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Functional Role of C-terminal Domains in the MSL2 Protein of *Drosophila melanogaster*

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Abstract—Dosage compensation complex (DCC), which consists of five proteins and two non-coding RNAs *roX*, specifically binds to the X chromosome in males, providing a higher level of gene expression necessary to compensate for the monosomy of the sex chromosome in male *Drosophila* compared to the two X chromosomes in females. The MSL2 protein contains the N-terminal RING domain, which acts as an E3 ligase in ubiquitination of proteins and is the only subunit of the complex expressed only in males. Functional role of the two C-terminal domains of the MSL2 protein, enriched with proline (P-domain) and basic amino acids (B-domain), was investigated. As a result, it was shown that the B-domain destabilizes the MSL2 protein, which is associated with the presence of two lysines ubiquitination of which is under control of the RING domain of MSL2. The unstructured proline-rich domain stimulates transcription of the *roX2* gene, which is necessary for effective formation of the dosage compensation complex.

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INTRODUCTION

Dosage compensation is the phenomenon of equalizing gene expression levels in organisms with different numbers of sex chromosomes. The mechanisms of dosage compensation in insects have been studied using the model organism *Drosophila melanogaster* [1-4]. Dosage compensation in Drosophila is based on formation of the RNA–protein complex, which is recruited to the male X chromosome and enhances gene expression by approximately two-fold. The dosage compensation complex (DCC) in Drosophila comprises five proteins (MSL1, MSL2, MSL3, MOF, and MLE) and two long non-coding RNAs (*roX1* and *roX2*). The subunits of DCC are highly conserved among animals, and the complex consisting of MSL1, MSL2, MSL3, and MOF proteins plays an important role in transcription regulation but not in dosage compensation in humans [5, 6].

Protein MSL2 is expressed exclusively in males and is considered as a key component of the dosage compensation complex [1, 2]. The MSL2 protein (Fig. 1a) consisting of 773 amino acids contains two highly conserved domains: N-terminal RING-domain and CXC-domain [4, 7]. The RING-domain is a conserved domain in MSL2 proteins of humans and Drosophila, which functions as a ubiquitin E3 ligase mediating ubiquitination of the specific substrates including core subunits of the dosage compensation complex [8, 9]. At the same time, the RING-domain is involved in interaction of MSL2 with the N-terminal coiled-coil domain of MSL1, which forms a homodimer [10-13]. MSL1 and MSL2 form core of the complex, which can specifically bind to certain male X-chromosome sites independent on other components of the dosage compensation complex [10]. MSL3 protein and acetyl transferase MOF interact with the C-terminal domain of MSL1 [14, 15]. The MLE helicase, belonging to the ATP-dependent RNA/DNA helicase family, specifically remodels secondary structure of the *roX* RNAs to increase their efficiency in formation of the dosage

Abbreviations: CES, chromatin entry sites; DCC, dosage compensation complex.

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compensation complex [16-18]. The second conserved domain of MSL2, CXC-domain (Zn3Cys9), is the only DNA binding domain found in the Drosophila dosage compensation complex proteins [19]. Structural analysis showed that the two CXC-domains can specifically bind to GA-repeats [20]. The incomplete dosage compensation complex comprising the core part of the MSL1–MSL2 complex binds to approximately 200 sites on the X chromosome, called chromatin entry sites (CES) [21] or high affinity sites (HAS) [22]. DNA elements rich in GA-repeats have been found in the CES region, which can bind the CXC-domain of the MSL2 protein [23]. The transcription factor CLAMP with its N-terminal zinc finger C2H2-type domain interacting with the unstructured region of amino acids 618-655 of the MSL2 protein [25-27] also binds to CES [24]. CLAMP has an N-terminal homodimerizing domain [28] and is involved in organizing distant contacts between the DCC binding sites [29]. It has been shown that the CXC and CLAMP-interacting domains of MSL2 jointly participate in the binding of MSL complex to the X chromosome in males [25].

The unstructured C-terminus of the MSL2 protein contains two regions: proline-rich (P-domain) and basic amino acid-rich (B-domain). The B-domain contains one of the numerous sites where self-ubiquitination of the MSL2 protein occurs *in vitro* [8]. It is hypothesized that the C-terminus specifically binds to *roX* RNA, providing efficient assembly of the MSL complex and inclusion of the MLE protein [30]. Some experimental data suggest that the C-terminus is involved in specific recognition of the GA-rich regions on the X chromosome by the CXC-domain in the male mammals [23]. Aim of this study was to elucidate functional role of the C-terminal regions in the MSL2 protein.

MATERIALS AND METHODS

Plasmid construction. For expression of the 3xFLAG-tagged full-length MSL2, wild-type and deletion variants corresponding to the P- and B-domains of the protein were fused with 3xFLAG at the C-terminus and cloned into an expression vector. The vector contains an *attB* site for *φC31*-dependent integration, a strong *Ubi-p63E* gene promoter with its 5′-UTR, last intron of the *dctcf* gene with its 3′-UTR, and SV40 polyadenylation signal. The intronless *yellow* gene was used as a reporter to screen for transformants. Details of the cloning procedures, primer sequences, and plasmids are available upon request.

Fly crosses and transgenic lines. *Drosophila melanogaster* lines were maintained at 25°C on standard yeast medium. Transgenic constructs were injected into preblastodermal embryos. Integration of constructs into the genome was achieved through the *φC31*-mediated site-specific integration at the 86F8 locus in the corresponding line with an *attP* site [31]. Flies obtained after injection were crossed with the y^1w^{1118} laboratory flies, and transgenic offspring were identified by cuticular structure pigmentation. Homozygous lines were obtained through a series of crosses via balancer chromosomes. Lines that were lethal in the homozygous state were maintained on balancer chromosomes. Details of crosses are available upon request.

Antibodies. Antibodies against MSL1 [423-1030], MSL2 [421-540], CLAMP [222-350] were raised in rabbits and purified from serum using ammonium sulfate fractionation followed by affinity purification on a CNBr-activated sepharose (GE Healthcare, USA) or Aminolink Resin (Thermo Fisher Scientific, USA) according to the standard protocols. Mouse monoclonal antibodies against FLAG epitope (clone M2) were obtained from Sigma (USA).

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 3 times for 5 s at 5 W. Next, 200 µl of 4×SDS-PAGE buffer was added, and the mixture was incubated for 10 min at 100°C and centrifuged at 16,000*g* for 10 min.

Immunostaining of polytene chromosomes. Drosophila 3rd instar larvae were raised at 18°C under standard conditions. Immunostaining of polytene chromosomes was performed as described previously [32]. The following primary antibodies were used: rabbit anti-MSl1 at dilution of 1 : 100, rabbit anti-MSL2 at dilution of 1 : 100, and mouse monoclonal anti-FLAG at dilution of 1 : 100. Secondary antibodies were goat anti-mouse conjugated with Alexa Fluor 488 used at dilution of 1 : 2000 and goat anti-rabbit conjugated with Alexa Fluor 555 used at dilution of 1 : 2000 (Invitrogen, USA). Polytene chromosomes were also stained with DAPI (AppliChem, USA). Images were captured using a Nikon Elclipse Ti fluorescent microscope equipped with a Nikon DS-Qi2 digital camera and processed using ImageJ 1.50c4 and Fiji bundle 2.0.0-rc-46 software. Three to four independent stainings were performed, and 4-5 samples of polytene chromosomes were obtained for each transgenic line expressing MSL2.

Chromatin immunoprecipitation. Chromatin preparation was performed according to the described protocols [33, 34] with some modifications. Samples of 500 mg each of adult flies were ground in a mortar in liquid nitrogen and resuspended in 10 ml of a buffer A (15 mM HEPES-KOH, pH 7.6, 60 mM KCl, 15 mM NaCl, 13 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5% NP-40, 0.5 mM DTT, supplemented with 0.5 mM PMSF and Calbiochem Complete Protease Inhibitor Cocktail V). The suspension was then homoge-

nized in a Dounce homogenizer with tight pestle and filtered through a 70-μm Nylon Cell Strainer (BD Biosciences, USA). The nuclei were pelleted by centrifugation at 4000*g*, 4°C, for 5 min in a buffer supplemented with sucrose, resuspended in a wash buffer (15 mM HEPES-KOH, pH 7.6, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, Calbiochem Complete Protease Inhibitor Cocktail V), and cross-linked using 1% formaldehyde for 15 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. The nuclei were washed with three 10-ml portions of wash buffer and resuspended in 1.5 ml of a nuclear lysis buffer (15 mM HEPES, pH 7.6, 140 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, Calbiochem Complete Protease Inhibitor Cocktail V). The suspension was sonicated (20×30 s with 60 s intervals, on ice at 50% output), and 50-μl aliquots were used to test the extent of sonication and to measure DNA concentration. Debris was removed by centrifugation at 14,000*g*, 4°C, for 10 min, and chromatin was pre-cleared with a Protein A agarose (Pierce, USA), blocked with BSA and salmon sperm DNA; 50-μl aliquots of such pre-cleared chromatin samples were stored as input material. 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 three rounds of washing with lysis buffer (as such and with 500 mM NaCl) and a single wash with TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), the DNA was eluted with an elution buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 1% SDS) at 65°C, proteins and RNA were removed by adding proteinase K and RNase A. DNA was purified using phenol-chloroform extraction followed by reprecipitation. Enrichment of specific DNA fragments was analyzed by real-time PCR using a QuantStudio 12K Flex Cycler (Applied Biosystems, USA).

At least three independent biological replicates were made for each chromatin sample. The results of chromatin immunoprecipitation are presented as percentage of input genomic DNA normalized to a positive control genomic site (a genomic site outside the CES to which the protein of interest binds). The tubulin-γ37C coding region (devoid of binding sites for the tested proteins) was used as a negative control; autosomal MSL1-binding region 26E3, MSL2-binding region 25A3, and CLAMP-binding region 39A1 were used as positive genomic controls.

RNA isolation and quantitative analysis. Total RNA was isolated from 2- to 3-day-old adult males and females using a TRI reagent (Molecular Research Center, USA) according to the manufacturer's instructions. RNA was treated with two units of Turbo DNase I (Ambion, USA) for 30 min at 37°C to eliminate genomic DNA. 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 corresponding to *roX1* and *roX2* 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 using a standard genomic DNA curve to account for differences in primer efficiencies. Individual expression values were normalized to RpL32 mRNA as a reference.

RESULTS

Study of functional role of the B- and P-domains of the MSL2 protein. The unstructured C-terminus of MSL2 (Fig. 1a) contains a proline-rich region (Proline-rich, P-domain 685-713 aa) and a region rich in basic amino acids (Basic-rich, B-domain, 715-728 aa). Both regions have a moderate level of conservation among different Drosophila species (Fig. 1b). However, several studies [30, 35-37] have provided experimental evidence that the C-terminal region of MSL2 interacts with *roX* RNA. Moreover, interaction of MSL2 with *roX* is important for the specific recruitment of DCCs to the male X chromosome [36, 37]. It was previously shown that deletion of the region 743-773 aa does not affect functions of the MSL2 protein *in vivo* [30]. Therefore, in this work, we investigated functional role of the adjacent P- and B-domains of the MSL2 protein.

For this purpose, MSL2 cDNA variants with deletions of sequences encoding regions 685-713 aa ($MSL2^{\Delta P}$) or 715-728 aa ($MSL2^{\Delta B}$) were obtained. To express the tested proteins, cDNA was inserted into an expression vector (Fig. 2a) under control of the strong promoter of the *Ubiquitin-p63E* (U) gene. cDNA for the MSL2 protein did not contain noncoding parts of the *msl-2* gene mRNA, which have binding motifs for the translation repressor Sxl in females [38]. As a result, the $U{:}msl\text{-}2^{WT}$ transgene is expressed at the same level in males and females.

The cDNAs to be cloned were fused in a single frame with the sequence encoding 3 copies of the FLAG epitope. The resulting transgenes ($U{:}msl\text{-}2^{\Delta P}$ and $U{:}msl\text{-}2^{\Delta B}$) were integrated into the 86Fb region on chromosome 3 using recombination system based on the *φC31* integrase [31]. As a control, we used the previously obtained *U:msl-2WT* (86Fb) line expressing wild-type MSL2 protein, $MSL2^{WT}$-FLAG [25]. To determine the level of expression of MSL2 mutants relative to the control,

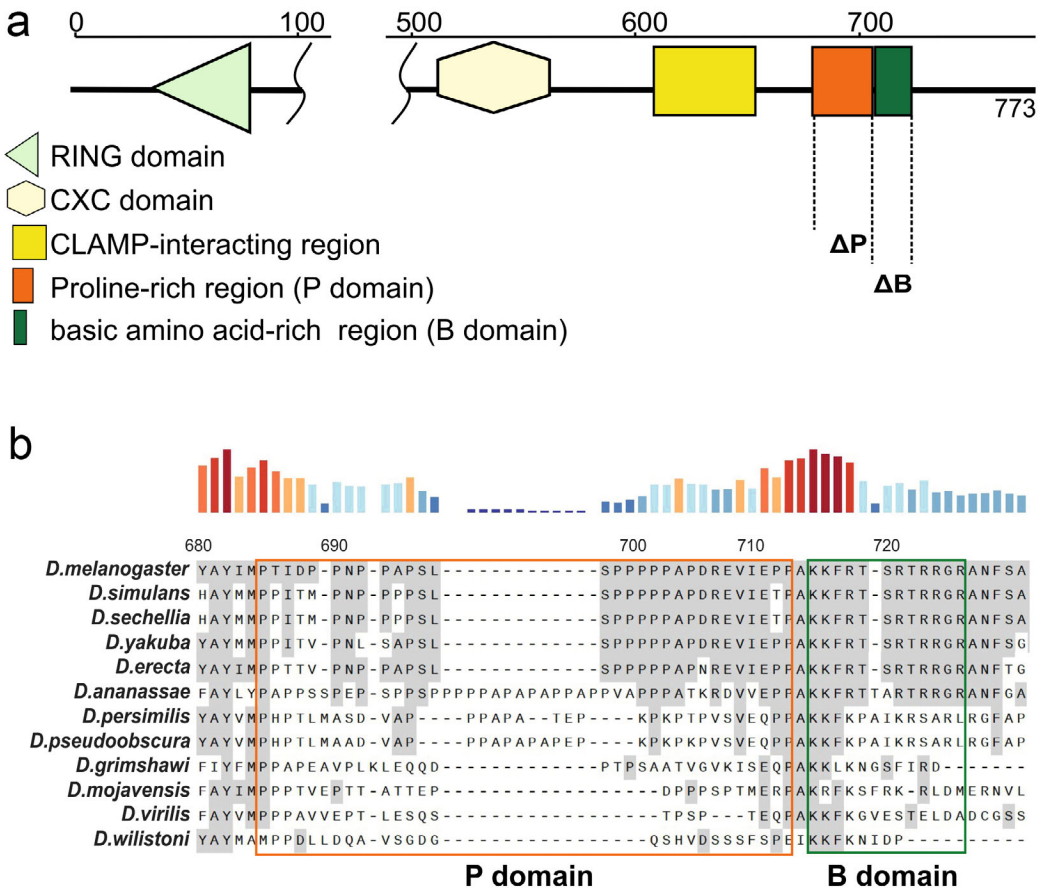

Fig. 1. Structural organization of the MSL2 protein. a) Scheme of the MSL2 protein. Main domains are shown: RING-, CXC-, CLAMP-binding, P- and B-domains. b) Clustal Omega sequence alignment of the C-terminal part of the MSL2 protein in the well-studied *Drosophilidae* species. P-domain is highlighted with an orange frame, and B-domain is highlighted with a green frame.

the amount of protein was determined using immunoblot analysis of the extracts obtained from the adult flies (Fig. 2b). It turned out that the MSL2$^{\Delta P}$-FLAG protein is expressed at the level comparable to the MSL2^{WT}-FLAG, while at the same time expression of the MSL2$^{\Delta B}$-FLAG increased 2-3-fold in comparison with the MSL2^{WT}-FLAG.

In the region 715-728 aa there are two sequentially located lysines (K715K716) ubiquitination of which *in vitro* is catalyzed by the RING-domain of the MSL2 protein [8]. The remaining lysines ubiquitinated *in vitro* by the RING-domain were localized in the region of aa 420-510. [8]. To determine contribution of the K715K716 lysines to stability of the MSL2 protein, constructs under the control of the *Ubiquitin-63E* promoter were obtained for transient expression in the S2 cell culture (Fig. 2c): MSL2^{WT}-FLAG (control), MSL2$^{\Delta RING}$-FLAG (deletion of the RING-domain in the MSL2 protein), and MSL2$^{\Delta B}$-FLAG. Expression levels of the MSL2 variants were detected using immunoblot analysis. The MSL2$^{\Delta B}$-FLAG and MSL2$^{\Delta RING}$-FLAG proteins were expressed at approximately the same level, several times higher than expression of the MSL2^{WT}-FLAG. Thus, it can be assumed that the amino acids K715K716 are the main targets for self-ubiquitination reducing stability of the MSL2 protein.

To clarify functional role of the P- and B-domains in the dosage compensation, ability of the mutant variants of the protein to restore survival of the males homozygous for the *msl2*^{y227} null mutation (2nd chromosome), which leads to complete inactivation of the *msl-2* gene, was investigated [7]. The *msl2*^{y227} mutation causes death of 100% of the males, predominantly at the embryonic and early larval stages and does not affect survival of the females. For the study, transgenic lines *msl2*^{y227}*/CyO* were obtained; *U:msl-2*/TM6,Tb*, in which *msl2*^{y227} and *U:msl-2** transgenes were bred respectively onto the *CyO* (2nd chromosome) and *TM6,Tb* (3rd chromosome) balancers. Expression of the MSL2 variants was examined only in the males that have one copy of the transgene (*U:msl-2*/TM6,Tb*). At the same time, comparison was made of the survival rate of males homozygous for the null mutation (*msl2*^{y227}/*msl2*^{y227}) relative to the *msl2*^{y227}*/CyO* (control) males with normal survival. As a result, it was shown that survival rate of the males expressing MSL2$^{\Delta P}$ and

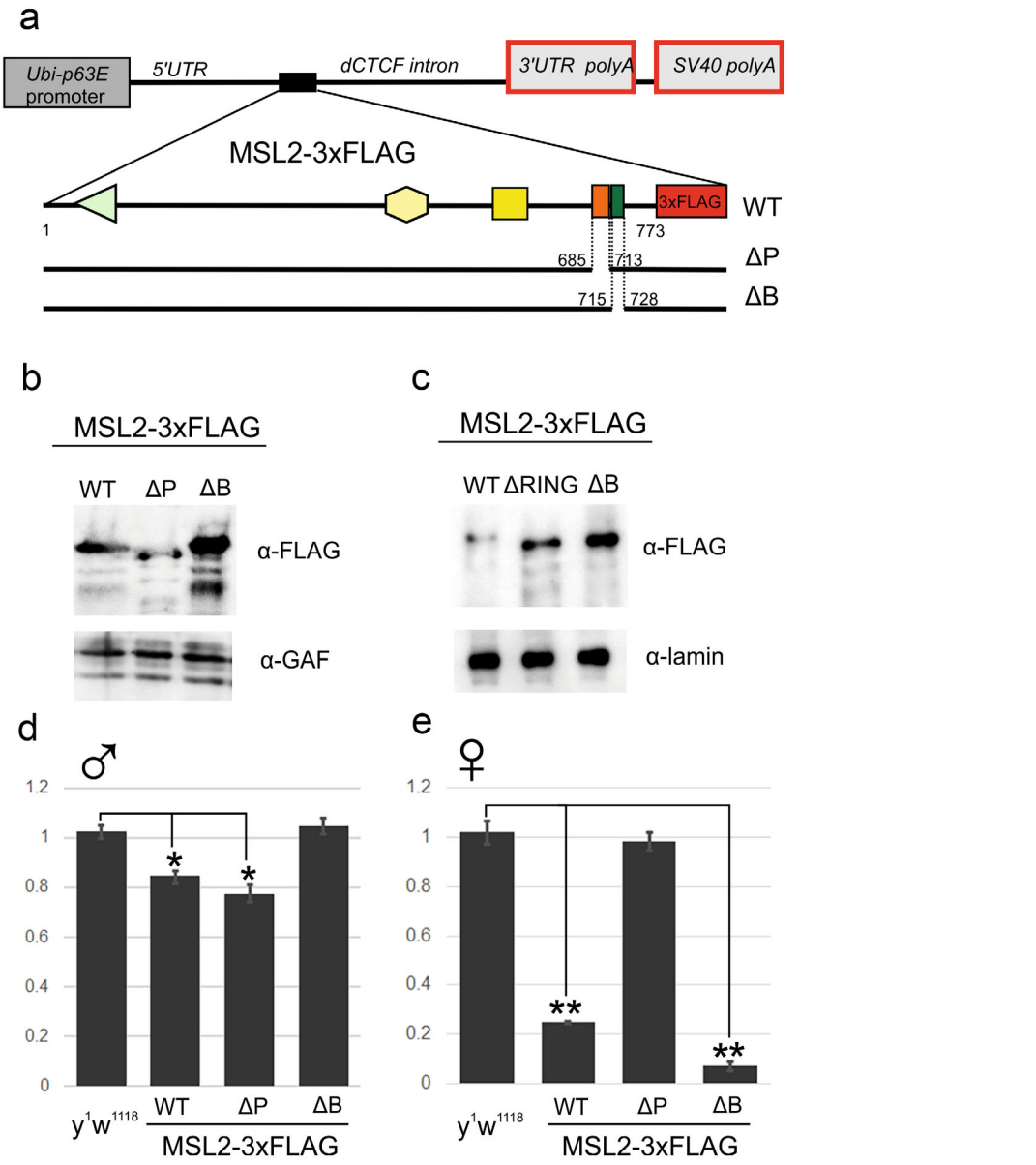

Fig. 2. Obtaining transgenic lines expressing mutant MSL2 proteins. a) Scheme of the used expression vector. Promoter and 5′-UTR of the *Ubiquitin-63E* gene, last intron and 3′-UTR of the *dctcf* gene, and polyadenylation signal from the SV40 virus are shown. MSL2 variants are presented below; dashed lines indicate locations of the introduced deletions. b) Immunoblot analysis of the protein extracts obtained from the adult flies expressing various MSL2 variants tagged with the 3×FLAG epitope (WT, ΔP, ΔB). Immunoblot analysis was performed using antibodies that specifically recognize FLAG and GAF (internal loading control). c) Comparison of the expression of MSL2^{WT}-FLAG, MSL2$^{\Delta RING}$-FLAG, and MSL2$^{\Delta B}$-FLAG proteins in S2 cells. Immunoblot analysis was performed using antibodies that specifically recognize FLAG and lamin (internal loading control). d) Comparison of viability (in relative percentage terms) of adult males *msl2*^{y227}/*msl2*^{y227}, in which the MSL2-3×FLAG variants were expressed (WT, ΔP, ΔB). Number of *msl2*^{y227}/*CyO* males expressing MSL2 variants was used as an internal control with normal viability. Ratio of adult males of line *y*^{1}*w*^{1118}; +/+ to males *y*^{1}*w*^{1118}; +/*CyO* was used as an indicator of survival of the wild-type line. Histogram shows the means with standard deviations obtained from three independent experiments. * $p < 0.05$. e) Viability (in relative percentage terms) of females homozygous for the transgene relative to the adult males expressing MSL2 variants. Histogram shows the means with standard deviations obtained from three independent experiments. ** $p < 0.01$.

MSL2^{WT} at the background of null mutation in the homozygote is slightly lower than that of the control males, while survival of the MSL2$^{\Delta B}$ is comparable to the control males (Fig. 2d, Table 1). Thus, deletion of the P-domain does not have a visible effect on the activity of MSL2 in dosage compensation, while, at the same time, MSL2$^{\Delta B}$ functions more efficiently than MSL2^{WT}, which is probably due to the greater stability of this MSL2 variant.

A sensitive model system for studying dosage compensation has previously been described, which is based on ectopic expression of MSL2 in females,

Table 1. Male survival study (positive function of dosage compensation)

Genotype	*Msl2^{y227}/ msl2^{y227}*	*msl2^{y227}/CyO*
U:MSL2^{WT}/TM6	138 ± 3.2	163 ± 1.1
U:MSL2$^{\Delta P}$/TM6	112 ± 5.2	145 ± 3.1
U:MSL2$^{\Delta B}$/TM6	90 ± 1.2	86 ± 2.3

Note. Analysis of the ratio of males with the *msl2^{y227}/msl2^{y227}*; *U:MSL2*/TM6* genotype to males with the *msl2^{y227}/CyO*; *U:MSL2*/TM6* genotype, which were obtained by crossing the (F0) males with the *msl2^{y227}/CyO*; *U:MSL2^{WT}/TM6* genotype with the *msl2^{y227}/msl2^{y227}*; *U:MSL2^{WT}/TM6* females.

Table 2. Female survival study (negative function of dosage compensation)

Genotype	MSL2/MSL2 males	MSL2/MSL2 females
U:MSL2^{WT}/TM6	141 ± 2.2	35 ± 0.6
U:MSL2$^{\Delta P}$/U:MSL2$^{\Delta P}$	162 ± 5.2	159 ± 3.1
U:MSL2$^{\Delta B}$/U:MSL2$^{\Delta B}$	114 ± 2.8	8 ± 2

Note. Analysis of the ratio of the *U:MSL2*/U:MSL2** females to *U:MSL2*/U:MSL2** or *U:MSL2*/TM6* males.

resulting in the assembly of a functional DCC [39, 40]. The more efficiently DCC assembles on the X chromosome, the more gene transcription increases, which directly correlates with the decrease in female viability as a result of imbalance in the gene expression profile. As expected (Fig. 2e, Table 2), the females carrying the homozygous *U:msl-2^{WT}* transgene are characterized by the reduced viability (about 25% relative to the males).

In the females homozygous for the *U:msl-2$^{\Delta B}$* transgene, there is a further decrease in survival. Surprisingly, the females homozygous for the *U:msl-2$^{\Delta P}$* transgene have close to normal survival rates. Thus, deletion of the P-domain in the MSL2 protein leads to the partial disruption of dosage compensation only in the more sensitive model system.

Comparison of MSL1 and MSL2 binding in males and females expressing MSL2 variants. To study efficiency of the DCC binding to the X chromosome of males, immunostaining of polytene chromosomes isolated from the salivary glands of Drosophila larvae is most often used, which makes it possible to visualize proteins on the interphase chromatin [10, 41-43]. In the line *msl2^{y227};U:MSL2^{WT}* proteins MSL1 and MSL2 efficiently bind only to the X chromosome (Fig. 3a). Similar results were obtained on the polytene chromosomes of the males of the line expressing MSL2$^{\Delta P}$. Thus, the results of binding of the MSL1 and MSL2 proteins to polytene chromosomes fully confirm the results of the functional test (Fig. 2d), according to which there are no disturbances in the process of formation of the dosage compensation complex in the males expressing MSL2$^{\Delta P}$.

A similar study was carried out on the polytene chromosomes from the salivary glands of the female larvae (Fig. 3, b, c) expressing variants of the MSL2 protein. In the larvae expressing MSL2^{WT}, the MSL1 and MSL2 proteins cover the entire X chromosome except for a few small regions. However, binding of the MSL proteins to the X chromosome is less intense in the females compared to the males. This is due to the fact that in the females expression of the MSL1 protein and *roX* RNA is much weaker. Binding of the MSL proteins is visually enhanced on the X chromosome of the larvae expressing MSL2$^{\Delta B}$, which can be explained by the significant increase in stability of the mutant protein. The results are consistent with the functional test, according to which survival rate of the females expressing MSL2$^{\Delta B}$ is significantly lower compared to the MSL2^{WT} females (Fig. 2e). Binding of MSL2$^{\Delta P}$ and MSL1 to the X chromosome of the *U:MSL2$^{\Delta P}$* females is significantly reduced. Intense staining with antibodies against MSL1 and FLAG (MSL2) is observed only in certain regions of the chromosome, which, apparently, coincide with the strongest CES. Thus, MSL2$^{\Delta P}$ disrupts effective binding of DCC to the X chromosome of the females.

Previous studies [10, 21, 22, 41, 44] showed that inactivation of MSL3, or MLE, or *roX* RNA resulted in the DCC recruitment to only small part of the regions corresponding to the main CES including the regions of the genes encoding *roX1* (3F) and *roX2* (10C). Thus, the MSL2$^{\Delta P}$ variant similarly leads to the decrease in efficiency of the DCC formation, which is visualized by preservation of the binding of the MSL1 and MSL2 proteins to the strongest CES on the X chromosome and decrease in the binding to the secondary sites of DCC recruitment.

To confirm this assumption, we compared binding of the MSL1, MSL2, and CLAMP proteins with the most well-studied CES on the X chromosome of the 2-3-day old males using chromatin immunoprecipitation (Fig. 3d). To compare DCC binding in the lines expressing MSL2^{WT} and MSL2$^{\Delta P}$, previously characterized representative CES of the complex were selected: PionX sites [23], HAS/CES sites [21, 22]. As a result, it was found that MSL2$^{\Delta P}$ and MSL2^{WT} bind to all sites with approximately the same efficiency. At the same time, at some sites there is an excessive accumulation of the MSL2$^{\Delta P}$ protein, which can be explained by partial redistribution of the complex in the line expressing MSL2$^{\Delta P}$. Similar results were obtained for MSL1. The CLAMP protein binds to the tested CES with equal efficiency

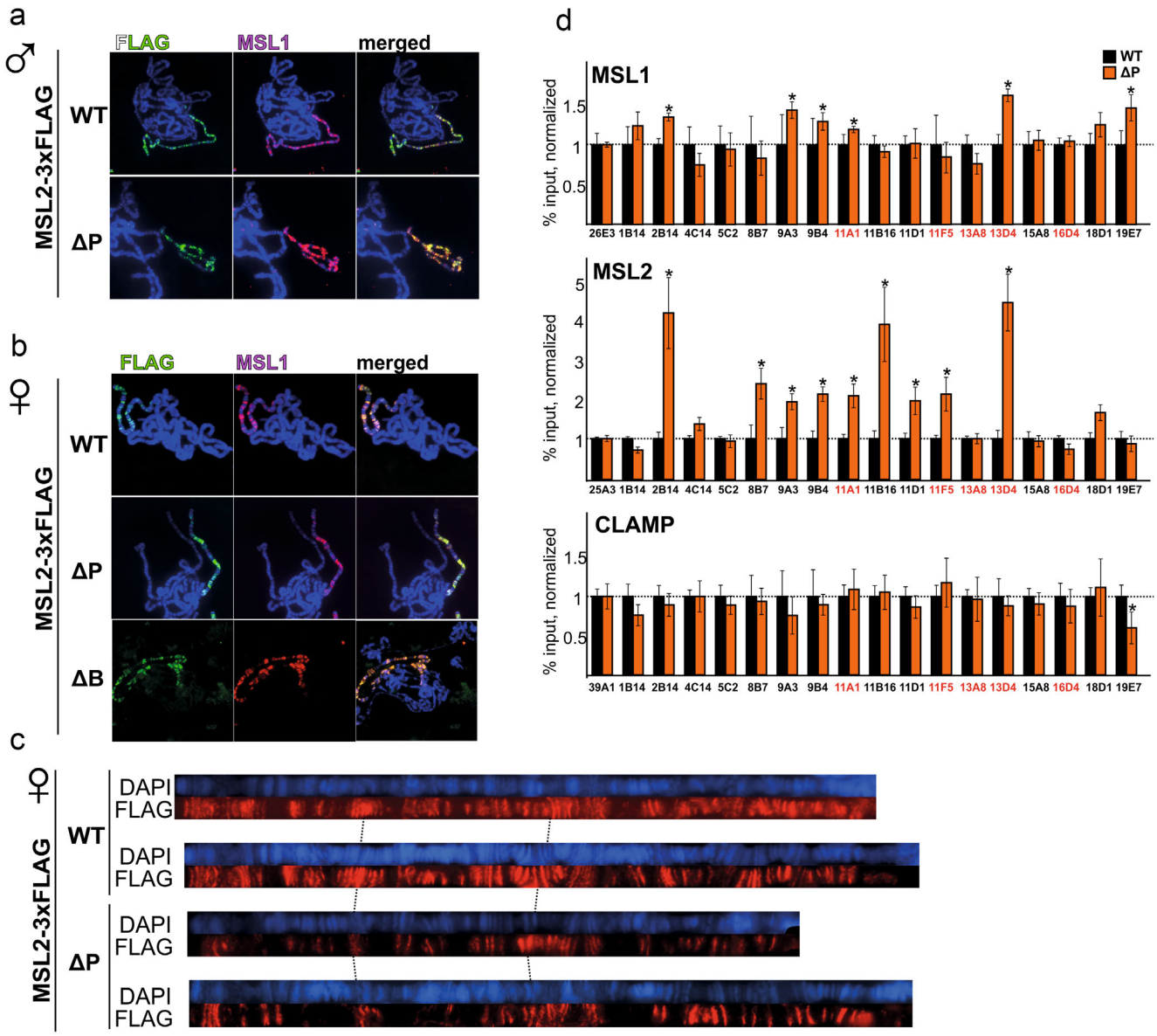

Fig. 3. a) Comparison of the MSL1 and MSL2 binding to the polytene chromosomes of the *msl2*^{y227} male larvae expressing different variants of MSL2 (MSL2^{WT}, MSL2$^{\Delta P}$). b) Comparison of the MSL1 and MSL2 binding to the polytene chromosomes of the female larvae heterozygous for the transgene expressing one of the MSL2 variants (MSL2^{WT}, MSL2$^{\Delta P}$, MSL2$^{\Delta B}$). The photographs show immunostaining with mouse anti-FLAG antibodies (MSL2, green) and rabbit anti-MSL1 antibodies (red). DNA staining – DAPI (blue). c) Comparison of distribution of the MSL2 protein along the polytene X chromosome in the females heterozygous for the transgene expressing one of the MSL2 variants (MSL2^{WT}, MSL2$^{\Delta P}$). Two independent stainings are shown. MSL2 staining – mouse anti-FLAG antibodies (red), DNA – DAPI (blue). d) Comparison of the MSL1, MSL2, and CLAMP proteins binding to CES in the males expressing MSL2 variants (WT and ΔP) at the *msl2*^{y227} background. Red letters indicate regions to which MSL2 is able to bind directly according to [23]. Results are presented as percent enrichment of DNA after immunoprecipitation to input DNA (% input), normalized relative to the corresponding positive control MSL1 (26E3), MSL2 (25A3), and CLAMP (39A1) binding sites on autosomes. The histograms show comparison of the level of MSL2$^{\Delta P}$ protein binding with the level of MSL2^{WT} binding (scaled to "1"). Whiskers show standard deviations of three independent experiments. * $p < 0.05$.

in all lines. Thus, the results obtained confirm that MSL2$^{\Delta P}$ maintains its effective binding to CES on the X chromosome.

P-domain determines ability of MSL2 to activate transcription. To study in more detail functional role of the P-domain in the process of dosage compensation, we examined expression of the *roX* RNAs, which are necessary for dosage compensation. In the wild-type females, *roX* RNAs are not expressed due to the absence of the MSL-containing complex that activates their transcription [45]. Expression of the wild-type MSL2 in females results in significant activation of the *roX2* RNA and, to a lesser extent, of the *roX1* RNA [46]. CES located near the *roX* genes are necessary

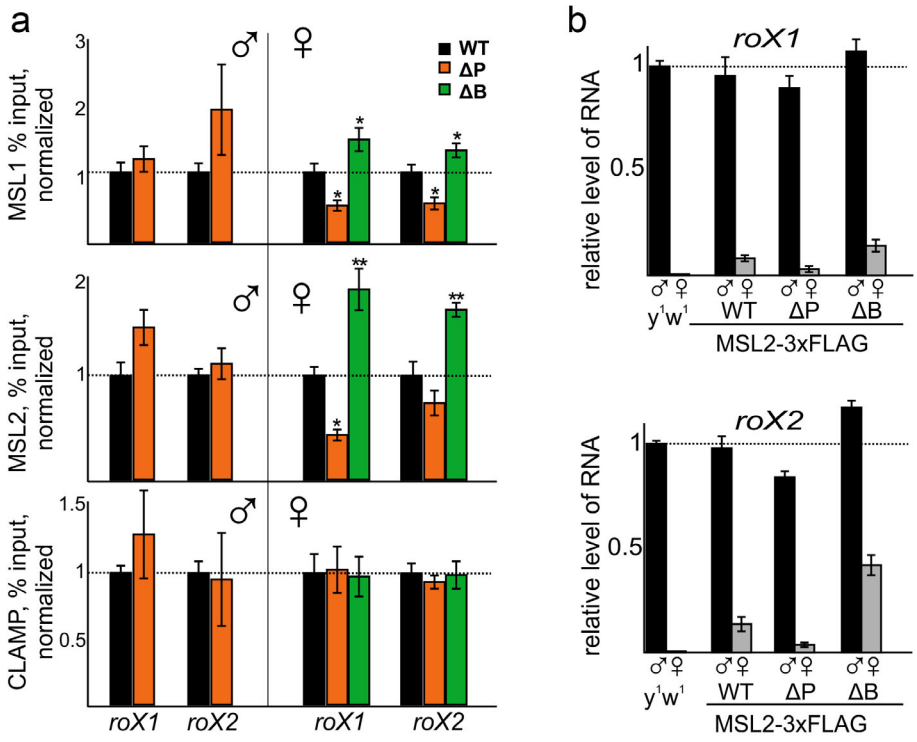

Fig. 4. Comparison of *roX1* and *roX2* in the females expressing MSL2^{WT}, MSL2$^{\Delta P}$, MSL2$^{\Delta B}$. a) Comparison of binding of MSL1, MSL2, CLAMP proteins in the lines expressing MSL2^{WT}, MSL2$^{\Delta B}$, MSL2$^{\Delta P}$ in the regions of the *roX1* and *roX2* genes. * $p < 0.05$; ** $p < 0.01$. b) Levels of *roX1* and *roX2* RNA expression in the larvae of male and female flies of the y^1w^{1118} line (wild type) and flies expressing MSL2^{WT}, MSL2$^{\Delta P}$, MSL2$^{\Delta B}$ at the background of the $msl2^{y227}$ null mutation. Histograms show changes in the mRNA levels of the tested *roX* genes in the lines expressing MSL2^{WT}, MSL2$^{\Delta P}$, MSL2$^{\Delta B}$, compared to the expression level in the males of the y^1w^{1118} line (corresponding to the "1" mark on the scale). Whiskers show standard deviations for three independent measurements.

to stimulate their transcription in males and to repress them in females [47, 48]. Chromatin immunoprecipitation showed that MSL2 and MSL1 bind efficiently to these sites in the female larvae expressing MSL2^{WT}, MSL2$^{\Delta B}$, and MSL2$^{\Delta P}$ proteins (Fig. 4a). Moreover, in the female larvae of the MSL2$^{\Delta B}$ line, the MSL1 protein binds approximately 1.5 times stronger to the roX1 CES and 1.2 times stronger to the roX2 CES compared to the control line MSL2^{WT}. A more pronounced increase in the binding to the studied CES was detected for the MSL2 protein (1.8 times stronger for the roX1 CES and 1.6 times stronger for the roX2 CES compared to the control line MSL2^{WT}). In the case of females of the MSL2$^{\Delta P}$ line, the MSL1 protein binds to the roX CES approximately 1.8-2 times weaker compared to the control line MSL2^{WT}, and the MSL2 protein also binds 2 times weaker with the roX1 CES, and approximately 1.2 times weaker with the roX2 CES. However, binding of the CLAMP protein in all lines remains the same. In the males of the MSL2$^{\Delta P}$ line, it was also not possible to detect statistically significant changes in the strength of binding of the MSL1, MSL2, CLAMP proteins to the roX CES compared to the control line MSL2^{WT}.

In the last part of the work, we confirmed that the MSL2 expression in females induces *roX* RNA transcription (Fig. 4b). However, the level of *roX* expression in such females is reproducibly lower compared to the males of the same lines. Expression of MSL2$^{\Delta B}$ in the females results in an approximately 2-fold increase in the *roX* RNA expression compared to the control MSL2^{WT} line. Since increased binding of the MSL1 and MSL2 proteins with the roX CES in females of this line was observed, we can conclude that there is a direct correlation between the efficiency of MSL1/MSL2 binding to the CES and activation of transcription of the *roX* genes. This result is consistent with the data for roX1 obtained for the MSL2$^{\Delta P}$ line: approximately twofold decrease in the binding of MSL1/MSL2 proteins is accompanied by the proportional decrease in the level of *roX1* expression. However, in the case of roX2 in the MSL2$^{\Delta P}$ line, a slightly different picture is observed: the level of *roX2* expression decreases 3.7-fold with slight decrease in the level of MSL2 binding (1.2-fold) compared to the control line MSL2^{WT}. The obtained difference can be explained by the relative accuracy of the qChIP method in measuring the amount of protein on chromatin. However, the significant difference obtained between the amount of MSL2 associated with CES and the level of *roX2* expression suggests that the P-domain in the MSL2 protein is involved in transcription activation of the *roX2* gene.

DISCUSSION

In this work, we investigated functional role of two domains (rich in prolines and basic amino acids) at the C-terminus of the MSL2 protein. As a result, it was demonstrated that these domains do not have a visible effect on the activity of DCC functioning in males. According to the currently dominant ideas [36, 37], C-terminus of the MSL2 protein interacts with the *roX* RNA, which is critical for the DCC assembly. It can be assumed that the C-terminal regions adjacent to the P- and B-domains are responsible for specific interaction of MSL2 with *roX* RNA, which requires further study.

The results obtained in this work suggest that the B-domain contains two lysines, which are the main residues in MSL2 subjected to autoubiquitination leading to the significant decrease of the protein stability. It can be assumed that upon interaction with the coiled-coil domain of the MSL1 protein catalytic activity of the RING-domain decreases, and, as a result, the MSL2 protein, which is in complex with MSL1, is stabilized. Thus, efficiency of the complex formation increases, and, at the same time, concentration of the free MSL2 decreases.

The MSL2 protein is capable of stimulating transcription within DCC and also has an independent function in activating transcription of a group of autosomal promoters [49]. In mammals, the MSL2 orthologue has been shown to ubiquitinate histone H3 at lysine 24 [5]. Histones are enriched with this mark in the areas of intense transcription. We have shown that the proline-rich region of MSL2 may be involved in activation of the *roX2* gene transcription by the dosage compensation complex. It is known that the unstructured proline-rich regions are capable of stimulating transcription by attracting and stabilizing transcription complexes at promoters. Interestingly, MSL1 also stimulates transcription independently on the dosage compensation complex by stabilizing binding to the promoters of the cyclin-dependent kinase CDK7, which accelerates release of the RNA polymerase II from the pausing state [50].

In conclusion, our data indicate that the reduced levels of *roX* RNA in the females expressing $MSL2^{\Delta P}$ negatively affect efficiency of DCC formation. This is manifested as a decrease in the amount of MSL1/MSL2 proteins associated with the roX CES and provides additional confirmation of the key role of the *roX* RNA in organizing dosage compensation.

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Contributions. E.A.T. planned and performed experiments, discussed the results; P.G.G. supervised the study, discussed the results, and edited the manuscript; O.G.M. planned and performed the experiments, discussed the results, supervised the study, and prepared the manuscript.

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