X-chromosome targeting and dosage compensation are mediated by distinct domains in MSL-3

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In *Drosophila*, dosage compensation of X-linked genes is achieved by transcriptional upregulation of the male X chromosome. Genetic and biochemical studies have demonstrated that male-specific lethal (MSL) proteins together with roX RNAs regulate this process. Here, we show that MSL-3 is essential for cell viability and that three domains in the protein have distinct roles in dosage compensation. The chromo-barrel domain (CBD) is not necessary for MSL targeting to the male X chromosome but is important for male viability and equalization of X-linked gene transcription. The polar region cooperates with the CBD in MSL-3 function, whereas the MRG domain is responsible for targeting the protein to the X chromosome. Our results demonstrate that MSL-3 localization to the male X chromosome and transcriptional upregulation of X-linked genes are two separable functions of the MSL-3 protein.

Keywords: dosage compensation; transcription; MSL-3; chromo-barrel domain; MRG

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INTRODUCTION

In heterogametic organisms, a specific compensatory mechanism, dosage compensation, is established to equalize X-chromosomal gene products between the two sexes. In *Drosophila melanogaster*, dosage compensation is achieved by doubling the level of expression of genes from the single male X chromosome. In *Drosophila*, this is mediated by the products of at least five protein-coding genes, collectively named male-specific lethal (MSL) proteins (MSL-1, MSL-2, MSL-3, MOF and MLE), and two non-coding RNAs (roX1 and roX2). These factors function together in a complex that is termed the dosage compensation complex (DCC) or MSL complex (reviewed by Lucchesi *et al*, 2005; Taipale & Akhtar, 2005). The DCC is specifically assembled in males, where it binds to hundreds of sites on the X chromosome. DCC binding to the X chromosome correlates with hyperacetylation of histone H4 at lysine 16 (H4K16Ac), mediated by the MYST histone

acetyltransferase MOF (Akhtar & Becker, 2000; Smith *et al*, 2000). Protein–RNA and protein–protein interactions among DCC components are important in regulating dosage compensation. For example, MLE, MOF and MSL-3 bind to RNA, and their localization to the X chromosome is sensitive to RNase treatment (Richter *et al*, 1996; Akhtar *et al*, 2000; Buscaino *et al*, 2003). MSL-1, MSL-3 and MOF interact *in vitro* and this interaction activates MOF acetyltransferase activity (Buscaino *et al*, 2003; Morales *et al*, 2004). In addition, MOF is able to acetylate MSL-1 and MSL-3 (Buscaino *et al*, 2003; Morales *et al*, 2004).

MSL-3 is a member of the MRG family of proteins, characterized by an amino-terminal chromo-barrel domain (CBD) flanked by a positively charged polar region and a carboxy-terminal MRG domain (Bertram & Pereira-Smith, 2001). The CBD is a protein motif structurally related to chromodomains (Nielsen *et al*, 2005). Different chromo-related domains have been shown to be involved in the recognition and binding of distinct chromatin components. For example, the chromodomains of HP1 and Polycomb interact with methylated histone tails by means of a conserved aromatic pocket (Lachner *et al*, 2001; Fischle *et al*, 2003) and their deletion *in vivo* abolishes chromosomal binding and results in lethality in flies (Messmer *et al*, 1992; Platero *et al*, 1995). In contrast, MOF and MSL-3 CBD are involved in RNA binding *in vitro* (Akhtar *et al*, 2000; Morales *et al*, 2005). However, the observation that the MSL-3 CBD contains an aromatic pocket similar to that responsible for the recognition and binding of methyl groups in the chromodomains of HP1 and Pc raises the possibility that the MSL-3 CBD could also recognize methyl groups (Nielsen *et al*, 2005).

The positively charged polar region of MSL-3 contains lysine 116, acetylated by MOF *in vitro* (Buscaino *et al*, 2003). Interestingly, the MOF CBD and the HP1 chromodomain are both flanked by a positively charged region (Muchardt *et al*, 2002; Nielsen *et al*, 2005). In HP1, the polar region, termed ‘hinge region’, can interact with RNA and cooperates with the chromodomain for targeting the protein to heterochromatic loci (Muchardt *et al*, 2002). These studies suggest that in different chromodomain protein families, the polar region cooperates with the chromodomain in a similar way.

The MRG domain is a novel protein motif of unknown function (Bertram & Pereira-Smith, 2001). Strikingly, all the characterized MRG family proteins are part of large chromatin remodelling

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complexes, all of which contain MYST family acetyltransferases (Doyon *et al*, 2004).

Although DCC localization to the X chromosome and male viability are correlated, it is not yet clear whether DCC binding to the male X chromosome is sufficient to achieve the twofold upregulation of X-linked genes or whether targeting and dosage compensation are two distinct events. To address this question, we have conducted a structure/function analysis of MSL-3 *in vivo*. Our results demonstrate that MSL-3 targeting and dosage compensation are mediated by distinct MSL-3 domains. We show that the CBD, unlike other chromo-related domains, is not important for DCC targeting to the X chromosome but is required for the proper compensation of X-linked genes and for male viability. We show the polar region cooperates with the CBD, whereas the MRG domain is responsible for targeting MSL-3 to the male X chromosome.

RESULTS AND DISCUSSION

Characterization of *msl-3⁰⁸³* allele

Both the Oishi and the Baker labs identified *msl-3* as a gene, the product of which is required for male viability in *Drosophila* (Uchida *et al*, 1981; Gorman *et al*, 1995). However, none of the identified alleles has been molecularly characterized in detail. Therefore, we first carried out a detailed characterization of an *msl-3* allele, *msl-3⁰⁸³*. Sequencing the genomic locus from *msl-3⁰⁸³* homozygous adult female flies showed that the *msl-3⁰⁸³* has a point mutation in the splicing junction of the first intron, which is mutated owing to a deletion of T104 (supplementary Fig S1A online). Western blot analysis using crude extract prepared from *msl-3⁰⁸³* homozygous embryos with an MSL-3-specific antibody raised against the full-length protein demonstrated that *msl-3⁰⁸³* flies do not produce any full-length or truncated protein (supplementary Fig S1B online). These results were confirmed by immunofluorescence studies on polytene chromosomes (supplementary Fig S1C online) and they demonstrate that *msl-3⁰⁸³* is a null allele of the *msl-3* gene. Next, we were interested to determine why mutations in *msl-3* gene lead to male lethality and whether the absence of MSL-3 produces the same phenotypes in different tissues. For this purpose, *msl-3⁰⁸³* mutant clones were generated in the eyes of adult flies with the *Minute* FLP/FRT system and in wing imaginal discs with the FLP/FRT system. Loss of MSL-3 in the eyes led to a male-specific phenotype, as clones of mutant cells are much smaller in males versus females (supplementary Fig S1D online). This phenotype indicated that *msl-3* is required for cell proliferation or survival. To examine whether *msl-3* mutation leads to cell death, *msl-3* mutant clones were generated in wing discs using Hs-FLP, and cell death was examined by staining discs with an antibody against activated caspase 9. As shown in supplementary Fig S1E online, we could detect *msl-3⁰⁸³* mitotic clones in female imaginal discs. In males, we could not identify any *msl-3⁰⁸³* mutant clones. Instead, a few apoptotic cells were present (supplementary Fig S1E online). These data show that *msl-3* gene is equally required in different tissues and they suggest that the lack of the *msl-3* gene induces cell death.

CBD and MRG domains are required for male viability

To determine the function of the different MSL-3 domains *in vivo*, we generated transgenic flies expressing MSL-3 derivatives under the control of a heat shock or tubulin promoter, tagged at the C terminus with an enhanced green fluorescent protein (EGFP) or

FLAG epitope (Fig 1A). The expression levels of all derivatives were compared with the wild-type (wt) MSL-3 protein by performing western blot analysis of the heads of adult males (supplementary Fig S2A–D online). To assess the function of the mutant proteins, the transgenic lines were assayed for their ability to rescue the male-specific lethal phenotype of *msl-3⁰⁸³* (Fig 1B). As expected, a transgenic construct encoding EGFP- or FLAG-tagged MSL-3 can efficiently rescue the *msl-3⁰⁸³* phenotype (Fig 1B, lanes 1–3).

This analysis showed that a point mutation in the aromatic pocket of the MSL-3 CBD (F56A-EGFP) did not severely affect MSL-3 function (Fig 1B, lane 5). This demonstrated that the proposed methyl binding properties of MSL-3 are not necessary for dosage compensation and male viability.

A recent report has suggested that the CBD is dispensable for MSL-3 function (Morales *et al*, 2005). It was therefore surprising that the complementation test showed that the CBD is essential for male viability because Δ CBD–EGFP male flies died as pupae and only a few males survived until adulthood (Fig 1B, lane 4). The flies that survived showed a range of phenotypic abnormalities (rough eyes, missing bristles and misshapen wings; Fig 1C).

We also found that a point mutation in K116 (K116G-FLAG) or deletion of the positively charged polar region (Δ POLAR–EGFP) does not affect the function of the protein (Fig 1B, lanes 6,7). Similar results have been observed in other contexts where mutations of lysine residues known to be acetylated *in vitro* do not produce any visible phenotypes *in vivo*, suggesting that other lysines are also acetylated *in vivo* and/or that there are several lysines that can be acetylated in the absence of the real substrate (Megee *et al*, 1990; Zhang *et al*, 1998; Feng *et al*, 2005).

Interestingly, we found that similar to HP1, the positively charged polar region cooperates with the CBD in MSL-3 function, as MRG–EGFP male flies died earlier than Δ CBD–EGFP male flies and they never reached adulthood (Fig 1B, lane 8). Finally, we found that the MRG domain is essential for male viability because Δ MRG–EGFP male flies die as first/second instar larvae (Fig 1B, lane 9).

MRG domain is required for targeting to the male X

Having documented that all MSL-3 domains are important for male viability, we next investigated whether the reason for loss of function of these derivatives was mistargeting of MSL-3 and/or other DCC components from the male X chromosome. Immunostaining and RNA fluorescence *in situ* hybridization (FISH) was performed on male third instar larvae expressing MSL-3 derivatives and homozygous mutant for *msl-3⁰⁸³* except for MRG–EGFP and Δ MRG–EGFP, where owing to early lethality the immunofluorescence was performed on male third instar larvae expressing the fusion proteins in a wt background. This analysis showed that deletion of the MRG domain impairs MSL-3 targeting to the male X chromosome without affecting its nuclear localization (Fig 2A,B) and that the MRG domain is sufficient to target the protein to the male X chromosome (Fig 3D). MSL-3 interacts with MSL-1 by means of its C terminus *in vitro* (Morales *et al*, 2005) and it is therefore likely that this interaction is important for MSL-3 localization to the X chromosome.

Interestingly, and in accord with a previous report (Morales *et al*, 2005), mutations in the CBD or in the polar region do not affect DCC localization to the male X chromosome (Fig 3A,C;

Fig 1 | Generation of MSL-3 derivatives and rescue test. (A) Schematic representation of MSL-3 domains and transgenic flies generated for this study. wt, enhanced green fluorescent protein (EGFP)-tagged and FLAG-tagged wild-type MSL-3 were generated as controls; chromo-barrel domain (CBD) mutants, deletion in the CBD and point mutation in the CBD of MSL-3; polar region mutants, deletion in the polar region and point mutation; CBD + polar region mutant, deletion of the CBD and the polar region; MRG mutant, deletion of the MRG motif. (B) Rescue test of *msl-3⁰⁸³* lethality by MSL-3 carboxy-terminal-tagged proteins (lanes 1-3), CBD MSL-3 mutant (lanes 4,5), polar region MSL-3 mutants (lanes 6,7), CBD and polar region MSL-3 mutant (lane 8) and MRG mutant (lane 9). The numbers of insertion analysed, the number of males and females counted and the average of the rescue frequency are indicated for each mutant. (C) Homozygous males expressing Δ CBD-EGFP in a homozygous *msl-3⁰⁸³* mutant background show different male-specific phenotypic abnormalities, including rough eyes (compare panels b,c,d with panel a); missed bristles (compare panels f,g,h with panel e) and misshapen wings (compare panels j,k,l with panel i). Panels a,e,i show normal phenotype of homozygous females expressing Δ CBD-EGFP.

supplementary Figs S3,S4 online). However, in Δ CBD-EGFP male flies, we observed an autosomal mislocalization, which varied in number and position (Fig 3A). Taken together, these data show that the MRG domain is necessary and sufficient for targeting of MSL-3 to the X chromosome, whereas the CBD, although essential for male viability, is not required for binding to the X chromosome.

CBD deletion impairs expression of X-linked genes

It was intriguing to find that the MSL proteins remained localized to the male X chromosome in Δ CBD-EGFP flies and yet these flies died as pupae (Figs 1B,3A,C). Mistargeting of MSL proteins to certain autosomal sites (Fig 3A) suggested that the male-specific lethal phenotype may be a result of transcriptional upregulation of

Fig 2 | The MRG domain is required for MSL-3 localization to the X chromosome. (A) Polytene chromosomes from MSL-3-EGFP (top) or Δ MRG-EGFP (bottom) male larvae were stained with antibodies against EGFP and MSL-1. (B) Salivary glands from MSL-3-EGFP (top) or Δ MRG-EGFP (bottom) male larvae were stained with antibodies against EGFP and MSL-1.

Fig 3 | Deletion of MSL-3 chromo-barrel domain does not affect targeting of MSL proteins to the male X chromosome. (A) Polytene chromosomes from Δ CBD-EGFP male larvae were stained with antibodies against EGFP, MSL-1, MOF, MSL-2 and MLE. Mislocalization of MSL proteins to autosomes is indicated by arrows. (B) Polytene chromosomes from F56A-EGFP male larvae were stained with antibodies against EGFP and MSL-1. (C) Intact salivary glands from MSL-3-EGFP (top) or Δ CBD-EGFP (bottom) male larvae were hybridized with roX1- and roX2-specific probes or hybridized with roX1- and roX2-specific probes and simultaneously stained with MSL-1 antibody. (D) Polytene chromosomes from MRG-EGFP male larvae were stained with antibodies against EGFP and MSL-1.

Fig 4 | Δ CBD-EGFP males are not able to fully compensate X-linked genes. Quantitative PCR analysis of *pgd*, *dspt6*, *sl*, *vap33-1*, *crm*, *CG32721*, *para*, *dspt4* and *pol2* in wild-type (wt) females (black), wt males (grey), MSL-3-EGFP females (dark green) and males (light green) and Δ CBD-EGFP females (dark blue) and males (light blue). Error bars represent the standard deviation of three different biological samples. Expression levels were normalized against the autosomal gene *rp49* and set to 100% for each gene in females.

autosomal genes and not of transcriptional downregulation of X-linked genes. To discriminate between these two possibilities, the expression levels of dosage-compensated genes (*pgd* and *dspt6*; Chiang & Kurnit, 2003) and genes enriched in MSL-3 and MSL-1 using chromatin immunoprecipitation assay (*sl*, *vap33-1*, *crm*, *CG32721*; Legube et al, 2006) were analysed by quantitative reverse transcription-PCR (RT-PCR; Fig 4). This analysis was performed in wt, MSL-3-EGFP and Δ CBD-EGFP larvae in an *msl-3⁰⁸³* homozygous mutant background. The X-linked *para* gene, which is dosage compensated in an MSL-independent manner (Chiang & Kurnit, 2003), and two autosomal genes, *dspt4* and *Pol2*, were included as controls. In contrast to wt and MSL-3-EGFP larvae, in male flies expressing Δ CBD-EGFP, all known dosage-compensated genes were consistently downregulated to around 60%. The downregulation was specific for compensated genes, as the expression levels of the *para* gene and of the autosomal genes *dspt4* and *pol2* were not affected (Fig 4). These data suggest that the observed male lethality of Δ CBD-EGFP flies is due to the loss of dosage compensation of X-linked genes.

CBD contributes to chromatin interaction *in vitro*

As the MSL-3 mutants lacking CBD can still be targeted to the X chromosome along with the other MSL proteins, their inability to rescue male viability and to ensure dosage compensation was rather puzzling. It was also intriguing that the Δ CBD mutants show mislocalization of MSL complex on autosomal sites. MSL-3 can interact with chromatin *in vitro* (Buscaino et al, 2003). We therefore suggested that although not a main contributor to MSL targeting on the X chromosome, the CBD may mediate MSL-3/ chromatin interaction and that in its absence the MSL/chromatin interaction is somewhat compromised. Therefore, to address this issue, we generated a set of recombinant MSL-3 deletion derivatives and tested their ability to bind to DNA and unmodified recombinant nucleosomes using electrophoretic mobility shift

assay (EMSA; Fig 5). Deletion of the CBD, but not deletion of the polar region, impairs the ability of the protein to bind to DNA and mononucleosomes (Fig 5, lanes 3,4 and 12,13). In addition, the CBD alone is able to bind to DNA and mononucleosome, although less efficiently (Fig 5, lanes 6,7 and 16,17).

The finding that the CBD is required for chromatin interaction *in vitro* and the observation that in Δ CBD-EGFP flies the DCC is mislocalized to autosomes suggest that in the absence of the CBD, the DCC is less stable and has a reduced affinity for the X chromosome. CBD also contributes to interaction of MSL-3 with RNA (Morales et al, 2005). Therefore, we propose that the interaction of MSL-3 with chromatin/RNA through its CBD is necessary for achieving the twofold upregulation of X-linked genes in male flies.

Interestingly, in the *Saccharomyces cerevisiae* NuA4 complex, deletion of Eaf3 (homologue of MSL-3) leads to a twofold downregulation of NuA4 target genes (Eisen et al, 2001) but the lack of Eaf3 does not affect the localization of Esa 1 (homologue of MOF) and does not change the overall level of H4 acetylation in the cells (Reid et al, 2004). However, acetylation is mistargeted to coding sequences instead of the promoters (Reid et al, 2004). It is therefore tempting to speculate that similar to Eaf3, MSL-3 could be required for correctly targeting H4K16 acetylation to specific chromosomal sites and that the CBD is required for this function.

Speculation

So far, all the lethal mutations in DCC components have been shown to impair the ability of the MSL complex to interact with the X chromosome. Our findings demonstrate for the first time that DCC targeting to the X chromosome and transcriptional regulation of X-linked genes are separable events and that once targeting of the dosage compensation components is achieved, further molecular interactions are needed for optimal transcription

Fig 5 | MSL-3 CBD is involved in DNA and chromatin interaction. (A) Schematic representation of recombinant MSL-3 derivatives generated for the DNA and nucleosome electrophoretic mobility shift assay. (B) Increasing amounts (9.21 and 13.82 pmol) of MSL-3 derivatives were incubated with radioactively labelled 146bp DNA fragment (lanes 1–8) or with nucleosomes assembled on the same radioactively labelled DNA fragment (lanes 10–17). Full-length MSL-3 (lanes 1,2; 10,11), ΔCBD (lanes 3,4; 12,13), ΔPOLAR (lanes 5,6; 14,15) or CBD alone (lanes 7,8; 16,17) are shown. Lane 9, free DNA; lane 18, nucleosomes. Arrows indicate the free probes. The protein–DNA and protein–nucleosome complexes are also indicated.

activity. The CBD is important for male viability and compensation of X-linked genes, but it is dispensable for targeting the protein to the X chromosome. Surprisingly, deletion of the CBD does not seem to affect global acetylation of H4K16, the only marker known to be associated with an active and fully functional DCC (Morales *et al*, 2005; data not shown). Therefore, in addition to acetylation of H4K16, a novel regulatory mechanism contributes to the twofold upregulation of the male X chromosome and this mechanism is essential for male viability.

METHODS

Fly stocks and crosses. Fly strains and genetic crosses used in this study are available as supplementary information online.

Western blot analysis for embryos and msl-3 transgenic flies. Homozygous embryos (*msl-3⁰⁸³/msl-3⁰⁸³*) were homogenized in SDS loading buffer. Crude extracts from fly heads were prepared as described (Gu *et al*, 2000). Antibodies against MSL-3 (1:5,000), EGFP (1:500; Torrey Pines Biolabs, Houston, TX, USA), FLAG (1:5,000; Sigma, Dorset, UK) and tubulin (1:10,000; Sigma) were used as indicated.

Immunofluorescence of polytene chromosome. Polytene chromosomes were prepared as described <http://www.igh.cnrs.fr/equip/cavalli/Immunostaining.rtf>. Apart from EGFP (1:50), all other antibodies were used at a dilution of 1:500.

RNA FISH and FISH probes. Antisense full-length roX1 (fluorescein-labelled) and roX2 (DIG-labelled) RNAs were prepared using appropriate labelling mix (Roche, Sussex, UK) and T7 RNA polymerase. RNA FISH was performed as described (Meller *et al*, 2000).

RNA extraction from salivary glands and RT-PCR. Salivary glands (male and female) from wt larvae, larvae of genotype *w⁻*; *hs msl-3-egfp/hs msl-3-egfp*; *msl-3⁰⁸³/msl-3⁰⁸³* and larvae of genotype *w⁻*; *hs ΔCBD-egfp*, *msl-3⁰⁸³/hs ΔCBD-egfp*, *msl-3⁰⁸³* were used. Total RNA was isolated using the Qiagen-RNeasy Mini Kit and 500 ng was reverse transcribed using Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Karlsruhe, Germany). RT-PCR was performed using the SYBR Green PCR master mix (Applied Biosystem, Foster City, CA, USA), 100 ng of each primer and 4 μl of the reverse-transcribed DNA.

EMSA. Proteins were expressed in bacteria and EMSA was performed as described (Akhtar *et al*, 2000). Cloning details are available on request.

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>).

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