Chromosome Research (2009) 17:603–619
DOI 10.1007/s10577-009-9054-x

X chromosomal regulation in flies: when less is more

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Abstract In *Drosophila*, dosage compensation of the single male X chromosome involves upregulation of expression of X linked genes. Dosage compensation complex or the male specific lethal (MSL) complex is intimately involved in this regulation. The MSL complex members decorate the male X chromosome by binding on hundreds of sites along the X chromosome. Recent genome wide analysis has brought new light into X chromosomal regulation. It is becoming increasingly clear that although the X chromosome achieves male specific regulation via the MSL complex members, a number of general factors also impinge on this regulation. Future studies integrating these aspects promise to shed more light into this epigenetic phenomenon.

Keywords X chromosome · MSL · *Drosophila* · transcription · chromatin · roX RNA · acetylation

Responsible Editor: Edith Heard.

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Abbreviations

AEL	After Egg Laying
CBD	Chromo barrel domain
CES	Chromatin entry site
DCC	Dosage compensation complex
DHS	Dnase hypersensitive site
HAS	High affinity sites
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ISWI	Imitation switch
LAS	Low affinity sites
MLE	Maleless
MRE	MSL recognition
MOF	Males absent on the first
MSL	Male specific lethal
NPC	Nuclear pore complex
NuRD	Nucleosome remodeling and deacetylase
NURF	Nucleosome remodeling factor
roX	RNA on X
SCF	Supercoiling factor
SXL	Sex lethal
UTR	Untranslated region

Introduction

Dosage compensation is an exquisite example of a chromosome wide phenomenon for regulating gene expression (Payer and Lee 2008; Meyer 2005; Straub and Becker 2007; Mendjan and Akhtar 2007). In

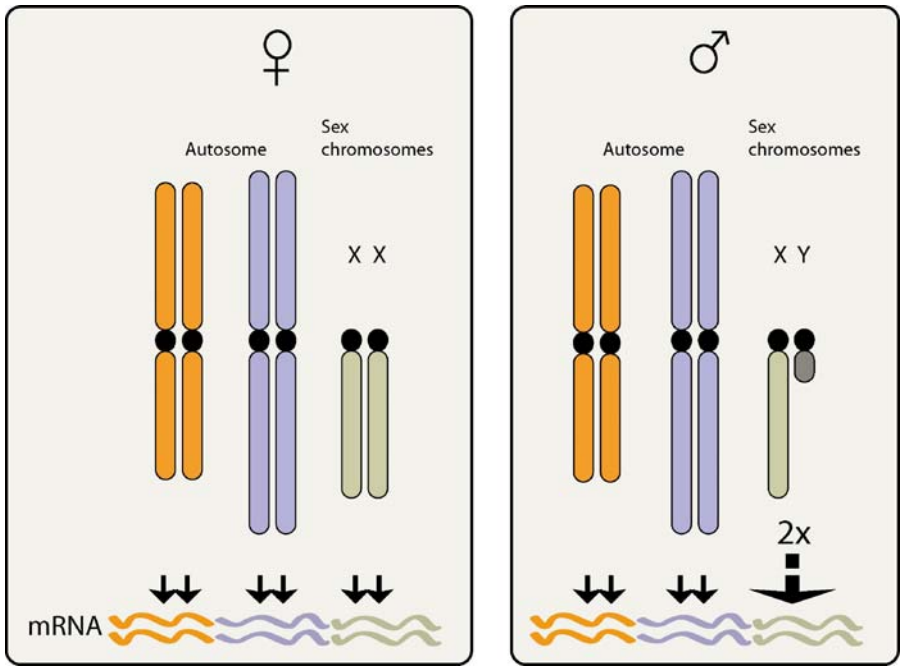

Fig. 1 Dosage compensation in *Drosophila melanogaster*. Female cells have two X chromosomes, while males have one X and one degenerated Y chromosome. The male X produces the same dose of RNA as the female to compensate for the absence of homologue

Drosophila this process involves upregulation of expression of X chromosomal genes in male flies by approximately two-fold in comparison to female flies which possess two X chromosomes (Fig. 1). In *Drosophila melanogaster*, dosage compensation is regulated by male specific lethal (MSL) factors whose product is essential for male survival. These factors are collectively called the MSL complex or the dosage compensation complex (DCC).

Domain architecture

Who interacts with whom?

MSL1 and MSL2

The MSL complex is a ribonucleoprotein complex that is composed of two long non-coding RNAs; roX1 or roX2 and at least five proteins namely MSL1, MSL2, MSL3, MOF and MLE (Fig. 2). All protein-coding genes are transcribed in both sexes (Kelley et al. 1995; Zhou et al. 1995; Gorman et al. 1995; Palmer et al. 1993; Kuroda et al. 1991; Hilfiker et al. 1997) however MSL2 mRNA translation is strictly inhibited by SXL, master sex regulator, in females (Beckmann et al. 2005). In the absence of MSL2, MSL1 protein is destabilized and presumably degraded because MSL1 protein is detected at very low level unless *msl2* is expressed ectopically in females (Kelley et al. 1995). MSL1 can be regarded as an assembly platform of the complex because it interacts with all other protein members, except for MLE (Scott et al. 2000). Leucine zipper like motif at the amino (N) terminus interacts with MSL2 (Li et al. 2005) and carboxyl (C) terminus binds MOF and MSL3 (Scott et al. 2000). MSL3 and MOF contact occurs on different parts of MSL1, MSL3 being close to the C terminus and MOF with PEHE domain (Morales et al. 2004). MSL1 also contains a coiled coil domain however the importance of this domain has not been determined. MSL2 has a RING finger domain along with a cysteine rich motif at its C terminus (Zhou et al. 1995). The RING domain has two zinc finger clusters and mutations of polar residues chelating the first zinc ion have been shown to disrupt the interaction of MSL2 with MSL1 (Copps et al. 1998). Although the RING finger is conserved in many species, the novel combination of RING domain and cysteine cluster has been proposed to have an important contribution for the birth of '*msl2* like' genes and a driving force for the formation of compensasome (Marín 2003). MSL3 has an MRG domain located at the C-terminus that mediates the interaction with MSL1 (Morales et al. 2005). MRG domains are highly conserved in MRG

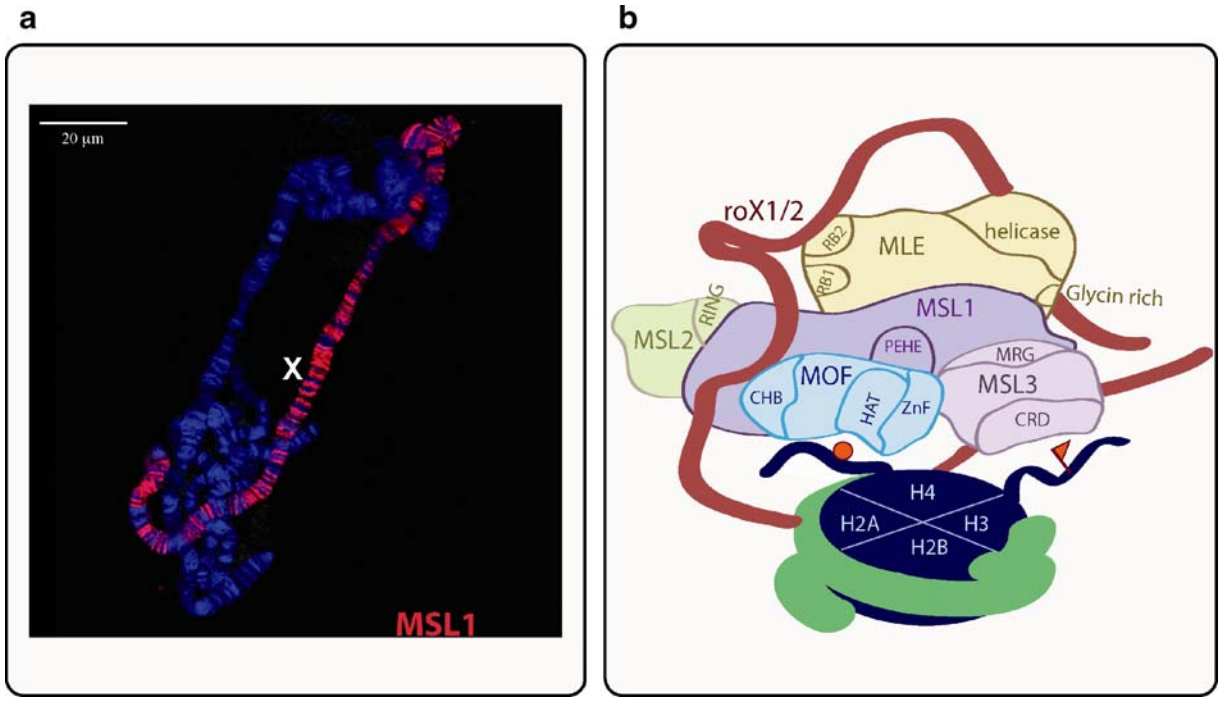

Fig. 2 MSL complex and known interactions; **a** MSL complex binds specifically to X chromosome in male cells. This is clearly seen in polytene squashes of salivary glands from third instar male larvae. DNA is stained with Hoechst and MSL1 is detected by specific antibody; **b** The MSL complex. MSL1 and MSL2 interact through RING domain of MSL2 and N terminus of MSL1. MOF chromobarrel (CHB) domain interacts with RNA. HAT (histone acetylase) domain acetylates H4K16 residue (represented by a red ball) and Zinc finger (ZnF) is important for the H4 specificity. MSL3 chromo- related domain (CRD) has been shown to bind to DNA and nucleosomes and been suggested to interact with tri-methylated H3 on K36 (H3K36me3 represented by red Flag). MSL3 and MOF bind MSL1 through ZnF and MRG domain, respectively. PEHE domain of MSL1 was shown to be crucial for MSL3 interaction. MLE has two RNA binding domains (RB1 and RB2) but only RB1 can bind RNA. Glycine rich region on the C terminus has a high affinity for RNAs. MLE could associate with the rest of the complex though RNA. The stoichiometry of the components and the mutual presence of roX RNAs are not known. The complex is not drawn to scale due to absence of any structural data therefore the figure must be seen as an artistic rendering of what is known

gene family and they are thought to be interaction platforms in large complexes that are usually chromatin related (Bowman et al. 2006). Interestingly the MRG domain of MSL3 is interrupted by non-conserved sequences and the importance of these MSL3 specific linkers are yet to be determined (Morales et al. 2005).

MOF and MSL3

The early observations of polytene squashes from the male larvae salivary glands revealed an interesting discovery that male X chromosome was enriched for a specific acetylation mark on the histone 4 lysine 16 (H4K16) (Turner et al. 1992). Observations of co-localization of this mark with MSL members and its absence in MSL mutants predicted a histone acetyltransferase (HAT) enzyme in the complex (Bone et al. 1994). Concordantly MOF, a member of MYST family of HATs, was shown to colocalize with MSL members and mark the male X chromosome on H4K16 (Gu et al. 1998, Hilfiker et al. 1997). MOF has a peculiar C2HC zinc finger motif, which mediates substrate recognition (Akhtar and Becker 2001), and its interaction with MSL1 (Morales et al. 2004). Although MOF binds to nucleosomes and its preferred substrate is H4 (Akhtar and Becker 2000; Smith et al. 2000), it can also acetylate MSL3 (Buscaino et al. 2003) and MSL1, nevertheless integration into the complex shifts the substrate specificity strongly to H4 (Morales et al. 2004). Both MOF and MSL3 have a chromo-related domain but accumulating evidence indicate that they may have different substrate specificities. Solution

structure of MOF chromo-related domain revealed that this fold has five beta strands forming a barrel shape, therefore named as chromo-barrel domain (CBD), which is structurally similar to Tudor domains. This fold is quite different from chromodomains that bind lysine methylated histone tails through a hydrophobic cage formed by three conserved aromatic residues (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002). These residues are not found in MOF and in fact Arg387 of CBD may clash with superimposed methylated lysine H3 peptide (Nielsen et al. 2005). Instead MOF chromo-barrel domain in the context of the full-length protein is important for RNA binding activity in vivo and in vitro and mutation of a conserved tyrosine disrupts this interaction (Akhtar et al. 2000). Nucleic acid binding activity of a chromo domain is not unique to MOF but also well documented for dMi-2 protein of NuRD remodeling-HDAC complex in *Drosophila* (Bouazoune et al. 2002). Curiously, the CBD used for structure determination was not able to bind RNA by itself leading to the hypothesis that the fold may need additional surrounding residues for RNA binding activity (Nielsen et al. 2005). Supportive evidence for this idea came from the structural elucidation of yeast homologue of MOF, Esa1 chromo-domain (Shimojo et al. 2008). Computationally predicted Esa1 chromo-domain folds similar to MOF CBD and does not show nucleotide binding activity in vitro however structure of N terminally extended domain has an extra beta sheet which induces a loop in the barrel. This "knotted" barrel has a high affinity for RNA in vitro (Shimojo et al. 2008). In the same vein, MOF chromo-barrel domain could adopt a slightly different form in the context of full protein such that it has a high affinity for RNA.

MSL3 chromo related domain is predicted to fold similar to chromobarrel domain but unlike MOF, it contains the typical hydrophobic residues that forms the aromatic cage (Nielsen et al. 2005). MSL3 has been shown to bind nucleosomes that are methylated on H3K36 and mutation of hydrophobic residues causes the loss of this interaction (Sural et al. 2008, Larschan et al. 2007). Besides, the chromodomain by itself can bind to a nucleosomal template (Buscaino et al. 2006). Chromodomain structures of Eaf3 and MRG15, yeast and human homologue of MSL3, were solved and showed to bind methylated H3K36 (Zhang et al. 2006a; Sun et al. 2008). Nevertheless, Eaf3 interaction is a rather weak one and was suggested to take assistance from PhD finger for the optimal binding (Xu et al. 2008; Li et al. 2007). MRG15 binding to H3K36me3 is predicted to be different from canonical chromodomains because a beta strand in chromo-barrel preoccupies the histone peptide-binding groove (Zhang et al. 2006a). Interestingly, MSL3 chromo-related domain can bind DNA in vitro suggesting that nucleosome interaction may occur partly through DNA (Buscaino et al. 2006). Additionally, MSL3 localization to X-chromosome is lost upon RNAse treatment and MSL3 can bind roX RNA both in vivo and in vitro (Morales et al. 2005; Buscaino et al. 2003) therefore nucleic acid binding surfaces can be used to bind both RNA and DNA. Acetylated MSL3 cannot immunoprecipitate significant amounts of roX2 RNA; hence this posttranslational modification may be used as a regulatory switch for the substrate specificity of MSL3 (Buscaino et al. 2003). Structure determination of MSL3 will delineate the properties of this peculiar domain.

MLE

The second known enzyme associated with the MSL complex is MLE. MLE bears a modified DEAD box motif, DEIH, which is one of the key signatures for RNA helicases (Kuroda et al. 1991). Mutations in the DEAD box and the ATP binding pocket are lethal for flies (Richter et al. 1996; Lee et al. 1997). MLE fulfills single stranded nucleic acid binding, double stranded nucleic acid binding, ATPase activity and homo-hetero duplex unwinding activities in vitro (Lee et al. 1997). It has two RNA binding domains in the N terminal region but only RB2 domain was shown to bind RNA (Izzo et al. 2008). RNA binding and deletion of C terminal glycine rich region increases the ATPase activity (Izzo et al. 2008). Therefore MLE may undergo continuous self-regulation through its own domains. MLE X chromosome localization is RNAse sensitive and it has a very salt susceptible, weak interaction with the rest of the complex, suggesting the possibility that it may bind MSL proteins through an RNA intermediate, presumably the roX RNAs (Copps et al. 1998; Richter et al. 1996). However till today, direct MLE interaction with roX RNAs have not been shown.

roX RNAs

RoX RNAs were discovered in two different enhancer trap screens; one to look for sex specific expression in

mushroom bodies of fly brains that causes dimorphic courtship behavior (Amrein and Axel 1997) and the other to look for differential expression of a reporter in mushroom bodies (Meller et al. 1997). These RNAs are male specific, confined to nucleus and expressed in all tissues of flies (Franke and Baker 1999; Amrein and Axel 1997; Meller et al. 1997). They both colocalize with MSL proteins along the X chromosome in males (Franke and Baker 1999). Although the big size and sequence difference, they are functionally redundant (Meller and Rattner 2002; Franke and Baker 1999). The only sequence similarity is a 30 bp sequence identity however deletion of this sequence has no phenotypic output (Kageyama et al. 2001). Many evidence suggest that roX RNAs exert their functions through a yet unpredictable tertiary structure or at least not in a strict sequence dependent manner. Additional to the inter-redundancy of roXs, there is also high intra-redundancy in each roX (Stuckenholz et al. 2003; Park et al. 2008). Successive ten percent deletions of roX1 and series of small deletions in roX2 do not change the male viability except for a region near 3′ end of roX1 that contains a predicted stem loop structure (Stuckenholz et al. 2003). Moreover, roX RNAs from other *Drosophila* species can be integrated into *D. melonagester* MSL complex in spite of the low sequence homology; 31% in the example of *D. willistoni* roX2 (Park et al. 2008). Recent findings suggest that there are evolutionary conserved "roX boxes" that may be the exchangeable functional units of rox RNAs (Park et al. 2008). Inarguably roX RNAs have physical contacts to the complex; they can be immunoprecipitated with MSL proteins and female expression of MSL2 causes stabilization of rox RNAs (Meller et al. 2000; Amrein and Axel 1997; Akhtar et al. 2000; Smith et al. 2000). Although, MLE, MOF and MSL3 have the capacity to bind roX RNAs, direct targets of roXs have not been determined but considering their size (roX1 3.7 kb and roX2 0.6 kb), it is plausible that they have several contact points. Interestingly, there is a time frame in the early hours of embryogenesis, where roX1 is transcribed in the absence of any detectable level of MSL components, but the rapid turnover of roX RNAs in the absence of the MSL complex led to the question how this stability is achieved. It was found that in the absence of maternal MLE, roX1 RNA is hardly detectable, therefore maternal stores of MLE can contribute to early stabilization of roX1 (Meller 2003).

Early events

What do females do?

The MSL complex formation is strictly inhibited in female cells, which is achieved by the master sex regulator, SXL protein. SXL is expressed only in females. In males, SXL pre-mRNA is spliced in a way that a premature stop codon is retained and the mRNA is degraded (Schütt and Nöthiger 2000). Many observations clearly showed MSL2 as the direct target of SXL. MSL2 mRNA has poly U stretches, binding sequences of SXL, in both UTRs and in males a 133 bp intron in 5′UTR, containing two of these binding sites are spliced out but retained in females (Bashaw and Baker 1995). Additionally transgenic constructs lacking poly U stretches enabled expression of MSL2 protein in females (Kelley et al. 1995). SXL modulates female specific intron retention by interfering with U2AF65 and U2AF35 snRNP interaction on the 3′ splice site (Merendino et al. 1999) and U1 recognition of the 5′ splice site (Förch et al. 2001). *Msl2* mRNA translation inhibition occurs a by a dual mechanism, one conducted through 3′UTR binding and inhibition of 43S recruitment and the second one through 5′ UTR binding and prevention of 43S pre-initiation complex scanning (Beckmann et al. 2005; Grskovic et al. 2003; Gebauer et al. 2003; Kelley et al. 1997). 3′UTR control of SXL requires a co repressor, UNR, which is present in both male and female cytoplasm but is specifically recruited to the *msl2* mRNA 3′UTR by SXL in females (Duncan et al. 2006; Abaza et al. 2006; Grskovic et al. 2003). Interestingly, UNR was recently found to have an important role in male dosage compensation (Patalano et al. 2009). Overexpression of UNR causes a preferential male lethality and loss of the MSL complex from the X chromosome. UNR also immnoprecipitates roX1 and roX2 however if this is a direct or indirect interaction has not been shown (Patalano et al. 2009).

Somatic versus germ line

Most of the available data on dosage compensation comes from observations of somatic cells, therefore much less is known how the male germ line deals with the dose problem. MLE had been known to function in spermatogenesis and consistently it can be

detected in male germ line cells (Rastelli and Kuroda 1998). Interestingly MSL1, MSL2 and MSL3 are not observed in these cells and MLE, along with H4K16 acetylation, is not concentrated on X but rather scattered throughout the genome (Rastelli and Kuroda 1998; Rastelli et al. 1995). Nevertheless, expression microarray analysis showed that male germ line do compensate for the imbalance despite the absence of the MSL complex although the number of escaping genes are higher than those on the somatic cells (Gupta et al. 2006). The possibility of an MSL independent dosage compensation mechanism was pointed out before because *msl* mutant males can complete embryogenesis, survive up to third instar larvae—early pupae and additionally some genes are compensated in the absence of MSL, like *runt* (Baker et al. 1994). This mechanism could be the result of a complex buffering system inherent in genetic networks or another uncharacterized protein complex may function in the early dosage compensation (Zhang and Oliver 2007). Although the nature and the timing of an MSL independent mechanism is elusive, it appears that MSL mediated activation begins at blastoderm, coinciding with zygotic transcription start in embryos (Meller 2003; Franke et al. 1996; McDowell et al. 1996). In males with homozygous *msl1, mle* or *msl3* mutant mothers, the onset of MSL detection on X chromosome is delayed; supporting the idea that maternal contribution may help balancing the low level of zygotic expression in initial stages of the MSL complex establishment (Franke et al. 1996; Rastelli et al. 1995). Initiation of dosage compensation relies on the expression of one of the roX RNAs (Meller 2003). In males, roX1 RNA transcription starts in the early stages of blastoderm (2 h After Egg Laying, AEL) and MSL2 localization to nuclei foci follows after. When roX1 is absent, MSL2 localization to the nuclear foci can only be seen after roX2 expression, which is nearly 6 h AEL. This may indicate that roX transcription may guide the MSL complex to the X chromosome.

Targeting the male X chromosome

Single gene versus global analyses

The advances of new technologies such as expression-arrays, high resolution tiling arrays and new generation sequencing technologies coupled with biochemical methods gave a totally new pace in understanding the mechanism of dosage compensation in *Drosophila*. MSL-chromatin interactions had often been carried out in polytene squashes of salivary glands by immunofluoresence but the resolution of this technique is low, therefore one big leap in the field occurred when MSL components were mapped in high resolution throughout the *Drosophila* genome by ChIP on chip method (Kind et al. 2008; Gilfillan et al. 2006; Legube et al. 2006; Alekseyenko et al. 2006; Alekseyenko et al. 2008; Straub et al. 2008). Although the immunoprecipitated proteins, cell type and embryonic stage are different in each case, common themes arose. First of all, not all genes on X are bound by the MSL complex and also there are a few autosomal sites that are clearly bound. The MSL complex members are mostly found on genes rather than intergenic sequences and when the binding profiles are averaged, a clear enrichment in the body and towards the end of the genes are observed (Gilfillan et al. 2006; Alekseyenko et al. 2006) **(**Fig. 3**)**. Most target genes seem to be actively expressed however there is no significant correlation between the expression level and MSL abundance (Legube et al. 2006; Alekseyenko et al. 2006). Although most MSL target genes are actively transcribed, transcription *per se* is not sufficient to explain MSL binding because many genes that are bound by elongating form of RNAPII and canonical elongation factors are devoid of the MSL complex (Gilfillan et al. 2006; Legube et al. 2006). Also, MSL1 binding profiles of 4–6 embryos and third instar larvae salivary gland are fairly similar (Legube et al. 2006), supporting the notion that most compensated genes are selected early during development and bind irrespective of developmental changes (Kotlikova et al. 2006). Remarkably, MOF seems to have a unique status in the complex (Kind et al. 2008). Promoter binding of MOF is distributed throughout the whole genome in both males and females in an MSL independent manner whereas 3′ enrichment is restricted to the X chromosome in males and is MSL dependent (Kind et al. 2008). This led to the hypothesis that MOF plays an important role on the promoters of both sexes, and the MSL complex members binds this HAT to skew its location towards the end of the genes for specific acetylation and up-regulation of male X chromosome (Kind et al. 2008). Realization of 3′ enrichment of the MSL complex members stimulated investigation of

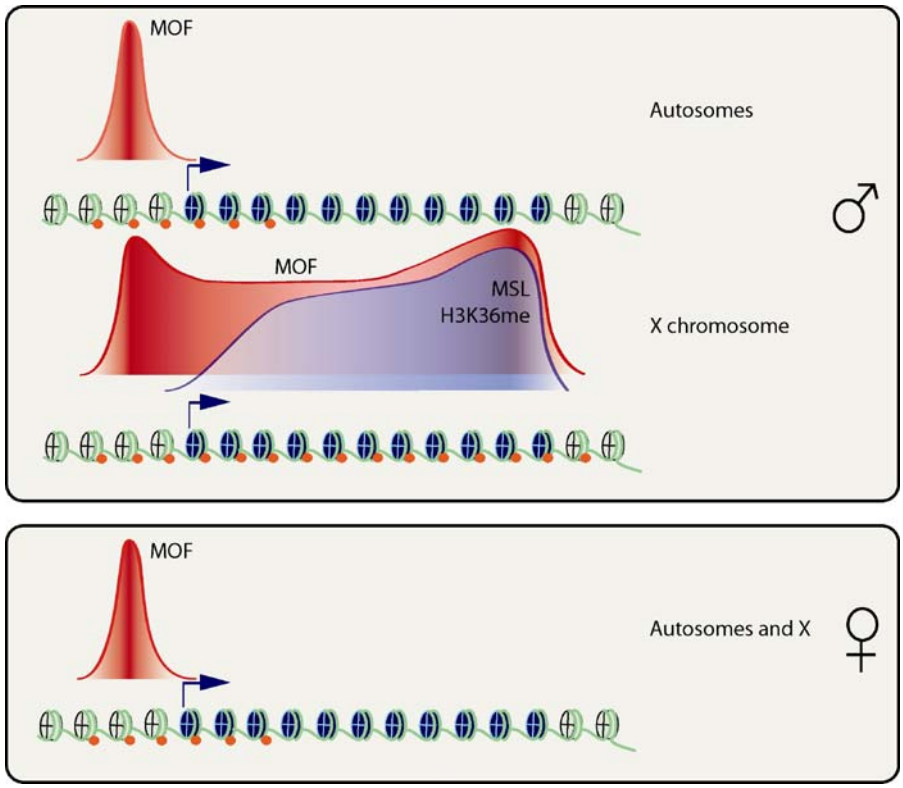

Fig. 3 Global profiles of MSL components and associated histone marks averaged on a single transcriptional unit. In male cells, MSL components are enriched in the body of the genes peaking at the end along with the H3K36me3 mark. MOF shows a distribution peaking at promoters and at the 3′end of the genes. MOF peaks at the promoters are independent of MSL complex and also found in female cells and autosomal genes in males. H4K16 acetylation distribution tends to be broader in comparison to MOF distribution on target genes. Red balls indicate histone acetylation and transcriptional unit nucleosomes are depicted in blue

associations to other well-known 3′ enriched epigenetic marks. Genome wide histone modifications from yeast and humans showed that Set2 dependent H3K36me3 is a conserved 3′ bias epigenetic mark associated with active genes (Barski et al. 2007; Pokholok et al. 2005). Mapping of H3K36me3 on X chromosome revealed that more than 90% of MSL targets are also enriched with this mark and there is a high correlation of MSL and H3K36me3 position on the gene (Larschan et al. 2007; Bell et al. 2008b). Interestingly, there seems to be a context dependent crosstalk between H3K36me3 and H4K16ac because reduction of Hypb, the enzyme required for final methylation state of H3K36 in *Drosophila*, causes a reduction of H4K16 acetylation mark on X-linked genes but not on autosomes (Bell et al. 2008b).

High affinity sites

One of the most obvious questions of dosage compensation is how the MSL complex recognizes the X chromosome specifically. An intriguing feature of the MSL complex is that partial complexes of MSL1 and MSL2 are able to bind 30–40 bands in polytene squashes in the absence of other MSL3, MOF or MLE, which have been named as Chromatin Entry Sites (CES) (Palmer et al. 1994; Lyman et al. 1997). MSL1 and MSL2 behave as the core of the complex because they are strictly dependent on each other for stability and they can localize to X without the other partners (Lyman et al. 1997; Kelley et al. 1995; Zhou et al. 1995). Surprisingly, roX1 and roX2 sites were the first CES to be mapped, due to their ability to recruit MSL components upon translocation to an autosomal site (Meller et al. 2000; Kelley et al. 1999). This feature of roX genes does not depend on their transcription but on a DNAse hypersensitive site (DHS) that can bind the MSL complex (Park et al. 2003; Kageyama et al. 2001). Initially, CES were thought to be the only sites for early MSL binding however, investigation of large X to autosome translocations showed that any segment of X was able to recruit MSL complexes even if they do not possess a previously mapped CES (Fagegaltier and Baker 2004; Oh et al. 2004). Moreover, translocated genomic segments from autosomes to X were devoid of MSL complexes (Oh et al. 2004). Identification of other MSL1 binding fragments by ChIP showed that only a subset of these fragments are able to recruit MSL1/2 when moved to autosomes, and the rest can do so

only in the presence of over expressed MSL1/2 (Dahlsveen et al. 2006). Therefore, X chromosome seems to have a gradient of potential to recruit the MSL complex, named as the "affinity model" (Fagegaltier and Baker 2004). Some sequences can recruit MSL independent of any apparent targeting determinant, called High Affinity Sites and other sequences, Low Affinity Sites, can only do so by the help of other mechanisms. The cipher of high affinity sites had remained a mystery due to absence of advance sequence algorithms and low number of mapped sites. But high throughput experiments discovered important clues about this mystery. High-resolution binding profiles of MSL1 and MSL2 in the absence of other MSL components revealed more than 130 Chromatin Entry sites (CES) or High Affinity sites (HAS) and a GA rich motif named as MRE motif (MSL Recognition) in these sites (Straub et al. 2008; Alekseyenko et al. 2008). Remarkably, this motif is slightly enriched on X chromosome and autosomal transposition of a minimal CES, containing as few as three MRE elements was able to recruit MSL, and up-regulate the upstream reporter gene (Alekseyenko et al. 2008). Since there are thousands of similar motifs scattered around the *Drosophila* genome, the choice of X chromosome still remains an unsolved issue. However, H3 depletion around the CES site indicates that accessibility could be an important player (Straub et al. 2008; Alekseyenko et al. 2008).

The importance of roX RNAs for the initial targeting have been shown in a number of cases (Ilik and Akhtar 2009). MSL protein complex *per se* have a weak affinity towards the chromatin but this is greatly enhanced with the presence and/or integration of roX RNAs (Li et al. 2008; Oh et al. 2003; Meller and Rattner 2002). In the absence of both roX RNAs, partial MSL complex can be located on a few X chromosome loci, autosomal loci and chromocenter (Li et al. 2008; Oh et al. 2003; Park et al. 2002; Meller and Rattner 2002). These sites have the intact complexes because all MSL proteins and H4K16 acetylation are seen coincidently (Meller and Rattner 2002; Oh et al. 2003). Moreover, overexpression of MSL1 and MSL2 can rescue male lethality to some extent (Oh et al. 2003). Nevertheless, there are opposing reports claiming that even though roX mutants are so severe that allows no detection by any means, they may still contribute to targeting and dosage compensation (Deng and Meller 2009; Deng et al. 2005). Therefore the role of roX RNAs in targeting is still an open issue.

Is the presence of entry sites on roX genes a coincidence? Compelling evidence suggest that the complex forms on the site of roX transcription in a co-transcriptional manner (Kelley et al. 2008; Oh et al. 2003). It is plausible that roX genes have acquired an entry site to establish the complex formation more efficiently. On the other hand, roX1 DHS was shown to play a role in roX1 transcription activation in males and MSL2 was shown to be important for this role as an independent task from the MSL complex (Bai et al. 2004; Rattner and Meller 2004). Similarly roX2 gene was found to have elements that bind MLE and regulates its transcription (Lee et al. 2004). Therefore, a complex regulatory network that contains components of the MSL complex may fine tune roX transcription and eventually formation of the MSL complex.

Beyond the high affinity sites

How is the complex located further from the high affinity sites? An intriguing observation upon roX gene translocation to autosomes was the spreading of the complex from the site of insertion in cis (Meller et al. 2000; Kelley et al. 1999). This spreading depends on the site of insertion, amount of the MSL complex, and presence of the competing roX transgene (Park et al. 2002). Because other large X to autosome translocations did not show any cis spreading, roX situation was pointed to be a unique feature of roX genes due to their function as the site of complex formation. (Fagegaltier and Baker 2004). MOF enzymatic activity is required for localization to low affinity sites but it is not known if this is due to its canonical histone acetylation activity or another protein acetylation event that may help maturation of the MSL complex (Buscaino et al. 2003; Gu et al. 2000). MLE helicase activity is also found to be important for LAS localization (Morra et al. 2008). Since MLE is required for the roX association into the complex (Meller et al. 2000), the phenotype can be a downstream result of an incomplete complex that is not able to spread further.

Enrichment of H3K36 methylation on actively transcribed genes prompted investigation of MSL3 as it contains a chromo related domain that could be a good candidate that can bind to this mark. By ChIP-chip analysis, it was seen that MSL3 chromodomain mutants, including the deletion mutant were enriched around the

high affinity sites suggesting the requirement of intact chromo domain for spreading (Sural et al. 2008). In view of these results, Sural et al, proposes a two-step model, in which sequence dependent initial targeting to X chromosome is established on MRE containing chromatin entry sites and is followed by H3K36me3-chromodomain mediated spreading, analogous to heterochromatin spreading (Sural et al. 2008). Surprisingly, transgenic flies carrying the chromodomain mutants have a range of phenotypes ranging from fully viable to developmentally delayed to severely affecting male viability (Sural et al. 2008; Buscaino et al. 2006). This already suggests that chromodomain mediated spreading from high affinity sites cannot be the sole mechanism for the MSL binding pattern throughout the X chromosome. Transcription was noticed as a good candidate inferred from the high correlation between active transcriptional state and MSL binding. Indeed when MOF gene, a low affinity site, is translocated to an autosomal site, it can recruit MSL1 only in the presence of an endogenous or an exogenous promoter (Kind and Akhtar 2007). When the promoter is absent, blocked or reversed, MSL1 cannot bind. Effect of transcription extends beyond the MOF gene because blocking of RNA polymerase II by alpha amanitin decreases the occupancy MSL components on X-linked genes (Kind and Akhtar 2007). Binding of MSL1 after a strong activation of X linked genes by a Gal4 induced promoter had also been observed before (Sass et al. 2003). The passage of RNA Polymerase II may either expose targeting sequences that are normally hidden or it can change the chromatin marks such that the gene becomes a better target for the MSL complex. All these models are not mutually exclusive and genes may have evolved different strategies to recruit MSL depending on their need to compensate, their inherent affinity towards MSL or their plasticity during the development. One attractive possibility could be that transcription in combination with MSL proteins that recognize chromatin marks on active genes could facilitate spreading along the X chromosome.

Evolutionary considerations

Dosage compensation arises as an inevitable consequence of sex chromosome evolution. In *Drosophila* species, the dose problem begins with the evolution of Y chromosome (Carvalho 2002). Although the exact nature of Y chromosome formation is under debate (Carvalho 2002) the current model predicts the random acquisition of a male determining gene on an ancient autosome and prevention of recombination of that locus (Steinemann and Steinemann 2005). This leads to a strong tendency to accumulate degenerative mutations like transposition, duplication and finally the heterochromatinization of the whole chromosome (Steinemann and Steinemann 2005). Therefore the male cell nucleus is forced to balance this hemizygocity by formation of a novel complex acting on the X chromosome. There are plethora of evidence that once the MSL complex evolved, it was co-opted in other *Drosophila* species, which have a different sex chromosome history (Marín et al. 1996). In *D. pseudoobscura*, a fusion event between an autosomal chromosome (Muller D element) and original X chromosome (Muller A element) led to formation of a metacentric X chromosome and the similarization of both arms in terms of sequence identity (Gallach et al. 2007). Interestingly, the autosomal homolog of D element is lost in males and the newly translocated hemizygous arm is bound by MSL complex and acetylated on H4K16 (Bone and Kuroda 1996). On the contrary, in *D. americana americana*, a similar fusion event occurred but the males kept the autosomal homologue (Neo-Y) chromosome (Charlesworth et al. 1997). Since the two homologues can still recombine, there is no sign of degeneration on the neo-Y and no MSL binding on the X chromosome (Bone and Kuroda 1996).

Perhaps the best tool to study the evolution of sex chromosomes and dosage compensation is the neo-sex chromosomes of *D.miranda*. *D.miranda* is closely related to *D. Pseudoobscura* and has the same metacentric X chromosome, which is fully dosage compensated. But in addition, a Robertsonian translocation of an autosome (Muller C element) to the Y chromosome generated a neo-Y chromosome (Macknight 1939). The fusion event is thought to occur about 2 million years ago and the homologue pair is kept in the male cells (neo-X chromosome) (Norman and Doane 1990). After the fusion event, neo-Y chromosome had undergone extensive random degeneration, including retrotransposition, duplication and nonsense mutations but most loci are still intact (Bachtrog 2006). Amazingly, the neo-X chromosome recruits MSL complex and acetylates H4K16 to the loci that are degenerating in the neo-Y homologue (Fig. 4) (Marín et al. 1996, Bone and

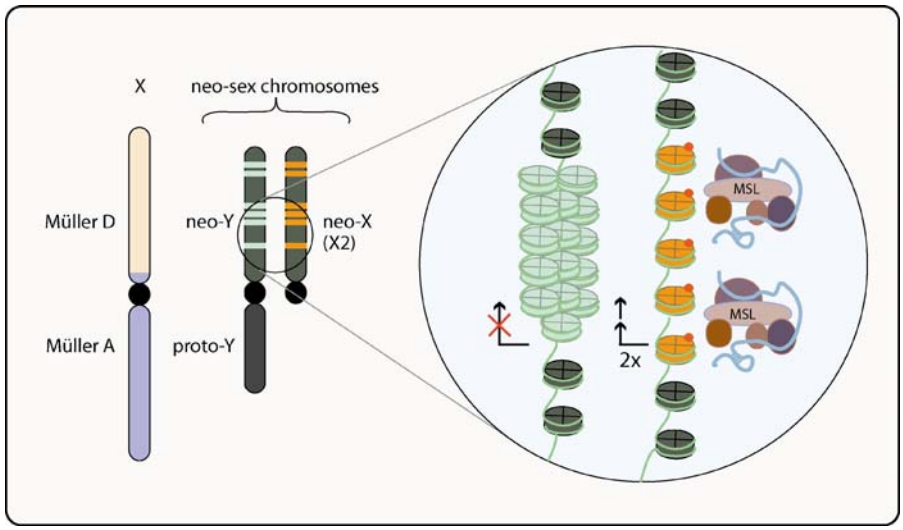

Fig. 4 Dosage compensation in *Drosophila miranda*. In male cells the proto X chromosome, (which is indicated by Muller A +Muller D) is compensated normally as in D.*melanogaster*. A translocation event of an autosomal arm onto the proto-Y chromosome created a neo-Y chromosome, which is in the process of degeneration. The degenerated loci are indicated as light colored bars. The autosomal homolog of neo-Y, also called neo-X or X2, shows upregulation at the loci in which there is degeneration on neo-Y. The upregulated loci are indicated in orange color and they correspond to similar loci as in the neo-Y. A hypothetical magnifier to one of these loci is shown. The degenerated loci on the neo-Y go under heterochromatinization due to retrotransposition and/or other means of molecular events leading to hemizygosity. In the homologous region on neo-X chromosome, the MSL complexes are recruited and upregulate the genes for dosage compensation

Kuroda 1996). Interestingly, the core promoters of the *lcp1-4* genes that are upregulated in the neo-X in response to degeneration in neo-Y show no apparent significant sequence alteration that could lead to two-fold upregulation (Steinemann and Steinemann 2007). Moreover, significant sequence variation of the neo-X chromosome from the old X shows that multiple selective sweeps of cis-acting regulatory regions did not occur (Yi et al. 2003). These results show that MSL complex recruitment may not require a strict gene-by-gene basis cis-acting sequence evolution.

Since dosage compensation in *Drosophila* is an old problem and various subgroups use the same complex to cover up hemizygousity, MSL components are expected to be under stabilizing (purifying) selection. But recent experiments show that even two closely related *Drosophila* species, *D. melanogaster* and *D. simulans* that diverged 2.5 million years ago have highly asymmetric rapid evolution of MSL genes (Rodriguez et al. 2007). Interestingly the MOF acetylation site on MSL3 in *D.melanogaster* is unique in the *Drosophila* species (Kelley 2004). It is possible that there are other selective forces that are acting on the MSL complex of *D.melanogaster* like the male killing bacteria *S.poulsonii* (Veneti et al. 2005). MSL proteins could be evolving away from recognition by these bacteria. Nevertheless since the protein complexes tend to coevolve; the whole complex could be trying to fine tune to escape from selection while also trying to do its essential function. Curiously, MOF has been shown to bind LTR retrotransposons in *D.melanogaster* and inhibit their transposition (Matyunina et al. 2008). Inhibition of retrotransposition could also be a strong selective force on this subgroup. These recent findings can provide an explanation why it is difficult to find a consensus sequence for the MSL binding among other species.

MSL-like proteins in other organisms

The protein components of the MSL complex of *D. melanogaster* have clear homologues from yeast to mammals, except that MSL1 and MSL2 are not found in yeast (Marín 2003). Yeast NuA4 HAT complex contains Esa1 and Eaf3, homologues of MOF and MSL3 respectively (Eisen et al. 2001). However, stringent sequence analysis showed that yeast NuA4 complex is not the direct ancestor of compensasome in *Drosophila*, rather a novel complex arose with the concomitant evolution of MSL1 and MSL2 (Marín

2003). Although humans have entirely different strategy for the dosage compensation problem, the MSL complex is kept in mammals as well (Mendjan et al. 2006a; Smith et al. 2005; Taipale et al. 2005). However it seems that human MSL complex has evolved other functions in DNA damage response pathway and inhibition of tumor genesis (Gupta et al. 2005; Taipale et al. 2005; Smith et al. 2005). Recently it was shown that hMSL2 is responsible for the mono-ubiquitination of p53 and subsequent extra nuclear localization (Kruse and Gu 2008). Even though mammals shows X-inactivation in female nucleus, the active homologue of X is two-fold up regulated to reach the stoichiometry of autosomal gene expression level (Nguyen and Disteche 2006). The role of the MSL complex in this upregulation is a tempting hypothesis.

How general factors may impinge on the mechanism

One of the first issues addressed by high throughput experiments was the global regulation of expression on X by the MSL complex. A significant amount of earlier data proposed an alternative mechanism named inverse dose model for the X versus autosome balance (Bhadra et al. 2005; Bhadra et al. 2000; Bhadra et al. 1999). This model suggests that X chromosome inherently possesses sequences that recruit transcription factors for a roughly two fold up-regulation and the MSL complex functions to titrate MOF from autosomes to X to inhibit over-expression of autosomes by overriding the effects of hyper-upregulation of X (Birchler et al. 2003). RNAi against the components of the MSL complex and stringent normalization analysis of expression arrays showed that the MSL complex was indeed acting to up-regulate X-linked genes to the autosomal levels arguing against the inverse dosage model (Straub et al. 2005; Hamada et al. 2005). Moreover artificial recruitment of the MSL complex upstream of a reporter gene can cause its up regulation and roX autosomal transgenes can overcome silent heterochromatin (Henry et al. 2001; Kelley and Kuroda 2003).

Specific enrichment of X chromosome by H4K16 acetylation led to the belief that this canonical activation mark could be largely responsible for the up regulation. Indeed MOF can activate transcription in vivo and in vitro (Akhtar and Becker 2000). Moreover H4K16 acetylation can decondense 30 nm chromatin fiber in vitro (Shogren-Knaak et al. 2006). A simple prediction was that opening the chromatin might enable loading more polymerase RNA Polymerase II onto compensated genes. However, polymerase profiles show that there are not more polymerases on compensated genes than non-compensated ones (Gilfillan et al. 2006). Recent findings of new components related to dosage compensation tell us that the story may be much more complicated.

Identification of nuclear pore components, Nup153 and Mtor, in MSL purifications, and their effect on X linked gene expression suggest a link between dosage compensation and nuclear architecture (Mendjan et al. 2006b). Numerous findings indicate that position of a gene in the nuclear volume could affect its transcriptional status (Deniaud and Bickmore 2009; Branco and Pombo 2007; Akhtar and Gasser 2007). Although nuclear periphery was long known accepted as a repressive zone and a host for heterochromatin, nuclear pore complexes (NPC) can be a docking site for an induced gene (Köhler and Hurt 2007; Brown and Silver 2007). Close approximation of the X-linked genes to NPC may create a transcription competent domain/environment and may even provide up regulation of genes that are not bound by MSL but still dosage compensated. Interestingly, human interphase chromosomes are found to be associated with lamins in domains that are clearly demarcated by insulators showing that the genome can indeed be organized in discrete structures under the nuclear envelope (Guelen et al. 2008).

Another protein found to be associated with MSL is the JIL-1 kinase. JIL-1 can co-immunoprecipitate with MSL components and it is enriched on male X chromosome, although it is also distributed on other chromosomes (Jin et al. 2000). JIL-1 is the main kinase that is responsible for H3S10 phosphorylation (Wang et al. 2001). Although this mark was known to be a mitotic marker, it is also enriched in euchromatic regions and can antagonize heterochromatin spreading (Zhang et al. 2006b; Wang et al. 2001). Recently JIL-1 was shown to be an important activator in many genes in *Drosophila* and can relieve the promoter proximal pausing of RNAPII, which is thought to be a checkpoint after the initiation of transcription (Ivaldi et al. 2007; Saunders et al. 2006). Conceptually, selective recruitment of JIL-1 kinase by MSL to the X-linked genes may relieve this pausing more than autosomes and female X; helping twice as much

transcription on male X. Albeit this attractive hypothesis, an opposing experiment demonstrated that RNA Polymerase II mediated transcription is independent of H3S10 phosphorylation and JIL-1 kinase affects transcription through maintaining the structural integrity of the chromosomes (Cai et al. 2008).

It seems that the male X chromosome is generally more sensitive to perturbations related to proteins that are responsible for general chromatin morphology. Two of these proteins are NURF, a chromatin remodeler, and Su(var)3–7, a protein responsible for heterochromatin formation by the help of HP1 (Spierer et al. 2005; Deuring et al. 2000). NURF is the founding member of ISWI family of remodelers and it contains ISWI protein as the catalytic subunit that enables sliding of nucleosomes (Bouazoune and Brehm 2006). Normally ISWI is not enriched on the male X chromosome or its mutations do not cause mislocalization of the MSL proteins, or the acetylation. Nevertheless, the male X chromosome looks much decondensed and broader in its absence and a functional MSL is required for this phenotype (Corona et al. 2002; Deuring et al. 2000). ISWI protein is also found in other complexes however this effect is related to NURF remodeler because aberrant phenotype of male X is repeated in NURF301 mutations, the main scaffold in NURF complex (Badenhorst et al. 2002). Recently it was found that roX null mutation could suppress the puffy appearance coming from the NURF mutations. Additionally, NURF can repress roX2 transcription in females (Bai et al. 2007). Similar to ISWI, Su(var)3–7 mutation causes male X chromosome decondensation, which can be suppressed by null MLE mutation (Spierer et al. 2008; Spierer et al. 2005). These antagonistic relations suggest that chromatin opening is not unchecked but is actually scrutinized by various complexes to maintain a sufficiently open—not more than necessary—state of chromatin. Actually, an analog system can be seen in a smaller scale on actively transcribing genes. Active genes have an increasing H3K36 di and trimethylation on the body of their genes and this mark is recognized by an HDAC complex, Rpd3S, which inhibits spurious transcription that may come from cryptic promoters (Carrozza et al. 2005). Interestingly, Rpd3S, the histone deacetylase and Set2, the enzyme required for H3K36 methylation play a role in dosage compensation (Buscaino et al. 2003; Bell et al. 2008a; Sural et al. 2008). Curiously, the components of the exosome, Dis3 and Rrp6, also copurify with MSL proteins (Mendjan et al. 2006b) suggesting that RNA degradation may be also be coupled to the system. In this sense exosome may degrade, antisense or cryptic transcripts that were generated uncontrolled due to open chromatin structure. Another fail-safe mechanism could be mediated by Supercoiling factor, of which genetic interaction with MSL has been shown (Furuhashi et al. 2006). Supercoilig Factor (SCF) was hypothesized to help decreasing the helical torsion that may have generated during chromatin remodeling however its role in dosage compensation is not determined yet (Furuhashi et al. 2006).

All these observations suggest that capabilities of MSL reach far beyond than expected before. Not only it behaves as a HAT complex but also acts as a mediator that fine tunes two fold upregulation by approaching to nuclear pore, cross talking with chromatin remodelers, heterochromatin proteins, and RNA degradation machines. Although the dazzling discoveries brought by powerful genetics, biochemistry and high throughput approaches, the new findings bring about their own mysteries the near future will be full of surprising discoveries.

Acknowledgements We are very grateful to Boryana Petrova for preparation of figures and Thomas Conrad for providing the polytene squash picture. We thank Kent Duncan and members of the lab for critical reading of the manuscript and helpful discussions.

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