

Dual sex-specific functions of *Drosophila* Upstream of N-ras in the control of X chromosome dosage compensation

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Dosage compensation in *Drosophila melanogaster* involves the assembly of the MSL-2-containing dosage compensation complex (DCC) on the single X chromosome of male flies. Translational repression of *msl-2* mRNA blocks this process in females. Previous work indicated that the ubiquitous protein Upstream of N-ras (UNR) is a necessary co-factor for *msl-2* repression in vitro. Here, we explore the function of UNR in vivo. Hypomorphic *Unr* mutant flies showed DCC assembly on high-affinity sites in the female X chromosomes, confirming that UNR inhibits dosage compensation in female flies. Unexpectedly, male mutant flies and UNR-depleted SL2 cells showed decreased DCC binding to the X chromosome, suggesting a role for UNR in DCC assembly or targeting. Consistent with this possibility, UNR overexpression resulted in moderate loss of DCC from the male X chromosome and predominant male lethality. Immunoprecipitation experiments revealed that UNR binds to *roX1* and *roX2*, the non-coding RNA components of the DCC, providing possible targets for UNR function in males. These results uncover dual sex-specific functions of UNR in dosage compensation: to repress DCC formation in female flies and to promote DCC assembly on the male X chromosome.

KEY WORDS: UNR, Dosage compensation, DCC, MSL, Translation, *roX1/2*

INTRODUCTION

Dosage compensation is the process that equalizes the level of X-linked gene expression between males (XY) and females (XX). In *Drosophila melanogaster*, dosage compensation occurs by increasing transcription of the single male X chromosome by ~2-fold (reviewed by Gilfillan et al., 2004; Lucchesi et al., 2005; Straub and Becker, 2007; Mendjan and Akhtar, 2007). Hyper-transcription requires the binding of the dosage compensation complex (DCC) to hundreds of sites along the male X chromosome. The DCC is composed of five proteins (MSL-1, MSL-2, MSL-3, MLE and MOF), the mutation of which causes male-specific lethality, and for this reason the DCC is also known as the male-specific lethal (MSL) complex. The DCC also contains two non-coding RNAs (*roX1* and *roX2*) that appear to have redundant functions (Meller et al., 1997; Franke and Baker, 1999). MSL-2 is a limiting RING finger protein that, together with MSL-1, nucleates the assembly of the DCC (Kelley et al., 1995; Bashaw and Baker, 1995). MLE (Maleless) is a helicase thought to be required for stable integration of *roX* RNAs into the DCC (Meller et al., 2000), whereas MSL-3 is a chromodomain protein, and MOF (Males absent on the first) is an acetyl-transferase that promotes the acetylation of histone H4 on lysine 16 (H4K16), a modification that specifically marks the compensated X chromosome (Akhtar and Becker, 2000; Smith et al., 2000). Other proteins, in addition to the DCC components, have been implicated in dosage compensation, including the H3S10 kinase JIL-1 (Wang et al., 2001), the DNA supercoiling factor (SCF) (Furuhashi et al., 2006), the chromatin-binding protein SU(VAR)3-7 (Spierer et al., 2005), and the nuclear pore components Mtor and NUP153 (Mendjan et al., 2006).

In female flies, dosage compensation is inhibited because the expression of *msl-2* is repressed by the female-specific RNA-binding protein Sex lethal (SXL). Enforced expression of MSL-2 leads to the assembly of the DCC on both female X chromosomes and to lethality (Kelley et al., 1995). SXL binds to both untranslated regions (UTRs) of *msl-2* pre-mRNA and inhibits first the splicing of a facultative intron in the 5' UTR of the transcript, and then its translation in the cytoplasm (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1998). Translational repression of *msl-2* by SXL occurs by a double-block mechanism whereby SXL bound to the 3' UTR inhibits the recruitment of the small ribosomal subunit, and SXL bound to the 5' UTR inhibits the scanning of those subunits that presumably have escaped the 3'-mediated control (Gebauer et al., 2003; Beckmann et al., 2005). Studies performed in cell-free translation extracts and cultured cells have shown that translational repression requires the recruitment of the co-repressor Upstream of N-ras (UNR) to sequences adjacent to the SXL binding sites in the 3' UTR (Abaza et al., 2006; Duncan et al., 2006). UNR is an evolutionarily conserved RNA-binding protein that contains five cold-shock domains (CSDs) and two glutamine (Q)-rich regions. The first CSD (CSD1) mediates interactions with SXL and *msl-2* mRNA, whereas the N-terminal third of the protein carries most of the translational repression function in vitro (Abaza and Gebauer, 2008). Although UNR is a ubiquitous, primarily cytoplasmic protein that is present in both males and females, it binds to *msl-2* only in females because its association depends on SXL. Thus, SXL provides a sex-specific function to UNR.

To gain insight into the roles of UNR in development, we have analyzed hypomorphic mutant flies that lack the C-terminal half of UNR, as well as flies that overexpress full-length UNR or a fragment containing CSDs 1 and 2. In *Unr* hypomorphic mutant females, the DCC was detected on a limited set of high-affinity sites on the X chromosomes, indicating that, as predicted from translation studies, UNR represses DCC formation in females. Unexpectedly, *Unr* mutant males showed decreased DCC recruitment to the X chromosome. Consistent with this, UNR knockdown in male *Drosophila* SL2 cells abrogated DCC binding without affecting the levels of DCC components or their nucleocytoplasmic distribution.

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In addition, flies overexpressing UNR showed preferential male lethality and DCC recruitment defects, and the X chromosome of both mutant and transgenic *Unr* males exhibited an altered morphology. Importantly, *roX1* and *roX2* RNAs co-immunoprecipitated with UNR in males, suggesting that UNR might function by targeting these non-coding RNAs. These results uncover new roles for UNR in the regulation of dosage compensation in males by a mechanism that is independent of *msl-2* translation.

MATERIALS AND METHODS

Fly strains

The hypomorphic mutant *Pbac{PB}UNR[c01923]* line was obtained from the FlyBase collection (FlyBase ID: FBst0010757) (Thibault et al., 2004). The insertion was precisely mapped by PCR from genomic DNA using *Unr*-specific primer UNR2658 (5'-CATAGAAGGTTTCTCTAGA-3') and PiggyBac-specific primer 3F1 (5'-CCTCGATATACCGATAAAAC-3'). *FLUNR* and *UNR1+2* transgenic flies were obtained by standard *P*-element transformation of *Oregon R* *w¹¹¹⁸* flies with the pUASp-*FLUNR*GFP and pUASp-*UNR1+2*GFP constructs, respectively. To generate these constructs, the coding sequences for full-length *Drosophila* UNR (amino acids 1-1039) or a fragment containing CSDs 1+2 (amino acids 197-372) were obtained by PCR amplification and subcloned into pEGFP-N1 (Clontech). The UNR-GFP fusions generated in this manner were then cloned into the *KpnI/XbaI* sites of pUASp (Rorth, 1998). At least three homozygote viable lines with insertions in the second and third chromosomes were recovered for *FLUNR*, and one on the X chromosome for *UNR1+2*. *FLUNR* and *UNR1+2* homozygous flies were crossed with *Gal4* driver lines to induce expression before gastrulation (*Act5C-Gal4*; FlyBase ID: FBtp0001406), at early gastrulation (*en-Gal4*), or in the salivary glands of third-instar larvae (*Sgs3-Gal4*; FlyBase ID: FBtp0016397).

Cell culture and RNAi treatment

Drosophila SL2 and Kc cells were grown at 25°C in Schneider medium (Gibco) supplemented with 100 units/ml penicillin/streptomycin and 10% FCS. RNAi against *Unr* was performed by incubating 2×10^6 SL2 cells with 22.5 µg of double-stranded (ds) RNAs corresponding to either the UNR coding region (nt 2139-2691, relative to the start codon) or the 3' UTR (nt 3120 to 3530 from the start codon) in 1.5 ml Schneider medium without serum. After a 40-minute incubation at 25°C, the volume was doubled with Schneider medium supplemented with 20% FCS and the cells were plated

in p35 dishes. Cells treated similarly but without double-stranded (ds) RNA addition were carried in parallel as control. Cells were recovered 6-9 days after plating, and the efficiency of UNR depletion tested by western blot. Both dsRNAs depleted UNR efficiently. No deleterious effect of RNAi treatment on cell viability was detected.

Western blot and immunoprecipitation

Adult and larval *Drosophila* extracts were prepared as described (Wilhelm et al., 2000) using a DIAx 900 homogenizer (Heidolph) at 4°C. Total protein extracts from SL2 and Kc cells were prepared in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1% SDS, 1% deoxycholic acid (DOC), 5 mM EDTA, 1% Triton X-100] supplemented with 1× Complete protease inhibitor cocktail (Roche). Briefly, cell pellets were resuspended in RIPA buffer, incubated on ice for 20 minutes and centrifuged to recover the supernatant. Cytoplasmic and nuclear protein preparations were obtained by first incubating cells in hypotonic buffer [10 mM HEPES pH 7.6, 10 mM KOAc, 0.5 mM Mg(OAc)₂, 5 mM DTT, 1% Triton X-100, 1× Complete protease inhibitor cocktail] for 5 minutes on ice. The cells were then homogenized, centrifuged and the supernatant recovered as the cytoplasmic fraction. The pellet was further washed in PBS, resuspended in RIPA buffer and processed as described above to obtain the nuclear fraction.

Protein extracts were resolved by SDS-PAGE, transferred to PVDF membranes and blocked with 5% dried milk powder in PBS. Incubation with primary antibodies was performed overnight at 4°C. Anti-MSL-2 antibodies were provided by P. Becker (Adolf-Butenandt-Institute, Munich, Germany) and used at 1:10 dilution. Antibodies to MSL-1 (1:1000), MLE (1:3000), MOF (1:500), MSL-3 (1:5000), Mtor (1:1000) and NUP153 (1:1000) were kindly provided by A. Akhtar (Mendjan et al., 2006) and used at the indicated dilutions. Anti-UNR (Abaza et al., 2006) and anti-tubulin (Sigma) were used at 1:500 and 1:10,000, respectively. Secondary detection was with protein A-HRP (Invitrogen) followed by chemiluminescence (ECL, Amersham) and autoradiography for UNR, MOF, NUP153 and tubulin, and with Alexa 660-conjugated anti-rat antibodies followed by detection with the Odyssey system (Molecular Probes) for all other antibodies.

Adult *Drosophila* extracts for immunoprecipitation were prepared by homogenizing frozen fly powder with one volume of 10 mM HEPES-KOH pH 7.4, 150 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 1× Complete protease inhibitor cocktail and 40 U/ml RNasin. Sixteen milligrams of extract were cross-linked by incubation for 10 minutes at 4°C with 1% formaldehyde and the reaction stopped with 250 mM glycine pH 7.0 for 5 minutes. One volume of RIPA buffer was added and the extract processed as described above for SL2 cells. Immunoprecipitation was performed with magnetic Dynabeads

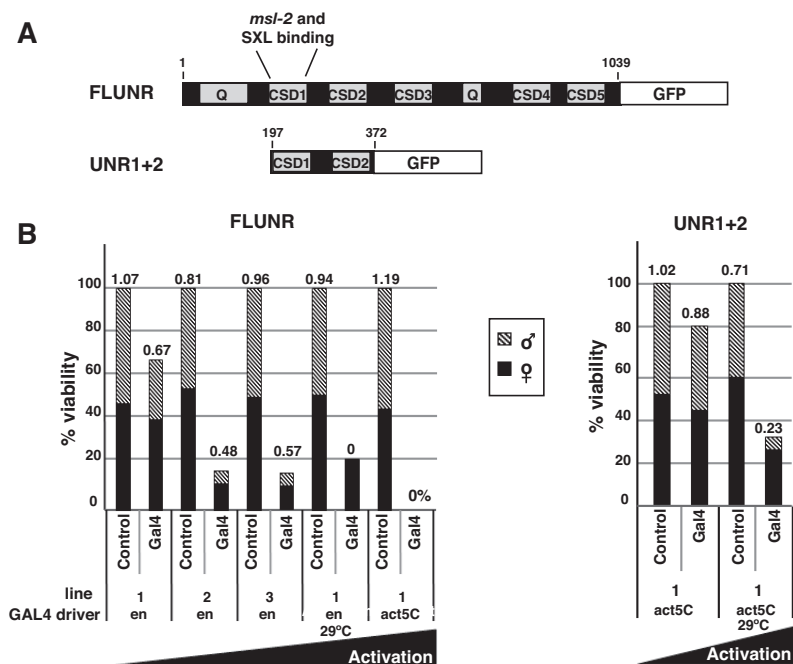


Fig. 1. UNR overexpression preferentially affects male viability. (A) Schematic of the UNR-GFP constructs FLUNR and UNR1+2 that were overexpressed in flies. The relevant domain (CSD1) for *msl-2* mRNA and SXL interactions is indicated, as well as the amino acids of UNR that are contained in each construct. CSD, cold-shock domain; Q, glutamine-rich region. (B) FLUNR and UNR1+2 were systemically overexpressed in flies using promoters of increasing strength. FLUNR and UNR1+2 homozygous flies were crossed to *Actin5C* (*Act5C*)-*Gal4* or *engrailed* (*en*)-*Gal4* driver lines balanced with *CyO*. F1 flies carrying *CyO* (control) or carrying the corresponding *Gal4* gene (*Gal4*) were scored. Numbers above the bars indicate the male-to-female ratio. Flies were grown at 25°C. In some cases, an increased temperature (29°C) was used to boost expression of the transgene.

coupled to protein A, pre-blocked with 100 ng/μl yeast tRNA. After immunoprecipitation, beads were washed with RIPA buffer, and eluates obtained by incubation with 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM DTT, 1% SDS. Eluates were treated with proteinase K and incubated at 70°C for 1 hour to reverse the cross-linking. RNA was extracted with Trizol (Invitrogen) followed by Turbo DNase treatment, and reverse transcription was performed with oligo dT₂₀ followed by PCR with specific oligos for *roX1*, *roX2*, *Dad* and *LIMK1*.

Immunostainings

Preparation and immunostaining of polytene chromosomes was performed as described (<http://www.igh.cnrs.fr/equip/cavalli/Lab%20Protocols/Immunostaining.pdf>). Immunostaining on SL2 cells was performed as described (Akhtar et al., 2000). Salivary glands were dissected in PBS, fixed in Brower Fix buffer (150 mM PIPES pH 6.9, 3 mM MgSO₄, 1.5 mM EGTA, 1.5% NP40) supplemented with 1.3% formaldehyde and stained as described for polytene chromosomes. Anti-MSL-2 antibodies used in immunostainings were kindly provided by P. Becker and used at 1:500. All other antibodies against DCC components were provided by A. Akhtar and used at the following dilutions: MSL-1 (1:500), MLE (1:500), MOF (1:500), MSL-3 (1:250), Mtor (1:500). Anti-histone H3 antibodies (Abcam) were used at 1:250. Secondary detection was with Cy3-conjugated anti-rat (Jackson Lab.) and Alexa 488-conjugated anti-rabbit (Molecular Probes) antibodies. Polytene chromosomes, salivary gland and SL2 cell images were captured with a Leica DFC350FX or Leica DMI 6000B digital camera, and TCS SPE and TCS SP2 microscopes with HCX PL APO CS 40×/1.25-0.75 or 63×/1.40-0.60 oil-immersion objectives. Images were taken using appropriate filter combinations and arranged using Photoshop (Adobe).

Staining of eye imaginal discs was performed in 0.1 M phosphate buffer, 0.3% Triton X-100 and 10% normal goat serum. Anti-MSL-2 was used at 1:500 and FITC-conjugated anti-rabbit (Calbiochem) was used as secondary antibody. All image acquisitions were performed using a Leica TCS-NT confocal laser-scanning microscope and Leica LCS software.

Fluorescent signal quantification

Fluorescent signal quantification of eye imaginal discs was performed on maximum projections of the same number of confocal sections taken with the same exposure time from a region posterior to the morphogenetic furrow, using the Leica LCS software. Conditions for image capture were set using eye discs from *w¹¹¹⁸* females as negative controls. Student's *t*-test was used to analyze the differences in signal intensity between samples. Fluorescent signal quantification of polytene chromosomes was performed on images taken with the same exposure time using ImageJ software.

Quantitative RT-PCR

Total RNA was isolated with Trizol from either SL2 cells or larvae, treated with DNase and further purified using Nucleospin columns (Macherey-Nagel). First-strand cDNAs were synthesized from 1 μg of total RNA with AMV-RT (Promega). The reaction mixture was serially diluted and amplified by quantitative PCR using the LightCycler DNA Master SYBR Green I Kit (Roche) and the following gene-specific primers:

roX1, 5'-ACAAATGAACCCAAAGCGTC-3' and 5'-GTATCATTGTC-TCGCTCGCA-3'; *roX2*, 5'-TCGATTAGAGCGAGATGACAA-3' and 5'-TAAAAGCATCTCGAATTCCGT-3'; and *rp49*, 5'-CCACAGT-CGGATCGATATG-3' and 5'-CACGTTGTGCACCAAGAACT-3'.

qPCR was performed on a LightCycler 480 (Roche) and the amplification curves were analyzed using the associated software. Appropriate dilutions and efficiencies of amplification were set for each primer. Quantitative values were normalized to the internal standard *rp49* (*RpL32* – FlyBase).

RESULTS

Overexpression of UNR preferentially affects male viability and X chromatin morphology

In *Drosophila* embryo extracts, an excess of UNR or a fragment containing CSD1 derepresses *msl-2* translation (Abaza and Gebauer, 2008). To investigate the roles of UNR in vivo, we attempted to interfere with UNR function by generating transgenic flies that overexpress full-length UNR (*FLUNR*) or a fragment containing CSDs 1 and 2 (*UNR1+2*). The proteins were fused to GFP (Fig. 1A) and their expression was driven by a GAL4-responsive promoter. Three independent lines were obtained for *FLUNR* and one for *UNR1+2* (Table 1). Homozygous transgenic flies were crossed with flies carrying the *Gal4* gene driven by promoters of increasing strength. Because the DCC is formed at the early blastoderm stage (Franke et al., 1996) and UNR is expressed early in embryogenesis (Abaza et al., 2006), we used the *engrailed* (*en*) and *Actin5C* (*Act5C*) promoters to drive GAL4 expression and specifically increase UNR levels at an early stage of development. Even limited UNR overexpression with the *en-Gal4* driver dramatically decreased the viability of transgenic flies as compared with control siblings of the same progeny in which the transgene was not expressed (Fig. 1B, left panel). Lethality occurred before the third-instar larvae stage. Analysis of the male-to-female ratio showed that lethality preferentially affected males (see numbers above the bars

Table 1. *FLUNR* and *UNR1+2* lines and crosses

Line	Crosses		Temp (°C)	Progeny				Total progeny	Male/ female ratio	
	Male	Female		Female <i>Gal4</i>	Male <i>Gal4</i>	Female <i>CyO</i>	Male <i>CyO</i>		<i>Gal4</i>	<i>CyO</i>
<i>FLUNR</i>										
1	<i>w;en-Gal4/CyO;+/+</i>	<i>w;UAS-FLUNR1/UAS-FLUNR1;+/+</i>	25	195	131	231	247	804	0.67	1.07
2	<i>w;en-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR2/UAS-FLUNR2</i>	25	44	21	200	161	426	0.48	0.81
3	<i>w;en-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR3/UAS-FLUNR3</i>	25	28	16	134	128	306	0.57	0.96
1	<i>w;en-Gal4/CyO;+/+</i>	<i>w;UAS-FLUNR1/UAS-FLUNR1;+/+</i>	29	15	0	34	32	81	0.00	0.94
2	<i>w;en-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR2/UAS-FLUNR2</i>	29	0	0	17	10	27	0.00	0.59
3	<i>w;en-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR3/UAS-FLUNR3</i>	29	2	0	19	22	43	0.00	1.16
1	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;UAS-FLUNR1/UAS-FLUNR1;+/+</i>	25	0	0	105	125	230	0.00	1.19
2	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR2/UAS-FLUNR2</i>	25	0	0	98	110	208	0.00	1.12
3	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR3/UAS-FLUNR3</i>	25	0	0	106	115	221	0.00	1.08
1	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;UAS-FLUNR1/UAS-FLUNR1;+/+</i>	29	0	0	13	13	26	0.00	1.00
2	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR2/UAS-FLUNR2</i>	29	0	0	52	51	103	0.00	0.98
3	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;+/+;UAS-FLUNR3/UAS-FLUNR3</i>	29	0	0	27	25	52	0.00	0.93
<i>UNR1+2</i>										
1	<i>w;en-Gal4/CyO;+/+</i>	<i>w;UAS-UNR1+2/UAS-UNR1+2;+/+;+/+</i>	25	195	224	207	203	829	1.15	0.98
1	<i>w;en-Gal4/CyO;+/+</i>	<i>w;UAS-UNR1+2/UAS-UNR1+2;+/+;+/+</i>	29	35	25	18	19	97	0.71	1.06
1	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;UAS-UNR1+2/UAS-UNR1+2;+/+;+/+</i>	25	224	196	256	260	936	0.88	1.02
1	<i>w;actV-Gal4/CyO;+/+</i>	<i>w;UAS-UNR1+2/UAS-UNR1+2;+/+;+/+</i>	27-29	39	9	90	64	202	0.23	0.71

Numbers in bold were used for the bar charts shown in Fig. 1B.

in the left panel of Fig. 1B). These effects were dose-dependent because increased UNR expression at 29°C with the *en-Gal4* driver yielded no male survivors (Fig. 1B and Table 1). Stronger overexpression of FLUNR with the *Act5C-Gal4* driver resulted in complete lethality for both sexes (Fig. 1B, left panel). Significantly, maximal overexpression yielded levels of UNR that were similar to those of the endogenous protein (see Fig. S1A in the supplementary material), indicating that the amount of UNR must be finely tuned during development. Lethality from UNR1+2 expression was only observed under the *Act5C-Gal4* driver (Fig. 1B, right panel; Table 1). Similar to the effect of FLUNR, expression of UNR1+2 resulted in preferential male lethality. These data suggest that UNR plays a crucial role during embryogenesis, particularly in male flies.

The early lethality of transgenic flies overexpressing UNR in a systemic manner prevented us from analyzing DCC formation on the polytene chromosomes of the salivary glands of third-instar larvae. To overcome this limitation, we overexpressed FLUNR exclusively in the salivary glands using the *Sgs3-Gal4* driver. Transgene expression was monitored by GFP fluorescence (see Fig. S1B in the supplementary material). Unexpectedly, whereas *FLUNR* transgenic females showed no abnormalities (data not shown), males showed diffuse MSL-2 and MOF staining of the X chromosome, and reduced staining for MLE (Fig. 2). Furthermore, DNA staining with Hoechst revealed that larvae overexpressing UNR exhibited regional decondensation of the X chromosome and showed partial loss of banding as compared with the non-activated *UAS-FLUNR* line (Fig. 2, arrows). This effect was dose-dependent, as it was more severe with stronger activation at an increased temperature. These data are consistent with a role for UNR in promoting DCC formation and correct X chromatin organization in males.

UNR inhibits DCC formation in female flies

To further examine the roles of UNR in development, we analyzed a hypomorphic *Unr* mutant obtained from the FlyBase collection. The *Pbac{PB}UNR[c01923]* line derives from a general gene disruption screening by insertion of a unique PiggyBac element (Thibault et al., 2004). We mapped the precise insertion of the PiggyBac element by PCR of genomic DNA followed by sequencing, and found that the insertion disrupted the UNR open reading frame yielding a truncated product of 638 amino acids that lacked the C-terminal Q-rich region and CSDs 4 and 5 (Fig. 3A). Western blot analysis confirmed the presence of the truncated UNR protein in both heterozygous and homozygous flies (Fig. 3B). Sixty percent of the homozygous flies survived beyond the pupae stage and died shortly after eclosion with no sex-specific bias, indicating that the N-terminal half of UNR suffices for development.

To check whether truncation of UNR resulted in derepression of *msl-2* translation and yielded significant DCC formation, we first stained eye imaginal discs of female mutants with anti-MSL-2 antibodies. Indeed, compared with wild-type females, increased staining of the X chromosome was observed in mutant females, visualized as a localized signal within the nuclei of the imaginal disc cells (Fig. 4Aa-f). Quantification of the MSL-2 signal confirmed these observations (Fig. 4A, bar chart). MSL-2 staining in mutant females did not reach the levels of wild-type males, suggesting that the N-terminal half of UNR supports strong inhibition of *msl-2* (Fig. 4Ac,f and bar chart). These results were corroborated by anti-MSL-2 staining of polytene chromosomes (Fig. 4Ag-k). Whereas no MSL-2 was observed on the X chromosomes of wild-type females (Fig. 4Ag,j), MSL-2 was detected on a few X chromosome sites in mutant females (Fig. 4Ah,k). Other DCC components, such as

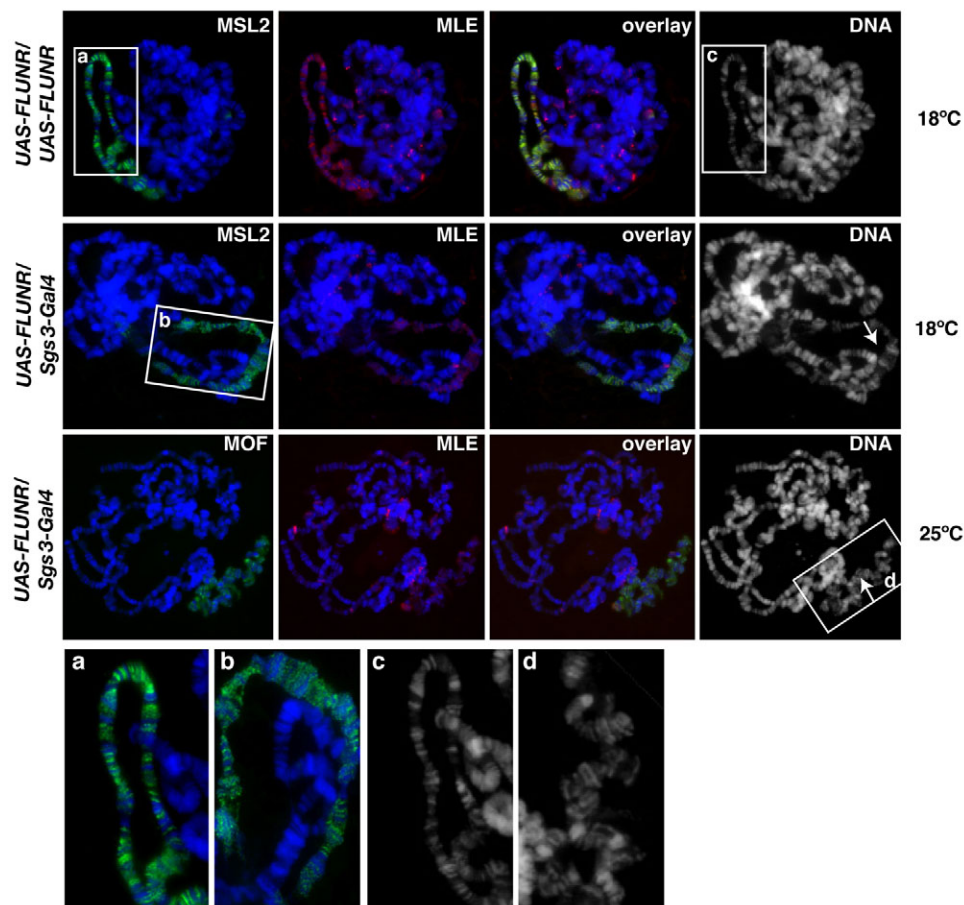


Fig. 2. Overexpression of UNR causes DCC targeting and chromatin defects. Polytene chromosome stainings of *FLUNR* male flies in which overexpression of UNR was specifically activated in the salivary glands (*UAS-FLUNR/Sgs3-Gal4*). The non-activated *FLUNR* line (*UAS-FLUNR/UAS-FLUNR*) was carried as control. The temperature of activation is indicated. Arrows indicate chromatin defects. Selected areas (a-d) are shown at higher magnification in the bottom row.

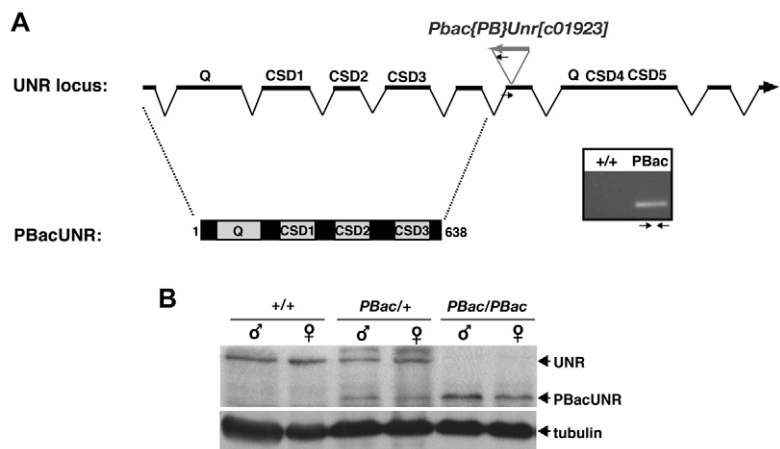


Fig. 3. Insertion of a PiggyBac element in the *Unr* locus. (A) Schematic of the PiggyBac (PBac) insertion in the *Drosophila Unr* gene. Introns and exons are represented as thin and thick lines, respectively. The locations of the sequences encoding the various domains of UNR are indicated within the exons. The PBac element is shown as a gray arrow. The insertion was mapped by PCR using the primers indicated by the small black arrows. The amplification products are shown in the inset (lane PBac). The domain structure of the truncated UNR fragment resulting from the insertion (PBacUNR) is shown. (B) Analysis of UNR products by western blot. Immunoblots were performed on cytoplasmic extracts from wild-type (+/+) or heterozygous (PBac/+) adult flies, or from homozygous (PBac/PBac) third-instar larvae. Tubulin is shown as a loading control.

MSL-1, MLE and MSL-3, were recruited to these sites, suggesting stable assembly of the DCC (Fig. 4B and data not shown). The sites closely mapped to previously identified high-affinity DCC binding sites (Dahlsveen et al., 2006; Lyman et al., 1997; Demakova et al., 2003) (Table 2). These results indicate that UNR inhibits DCC formation in female flies.

Truncation of UNR decreases DCC binding to the male X chromosome

We next analyzed DCC formation in male mutants. Consistent with the results in transgenic flies that overexpressed UNR, staining of eye imaginal discs with anti-MSL-2 antibodies showed a dramatically reduced and diffuse signal in mutant males as compared with the wild type (Fig. 5A). These results were confirmed by staining of polytene chromosomes (Fig. 5B). Measurement of the MSL-2 signal (Fig. 5Bd-f), normalized to the total amount of DNA (Hoechst signal, Fig. 5Ba-c), showed 30% staining in wild-type males, as expected from the fact that the X chromosome represents about a third of the total chromatin in a single nucleus (Fig. 5B, bar chart). However, the signal in homozygous mutant males was 3-fold lower than in the wild type.

Table 2. Cytological location of MSL2 binding sites on the X chromosomes of *Pbac Unr* mutant females

Cytological position*	Frequency†	Intensity
1C-D	2/5	Low
2F	5/5	Strong
3F	3/5	Strong
4E	2/5	Very low
5D	5/5	Strong
7A	5/5	Strong
7E	5/5	Regular
8A	3/5	Low
10B-C	2/5	Low
11A	5/5	Strong
11C cluster	5/5	Regular
13A 1-6	5/5	Very strong
13A 6-12	5/5	Very strong
13D	5/5	Strong
14B	5/5	Very low
16D	5/5	Low
17F	4/4	Strong
18D	2/2	Regular
19F	2/2	Strong

*Approximate location.
†Number of times where position was detected over number of chromosomes examined.

Furthermore, the morphology of the X chromosome in mutant males often appeared irregular, with regions of decondensed or knotted chromatin (Fig. 5B,C, arrowheads). Consistent with the reduced MSL-2 content of the X chromosome, the DCC components MSL-1, MSL-3 and MLE were also severely reduced (Fig. 5C). In addition to the X chromosome, MLE targets a number of autosomal sites (Kotlikova et al., 2006). Significantly, autosomal MLE staining was less affected, suggesting that the effect of UNR is specific to the X chromosome (Fig. 5C, asterisks). Histone H3 staining was similar in wild-type and mutant male X chromosomes, suggesting that the observed decrease in DCC staining is not a consequence of general chromatin defects (Fig. 5C). These results reveal a new function for *Drosophila* UNR in the efficient assembly or recruitment of the DCC to the male X chromosome.

UNR affects DCC binding without altering the levels or nucleocytoplasmic distribution of DCC components

To further examine the role of UNR in DCC formation, we knocked-down UNR from *Drosophila* male SL2 cells. Western blot analysis indicated that UNR was efficiently depleted after RNAi treatment (see Fig. 6B, UNR panel). Staining of untreated SL2 cells with anti-MSL-2 antibodies showed a strong localized signal within the nucleus corresponding to the X chromosome territory (Fig. 6A). Consistent with the results in flies, UNR depletion resulted in a less intense MSL-2 signal, which was dispersed within the nucleoplasm (Fig. 6A). Importantly, both the level and nucleocytoplasmic distribution of MSL-2 were unaffected, indicating that UNR is required for correct DCC assembly or targeting in males independently of changes in MSL-2 expression or localization (Fig. 6B, MSL-2 panel).

Previous studies have reported reduced DCC targeting after RNAi treatment of SL2 cells against the nuclear pore components Mtor and NUP153 (Mendjan et al., 2006), or after alteration of the balance between DCC components (Demakova et al., 2003). We checked whether the levels and distribution of these proteins were affected by the depletion of UNR. Staining for Mtor and NUP153 showed no significant differences between untreated and UNR RNAi-treated cells (Fig. 6A and data not shown). In addition, western blot analysis indicated that both the amount and distribution of all DCC protein components, and of Mtor and NUP153, remained unchanged (Fig. 6B). These data indicate that UNR promotes DCC binding to the male X chromosome by a mechanism that is independent of translation of DCC components.

Loss of DCC staining on the X has also been observed in *roX* mutants, which lack the RNA component of the DCC (Franke and Baker, 1999; Meller et al., 2000; Meller and Rattner, 2002). We therefore tested whether depletion of UNR resulted in loss of *roX2*. No loss of *roX2* was detected by qRT-PCR of total RNA from UNR-depleted cells (see Fig. S2 in the supplementary material). Similarly, the levels of *roX1* and *roX2* in *Unr* mutant males were nearly identical to those in wild-type males (see Fig. S2 in the supplementary material). Thus, UNR does not affect the expression of *roX* RNAs.

UNR binds to *roX* RNAs

Because the binding of MLE was more affected in *Unr* mutants compared with other DCC components, and MLE is required for proper integration of *roX* RNAs into the DCC, we thought that UNR might cooperate with MLE in this function. We tested whether UNR binds to *roX* RNAs by immunoprecipitation of UNR from wild-type adult extracts and amplification of *roX* from the pellet. The results showed that UNR indeed binds to both *roX1* and *roX2* RNAs in male flies (Fig. 7). The binding to the *roX* RNAs is specific because *Daughters against dpp* (*Dad*) and *LIM-kinase 1* (*LIMK1*), two

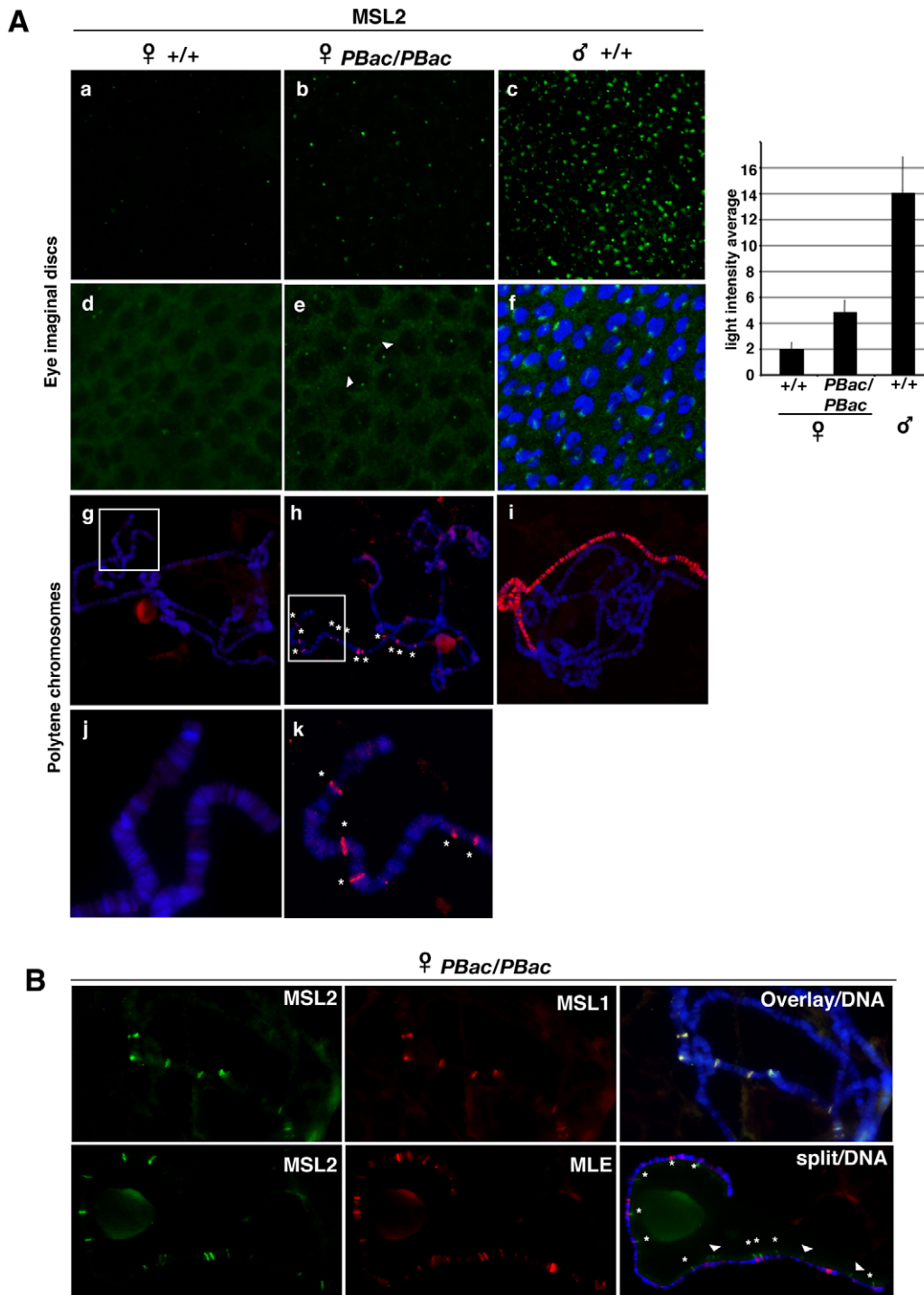


Fig. 4. UNR inhibits DCC formation in females.

(A) MSL-2 expression in *Unr* hypomorphic mutant females. Eye imaginal discs (a-f) and polytene chromosomes (g-k) of third-instar *Drosophila* larvae were stained with anti-MSL-2 antibodies. MSL-2 was derepressed and assembled on the X chromosomes of mutant females (b,e,h,k). Staining of wild-type females (a,d,g,i) and males (c,f,i) is shown for comparison. The boxed regions in g and h are shown at higher magnification in j and k, respectively. a-c and d-f represent independent staining sets. a-e correspond to maximal projections and f represents a single section. (d-f) Higher magnification images in which the background has been increased to highlight the nuclei (also revealed by DAPI staining in f). Arrowheads indicate MSL-2 on the X chromosome of cells from female mutant eye imaginal discs. MSL-2 staining is stronger in wild-type male cells, where the X chromosome domain is detected as an extended signal on one side of the nucleus (f). Asterisks denote MSL-2 assembled on X chromosome high-affinity sites. The bar chart indicates the geometric averages of MSL-2 light intensity from eye imaginal disc images from arbitrary values calculated with Leica LCS software. Quantifications were performed on 6, 9 and 8 images (same exposure time) for *+/+* females, *PBac/PBac* females and *+/+* males, respectively. (B) The DCC assembles on X chromosome high-affinity sites of female *Unr* hypomorphic mutants. Except for a few sites (arrowheads), most of the MSL-2 signal colocalizes with MLE (asterisks).

mRNAs that are abundant in males (Britta Hartmann and Juan Valcárcel, personal communication; see also Fig. 7), are not immunoprecipitated by UNR. These data suggest that UNR promotes DCC formation in males by targeting the non-coding *roX* RNAs.

DISCUSSION

UNR is an evolutionarily conserved RNA-binding protein that is necessary to repress *msl-2* translation in *Drosophila* (Abaza et al., 2006; Duncan et al., 2006). In this work, we have analyzed the *in vivo* roles of UNR by characterizing a hypomorphic *Unr* mutant and testing the effect of overexpressing UNR in flies. Our results uncover dual, mechanistically distinct roles for UNR in the regulation of dosage compensation in males and females.

Specific recruitment of UNR to the 3' UTR of *msl-2* mRNA by SXL is required for repression of *msl-2* translation both *in vitro* and in cell culture (Abaza et al., 2006; Duncan et al., 2006). A prediction from these results is that UNR represses dosage compensation in female flies. Indeed, in hypomorphic mutant females lacking the C-terminal half of UNR, the DCC assembles on a set of X chromosomal sites (Fig. 4). These sites map closely with positions previously described as being high-affinity sites, which are occupied

by the DCC in conditions of low complex concentration (e.g. Demakova et al., 2003). These observations suggest partial derepression of *msl-2* translation in mutant females. Two of the high-affinity sites correspond to the loci for *roX1* and *roX2* RNAs (Table 2, cytological positions 3F and 10C, respectively). Expression of these RNAs requires MSL-2 and their stability depends on their association to the DCC complex (Meller et al., 2000; Rattner and Meller, 2004). The fact that *roX* levels were similarly low in mutant and wild-type females supports the notion that *msl-2* translational derepression in the mutant is only partial (see Fig. S2 in the supplementary material). These results indicate that the N-terminal half of UNR exerts strong translational inhibition *in vivo*, and are consistent with *in vitro* data showing that amino acids 1-397 of UNR are sufficient for translational repression in functional tethering assays (Abaza and Gebauer, 2008). Appropriate UNR levels are essential for viability and development because moderate (~2-fold) overexpression of UNR results in complete lethality early in development for both males and females (Fig. 1). Accordingly, keeping the correct stoichiometry between UNR and SXL is important for translational control of *msl-2*, and might be necessary for the regulation of other substrates (Abaza and Gebauer, 2008).

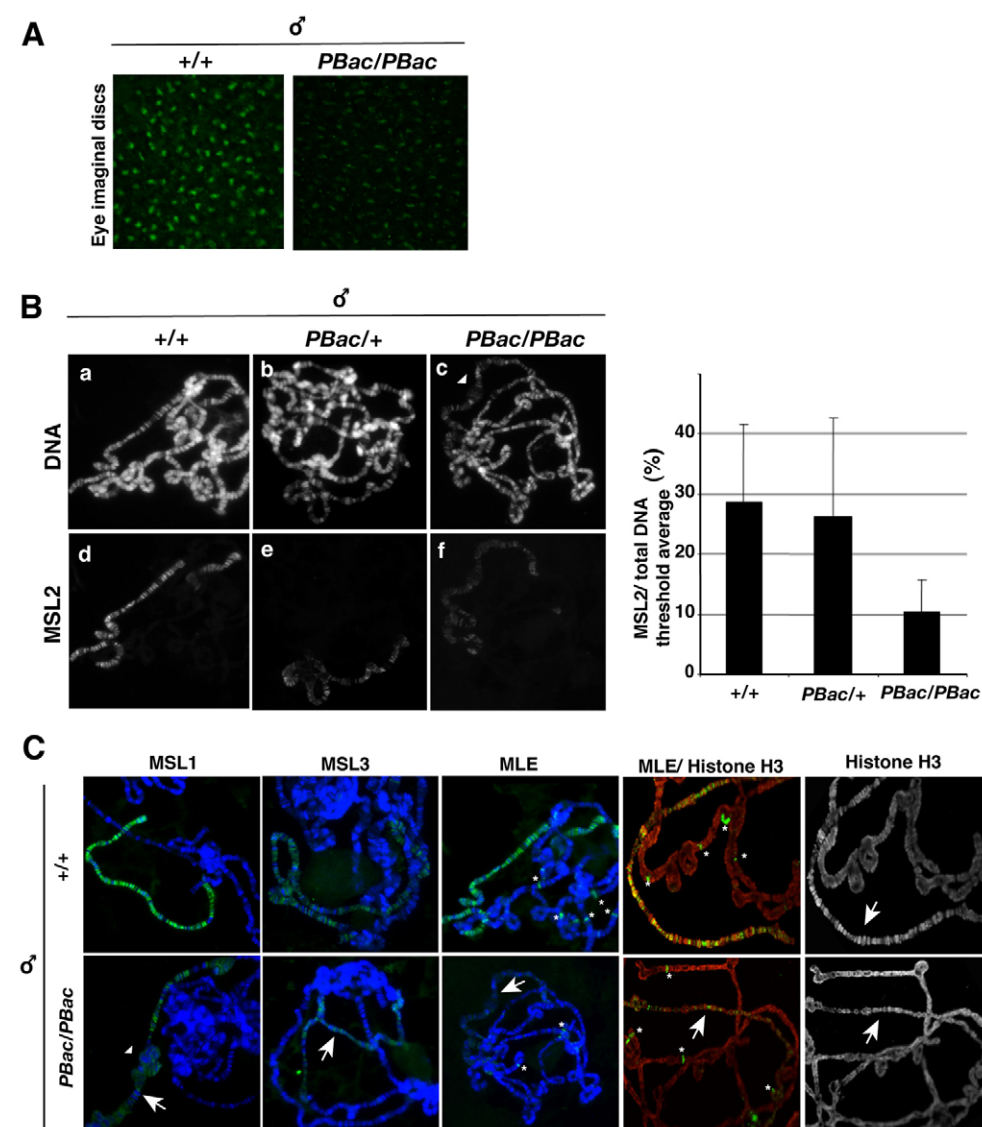


Fig. 5. Truncation of UNR decreases DCC binding to the male X chromosome.

(A) Staining of male *Drosophila* eye imaginal discs with anti-MSL-2 antibodies. (B) Staining of male polytene chromosomes with Hoechst (a-c) and with anti-MSL-2 antibodies (d-f). The arrowhead in c indicates a bloated region of the X chromosome. The bar chart shows quantification of MSL-2 intensity corrected for the total amount of DNA. Quantifications were performed using ImageJ software on 25, 14 and 30 images (same exposure time) for +/+, PBac/+ and PBac/PBac males, respectively. (C) Decreased recruitment of MSL-2 to the male X chromosome is accompanied by reduced binding of other DCC components. Co-staining of MLE (green) with histone H3 (red) is also shown. A black-and-white image of histone H3 staining alone is shown to illustrate the intensity of the signal. Arrows indicate the X chromosome, the arrowhead shows a region of the X chromosome with knotted chromatin, and asterisks denote MLE binding sites on the autosomes.

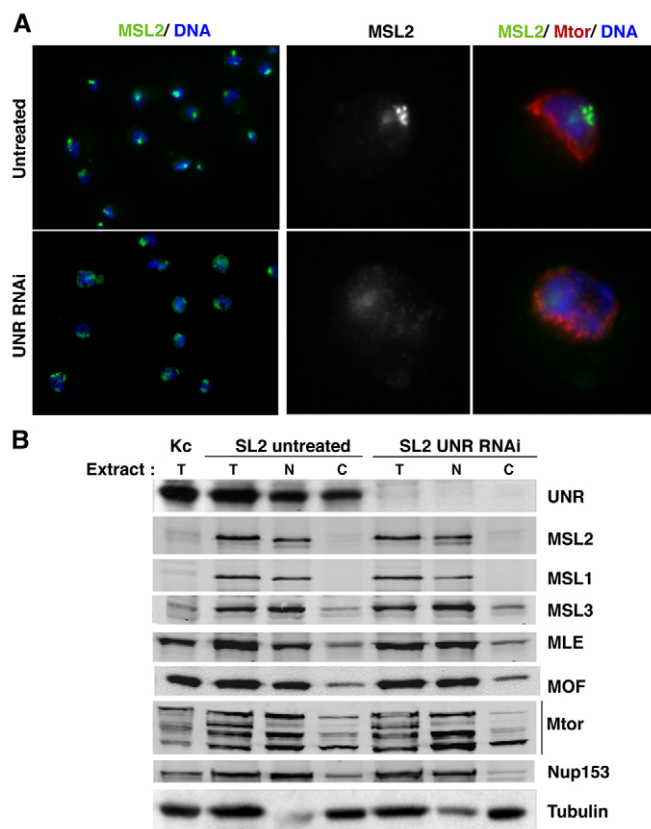


Fig. 6. UNR depletion in SL2 cells abrogates DCC binding to the X chromosome without affecting DCC protein levels or distribution. (A) Staining of untreated and UNR RNAi-treated *Drosophila* SL2 cells with antibodies against MSL-2 (green) and Mtor (red). DNA is shown in blue (Hoechst). The left panel shows a wide-field view of the cell preparation; the middle and right-hand panels show staining of a single cell. (B) UNR depletion does not alter the levels or nucleocytoplasmic distribution of DCC components or of nuclear pore proteins involved in DCC targeting. Western blots were performed on total (T), nuclear (N) and cytoplasmic (C) extracts of the indicated cells. Tubulin is shown as a loading control. Untreated Kc female *Drosophila* cells were carried in parallel as a negative control for MSL-2 and MSL-1 detection.

Unexpectedly, *Unr* mutant males showed decreased MSL-2 staining on the X chromosome, and UNR-depleted SL2 cells showed MSL-2 delocalization from the X chromosome and redistribution in the nucleoplasm (Figs 5 and 6). Reduced MSL-2 targeting to the X chromosome correlated with defective recruitment of other DCC components (Fig. 5). These effects were independent of variations in MSL-2 levels, consistent with the observation that UNR does not bind to *msl-2* mRNA in males (Abaza et al., 2006). Because DCC targeting defects have been observed under conditions of unbalanced concentrations of MSL proteins or disturbed MSL/*roX* ratios, we reasoned that UNR might regulate the levels of other DCC constituents in males (Demakova et al., 2003; Dahlsveen et al., 2006; Meller et al., 2000; Oh et al., 2003). Strikingly, however, the levels and nucleocytoplasmic distribution of all DCC protein components remained unaltered in UNR-depleted cells (Fig. 6). Similarly, the levels of *roX* RNAs in *Unr* mutant flies or UNR-depleted cells were indistinguishable from those in the wild type

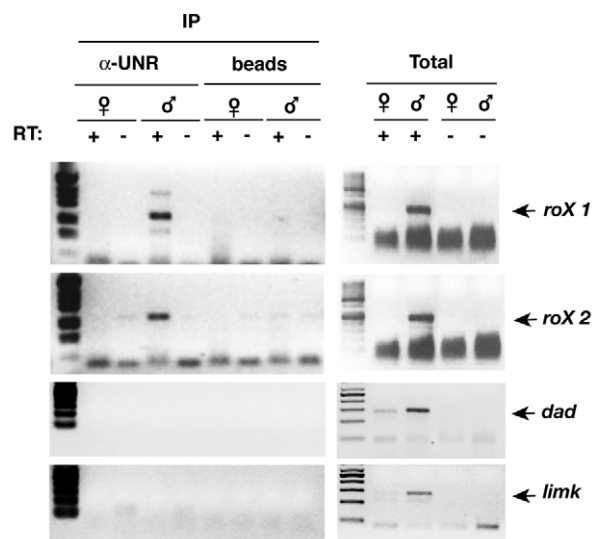


Fig. 7. UNR binds to *roX* RNAs. (Left) Immunoprecipitation (IP) of UNR from adult male and female *Drosophila* extracts followed by RT-PCR amplification of *roX* RNAs from the pellet. Two other abundant mRNAs in males, *Dad* and *LIMK1* (*limk*), were amplified as controls. Beads with no antibody, as well as parallel reactions with no reverse transcriptase (RT), were also included as negative controls. Left-hand lane, 1 kb DNA ladder (Invitrogen). (Right) Amplification of transcripts from total RNA preparations to show their relative abundance. Left-hand lane, 50 bp DNA ladder (Invitrogen).

(see Fig. S2 in the supplementary material). We conclude that UNR does not interfere with the expression or localization of DCC components.

In principle, UNR could affect DCC recruitment in males either directly or indirectly. A direct effect could be mediated by MLE and *roX*. Compared with other DCC proteins, binding of MLE to the X chromosome was more severely affected by *Unr* mutation or overexpression (Figs 2 and 5). MLE is loosely associated with the DCC: the presence of MLE in purified DCC complexes requires protection from RNA degradation and low salt conditions (Smith et al., 2000). In addition, RNase treatment of polytene chromosomes removes MLE from the DCC, suggesting that MLE recruitment to the X chromosome requires *roX* RNAs (Richter et al., 1996). Conversely, MLE is an RNA helicase necessary for *roX* incorporation into the DCC and its helicase activity is necessary for spreading of the DCC along the X chromosome (Meller et al., 2000; Morra et al., 2008). Thus, the binding of MLE and of *roX* RNAs to the X chromosome appear to be interdependent. A possible explanation for the role of UNR in males is that UNR affects the function of these DCC components. UNR is a CSD-containing protein and, in bacteria, CSD proteins associate with RNA helicases to modify the structure of RNA and regulate gene expression (reviewed by Horn et al., 2007). Indeed, mammalian UNR binds to the IRES of *Apaf1* mRNA and modifies its conformation (Mitchell et al., 2003). Therefore, UNR might associate with MLE in order to promote the appropriate structure of the *roX* RNAs for incorporation into the DCC or for subsequent spreading along the X chromosome. In support of this hypothesis, UNR specifically binds to both *roX1* and *roX2* RNAs in males (Fig. 7). In addition, as previously observed in blastoderm embryos, a fraction of UNR localizes to the nucleus of SL2 and salivary gland cells, where both MLE and *roX* concentrate (see Fig. S3 in the supplementary material) (Abaza et al., 2006).

UNR could also function indirectly, via the regulation of chromatin structure, to promote DCC recruitment to the X chromosome. The *Unr* hypomorphic mutant and the transgenic *Unr* flies show abnormal packaging of the male X chromosome, consisting of bloated or knotted chromatin. The observation that staining of histone H3 appears normal suggests that the first level of chromatin compaction remains unaltered in *Unr* mutants (Fig. 5C). In order to regulate chromatin structure, UNR could interact with chromatin remodeling factors. For example, a member of the trithorax group, ALL-1 (MLL – Human Gene Nomenclature Database), was found to interact with human UNR (CSDE1) in a yeast two-hybrid assay (Leshkowitz et al., 1996). Alternatively, UNR could control the expression of chromatin regulators that influence X chromosome morphology, such as ISWI, NURF, JIL-1 or SU(VAR)3-7 (Deng et al., 2005; Corona et al., 2007; Carré et al., 2008). It is interesting to note that although mutations of most of these factors do not concur with loss of DCC binding, null mutations of *Su(var)3-7* result in both a bloated X chromosome and depletion of the DCC from the X chromosome (Spierer et al., 2008). Thus, UNR could regulate the expression of SU(VAR)3-7 – or of other regulators with similar functions – in order to modulate DCC recruitment. In summary, at this point our results do not allow us to conclude whether the chromatin-packaging and DCC-binding defects observed in males are dissociable events. Nevertheless, the fact that UNR binds to *roX* RNAs implicates a direct role of UNR in DCC recruitment. Further studies are necessary to clarify the relationship between the multiple nuclear functions of UNR.

Our results show that UNR performs opposing functions in the regulation of dosage compensation in males and females. Dosage compensation is evolutionarily linked to sex determination. In *D. melanogaster*, a single master protein regulates both processes: SXL determines the female sexual fate and represses dosage compensation. However, SXL is not sex-specifically expressed in other distant species of Diptera, raising the possibility that the use of SXL for sex determination is a recent adaptation of the *Drosophila* genus (Pomiankowski et al., 2004). Perhaps, SXL made use of an existing regulator of dosage compensation, namely UNR, and adapted its function to a new role in females. Further genetic studies and biochemical analyses will help to identify the interactors and substrates that mediate the diverse roles of UNR.

We thank the laboratory of Cayetano González for advice on *Drosophila* methods and for crucial help during the initial stages of this project; Clement Carré, Martine Simonelig, Alain Debec and Carmen Segarra for advice on *Drosophila* protocols; Timo Zimmerman and members of the laboratory of Isabel Vernós for advice on microscopy; Dolores Martín Bermudo for the *engrailed-Gal4* driver line; Asifa Akhtar and Peter Becker for kindly sharing their antibodies; Asifa Akhtar, Peter Becker, Tobias Straub, Ramin Shiekhattar, Clement Carré, Juan Valcárcel and members of the laboratory of F. Gebauer for useful suggestions and/or for critical reading of the manuscript. This work was supported by grants BMC2003-04108 and BFU2006-01874 from the Spanish Ministry of Education and Science, and grant 2005SGR00669 from the Department of Universities, Information and Sciences of the Generalitat de Catalunya (DURSI).

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/4/689/DC1>

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