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RESEARCH REPORT

# The simultaneous interaction of MSL2 with CLAMP and DNA provides redundancy in the initiation of dosage compensation in *Drosophila* males

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## ABSTRACT

The binding of the *Drosophila* male-specific lethal dosage compensation complex (DCC) exclusively to the male X chromosome provides an excellent model system to understand mechanisms of selective recruitment of protein complexes to chromatin. Previous studies showed that the male-specific organizer of the complex, MSL2, and the ubiquitous DNA-binding protein CLAMP are key players in the specificity of X chromosome binding. The CXC domain of MSL2 binds to genomic sites of DCC recruitment *in vitro*. Another conserved domain of MSL2, named Clamp-binding domain (CBD) directly interacts with the N-terminal zinc-finger domain of CLAMP. Here, we found that inactivation of CBD or CXC individually only modestly affected recruitment of the DCC to the X chromosome in males. However, combination of these two genetic lesions within the same MSL2 mutant resulted in an increased loss of DCC recruitment to the X chromosome. Thus, proper MSL2 positioning requires an interaction with either CLAMP or DNA to initiate dosage compensation in *Drosophila* males.

**KEY WORDS: MSL, CLAMP, Sex determination, Transcription factor, C2H2 zinc finger, CXC**

## INTRODUCTION

In *Drosophila* males, a multisubunit dosage compensation complex (DCC) is responsible for a precise compensatory increase in the expression of genes on the X chromosome (Kuroda et al., 2016; Lucchesi, 2018; Samata and Akhtar, 2018). The DCC consists of five proteins, MSL1, MSL2, MSL3, MOF and MLE, and includes two redundant non-coding RNAs, *roX1* (3.7 kb) and *roX2* (0.6 kb) that perform similar functions (Lucchesi, 2018; Samata and Akhtar, 2018). MSL2 is expressed only in males and is the core DCC component, suggesting a key role in binding specificity of DCC to the X chromosome (Kuroda et al., 2016). The interaction between MSL1 and MSL2 proteins forms the structural core of the DCC, and the C-terminal PEHE domain of MSL1 is responsible for interactions with MSL3 and MOF (Li et al., 2005; Kadlec et al., 2011; Hallacli et al., 2012). The MLE protein, an ATP-dependent RNA/DNA helicase of the DEAD subfamily, interacts with two noncoding roX RNAs, *roX1* and *roX2*, to induce their unwinding (Maenner et al., 2013; Ilik et al., 2017), which allows them to bind to MSL2. Thus, roX RNAs link MLE and MSL2 proteins into a single complex.

One of the key questions is what is responsible for the specificity of DCC binding to the X chromosome of males. An incomplete DCC containing only MSL1 and MSL2 binds to an invariant set of about 200 sites on the X chromosome, which are called the primary chromatin entry sites (CES) (Alekseyenko et al., 2008) or high-affinity sites (HAS) (Straub et al., 2008). Structural analysis showed that the CXC domain of MSL2 specifically recognizes a GA-rich sequence motif within the HAS/CES (Fauth et al., 2010; Zheng et al., 2014).

Using *in vitro* genome-wide DNA-binding assays, it was demonstrated (Villa et al., 2016) that MSL2 alone specifically binds to a subclass of HAS/CES named PionX (pioneering sites on the X). A zinc-finger protein called CLAMP (Chromatin Linked Adaptor for MSL Proteins) was found to be important for binding of the DCC on the X chromosome (Larschan et al., 2012; Soruco et al., 2013). CLAMP is an essential protein in *Drosophila* that is expressed in both sexes and binds thousands of GA–rich sequences across the genome (Soruco et al., 2013; Kuzu et al., 2016; Urban et al., 2017a). CLAMP directly interacts with MSL2 and opens chromatin at HAS, which promotes DCC recruitment (Urban et al., 2017a; Albig et al., 2019).

Here, we examined the role of the CXC and CLAMP-binding domains of MSL2 in recruiting the DCC to the X chromosome. We show that these two domains have overlapping and partially redundant functions in ensuring proper positioning of the DCC on the X chromosomes in *Drosophila* males, thus permitting its proper function.

## RESULTS AND DISCUSSION

### Mutations and deletions of CXC and CLAMP-binding domain (CBD) do not affect the stability of MSL2 mutants

The MSL2 protein is crucial for recruitment of DCC to the X chromosome in males (Lyman et al., 1997; Straub et al., 2013). The main aim of this work was to assess the roles of the CLAMP-binding domain (CBD) and CXC in the ability of MSL2 to recruit DCC to the X chromosome (Fig. 1A, Fig. S1).

In a previous study (Albig et al., 2019), CBD was mapped within the 620-685 aa region of MSL2. In order to make highly precise mutations in MSL2, we have refined the interacting regions within the MSL2 and CLAMP proteins using a glutathione S-transferase (GST) pulldown assay *in vitro* (Fig. 1B). We tagged the 573-708 aa region of MSL2 and the 1-196 aa region of CLAMP with either GST or Thioredoxin-6xHis-tag. The GST protein and the 6xHis-tagged MSL2 RING domain (1-196 aa) were used as negative

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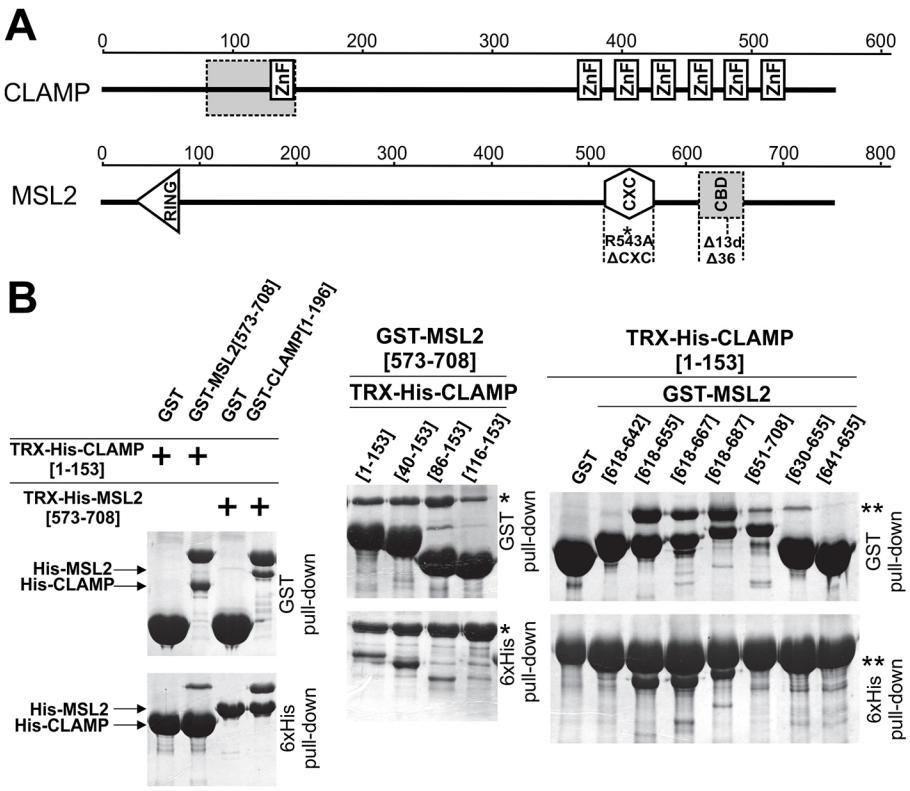


**Fig. 1. Mapping CBD in MSL2 and transgenic lines expressing MSL2 mutations.** (A) Schematic of full-length CLAMP and MSL2 proteins. The scale shows the number of amino acid residues. Boxes with dashed lines indicate regions involved in interaction between MSL2 and CLAMP. Dashed lines indicate deletions in full-size MSL2 protein that were used in this work. ZnF, zinc-finger domain. (B) Mapping of interacting domains of CLAMP and MSL2 proteins in *in vitro* pulldown assays. The positions of amino acids are given in square brackets. Asterisk marks position of GST-MSL2[573-708]. Two asterisks mark position of Thioredoxin-6xHis-CLAMP[1-153].

controls. Both GST- and 6xHis-tag pulldown assays confirmed interaction between 1-196 aa of CLAMP and 573-708 aa of MSL2. To refine the interacting domains further, we developed a set of deletion derivatives of the GST-tagged MSL2 and Thioredoxin-6xHis-tagged CLAMP. The pulldown experiments showed that a region between aa 86 and 153 of CLAMP containing the zinc-finger and the preceding 30 aa interact with MSL2. It was also found that 618-655 aa of MSL2 are crucial for its interaction with CLAMP. It turned out that the CLAMP-interacting region in MSL2 is highly conserved within the *Drosophila* family, similar to the RING and CXC domains (Fig. S1).

To characterize the domains of MSL2 that contribute to the recruitment of DCC to the X chromosome, we expressed in flies different cDNA variants of MSL2, tagged with 3×FLAG (Fig. 1A). To better define CBD, we made a deletion not only of the whole 620-655 aa region (MSL2$^{\Delta 36}$), but also of the distal 642-654 aa (MSL2$^{\Delta 13d}$) part. To ablate activity of the CXC domain, we used a previously characterized point mutation in this domain (Li et al., 2008; Villa et al., 2016) that substitutes the arginine at position 543, which is essential for DNA binding, by alanine [R543A (R*)]. We also made constructs expressing the MSL2$^{\Delta CXC}$ mutant lacking the CXC domain, and the MSL2$^{R*\Delta 13d}$ mutant, which combines R* and Δ13d.

A strong ubiquitin (Ubi) promoter was used to express cDNAs encoding these MSL2 variants. Overexpression of MSL2 under this Ubi promoter allowed problems associated with low stability MSL2 mutant variants to be overcome. All transgenes were inserted at the same 86Fb region on the 3rd chromosome, using a ϕC31-based integration system (Bischof et al., 2007). Immunoblot analysis showed that all MSL2 variants were present in the transgenic flies at nearly equivalent levels (Fig. S2). The relative stability of MSL2 variants makes it possible to compare their ability to recruit DCC on the X chromosome.

### CXC and CBD functions are partly redundant in the ability of MSL2 to recruit DCC to the X chromosome in males

To test role of CXC and CBD in specific recruitment of the DCC in males, we introduced transgenes into the null *msl2*$^{\gamma 227}$ background (Zhou et al., 1995). Unexpectedly, all the single-mutant variants of MSL2 (MSL2$^{\Delta 36}$, MSL2$^{\Delta 13d}$, MSL2$^{\Delta CXC}$ and MSL2$^{R*}$) were able to complement the *msl2*$^{\gamma 227}$ null mutation and considerably restored viability in males homozygous for the null mutation (Fig. 2A). Only *msl2*$^{\gamma 227}$ null males carrying the MSL2$^{R*\Delta 13d}$ transgene showed very low viability, and early mortality (5-7 days after eclosion). Thus, dosage compensation is strongly compromised in the MSL2$^{R*\Delta 13d}$ mutant, which lacks the ability to bind to both CLAMP and DNA.

Next, we tested binding of DCC to the polytene chromosomes in males carrying different variants of the MSL2 protein (Fig. 2B, Figs S3, S4). In all cases, binding of MSL1 was correlated with the binding of the MSL2 variants. In male MSL2$^{\Delta 13d}$, MSL2$^{R*}$ or MSL2$^{\Delta CXC}$ larvae, we observed reduced binding of MSL2-FLAG and MSL1 to the X chromosome (Fig. S4). The binding of MSL1 and MSL2-FLAG was much more strongly diminished in MSL2$^{R*\Delta 13d}$ males. Additional MSL1/MSL2 bands were found on the polytene autosomes in all tested transgenic lines (Fig. 2B, Fig. S4) suggesting that MSL complexes started to bind to the lower-affinity sites on autosomes.

To compare binding of MSL complex in different mutants, we selected previously characterized representative binding sites for MSL complex: PionX sites (Villa et al., 2016), CES sites (Alekseyenko et al., 2008), and several sites on the autosomes that have MSL2 signal (Straub et al., 2013). We found that MSL2-FLAG and MSL1 bound to all PionX and CES sites in males expressing wild-type MSL2 (Fig. 2C). The MSL2$^{R*}$ and MSL2$^{\Delta 13d}$ had the same binding patterns at all tested sites, with no difference between PionX and CES (Fig. 2C, Fig. S5): at 12 sites, binding of MSL1 and

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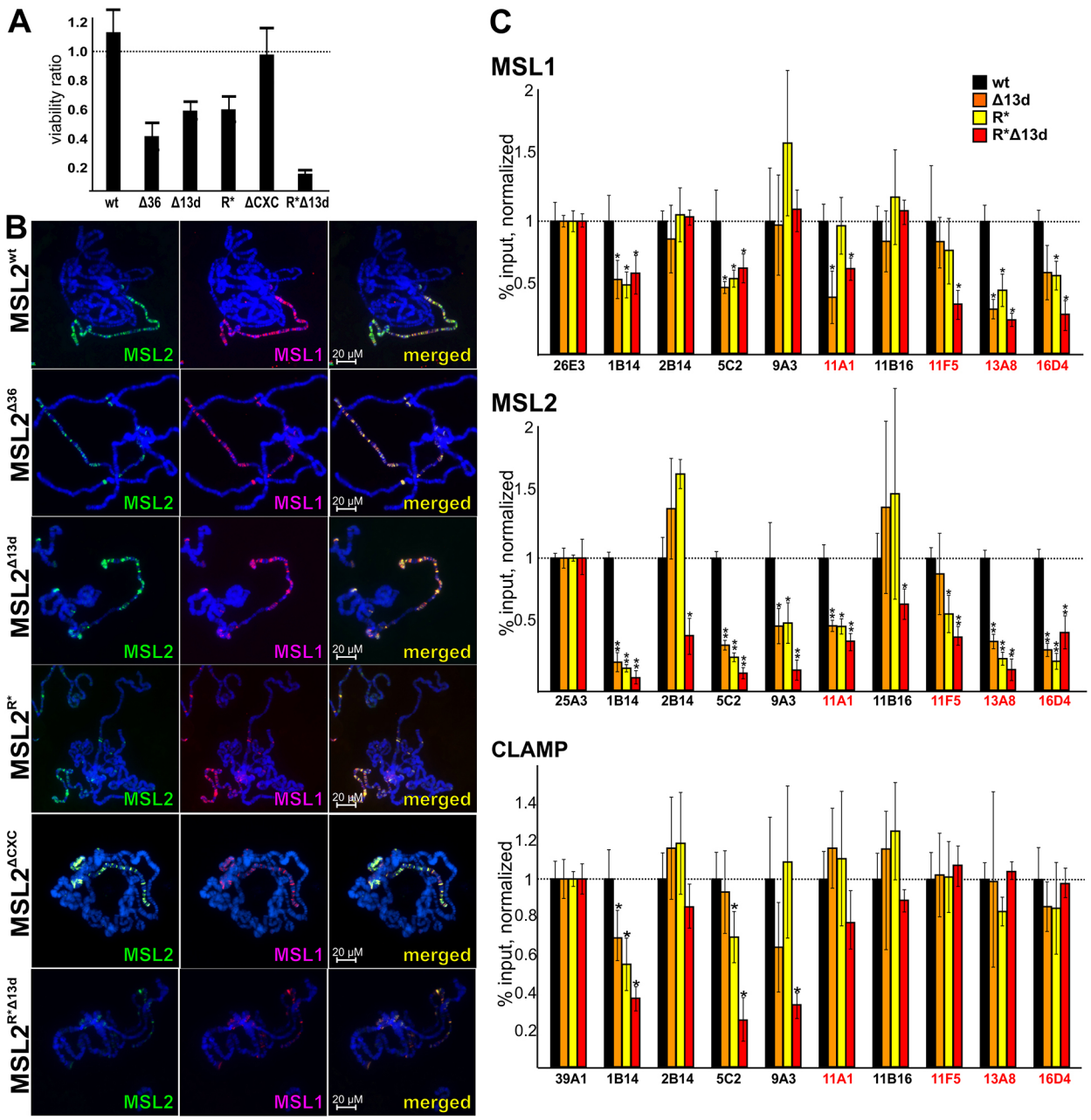


**Fig. 2. Testing functional activity of MSL2 mutants in males.** (A) Viability (as a relative percentage) of male adult flies after expression of MSL2 derivatives on the *msl2*$^{\gamma 227}$/*msl2*$^{\gamma 227}$ (*msl2*-null) background. Flies expressing corresponding MSL2 derivatives in heterozygous *msl2*$^{\gamma 227}$/*CyO* flies were used as internal controls with 100% viability. Results are expressed as mean±s.d. of three independent crosses. (B) MSL1 and MSL2 localization on the polytene chromosomes from 3rd day male larvae on the *msl2*-null background expressing different FLAG-tagged variants of MSL2 protein. Panels show immunostaining of 3×FLAG-MSL2 protein with mouse anti-FLAG antibody (green) and MSL1 protein with corresponding rabbit antibody (magenta). DNA was stained with DAPI (blue). (C) Comparison of binding of MSL1, MSL2 and CLAMP at different CES and PionX (marked with red text) regions in the MSL2-expressing flies on the *msl2*$^{\gamma 227}$ background. Histograms show ChIP enrichments at the CES regions on chromatin isolated from male flies expressing different MSL2 variants: MS2$^{wt}$(wt), MSL2$^{\Delta 13d}$(Δ13d), MSL2$^{R*}$(R*), MSL2$^{R*\Delta 13d}$(R*Δ13d). The results are presented as a percentage of input genomic DNA normalized to corresponding positive autosomal genomic regions for MSL1 (26E3) and MSL2 (25A3), and compared with binding levels in the flies expressing wild-type MSL2 protein (corresponding to the '1' on the scale). Error bars show s.d. of quadruplicate PCR measurements for three independent experiments. *$P$<0.05, **$P$<0.01.

the MSL2 mutant proteins was reduced nearly 50%; at three sites (2B14, 11B16, and 13D4) binding of both proteins did not change in mutant larvae. The point mutation R543A (R*) in the CXC domain and its complete deletion (ΔCXC) displayed similar patterns of MSL1/MSL2/CLAMP binding to the selected sites (Fig. S6).

Similar to the results obtained with polytene chromosomes, the MSL2$^{R*\Delta 13d}$ double mutant, which ablates interaction with both CLAMP and DNA, showed diminished chromatin immunoprecipitation (ChIP) enrichment compared with MSL2$^{R*}$ or MSL2$^{\Delta 13d}$ single mutants at most locations (Fig. 2C). At the 16D4 site, both single and double MSL2 mutants had similar significant reductions in binding. In two cases, 2B14 and 11B16, only the binding of MSL2$^{R*\Delta 13d}$ was reduced. Binding of MSL1 only partially correlated with recruitment of MSL2 variants to the same sites. This might be explained by the ability of MSL1 to be recruited as a component of alternative complexes. In most cases,

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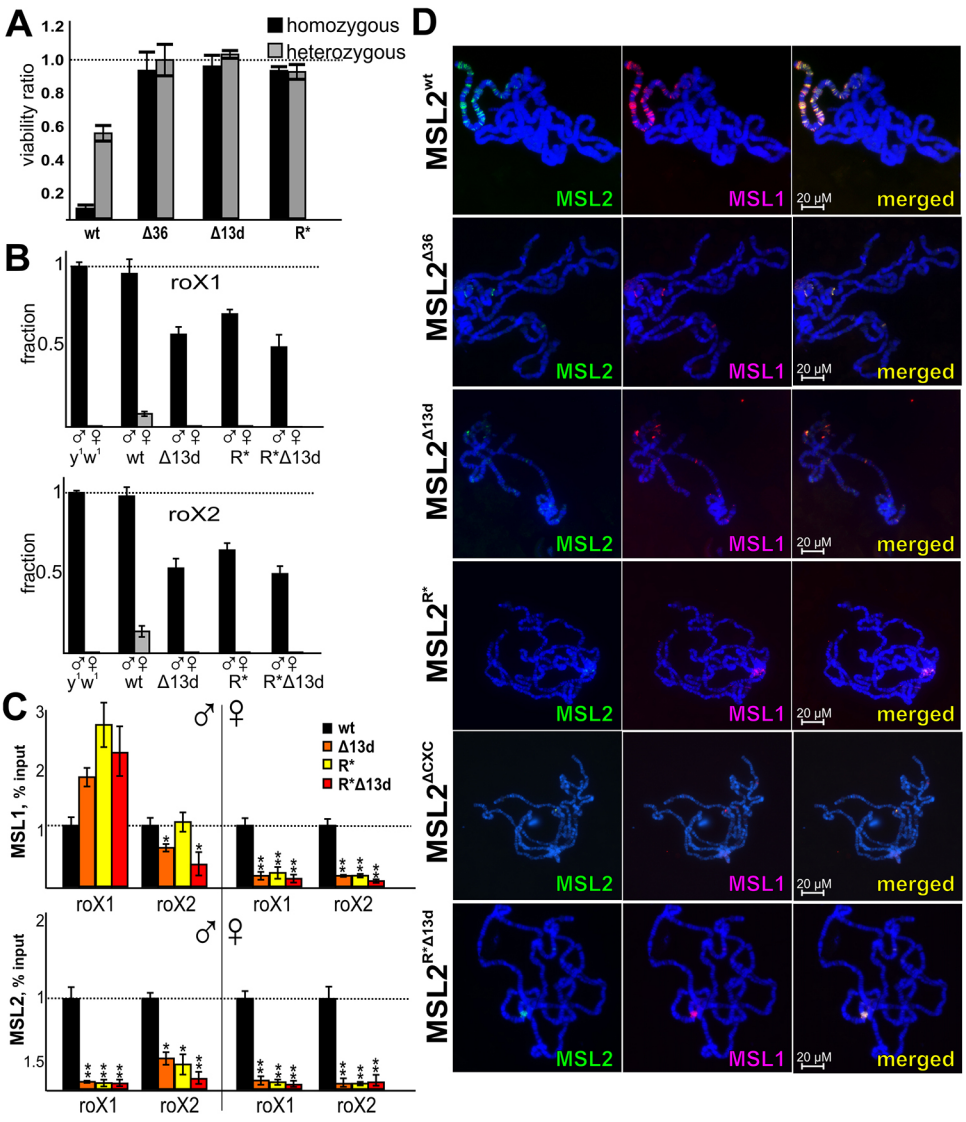


**Fig. 3. Testing functional activity of MSL2 mutants in females.** (A) Viability (as a relative percentage) of females to male adult flies after expression of MSL2 derivatives (wt, Δ13d, R*, R*Δ13d). Results are expressed as mean±s.d. of three independent crosses in homozygous (black) and heterozygous (gray) lines. (B) Expression levels of the *roX1* and *roX2* RNAs in male and female larvae in the $y^1w^1$ flies (wild-type background) and MSL2-expressing flies (wt, Δ13d, R*, R*Δ13d) on the $msl2^{y227}$ background. Individual transcript levels were determined by RT-qPCR with corresponding primers normalized relative to *RpL32* for the amount of input cDNA. Histogram shows the changes of mRNAs for the tested roX genes compared with expression levels in wild-type flies ($y^1w^1$, '1' on the scale). Error bars show s.d. ($n$=3). (C) Comparison of binding of MSL1 and MSL2 in the *roX1* and *roX2* CES regions in the MSL2-expressing flies on the $msl2^{y227}$ background. (D) Distribution of the MSL complex on the polytene chromosomes from 3rd day female larvae expressing different FLAG-tagged variants of MSL2 protein. Other designations are as in Fig. 2.

CLAMP binding did not change in lines expressing different MSL2 variants (Fig. 2C). However, strong reduction of the CLAMP binding was observed at the 1B14, 5C2 and 9A3 sites in males expressing $MSL2^{R*\Delta13d}$. Thus, in some cases, CLAMP binding depends on recruitment of the DCC, correlating with the synergy observed between MSL2 and CLAMP occupancy (Soruco et al., 2013; Albig et al., 2019).

Interestingly, at some autosomal sites, binding of MSL1 (in $MSL2^{R*}$ and $MSL2^{\Delta13d}$ flies), but not the MSL2 mutant proteins, was reduced (Fig. S7). This result suggests an existence of alternative mechanisms of MSL2 recruitment to sites outside of the DCC.

### CXC and CBD cooperate in recruiting DCC to the X chromosome in females

Ectopic expression of MSL2 in females resulted in assembly of a functional DCC (Kelley et al., 1997) that led to a strong elevation of gene expression and, as a consequence, a decrease in viability. As expected, females carrying homozygous $MSL2^{wt}$ transgenes had low viability (Fig. 3A). In contrast, we observed normal viability in females carrying homozygous transgenes expressing mutant versions of MSL2 affecting either CXC or CBD. These results suggest that functional activity of these transgenic MSL2 variants was at least partially compromised.

In wild-type female larvae, the roX RNAs are not expressed (Meller et al., 1997). Expression of wild-type MSL2 in females resulted in a significant activation of the *roX2* RNA and, to a lesser extent, the *roX1* RNA (Rattner and Meller, 2004). We confirmed that the expression of MSL2 in females induced transcription of both roX RNAs (Fig. 3B). However, expression of the roX RNAs in females was lower than in corresponding males, as expected. The roX RNAs were also not expressed in females expressing mutant versions of MSL2 with mutations in either CXC or CBD, or both.

The HAS/CES located near the roX genes are required for stimulation of their transcription in males and repression in females (Bai et al., 2004; Urban et al., 2017b). We performed ChIP to determine whether MSL2 mutants are normally recruited to the known HAS/CES adjacent to the roX genes (Fig. 3C). ChIP showed that MSL2 and MSL1 were bound to these HAS/CES in the $MSL2^{wt}$ transgenic female larvae. In contrast, the MSL2 mutants in which the CXC domain was disrupted or contained deletions of the CBD failed to recruit MSL2 or MSL1. These results demonstrate a direct correlation between the binding of MSL1/MSL2 and the activation of the roX genes in females.

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Polytene chromosomes in the females are a useful model system to define protein domains that are essential for recruiting the DCC to the X chromosome (Zhou et al., 1995; Li et al., 2008; Morra et al., 2011). Transgenic expression of MSL2 in females led to localization to the X chromosomes of both the MSL2 and MSL1 proteins, confirming that MSL2 expressed under the Ubi promoter is able to recruit DCC to the X chromosome in females (Fig. 3D). Inactivation of the CXC domain in the $MSL2^{R*}$ or $MSL2^{\Delta CXC}$ transgenic larvae led to binding of either mutant to several sites on different chromosomes and to the centromeric region (Fig. 3D, Fig. S8). MSL1 was colocalized at the same sites, suggesting that MSL2 lacking a functional CXC domain redirects recruitment of MSL complex to new sites. Deletions in CBD ($MSL2^{\Delta 36}$, $MSL2^{\Delta 13d}$ and $MSL2^{R*\Delta 13d}$) resulted in an almost complete absence of binding sites for the mutant variants of MSL2 and MSL1. These results suggest that CXC and CBD function cooperatively to recruit DCC onto the X chromosome.

### A model of specific recruitment of the DCC to the HAS/CES

MSL2 was previously suggested as a key protein in recruitment of the DCC to the X chromosome (Lyman et al., 1997; Li et al., 2008; Villa et al., 2016). Unexpectedly, deletion of the CXC domain and the R543A point substitution showed similar weak effects on the recruitment of the DCC in males. This result is in accordance with a previous observation that mutations of two cysteines, forming architecture of the CXC domain, did not significantly reduce male survival (Lyman et al., 1997). At the same time, mutant MSL2 lacking both activities (DNA binding and interaction with CLAMP) failed to rescue the null $msl2^{\gamma 227}$ mutant in males effectively. Thus, the MSL2-CLAMP interaction and recognition of DNA by the CXC domain cooperatively contribute to the specificity of DCC recruitment to CES/HAS *in vivo*.

The phenotype of $MSL2^{R*\Delta 13d}$ flies includes poor male survival and decreased targeting of DCC to the X chromosome, similar to that observed in flies lacking both roX genes (Meller and Rattner, 2002). It seems likely that an important role in the initial recruitment of the DCC to the HAS/CES is played by MLE, which binds to the core MSL complex through the roX RNAs (Li et al., 2008; Straub et al., 2013; Quinn et al., 2014). The CXC and CLAMP-binding domains are essential for the initial recruitment of the MSL sub-complex (which likely also includes MSL1, MSL3 and MOF) to the regulatory regions of the roX genes. For this reason, specific binding of MSL sub-complex (MSL1/MSL2) has become completely dependent on the CXC and CBD in females. In males, roX gene expression is less dependent on binding of the MSL complex (Meller et al., 1997; Bai et al., 2004). This might be a likely explanation for why inactivating the CXC or CBD only partially reduced recruitment of the DCC to the X chromosomes in males, but is fully required in females.

Importantly, even the double MSL2 mutant can partially support recruitment of the DCC to the X chromosome. The mutant $MSL2^{R*\Delta 13d}$ still organized DCC by forming the core complex with MSL1 through the RING domain and recruiting MLE via roX RNA. Previously, the N-terminal domain (84 aa) of MSL1 was shown to be required for recognition of the X chromosome by the DCC (Scott et al., 2000; Li et al., 2005). To explain functional redundancy of MSL2 domains in males, we propose a model in which multiple protein-protein and CXC-DNA interactions are responsible for the specificity of DCC recruitment to the male X chromosome (Fig. 4). In the future, identification of additional proteins that directly interact with the components of DCC, will be key to understanding the mechanisms of selective recruitment of the DCC to HAS/CES on the X chromosome.

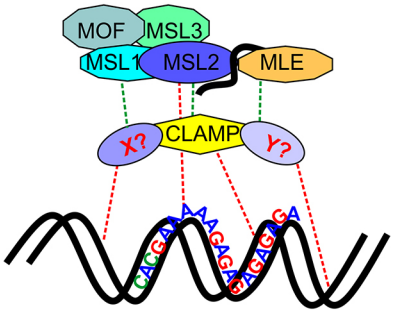


**Fig. 4. Model explaining redundancy and cooperativity of CBD and CXC in functional activity of MSL2.** Red dashed lines show potential DNA-protein contacts, green dashed lines show protein-protein interactions.

## MATERIALS AND METHODS

### Plasmid construction

For *in vitro* experiments, protein fragments were either PCR-amplified using corresponding primers, or digested from MSL2 or CLAMP cDNA and subcloned into pGEX-4T1 (GE Healthcare) or into a vector derived from pACYC and pET28a(+) (Novagen) bearing p15A replication origin, Kanamycin resistance gene, and pET28a(+) MCS.

To express 3×FLAG-tagged MSL2 and 3×HA-tagged CLAMP in the S2 cells, protein-coding sequences were cloned in frame with 3×FLAG and 3×HA, excised, and subcloned into the pAc5.1 plasmid (Life Technologies). Plasmids for the yeast two-hybrid assay were prepared using the full-sized and truncated versions with separate domains of MSL2 and CLAMP as C-termini fused with pGBT9 and pGAD424 vectors from Clontech. PCR-directed mutagenesis was used to make constructs with mutated MSL2 (corresponding primers are given in Table S1). Different full-sized variants of MSL2 were fused with 3×FLAG at the C terminus and cloned into an expression vector. This vector contains an *attB* site for ϕC31-mediated recombination, the *Ubi67c* promoter with its 5′UTR, a 3′UTR with SV40 polyadenylation signal, and the intronless *yellow* gene as a reporter for detection of transformants. Details of the cloning procedures, primers, and plasmids used for plasmid construction are available in Table S2.

### Pulldown assays

BL21 cells co-transformed with plasmids expressing GST-tagged and 6×His-tagged derivatives of MSL2 and CLAMP were grown in LB media to an A600 of 1.0 at 37°C and then induced with 1 mM IPTG at 18°C overnight. $ZnCl_2$ was added to final concentration 100 µM before induction. Cells were disrupted by sonication, centrifuged at 13,000 ***g*** for 30 min, applied to resin for 10 min at 4°C, followed by four washes with buffer C (see below) containing 500 mM NaCl. GST pulldown was performed with Immobilized Glutathione Agarose (Pierce) in buffer C [20 mM HEPES-KOH, pH 7.7, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% NP40 and 10% (w/w) glycerol]. The bound proteins were eluted with 50 mM reduced glutathione, 100 mM Tris-HCl, pH 8.0, 100 mM NaCl for 15 min. The 6×His-pulldown was performed similarly, with Zn-IDA resin (Cube Biotech) in buffer A (50 mM HEPES-KOH, pH 7.6, with 500 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 20 mM imidazole, 5% glycerol, 0.1% NP-40 and 5 mM β-mercaptoethanol) containing 1 mM PMSF and Calbiochem Complete Protease Inhibitor Cocktail VII (5 µl/ml), washed with buffer A containing 30 mM imidazole, and proteins were eluted with buffer B (50 mM HEPES-KOH, pH 7.6, with 500 mM NaCl, 250 mM imidazole and 5 mM β-mercaptoethanol), 20 min at +4°C.

### Antibodies

Antibodies against MSL1 (aa 423-1030), MSL2 (aa 421-540), CLAMP (aa 222-350) were raised in rabbits and purified from the sera by ammonium sulfate fractionation followed by affinity purification on CNBr-activated Sepharose (GE Healthcare) or Aminolink Resin (Thermo Fisher Scientific) according to standard protocols. Affinity-purified antibodies were tested with immunoblot analysis (Fig. S9). Mouse monoclonal anti-FLAG (clone

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M2, F1804, 1:500) and anti-HA (clone HA-7, H3663, 1:200) antibodies were from Sigma-Aldrich.

### Cell culture, transfection

*Drosophila* S2 cells were grown in SFX medium (HyClone) at 25°C. Transfection of plasmids was performed with the Cellfectin II reagent (Invitrogen) according to the manufacturer's instructions. Typically, cells were transfected in six-well plates and grown for 24-48 h before harvesting.

### Co-immunoprecipitation assay

S2 cells grown in SFX medium were co-transfected by 3×FLAG-MSL2 (with deletions) and 3×HA-CLAMP plasmids with Cellfectin II (Life Technologies), as recommended by the manufacturer. After transfection, the cells were incubated for 48 h and then collected by centrifugation at 700 ***g*** for 5 min, washed once with 1×PBS and re-suspended in 20 packed cell volumes of hypotonic lysis buffer (20 mM Tris-HCl, pH 7.4, with 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT and Calbiochem Complete Protease Inhibitor Cocktail V). After incubation on ice for 10 min, the cells were sonicated (2×15 s on ice at 20% output, 40% duty cycle), NaCl was added to a final concentration of 420 mM, and incubation on ice continued for 60 min, with periodic mixing. Sonication was repeated as above to reduce viscosity, cell debris was pelleted by centrifugation at 10,000 ***g*** for 30 min at 4°C, and the supernatant was collected for immunoprecipitation with anti-CLAMP antibodies, incubated with Protein A Agarose (Pierce), and equilibrated with incubation buffer-150 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol and 0.1% NP-40). Rabbit IgG conjugated to Protein A Agarose beads (by incubating in the same buffer on a rotary shaker at 4°C for 1 h) were used as negative control. The protein extract (50 µg protein) was adjusted to a volume of 500 µl with buffer-150, mixed with antibody-conjugated beads (30 µl), and incubated on a rotary shaker overnight at 4°C. The beads were then washed with one portion of incubation buffer-500 (buffer-150 with 500 mM NaCl), one portion of incubation buffer-300 (buffer-150 with 300 mM NaCl), and one portion of incubation buffer-150, re-suspended in SDS-PAGE loading buffer, boiled, and analyzed by western blotting. Proteins were detected using the ECL Plus Western Blotting substrate (Pierce) with anti-FLAG (clone M2, F1804, Sigma-Aldrich, 1:500) and anti-HA (clone HA-7, H3663, Sigma-Aldrich, 1:200) antibodies.

### Fly crosses and transgenic lines

*Drosophila* strains were grown at 25°C under standard culture conditions. The transgenic constructs were injected into preblastoderm embryos using the ϕC31-mediated site-specific integration system at locus 86Fb (Bischof et al., 2007). The emerging adults were crossed with the *y ac* $w^{1118}$ flies, and the progeny carrying the transgene in the 86Fb region were identified by $y^+$ pigmented cuticle.

Viability (as a relative percentage) of $msl2^{\gamma 227}/msl2^{\gamma 227}$ males expressing different MSL2 derivatives (MSL2*) was calculated as a ratio of males to females in $msl2^{\gamma 227}/msl2^{\gamma 227}$; MSL2*/MSL2* lines. Viability (as a relative percentage) of females expressing different variants of MSL2 (MSL2*) were calculated as a ratio of females to males in MSL2*/TM6,Tb (heterozygous) or MSL2*/MSL2* (homozygous) lines. At least 300 males and females were counted in each of three independent experiments.

### Fly extract preparation

Twenty adult flies were homogenized with a pestle in 200 µl of 1×PBS containing 1% β-mercaptoethanol, 10 mM PMSF and 1:100 Calbiochem Complete Protease Inhibitor Cocktail VII. The suspension was sonicated three times for 5 s at 5 W. Then, 200 µl of 4×SDS-PAGE sample buffer was added and the mixture was incubated for 10 min at 100°C and centrifuged at 16,000 ***g*** for 10 min.

### RNA isolation and quantitative analysis

Total RNA was isolated from 2- to 3-day-old adult males and females using the TRI reagent (Molecular Research Center) according to the manufacturer's instructions. RNA was treated with two units of Turbo DNase I (Ambion) for 30 min at 37°C to eliminate genomic DNA. The synthesis of cDNA was performed using 2 µg of RNA, 50 U of ArrayScript reverse transcriptase (Ambion) and 1 µM of oligo(dT) as a primer. The amounts of specific cDNA fragments were quantified by real-time PCR with Taqman probes. At least three independent measurements were made for each RNA sample. Relative levels of mRNA expression were calculated in the linear amplification range by calibration to a standard genomic DNA curve to account for differences in primer efficiencies. Individual expression values were normalized with reference to *RpL32* mRNA.

The sequences of primers and oligonucleotides used in this work are given in Table S1.

### Immunostaining of polytene chromosomes

*Drosophila* 3rd instar larvae were cultured at 18°C under standard conditions. Polytene chromosome staining was performed as described (Murawska and Brehm, 2012). The following primary antibodies were used: rabbit anti-MSL1at 1:100 dilution, rabbit anti-MSL2 at 1:100 dilution, and monoclonal mouse anti-FLAG at 1:100 dilution. The secondary antibodies were Alexa Fluor 488 goat anti-mouse 1:2000 and Alexa Fluor 555 goat anti-rabbit 1:2000 (Invitrogen). The polytene chromosomes were co-stained with DAPI (AppliChem). Images were acquired on the Nikon Elclipse T*i* fluorescent microscope using Nikon DS-Qi2 digital camera, processed with ImageJ 1.50c4 and Fiji bundle 2.0.0-rc-46. Three or four independent staining and four or five samples of polytene chromosomes were performed with each MSL2-expressing transgenic line.

### Chromatin immunoprecipitation

Chromatin preparation was performed as described (Maksimenko et al., 2015; Zolotarev et al., 2017) with some modifications. Samples containing 10-20 µg of DNA equivalent in 1 ml of nuclear lysis buffer were incubated overnight, at 4°C, with rabbit antibodies against MSL1 (1:500), MSL2 (1:200) and CLAMP (1:200), or with nonspecific IgG purified from rabbit preimmune sera (control). Chromatin–antibody complexes were collected using blocked Protein A agarose at 4°C over 5 h. After several rounds of washing with one portion of lysis buffer, three portions of lysis buffer with 500 mM NaCl and one portion of TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), the DNA was eluted with elution buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 1% SDS), the cross-links were reversed, and the precipitated DNA was extracted with phenol–chloroform. The enrichment of specific DNA fragments was analyzed by real-time PCR using a QuantStudio 12K Flex Cycler (Applied Biosystems). The primers used for PCR in ChIP experiments for genome fragments are shown in Table S1.

At least three independent biological replicates were made for each chromatin sample. The results of chromatin immunoprecipitation are presented as a percentage of input genomic DNA normalized to a positive control genomic site. Error bars show standard deviations of quadruplicate PCR measurements for three independent experiments. The *tubulin-γ37C* coding region (devoid of binding sites for the test proteins) were used as negative controls; Autosomal MSL1-binding region 26E3, MSL2-binding region 25A3 and CLAMP-binding region 39A1 were used as positive genomic controls.

#### Acknowledgements

We thank Farhod Hasanov and Aleksander Parshikov for fly injections, and Olga Kyrchanova for help with fly genetics. This study was performed using the equipment of the IGB RAS Core Facilities Centre, supported by the Ministry of Science and Education of the Russian Federation.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: P.G., O.M.; Methodology: O.M.; Formal analysis: E.T., O.M.; Investigation: E.T., A.F., A.B., V.M., O.M.; Writing - original draft: P.G., O.M.; Writing - review & editing: E.N.L., P.G., O.M.; Visualization: E.T., A.F., A.B., V.M.; Supervision: P.G., O.M.; Funding acquisition: O.M.

#### Funding

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#### Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.179663.supplemental

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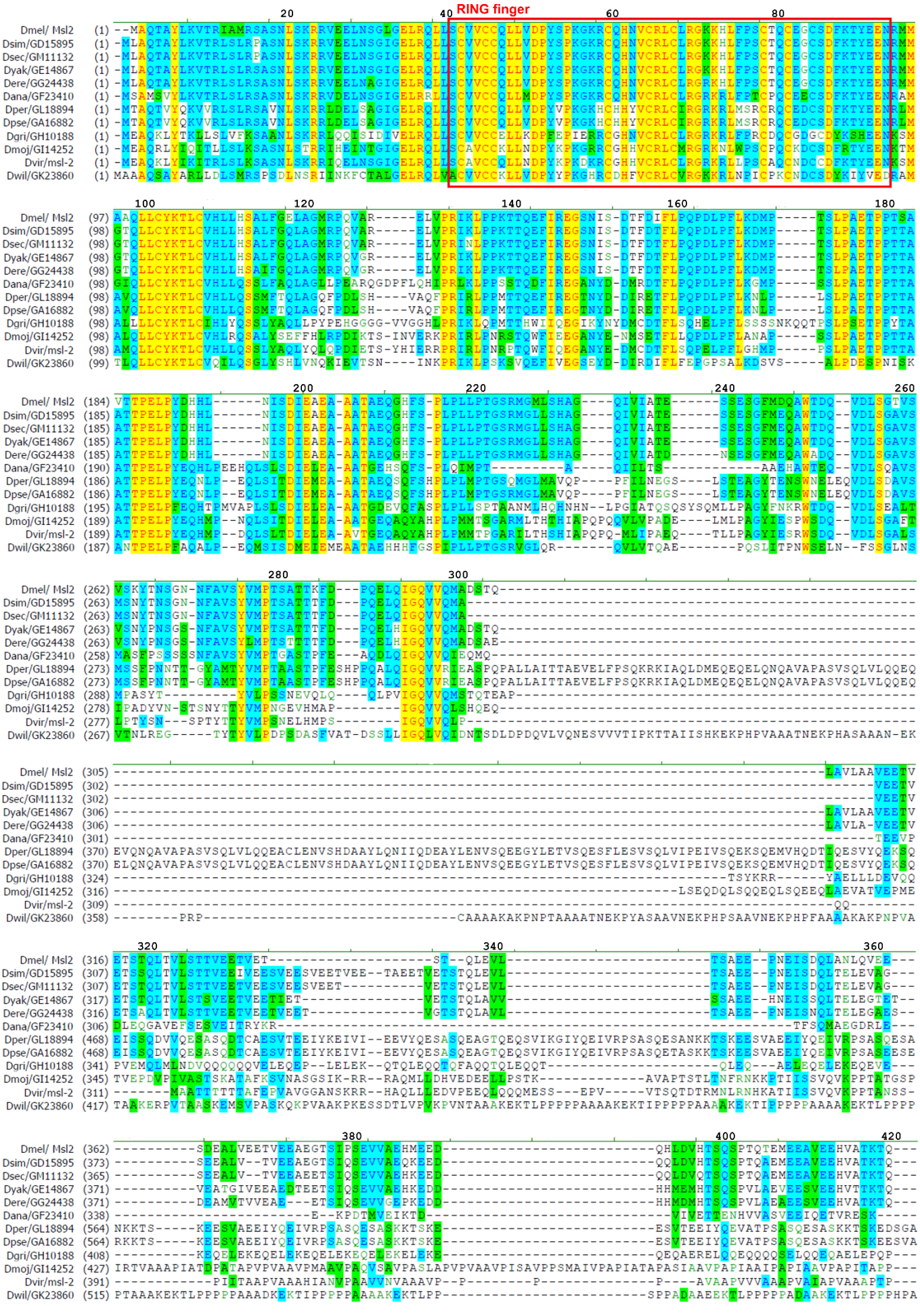
# Supplementary Figures



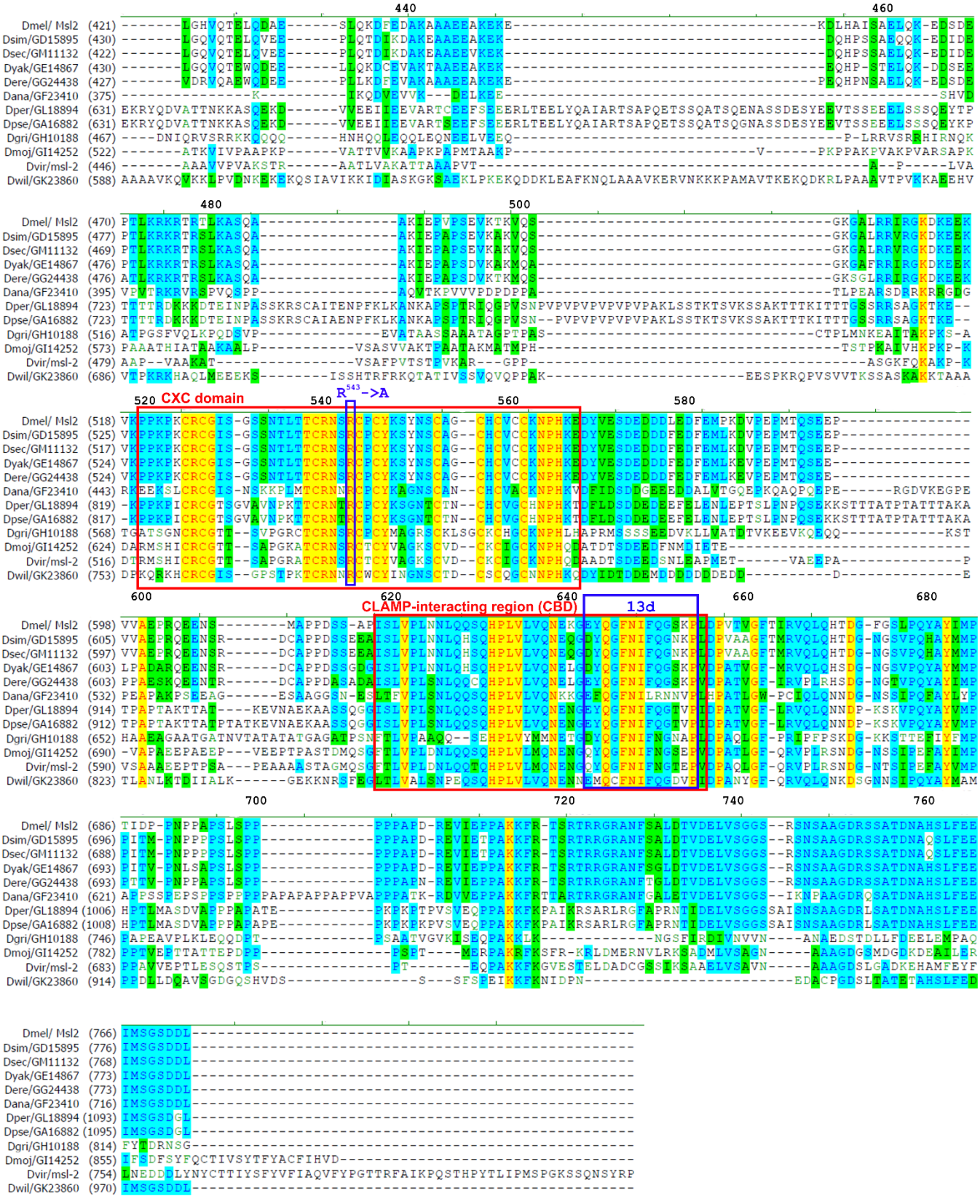



**Figure S1.** Alignment of MSL2 protein among Drosophilidae. Different regions are shown in the red frames. Also R543 amino acid residue and minimal 13 aa CLAMP-interacting region are marked in blue frames.

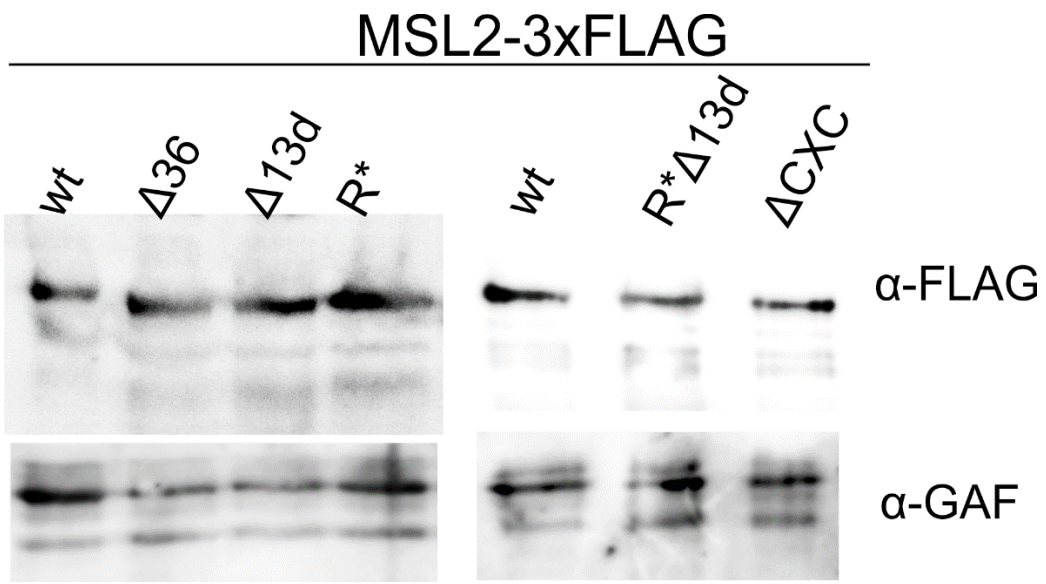



**Figure S2.**

Western blotting of protein extracts prepared from adult flies expressing different variants of 3xFLAG-tagged MSL2 protein (wild-type (wt), deletion of 36 (Δ36) and 13 (Δ13d) aa from CLAMP-binding region, substitution of arginine at the 543 position by alanine (R*), simultaneous substitution of arginine at the 543 position by alanine and deletion of 13 aa from CLAMP-binding region (R*Δ13d), deletion of CXC domain (ΔCXC)).

Immunoblot analysis was performed with FLAG and GAF antibodies (internal control).

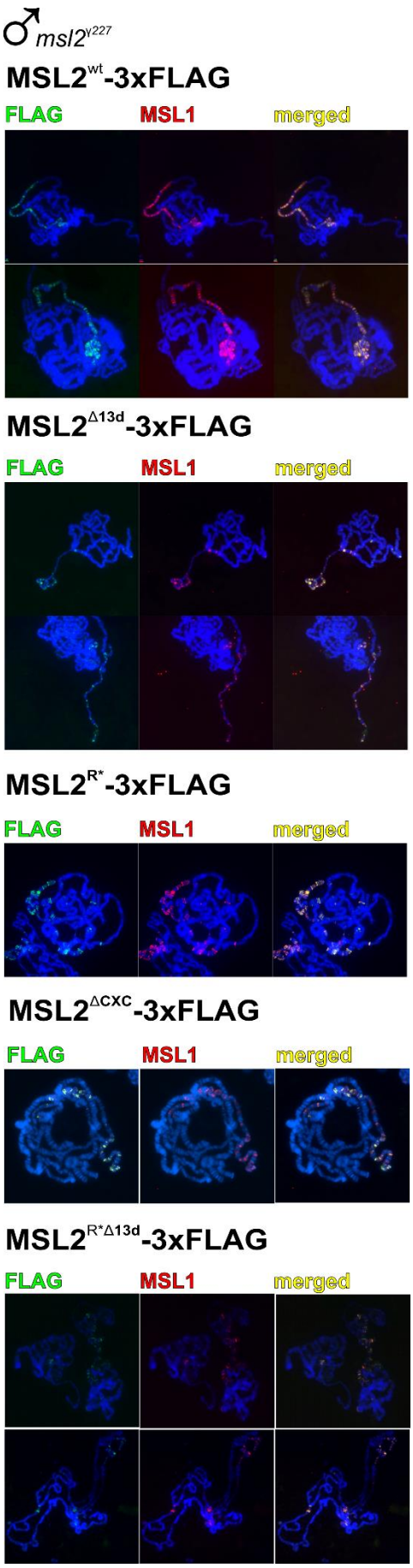



**Figure S3.** MSL1 and MSL2 localization on the polytene chromosomes from salivary glands of male 3rd instar larvae in the *msl2*-null (*msl2*$^{\gamma 227}$) background that express different FLAG-tagged variants of MSL2 protein: wild-type (wt), deletion of 13 aa from CLAMP-binding region (Δ13d), substitution of arginine at the 543 position by alanine (R*), deletion of CXC domain

(ΔCXC), simultaneous substitution of arginine at the 543 position by alanine and deletion of 13 aa from CLAMP-binding region (R*Δ13d). Panels show immunostaining of 3xFLAG-MSL2 protein with mouse anti-FLAG antibody (green) and MSL1 protein with corresponding rabbit antibody (red) on polytene chromosomes. DNA is stained with DAPI (blue).

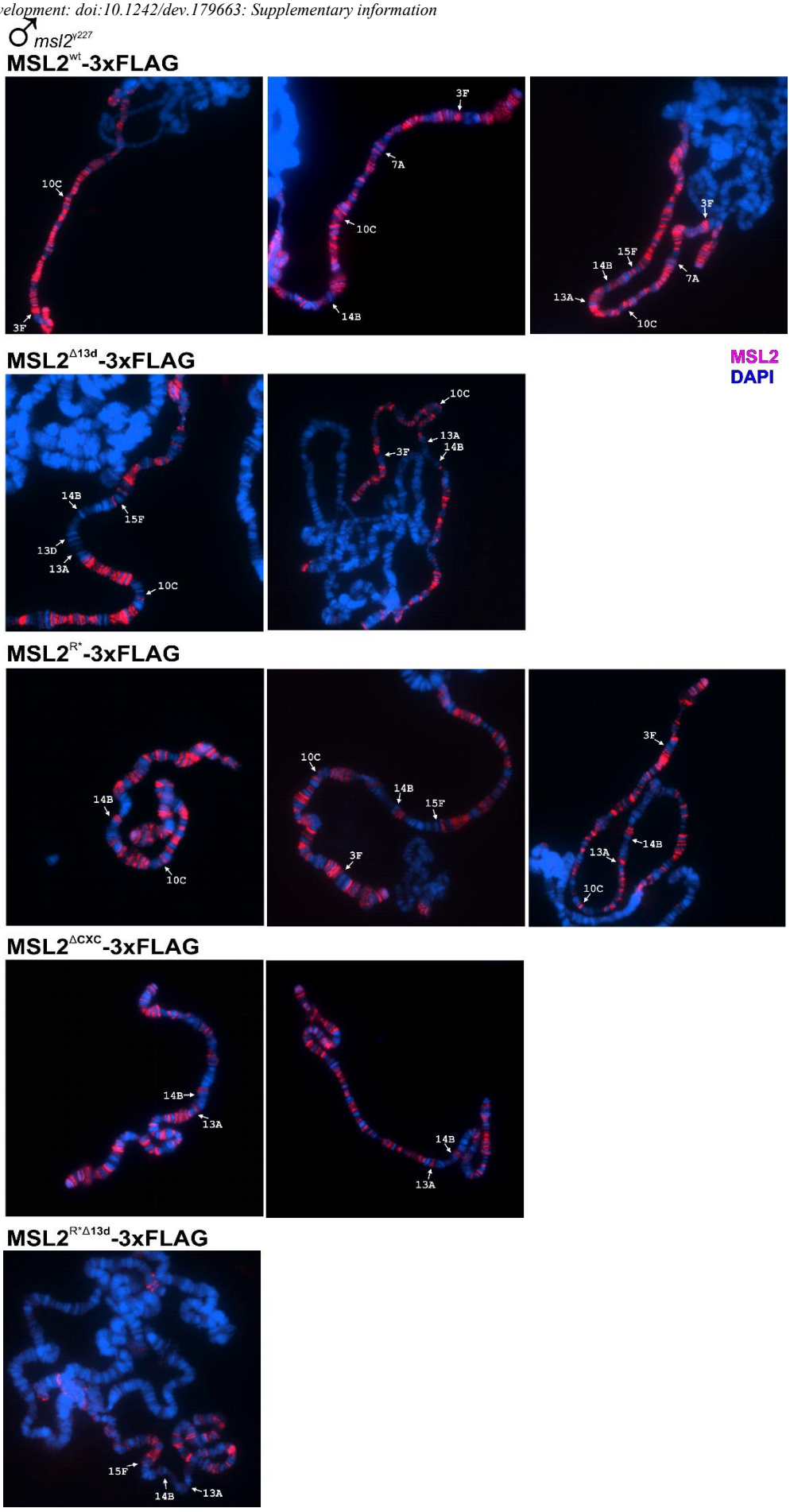



**Figure S4.** Comparing several sites of MSL complex binding at the high resolution cytological map on the polytene chromosomes from salivary glands of male 3rd instar larvae in the *msl2*-null (*msl2*$^{\gamma 227}$) background that expressed different FLAG-tagged variants of MSL2 protein: wild-

type (wt), deletion of 13 aa from CLAMP-binding region (Δ13d), substitution of arginine at the 543 position by alanine (R*), deletion of CXC domain (ΔCXC), simultaneous substitution of arginine at the 543 position by alanine and deletion of 13 aa from CLAMP-binding region (R*Δ13d). Panels show immunostaining of 3xFLAG-MSL2 protein with rabbit anti-MSL2 antibody (red) on polytene chromosomes. DNA is stained with DAPI (blue).

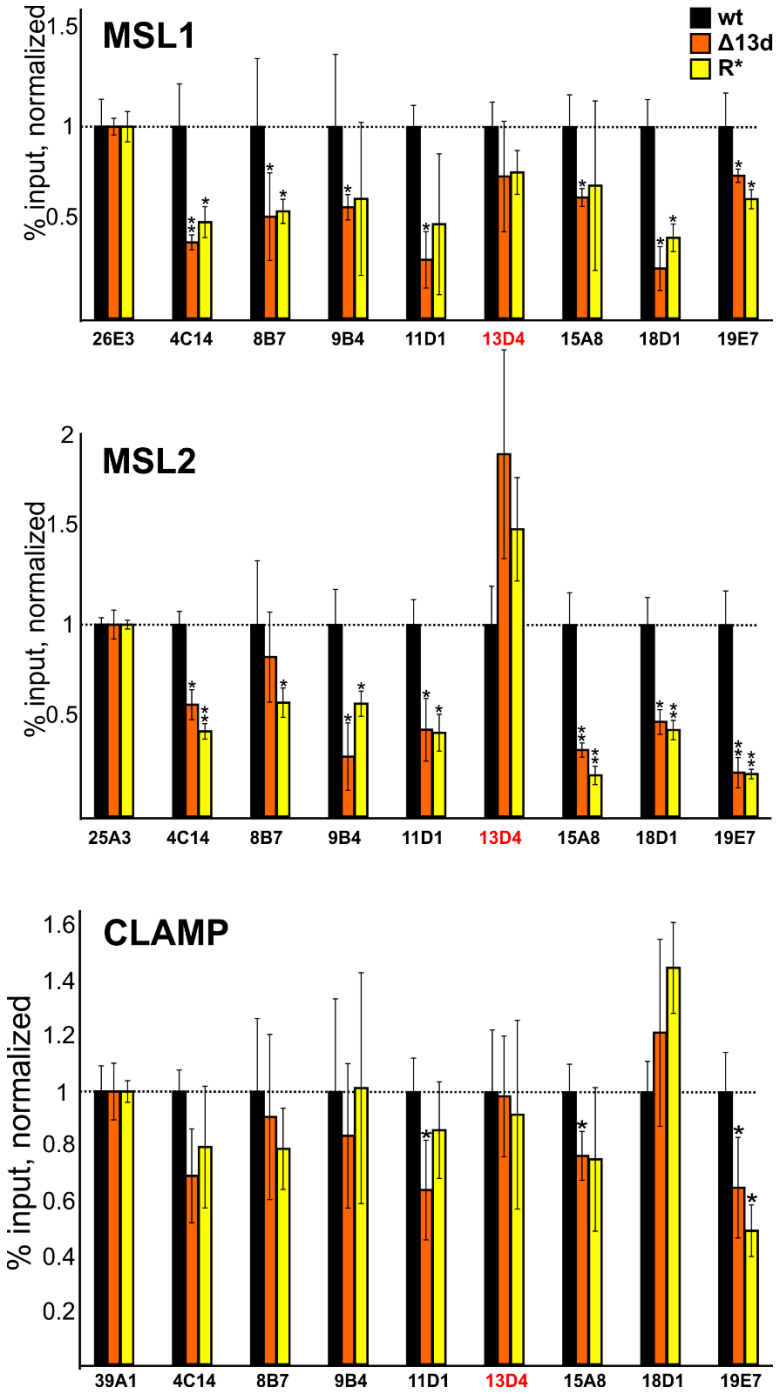



**Figure S5.** Comparing binding of MSL1, MSL2, and CLAMP at different CES and PionX (marked with red) regions in the MSL2-expressing males in the *msl2*$^{\gamma 227}$ background. Histograms show ChIP enrichments at the CES regions on chromatin isolated from male flies expressing different variants (wt, Δ13d, R*) of MSL2 protein. The results are presented as a percentage of input genomic DNA normalized to corresponding positive autosomal genome regions for MSL1(26E3) and MSL2 (25A3), and compared with binding levels in the flies with expressed wild type MSL2 protein (corresponding to the "1" on the scale). Error bars show standard deviations of quadruplicate PCR measurements for three independent experiments. Asterisks indicate significance levels: *$p < 0.05$, **$p < 0.01$.

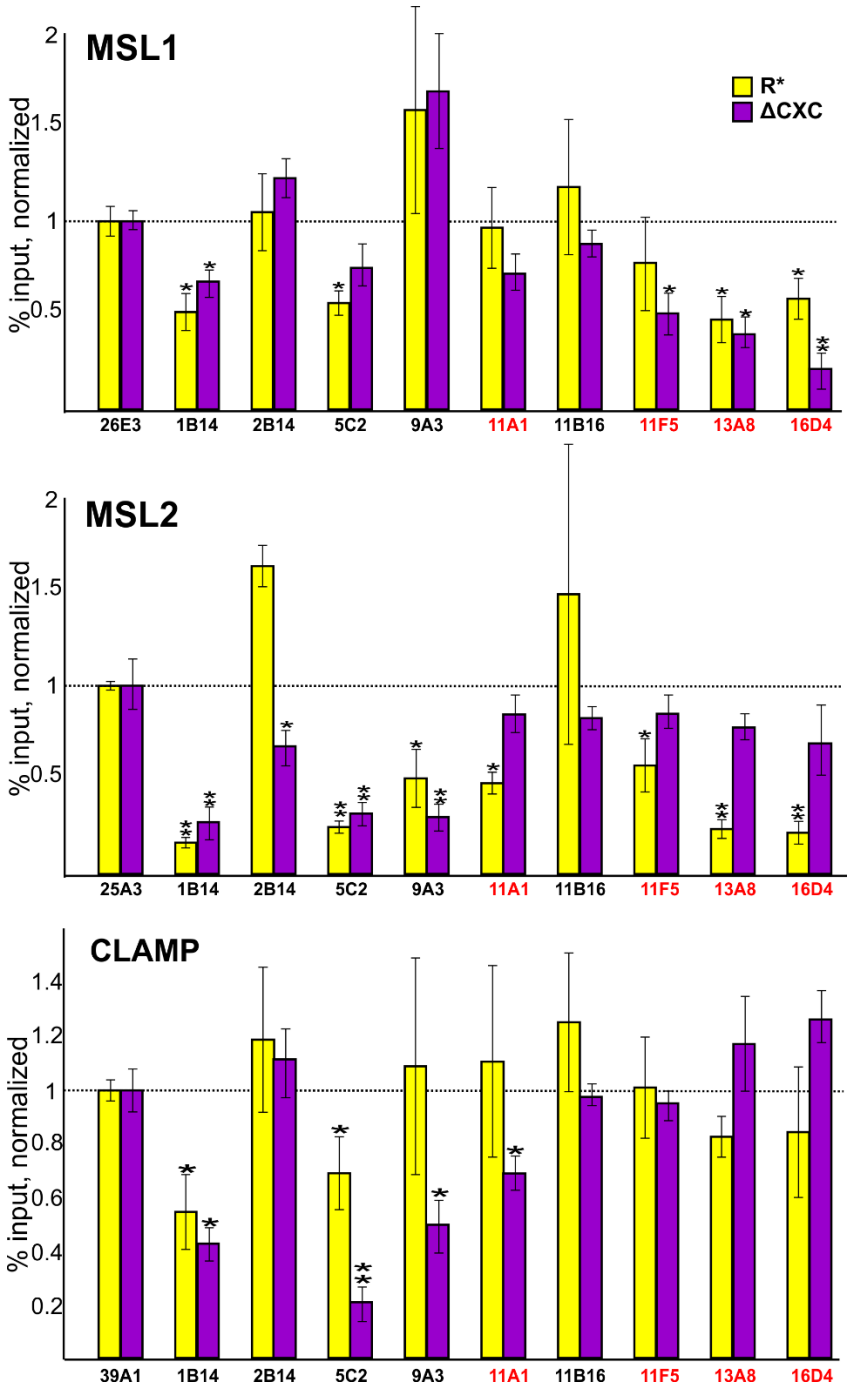



**Figure S6.** Comparing binding of MSL1, MSL2, and CLAMP at different CES and PionX (marked with red) regions in the MSL2-expressing flies in the *msl2*$^{\gamma 227}$ background. Histograms show comparison of ChIP enrichments at the CES regions on chromatin isolated from male flies expressed MSL2 protein with substitution of arginine at the 543 position by alanine (R*) and deletion of CXC domain (ΔCXC). The results are presented as a percentage of input genomic DNA normalized to corresponding positive autosomal genome regions for MSL1(26E3), MSL2 (25A3) and CLAMP (39A1), and compared with binding levels in the flies with expressed wild type MSL2 protein (corresponding to the "1" on the scale). Error bars show standard deviations of quadruplicate PCR measurements for three independent experiments. Asterisks indicate significance levels: *$p < 0.05$, **$p < 0.01$.

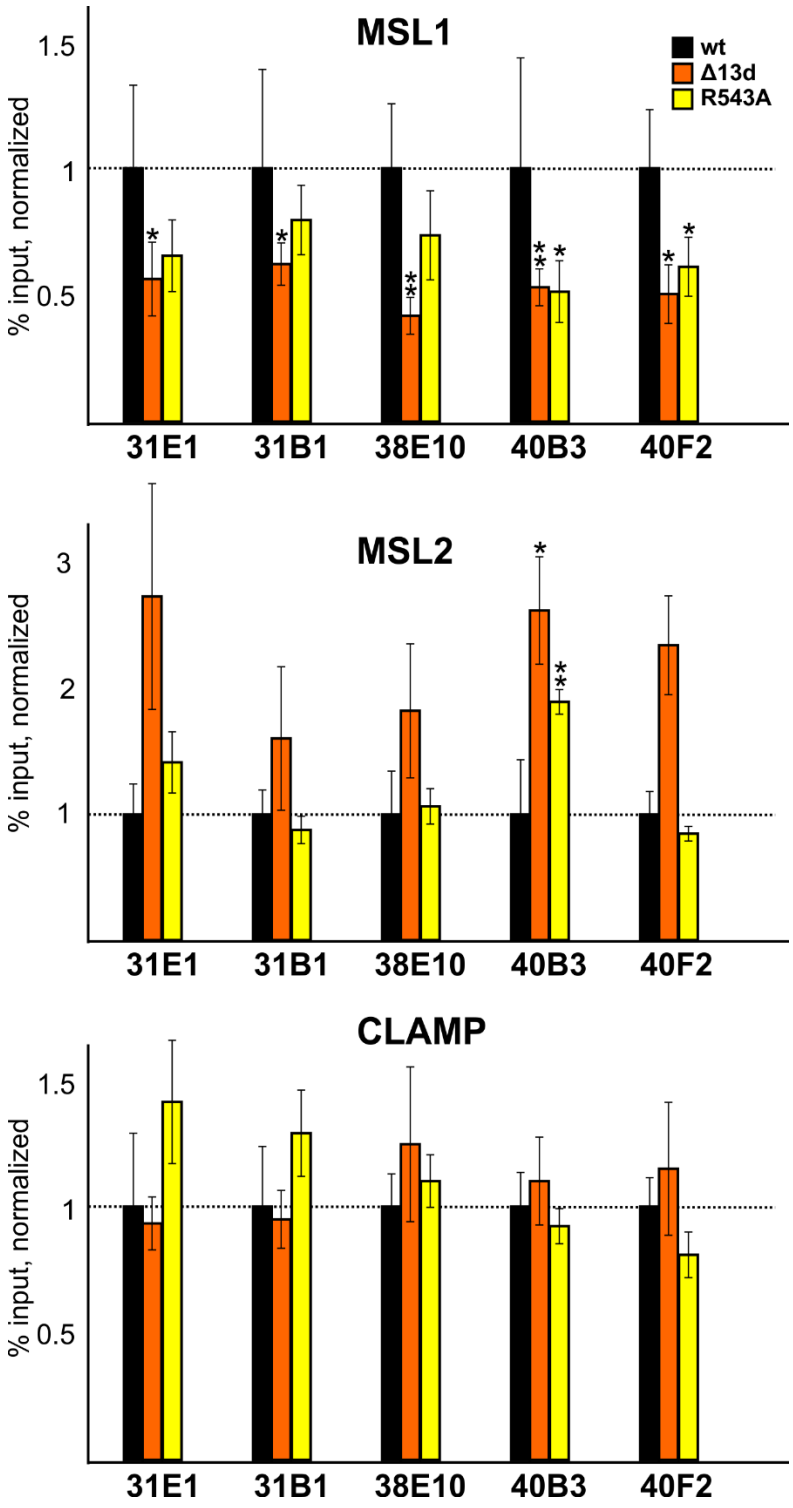



**Figure S7.** Comparing binding of MSL1, MSL2 and CLAMP at different autosomal genome regions in the MSL2-expressing flies in the *msl2*$^{\gamma 227}$ background. Histograms show ChIP enrichments at the CES regions on chromatin isolated from male flies expressing different variants (wt, Δ13d, R543A) of MSL2 protein. The results are presented as a percentage of input genomic DNA normalized to corresponding positive autosomal genome regions for MSL1(26E3), MSL2 (25A3), and compared with binding levels in the flies with expressed wild type MSL2 protein (corresponding to the "1" on the scale). Error bars show standard deviations of quadruplicate PCR measurements for three independent experiments. Asterisks indicate significance levels: *$p < 0.05$, **$p < 0.01$.

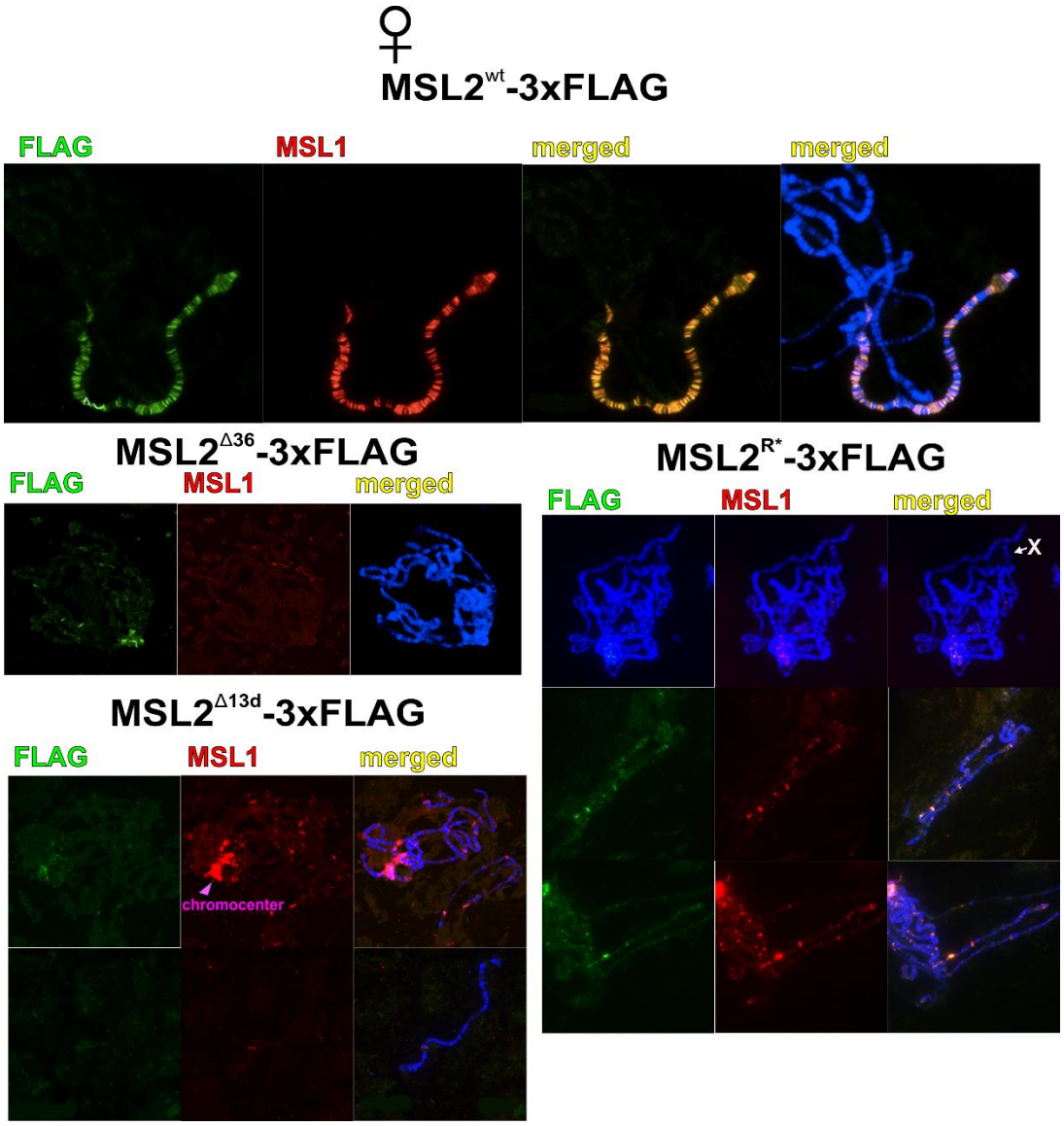



**Figure S8.** Distribution of MSL complex on the polytene chromosomes extracted from salivary glands of female 3rd instar larvae that express different FLAG-tagged variants of MSL2 protein: wild-type (wt), deletion of 36 (Δ36) and 13 (Δ13d) aa from CLAMP-binding region, substitution of arginine at the 543 position by alanine (R*). Panels show immunostaining of 3xFLAG-MSL2 protein with mouse anti-FLAG antibody (green) and MSL1 protein with corresponding rabbit antibody (red) on polytene chromosomes. DNA is stained with DAPI (blue).

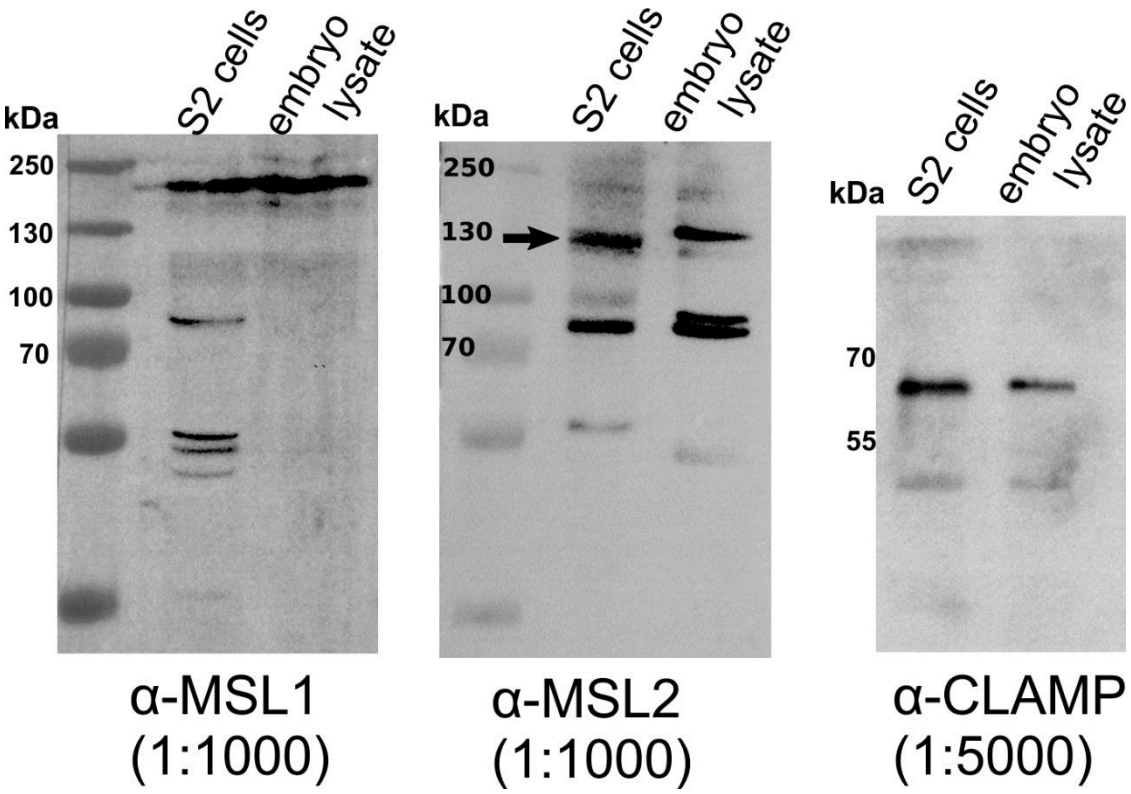



**Figure S9. Immunoblot analysis of affinity-purified antibodies for MSL1, MSL2, CLAMP proteins.**

# Supplementary Table

**Table S1. The list of used oligonucleotides.**

| **Primers for cloning of msl2 deletions** | | | |
|---|---|---|---|
| **Name** | **dir** | **rev** | |
| msl2_full-sized | TTCGATATCATGGCCCAGACGGCATACTT | CCCGGGCAAGTCATCCGAGCCCGA | |
| msl2_d13 | AAAGGCGAGGATCCGGTCACCGTTGG | GACCGGATCCTCGCCTTTCTCATTCT | |
| msl2_dB | CCACCGGCCGCCAACTTCTCGGCCCT | GAAGTTGGCGGCCGGTGGCTCGATGA | |
| msl2_dP | TATATAATGGCCAAGAAATTCAGGACC | TTTCTTGGCCATTATATAAGCATACT | |
| **Primers for RT-qPCR** | | | |
| **Name** | **dir** | **rev** | **Taqman probe** |
| RoX1 | CTTGTGCTTTCTCCTGAATGTG | TGTATTAGGCGGAGCTTCTTG | FAM-AGCCTATGAAATCCGGTCCAACCC-BHQ1 |
| RoX2 | TTCGAAACGTTCTCCGAAGC | AGTCGTACTCATCTCACTGTCC | FAM-AGCAAGAGTAACGATTTCCGCATAGTCG-BHQ1 |
| RpL34 | ACAACACACGCTCCAACA | GGGTGATACCCTTCAACTTCTC | FAM-TGGTAAACCAGACGACCACCGG-BHQ |
| **Primers for ChIP analysis** | | | |
| **Genome region** | **dir** | **rev** | |
| RoX1 | AGGTCTGCAAGGTTCAGTTTAT | ATTCTTAAGGGTGGCGTCTTC | |
| RoX2 | GGCTTAGAGAGAGATGGCAATAC | AGTTCTGGTCACCCTGGA | |
| CES1B14 | CAGGACAAGACTAGGACAAAGG | GGTTTGCGGATTGAGGATTATTG | |
| CES2B14 | CCCTCATATGCTTCTTCTGGTC | ACGTACGATCACGCACATATC | |
| CES4C14 | ATTGGTATTCGGGCAAGGG | GAGCATATTTCTCTCGAGGATGG | |
| CES5C2 | CAGAAATTCGAAGCGATCTCAAC | AATCGACTGCTAGGTTGGTAAA | |
| CES8B7 | CAGCAGAACGCTCTTTGATTT | CCAACATTCGCTTCACACAC | |
| CES9A3 | TAATGCTGGATCTCGCTCAC | CTGATCGCCGGTCATAGAAA | |
| CES9B4 | CGTGCCGCCTAACTATCTAAC | GGGATGGAAAGAGAGAGCAAA | |
| CES11A1 | GCCCGGAACTCCTTTAGTATG | TGATGCCACTGGATGAGTATG | |
| CES11B16 | GGTGGTGGACATCTCGTTAAT | ACCAGCGAATATCGAGCATAAA | |
| CES11D1 | AAGCCACTGATGCGTACAA | CAGAACGGCTGGCAAGATA | |
| CES11F5 | TACAGTAGCTGAGAGCTGTACT | GTTTGGACTTGCGCCTTTAC | |
| CES13A8 | AATCTGAGCGAGATGGAAGAAC | GTGTCTAGTGGTTGGCTATGAC | |
| CES13D4 | GCCGTGATTGTGGATCTCTT | CTCCAGACGTGCTGAATTGT | |
| CES15A8 | CCAGGCTAAATAGTTCGCTACA | CGTAGTTGCATCTCGCTCTAAT | |
| CES16D4 | CATCAACGACCTTGTCACATTATC | GCACGAATCAGACAGAGAAGTA | |
| CES18D11 | GCGCTTGCAATAGCTTCAATTA | GGAAATCCAAACAGTACAACTTCG | |
| CES19E7 | GAGCGAGAGAGATTGCCAAATA | CGAATCGTGGAGTCTGAGAAAT | |
| 26E3 | CGTAACGGCACCCCTCAA | ACCGCACCGCACTACAAG | |
| 25A3 | GCTCCAGGAACCGATCTATTG | CTTGGCTTCCACTGAAGTTAGA | |
| 39A1 | CTCTTGTTCACACAGCCATTTC | TACTCTCTTCGGGCGGTATAA | |
| 31E1 | TGGATATGGCTTCTGGTTCATC | GGGCTGCATTCGGAGTTTA | |
| 31B1 | TTGTAAGGCGACTCGACTATTT | TGCTTTCGGCTGACTAATGA | |
| 38E10 | CGACACAAGTACCAGCTCTAAT | CGGCGAGGTACTTACCATATTC | |
| 40B3 | GCAGAAGGGAGAACTGTGAAATA | CTTCGCGGAGGGTTAATTGT | |
| 40F2 | CAGAACAACGCTCAGAGATAGA | GGGCTCTAAGAAATCCTACCAG | |

**Table S2. Cloning procedures**

| Pull down assay | | | |
|---|---|---|---|
| | **dir** | **rev** | **Restriction sites** |
| TRX-His-MSL2 [573-708] | ttggaattcatggaggactacgttg | agtgtcgacctattccctgtcaggagca | EcoRI SalI |
| TRX-His-CLAMP [1-153] | ctggaattcatggaagaccttaccaa | aacgtcgacttccccgtctgtatgcat | EcoRI SalI |
| TRX-His-CLAMP [40-153] | ctggaattcatgaaaacggagcagcagc | aacgtcgacttccccgtctgtatgcat | EcoRI SalI |
| TRX-His-CLAMP [86-153] | - | - | HincII SalI |
| TRX-His-CLAMP [116-153] | - | - | MunI SalI |
| GST-CLAMP [1-196] | tacccgggatggaagaccttaccaaaaac | aacgtcgacagacacaatctgtatctgg | SmaI SalI |
| GST-MSL2 [573-708] | ttggaattcatggaggactacgttg | agtgtcgacctattccctgtcaggagca | EcoRI SalI |
| GST-MSL2 [618-642] | - | - | BamHI RsaI |
| GST-MSL2 [618-655] | - | - | BamHI DpnI |
| GST-MSL2 [618-667] | - | - | BamHI PvuII |
| GST-MSL2 [618-687] | - | - | BamHI MunI |
| GST-MSL2 [651-708] | cagggatccaagcccttgatccggtc | agtgtcgacctattccctgtcaggagca | BamHI SalI |
| GST-MSL2 [630-655] | ctcggatccatgcagcatcctttggt | gtggaattcctaatcaaggggcttg | BamHI EcoRI |
| GST-MSL2 [641-655] | - | gtggaattcctaatcaaggggcttg | BamHI EcoRI |
| **Fly constructs** | | | |
| pSK msl2-Fx3 full | ttcgatatcatggcccagacggcatactt | tctcccgggcaagtcatccgagcccga | EcoRV SmaI |
| pSK msl2-Fx3 Δ36 | accgatatcatggagggcatggatctg | tggactagttaggcttatgggggcagaa | EcoRV SpeI |
| | ctcactagtcttgatccggtcaccgt | tctcccgggcaagtcatccgagcccga | SpeI SmaI |
| pSK msl2-Fx3 Δ13d | ttcgatatcatggcccagacggcatactt | gaccggatcctcgcctttctcattct | EcoRV SmaI |
| | aaaggcgaggatccggtcaccgttgg | tctcccgggcaagtcatccgagcccga | |
| pSK msl2-Fx3 R543A | atcgaattccgcctgtcctt | tctcccgggcaagtcatccgagcccga | EcoRI SmaI |
| pSK msl2-Fx3 ΔCXC | ttcgatatcatggcccagacggcatactt | cggattcttcttcggcttcggaggct | EcoRV SmaI |
| | aagccgaagaagaatccgcacaagga | cccgggcaagtcatccgagcccga | |
| pSK msl2-Fx3 R543A ΔCXC | atcgaattccgcctgtcctt | tctcccgggcaagtcatccgagcccga | EcoRI SmaI |
| yil Ubi msl2-Fx3 full attB | - | - | EcoRV NotI |
| yil Ubi msl2-Fx3 Δ36 attB | - | - | EcoRV NotI |
| yil Ubi msl2-Fx3 Δ13d attB | - | - | EcoRV NotI |
| yil Ubi msl2-Fx3 R543A attB | - | - | EcoRV NotI |
| yil Ubi msl2-Fx3 ΔCXC attB | - | - | EcoRV NotI |
| yil Ubi msl2-Fx3 R543A ΔCXC attB | - | - | EcoRV NotI |