequences and initiates through DNA methylation retrotransposon silencing followed by recruitment of H4K20me3 methylation catalyzed by the SUV4-20 histone methyltransferase. H4K20me3 controls maintenance of retrotransposon silencing in somatic cells. In *Dnmt2* and *Suv4-20* null cells retrotransposons are over-expressed. Overexpression of retrotransposons also leads to their mobility in *Dnmt2* null mutant flies, however the mobility was found to be restricted to the heterochromatin sequences.

Telomere Associated Sequences of right arms of the second and third chromosomal telomeres were found to be rich in DNMT2 controlled *Invader4* LTR sequences. DNMT2 is essential for the integrity of these two telomere associated sequences. Loss of function mutations for *Dnmt2* leads to complete loss of *Invader4* LTR repeats from both the telomeres, without affecting the telomeric retrotransposon array (HTT) or telomere cap function. The loss of subtelomeric *Invader4* clusters is permanent and rather slow process.

Together this work produced new tools to dissect out differential gene silencing processes in *Drosophila* and identified novel DNMT2/SUV4-20 dependent somatic control of retrotransposon expression. Also the study reveals an *in vivo* DNA methylation activity of DNMT2 in *Drosophila* and suggests for the first time the role of DNA methylation in control of genome stability in *Drosophila*.

Zusammenfassung

Durch vorangegangene Untersuchungen ist bekannt, dass in *Drosophila* verschiedene genomische Regionen unterschiedlich in ihrer Expression reguliert werden. Um das funktionelle *Genesilencing* in den hetero- und euchromatischen Bereichen besser verstehen zu können, wurden in der vorliegenden Arbeit 67 *white*-varigierende-Linien zur Untersuchung funktioneller *Gensilencing*-Prozesse genutzt. Durch Erzeugung neuer Mutantenallele der einzigen DNA-Methyltransferase in *Drosophila* (DNMT2) war es möglich die Rolle der DNA-Methylierung in diesen *Silencing*-Prozessen aufzuklären. Dadurch konnten fünf neue, voneinander unabhängige *Silencing*-Prozesse gefunden werden, von denen drei DNA-Methylierungs-abhängig sind. Durch die erzeugten *Dnmt2*-Nullmutanten wurde gezeigt, dass in *Drosophila* DNMT2-abhängige DNA-Methylierung hauptsächlich in frühen Embryonalstadien auftritt und dort das somatische *Retrotransposonsilencing* induziert. Als Zielsequenzen für DNMT2 konnten LTR-Sequenzen der Retrotransposons identifiziert werden. Des Weiteren wurde durch molekulare und genetische Analysen gezeigt, dass es sich beim *Retrotransopsonsilencing* in *Drosophila* um einen sequenziellen Prozess handelt, an dem sowohl DNMT2 als auch SUV4-20 beteiligt sind. Dabei methyliert DNMT2 in frühen Embryonen die LTR-Sequenzen der Retrotransposons. Anschließend führt dies zu einer H4K20 Tri-Methylierung, welche durch SUV4-20 katalysiert wird. Die H4K20me3 hält das *Retrotransposonsilencing* in späteren Entwicklungsstadien aufrecht. In *Dnmt2*- und *Suv4-20*-Nullmutanten kommt es zu einer erhöhten Expression von Retrotransposons. Diese erhöhte Aktivität spiegelt sich auch in einer gesteigerten Mobilität der Retroelemente wieder, welche ausschließlich auf das perizentrische Heterochromatin beschränkt ist.

Die *Telomere assosiated sequences* (TAS) an den rechten Armen der Chromosomen 2 und 3 weisen eine Häufung der durch DNMT2 kontrollierten *Invader4*-LTR-Sequenzen auf. DNMT2 ist für die Integrität dieser genomischen Bereiche essentiell. In *Dnmt2*-Nullmutanten kommt es zu einem kompletten Verlust der TAS an diesen beiden Telomeren. Andere Bereiche des Telomers, wie HTT-Region und

Telomerkappe, sind davon nicht betroffen. Der Verlust des TAS-Bereiches ist permanent und erstreckt sich über mehrere Generationen.

In dieser Arbeit wurden neue Werkzeuge zur Aufklärung von Genesilencingprozessen generiert, mit denen u.a. ein DNMT2/SUV4-20 abhängiger Mechanismus zur Kontrolle der somatischen Retrotransposonaktivität aufgeklärt werden konnte. Weiterhin wurde zum ersten Mal *in vivo* eine DNA-Methylierungsaktivität in *Drosophila* nachgewiesen. Außerdem wurde erstmalig gezeigt, dass diese einen Einfluss auf die Genomstabilität in *Drosophila* besitzt.

1. Introduction

Genetic information is stored in the genomic DNA and is dictated by the order of four nucleotides adenine, cytosine, thymine and guanine. Complete genome sequencing projects have revealed that human and *Drosophila* genomes are comprised of at least $6x10^6$ kb and 180Mb nucleotides which correspond to a linear length of 1.8m and 0.5m, respectively. This implies that genetic material of a cell exist as a compact mass, occupying a limited volume; and its various activities such as replication and transcription, must be accomplished within these confines. The organization of DNA should also accommodate the stable transition of active and inactive chromatin states.

Compact packaging of DNA within nucleus is not just a one step process, but there are hierarchies of organization. First level of organization of genomic DNA within the nucleus is achieved by nucleosome formation. The nucleosome is the fundamental unit of chromatin containing 147bp DNA wrapped around an octamer of small basic proteins into a bead like structure (Luger *et al*., 1997). The octamer consist of core histones H2A, H2B, H3 and H4; while DNA lies on the surface of this octamer. The second level of organization is accomplished by the coiling of series of nucleosomes into a helical array to constitute the ~30nm fibre that is found in both interphase chromatin and mitotic chromosomes. Final packaging of chromatin is determined by a third level of organization, the packaging of the fibre itself, which is achieved by chemical modifications of DNA or histone proteins. This final packaging differentiates euchromatin from heterochromatin (Lam, Kato and Watanabe, 2004).

Euchromatin is characterised by open, transcriptionally active structures and includes the majority of genes. In contrast, heterochromatin remains condensed throughout the cell cycle, a behaviour which is referred to as negatively heteropycnotic (Heitz, 1928), and becomes replicated in late interphase (Lima di Faria and Jaworska, 1968; Gilbert, 2002). The heterochromatin can be furthermore differentiated into constitutive and facultative heterochromatin. Constitutive heterochromatin is the late replicating material that remains condensed throughout the cell cycle and it is rich in

highly repetitive as well as moderately repetitive sequences predominantly consisting in transposable elements (Gatti and Pimpinelli, 1992; Lohe *et al.,* 1993; Pimpinelli *et al.,* 1995; Laurent *et al.,* 1997). In contrast, facultative heterochromatin represents transiently condensed and silenced euchromatin. Best known examples are the inactivated X chromosome in female mammals, and the heterochromatic paternal chromosome compliment in the mealybug males.

The heterochromatin is involved in control of several important functions nucleolus organisation (Cremer *et al.,* 2004), chromosome segregation and crossing over in mitosis and meiosis (Hawley *et al.,* 1992; Wines and Henikoff, 1992; Westphal and Reuter, 2002), genome variability (Dimitri and Junakovic, 1999; Birchler *et al*., 2000) as well as in protecting the genome towards transposable elements (Henikoff and Matzke, 1997) and in gene silencing (Wakimoto, 1998; Wallrath, 1998). Telomeric and pericentric regions of most eukaryotic genomes are packaged into heterochromatin (Weiler and Wakimoto, 1995). In contrast, most regions between centromeres and telomeres are packaged into euchromatin, the gene-rich, early replicating material that decondenses during interphase. When euchromatic genes are brought into juxtaposition with heterochromatin by chromosomal rearrangement or transposition, they can exhibit position effect variegation (PEV), a silencing of the gene in a subset of the cells in which it is normally expressed (Weiler and Wakimoto, 1995). Such silencing is not gene specific, and can affect flanking DNA up to 100 kb from the breakpoint (Weiler and Wakimoto, 1995). Nuclease digestion analysis of the *hsp26* transgene subjected to the PEV has demonstrated that PEV involves local nucleosome remodelling (Wallrath and Elgin, 1995). Similarly a spreading of the inactive histone modification marks has been shown to be causally connected with PEV (Rudolph *et al.,* 2007). With an inducible *LacZ* gene subjected to PEV it was shown that heterochromatin silencing is early initiated at the onset of the gastrulation (Lu *et al.,* 1998) while recently a coordinated action of demethylases and HMTases has been suggested for the initiation of heterochromatin formation during cellular blastoderm stage of the *Drosophila* embryonic development (Rudolph *et al.,* 2007).

In(1)w^{m4} is the classical example of PEV rearrangement of *white* gene in *Drosophila* (Muller, 1930), where the *white* gene is juxtaposed near to the pericentric heterochromain by inversion of the *Drosophila* X chromosome, resulting in a variegating eye colour phenotype. The variegating phenotype in *w^{m4}* is extremely sensitive to the modifying factors. With this genetic model suppressors [*Su(var)s*] and enhancers [*E(var)s*] of PEV were identified (Wustmann *et al.*, 1989). Locke and coworkers (1988) postulated *Su(var)s* as genes which participate directly or indirectly in the establishment of heterochromatin. In contrast *E(var)s* should promote the formation of euchromatin. Studies with the *In(1)w^{m4}* PEV rearrangement have pioneered identification and molecular analysis of functions controlling heterochromatin formation (Reuter and Wolff, 1981; Schotta *et al.*, 2003).). Mutations of *Su(var)3-9*, *Su(var)2-5* (HP1) and *E(z)* are all suppressors of *white* variegation in *In(1)w^{m4}*. Heterochromatic gene silencing in PEV depends primarily on SU(VAR)3-9 dependent H3-K9 di-methylation (Schotta *et al.*, 2002). The mark is recognized by HP1 which restricts SU(VAR)3-9 and SU(VAR)3-7 through protein-protein interaction to chromatin marked by dimethyl H3-K9 (Ebert *et al.*, 2006). HP1 also recruits the SUV4-20 HMTase catalysing H4-K20 tri-methylation in pericentric heterochromatin (Schotta *et al.*, 2004). E(Z) also contributes to heterochromatin formation by catalysing H3-K27 mono-, di- and tri-methylation (Ebert *et al.* 2004). Mutations in factors controlling active histone modification marks revealed the dynamic balance between euchromatin and heterochromatin (Ebert *et al.*, 2004).

In parallel to the classical PEV studies in *Drosophila*, transgene silencing has also been used to identify *Su(var)s* or *E(var)s* for gene silencing processes in *Drosophila* and other organisms. The transgene approach demonstrated differential control of gene silencing processes in *Drosophila* depending on the neighbouring chromatin composition at the point of insertion (Wallrath and Elgin, 1998). In *Drosophila,* although the *white* gene has been efficiently used as a reporter gene to dissect out the factors responsible for gene silencing at pericentric, telomeric and fourth chromosomal heterochromatin, other eukaryotic regions undergoing gene silencing have been largely ignored (Cryderman *et al.,* 1999). More such variegating inserts

tagging the transposon sequences are needed to be studied in detail to identify different gene silencing pathways existing in *Drosophila*.

The epigenetic control of all the gene silencing processes including PEV, depend on DNA methylation and posttranslational histone modification such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination. Different histone modifications in specific combinations represent a code which defines the chromatin status. This 'epigenetic histone code' is not universal; rather it exists in diverse dialects in different species (Ebert *et al*., 2006). The histone code is recognised by specific histone modifying enzymes and generates new nucleosome binding sites recognised by non-histone proteins which consequently activate cellular processes. Alternatively, the nucleosome-remodelling factors are also involved in nucleosome-repositioning, altering histone-DNA interactions, disassembly of nucleosomes and the exchange of histones with variants of different properties at the level of single nucleosomes (Varga-Weisz and Becker, 2006). Thus establishment, maintenance and inheritance of specific chromatin structures is a result of interaction of various proteins such as histone modifying enzymes, DNA methyltransferases, chromatin remodelling factors, transcription factors as well as components of RNAi machinery (Volpe *et al*., 2002).

While several dialects of histone epigenetic codes have been suggested (Jenuwein and Allis, 2002; Ebert *et al*., 2006), 5 methyl cytosine is the only epigenetic modification known to occur on eukaryotic DNA (Bird, 2002). Post synthetic addition of the methyl group to the 5^{th} position of cytosine changes the appearance of the major groove of DNA and hence inhibits the binding of the DNA binding proteins which would activate those DNA sequences. This DNA modification plays an important role in genomic imprinting (Li, 2002; Kohler and Grossniklaus, 2005), X-chromosome inactivation (Goto *et al*., 1998; Li, 2002) and mammalian development (Li *et al*., 1992; Bird, 2002) especially during embryogenesis. Furthermore DNA methylation plays a role in stabilisation of the genome and the irreversible silencing of transposons (Yoder *et al*., 1997; Miura *et al*., 2001) as well as of endogenous retroviruses (Walsh *et al*., 1998) through chromatin remodelling (Bird, 2002; Li,

2002). In animals and plants DNA methylation has been shown to regulate gene expression along with histone methylation marks (Li, 2002; Naumann *et al*., 2005). However the mutual dependence of these two marks in regulation of gene expression is still a matter of debate because of the complexity of proteins involved in the processes (Naumann *et al*., 2005; Lindroth *et al*., 2004). Thus DNA methylation is a major epigenetic mark for transcriptional gene regulation however the exact mechanisms are still obscure. Aberration of the DNA methylation machinery leads to several human cancers (Ehrlich, 2002; Jones and Baylin, 2002) and diseases like ICF (immunodeficiency, centromere instability and facial anomalies) and Rett syndrome (Robertson and Wolffe, 2000). Genome wide efforts to map DNA methylation of the human cell lines showed that CpG islands remains unmethylated, even in transcriptionally inactive gene promoters but the gene region were highly methylated (Weber *et al.,* 2005; Weber *et al.,* 2007). Thus the corresponding DNA methylation systems are rather complex and an evolutionary conserved function has not been identified yet. Presence of several components of DNA methylation machinery in higher vertebrates makes it even more complicated to understand the DNA methylation in higher organisms.

DNA methyltransferases are the enzymes responsible for DNA methylation. In vertebrate, 3 families of DNA methyltransferases (MTases) comprising (so far) 4 members have been identified: DNMT1, DNMT2, DNMT3A and DNMT3B. In addition, DNMT3L has been identified as a stimulator of the DNMT3A and DNMT3B enzymes. All these enzymes contain a C terminal domain of approximately 400–500 amino acid residues, which is characterized by the presence of 10 conserved amino acid motifs, shared between prokaryotic and eukaryotic DNA-(cytosine-C5)-MTases (Fig 1.1; Cheng, 1995; Jeltsch, 2002). The catalytic centre and coenzyme binding site of MTases reside within this domain. In addition, the DNMT1 and the DNMT3 enzymes harbor large N-terminal regulatory parts (Fig 1.1; Chen and Li 2004; Hermann *et al*. 2004a).

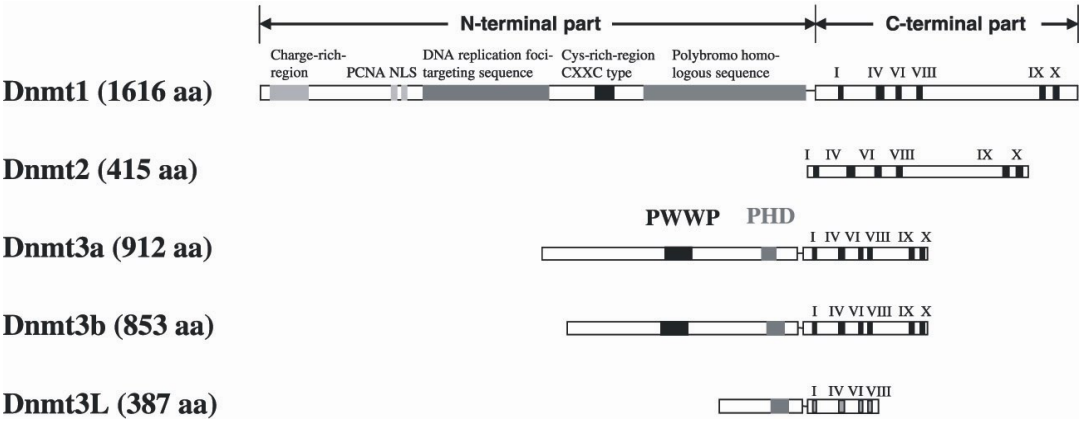

Figure 1.1 Domain organisation of the DNA methyltransferases. DNMTases are divided into an N-terminal part and a C-terminal part. The C-terminal part is highly conserved in all DNMTases and contains 10 conserved catalytic amino acid motifs (indicated by Roman numerals) characteristic for this enzyme family. The N terminus is highly variable and is absent in DNMT2 (Adapted from Jeltsch, 2006a).

The N-terminal regulatory domain of DNMT1 contains different motifs and subdomains which interact with many other proteins (Chuang *et al*., 1997; Fuks *et al*., 2003; Liu and Fisher, 2004; Margot *et al*., 2003; Pradhan and Kim 2002; Robertson *et al*., 2000; Rountree *et al*., 2000). While N terminal regulatory domain of DNMT3a and DNMT3b contain an ATRX-like Cys-rich domain (also called PHD domain) and a PWWP domain, which are involved in interactions with other proteins and targeting to heterochromatin (Aapola *et al*., 2002; Bachman *et al*., 2001; Chen and Li, 2004; Fuks *et al*., 2003; Ge *et al*., 2004). It seems that the N-terminus is forming a platform for binding of proteins involved in chromatin condensation, gene regulation and DNA replication.

DNMT1 has a strong preference for methylation of hemimethylated CG sites (Fatemi *et al.*, 2001; Gruenbaum *et al*., 1982; Hermann *et al*., 2004), which implicates a function in maintenance of the methylation pattern of the DNA after replication. DNMT1 knock-out mice die during embryogenesis; embryos show almost complete loss of DNA methylation (Li *et al*., 1992). Interestingly, the catalytic domain of DNMT1 is inactive in the absence of the N-terminus of the protein (Fatemi *et al*., 2001), which implies an important regulatory function of the N-terminal domain on

enzyme activity. The mammalian DNMT3 enzyme family consists of three different proteins, DNMT3A, DNMT3B andDNMT3L. The regulatory N-terminus of DNMT3A and DNMT3B is not essential for catalysis (Gowher and Jeltsch, 2002; Reither *et al*., 2003). Despite significant amino acid sequence and biochemical similarities, DNMT3A and DNMT3B have distinct biological roles. DNMT3B is responsible for methylation of pericentromeric satellite regions (Hansen *et al*., 1999; Okano *et al*., 1999; Xu *et al*., 1999). *Dnmt3B*$^{-/-}$ knock-out mice die during the late embryonic stage and the embryos lack methylation in pericentromeric repeat regions (Okano *et al*., 1999). Loss of DNMT3B activity in human leads to ICF (immunodeficiency, centromere instability, facial anomalies) syndrome, a genetic disorder that is accompanied by low methylation in the pericentromeric satellite regions of chromosomes 1, 9 and 16 (Ehrlich, 2003). *Dnmt3A* knock-out mice show developmental abnormalities and die a few weeks after birth (Okano *et al*., 1999). This enzyme has been associated with the methylation of single copy genes and retrotransposons (Bourc'his and Bestor 2004; Bourc'his *et al*., 2001; Hata *et al*., 2002) and it is required for the establishment of the genomic imprint during germ cell development (Kaneda *et al*., 2004). The N-terminal part of DNMT3L is shorter than those of DNMT3A and DNMT3B and only contains the PHD domain. The C-terminal part of this protein is truncated and all its "catalytic" motifs are crippled, indicating that it cannot be an active DNA MTase. DNMT3L acts as a stimulator of the catalytic activity of DNMT3A and DNMT3B activity (Chedin *et al*., 2002; Gowher *et al*., 2005; Suetake *et al*., 2004).

DNMT2 is the smallest enzyme among the eukaryotic MTases and it comprises only the catalytic domain. Unlike the above three enzymes, it lacks the N-terminal regulatory region (Fig 1.1; Li, 2002). DNMT2 is a highly conserved protein found in all the eukaryotic species except in *C. elegans* and *S. cerevisae.* DNMT2 proteins are widely evolutionary conserved from vertebrates, like *Homo sapiens*, *Mus musculus*, *Xenopus laevis* and *Danio rerio*, to plants like *Arabidopsis thaliana* and were also found in fungi like *Dictyostelium*, fission yeast *Schizosaccharomyces pombe* and in

several insect species like *Bombyx mori* and *Drosophila melanogaster* (Fig 1.2; Dong *et al*., 2001).

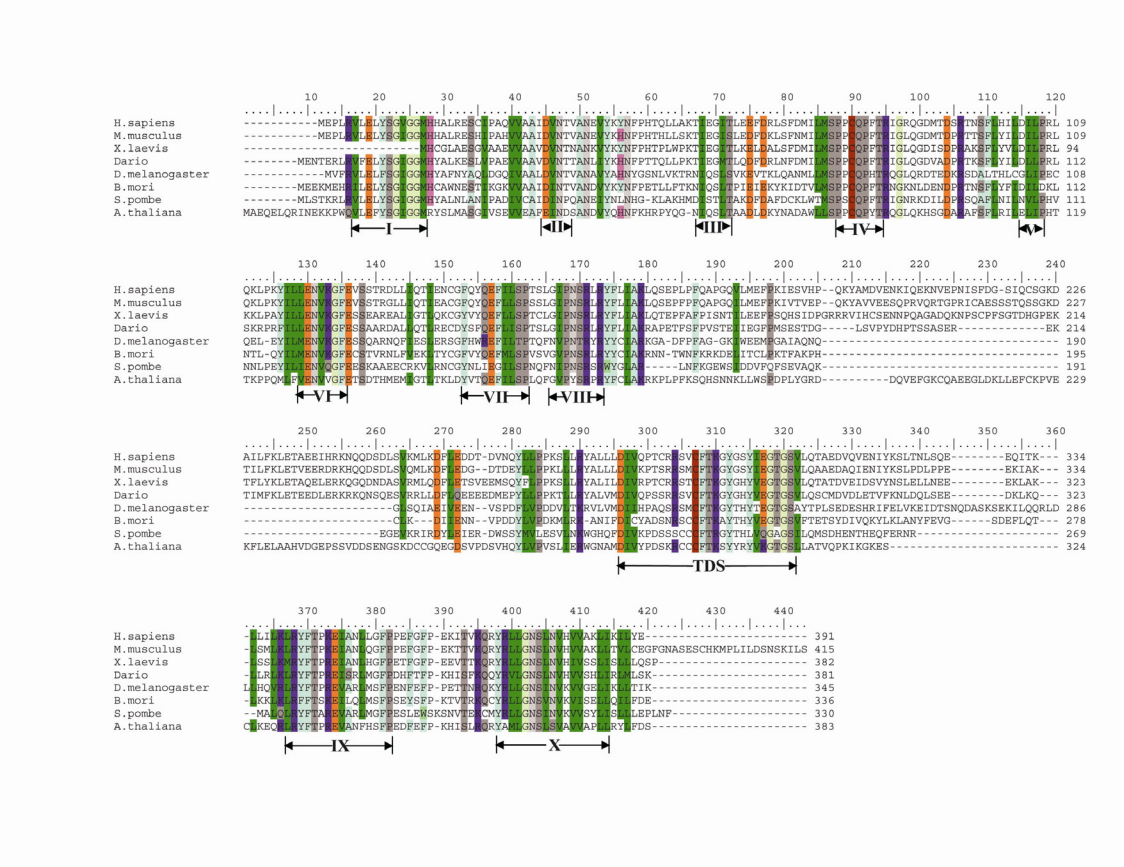

Figure 1.2 Alignment of DNMT2 proteins. DNMT2 protein sequences from human, mouse, *Xenopus*, zebra fish, *Drosophila*, *Bombyx mori*, *S. pombe* and *Arabidopsis* were aligned. Roman numbers from I to X represents the 10 highly conserved motifs of all the DNA methyltransferases. (Adapted from Dong *et al*., 2002)

Even though DNMT2 proteins show a perfect conservation of all ten catalytic DNA methyltransferase motifs (Kumar *et al*., 1994), it has been suggested to be an inactive DNA methyltransferase (Okano *et al.*, 1998; Dong *et al.*, 2001; Goll and Bestor, 2005). Recent studies have demonstrated residual DNA methylation activity (Hermann *et al*., 2003; Kunert *et al*., 2003; Narsa Reddy *et al*., 2003; Liu *et al*., 2003; Tang *et al*., 2003), while strong tRNA methylation activity of the DNMT2 protein (Goll *et al.*, 2006; Jurkowski *et al.*, 2008). This suggests a dual substrate specificity

of DNMT2 (Jeltsch *et al.,* 2006b), still its biological function remains enigmatic. *Dnmt2* knock out showed variable phenotypic effects in different organisms. Loss of function mutations in mice did not show any obvious effect on embryonic stem cell viability or genomic methylation patterns (Okano *et al.*, 1998), while several developmental defects were observed in zebrafish *Dnmt2* knock outs (Rai *et al.,* 2007). In *D. melanogaster*, *Dnmt2* has been associated to longevity (Lin *et al*., 2004).

For a long time the *Drosophila* was thought to be completely devoid of DNA methylation (Rae *et al.,* 1979; Patel, and Gopinathan, 1987). However several independent studies provided indications for the existence of a functional DNA-methylation system in *Drosophila* (Hung *et al.,* 1999, Tweedie *et al.,* 1999; Lyko *et al.,* 2000; Kunert *et al.,* 2003 ;). DNA methylation has been suggested to be present only in very low amounts and restricted to early stages of *Drosophila* development (Lyko *et al.,* 2000). DNMT2 is the only single DNA methyltransferase in *Drosophila.* Similar to *Drosophila*, the protozoan parasite *Entamoeba histolytica* and the myxomcete *Dictyostelium* also possess single DNA methyltransferase homologous to DNMT2, EHMETH and DNMA, respectively (Fisher *et al*., 2004; Kuhlmann *et al*., 2006). DNMT2 protein of *Drosophila melanogaster* and *Drosophila simulans* show 96% similarity to each other and these both proteins show 54% similarity to *H. sapiens* and *Mus musculus* (Marhold *et al*., 2004a). Vertebrate DNMT2 contains an additional stretch of about sixty amino acids between the catalytic motif VIII and the target recognition domain that is not present in the insect proteins. Furthermore, the *Drosophila* DNMT2 proteins contain a small block of amino acids between the target recognition domain and the catalytic motif IX that is not present in the proteins of mouse and human. Other then DNMT2, only single methyl cytosine binding protein, MBD2/3 has been identified in *Drosophila* which suggest an existence of a functional DNA methylation system in *Drosophila* (Marhold *et al.,* 2002). *Drosophila* with its relatively simple DNA methylation system appears to be an attractive model organism to understand the complex mechanisms of DNA methylation in eukaryotes.

However the function of DNMT2 mediated DNA methylation in *Drosophila* is still elusive. Loss of function mutation flies are viable and fertile without any obvious

phenotypic effects (Goll *et al.,* 2006). DNMT2 mediated DNA methylation has been shown to be involved in tumor formation in *Drosophila* (Marco D. *et al.,* 2006). Another proposed function of DNMT2 is the regulation of *Drosophila* life span by acting upstream of the expression of at least 3 small *Hsp* genes (Lin *et al*., 2004). In this study overexpression of DNMT2 causes increased life span of the flies, while a hypomorphic mutant for *Dnmt2* showed a decreased life span. Like other DNMTases, DNMT2 mediated DNA methylation has been suggested to target transposable elements and repetitive DNA sequences in *Drosophila* and *Dictyostelium* (Salzberg *et al*., 2004; Kuhlmann *et al.,* 2006).

Transposable elements are abundant yet not fully understood components of the eukaryotic genome. They have been implied in genome evolution (Kazazian, 2004), and telomere stabilization in *Drosophila* (Mason and Biessmann 1995). Yet they have been considered to be deleterious for the organisms because of their ability to jump into the other parts of genome (Lim and Simmons 1994). The tight control of their mobilization is thus an important feature of their regulation to prevent increased mutation rates due to novel insertions. With the complete genome sequencing of *D. melanogaster* distribution of transposable elements (Kaminker *et al.,* 2002; Bergman *et al.,* 2006) and possible mechanisms of their regulation can be analyzed in detail (Brennecke *et al.,* 2007).

Eukaryotic transposable elements are divided into retrotransposons that transpose via an RNA intermediate, and the transposons that transpose by DNA excision and repair (cut and paste mechanism). Within the retrotransposons, the major division is between those that possess long terminal repeats (LTR elements) and those that do not (LINE and SINE elements). Among the transposons, the majority transpose via a DNA intermediate, encode their own transposase and are flanked by relatively short terminally inverted repeat structures (TIR elements). Approximately 5.5% of *Drosophila* genome sequence consists of complete or complete transposable elements (Bergman *et al.,* 2006). A detailed genome analysis revealed a non random distribution of the transposable elements in discrete high density clusters. Overall 24 high density regions were identified, most of them mapping to the pericentromeric

region and the 4th chromosomes, except for 3 clusters which were mapped to the non pericentromeric region of X and 3R chromosome arms (Bergman *et al.,* 2006). Both the pericentromeric (*flamenco* locus) and non pericentromeric (3R TAS) clusters of transposable elements have been suggested to function as master loci for the regulation of retrotransposon expression through an RNAi pathway (Brennecke *et al.,* 2007; Yin and Lin., 2007). Several components of the RNAi gene silencing pathway have been shown to control retrotransposon expression in *Drosophila* germ line tissue (Aravin *et al.,* 2004; Klenov *et al.,* 2007). However RNAi independent control of retrotransposon has been suggested in *Drosophila* somatic tissues (Klenov *et al.,* 2007, Desset *et al.,* 2008).

Retrotransposons have also been implicated in maintaining the telomere length in *Drosophila*. Telomeres are specialized nucleoprotein structure on the ends of the linear chromosome, required for the accomplishment of the chromosome end replication. Most of the organisms use a specialized reverse transcriptase, telomerase dependent mechanism, which uses an RNA template to add small DNA sequences at the ends of the chromosome after each cycle of replication (Greider and Blackburn, 1985). *Drosophila* telomeres do not possess arrays of simple telomeric repeats, generated by telomerase. Instead, *Drosophila* maintains its telomeres by transposition of specific retrotransposons, *HeT-A*, *TART*, and *TAHRE*, referred as HTT, to chromosome ends (Mason and Biessmann, 1995; Abad *et al*., 2004). Proximal to the terminal retrotransposon array *Drosophila* telomeres carry an irregular array of repeated sequences often termed the subtelomeric repeat, or the telomere associated sequence (TAS) (Karpen and Spradling 1992; Walter *et al.,* 1995). While TAS have been identified in a wide variety of species (Pryde *et al.,* 1997), their sequence and arrangement varies considerably from one species to another and even from one telomere to another within the same cell (Abad *et al.,* 2004). Genetic and molecular analysis revealed that TAS is the heterochromatic component of the telomeres (Mason *et al.,* 2004; Yin and Lin, 2007). To the distal end HTT array is protected with a protein complex, referred to as telomere cap. Major components of this protein complex include several DNA damage response proteins, HP1 and HOAP (Mason,

Capkova, and Biessmann, 2008). All the components of this protein cap are essential for integrity of *Drosophila* telomeres, loss of one or more components leads to telomere fusions. DNA methylation has been shown to be involved in regulating the telomere length in mammalian cells (Gonzalo *et al*., 2006); however the possible role of DNA methylation in controlling the telomere stability has not been studied in *Drosophila*.

In the present study genetic analysis of differential gene silencing process has been performed using a transgene approach. Silenced region of the genome were tagged by variegating *white* transgenes and tested for the modifier effect of known PEV modifier gene mutations. A null mutation for *Dnmt2* was generated to assess the role of DNA methylation in *Drosophila*. In this study a detailed analysis of DNMT2 dependent gene silencing processes was performed. Role of DNMT2 in control of retrotransposition and telomere control was also evaluated.

2. Materials and Methods

2.1 Genetic methods

2.1.1 *Drosophila* stock maintenance, culture conditions and embryo collection

All *Drosophila* stocks were reared and maintained in vials or in 0.5 l glass bottles containing standard medium (1% agar, 2.5% syrup, 3.5% raisins, 3% semolina, 5% yeast). The stocks and crosses were kept at 18 °C and at 25 °C, respectively. To avoid fungal and bacterial contamination 0.1% Nipagin and 100mM Ampicilline were added to the medium.

For embryo collection freshly hatched flies were reared in plastic cages, covered by the gauge on the one side and the embryo collection plate on the other side. Solid medium containing 10% Sugar, 2.5% agar, 0.1% Nipagine and 0.1% acetic acid supplemented with active coal is used for the for the egg laying. The cages are maintained at 25 °C, and the embryos were collected in desired time intervals.

2.1.2 Generation of new mutant alleles using P element remobilization

New mutant alleles of *Dnmt2, Suv4-20* and *Mbd2/3* were generated by remobilization of GE15695, BG00814 and *P{Epgy2}EY04582* P elements using an external transposase, Δ*2-3*. Using genetically controlled mating schemes (Fig 2.1a, b, c) respective P element was brought together with Δ*2-3* transposase on the third chromosome linked with *Dropped* eye phenotype. These males were mated with a balancer stock for the respective chromosomes. Exceptional white eyed progenies (because of the P element jump out) were back crossed to establish a genetically

stable stock and were analysed for the resulting DNA lesion using genomic primer pairs. Primers used to characterize genetic lesions are listed in Table 2.1. Balancers and associated mutation have been described in Lindsley and Zimm (1992) and Flybase (2008)

a

w/w; SM6/Sco;+/+ **X** *w/Y; +/+; DrΔ2-3/TM6c*

w/w; GE15695/ GE15695; +/+ **X** *w/Y; SM6/+; DrΔ2-3/+*

w/w; SM6/Sco; +/+ **X** *w/Y; GE15695/SM6; DrΔ2-3/+*

analyse *w/Y; GE15695(w^-)/SM6; +/+* exception flies

b

w/w; +/+; P{Epgy2}EY04582/ P{Epgy2}EY04582 **X** *w/Y; +/+; DrΔ2-3/TM6c*

w/w; +/+; TM6c/TM2 **X** *w/Y; +/+; DrΔ2-3/ P{Epgy2}EY04582*

analyse *w/Y; +/+; P{Epgy2}EY04582(w^-)/TM6c* exception flies

c

BG00814/ BG00814; +/+; +/+ **X** *w/Y; +/+; DrΔ2-3/TM6c*

FM7/CLB; +/+; +/+ **X** *BG00814/Y; +/+; DrΔ2-3/+*

analyse *BG00814 (w^-)/Y; +/+; +/+* exception flies

Figure 2.1.P element remobilization scheme to generate new (a) *Dnmt2,* (b) *Mbd2/3* and (c) *Suv4-20* mutant alleles.

Table 2.1 List of Primers used to identify mutational lesions after P element mediated mutagenesis.

Gene	Primers*
Dnmt2	Mt2_in_fwd, Mt2_in_rev, Mt2_fp1, Mt2_fp2
Suv4-20	420Fwd1, 420Rev1, 420Fwd3, 420Rev3, 420Fwd2, 420Rev2
Mbd2/3	Mbdfwd, mbdrev, mbdfwd1, mbdrev1, mbdfwd2, mbdrev2

*For primer sequences refer to appendix 2.

2.1.3 Alternative gene silencing pathways in *Drosophila*

To understand differential gene silencing mechanisms throughout *Drosophila* genome, 67 variegating P-element inserts were collected from DROSDEL EU project. 43 of these variegating inserts were precisely mapped on *Drosophila* chromosomes (Appendix 1). Representative examples of differentially located P element were mated with males or females from strong null mutant alleles of heterochromatin associated genes and the newly generated *Dnmt2* and *Mbd2/3* mutant alleles (Tab 2.1). Progenies resulting from mating w^{1118} males or females with the variegating inserts were used as an external control for variegating eye phenotype. F1 progenies resulting from these crosses were evaluated by analysing the eye colour on a ZEISS Stemi SV6 binocular eyepiece and by taking photos in Adobe Photoshop™ 3.0 with a Sony DXC-930P camera.

Table 2.1 Gene mutations used in the study

Mutation	Affected Protein/ Function	Reference
Su(var)3-9^{0}	HP1	Schotta *et al*., 2002
Su(var)2-5^{05}	SU(VAR)3-9/ H3K9me3	Wustmann *et al*., 1989
E(z)5^{5}	Enhancer of Zeste/ H3K27 me1, me2, me3	LaJeunesse and Shearn, 1996
Su(var)3-1^{suc40}	JIL1 Kinase/ H3S10P	Ebert *et al*., 2004
Suv4-20^{SP}	SUV4-20/ H4K20me3	Present work
SetDB1^{1473}	SETDB1/ H3K9me2	Clough *et al*., 2007
Dnmt2^{149}	DNMT2/5mC	Present work
Mbd2/3^{19}	MBD2/3	Present work
w^{1118}	WHITE/ Transporter for the red eye pigment	Hazelrigg *et al*., 1984

2.1.4 Zygotic control of *Dnmt2* mediated retrotransposon expression

Genetic analysis was used to determine if the DNMT2 mediated control of retrotransposon expression if maternally or zygotically controlled. *Invader4* variegating insert *p(RS5)5-HA-1992* females were mated with *Dnmt2* null males and vice verse. F1 progeny obtained from either crosses were scored for the suppressor effect on the variegating P element insert.

2.1.5 Somatic cells specific up regulation of retrotransposon

Somatic or germ line specific activity of DNMT2 mediated regulation of retrotransposon was assayed by genetic system described before for *SpnE* RNAi mutations (Klenov *et al*., 2007). Transgenic flies carrying *LacZ* reporter gene under control of *Copia* LTR promoter activity on X chromosome were obtained from Gvodzev group, Russia. Stabilised stocks carrying this *LacZ* transgene in *Dnmt2* null mutant background were established through controlled mating scheme (Fig 2.2). As a control of basal activity of *Copia* LTR promoter, *LacZ* transgene stabilised in second chromosome balancer stock or in w^{1118} background were used.

CopiaLTR:LacZ; SM6/Sco; +/+ **X** *w/Y; Dnmt*^{149}*/ Dnmt*^{149}*; +/+*

CopiaLTR:LacZ; SM6/Sco; +/+ **X** *CopiaLTR:LacZ /Y; Dnmt*^{149}*/SM6; +/+*

CopiaLTR:LacZ; Dnmt^{149}*/SM6; +/+* **X** *CopiaLTR:LacZ /Y; Dnmt*^{149}*/SM6; +/+*

CopiaLTR:LacZ; Dnmt^{149}

Figure 2.2 Mating scheme to generate stabilised *CopiaLTR:LacZ* transgene stock in *Dnmt2* null mutant background.

Transgenic stocks were fed with yeast, larval somatic tissues and the adult germ line tissues were dissected out and biochemical assay for *LacZ* was performed

2.2 Molecular methods

2.2.1 Isolation of genomic DNA/RNA

2.2.1.1 Maxi preparation of genomic DNA

The protocol 23.1* "Quick genomic DNA preparation" of Huang *et al*., (2000) was used for isolation of genomic DNA in a large scale from adult flies with the following modifications. The samples were incubated at 70°C for 30 minutes after grinding. The centrifugation steps were performed at 13000 rpm and the DNA pellet was dissolved in 150 µl Aqua bidest.

2.2.1.2 DNA-Isolation from a single fly

Lower amounts of genomic DNA from single adult flies were isolated according to the "single fly prep"-method of Gloor and Engels (1992). The DNA which was used for polymerase chain reactions (PCR) was not more than 10% of the final volume of PCR.

2.2.1.3 Isolation of total RNA and preparation of cDNA

Total RNA was isolated using Trizol®-Method as described by manufacturer (GibcoBRL). Approximately 100mg embryos (0-2 h) or 100 adult heads from wild type and *Dnmt2* null mutant flies were homogenised in Trizol®, followed by centrifugation at 10000 rpm for 5 min to remove the cell debris. 200µl chloroform was added to the supernatant, mixed vigorously and incubated at room temperature

for 5 min, followed by centrifugation at 8000 rpm. Aqueous phase (upper phase) was transferred to a fresh eppendorf tube, and the RNA was precipitated with 1 volume of Isopropanol by centrifugation at 15000 rpm for 15 min at 4°C. RNA pellet was washed with 70% Ethanol; air dried at room temperature and finally resuspended in 100µl DEPC treated sterilised water.

cDNA was prepared from 2.0µg RNA using 1.0U MMLV-RT enzyme, 2.0 µl Random primer and 25ng dNTPs. The cDNA thus obtained was used or the semi quantitative or real time PCR using specific retrotransposon sequence primers (Tab 2.2).

Table 2.2 Primers used for RT PCR analysis

Analysed Sequence	Primer*
Dnmt2	Mt2RAFwd, Mt2RBFwd, Mt2RABRev, cMt2Fwd, cMtRev, HD11, HD12
Suv4-20	Suv420Fwd4, Suv420Rev4
Invader4	Inv4Fwd2, InvRev2
Gypsy	Gypsy_PolFwd, Gypsy_PolRev
Copia	CopiaRNAFwd, Copia RNARev
Rover	Rov_PolFwd, Rov_Pol Rev
Lamine	LamFwd, LamRev

*For primer sequences refer to appendix 2.

2.2.2 Standard molecular methods

Polymerase Chain Reaction was performed using standard protocol (Seiki, 1990) using Taq-Polymerase (GibcoBRL) or *Pfu*-Polymerase (Stratagene). PCR fragments were separated on 0.5%- 2.0% agarose gel containing 0.01% Ethidium Bromide and eluted out of the gel using NucleoSpin® Extract II kit (Macherey-Nagel). Eluted DNA fragments were ligated into pGEM-T vector using fast ligation pGEM-T vector kit

(Promega). Cloned vectors were transformed into *E. coli* strain *DH5α* (Invitrogen GmBH) according to standard protocol (Sambrook *et al*., 1989).
Plasmid isolation was performed by alkali lysis method (Birmboim and Doley 1979) or by mini plasmid kit (Macherey-Nagel) according to manufacturer's instructions.
Restriction analysis was performed by enzymes obtained from MBI Fermentas with appropriate buffers and incubation conditions as instructed by manufacturer.
The sequencing was performed with the Big-Dye kit from Applied Biosystems in a TGradient (Biometra®, Göttingen) or in a T3Thermocycler (Biometra®, Göttingen) using appropriate primers. Analysis of the sequencing data was done by ABI 3130x/ Genetic Analyzer with the Sequence Analysis Software v5.2 (ABI Applied Biosystems, Darmstadt).

2.2.3 Inverse PCR

Inverse PCR was performed to locate P-element insertions in the genome using protocol described in Huang *et al.* (2000) with some modifications. Genomic DNA was prepared from the flies carrying a P-element. The DNA was dissolved in 150µl Aqua bidest and digested with different frequent cutter enzymes (MBI Fermentas) at 37°C over night. The digested DNA was ligated (T4 DNA ligase, MBI Fermentas) and resuspended in 100µl Aqua bidest. The ligated DNA was subjected to PCR using the primers specific for 5' and 3' end of the P-element (Tab 2.3). Products from this PCR were subjected to nested PCR using primers binding downstream of the first primers (Tab 2.3). The fragments were eluted from the agarose gel and either subcloned into pGEM-T vector for sequencing or sequenced directly using primers used in the second PCR. Subsequently the adjacent genomic sequences of the P-element were compared with whole genome of *Drosophila melanogaster* in the BLAST (Altschul *et al*., 1997) programme of FlyBase (http://flybase.bio.indiana.edu/) to identify the insertion position.

Table 2.3 Primers used for inverse PCR

P element end	Primers*
First PCR	
5' End	5' P fwd1, 5' P rev1
3' End	3' P fwd1, 3' P rev1
Second PCR	
5' End	5' P fwd2, 5' P rev2
3' End	3' P fwd2, 3' P rev2

*For primer sequences refer to appendix 2.

2.2.4 Real Time QPCR

The Real-time PCR analysis was performed as described by Dellino *et al*. (supplemental Data, 2004) with the following modifications. 5 µl of each DNA sample was amplified in 20 µl reaction mixtures in the presence of 10 µl iQ™ SYBR®-Green Supermix 2x Mix for Real-Time PCR (Bio-Rad Laboratories) and 4 pM appropriate primers.

The Real-time PCR was performed in 96-well plates with the iCycler Real-Time PCR Optical Detection System controlled by iCycler™ iQ Optical System Software Version 3.0a (Bio-Rad). The calculations are based on the description of Dellino *et al*. (supplemental Data, 2004). All experiments were repeated at least twice, and mean values and standard deviations were calculated.

2.2.5 DNA methylation analysis

2.2.5.1 Bisulphite Sequencing

Cytosine methylation was assayed by the bisulphite genomic sequencing as described in Grunau *et al*, 2001. 2 µg of genomic DNA isolated from early wild type and

Dnmt2^{149} embryos (0-2h) was digested with *MspI* and denatured by alkali treatment. Denatured DNA was incubated in 1.2 ml freshly prepared 3.1M sodium bisulphite/0.5mM hydoquinone (Sigma), pH 5.0 at 95C for 1 h, followed by desalting (QIAaexII, Quiagen), desulphonation and neutralization. DNA was precipitated, resuspended in 100 µl of 1mM Tris, pH 8.0 and stored at -20°C. desired sequences were PCR amplified using appropriate primers (Tab 2.4) and cloned into pGEM-T vector (Promega) for sequencing. For each genotype, 10-15 independent clones were sequenced. Raw sequencing data obtained from ABI sequencer was analysed for cytosine to thymine conversion in Bioedit Sequence editor v7.0 software.
As a positive control of the bisulphite reaction pGEM-T vector ligated with *Invader4* sequence was subjected to bisulphite treatment in parallel to the genomic DNA.

2.2.5.2 Restriction analysis

Genomic DNA was isolated from precisely staged wild type and *Dnmt2* null embryos. Approximately 1.0 µg DNA was digested with 2U *Sge*I (Fermentas GMBH)**,** 2U *Sau*3AI (*Bsp*143I) or 2U *Mbo*I enzyme. DNA was digested at 37C for either 3hrs (*Sge*I) or overnight (*Sau*3AI or *Mbo*I) and then loaded directly on 1% agarose gel or used for semi quantitative PCR to amplify desired sequences (Tab 2.4). 20-25 cycles of PCR amplification cycle were used. Parallel to the DNA samples with respective enzyme, samples without enzyme were used as control.

Table 2.4: Primers used for DNA methylation analysis

Sequence	**Primers***
Invader4LTR	Inv4Fwd1, Inv4Rev1
Invader4{}511	Inv4_511Fwd1, Inv4_511Rev1
Doc	DocRTFwd, DocRTREv
RP49	RP49Fwd, RP49Rev

*For primer sequences refer to appendix 2.

2.2.6 Chromatin Immunoprecipitation (ChIP)

For chromatin immunoprecipitation with one antibody, 200 adult heads or 300mg precisely staged embryos were cross linked using 1.8% Formaldehyde in X linking buffer (50 mM HEPES, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl) for 10-20 min at room temperature on a rotating platform. Cross-linking was stopped by adding 125mM glycin at 4°C for 5 minutes. Fixed tissue was washed with ChIP wash buffer A (10 mM HEPES, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and ChIP wash buffer B (10 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100) at 4°C each for 10 minutes on rotating wheel followed by sonification (10 times each for 30 seconds) in TEN140 (10 mM Tris-HCl, 1 mM EDTA, 140 mM NaCl) and 30 µl 1.0 mM PMSF (SERVA). Cell debris was removed by centrifugation at 15000 rpm for min at 4°C. About 13% of the supernatent was used as input chromatin and the rest was divided in to two or three equal parts. One part was incubated with 7-10 µg of appropriate antibody while other was used as no antibody control.

After over night-incubation of all fractions on the rotating platform at 4°C, 40 µl of 50% Protein G Sepharose™ beads suspension in RIPA buffer (140 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC) were added to the fractions and incubated for at least 3 hours at 4°C while rotating. The antibody complexes were bound to Protein G Sepharose™ beads and washed 6 times with RIPA, once with 1 ml LiCl ChIP buffer (250 mM LiCl, 10 mM Tris-HCl, 1 mM EDTA, 0.5% NP40, 0.5% DOC) and finally twice with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). The Protein G Sepharose™ beads were resuspended in 100 µl TE-buffer (Marligen Biosciences) and treated with RNase A (50µg/ml, Marligen Biosciences) at 37°C for 30 minutes and 50 µg/ml Proteinase K (Fermentas) at 37°C over night.

Reverse cross linking was performed at 65°C for 6 hours and the DNA was extracted with 150 µl phenol-chloroform-isoamyl alcohol-mix (25:24:1), the organic phase

were back-extracted with 50 µl 50 mM Tris-HCl pH 8.0. The aqueous phases were combined and extracted with 200 µl chloroform. The DNA was precipitated in 0.3 M Sodiumacetate (pH 5), with 3 µl glycogen (20 mg/ml) and 3 volumes of 96% Ethanol at -70°C for at least one hour. After 10 minutes centrifugation at 15500 rpm and washing the pellet with 70% Ethanol DNA was dissolved in 150 µl Aqua bidest for the Real-time PCR Analysis using appropriate primers (Tab 2.5).

Table 2.5 Primers used for ChIP analysis

Analysed Sequence	**Primers***
Satellite	satFwd, satRev
Tubuline	tubFwd, tubRev
Invader4	Inv4Fwd1, Inv4Rev1, Inv4Fwd2, Inv4Rev2
Rover	RovPolFwd, RovPolRev, RovgagFwd, RovgagRev
Gypsy	GypPolFwd, GypPolRev
Copia	CopRNAFwd, CopRNARev, CopLTRFwd, CopLTRRev
pP(RS5)	RS5fwd3, RS5rev3, RS5fwd4, RS5rev4

*For primer sequences refer to Appendix 2.

2.3 Biochemical methods

2.3.1 Preparation of nuclear protein extracts of *Drosophila melanogaster* larvae

100 third instar larvae of *Drosophila melanogaster* were collected for the preparation of nuclear extracts. The larvae were frozen in liquid nitrogen and immediately stored at -80°C until further use or were used immediately. The preparation of the nuclei was performed on ice. The larvae were suspended and crushed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4) with 1 mM PMSF (SERVA) in a Dounce-homogeniser. Afterwards the nuclei were centrifuged through

a saccharose gradient (350 mM saccharose in PBS) at 4000 rpm for 10 minutes. The nuclei-pellet was dissolved in 100 µl PBS.
A corresponding volume of loading buffer (Rotiload; Roth) was added to the nuclear extracts. The samples were then denatured at 95°C for 5 minutes and used for gel electrophoresis. Denatured samples were stored at -20°C.

2.3.2 Protein gel electrophoresis

The separation of the proteins according to the molecular weight was performed in discontinuous SDS-Polyacrylamid gels (Laemmli, 1970). The concentration of polyacrylamid in the stacking gel was 5% and in the resolving gel 15-16%. The electrophoresis was made in ELPHO-buffer (192 mM glycin, 25 mM Tris, 0.1% SDS) at 15-20 mA (300 V) for 8 x 9 cm gels. Page Ruler™ Prestained Protein ladder (MBI Fermentas) was used as marker.
After the electrophoresis the proteins were stained with Coomassie-solution (0.25% Coomassie Brilliant Blue R250 in 50% methanol, 10% acetic acid) and then the gel was destained by distilled water.

2.3.4 Western blot analysis

After the electrophoresis, the proteins were blotted on PVDF membrane (Westran®S; Schleicher & Schuell) in Hoefer SemiPhor semi dry blotting unit at 1.5 mA/cm^2 (300 V) with blotting buffer (192 mM glycin, 25 mM Tris, 20% methanol) for one hour. The blotted PVDF-membrane was blocked with 5% milk powder (ROTH) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4) for one hour and then probed with the appropriate dilution of primary antibody (Tab 2.6) in 5% milk powder in PBS at 4°C over night. The membrane was washed with PBST (PBS containing 0.1% Tween 20) three times 20 minutes each. Subsequently the blots were incubated with appropriate dilution of horseradish-peroxidase conjugated secondary antibody (Tab 2.6) in 5% milk powder in PBS at 37°C for 1-2 hours.

Again the membrane was washed with PBST three times for 20 minutes each. The detection of the peroxidase-coupled antibodies was performed with the enhanced chemiluminescence (ECL) Western Blotting Detection System (Amersham Bioscience) according to the manufacturer's instructions.

2.3.5 *LacZ* staining

LacZ stainings were performed as described before (Lu *et al*., 1998). In brief the larval tissues or adult ovary and testis were dissected in PBS and incubated with *LacZ* staining solution (3.1 mM potassium ferricyanide, 3.1 mM potassium Ferrocyanide, 10 mM sodium phosphate, pH 6.8, 0.15 M NaCl, 1 mM MgCl2, 10% DMSO and 0.1% X-gal (5-bromo-4-chloro-3-indolyl-b-Dgalactoside:Gold Biotechnology) for at least 3 h. X-gal was stored at -20°C as a 10% stock solution in dimethylformamide and added immediately prior to staining. The stained tissues were analysed by ZEISS Stemi SV6 binocular eyepiece and by taking photos in Adobe Photoshop™ 3.0 with a Sony DXC-930P camera.

2.4 Immunocytological methods

2.4.1 Immunostaining of polytene chromosomes and the embryos.

The preparation of polytene chromosomes from salivary glands of *Drosophila melanogaster* was performed as described in Alfageme *et al*., (1980) with the following modifications. The salivary glands of third instar larvae were dissected in preparation buffer (0.7% NaCl, 1.0% NP-40) and treated with fixation buffer (2% formaldehyde, 1% Triton X-100, 0.7% NaCl) for two minutes to prevent the loss of chromosomal proteins during subsequent treatment. The glands were then transferred to a drop of spreading buffer (45% acetic acid, 2% formaldehyde), incubated for three minutes and finally squashed between a microscope slide and a siliconised cover slip.

The preparations were frozen in liquid nitrogen and the cover slips were removed. The solutions were always prepared fresh and stored on ice while using. The preparations were stored in PBST (PBS containing 0.1% Tween 20) at 4°C until antibody incubation.

The polytene chromosomes were incubated with the primary antibodies (Tab 2.6) diluted in 5% milk powder in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4) at 4°C over night in a humid chamber. Then the preparations were washed, to eliminate weakly bound antibodies, three times with PBST each 10 minutes followed by two hours incubation with fluorescence labelled secondary antibody diluted (Tab 2.6) in 5% milk powder in PBS at 37°C in a humid chamber. After further three washing steps, each 10 minutes, in PBST the polytene chromosomes were treated with 4', 6'-diaminodino-2-phenylindole (DAPI) to stain the DNA for 5 minutes. The preparations were washed with PBST twice each 5 minutes, dried, mounted with CITIFLUOR Glycerol/PBS solution AF1 (Agar SCIENTIFIC) and finally sealed with nail varnish. The preparations were stored at 4°C until analysis. The analysis/examination of the polytene chromosomes immunostainings was performed by a confocal microscope (LSM 510; Zeiss) by using standard filters for DAPI and *Alexa* fluor®488 and Laser Scanning Microscope LSM 510 Version 3.2 SP2 software (Carl Zeiss). The pictures were arranged with the LSM 5 Image Browser and Adobe Photoshop Version 8.0.

2.4.2 Fluorecent *in situ* hybridization

Polytene chromosomes were prepared as similar to as described above except that the spreads were made without fixation. The spreaded chromosomes were boiled in 2xSSC at 70°C for 30 min, followed by denaturation in 0.07M NaOH for 1.5min. The chromosomes were dehydrated by serial washings in 70, 80 and 96% Ethanol.

For preparation of digoxigenin labelled probe, the desired sequence (*Invader4 LTR*, *381bp* repeat and *HeT-A*) was amplified either directly from the genomic DNA or from the cloning vector carrying the desired sequence, in the presence of digoxigenin

labelled dNTPs. The labelled probe is denatured for 5min at 80°C in the presence of hybridization buffer (50% Formamide, 10% Dextran Sulphate and 2X SSC) and Salomon sperm DNA (Invitrogen). The denatured and labelled probe is hybridized with the denatured chromosomes at 37°C in humid chamber. After an overnight incubation the unhybridized probe is washed away by 3x 5 min washings in 2x SSC. The chromosomes are blocked for 30 min in blocking solution (4XSSC, 0.1% Triton X 100, 5% BSA) at 37°C, followed by incubation with FITC coupled anti-DIG-antibody for 1h at 37° in the humid chambers. Unbound antibody is removed by 3x 5min washings with 4x SSC+0.1% Triton-X100. The chromosomes were counterstained with DAPI, mounted and analysed same as for the immunostainings.

Table 2.6 List of antibodies used for the biochemical and immunocytological analyses.

Antibody	Dilution for western blot analysis	Manufacturer	Dilution for cytology
rabbit polyclonal DNMT2	1:500	F.Lyko	ND
mouse monoclonal α-Tubuline	1:50000	Sigma	ND
rabbit polyclonal HP1	1:4000	Abcam	1:800
rabbit polyclonal H2B	1:10000	Upstate	ND
rabbit polyclonal H3K9 me1	ND	Upstate	1:100
rabbit polyclonal H3K9 me2	ND	Upstate	1:100
rabbit polyclonal H3K9 me3	ND	Upstate	1:50
rabbit polyclonal H3K27 me1	ND	Upstate	1:100
rabbit polyclonal H3K27 me2	1:500	Upstate	1:100
rabbit polyclonal H3K27 me3	1:1000	Upstate	1:100
rabbit polyclonal H4K20 me1	1:1000	Abcam	1:50
rabbit polyclonal H4K20 me2	1:1000	Abcam	1:50
rabbit polyclonal H4K20 me3	1:1000	Upstate	1:100
rabbit polyclonal SU(VAR)3-9	ND	Schotta, G	1:50
Goat Anti-mouse-HRPO	1:2000	Abcam	ND
Goat Anti-rabbit- HRPO	1:4000	Abcam	ND
Goat Anti-rabbit-Alexa Fluor® 488	ND	Invitrogen	1:100
Sheep Anti DIG FITC	ND		1:50

3. Results

3.1 Alternative gene silencing pathways in *Drosophila*

3.1.1 Tagging the silenced regions of *Drosophila* genome

For genetic dissection of epigenetic processes different experimental systems have been used in *Drosophila*, e.g. phenotypic effects caused by position-effect variegation [PEV] (Reuter and Wolf, 1981; Spierer and Spierer, 1992; Schotta *et al*., 2003), transgene silencing (Wallrath and Elgin, 1995; Cryderman *et al*.,., 1998; Cryderman *et al*.,., 1999) or Polycomb-dependent silencing (Pirrotta and Rastelli, 1994; Dellino *et al*., 2004). Genetic dissection of position-effect variegation in *Drosophila* pioneered identification and molecular analysis of functions controlling heterochromatin formation (Schotta *et al*., 2003). Position effect variegation (PEV) reflects gene silencing caused by variable spreading of the heterochromatic chromatin states into the neighbouring euchromatic regions. Similarly when a P-element the carrying white transgene is inserted within or near to the heterochromatic or silenced genome region it shows the variegated eye phenotype. Hence each variegated insertion actually tags the genomic regions undergoing gene silencing. Using this strategy overall 67 *white* variegating lines were identified from the Drosdel Genome Sequencing Project or other stock collection (Crydermann *et al*., 1999). Out of 67 lines 43 were mapped precisely on the genome using inverse PCR or immuno FISH. 6 inserts were found in the telomere, 3 in pericentric heterochromatin, 6 on the fourth chromosome while 28 are found in euchromatic regions (Appendix 1).

3.1.2 Genetic dissection of silencing processes in *Drosophila* genome

Genetic dissection of position-effect variegation in *Drosophila*, using the *In(1)w*^{m4} rearrangement in which the *white* gene is juxtaposed to pericentric heterochromatin through an inversion, pioneered identification and molecular analysis of functions controlling heterochromatin formation (Schotta *et al*., 2003). The inactivation of *white* gene by heterochromatisation is clonally induced which results in a variegated phenotype. With this genetic model suppressors [*Su(var)s*] and enhancers [*E(var)s*] of PEV were identified

(Wustmann *et al*., 1989). Locke and coworkers (1988) postulated *Su(var)s* as genes which participate directly or indirectly in the establishment of heterochromatin. In contrast *E(var)s* should promote the formation of euchromatin. Heterochromatic gene silencing in PEV depends primarily on SU(VAR)3-9 dependent H3-K9 di-methylation (Schotta *et al*., 2002). The mark is recognized by HP1 which restricts SU(VAR)3-9, so far one of the strongest identified suppressor of PEV, and SU(VAR)3-7 through protein-protein interaction to chromatin marked by H3K9me2 (Ebert *et al*., 2006). HP1 also recruits the SUV4-20 HMTase catalysing H4K20me3 in pericentric heterochromatin (Schotta *et al*., 2004). E(Z) also contributes to heterochromatin formation by catalysing H3K27 mono-, di- and tri-methylation (Ebert *et al*., 2004). Mutations of *Su(var)3-9*, *Su(var)2-5* (HP1) and *Enhancer of zeste* are all suppressors of *white* variegation in $In(1)w^{m4}$. Mutations in factors controlling active histone modification marks revealed the dynamic balance between euchromatin and heterochromatin (Ebert *et al*., 2004). With genome tagging approach it is possible to investigate if all the gene silencing pathways are identical or different at different genomic locations. Genetic screens were performed in which the strong null alleles of the known *Su(var)* genes were tested for their effect on each of the variegating insert lines. Mutations for the genes encoding HMTases like *Su(var)3-9, Enhancer of Zeste*, and *SetDB1* and the heterochromatin associated proteins like *HP1*, *Su(var)3-7*, and *Jil1 kinase* were used. To further extend our knowledge about gene silencing processes we generated mutants for the only DNA methyltransferase encoding gene in *Drosophila*, DNMT2 and also for the another HMTase SUV4-20 which has been shown to be involved in the heterochromatic gene silencing in *Drosophila* (Schotta *et al*., 2004).

3.1.2.1 DNA methylation is an early process in *Drosophila* and is controlled by DNMT2

Only a single DNA methyltransferase encoding gene has been found in *Drosophila* after completion of the genome sequencing project. This gene codes for the DNMT2 class of the DNA methyltransferase. To understand the role of DNA methyltransferase in *Drosophila* it was necessary to generate a null mutant for this gene. A P element, *pGE15695* inserted 108 bp upstream of the *Dnmt2* gene was obtained from the Genexel P element collection (Jurkowski *et al*., 2008). The P element was then mobilized using the *Δ2-3* P element as an internal transposase source (Fig 3.2, Materials and Methods). Approximately 1100 independent chromosomes were analyzed and 4 exceptional flies with chromosomal lesion disrupting the *Dnmt2* gene were identified. All of these lines showed an insertion of variable length from the 5' end of the original P element into the coding region of *Dnmt2* (Appendix

3). These four alleles were named as *Dnmt2*^{147}, *Dnmt2*^{149}, *Dnmt2*^{162} *and Dnmt2*^{105}. Among these five alleles *Dnmt2*^{105} showed a strong reduction of the transcript while *Dnmt2*^{149} showed complete loss of the transcript. Other alleles showed only weak differences in the *Dnmt2* transcription (Fig 3.1).

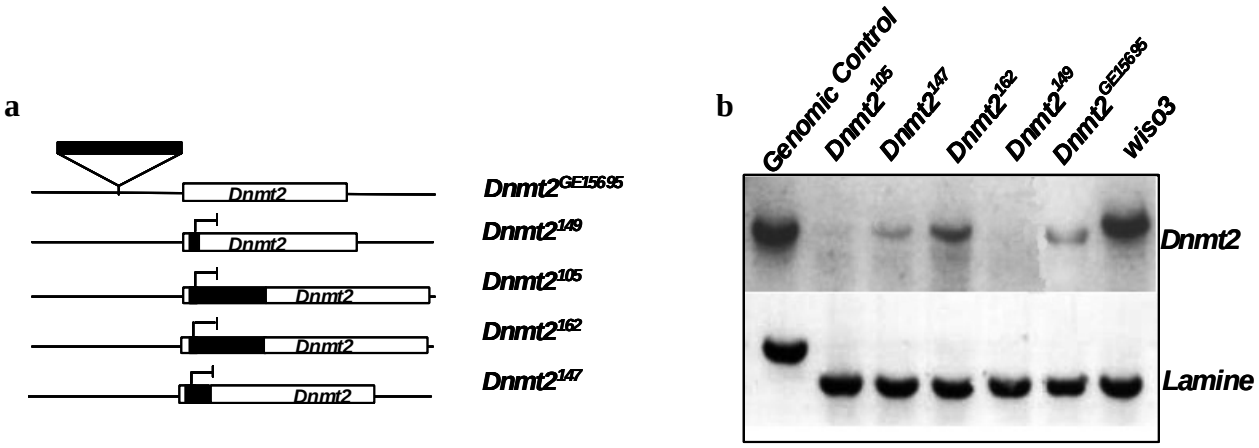

Figure 3.1 Characterization of new *Dnmt2* mutant alleles. (**a**) P element *GE15695*, inserted 128bp upstream of *Dnmt2* ORF was remobilized to generate four new *Dnmt2* alleles. All the four alleles possess an insertion of variable length of 5'end of P element at the same point within *Dnmt2* ORF. (**b**) Transcript analysis using *Dnmt2* specific primers showed complete loss of transcripts in *Dnmt2*^{149} allele, while strong reduction of Dnmt2 transcripts was detected in *Dnmt2*^{105}. Other alleles were not significantly affected at transcript levels. *Lamin* specific primers were used as loading control. Open box represents the *Dnmt2* ORF while black bars represent the P element sequences.

No DNMT2 protein was detectable in *Dnmt2*^{149} while only trace amounts were detected in *Dnmt2*^{105} mutant allele as revealed by western blot analysis using α-DNMT2 peptide antibody (Fig 3.2a, kind gift from F. Lyko). For further analysis the *Dnmt2* null allele *Dnmt2*^{149} was used. *Dnmt2*^{149} showed an insertion of 59 bp from the 5' end of the P element with 6 bp insertion site duplication within *Dnmt2* ORF (Fig 3.2b)

All *Dnmt2* mutant alleles were homozygous viable and fertile, indicating that unlike canonical DNA methyltransferases in vertebrates; DNMT2 does not play an essential role in *Drosophila* development.

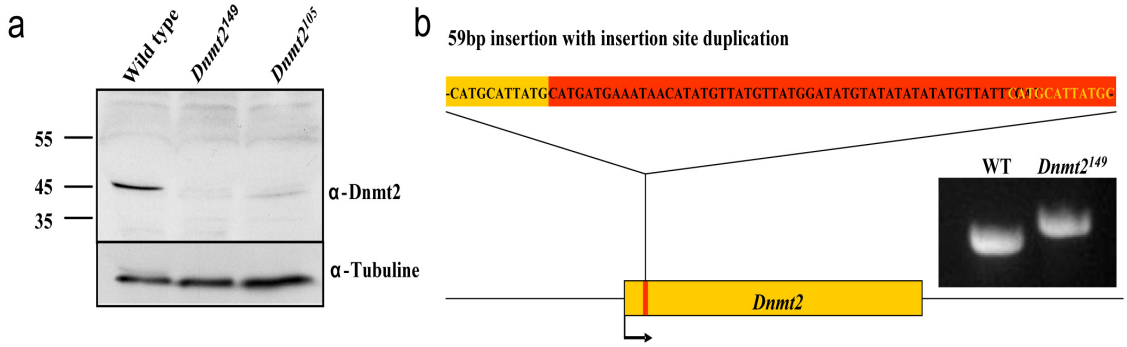

Figure 3.2 Characterization of new *Dnmt2* mutant alleles. (**a**) Western blot analysis using anti DNMT2 antibody revealed complete loss of DNMT2 in *Dnmt2*^{149} allele, while only trace amounts could be detected in *Dnmt2*^{105}. Tubuline was used as a loading control. (**b**) An insertion of 59bp from the P element into the *Dnmt2* ORF leads to an early stop and also a frame shift. 59bp insertion could be detected using *Dnmt2* specific primers.

DNMT2 has been suggested to possess dual activity as DNA and RNA methyltransferase (Jeltsch *et al*., 2006b). tRNA methylation activity has been recently demonstrated (Goll *et al*., 2006). As reported earlier the *Dnmt2* null mutant RNA showed a complete loss of RNA methylation (Jurkowski *et al*., 2008). *In vivo* DNA methyltransferase activity of DNMT2 was studied by analysing global DNA methylation levels in *Dnmt2* null mutant embryos as compared to the wild type embryos using DNA methylation dependent *Sge*I restriction enzyme, cleaving DNA at methylated CpNpNpG sites (Fermentas AG, non-commercial product). Only DNA isolated from 2-3h wild type embryos but not from 15-16h embryos or adult flies is significantly digested indicating that significant DNA methylation in *Drosophila* is only detected in early embryonic development (Fig.3.3).

*Sge*I does not digest genomic DNA isolated from *Dnmt2* null embryos. The results indicate that significant DNMT2 dependent DNA methylation in *Drosophila* is detected only during early embryogenesis but not in later developmental stages. Although these data affirm a dual activity of DNMT2 as an RNA and DNA methyltransferase in *Drosophila* it does not reveal the functional significance of these two activities. DNA methylation has also been involved in gene silencing processes in higher organisms (Bird, 2002).To analyze whether DNMT2 mediated DNA methylation has also some functional relevance in gene silencing processes in *Drosophila*, *Dnmt2*^{149} allele was tested for its effect on the variegating P element inserts.

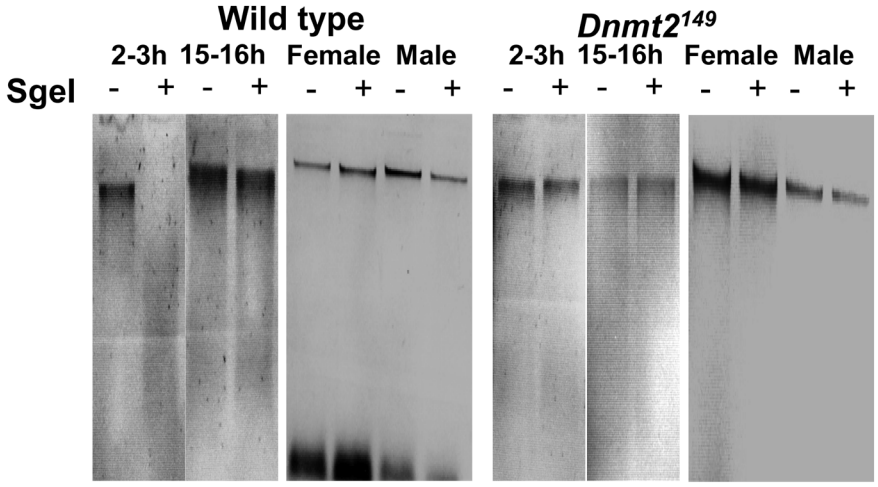

Figure 3.3 Analysis of global DNA methylation with the 5 methylcytosine dependent restriction enzyme *Sge*I. DNA was isolated from wild type and *Dnmt2* null 2-3h and 15-16h old embryos and adult females and males. DNA not treated with *Sge*I is loaded as control (- lane). Note that DNA isolated from *Dnmt2* null embryos is not digested by *Sge*I indicating absence of DNA methylation, while early embryonic DNA from wild type embryos is readily digested.

3.1.3 Alternative gene silencing pathways coexist in *Drosophila* genome

Systematic genetic tests for modifying effects of PEV *Su(var)* mutations and a *Dnmt2* null allele on the variegating transgenes revealed at least five coexisting alternative gene silencing processes within the *Drosophila* genome (Fig 3.4). In agreement with the classical PEV experiments *Su(var)3-9, Su(var)2-5* (HP1), *Suv4-20, Su(var)3-1* (Jil1 Kinase) *and E(Z)* mutations showed strong dominant suppressor effect on p(1-67), an insert within X chromosomal pericentric heterochromatin. However both *Dnmt2* and *SetDB1* mutations did not show dominant or recessive effect. This reflects a silencing pathway which is initiated by SU(VAR)3-9 dependent H3K9 dimethylation, a mark recognized by HP1 which anchors SU(VAR)3-9 and the H4K20me3 catalyzing SUV4-20 to the heterochromatic sequences (Schotta *et al*., 2002; 2004). Spreading of the heterochromatic H3K9me2 mark into heterochromatin is blocked by the Jil1 kinase, *Su(var)3-1* mutations (Ebert *et al.*, 2004). In addition E(Z) dependent H3K27 methylation contributes to heterochromatin formation (Ebert *et al.*, 2004). In the 4th chromosome of *Drosophila* gene silencing depends on the SetDB1 HMTase and HP1 (Seum *et al.*, 2007). Variegated inserts on the 4th chromosome were consequently suppressed only by *Su(var)2-5* (HP1), *SetDB1* and *Su(var)3-1* mutations whereas all other tested mutations show no effects. This reflects another heterochromatin

gene silencing pathway specific for the 4th chromosomal heterochromatin which is initiated by SETDB1 mediated H3K9me2 and then recognized by HP1, while the Jil1 Kinase appears to be required for spreading of heterochromatin. Although SU(VAR)3-9 binds on 4th chromosome through HP1 interaction, it is not required for heterochromatin formation in the 4th chromosome (Schotta *et al*., 2002). Telomeric heterochromatin has been shown to have completely different organization as compared to the pericentric and 4th chromosomal heterochromatin (Crydermann *et al*., 1999, Mason *et al*., 2001). Four independent telomeric insertions were analysed. An insert within *HeT-A* retrotransposon on 3R telomere, *pP{RS5}5-HA-2057* and 2L TAS repeat, *pP{hsp26-pt-T}39C5* were not suppressed by any of the tested mutations (not shown). Two independent inserts on the X telomere *pP{RS5}5-HA-1902* and *pP{RS5}5-HA-1994* were mapped within *HeT-A* 5'UTR and 13bp upstream of *HeT-A* 5'UTR respectively. Only *SetDB1* and *Dnmt2* mutations suppressed these inserts whereas no effect was shown by other mutations. This reflects yet another unknown silencing pathway which requires putative DNA methylase activity of DNMT2 and SETDB1 mediated H3K9me2. Furthermore inserts in the euchromatic retrotransposon sequences were tested. Remarkably the inserts within *Invader4* retrotransposon were strongly suppressed by *Dnmt2, Suv4-20* and *Su(var)3-1* mutations while unaffected by the other tested mutations. This depicts another silencing pathway in which DNMT2 and SUV4-20 mediated H4K20me3 interact to maintain a silent state of the retrotransposon sequences.

Like in plants, presence of transgenic repeats can also induce heterochromatin formation in *Drosophila* (Dorer and Henikoff, 1995). Molecular factors involved in this repeat induced heterochromatin formation are unknown. One of the repeat induced variegated transgenic line, carrying three *white* repeats (Dorer and Henikoff, 1997) was suppressed by *Su(var)2-5, SetDB1, Su(var)3-1* and *Dnmt2* mutations, while it remained unaffected by *Su(var)3-9, Suv4-20* and *E(Z)* mutations. This represents another gene silencing pathway where DNMT2 is involved and is maintained by SETDB1 mediated H3K9me2 and HP1 protein binding. JIL1-Kinase is again required for spreading of heterochromatin in to the neighbouring genes.

Thus the genome tagging approach could be successfully used for a dissection of several alternative gene silencing pathways. These studies also showed for the first time that *Dnmt2* in *Drosophila* is dispensable for development but, like other DNA methyltransferases in vertebrates, has an important role in gene silencing mechanisms. In the present study the *Dnmt2* mediated gene silencing pathways in *Drosophila* are studied in detail.

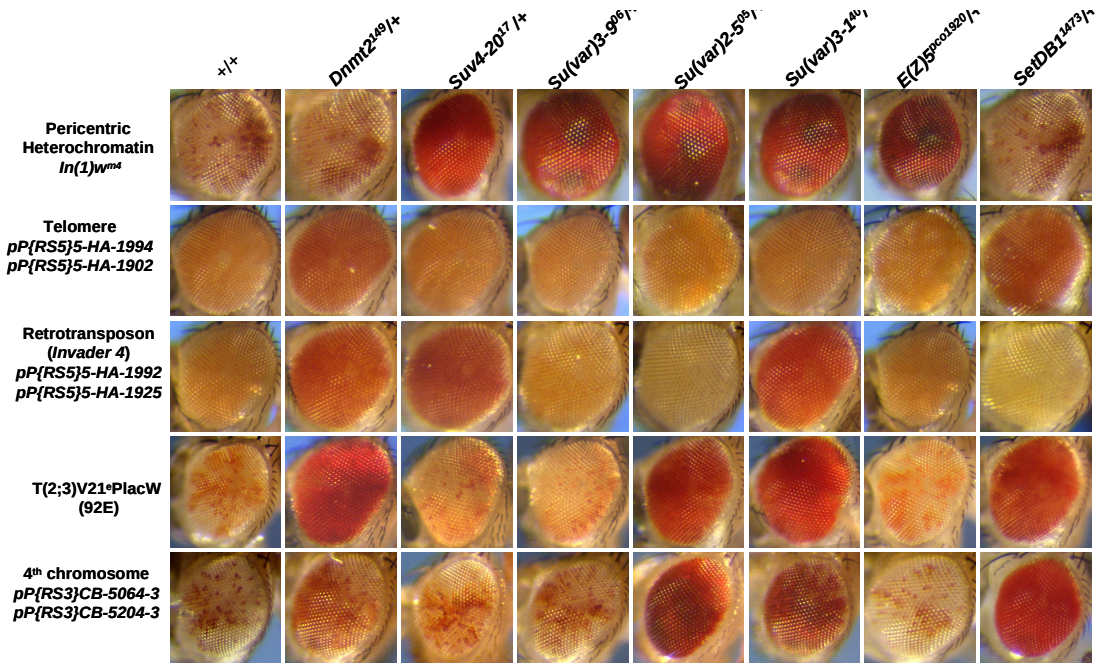

Figure 3.4 Alternative gene silencing pathways in *D. melanogaster*. Representative variegating *white* P elements inserted into pericentric heterochromatin, telomeres, retrotransposon, in repeats and in the fourth chromosome are shown (control panel). Homozygous or heterozygous *Dnmt2*^{149}, *Suv4-20*^{17}, *Su(var)3-9*^{06}, *Su(var)2-5*^{05}, *Su(var)3-1*^{40}, *E(z)5*^{pco1920} and *SetDB*^{1473} mutant females were mated with males carrying the variegating P element insertions. Transheterozygous male offspring were analyzed for a modifier effect of any of the mutations studied. *Dnmt2* mutations show a dominant suppressor effect on *white* gene silencing in P elements inserted into *HeT-A* telomeric sequences, into retrotransposons and in repeat dependent *white* gene silencing.

3.2 DNA methyltransferase activity of DNMT2 is required for retrotransposon silencing

3.2.1 *Dnmt2* is responsible for retrotransposon silencing in the somatic tissues

Genetic approach could identify a possible role of the putative DNA methyltransferase DNMT2 in retrotransposon silencing. To test if this effect of a *Dnmt2* mutation is restricted only to a certain specific retrotransposon at specific location or is more general to different retrotransposons, levels of different LTR retrotransposons expression were analysed in the RNA isolated from adult heads and embryos of the wild type and the *Dnmt2*^{149} animals. As revealed by both semi quantitative and quantitative PCR all the tested retrotransposons showed at least 2 fold up regulation in the *Dnmt2*^{149} mutants in the embryonic as well as adult

somatic tissues (Fig 3.5). No difference in the expression of retrotransposon in the germ line tissue is detected (not shown).

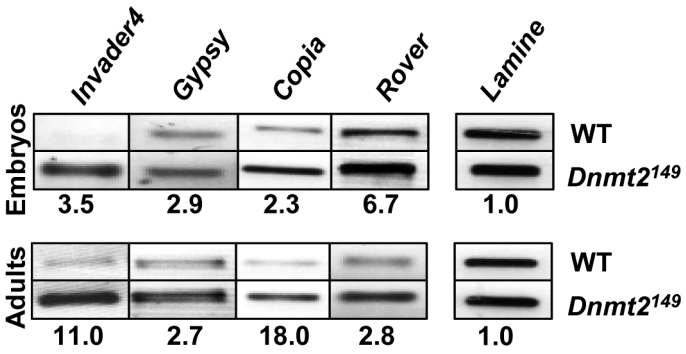

Figure 3.5 *Dnmt2* Controls retrotransposon expression in the somatic tissues. Semi quantitative and quantitative RT-PCR analysis shows in a *Dnmt2* null genotype significant overexpression of *Invader4*, *Copia*, *Rover* and *Gypsy* retrotransposons in embryos and adult heads. *Lamine* was used as a loading control.

This shows that DNMT2 controls the levels of retrotransposons in *Drosophila* only in somatic tissue. Activity of retrotransposons has been shown to be controlled by RNAi components like the PIWI family of proteins in *Drosophila* germ line tissues (Klenov *et al.*, 2007). To test if RNAi components are also involved in retrotransposon silencing in the soma, different RNAi mutations were crossed with the different variegating inserts in the *Invader4* retrotransposon or at other genomic locations. None of the RNAi mutations affected *white* variegation in the tested inserts (Cristina Onorati, personal communication). This finding suggested that RNAi components are only needed for retrotransposon silencing in the germ line and that this pathway does not play any role in the somatic gene silencing processes. To affirm this differential control of retrotransposon silencing by a DNMT2 dependent or an RNAi controlled pathway in somatic and in the germ line tissue, respectively, a *LacZ* transgene expressed under control of *copia LTR* sequences was used (Klenov *et al*., 2007). *Dnmt2* null larval somatic tissues (salivary glands, imaginal discs, brain) showed a strong *LacZ* reactivation as compared to the wild type. In ovaries only the somatic follicle cells showed *LacZ* activation but *LacZ* expression was unaffected in the germ line nurse cells (Fig 3.6). The RNAi mutants on the contrary showed an activation of *LacZ* only in the germ line nurse cells, all the other tested somatic tissue were unaffected (Klenov *et al*., 2007).

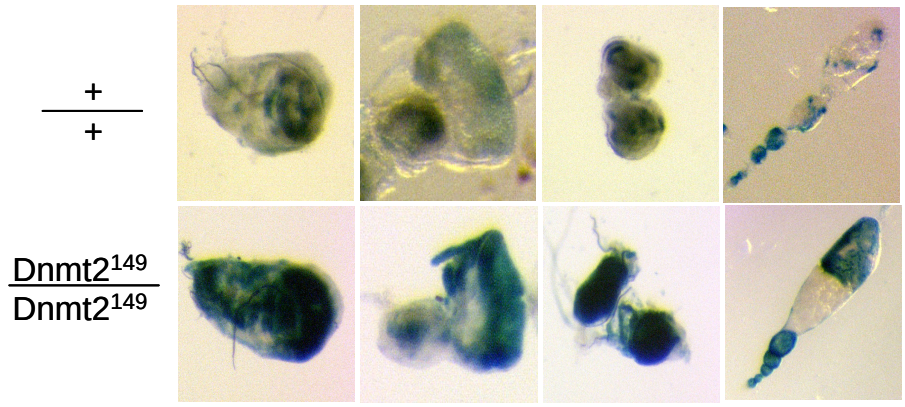

Figure 3.6: ***Dnmt2* dependent somatic tissue specific control of retrotransposon activity**. β-galactosidase staining in the somatic (wing, eye and legs imaginal discs) and germ line (ovaries) tissue in the wild type (upper penal) and *Dnmt2* null mutant (lower penal) carrying *Copia-LTR:LacZ* transgene. Strong activation of *LacZ* activity can be observed in *Dnmt2* null somatic tissue. In ovaries only the somatic follicle cells shows *LacZ* activation, while it remains unaffected in the germ line nurse cells.

3.2.2 *Dnmt2* mediated silencing defects are due to its DNA methyltransferase activity

DNMT2 has been postulated to have dual specificity to function as a DNA or RNA methyltransferase (Jeltsch *et al*., 2006b). The silencing defects induced by loss of *Dnmt2* could be an indirect effect of its RNA methyltransferase activity or the direct manifestation of its DNA methylation activity at the target sequences. To test if these silencing defects are because of the DNA methylation, affords were made to identify the target sequences for DNMT2 mediated DNA methylation. For this precise mapping of the variegated P elements inserts into the *Invader4* retrotransposons. Altogether 19 such lines represent insertion into *Invader4* and with an exception of one single insert, all of them were dominantly suppressed by loss of *Dnmt2*. All these lines were mapped to the 5'LTR of the *Invader4* retrotransposon, which suggest that LTR sequences are the potential target sites for DNMT2 mediated DNA methylation (Fig 3.7). LTRs are known to act as regulatory sequences for several retrotransposons (Codani-Simonart *et al*., 1993).

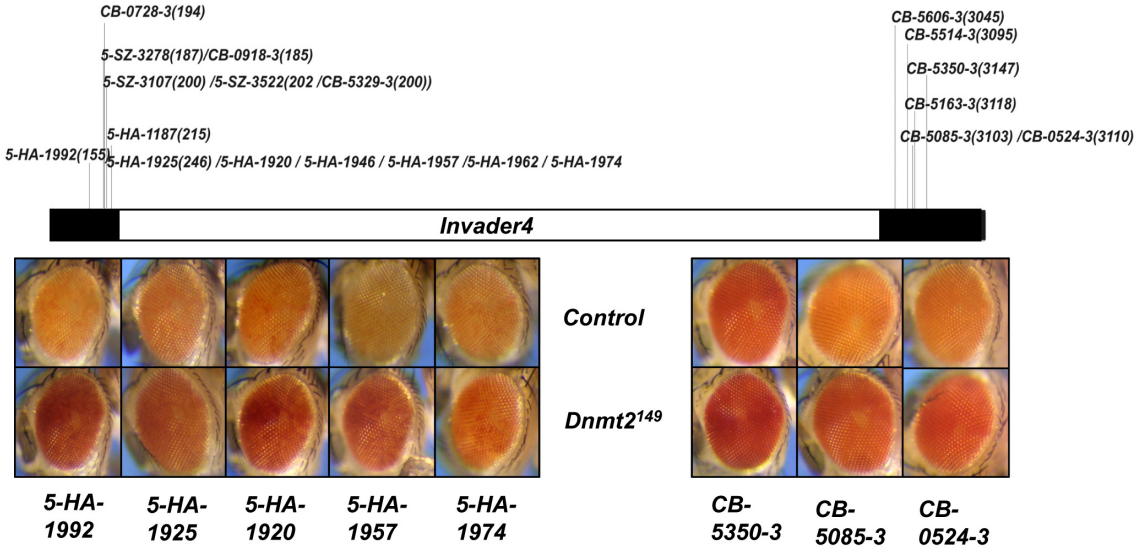

Figure 3.7 *Invader4* LTR is the target for DNMT2 dependent silencing. Full length *Invader4* flanked by 3' and 5' LTR (filled boxes) have been shown. All the variegating *Invader4* inserts, suppressed by loss of *Dnmt2*, were mapped to the 5' or 3' LTR of the *Invader4*. Exact location of each insert within *Invader4* LTR is shown in brackets.

Bisulphite sequencing of the *Invader4* LTR sequence revealed DNA methylation at several cytosine residues in the DNA isolated from the wild type early embryos (up to 3 hrs AEL). No DNA methylation was observed in the DNA isolated from *Dnmt2* mutant embryos of the same age (Fig 3.8).

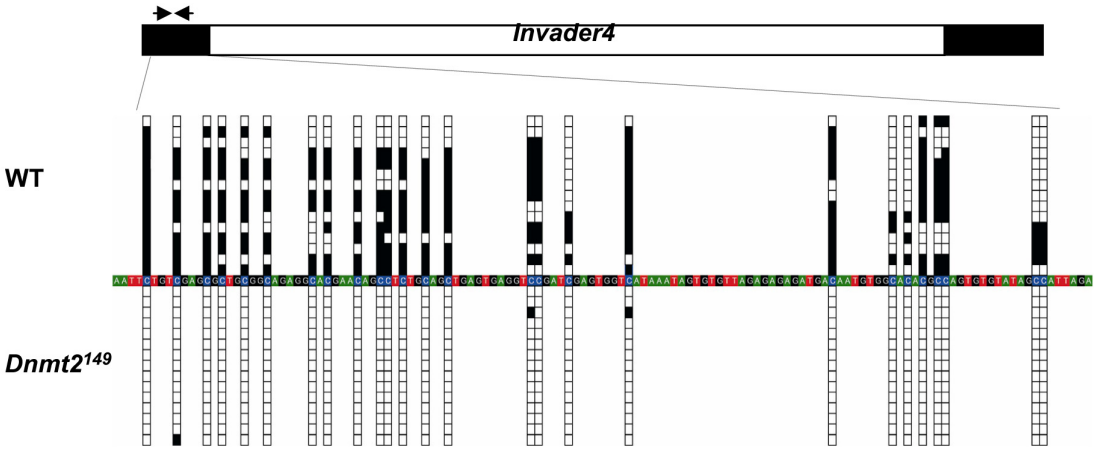

Figure 3.8 DNA methylation analysis by bisulphite sequencing. Bisulphite sequence analysis of *Invader4* LTR sequence (filled boxes) from wild type and *Dnmt2*^{149} 2h old embryos. 15 independent clones were sequenced from each genotype. Filled bars represent methylated cytosine residues while empty bars denote unmethylated cytosine residue. None of the clones isolated from *Dnmt2*^{149} mutant embryos showed significant DNA methylation at any of the cytosine residue. Position of primers used is indicated by arrowheads.

Next *Sge*I enzyme, which only restricts DNA containing 5-methyl cytosine was used to analyse DNA methylation. The 5´LTR sequence of *Invader4* and *Doc* retrotransposons contain several recognition sites of *Sge*I and DNA digestion was evaluated by PCR analysis. Significant digestion of the 5´LTR sequences is only found in DNA from wild type early embryos but not from older embryos and *Dnmt2* null early or late embryos (Fig. 3.9). As a negative control for the *Sge*I digestion *rp49* sequences were analysed.

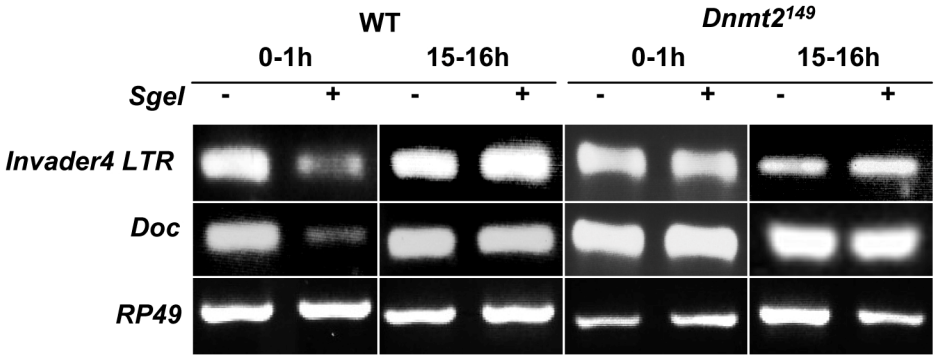

Figure 3.9 DNA methylation analysis using 5 methyl cytosine dependent restriction enzyme *Sge*I. DNA isolated from 0-1h and 15-16h old wild type and *Dnmt2*^{149} embryos was digested with DNA methylation dependent *Sge*I enzyme followed by PCR amplification using *Invader4*, *Doc* and *rp49* sequences. No digestion of DNA is found in *Dnmt2* null embryos. DNA without enzyme was used as control (- lane).

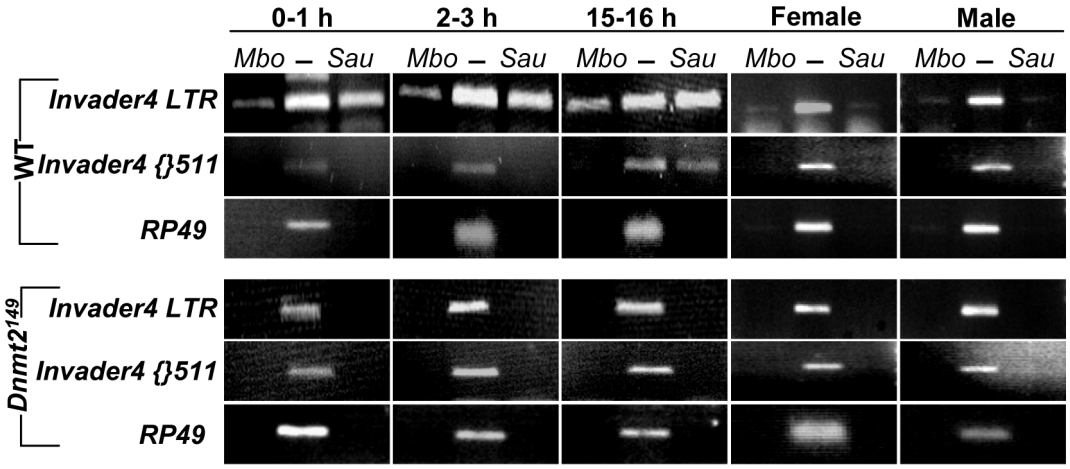

Figure 3.10 DNA methylation analysis using 5 methyl cytosine sensitive restriction enzyme Sau3AI. Methylation insensitive isochizomer *Mbo*I is used as a control. Undigested DNA is used as internal control. DNA isolated from 2-3 and 15-16 h old embryos and adult flies is digested to completion with *Sau3*AI, followed by PCR amplification of *Invader4 LTR* sequences from all *Inavder4* genomic copies or the individual *Invader4{}511* element. *RP49* is used as control for complete digestion.

Finally DNA methylation sensitive restriction enzyme *Sau*3AI was used to monitor DNA methylation within 5´LTR sequence of *Invader4*, which contains a single *Sau3*AI recognition site. In wild type embryonic DNA *Sau3*AI digestion of *Invader4* 5´LTR´s is significantly

impaired whereas these sequences are completely digested in DNA isolated from *Dnmt2* null embryos (Fig. 3.10). As a control *Mbo*I the methylation independent isochizomer of *Sau3*AI was used. Weak amplification of *Invader4* 5´LTR´s in wild type after *Mbo*I digestion is due to defective subtelomeric *Invader4* elements with a mutated *Sau3*AI restriction site. These elements are lost as shown later due to genomic instability in *Dnmt2* null flies. The cytosine in the *Sau3*AI restriction site of *Invader4* elements only shows significantly methylation during embryonic development but not in adult flies (Fig. 3.10). Monitoring cytosine methylation at the *Sau3*AI restriction site at individual *Invader4* elements revealed significant differences in the developmental specific methylation profile between different elements as shown for *Invader4{}511* located at 42B (Flybase 2008). *Sau3*AI digests the *rp49* fragment in wild type and *Dnmt2* null mutant embryos showing that the cytosine in this restriction site is not methylated in wild type embryos (Fig. 3.10).

3.2.3.DNMT2 mediated DNA methylation is an early zygotic activity.

Bisulphite sequencing and the methylation specific restriction digestion of DNA indicates that DNA methylation is an early process in *Drosophila*. This could be either because of the early zygotic expression of the DNMT2 protein or due to the maternal load of the DNMT2 protein in the egg. To differentiate if DNMT2 mediated gene silencing defects are due to maternally or zygotically expressed protein genetic methods were used. Reciprocal crosses between *Dnmt2* null and *Invader4* variegating insert *p(RS5)5-HA-1992* animals were performed. The progenies were evaluated for the suppressor effect on *white* variegation of the P element. The progenies produced from the $Dnmt2^{149}$ mothers were identical to the progenies produced from the $Dnmt2^{149}$ fathers in their suppressor effect (Fig 3.11a) which excludes any maternal contribution of DNMT2 protein. A robust expression of Dnmt2 has recently been demonstrated (Schaffer *et al*., 2008). Together this data suggest that DNMT2 induced silencing occurs due to early zygotically expressed DNMT2 protein, and is independent of any maternal contribution. To analyse if this early expression can also lead to early DNA methylation of the target sequences we collected the precisely staged embryos produced by crossing $Dnmt2^{149}$ mothers with the wild type males. The DNA isolated from these embryos was digested with the methylation sensitive enzyme *Sau*3AI, followed by PCR amplification of the target *Invader4* LTR sequence (Fig3.11b).

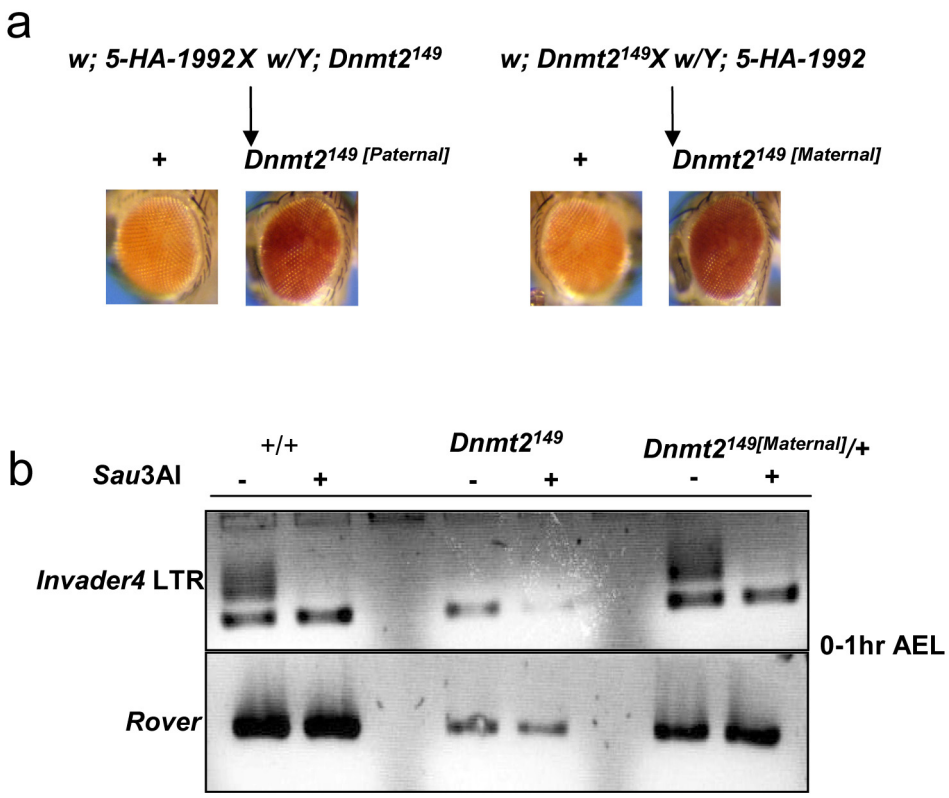

Figure 3.11 Retrotransposon silencing is under zygotic control of *Dnmt2*. Variegating *Invader4* insert *5-HA-1992* flies were mated with *Dnmt2* in a reciprocal cross. Progenies resulting from either crosses showed dominant suppressor effect. DNA isolated from *Dnmt2/+* early embryos laid by *Dnmt2* null mothers mated with *5-HA-1992* fathers was digested with DNA methylation sensitive enzyme *Sau3*AI followed by PCR amplification of the Invader4 LTR sequences. Wild type and Dnmt2 null embryonic DNA was used a control. Rover sequences lacking *Sau3*AI recognition site was used as negative control. Note the amplification of the *Invader4* LTR sequences in early *Dnmt2/+* embryos because of DNA methylation

Like wild type DNA, the DNA from the heterozygote embryos showed a strong inhibition to the *Sau3*AI digestion, which suggests a restoration of the DNA methylation which can be explained by the activity of the early expressed paternal *Dnmt2* allele.

3.2.4 *Dnmt2* is associated with chromatin during early embryonic development

A large fraction of DNMT2 has been shown to be localised within cytoplasm in *Drosophila* cell lines (Goll *et al*., 2006), however a lower but significant amounts of this protein are also observed in the nuclear fraction isolated from the *Drosophila* embryos (Schaffer *et al*., 2008). Direct binding of DNMT2 to its target sequences was demonstrated by chromatin immunoprecipitation using anti DNMT2 antibody (kindly provided by Frank Lyko) on the

chromatin isolated from precisely staged 0-2 and 2-4 h embryos. For identifying the binding of DNMT2 to a single *Invader4* retrotransposon a stabilised variegated P element insertion line, in which the element was inserted in to the *Invader4 LTR* sequence on the Y chromosome, was used in wild type and in the *Dnmt2* null mutant background. Significant enrichment of DNMT2 at the retrotransposon sequences and at the P element was observed in the wild type 0-3 h embryos while only background levels of DNMT2 protein could be detected in the *Dnmt2* null embryos of the same age. A strong reduction of the DNMT2 levels was observed in the wild type 2-4 h embryos, which was almost equal to the background levels in *Dnmt2* null mutant embryos (Fig 3.12**)**.

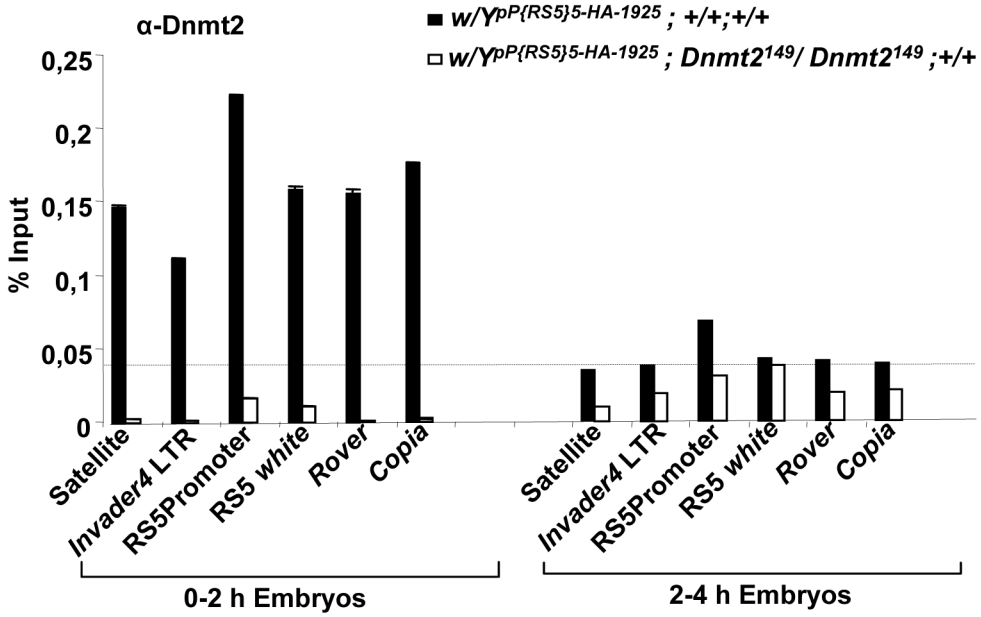

Figure 3.12 Chromatin association of DNMT2 protein. ChIP analysis of the wild type (black bars) and *Dnmt2* null (white bars) 0-2 h and 2-4 h embryos carrying *pP(RS5)5-HA-1925* transgene, using DNMT2 specific antibodies. *Satellite*, *Invader4*, *Rover*, *Copia* and P element linked *white* gene and its promoter sequences were analysed. Significant enrichment of DNMT2 could be found at the analysed sequences only in 0-2 h embryos.

This data suggests nuclear localisation and chromatin association of DNMT2 during early stages of *Drosophila* development; however nuclear localisation of DNMT2 appears to be strongly reduced during progressive developmental stages in *Drosophila* embryos.

3.3 Histone H4K20me3 modification catalysed by SUV4-20 is a functional component of the DNMT2 mediated gene silencing pathway

3.3.1 Loss of DNMT2 causes reduction of H4K20me3 histone modification mark

Genetic analysis indicated the role of DNA methylation in the regulation of retrotransposon and transgene repeat silencing in *Drosophila* (Phalke *et al*., submitted). Molecular analyses by RT-PCR showed that loss of DNA methylation causes an upregulation of all tested LTR retrotransposons in embryos and adult tissues (Phalke *et al*., submitted). Impairment of retrotransposon silencing becomes visible in adult flies by suppression of *white* variegation, however as shown by *Sge*I analysis the global DNA methylation levels drop down severely after the cellular blastoderm stage of development (Phalke *et al*., submitted).

This suggests maintenance of DNA methylation induced retrotransposon silencing in consecutive stages of the development by an independent molecular mechanism. Epigenetic control of chromatin stages by covalent histone modifications could be a potential candidate for such a molecular process. Polytene chromosomes of *Drosophila* third instar larvae allow detailed cytological analysis of covalent histone modifications involved in the control of gene expression pattern during development (Ebert *et al*., 2006). Since DNA methylation is involved in gene silencing, distribution of all the covalent histone modifications marks indexing the inactive chromatin were compared by cytological analysis of salivary glands polytene chromosomes and by western blot analysis of null mutant $Dnmt2^{149}$ and wild type third instar larvae.

In *Drosophila* inactive chromatin is defined by H3K9 mono-, di- and trimethylation, H3K27 mono-, di- and trimethylation and H4K20 trimethylation (Schotta *et al*., 2002, Schotta *et al*., 2004, Ebert *et al*., 2004). All of these marks are accumulated but not restricted to pericentric heterochromatin (Ebert *et al*., 2006). Mono-, di- and trimethyl H3K9 is also found at a number of sites along the chromosomes and at telomeres. Trimethyl H3K9, is detected mainly at a central region within chromocenter (Fig 3.13; Ebert *et al*., 2004). Several marks of inactive chromatin are found in heterochromatin and at euchromatic bands. Pericentric heterochromatin and almost all bands show staining for mono- and dimethyl H3K27 whereas trimethyl H3K27 is found only in a subset of about 100 bands and at the chromocenter core (Fig 3.14; Ebert *et al*., 2006). Also mono-, di- and trimethyl H4K20 is found in chromocenter

heterochromatin and in a high number of bands along euchromatic arms (Fig 3.16; Ebert *et al*., 2006). In a *Dnmt2* null background significant reduction of heterochromatic H4K20me3 mark at chromocenter and a high number of euchromatic bands is detected on the salivary glands polytene chromosomes (Fig 3.16). All other covalent histone modification marks (including H3K9 mono- di- and trimethylation, H3K27 mono- di- and trimethylation and H4K20 mono- and dimethylation) and binding of heterochromatic associated proteins SU(VAR)3-9 and HP1 found to be unaffected in homozygous *Dnmt2*^{149} animals (Fig 3.13, 3.14, 3.15, 3.16).

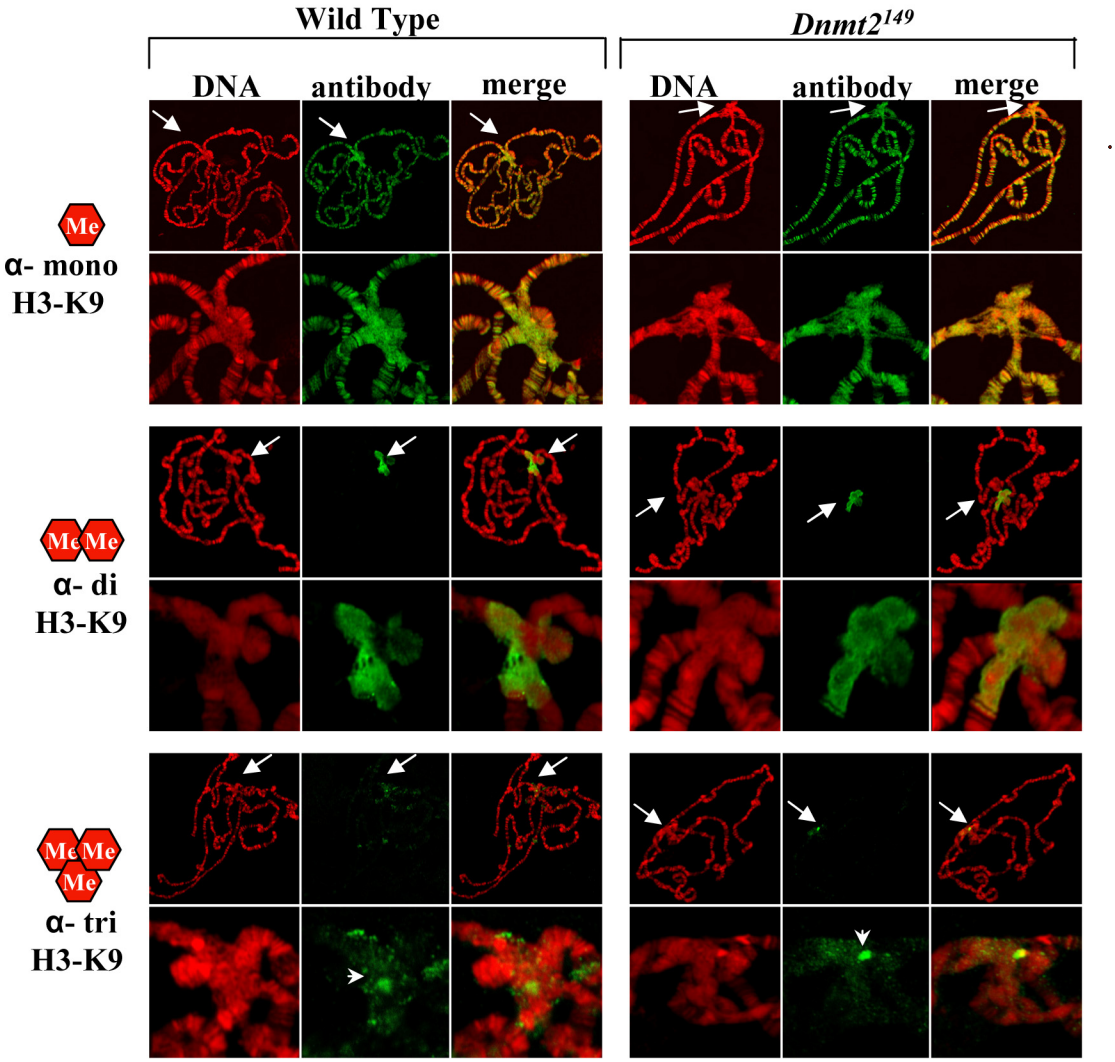

Figure 3.13 Detection of H3K9 methylation in polytene chromosomes of wild type and *Dnmt2* null larvae. All the three methylation states (mono, di and tri methylation) were analysed (green). Chromosomes were counterstained with DAPI (red). Arrow indicates the chromocenter, arrow heads indicate localised distribution of H3K9me3 mark within chromocenter heterochromatin. For each methylation state the lower penal shows the enlargement of the chromocenter. No differences can be seen in the wild type and the *Dnmt2* null chromosomes for the distribution of this mark.

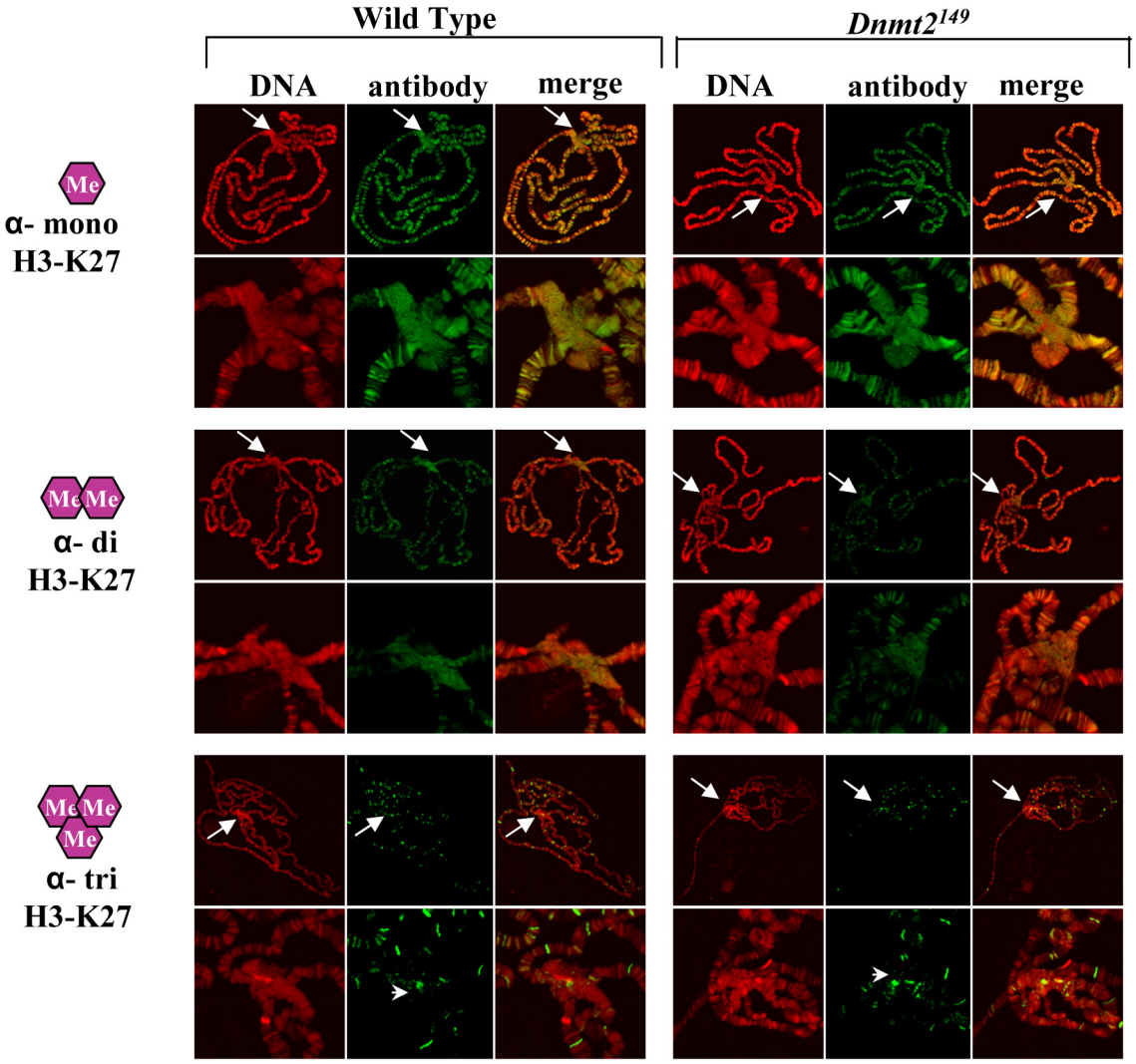

Figure 3.14 Detection of H3K27 methylation in polytene chromosomes of wild type and *Dnmt2* null larvae. All the three methylation states (mono, di and tri methylation) were analysed (green). Chromosomes were counterstained with DAPI (red). Arrow indicates the chromocenter, arrow heads indicate localised distribution of H3K27me3 mark within chromocenter heterochromatin. For each methylation state the lower penal shows the enlargement of the chromocenter. No differences can be seen in the wild type and the *Dnmt2* null chromosomes for the distribution of this mark.

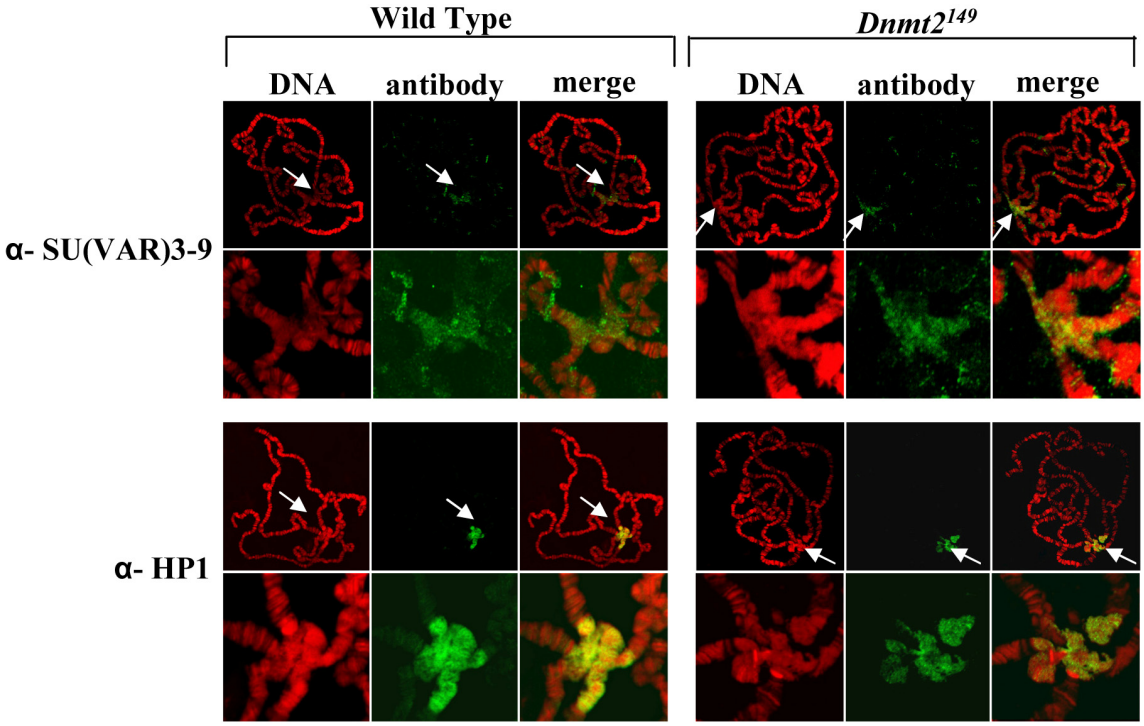

Figure 3.15 Detection of Heterochromatin associated proteins in polytene chromosomes of wild type and *Dnmt2* null larvae. Distribution of SU(VAR)3-9 and HP1 were analysed (green). Chromosomes were counterstained with DAPI (red). Arrow indicates the chromocenter. For each protein the lower penal shows the enlargement of the chromocenter. No differences can be seen in the wild type and the *Dnmt2* null chromosomes for the distribution of these proteins.

Immunocytological analysis was confirmed by western blot analysis of the nuclear protein extracts from third instar larvae (Walluscheck, 2006). Protein immunoblots affirmed a decrease of H4-K20 tri-methylation in the *Dnmt2* null mutant, while amounts of H4-K20 mono-and di-methylation remains unchanged.

Together these data suggest that maintenance of retrotransposon silencing initiated by DNMT2 mediated DNA methylation is controlled by histone 4 Lysine 20 trimethylation mark.

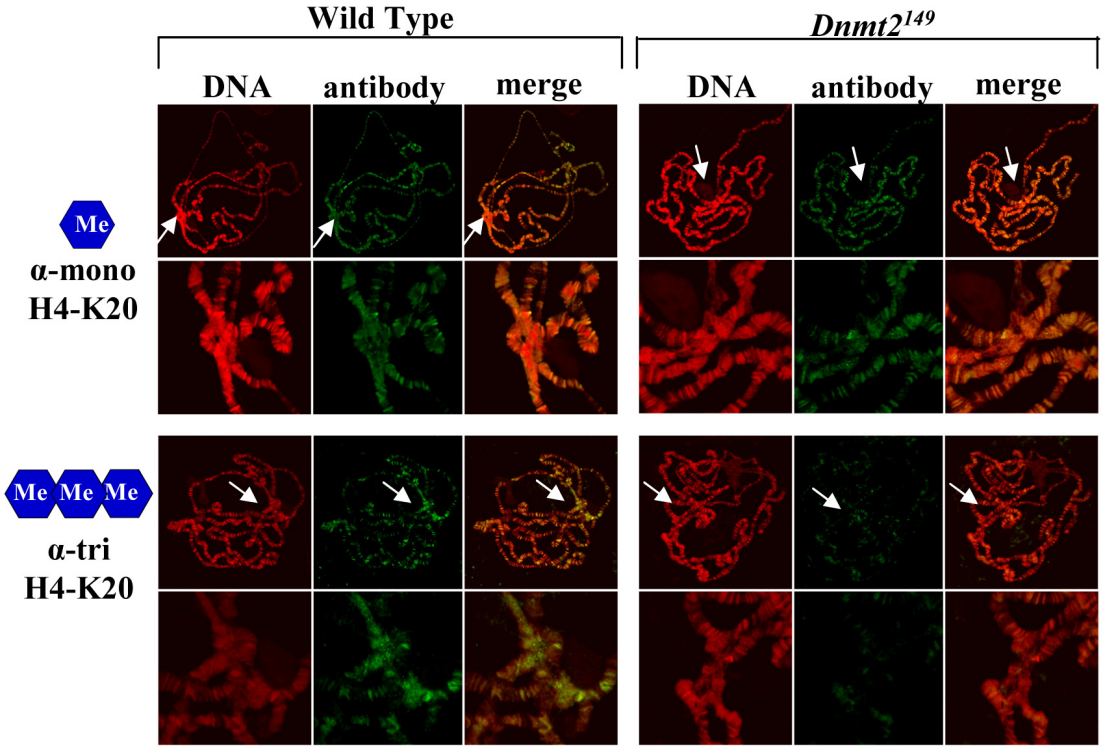

Figure 3.16 Detection of H4K20 methylation in polytene chromosomes of wild type and *Dnmt2* null larvae. Mono and tri methylation states were analysed (green). Chromosomes were counterstained with DAPI (red). Arrow indicates the chromocenter. For each methylation mark the lower penal shows the enlargement of the chromocenter. Note severe reduction of H4K20me3 mark in *Dnmt2* null chromosomes.

3.3.2 SUV4-20 histone methyltransferase is essential for retrotransposon silencing

Comparative immunocytological analysis showed that at many sites in the genome DNA methylation is essential for establishment of H4K20me3 histone methylation for which a maintenance function of the silenced chromatin state is suggested. Considering the fact that gene silencing defects observed in the *Dnmt2* null animals could be resulted by loss of consequent H4K20me3, mutations in the gene encoding histone 4 Lysine 20 specific histone methyltransferase should elicit identical molecular phenotype as *Dnmt2* mutations. *Suv4-20* has been demonstrated to possess histone 4 Lysine 20 trimethylation activity (Schotta *et al*., 2004). *BG00814* represents a strong hypomorphic insertional allele of *Suv4-20* which leads to strong reduction of H4K20me3 from all the sites on the polytene chromosome (Schotta *et al*.,

2004). Transcript analysis on total RNA isolated from embryos and adult heads of *BG00814* flies showed a strong upregulation of *Invader 4, Copia, Rover* and *Gypsy* retrotransposons (Fig 3.17).

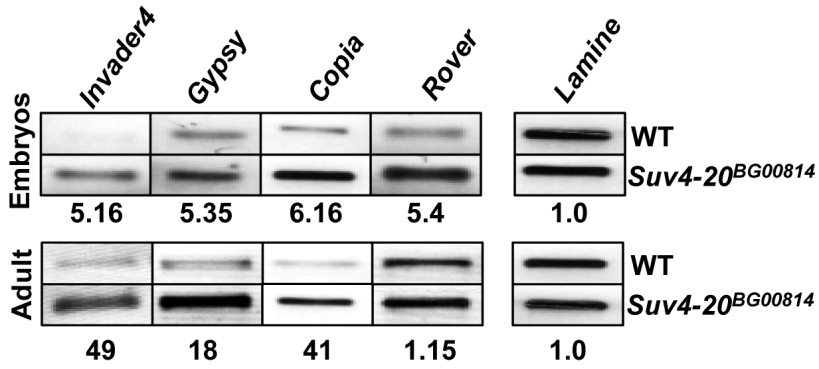

Figure 3.17 *Suv4-20* is involved in the control of retrotransposon expression. Semi quantitative and quantitative RT-PCR analysis of retrotransposon expression in wild type and *Suv4-20*^{BG00814} mutant embryos and the adult heads. *Invader4*, *Copia*, *Gypsy* and *Rover* were analyzed. *Lamine* was used as the loading control. Significant upregulation of all tested retrotransposons is observed in *Suv4-20*^{BG00814} embryos and in adult heads.

This data suggests that SUV4-20 mediated H4K20me3 is a component of *Dnmt2* mediated gene silencing processes. Immunostaining of wild type embryos using anti H4K20me3 antibody revealed a rather late appearance of this covalent histone modification mark during development (Fig 3.18). The signals were first observed in the gastrulating embryos (stage 6); coincidently this is the stage when global levels of DNMT2 mediated DNA methylation drops down severely in *Drosophila* embryos. Significant reduction of H4K20me3 was seen in the *Dnmt2* null embryos.

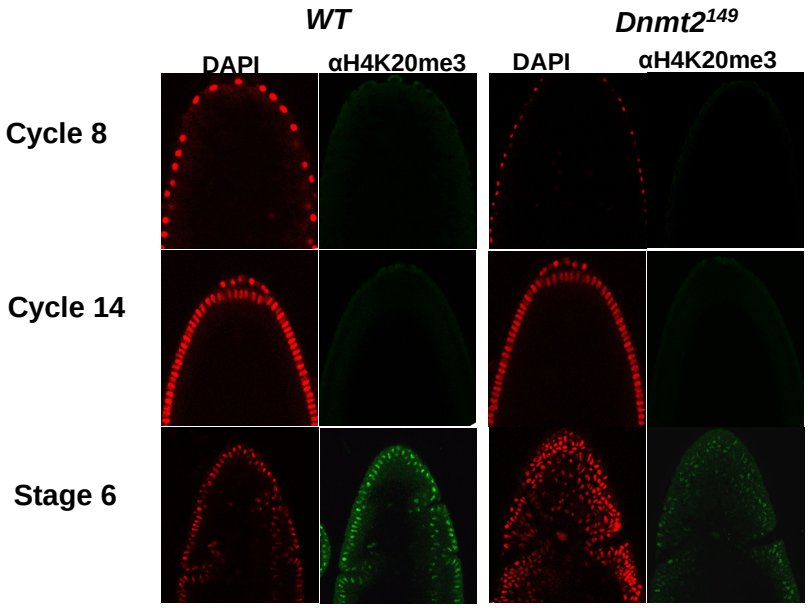

Figure 3.18 Histone 4 Lysine 20 tri methylation during *Drosophila* embryonic development. Wild type and *Dnmt2* null embryos of three different stages of early embryonic developmental are shown. Note the appearance of H4K20me3 mark (green) after the beginning of the gastrulation. Strong reduction of H4K20me3 can be seen in Dnmt2 null embryos.

BG00814 allele contains a *white* marked P element inserted into the *Suv4-20* ORF. Presence of the *white* transgene made it impossible to analyse the effect of loss of *Suv4-20* on *white* gene silencing. New *Suv4-20* mutant allele was generated by remobilization of the *BG00814* element and is characterized by an insertion of 17 bp within 3'end of the *Suv4-20 ORF* (Fig 3.19a, Materials and Methods).

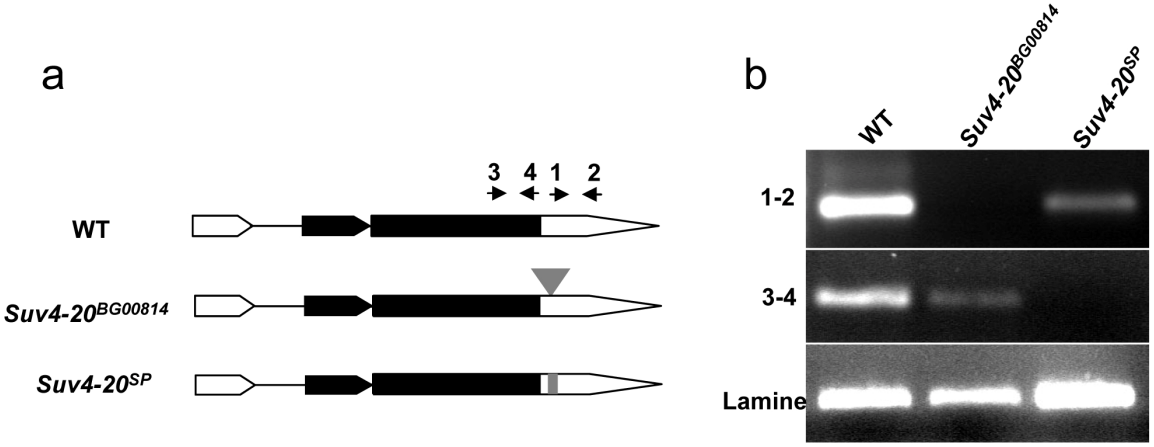

Figure 3.19 Characterization of the new *Suv4-20* mutant allele (a) *Suv4-20*^{SP} was generated by remobilization of *pBG00814* inserted within 3'end of *Suv4-20* ORF (grey triangle), and is characterized by an insertion of 17bp (grey box) from the P element. (b) RT-PCR analysis revealed a strong reduction of *Suv4-20* specific transcripts in *Suv4-20*^{BG00814} and *Suv4-20*^{SP} alleles. *Suv4-20* specific primers used for the transcript analysis are indicated by black arrows. Lamine was used as a loading control.

The resulting putative *Suv4-20* null allele (*Suv4-20*^{SP}) showed complete loss of *Suv4-20* specific transcript (Figure 3.19b). Like *Dnmt2* null mutants, the putative *Suv4-20* null mutant flies are viable and fertile which shows that SUV4-20 is not essential for *Drosophila* development. *Suv4-20*^{SP} allele showed a strong suppressor effect on *white* variegation in the *In(1)w*^{m4} rearrangement, which is in agreement with the earlier work which showed SUV4-20 as a component of the SU(VAR)3-9/HP1 heterochromatic gene silencing pathway. *Suv4-20*^{SP} allele also showed a strong suppressor effect on the variegated P element inserts in the *Invader4* retrotransposon (Fig 3.20).

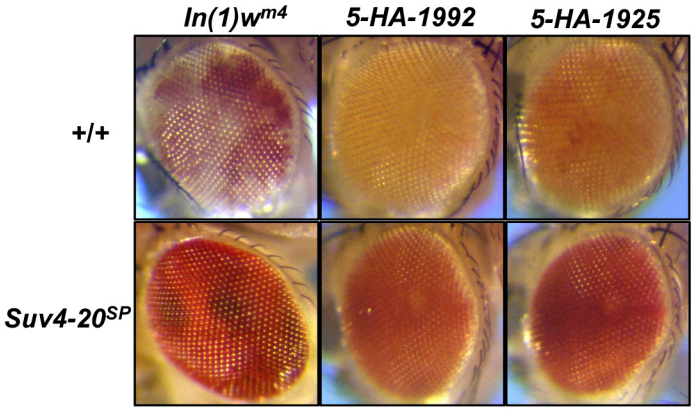

Figure 3.20 *Suv4-20* is a functional component of two silencing pathways. New mutant allele *Suv4-20*^{SP} is a dominant suppressor of classical PEV in *In(1)w*^{m4} rearrangement and also for the variegating *white* inserts within *Invader4* retrotransposons.

The *Suv 4-20* null mutant allele showed a complete loss of H4K20me3 mark on the polytene chromosomes which can be further confirmed by western blot analysis (Fig 3.21).

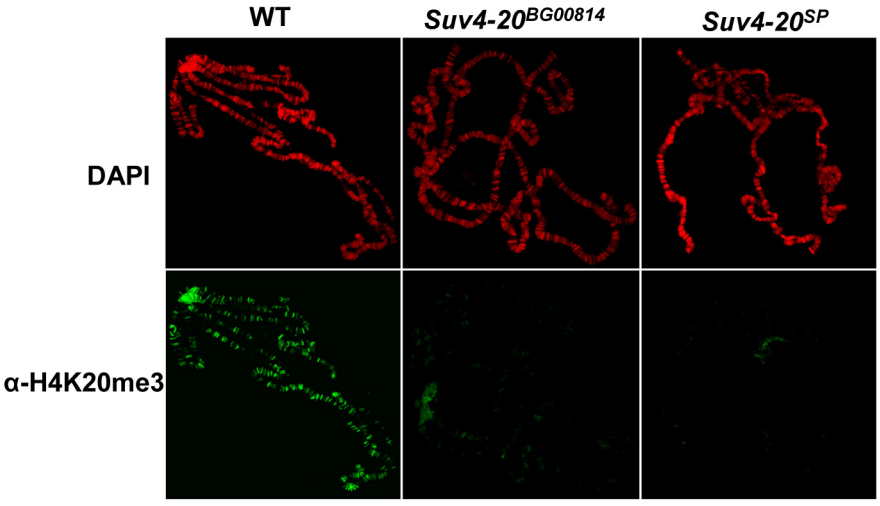

Figure 3.21 SUV4-20 controls H4K20me3 histone modification mark. Immunostaining using anti H4K20me3 antibody (green) showed strong reduction of H4K20me3 from the *Suv4-20*^{BG00814} *and Suv4-20*^{SP} polytene chromosomes. Chromosomes were counterstained with DAPI (Red).

Together these data suggest that SUV4-20 controls all H4K20 trimethylation mark in *Drosophila* and is a component of at least two independent gene silencing pathways, one with SU(VAR)3-9/HP1 pathways for heterochromatic gene silencing and the other in DNA methylation dependent retrotransposon silencing in *Drosophila*.

3.3.3 Silencing of the retrotransposons is correlated with the distribution of H4K20me3 mark

Genetic and molecular analysis demonstrated an involvement of SUV4-20 in DNMT2 mediated gene silencing pathway. Global reduction of H4K20me3 mark is observed in *Dnmt2* null mutant flies. In order to correlate the loss of H4K20me3 specifically with silencing of retrotransposons, chromatin immunoprecipitation (ChIP) was performed on the chromatin isolated from wild type, $Dnmt2^{149}$ and $Suv4\text{-}20^{BG00814}$ adult heads, using an anti H4K20me3 specific antibody and enrichment of this mark was analyzed on the selected retrotransposon sequences. Strong enrichment of H4K20me3 is observed at heterochromatic *satellite* sequences, *Invader 4*, *Rover* and *Copia LTR* sequences, which was significantly reduced in *Suv4-20* and *Dnmt2* null mutant tissues (Fig 3.22).

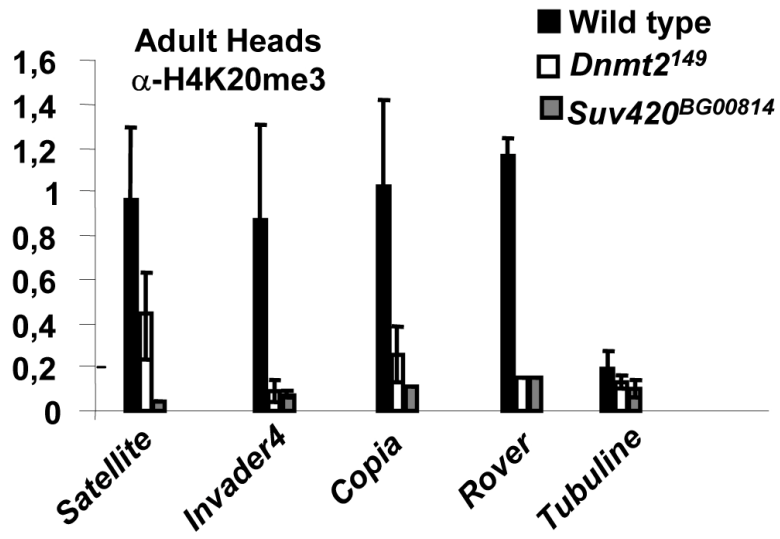

Figure 3.22 Chromatin immunoprecipitation using an H4K20me3 specific antibody. Chromatin was isolated from heads of adult wild type, $Dnmt2^{149}$ and $Suv4\text{-}20^{BG00814}$ males. *Invader4*, *Copia*, *Gypsy* and *Rover* sequences were tested. *Satellite* and *tubuline* were used as positive and the negative controls, respectively. Significant reduction of H4K20me3 is found in *Suv4-20* and *Dnmt2* mutant strains.

Reduction of H4K20me3 at heterochromatic satellite sequences is more pronounced in *Suv4-20* than in *Dnmt2* mutants reflecting the predominant impact of the SU(VAR)3-9/HP1/SUV4-

20 pathway on H4K20 trimethylation at satellite sequences. *Tubuline,* an active gene, was used as a negative control.

3.3.4 *Drosophila* MBD2/3, the 5-methyl cytosine binding proteins is not involved in DNMT2 dependent gene silencing pathway

Genetic and molecular analysis demonstrated the existence of at least one gene silencing pathway, initiated by DNMT2 mediated DNA methylation and maintained through histone 4 lysin 20 trimethylation for the regulation of retrotransposons in the somatic cells. In this pathway, however, it is not yet clear how the DNA methylation mark is read out by the SUV4-20 or other histone methyltransferase. In higher eukaryotes, a functional role of Methyl DNA Binding proteins (MBD) for recognizing the DNA methylation mark on the DNA and then recruiting the silencing machinery to those sites has been demonstrated (Bird and Wolffe, 1999). In contrast to vertebrates only one MBD homologous protein is present in *Drosophila*, named as MBD2/3 (Tweedie *et al*., 1999). We tested whether MBD2/3 protein is an essential component of the DNMT2 mediated gene silencing pathway in reading out the DNA methylation and then recruiting the other components of silencing machinery. For this analysis a new mutation in MBD2/3 was generated by remobilization of *P{Epgy2}EY04582* P element inserted into *Mbd2/3* gene (see Materials and Methods; Marhold *et al*., 2004b). In a screening of 50 independent chromosomes, one showed an insertion of X bp in the 5'end of the M*bd2/3* ORF, resulting into a very early stop codon.

The new putative null mutation allele of *Mbd2/3* gene, $Mbd2/3^{19}$ was tested for its effect on white variegation in different P element inserts. This mutation suppressed the white variegation of a P element inserted within pericentric heterochromatin, but did not affect the inserts within telomeres or *Invader4* retrotransposons.

This concludes that even though MBD2/3 shares homology to the vertebrate methyl DNA binding protein, functional similarity is not clear.

3.4 Loss of DNMT2 leads to the retrotransposon mobility and telomere instability

3.4.1 Loss of *Dnmt2* leads to the mobility of the *Invader4* retrotransposon

Genetic and molecular analysis demonstrated a DNMT2 mediated DNA methylation dependent control of retrotransposon silencing in somatic tissues of *Drosophila*. Strong overexpression of retrotransposons was detected in $Dnmt2^{149}$ mutant tissue; however this overexpression does not lead to any functional consequences. Active transcription of retrotransposons has been correlated with their mobility (Desset *et al*., 2003). Therefore it was necessary to analyze the distribution of retrotransposons in the wild type and the $Dnmt2^{149}$ chromosomes. Most of the variegating P element inserts which were suppressed by loss of *Dnmt2* are localized to the 5'LTR sequences of the *Invader4* retrotransposon; hence the distribution of *Invader4* was studied in detail. Two probes detecting *Invader4* LTR (200bp) and *Invader4* coding region (500bp) were used for Fluorescent *in situ* Hybridization (FISH) on polytene chromosomes from wild type and $Dnmt2^{149}$ mutant larval tissue. Use of *Invader4* retrotransposon was advantageous because of the fact that only 9 copies of this retrotransposon have been reported in the *Drosophila* euchromatin, out of which only 2 of them are intact (Flybase 2008).

In agreement with the Flybase annotation of *Invader4* retrotransposons both probes showed two signals at 39E on 2L chromosome and 102F on 4^{th} chromosome, which represent the two annotated full length euchromatic *Invader4* copies, *Invader4{}511* and *Invader4{}1541*. in addition to these two signals one clear signal was observed within the pericentric heterochromatin which represent the 2R heterochromatin full length copy *Invader4{}RR48393* (Fig 3.23). Beside these signals both probes showed additional sporadic signals within pericentric heterochromatin. *Invader4 LTR* probe showed unique signals at the tip of 2R and the 3R chromosome arms, which were not found with *Invader4* coding region probe. These data suggest the presence of at least three full length copies and several defective copies (specifically LTR sequences) of *Invader4* in *Drosophila* genome.

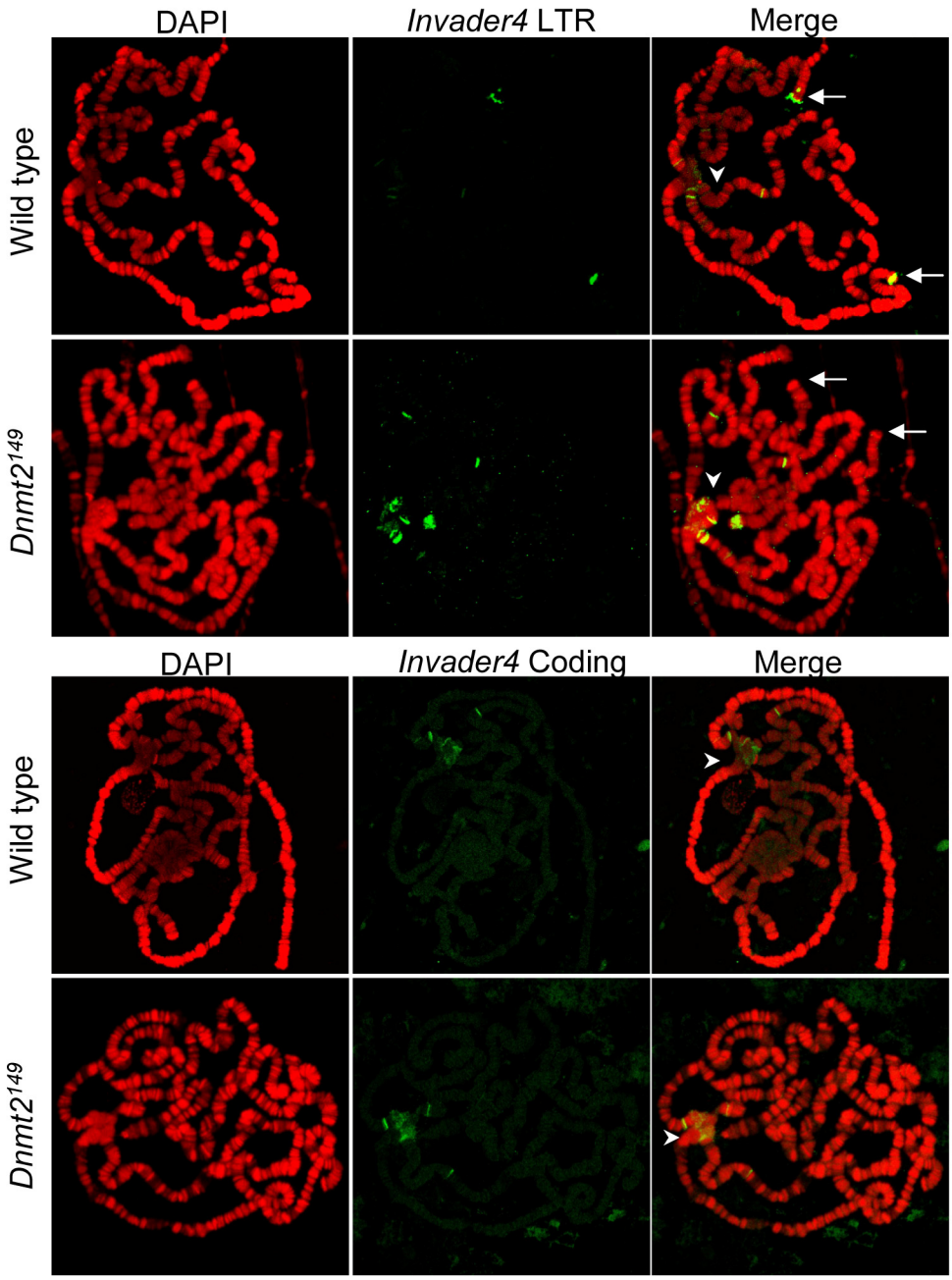

Figure 3.23 Immuno FISH to monitor *Invader4* distribution on the polytene chromosomes. *Invader4* LTR or coding region probes (green) were hybridized with wild type or *Dnmt2* null polytene chromosomes. LTR probes showed complete loss of telomeric signals in *Dnmt2* null chromosomes (arrows), while both probes showed an increase in the pericentric heterochromatin signals on *Dnmt2* deficient chromosomes (arrow heads).

On the *Dnmt2*^{149} chromosomes, the three signals were identical to that of the wild type signals, but a significant increase in the sporadic signals in the pericentric heterochromatin with both *Invader4* probes is observed. Most noticeable are the loss of the telomeric signals identified with *Invader4* LTR probes.

Thus the FISH experiments suggested possible restricted mobility of the *Invader4* retrotransposon in the *Dnmt2*^{149}, within pericentric heterochromatin. To further confirm this mobility, an inverse PCR analysis was performed to identify the *Invader4* sequence locations in the wild type and the *Dnmt2*^{149} flies (see Materials and Methods; Nickel, 2008). Significant differences were observed in the distribution of *Invader4* in the two genotypes. Several new fragments, representing the new *Invader4* insertion sites were obtained in *Dnmt2* null mutant DNA, while some fragments were lost. Cloning and further sequencing of the *Dnmt2*^{149} specific fragments could only retrieve yet unannotated heterochromatin sequences, which is in agreement with the *Invader4* FISH analysis which showed an increase in the heterochromatin signals in the *Dnmt2* null background. Thus these data suggest a mobility of *Invader4* retrotransposons in the *Dnmt2* null mutant genotype, which by some unknown mechanisms is restricted to the pericentric heterochromatin.

3.4.2 Loss of *Dnmt2* leads to instability of telomere associated sequences on 2R and 3R chromosome arms

Because of unique distribution of *Invader4 LTR* sequences on the *Drosophila* telomeres and significant changes in *Dnmt2* null flies, sequence analysis of *Invader4 LTR* was performed using complete *Drosophila* sequence database. Overall 48 homology regions were identified, in the Release 5.8 complete assembled genome sequence of the *D. melanogaster*. Among these 48 regions, 32 are the annotated euchromatic or heterochromatic *Invader4* copies while 16 lie among unannotated sequences. Of the 32 annotated copies 3 are the full length copies, two in the euchromatin (39E and 102F) and one in heterochromatin (2R Het), 22 copies are annotated as defective elements on the TAS region of 2R and 3R chromosome, while 7 copies represent the defective elements elsewhere in the genome (Flybase 2008).

Presence of the *Invader4* cluster on the 3R telomere is in agreement with telomeric signals detected in the FISH analysis, however the Release 5.8 of *Drosophila* genome sequence showed only one *Invader4* copy on the 2R telomere associated sequence, which could be due to incomplete sequencing of this chromosome end. Several different wild type strains of *Drosophila* were tested for the presence of telomeric *Invader4* copies and all including flies from natural population (*D. melanogaster Canaberra*) showed the identical pattern of *Invader4* on the polytene chromosomes (Nickel, 2008).

FISH experiments showed a complete loss of the *Invader4* clusters from both the 2R and the 3R telomeric ends in *Dnmt2* null chromosomes, which was consistent in all the other *Dnmt2*

mutant alleles. To exclude any background effects, the original P insertion line *GE15695*, used to generate *Dnmt2*^{149}, and one of the wild type revertant *Dnmt2*^{215} were used as control and the loss was found to be *Dnmt2* null specific (Nickel, 2008).

To determine if this loss represents a terminal deletion of the chromosomes or it involves an internal deletion, presence of the telomeric retrotransposon *HeT-A* was evaluated at the chromosome ends in the wild type and the *Dnmt2*^{149} chromosomes. No significant difference could be observed in the *HeT-A* distribution on these two telomere ends in both genotypes. Similarly HP1 binding remains unaffected in *Dnmt2* null mutant chromosomes (Fig 3.24).

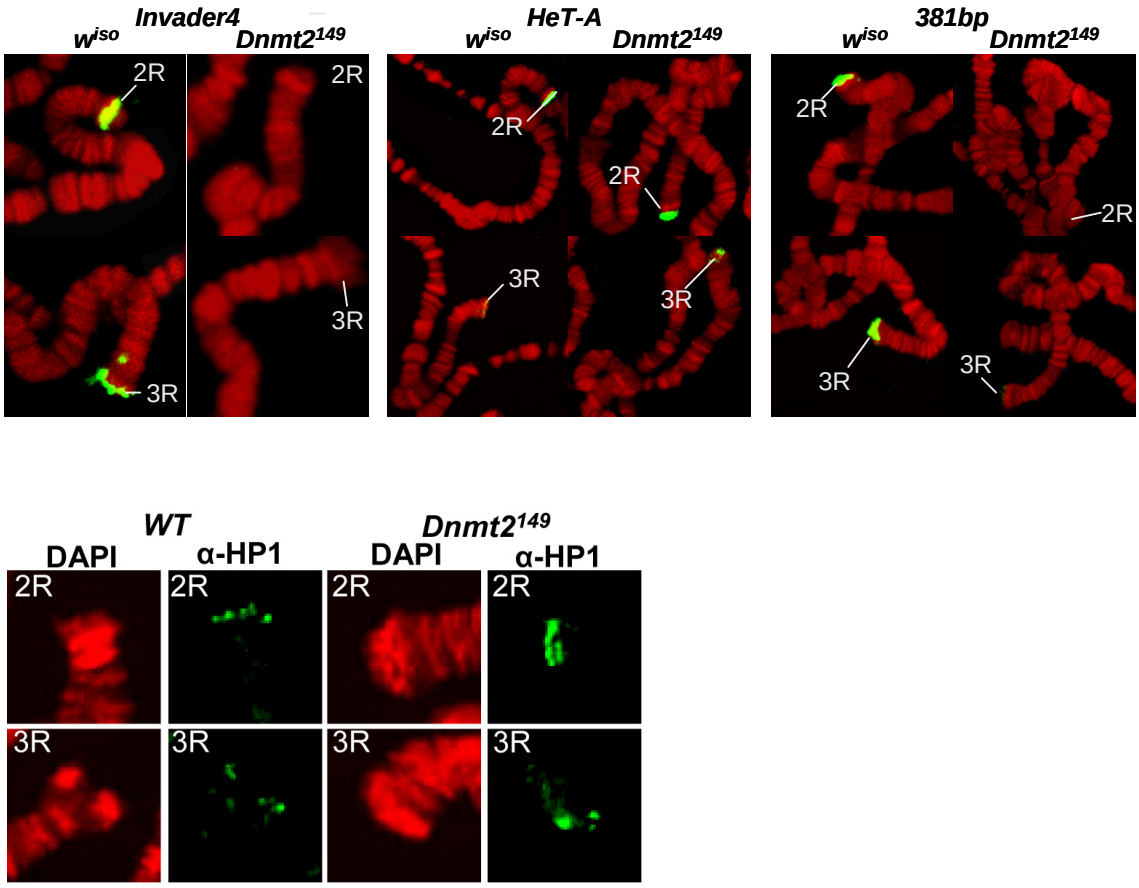

Figure 3.24 Loss of Dnmt2 leads to deletion of TAS sequences. (**a**) Fluorescent *in situ* hybridization analysis of wild type and *Dnmt2* null mutant salivary gland polytene chromosome using *Invader4* LTR, 381bp repeats and *HeT-A* specific probes. Note the complete loss of telomeric *Invader4* LTR and 381bp signals in *Dnmt2* null 2R and 3R telomeres, while *HeT-A* is not deleted. (**b**) Telomere capping is not affected in *Dnmt2* null chromosomes as shown by antibody staining for HP1 in wild type and *Dnmt2* null mutant chromosomes. Telomere localization of HP1 is not affected at 2R or 3R telomeres in *Dnmt2* null chromosomes.

Together this data argue against a terminal deletion in the *Dnmt2* null chromosomes, and favours the internal deletion of the *Invader4* cluster and also that the telomere capping remains unaffected. A detailed sequence analysis of the subtelomeric 3R *Invader4* cluster

suggested a rather complex structure of this repeat. Subtelomeric Telomere Associated Sequences (TAS) on chromosome arm 2R and 3R have been shown to be highly homologous (Yin and Lin, 2007) with a repeat size of 1000bp and 984 bp respectively. Sequence database search reveals presence of six 984bp repeats on the 3R telomere, which consist of 381bp 3R and 2R specific repeat sequence and 603bp of three crippled *Invader4 5'LTR* copies. A complete LTR of *Invader4* retrotransposon is about 350bp, the 603bp of the 3R TAS repeat consist of three broken fragments of *Invader4 LTR* (First segment from 1 bp to 249 bp, second from 77bp to 172bp and the third segment from 77bp to 300bp) and X bp of *Invader4* homology sequence (Fig 3.25).

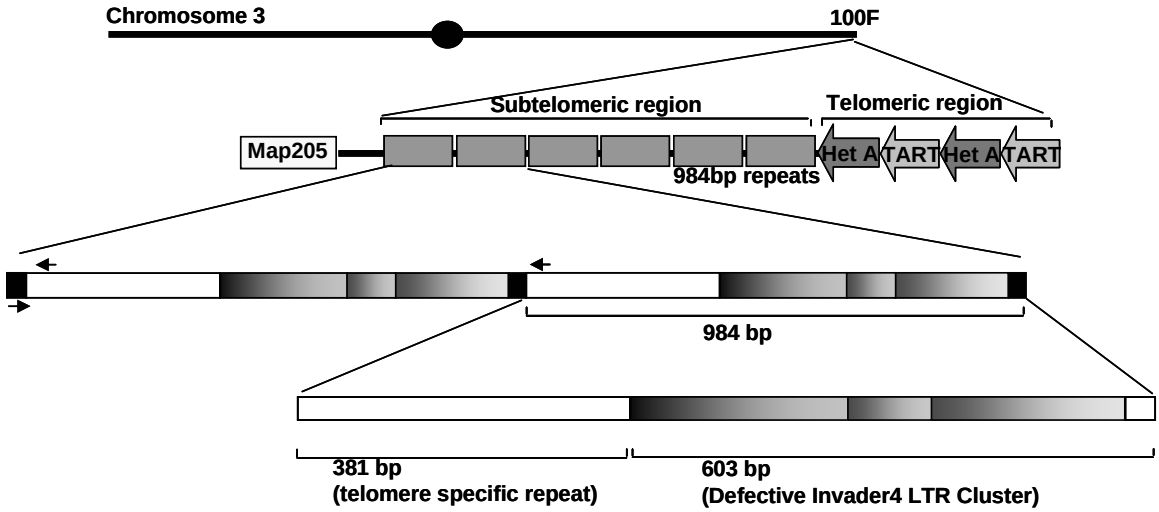

Figure 3.25 Schematic representation of 3R telomere associated sequences. Six copies of 984bp repeats are arranged proximal to the *HeT-A* sequences. Each 984bp repeat consist of unique 381bp repeat and 503bp of three rearranged crippled copies of *Invader4* LTRs. Shaded box represents the length of each *Invader4* LTR sequence.

FISH analysis also showed a loss of 381bp 2R and 3R specific telomere repeat in *Dnmt2* null chromosomes (Fig 3.25), which supports a complete internal deletion of the TAS repeat in the absence of DNMT2. Finally molecular evidence for the internal deletion comes from PCR analysis performed using primer binding to the 3' end of *Invader4* segment (3RtelFwd) and to the 5' end of the 381 bp sequence (3RtelRev). Wild type DNA showed two expected amplification products of 150bp and 1000bp, due to presence of the repeats, while $Dnmt2^{149}$ DNA showed only one 150bp fragment (Fig 3.26).

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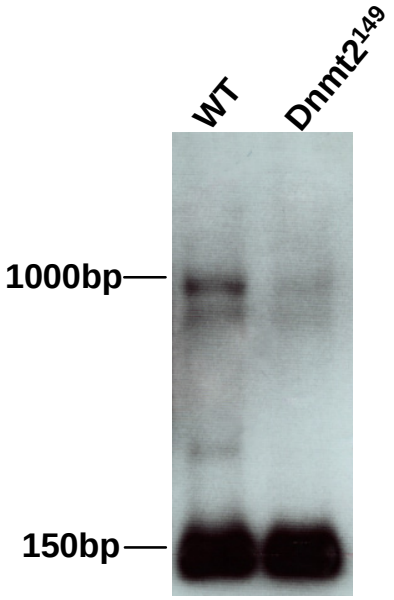

Figure 3.26. ***Dnmt2* null mutants leads to the deletion of 3R TAS repeats.** Molecular analysis using primers specifically amplifying the 3R TAS repeats. Repetitive structure of the 3R TAS give two major fragments of 150bp and 1000bp in wild type DNA, while only 150bp fragment was seen in the *Dnmt2* null mutant DNA demonstrating deletion of the repeats.

This suggests a complete loss of 984 bp repeats, except for few base pairs intact from the first 381bp repeat.

3.4.2 Loss of 2R and 3R TAS is rapid and irreversible process

With a series of crosses the kinetics of the *invader 4* cluster loss at telomere 3R in a *Dnmt2* null genotype was studied and asked whether the observed loss is genetically stable after the chromosomes are placed back into a wild type background. Chromosomes from the *Dnmt2*^{149}(2R^{-})/*Dnmt2*^{149}(2R^{-}); +(3R^{-})/+(3R^{-}) strain with deletions of the 2R and 3R telomere *invader 4* clusters (symbolized 2R^{-} and 3R^{-}) were transferred by a two generation mating scheme with the help of crossover balancer chromosomes into the two new strains *SM6*(2R^{+})/*Sco*(2R^{+}); +(3R^{-})/+(3R^{-}) and *Dnmt2*^{149}(2R^{-})/*Dnmt2*^{149}(2R^{-}); *TM6*(3R^{+})/*TM2*(3R^{+}). The *SM6*, and *TM2* balancer chromosomes contain like wild type chromosomes *invader 4* clusters in their telomere-associated sequences while on *TM6* balancer chromosome the 3R subtelomeric *Invader4* cluster is relocated by an inversion into a central position with chromosome 3 and is separated from *HeT-A* and *TART* telomeric elements.

The first strain is wild type for *Dnmt2* and we tested over successive generations whether telomere 3R *Invader4* clusters are established again. In the second strain we studied kinetics of *Invader4* cluster loss in the *TM6* and *TM2* third chromosome balancers in *Dnmt2* null genotype. FISH analysis revealed that once deleted *Invader4* clusters at 3R telomeres cannot

be re-established at least until 10 generations (Personal communication Olaf Nickel). An intact *Invader4* cluster from the 3R telomere in TM2 chromosomes and at an internal position on TM6 chromosome was observed even after six generations. A completely different behaviour is found for the *TM6* chromosome. In this new position the *Invader4* cluster is stable and is not lost in *Dnmt2* null background suggesting a functional interrelationship between the *Invader4* subtelomeric cluster and the *HeT-A* and *TART* telomeric elements in control of telomere stability and integrity.

4. Discussion

Differential packaging of DNA with histone and non-histone proteins into chromatin defines its accessibility for transcription during development. Establishment and maintenance of chromatin states depends on DNA methylation, histone modification, nucleosome remodelling and non-coding RNA molecules. These epigenetic processes define pattern of gene expression and are key mechanisms in control of developmental programs. In *Drosophila,* Position Effect Variegation (Reuter and Spierer 1992, Wallrath 1998, Schotta *et al*., 2003), transgene silencing (Wallrath and Elgin, 1995; Cryderman *et al*., 1998; Cryderman *et al.,* 1999) and Polycomb gene silencing (Pirrotta and Rastelli, 1994; Dellino *et al*., 2004) have dissected more local events of gene silencing in the *Drosophila* genome. As a result of all these studies finally differential molecular mechanisms of gene silencing has been suggested (Weiler and Wakimoto 1995; Cryderman *et al.,* 1999). This differential regulation is controlled by overlapping or independent processes. As shown by the studies performed in this work even the poorly understood DNA methylation is required for the regulation of retrotransposon expression in the somatic cells of *Drosophila*.

4.1 *Drosophila* DNA methyltransferase DNMT2 is involved in gene silencing processes

Existence of DNA methylation in *Drosophila* has been discussed controversially for a long period of time. There are several reports that demonstrate the absence of 5-methylcytosine from pupal and adult stages of fly development (Patel and Gopinathan, 1987; Tweedie *et al.*, 1999). With the finding of presence of DNA methyltransferase homologue, DNMT2 in *Drosophila*, studies on DNA methylation in *Drosophila* were restarted. (Hung *et al*., 1999; Tweedie *et al.,* 1999; Lyko *et al*., 2001). Very low levels of overall DNA methylation, restricted only to early embryogenesis made detection of DNA methylation in *Drosophila* quite challenging. However several independent methods such as sensitive chromatographic approach, capillary electrophoresis, thin layer chromatography, nearest neighbour analysis, bisulphite sequencing and immunocytology have recently confirmed the presence of DNA methylation in *Drosophila* genomic DNA (Gowher *et al.,* 2000; Marhold *et al*., 2004; Lyko *et al.,* 2000, Kunert *et al.,* 2003). To understand the DNA methylation system of *Drosophila,* mutations for *Dnmt2,* the gene encoding only DNA methyltransferase, were generated. In

contrary to vertebrates, *Dnmt2* was not essential for *Drosophila* development (Goll *et al*., 2006; Phalke *et al.*, submitted) and none of these mutant flies showed any visible phenotypes. A detailed genetic and molecular analysis however revealed involvement of DNMT2 in retrotransposon silencing (Phalke *et al*., submitted). In vertebrates and plants DNA methylation has been connected with heterochromatin formation (Tamaru and Selker 2001; Jackson et al., 2002; Lehnertz et al., 2003), hence first the role of *Dnmt2* was analysed in heterochromatin formation in *Drosophila*. Genetic tests using $In(1)w^{m4}$ PEV rearrangement excluded a function of DNMT2 in heterochromatic gene silencing. However further genetic analysis using variegating inserts within other regions including telomere and retrotransposons sequences indeed provided first indications for the role of DNMT2 in gene silencing pathways in *Drosophila*. Furthermore a general up regulation of retrotransposon expression was observed in the somatic tissues of *Dnmt2* null mutant flies. Together these preliminary results indeed showed for the first time a role of DNMT2 in control of retrotransposon expression in *Drosophila* somatic tissues. DNA methylation has been causally connected with transposon silencing in higher organism as well as in plants (Bird, 2001; Naumann *et al*., 2005), therefore a direct control of retrotransposon expression by DNMT2 mediated DNA methylation can be suggested. However several independent studies suggested only very weak, *in vitro*, DNA methyltransferase activity of DNMT2, on the contrary robust tRNA-Asp methyltransferase activity of this enzyme could demonstrated *in vitro* and *in vivo* (Goll *et al*., 2006, Jurowski *et al*., 2008). This leads to two basic queries; whether DNMT2 is really a DNA methyltransferase *in vivo* and if DNMT2 mediated DNA methylation is responsible for the control of retrotransposon silencing in *Drosophila*.

To address the first question global levels of DNA methylation were assayed in wild type and *Dnmt2* null mutant flies by DNA methylation dependent restriction enzyme analysis. This analysis showed a complete loss of DNA methylation in *Dnmt2* null mutant embryos, confirming that DNMT2 is the only enzyme responsible for all DNA methylation in *Drosophila*. In wild type embryos, global levels of DNA methylation seem to be significantly high during early embryogenesis but it drops down severely within consecutive developmental stages. Absence of any maintenance DNA methyltransferase explains the severe reduction of DNA methylation along with *Drosophila* development and also argues for the existence of another molecular maintenance function.

In previous studies retrotransposons sequences have been enriched over anti 5 methyl cytosine antibody affinity columns (Salzburg *et al.*, 2004) demonstrating methylation of these sequences in *Drosophila*. All of the variegating *white* transgene within retrotransposon

sequences were mapped within LTR sequences indicating LTRs to be the target sites for DNMT2 mediated activity. For a direct evaluation of DNA methylation on retrotransposon LTR sequences bisulphite sequencing and restriction digestion analysis were used. Bisulphite sequence analysis of the *Invader4* LTR demonstrated a DNMT2 dependent DNA methylation. Restriction analysis using both DNA methylation dependent and sensitive restriction enzymes showed DNA methylation on the target retrotransposon sequences. Interestingly, overall *Invader4* LTRs showed DNA methylation in very early embryos which was reduced significantly in the older embryos, but differences were observed in the onset of DNA methylation when an individual *Invader4* element was analysed. Some elements showed DNA methylation only in later stages of embryogenesis, and remain unmethylated during early stages of development and in the adult flies. Thus, taken together DNMT2 indeed is an *in vivo* DNA methyltransferase in *Drosophila*. Previous attempts to analyse an *in vitro* DNA methylation activity of DNMT2 might have failed because of inappropriate knowledge about DNMT2 target sequences and the absence of certain specific cofactors which might be present only in early embryonic nuclear extracts.

In cultured cell lines DNMT2 protein has been shown to reside strictly within the cellular cytoplasm (Goll *et al.*, 2006). However its nuclear localisation has been demonstrated *in vivo* by immunocytological, biochemical methods (Schaffer *et al*., 2008). Similarly chromatin immunoprecipitation using anti DNMT2 antibody indeed showed nuclear localisation of the DNMT2 protein to the target retrotransposon sequences during early embryogenesis, however the protein levels associated with the chromatin were severely reduced in the older embryo. This could represent a developmentally regulated nuclear localisation on DNMT2 protein, however a limited application of anti DNMT2 peptide antibody hinders a detailed *in vivo* analysis of DNMT2 distribution in *Drosophila* cells. GFP tagged DNMT2 protein demonstrated only a weak and transient nuclear localisation of DNMT2 within *Drosophila* cell nuclei (Schaffer *et al*., 2008). Large GFP tag could hinder proper folding of rather small DNMT2 protein which could lead to ectopic localisation of DNMT2. Hence new antibodies against whole DNMT2 protein or DNMT2 fusion proteins with shorter peptide tags should be generated to analyse *in vivo* distribution of DNMT2.

4.2 DNMT2/SUV4-20 mediated novel somatic retrotransposon silencing pathway in *Drosophila*

Retrotransposon expression could be deleterious to the cells due to their tendency to jump within other genomic region. A germ line specific control of retrotransposon expression has been demonstrated to be mediated by components of RNAi while an independent control of somatic retrotransposon expression has been suggested (Klenov *et al*., 2007). On the contrary other reports have suggested a somatic gene silencing control mediated by RNAi components (Pal Bhadra *et al*., 2006). Mutations in RNAi components were reported to dominantly suppress variegating *white* transgene inserted within pericentric heterochromatin, 4^{th} chromosomes and within telomeres and also to cause mislocalisation of HP1 and other heterochromatin associated histone modifications. However no modifier effects by any of the tested mutations for components of the RNAi machinery could be detected in any of the *white* variegating P element insertion lines which does not support the involvement of RNAi component in somatic gene silencing pathways, yet a role of maternal components of the RNAi machinery in somatic retrotransposon silencing cannot be ruled out. Homozygous viable mutations of genes encoding components of the RNAi machinery impair completely development of germ line cells and no eggs are produced making it impossible to study a possible maternal effect of the encoded products on early induced DNMT2 dependent DNA methylation at LTR sequences of retrotransposons. Theoretically maternally provided products of the RNAi machinery might control association of DNMT2 with LTR sequences through small antisense RNAs as indicated by recent findings that in S2 cells such molecules could be detected (Ghildiyal *et al*., 2008).

Molecular analysis indeed showed that DNA methylation activity of DNMT2 is required for the silencing of retrotransposons at least in somatic tissues of *Drosophila*. Previous studies (Lyko *et al.,* 2000a) and *SgeI* restriction analysis showed that global DNA methylation levels drops down severely after early embryonic stages and becomes undetectable in adult flies. Hence DNA methylation can only lead to establishment of silencing at the target sites during early stages of development. During later stages of development an independent maintenance function has to be suggested. Genetic analysis identified SUV4-20 and SETDB1 as genetic interaction partners of DNMT2. Genetic PEV tests showed that *Dnmt2* and *Suv4-20* mutations are dominant suppressors the inserts within LTR sequence of *Invader4* retrotransposons, while the inserts within regulatory regions of *HeT-A* were dominantly suppressed by *Dnmt2*

and *SetDB1* mutations. *Suv4-20* and *SetDB1* encode for histone methyltransferases which have been shown to catalyse H4K20me3 and H3K9me2, respectively (Schotta *et al*., 2004; Seum *et al*., 2007). Both histone modifications index silenced chromatin states and can serve for the maintenance of DNMT2 mediated gene silencing processes. Indeed a strong reduction of H4K20me3 was detected in *Dnmt2* null mutant polytene chromosome by comparative immunocytology which was further confirmed by western blot analysis. *Dnmt2* null polytene chromosomes showed only a moderate reduction of H4K20me3 histone modification mark at chromocenter heterochromatin while at other chromosomal bands the loss was stronger, similarly ChIP analysis also demonstrated strong reduction of this mark at the target retrotransposon sequences, but only moderate reduction at pericentromeric satellite repeats. This reflects that the SU(VAR)3-9/HP1/SUV4-20 heterochromatic gene silencing pathway remains unaffected in *Dnmt2* null flies. SUV4-20 is the only histone methyltransferase responsible for all H4K20me3 histone modification, while recently it has also been suggested to catalyse H4K20me2 (Yang *et al*., 2008). *Suv4-20* mutations showed a complete loss of H4K20me3 as analysed by immunocyology and ChIP. *Suv4-20* mutations are strong suppressor of w^{m4} PEV for the inserts within pericentric heterochromatin as well as within retrotransposon sequences and showed an up regulation of retrotransposon sequences in the somatic tissues. This suggests a dual function of SUV4-20 in two independent silencing pathways in *Drosophila*. In the SU(VAR)3-9/HP1 dependent heterochromatin gene silencing pathway HP1 binds to the SU(VAR)3-9 dependent H3K9me2 histone modification mark through its chromodomain and restricts SU(VAR)3-9 to the pericentric heterochromatin. HP1 also recruits SUV4-20 to the pericentric heterochromatin and stabilizes the silenced chromatin state. While in DNMT2 dependent retrotransposon silencing DNMT2 protein is expressed early in the zygotic cells and catalyses cytosine methylation on the target retrotransposon sequences. DNMT2 protein then interacts with other unknown proteins directly or through some 5 methyl cytosine binding proteins, which recruits SUV4-20 for the maintenance of the silencing during subsequent stages of development. Monitoring *Drosophila* embryonic developmental stages for appearance of H4K20me3 mark demonstrated that contrary to the H3K9 methylation marks, H4K20me3 histone modification appears relatively late during *Drosophila* development. Differentiation of euchromatin and heterochromatin occurs during stage five, when cellularization has already been started. After cellular blastoderm stage the embryo enters the gastrulation phase of development, it is at this stage of development that H4K20me3 mark can be first detected. Coincidently this is the developmental stage when global levels of DNA methylation drop down severely. This affirms a sequential pathway for

retrotransposon silencing established by DNMT2 mediated DNA methylation while it is maintained by SUV4-20 mediated H4K20me3 histone modification mark (Fig 4.1).

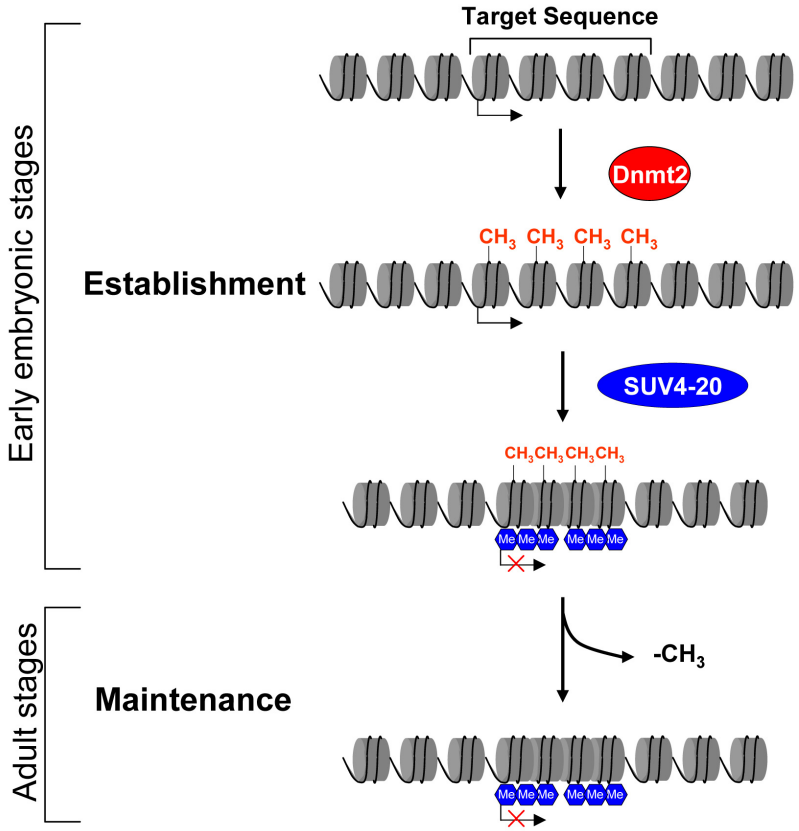

Figure 4.1 Model for sequential DNMT2/Suv4-20 mediated retrotransposon silencing pathway. DNMT2 is nuclear during early embryogenesis and can recognizes and methylates the target sequences. In post blastoderm stages SUV4-20 is recruited to the methylated target sequences with direct or indirect interaction with DNMT2 and mediates H4K20me3. During consecutive stages of development DNA methylation is removed by passive demethylation and H4K20me3 maintains the silencing throughout further development.

Similar to the involvement of SUV4-20 in the DNMT2 mediated retrotransposon silencing pathway, genetic analysis also suggested an involvement of SETDB1 in DNMT2 mediated silencing at *HeT-A* and transgene repeat sequences. SETDB1 has been demonstrated to possess an H3K9me2 activity *in vitro* and *in vivo* in *Drosophila*. In *Dnmt2* null flies, no significant differences could be observed in global H3K9 methylation marks, however local reduction of H3K9me2 histone modification can not be excluded at the target *HeT-A* sequences.

All DNMT2 dependent silencing processes seem to be established early during development and during later development maintained by different histone modification at different target sequences. However how 5 methyl cytosine is read out in to histone modification marks is yet unknown. In vertebrates DNMTases have been shown to interact directly with other proteins

to assemble the silencing machinery (Viré *et al*., 2006; Rountree *et al*., 2000) or 5 methyl cytosine binding proteins have been suggested to be a link between DNA methylation and the histone modifications (Jones *et al*., 1998; Nan *et al*., 1998). DNMT2 protein lacks an N terminal domain which has been attributed to protein-protein and protein-DNA interactions, thus a direct interaction with other silencing components is rather unlikely.

Another possibility for the read out of DNA methylation silencing machinery is through the 5-methylcytosine binding protein. *Drosophila* genome contains only a single gene encoding an MBD like protein, MBD2/3. This protein shows a significant homology to MBD2 and MBD3 of vertebrates. Previous studies have demonstrated an *in vitro* binding of MBD2/3 to methylated cytosine in a CpT and CpA context and have been suggested to be a functional component of the DNA methylation system in *Drosophila* (Marhold *et al.,* 2004b). MBD2/3 has also been shown to be associated with some fly homologues of the vertebrate MI-2 complex (Ballestar *et al.,* 2001; Tweedie *et al.,* 1999). However the suppressor effect of *Mbd2/3* mutation on variegating *white* transgene inserts within pericentric heterochromatin rather affirms a functional role of MBD2/3 in the SU(VAR)3-9/HP1 heterochromatic gene silencing pathway as suggested earlier (Marhold *et al.,* 2004). On the contrary the variegation of inserts within retrotransposon sequences remains unaffected. Taken together the results of our genetic analysis suggest that MBD2/3 is most likely not responsible for the *in vivo* read out of DNA methylation into maintenance histone methylation.

Thus the DNA methylation system seems to be more complicated in *Drosophila*. The protein with high similarity with the canonical MBD proteins does not seem to be functionally conserved. This raised the possibility of some yet unknown, crippled 5-methylcytosine binding proteins existing in *Drosophila* genome. Some histone methyltransferases like SETDB1 have been shown to possess an MBD domain in its N terminus. Whether other histone methyltransferases could also possess similar homologous sequences should be analysed further. Moreover actual *in vivo* and *in vitro* binding of these proteins to the methylated DNA should also be studied in detail. Isolation of new modifier mutations affecting PEV of the white inserts within retrotransposon sequences could help to find out functional interaction partners of *Dnmt2* mediated gene silencing pathways.

4.3 DNMT2 controls *Invader4* mobility and maintains telomere integrity

Molecular analysis demonstrated a new DNMT2/SUV4-20 mediated retrotransposon silencing in somatic tissues of *Drosophila*. Both *Dnmt2* and *Suv4-20* mutations showed an up regulation of retrotransposon sequences in the soma. Expression of retrotransposons has been associated with their mobility. Even though loss of *Dnmt2* leads to an up regulation of retrotransposons, mobility was not very obvious as observed at least for the LTR retrotransposon *Invader4*, which further affirms that DNMT2 mediated control is only somatic tissue specific and might not interfere with the germ line specific gene silencing programming. Although no clear new insertions of *Invader4* could be detected within euchromatic region, FISH and Inverse PCR analysis revealed new variable insertions of *Invader4* within pericentric heterochromatin. This could reflect its restricted somatic mobility; however other mechanisms must exist to specifically direct them into the heterochromatin. Some retrotransposons are known to transpose within themselves or to certain specific target sequences (Flavell, 2001; Gao *et al*., 2008), *Invader4* retrotransposon might be able to transpose preferentially into certain heterochromatin specific sequences.

The most remarkable feature of *Invader4* distribution within *Drosophila* genome is the presence of several defective *Invader4* LTR copies in the telomere associated region of chromosome arms 3R and 2R. A unique arrangement of *Invader4* LTRs interspersed with unique satellite sequences constitutes the telomere associated sequences (TAS) of 3R and 2R. Stability of these TAS regions is particularly dependent on DNMT2. The TAS region is an integral part of telomeres of all the organisms, and has been suggested to represent a template for formation telomeric heterochromatin which is well demonstrated by variegation of the reporter transgenes inserted within TAS region (Karpen and Spradling 1992; Levis *et al.,* 1993; Wallrath and Elgin 1995; Cryderman *et al.,* 1999; Marin *et al.,* 2000; Golubovsky *et al.,* 2001). Although telomere position effects have been studied in details over many years, its exact nature, the functional components and the functional relevance are unknown as yet. Equilibrium of the expression of telomeric retrotransposons has been shown to be controlled by activating effect of their 3' UTR promoter and the silencing effect of the TAS heterochromatin (Mason *et al*., 2002).

Recently at least four piRNAs have been mapped to the TAS repeats of 3R telomere; one of these piRNA has been associated with germ line stem cell defects (Yin and Lin, 2007;

Srinivasan and Lin, 2003). Loss of the PIWI protein leads to depletion of this piRNA and causes germ line defects and lethality. Rescue of piRNA expression can rescue *piwi* mutant phenotype. *Dnmt2 loss of function* mutation showed a deletion of the 3R and 2R TAS region, yet the flies are viable and fertile, which suggest an intact piRNA coding sequences. Molecular analysis indeed showed that at least one of the TAS repeat is partially retained in these flies, which is sufficient for the expression of piRNA.

Genetic analysis has demonstrated that the variegated expression of an insert within 3R TAS is dominantly suppressed in *Dnmt2* and *Suv4-20* null mutant. This suggests that these two factors are functional components of heterochromatin at 3R TAS. Enrichment of H4K20me3 histone modification could be detected at the 3R TAS region (Wacker J., 2008, Hortig F., personal communication). Similarly DNA methylation sensitive restriction analysis showed DNMT2 dependent DNA methylation at the *white* gene of the *P element* inserted within 3R TAS repeats (Hortig F., personal communication). This suggests that DNMT2 mediated 5 methyl cytosine is required to initiate telomeric heterochromatin formation at the 3R TAS region. In loss of function *Dnmt2* mutation formation of telomeric heterochromatin is impaired, which leads to the instability of the TAS region and the repetitive sequences recombine out to give deletions of the TAS region. This model suggests that loss of TAS sequences in *Dnmt2* null a mutation is a gradual rather than rapid process, however once lost, these sequences can not be recovered even in the wild type background. Systematic genetic tests coupled with FISH analysis indeed support this hypothesis. Intact TAS sequences could be detected at least until five generations after *Dnmt2* null chromosomes were introduced in otherwise wild type background, while no new TAS sequences could be recovered when a TAS deleted third chromosome from *Dnmt2* null flies was introduced in the wild type background.

Interestingly although *Suv4-20* mutations dominantly suppress *white* variegation of the inserts within 3R telomere associated sequences, it causes an increase in the *Invader4* clusters on 2R and 3R telomeres as demonstrated by FISH analysis (Nickel, 2008). This could suggests that in *Suv4-20* mutations DNA methylation recruits some other factors instead of SUV4-20 to the telomere associated sequences, which might not be sufficient for the maintenance of DNA methylation silencing, but can lead to homing of the *Invader4* retrotransposons to the TAS. Presence of SUV4-20 might be essential for heterochromatin formation at the telomeres and also for maintenance of the length of TAS sequences. However no molecular evidences are available as yet to confirm a role of SUV4-20 in controlling TAS elongation, which could be studied in detail using the mutant material generated in this study.

Since TAS repeats from 3R telomeres have been shown to encode essential piRNAs and *Dnmt2* seems to control the integrity of these repeats, a functional connection should be analysed. It would be interesting to analyse if loss of *Dnmt2* have any effects on the piRNAs.

Other than genomic stability on autosomal telomere, genetic analysis also suggests a similar situation on *Drosophila* Y chromosome. Several *Invader4* variegating inserts were mapped to the Y chromosome and were dominantly suppressed by loss of *Dnmt2*. isolation of multiple inserts on Y chromosomal Invader4 sequences suggest an existence of a cluster of Inavder4 LTR sequences similar to that of 3R and 2R telomere associated cluster, however limited sequence information of the Y chromosome does not allow a detailed molecular analysis of these repeats. A *white+* transgene inserted within Y chromosomal *Invader4* LTR sequence maintained in *Dnmt2* null background often show a *white*$^{-}$ fertile males which could represent deletion of these Y chromosomal *Invader4* clusters. However a detailed analysis on *Drosophila* mitotic chromosomes should be performed to analyse the Y linked *Invader4* cluster.

4.4 *Dnmt2* dependent and independent gene silencing pathways in *Drosophila* genome

Previous studies have indicated differential control of gene silencing in the major heterochromatin blocks of *Drosophila genome* (Cryderman *et al*., 1999). The genetic approach used in this study not only highlights the control of major heterochromatin blocks in *Drosophila*, but also provides an important tool to tag new genomic region undergoing gene silencing throughout the genome at both heterochromatin and euchromatin. Genetic data using newly isolated variegating inserts studied in this work showed an existence of at least five independent or overlapping silencing pathways in *Drosophila* (Fig 4.2).

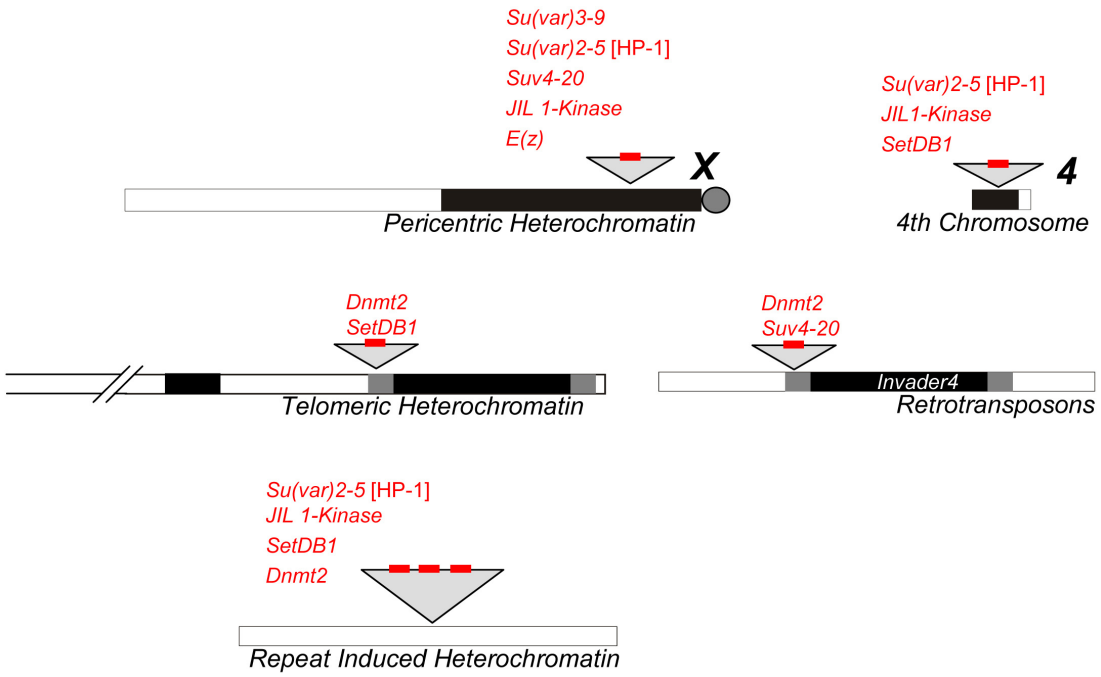

Figure 4.2 At least five alternative gene silencing pathways coexist in *Drosophila* genome. Variegating P elements (grey triangles) carrying *white* transgenes (Red boxes) are shown within different silenced genomic loci. Genes controlling silencing at each region are indicated.

Silencing of the inserts within pericentric heterochromatin reflects one of classical heterochromatin silencing pathway, in which SU(VAR)3-9 mediated histone H3K9me2 modification provides the binding site for HP1, which in turn restricts SU(VAR)3-9 mediated H3K9me2 only to the pericentric heterochromatin. Furthermore HP1 also recruits SUV4-20 protein which mediates histone H4K20me3 modification for the stabilization of the heterochromatin assembly. Enhancer of Zeste [E(Z)] also contributes to pericentric heterochromatin formation by catalyzing histone H3K27me1, me2, and me3 modifications. Spreading of heterochromatic chromatin modification marks into euchromatin is prevented by the JIL1 Kinase mutation, *Su(var)3-1*. On the 4th chromosome instead of SU(VAR)3-9, SETDB1 acts as Histone H3 Lysine 9 methyltransferase, and provides the binding sites for HP1. None of the known SU(VAR)s are required for establishment of 4th chromosomal heterochromatin. Jil1 Kinase mutation *Su(var)3-1* are again required to prevent spreading of heterochromatin in the euchromatin.

Several independent insertions tagging HTT (Telomeric *HeT-A*, *TART* and *TAHRE* arrays) and TAS (Telomere Associated Sequences) region of *Drosophila* telomeres were collected. In previous studies none of the variegating inserts were identified within HTT region and it was assumed that HTT region are transcriptionally active and do not lead to the variegation of the reporter gene inserted within them (Mason *et al.*, 2002). However at least three variegating P

element insertion were identified within 3'UTR or within *HeT-A* ORF on X and 3R chromosome respectively, indicating silencing of these telomeric regions as well. On the contrary only two variegating inserts was mapped to the telomeric heterochromatin sequences, TAS, on the 3R chromosome arm. Another known insert within TAS region of 2L telomere was also included in the study. Altogether a genetic analysis of all these 5 independent telomeric inserts showed strong differences in the factors controlling gene silencing at each region of the telomere. 3'UTR region of *HeT-A* which has been shown to possess promoter activity is controlled by DNMT2 and maintained by SETDB1 mediated Histone H3K9me2 modification. HP1, SU(VAR)3-9, SUV4-20, E(Z) or JIL1-Kinase do not influence gene silencing at *HeT-A* promoter elements. Although an insertion within *HeT-A* ORF showed strong variegation in a heterozygous constitution, it was not affected by any of the tested *Su(var)* mutations, which indicates another yet unknown mechanism operating at *HeT-A* ORF sequences. It is known that TAS region of telomere vary strongly from one organism to other or even from one chromosome to other in the same organism (Pryde et al., 1997; Abad et al., 2004). In *Drosophila*, TAS region on 2L telomere is composed of unique 300bp satellite repeat sequences, while 2R and 3R TAS region consist of unique 381bp satellite sequences interspersed with defective *Invader4* LTR sequences. Genetic analysis of the variegating inserts within 2L and 3R TAS region showed that other than the differences in sequence composition, TAS regions also differ significantly in the molecular pathways controlling heterochromatin formation on different telomeres within a cell. Only the polycomb group protein SU(Z) has been shown to influence gene silencing on 2L TAS region (Crydermann *et al.,* 1999), while all other heterochromatin proteins showed no effect at all. DNMT2 and SUV4-20 seems to be the functional components of 3R TAS heterochromatin. Even though an enrichment of inactive histone modification marks like H3K9me2, and H3K27me2 has been shown (Yin and Lin., 2007), genetic data did not support any functional role of the proteins responsible for these modifications. Neither *E(z)* nor *Su(var)3-9* or *SetDB1* mutations showed any modifier effect on white variegated transgenes inserted within these sequences. Thus it can be suggested that DNMT2 mediated DNA methylation establishes gene silencing on *Drosophila* telomere, which is then maintained by SetDB1 mediated H3K9me2 in the HTT region and by SUV4-20 mediated H4K20me3 in the TAS region of at least 2R and 3R telomeres.

Several variegating inserts were mapped to *Invader4* retrotransposons on different chromosome arms. Genetic analysis of these inserts at different locations suggested a common DNMT2/SUV4-20 dependent gene silencing pathway. However several inserts

within other retrotransposon like *Doc* or *Copia* were not affected by loss of DNMT2 or SUV4-20 (Walluscheck; 2006), which indicate that local surrounding sequences might also play an important role in the assembly of the silencing machinery.

Formation of the facultative heterochromatin, tested by repeat induced silencing of *white* transgene, represented yet another unique pathway for gene silencing (Dorer and Henikoff, 1997). Genetic data reveals an important role of *Dnmt2* in the establishment of the silencing of these repeats. However unlike retrotransposon silencing, SETDB1 and HP1 seems to cooperate with DNMT2 for the formation of heterochromatin at these repeats.

Thus taken together at least three of five alternative gene silencing pathways identified in this work seem to be dependent on *Dnmt2*. Yet our current knowledge about *Dnmt2* is limited. Similar to SUV4-20, other functional interaction partner of DNMT2 could be identified on the basis of genetic tests. Several other unknown factors might corroborate together to read out a DNA methylation marks into histone methylations. Several yet unmapped weak or strong suppressors of *In(1)w*^{m4} PEV with unknown functions have been analyzed for their modifier effects on the variegation *white* transgenes inserted into the retrotransposon sequences. At least three of these *Su(var)* mutations were identified as dominant suppressors of the *white* inserts into the *Invader4* LTR sequences (Amit Sharma, personal communication). These Su(var)s could represent the functional interaction partners of *Dnmt2* mediated retrotransposon silencing pathways. Further molecular and genetic tests can be performed to identify other components of this newly identified silencing process.

Moreover, although present work identified five alternative gene silencing pathways, several other unmapped inserts which were not affected by any of the tested mutations indicates existence of several other unknown factors and gene silencing pathways in *Drosophila*. Material generated in this work can be utilized to classify these new silencing processes at different genomic location.

5. List of publications

Naumann K, Fischer A, Hofmann I, Krauss V, **Phalke S**, Irmler K, Hause G, Aurich AC, Dorn R, Jenuwein T, Reuter G. Pivotal role of *At*SUVH2 in control of heterochromatic histone methylation and gene silencing in *Arabidopsis*. *EMBO J*, 24, 1418-1429, 2005

Rudolph T, Yonezawa M, Lein S, Heidrich K, Kubicek S, Schäfer C, **Phalke S**, Walther M, Schmidt A, Jenuwein T, and Reuter G (2007) Heterochromatin formation in *Drosophila* Is initiated through active removal of H3K4 methylation by the LSD1 homolog SU(VAR)3-3. *Molecular Cell***,** 26**,** 103-115**,** 2007

Jurkowski, P, Meusburger, M, **Phalke, S**, Helm, M, Nellen, W, Reuter, G and Jeltsch, A Human DNMT2 methylates $tRNA^{Asp}$ molecules using a DNA methyltransferase-like catalytic mechanism. *RNA*, 14, 1663-1670, 2008.

Phalke, S., Nickel, O., Walluscheck, D., Hortig, F. and Reuter, G. Epigenetic control of retrotransposon silencing and telomere integrity in somatic cells of *Drosophila* depends on the cytosine 5 methyltransferase DNMT2**, Submitted** (2008).

Appendix 1. List of variegating P element inserts.

Insert name	P element	Chromosome	Genomic location
5-HA-1994	pP{RS5}	X	1A1; Telomere; Near *HeT-A*
5-HA-1902	pP{RS5}	X	1A1; *HeT-A*
5-HA-2098	pP{RS5}	X	Pericentric heterochromatin
p{9-5}		X	Pericentric heterochromatin
p{S-W-(S)1-67}		X	Pericentric heterochromatin
5-HA-5104	pP{RS5}	2	Unknown
5-HA-5105	pP{RS5}	2	Unknown
CB-6731-3	pP{RS3}	2	*Doc*
5-HA-1132	pP{RS5}	2L	Unknown
CB-5487-3	pP{RS3}	2L	24A1; 3606527 (in *odd* gene)
5-HA-2669	pP{RS5}	2L	38C1/2
5-HA-1504	pP{RS5}	2L	40C
39C5	pP{hsp26-pt-T}	2L	0.4kb Satellite repeat (Cryderman *et al*., 1999)
CB-0787-3	pP{RS3}	2L	33C1, *Invader4*; within *Jhl21*
CB-5329-3	pP{RS3}	2L	25F5*; Invader4*
5-SZ-3107	pP{RS5}	2R	*Invader4*
5-HA-2080	pP{RS5}	2R	42A
5-HA-5084	pP{RS5}	2R	43B1/2 (in *CG11107)*
5-HA-5106	pP{RS5}	2R	44D*; 1360{}786 Hoppel*
CB-6754-3	pP{RS3}	2R	42A *Doc{}772*
5-HA-1981	pP{RS5}	2R	*Rt1a*; 42B
5-HA-3b	pP{RS5}	2R	42B, *Hoppel 1360{}780*
pP{$K10^+$, w^+}51	pP{$K10^+$, w^+}	2L	36D, U
CB-0524-3	pP{RS3}	3L	75A4*; Invader4*
CB-5085-3	pP{RS3}	3R	89B7, *Invader4;* within *CG6072*
5-HA-1992	pP{RS5}	3R	100F; Subtelomeric, *Invader4*
CB-0686-3	pP{RS3}	3R	100F; Subtelomeric, *Invader4*
5-HA-2057	pP{RS5}	3R	*HeT-A*
CB-6857-3	pP{RS3}	3	U
CB-5350-3	pP{RS3}	3R	*Invader4*

5-HA-2055	pP{RS5}	3R	92A11, *CG16718*
CB-5708-3	pP{RS5}	4	Unique Sequence *CG32021,* Between *1360 Hoppel* and *INE-1*
UM-8050-3	P{RS5}	4	Unique Sequence CG11148
CB-5204-3	P{RS3}	4	Unique Sequence CG17964
118E10	pP{hsp26-pt-T}	4	101; Pericentric heterochromatin; *Hoppel* (Sun *et al*., 2000)
118E15	pP{hsp26-pt-T}	4	102F8 (Dyrk3 gene)
39C12	pP{hsp26-pt-T}	4	102B
5-HA-1172	pP{RS5}	4	*Invader4*
5-HA-1610	pP{RS5}	Y	*Invader4*
5-HA-1925	pP{RS5}	Y	*Invader4*
5-HA-1920	pP{RS5}	Y	*Invader4*
5-HA-1962	pP{RS5}	Y	*Invader4*
5-HA-1974	pP{RS5}	Y	*Invader4*
5-HA-1957	pP{RS5}	Y	*Invader4*
5-HA-1318	pP{RS5}	Y	*Invader4*
5-HA-1222	pP{RS5}	Y	*Invader4*
5-HA-1905	pP{RS5}	*Y*	*Micropia*
5-HA-2486	pP{RS5}	Y	Unknown
5-HA-2107	pP{RS5}	Y	*Copia*
5-HA-3278	pP{RS5}	U	*Invader4*
5-SZ-3522	pP{RS5}	U	*Invader4*
5-HA-1172 (305)	pP{RS5}	Y	Unknown
5-HA-1172 (300)	pP{RS5}	Y	Unknown
5-HA-1172 (20)	pP{RS5}	Y	Unknown
5-HA-1172 (74)	pP{RS5}	Y	Unknown
5-HA-1172 (53)	pP{RS5}	Y	Unknown
5-HA-1172 (159)	pP{RS5}	Y	Unknown
5-HA-3b2	pP{RS5}	Y	Unknown
5-HA-3b3	pP{RS5}	Y	Unknown
Fel1	pP{RS5}	Y	Unknown
Fel2	pP{RS5}	Y	Unknown
5-HA-2486	pP{RS5}	Y	Unknown

5-HA-2485(M295)	pP{RS5}	Y	Unknown
5-HA-2486	pP{RS5}	Y	Unknown
CB-0776-3	pP{RS3}	Unknown	Unknown

U = unannotated sequences

Appendix 2. List of primers

Primers used for molecular analysis of the P element mediated mutations	
Mt2_in_fwd	att gga tgg aca aat agt tgc
Mt2_in_rev`	cac cga caa cct tta cat taa tac
Mt2_fp1	cct gtg ctt ggt tat ctc ct
Mt2_fp2	tgc cac tca caa cgt gta gt
420Fwd1	att gct gtt ctg gtt gcc gct gc
420Rev1	acc taa aca gcg gac gag cag cc
420Fwd3	gat ggc ttg gct ttg gct gcg t
420Rev3	act gac ttc agt ggt cga agc atc aac g
420Fwd2	cca ctt gaa cca cca ctt gta tcg ag
420Rev2	ctt tac acc aag caa gcc cct ct
Mbdfwd	atg caa atg aac ccg agc
Mbdrev	tgt ctt gag tgc atc ctg c
mbdfwd1	tct agc atc act att gtg cca tcc g
mbdrev1	ctg agt tcg atc tgt tgc tgc tgg
mbdfwd2	cca tcc gca gta cgg cca
mbdrev2	gga gtt gga ttt ggt tta tta cac act agt

Primers used for RT-PCR, ChIP and Restriction analysis	
cMt2_rev	atg tcg acg ttt tat cgt cag caa ttt aat a
cMt2_fwd	ttg gtc gac tca tgc ctt taa ttg tga g
Mt2_RA_fwd	atg cgg ccg cat gcc ttt aat tgt
Mt2_RB_Fwd	atg cgg ccg cat gca tta tgc ctt
Mt2_RAB_REV	tag cgg ccg cat ttt atc gtc agc a
420Fwd4	gctgctcgtccgctgtttaggtc
420Rev4	gcaggtggcagtctgaatcgtagaagt
INVDR_fwd2	gat tgg cca aga cat att aga gct tgg
INVDR_rev2	gca tgt ttt cct tgc ttg tac gct c
Rov_gag_fwd	atg gca aca gca agc cct ata ata cta tc
Rov_gag_rev	gtt ccg aaa atc ctc caa aag ttg at
Rov_pol_fwd	cgg aca gca gaa act tcg aat agt cat aga
Rov_pol_Rev	ggt tgg ctt gag ata att tct cga aga ct
Gyp_env_fwd	tta aag gtt gtg gcg ggt act ccc
Gyp_env_rev	ctt caa gtc ggc atg atc aag aat tgt
Gyp_pol_fwd	gag att tgc ttc gaa aga gca cca
Gyp_pol_rev	agg aac acg aat gcc aag ata aag a
CopRNA_Fwd	gct ctt tta gcc gag caa gat gtg
CopRNA_Rev	ccc ttg aat att ttc ttt ggt tta gtt acc
DocGagFwd	cga gtg cag tga cct cac tgg caa ct
DocGagRev	tgt tcg gtc gca gaa tca gcc aa
DocRTFwd	acg gat atc tct gtg gaa cgc aaa t
DocRTRev	aat ggg tgt gct tgg cgt tat agt c
INVDR_FWD1	ttc tga tga aat tgt aat att gcg gta
INVDR_REV1	cac tga agg gat ctt ctt aca tgt cc
CopiaLTR1FWD	tgttggaatatactattcaacctac
CopiaLTR1REV	taattgtttattaggcatggactg

RP49_fwd	gat gac cat ccg ccc agc ata c
RP49_rev	agt aaa cgc ggt tct gca tga gc
RS5FWD3	tatagcgagcacagctaccagaataatctg
RS5REV3	aagagaactctgaataggtggatagcgtca
RS5Fwd4	tga cgc tat cga cct att cag agt tct ctt
RS5Rev4	ctt gca tgc ccc gtt att ctc tat tc
Tub_fwd	agcaaattacttgcagaattgg
Tub_rev	gattagtgcgattaggacttg
SatFwd	ata ggg atc gtt agc act gg
SatRev	att tta tta cga gct cag tga g
Inv4_511Fwd1	cgt cgt gtt cta aac tgt gct aga caa
Inv4_511Rev1	gtc ggc gaa aca gga gtc tca tcc ata tt

Primers used for Inverse PCR and FISH analysis	
5’ P fwd1	acc cac ttt aat gat tcg cag
5’ P rev1	ttt act cca gtc aca gct ttg
3’ P fwd1	ata cta ttc ctt tca ctc gca c
3’ P rev1	tgt cgt att gag tct gag tga g
5’ P fwd2	cac aca acc ttt cct ctc aac
5’ P rev2	acc agt ggg agt acc caa aca g
3’ P fwd2	acc agt ggg agt acc caa aca g
3’ P rev2	tga tta acc ctt agc atg tcc g
3RTelFwd1	gct gcc gca cca aat tac aaa ctg a
3RTelRev1	taa ttt ctt tta tac acc ggc aag aac aaa acg
InvIPCR1	aat gcg aac agt cac gct gcg ac
InvIPCR1	gag gca cga aca acc tct gca g
3RtelSatRev	gcaatgcttttacaccaaaaacagagaac
3RtelSatFwd	cgttttgttcttgccggtgtataaaagaaatta
3RtelFwd2	taa cga cgc gta cac ata tgt cgc agc gt
3RtelRev2	tgc ctgc aga ggt tgt tcg tgcctc

Appendix 3. Insertion sequences within *Dnmt2* ORF in *Dnmt2* alleles

Dnmt2^{149}
CATGATGAAATAACATATGTTATGTTATGGATATGTATATATATATGTTATTTCAT

Dnmt2^{147}
CATGATGAAATAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAAGTATA
CACTTAAATTCAGTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTGTGCGGACGAA
TTTTTTTTTGAAAACATTAACCCTTACGTGGAATAAAAAAAAATGAAATATTCAT

Dnmt2^{162}
CATGATGAAATAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAAGTATA
CACTTAAATTCAGTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTGTGCGGACNAA
TTTTTTTTTGAAAACATTAACCCTTACGTGGAATAAAAAAAAATGAAATATTGCA
AATTTTGCTGCAAAGCTGTGACTGGAGTAAAATTAATTCACGTGCCGAAGTGTGC
TATTAAGAGAAAATTGTGGGAGCAGAGCCTTGGGTGCAGCCTTGGTGAAAACTCC
CAAATTTGTGATACCCACTTTAATGATTCGCAGTGGAAGGCTGCACCTGCNAAAG
GTCAGACATTTAAAAGGAGGCGACTCAACGCAGATGCCGTACCTAGTAAAGTGA
TAGAGCCTGAACCAGAAAAGATAAAAGAAGGCTATACCAGTGGGAGTACACAAA
CNGAGTAAGTTTGAATAGTAAAAAAAATCATTTATGTAAACNATNACCTGACTGT
GCGTTAGGTCCTGGTCATTGGTTAATGGAAATGAGAGCTTGGGGGGAAAAATTCG
TACTTTGGAGTACNAATGCGTCCTTT

Dnmt2^{105}
CATGATGAAATAACACTAGAGGTCGGAGTACTGTCCTCCGACGCAAGGCGGAGT
ACTGTCCTCCGGGCTGCGGAGTACTGTCCTCCGGCAAGGTCGGAGTACTGTCCTC
CGACACTAGAGGTCGGAGTACTGTCCTCCGACGCAAGGTCGGAGTACTGTCCTCC
GACACTAGAGGTCGGAGTACTGTCCTCCGACGCAAGGTCGGAGTACTGTCCTCCG
ACACTAGAGGTCGGAGTACTGTCCTCCGACGCAAGGCGGAGTACTGTCCTCCGGG
CTGGCGGAGTACTGTCCTCCGGCAAGGGTCGAGGGTACCGAGAGAGCGCCNGAG
TATAAATAGAGGCGCTTCNTCTACGGAGCGACAATTCAATTTAAACAAGCAAAGT
GAACACGTCNAGATCTCTGNAGCCAAACTTTGNGTACTCCAAATTATTAAAAATA
AAACTTTAAAAATAAATTCGTCTAATTAATATTATGAGTTAATTCAAACCCCNCG
GA

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Acknowledgements

I am deeply indebted to my supervisor Prof. Dr. Gunter Reuter, not only for introducing me to the interesting field of *Drosophila* genetics but also for his motivating words, constructive criticism throughout and constant help in academic as well as personal affairs.

I would like to thank Dr. Rainer Dorn for all scientific discussions throughout my PhD work and Prof. Frank Lyko for kindly providing the DNMT2 specific antibody. I am very grateful to Dr. Andreas Fischer and Dr. Anja Ebert for their support during initial period of my PhD.

A special thanks to Amit and Deepak for their moral support throughout and great fun filled discussions during coffee sessions which made the journey more enjoyable. Many thanks to Sandy, Sandro, Manu, Christian, Claudia, Jessi, Kirsten, Janina and all the other members of Developmental Genetics group for all their support and providing a wonderful working atmosphere in the lab.

I am thankful to Diana, Olaf and Frank for embarking with me on this thesis journey. Their contributions have been of great value to the success of my thesis.

I also owe a lot to Maria for providing all the fly stocks whenever I needed them, to Mrs Abe for always being so prompt in providing the fly food whenever needed and to Katherine for teaching me the perfect Deutsch.

I have to say 'thank-you' to all my Indian friends in Halle especially Tushar, Ganesh, Ankush, and Nitin for always being there during all brights and blues.

I would like to take this as an opportunity to thank my parents, brother and sister for their everlasting love, moral support and inspiration to move ahead in life. It is their love and support for what I am today.

No words are sufficient to express my gratitude to my wife Priyanka who supported me in all the odds. Her constant love, unconditional support and positive criticism always helped me to take things in better stride. Also I would like to thank my daughter Gauri who kept me stress free, even after a very hectic day, just by her cute smile.

Last but not the least I thank DFG for the financial support and *Drosophila* without whom I would not have achieved anything.

SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Desweiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsverordnung. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrades.

Halle, den 23.09.08

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Sameer Phalke

STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I herby declare that I have not submitted this thesis material, either in full or in part, for a degree at this or any other institution.

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