

DNA supercoiling factor contributes to dosage compensation in *Drosophila*

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DNA supercoiling factor (SCF) is a protein capable of generating negative supercoils in DNA in conjunction with topoisomerase II. To clarify the biological functions of SCF, we introduced a heritable SCF RNAi into *Drosophila*. Upon knockdown of SCF, we observed male lethality and male-specific reduction in the expression levels of X-linked genes. SCF functionally interacts with components of the MSL complex, which are required for dosage compensation via hypertranscription of the male X chromosome. Moreover, SCF colocalizes with the MSL complex along the male X chromosome. Upon overexpression of SCF, the male X chromosome had a bloated appearance. This phenotype was dependent on the histone acetyltransferase MOF and was suppressed by simultaneous overexpression of ISWI. These findings demonstrate that SCF plays a role in transcriptional activation via alteration of chromatin structure and provide evidence that SCF contributes to dosage compensation.

KEY WORDS: Supercoiling factor, Dosage compensation, Chromatin remodeling, MSL complex, *Drosophila*

INTRODUCTION

Both female X chromosomes are expressed in *Drosophila*. To achieve dosage compensation, the single male X chromosome is hypertranscribed approximately twofold relative to the female X chromosomes. The dosage-compensated X chromosome is less condensed than the autosomes, which correlates with acetylation of the lysine 16 residue of histone H4 (H4K16) by MOF, a histone acetyl transferase (Turner et al., 1992; Bone et al., 1994; Smith et al., 2001). Although little is known about the molecular mechanisms underlying the approximately twofold upregulation of X-linked genes, it is likely that differences in chromatin structure play a key role in dosage compensation.

At least five genes, *mnl-1*, *mnl-2*, *mnl-3*, *mle* and *mof*, have been identified in genetic screens for male-specific lethality (Fukunaga et al., 1975; Belote and Lucchesi, 1980; Uchida et al., 1981; Lucchesi et al., 1982; Hilfiker et al., 1997). In males, the products of these genes form an MSL complex that binds to numerous sites along the X chromosome, but in females the complex fails to assemble as a result of a translational block of the MSL-2 transcript by a protein called Sex-lethal (Bashaw and Baker, 1995; Kelley et al., 1995). In addition to these components, two noncoding RNAs, *rox1* and *rox2*, have also been identified as members of the dosage compensation complex (Amrein and Axel, 1997; Meller et al., 1997; Franke and Baker, 1999). An essential histone H3 kinase, JIL-1, also associates with the MSL complex (Jin et al., 2000; Wang et al., 2001).

Mutations in the genes that encode the *Drosophila* ISWI and NURF301 [E(bx) – FlyBase] components of the chromatin remodeling complex NURF cause an X chromosome defect in males (the X chromosome is much less compact than normal), and the phenotype can be alleviated by lowering acetylation levels on the X chromosome. This suggests that ISWI ATPase activity and H4K16 acetylation counteract one another on the dosage-compensated X

chromosome (Deuring et al., 2000; Corona et al., 2002; Badenhorst et al., 2002). The chromosome defect in the mutant strains is likely to be the result of an imbalance caused by acetylation by MOF leading to a more open chromatin state, without ISWI working toward a more condensed chromatin state (Deuring et al., 2000). However, it remains unclear whether the phenomenon is caused by functional antagonism between the two factors already identified (MOF and ISWI), or whether additional factors are also involved.

DNA supercoiling factor (SCF) was first identified in the silkworm as a protein that generates negative supercoils in DNA in conjunction with eukaryotic topoisomerase II (Ohta and Hirose, 1990). A subsequent study revealed that a *Drosophila* ortholog of SCF interacts with topoisomerase II in the nucleus and localizes to puffs on polytene chromosomes (Kobayashi et al., 1998), suggesting a role for SCF in transcription on chromatin. Interestingly, recent studies have shown that an ability to generate superhelical torsion in DNA is shared by the ATP-dependent chromatin remodeling factors SWI/SNF, ISWI and Mi-2 (Havas et al., 2000). However, the supercoiling activities of these molecules, including that of SCF, were demonstrated only in in vitro assay systems, and little is known of their biological functions.

In this study, we analyzed the role of SCF in vivo. Although SCF may have a general function in the formation and maintenance of active chromatin, here we focused on the specific role of SCF in males. We chose this focus because knockdown of SCF resulted in male-specific reduction of X-linked gene expression and a male lethal phenotype. Our results illustrate a role for SCF in hypertranscription of X-linked genes via the alteration of chromatin structure after association of the MSL complex and acetylation of H4K16 along the male X chromosome. We propose that SCF counteracts ISWI action and forms (and/or maintains) transcriptionally active open chromatin.

MATERIALS AND METHODS

Plasmid DNA construction

To construct *UAS-IRscf*, a 1220 bp fragment containing the *scf* coding sequence was amplified using the primers 5'-CGAAATATCTCAATCACAC-3' and 5'-CAATCTTAATCTCAGGGATTC-3', and subcloned into the T-vector pT7Blue (Novagen). A clone of correct sequence was chosen and designated pSCF-CR. A 480 bp fragment was then amplified, using pSCF-CR as template, with additional *HindIII* and *EcoRI* sites introduced

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at the 5' end of the *scf* coding sub-fragment (the primers used were 5'-GCCAAGCTTGAATTTCGATGACAATCTCAC-3' and 5'-CAATCT-TAATCTCAGGGATTC-3'). In parallel, the primer pair 5'-TCTTC-TAGAAGCAGCGCAAC-3' and 5'-CTGTTCTAGATGTACTCCAGC-3' was used to amplify a fragment of the green fluorescent protein (GFP) coding sequence from pEGFP-N1 (Clontech), resulting in a 150 bp fragment with *Xba*I sites at both ends. pSCF-CR was linearized by digestion with *Hind*III (in the vector cloning site) and *Xba*I (in the *scf* fragment) and then re-circularized together with the *Hind*III-*Xba*I-digested 480 bp *scf* fragment and the 150 bp *Xba*I-*Xba*I GFP fragment. Finally, the resulting fragment containing the *scf* inverted repeat separated by the GFP spacer was excised using *Eco*RI and *Kpn*I and cloned into the *Drosophila* transformation vector pUAST (Brand and Perrimon, 1993).

To construct Hsp83-SCF, the *scf* cDNA was amplified using a 5' *Sal*I site- and FLAG-tag-containing primer (5'-ACAATGTCGACAACATGGACT-ACAAGGACGACGATGACAAGATGCAGACTGTCTACGGCTTC-3'), and a 3' *Not*I site-containing primer (5'-TTCTAGCGCGCCGCTAG-AACTCATCGTGGCG-3'). The resulting fragment was subcloned into pT7Blue and checked by sequencing. The *scf* cDNA fragment was then excised with *Sal*I and *Not*I, and cloned into *Xho*I and *Not*I-digested pCaSpeR-Hsp83 (Horabin and Schedl, 1993).

Fly stocks and genetics

Flies were raised on standard agar/cornmeal/yeast medium. *UAS-IRscf* and *Hsp83-SCF* transgenic flies were produced by P-element-mediated transformation using the *yw* strain as a host, and several independent lines were obtained. For each *Hsp83-SCF* transgenic line, the levels of SCF were examined by immunoblotting. For rescue experiments, a line expressing a similar level to endogenous SCF was selected. More robust levels of expression were detected in another line, which exhibits an X chromosome defect in males. The *Act5C-GAL4* driver strain was a gift from Yasushi Hiromi, National Institute of Genetics, Japan. *Df(3)Rac1* that lacks the *scf* locus (Ng et al., 2002) was a gift from Julian Ng. The *msl-1^{γ216}* mutant line was obtained from the Bloomington Stock Center. The [*H83M2-6I*] transgenic line bearing the ectopic *msl-2* gene under control of the *Hsp83* promoter, and the *mof^l* mutant line were gifts from Yuji Kageyama, Nana Institute of Science and Technology, Japan. *P[ISWI⁺]* (Deuring et al., 2000) was a gift from J. Tamkun. The *P[ISWI⁺]* chromosome was introduced into the *Hsp83-SCF* line in order to overexpress both SCF and ISWI.

For RNAi assays, several independent *UAS-IRscf* lines were crossed to the *Act5C-GAL4* driver strain at 25°C or 18°C. To generate control individuals that carry only *Act5C-GAL4* for immunoblot, immunostaining and quantitative RT-PCR analyses, *yw* was crossed to *yw;Act5C-GAL4/TM6B*.

For immunostaining of *mof* mutant polytene chromosomes, *mof^l* male larvae were generated by crossing *yw/Y* males to *mof^l/mof^l* females. To overexpress SCF in *mof^l*, the *P[Hsp83-SCF]* transgene on the third chromosome was introduced into *mof^l*; *CyO*, *P[w⁺, mof⁺]/+* and F1 *mof^l/mof^l*; *P[Hsp83-SCF]* females were crossed to *yw/Y*; *P[Hsp83-SCF]* males.

Immunoblot analysis

Immunoblot analyses were performed using standard protocols (Sambrook et al., 1989). To prepare larval extracts, third instar larvae were homogenized in Laemmli sample buffer. Extracts were separated by SDS-PAGE, transferred to a PVDF membrane (Roche), probed with antibodies against SCF (1:1000), MSL-1 (1:500), MSL-2 (1:500), MSL-3 (1:1000), MLE (1:500), MOF (1:500), ISWI (1:5000) or α -tubulin (1:1000) (all supplied by Sigma), followed by horseradish peroxidase-linked anti-rabbit (1:5000), anti-mouse (1:3000) or anti-goat (1:3000) IgG (Santa Cruz Biotechnology), and detected using SuperSignal (Pierce).

Immunostaining of polytene chromosomes

Preparation of chromosomes and immunostaining were performed essentially as described previously (Pile and Wassarman, 2002), with the following modifications. Salivary glands were dissected in PBS and placed in a fixative containing 3.7% formaldehyde, 1% Triton X-100 and PBS for 20 seconds, and then transferred to a fixative containing 3.7% formaldehyde, 45% acetic acid for 90 seconds before squashing. For spreads stained with

anti-H4Ac16, salivary glands were dissected in PBS containing 5 mM sodium butyrate as described by Turner et al. (Turner et al., 1992). Polytene chromosomes were then treated with purified antibodies against SCF (1:25), MSL-1 (1:100), H4Ac16 (1:50, Serotec) or MSL-3 (1:1000), followed by donkey anti-rabbit Cy2 (1:500) or anti-goat Cy3 (1:2000, Jackson ImmunoResearch). We included appropriate controls in order to verify that the signals are dependent on, and specific to, the primary antibody used, and that there is no cross-reactivity of secondary antibodies under these conditions. For staining with anti-SCF antibody, wash buffer containing 300 mM or 400 mM NaCl, 0.2% Triton X-100, 0.2% NP-40 and PBS was used after primary antibody treatment. Finally, the samples were mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories). At least six squash preparations were analyzed for each antibody staining, and we verified that the staining pattern was reproducible in the independent experiments.

Quantitative RT-PCR

Total RNA was isolated using Sepazol RNAi (Nakarai) from the six animals collected at the time of puparium formation. The RNA was then treated with RNase-free DNaseI (TaKaRa). To synthesize first strand cDNA, 1 μ g of the total RNA template was reverse transcribed with AMV-RTase (1st Strand cDNA Synthesis Kit for RT-PCR; Roche). After the reverse transcription reaction, the reaction mixture was diluted (from 1:10 to 1:50) and amplified by quantitative PCR using the LightCycler-DNA Master SYBR Green I Kit (Roche) and the following gene-specific primers:

β 1-tubulin (*β Tub56D* – FlyBase), 5'-AGTTCACCGCTATGTTCA-3' and 5'-CGCAAAACATTGATCGAG-3';
BR-C (*br* – FlyBase), 5'-ATGGACATGGTCTGCTCTAA-3' and 5'-GCTGCATGGAACATCTTGT-3';
Pgd, 5'-GCCGGAGCTGTCTAATCTG-3' and 5'-CAGCAGCTCAT-AGGTGTG-3';
Rp49 (*RpL32* – FlyBase), 5'-CCACCAGTCGGATCGATATG-3' and 5'-CACGTTGTGCACCAGGAAC-3';
Sgs4, 5'-GCGGATGATTTTGAAGGAC-3' and 5'-CTTTTTGTGGCT-GAGTCTTC-3';
Dspt4 (*spt4* – FlyBase), 5'-AGTGGCAAAGATTGTCCC-3' and 5'-ATCGTTGACTTCTGTCCC-3';
Zw, 5'-TCTACTCTCGACGTCTTCT-3' and 5'-ATTGTTCTCTCG-CACTTGC-3';
Gs2, 5'-TGCAGGAGAACATCGTTCAG-3' and 5'-TCCATCGTAGT-TCCAAACGG-3';
mRpL16, 5'-GTATTTTCGCTCCGCCTATTA-3' and 5'-CTTATGGAGC-AGCGTGTGT-3';
blue, 5'-TGGAGCAGCAGCGAAATGA-3' and 5'-TGTGTGCTCTG-ACTCGTTGTA-3'; and
trio, 5'-GCCACCAAGTTCGCTCTGTA-3' and 5'-GGCCTTGATGAT-CTCCTTGA-3'.

The quantification value was then normalized to the value of the internal standard *β 1-tubulin* as described previously (Bhadra et al., 1999). Each quantitative RT-PCR experiment was repeated at least three times using independently prepared samples. For quantification of *rox1* and *rox2* expression levels (Fig. 2A), the reverse transcription reaction products prepared as described above were diluted (from 1:3 to 1:27) and amplified by PCR using TaKaRa Ex Taq (TaKaRa) and the following gene-specific primers: *rox1*, 5'-CAAATGCTCTGCAGAAGAGG-3' and 5'-ATGGT-TGGTTATTCGGGTGG-3'; *rox2*, 5'-AGATGTTGCGGCATTCGCGG-3' and 5'-TGCGACTTGTACAATGTTGCG-3'. The PCR products were resolved by gel electrophoresis, stained with SYBR Green (Molecular Probes), and analyzed using an LAS-1000 luminescent image analyzer (Fujifilm).

RESULTS

Knockdown of SCF severely affects male viability

To analyze the biological function of SCF, we attempted to perform RNA interference (RNAi) in vivo. Recently, a method developed to express dsRNA as an extended hairpin loop was shown to be successful for RNAi-based gene knockdown in the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*

(Tavernarakis et al., 2000; Kennerdell and Carthew, 2000; Piccin et al., 2001). We employed this method to knockdown *scf* function throughout development. To do this, we generated transgenic fly lines carrying a *UAS-IRscf* insert consisting of the GAL4-upstream activating sequence (UAS) and an inverted repeat (IR) of a part of the *scf* gene. Several independent transformants were crossed with flies carrying the yeast GAL4 gene driven by the *Actin5C* promoter (*Actin5C-GAL4*) in order to constitutively activate transcription from the hairpin-encoding transgene. To estimate the effect of RNAi in the progeny, we examined expression levels of SCF in late third-instar larvae by immunoblot analysis. When the transgenic line 2-1 carrying the *UAS-IRscf* construct was crossed with the *Actin5C-GAL4* driver line, SCF protein levels were markedly reduced in male and female progeny that carried both the *GAL4* driver and the *UAS-IRscf* transgene (Fig. 1A). Comparable results were obtained for transgenic lines 3-1 and 3-2 (data not shown). Next, we examined the effect of *scf* knockdown on viability, and found a specific and

dramatic reduction in the viability of males carrying both *GAL4* and *UAS-IRscf* (Fig. 1B). Most of these males died during the pupal stage, and the few individuals that did survive to adulthood died shortly after eclosion. The effects on male viability do not appear to be due to position effects, as the three independent transgenic lines (2-1 on the second chromosome, and 3-1 and 3-2 on the third chromosome) exhibited this phenotype. Whereas male viability was severely affected in all three lines, the effects on female viability were less pronounced and varied among the lines tested. No significant effect on females was observed for the 2-1 transgenic line (Fig. 1B). In an experiment with the 2-1 line, we changed the temperature from 25°C to 18°C because the activation levels in the GAL4/UAS system are dramatically affected by temperature, and RNAi using this system at 18°C is less effective than that at 25°C (Fig. 1A, lanes 9-12). We found that the reduction in male viability was significantly suppressed at 18°C (Fig. 1B). These data show that males are more sensitive than females to SCF knockdown. The similar decrease in the amount of SCF protein in both sexes upon RNAi (Fig. 1A) excludes the possibility that the observed male lethality is due to a difference in the extent of RNAi in the two sexes.

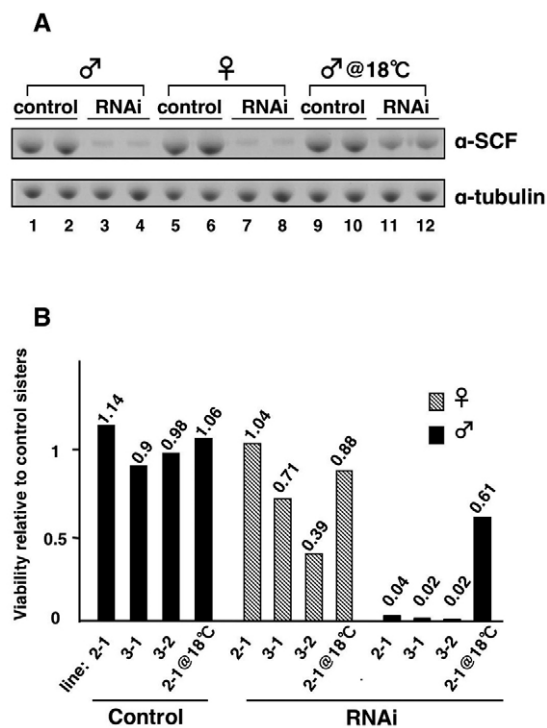


Fig. 1. Effect of *scf* RNAi on expression of SCF and viability. (A) Comparison of the RNAi effect on expression of SCF between males and females. To generate control individuals (carrying only *GAL4*) or RNAi-induced individuals (carrying both *GAL4* and *UAS-IRscf*), the *Act5C-GAL4* driver was crossed to *yw* or *yw*; [*UAS-IRscf*] (line 2-1) at 25°C (lanes 1 to 8) or 18°C (lanes 9 to 12). Two individuals (late third-instar larvae) of each sample type indicated were randomly selected. Proteins in a 12 μ l or 1.5 μ l aliquot of the extract from each animal were resolved by SDS-PAGE, blotted onto a membrane, and the membrane probed with anti-SCF (upper panel) or anti- α -tubulin antibody (lower panel, loading control). (B) Effect of *scf* RNAi on viability of males and females. Each *UAS-IRscf* line indicated (balanced with *CyO*, *y⁺* or *TM3*, *y⁺*) was crossed to *Act5C-GAL4* driver. F1 animals carrying only *GAL4* (control), or carrying both *GAL4* and *UAS-IRscf* (RNAi), were sorted and counted. Numbers above the bars indicate viability relative to the control sisters carrying only *GAL4*. The reduction in male viability observed at 25°C was significantly suppressed at 18°C in the experiment using the 2-1 line. The number of control sisters examined for the lines 2-1, 3-1, 3-2, and 2-1 at 18°C was 180, 164, 59 and 160, respectively.

SCF functionally interacts with the dosage compensation complex in vivo

In *Drosophila*, a loss-of-function mutation in any of the genes known to encode components of the MSL complex abolishes painting of the X chromosome with the complex, abolishes acetylation of H4K16, and causes male-specific lethality (Palmer et al., 1994; Gorman et al., 1995; Kelley et al., 1995; Gu et al., 2000). This led us to the idea that SCF might be involved in dosage compensation. To test this possibility, we first examined the effect of SCF knockdown on the expression levels of each MSL component (Fig. 2A). Almost no difference in the level of MSL-1, MSL-2, MSL-3, MLE or MOF protein or *rox1* or *rox2* RNA was observed between the SCF-reduced background and the control. This suggests that the male lethal phenotype caused by knockdown of SCF is not due to reduced expression of the MSL components.

We next examined the possibility that SCF is involved in dosage compensation via an interaction with the MSL complex. Two different analyses were performed to test for genetic interactions between *scf* and the *msl* alleles. First, we examined the ability of an *scf* deficiency to prevent the lethality caused by the forced expression of MSL-2 protein in females (Fig. 2B). The MSL-2 protein is not normally expressed in females and its ectopic expression results in the appearance of the MSL complex on both X chromosomes, leading to an abnormal regulation of X-linked genes and developmental delay or decreased viability in females (Kelley et al., 1995). Suppression of the misexpression phenotype provides evidence that the mutation is involved in dosage compensation via function of the MSL complex. Previous studies have shown that the abnormal development is rescued in homozygotes for *mle*, *msh-3* or *rox* mutant alleles, and in heterozygotes for *msh-1* or *mof* mutant alleles (Kelley et al., 1995; Hilfiker et al., 1997; Meller and Rattner, 2002). We found that the presence of the *scf* deficiency also significantly rescued females ectopically expressing MSL-2 (Fig. 2B). Moreover, the rescue was suppressed by introducing an *Hsp83*-driven *scf*cDNA fragment. We also note that, in this case, the control sisters were more severely affected by the misexpression of MSL-2 than lines carrying no *scf* transgene.

Next, we examined the effect of combining the *scf*-deficient allele and the *msh-1* mutant allele on male viability (Table 1). We observed a significant reduction in male viability in double heterozygotes ($P < 0.001$, χ^2 test; see Table 1, row three) when compared with each

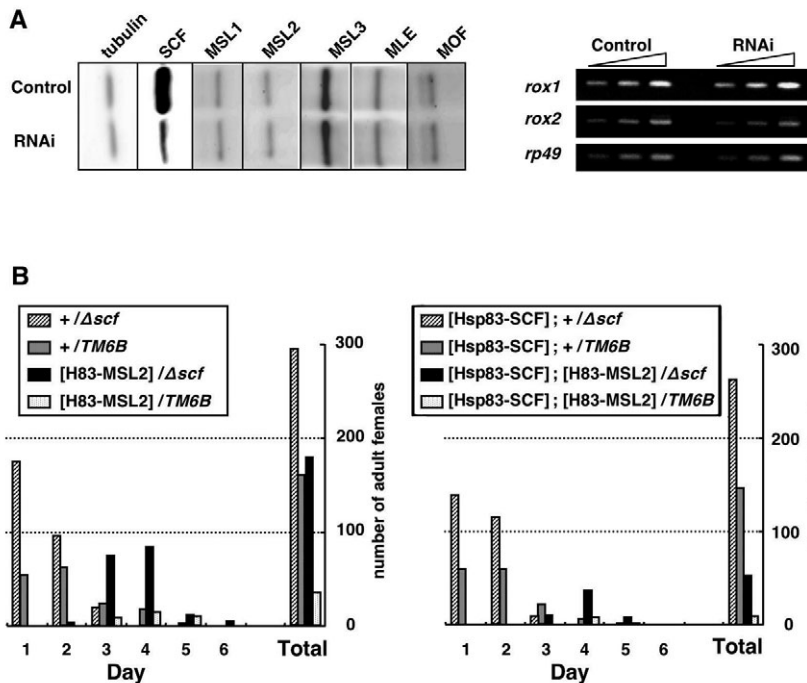


Fig. 2. Genetic interactions between SCF and the MSL complex. (A) Expression levels of MSL proteins and *rox* RNAs are not affected by knockdown of SCF. Control individuals (carrying only *GAL4*) and RNAi-induced individuals (carrying both *GAL4* and *UAS-IRscf*) were sampled from third-instar male larvae. The expression levels of MSL-1, MSL-2, MSL-3, MLE, MOF and tubulin were examined by immunoblotting (left panel), and *rox1* and *rox2* RNA expression levels were analyzed by RT-PCR (right panel) with *rp49* as a control. First-strand cDNA synthesized from each RNA sample was diluted (1:3, 1:9 and 1:27) and used as PCR template. (B) Effect of the *scf* deficiency on the survival of females expressing *msl-2* under the control of a constitutive promoter. To generate the progeny females listed in the boxes, [H83MSL2]/+ males were crossed to $\Delta scf/TM6B$ females (left) or [Hsp83-SCF]; $\Delta scf/TM6B$ females (right). Day 1 represents the day when the first population eclosed. Females bearing the [H83MSL2] transgene displayed developmental delay and decreased viability compared with their sisters lacking the transgene. The *scf* deficiency significantly rescued these females (left graph). Moreover, the rescue was suppressed by introducing *Hsp83*-driven *scf* cDNA (right graph).

single allele (Table 1, rows one and two). Furthermore, the reduction in the viability of these animals was also significantly suppressed by introducing the *Hsp83*-driven *scf* cDNA fragment (Table 1, row four). These data demonstrate a functional interaction between SCF and the MSL complex.

SCF colocalizes with the MSL complex along the male X chromosome

A sex-specific pattern of SCF localization on chromosomes would provide additional evidence that it is involved in dosage compensation. To test this, we analyzed the distribution of SCF on polytene chromosomes. Although a previous study showed that SCF localizes to transcriptionally active loci on polytene chromosomes, such as interbands or puffs (Kobayashi et al., 1998), differences between the distribution of SCF in males and females, and between the X-chromosome and autosomes remain to be addressed. We performed immunostaining of SCF on polytene chromosomes prepared from both males and females (Fig. 3A-E).

We found many extra SCF signals along the male X chromosome that could be clearly identified by co-detection of MSL-3 (Fig. 3C), in comparison to what was observed for the female X chromosome (Fig. 3, compare B with E). On the male X chromosome, most of the SCF signals were detectable as fine bands or dots. These signals were weak

when compared with the robust staining observed in discrete interbands and puffs of autosomes, and at some sites on the X chromosome. Whereas the intense staining pattern was the same in both males and females (e.g. see arrowheads in Fig. 3B,E), the 'milky way'-like staining pattern consisting of many faint signals between the robust staining sites was specific to the male X chromosome (Fig. 3B,E). This suggests the presence of a relatively small amount of SCF on each of the many male X-specific faint signals. Furthermore, the male X-specific faint signals of SCF, but not the robust bands, colocalized with MSL-3, which is a component of the MSL complex (Fig. 3F-I). These observations support the idea that SCF regulates gene expression in the context of dosage compensation.

Knockdown of SCF does not disrupt localization of MSLs on the male X chromosome

To investigate the possible role of SCF in MSL complex-mediated dosage compensation, we first examined whether the knockdown of SCF by RNAi affects binding of the MSL complex to the X chromosome. We performed immunostaining of polytene chromosomes with anti-MSL-1 antibody because MSL-1 plays a central role in assembly of the MSL complex and is thus a good indicator of complex formation (Scott et al., 2000). The MSL-1 protein localized to the male X chromosome even under *scf* RNAi

Table 1. Genetic interaction between *scf* and *msl-1*

Cross	F1 genotype	Males	Females	Male/female ratio	P value*
1 [†]	$\Delta scf/+$	309	312	0.99	NA
2 [‡]	<i>msl-1/+</i>	268	323	0.83	$P > 0.01$
3 [§]	<i>msl-1/+; \Delta scf/+</i>	190	386	0.49	$P < 0.001$
4 [¶]	<i>msl-1/[Hsp83-SCF]; \Delta scf/+</i>	191	266	0.72	$P > 0.01$

* Compared with $\Delta scf/+$, using χ^2 test.

[†] *yw; \Delta scf/TM6B \times +/CyO*.

[‡] *yw; +/TM6B \times msl-1/CyO*.

[§] *yw; \Delta scf/TM6B \times msl-1/CyO*.

[¶] *yw; [w⁺, Hsp83-SCF]; \Delta scf/TM6B \times msl-1/CyO*.

NA, not applicable.

conditions (Fig. 3, compare J with K). Consistent with this, immunostaining with anti-acetylated H4K16 antibody showed that acetylation of H4K16 along the male X chromosome was not affected by RNAi (Fig. 3, compare L with M). These results indicate that SCF is not necessary for association of the MSL complex with chromatin or for the subsequent H4K16 acetylation on the male X chromosome.

Histone acetyltransferase MOF is required for the proper localization of SCF along the male X chromosome

Taken together, the data presented thus far indicate that, if SCF participates in dosage compensation, it does so only after association of the MSL complex and H4K16 acetylation by MOF on the male X chromosome. To test this, we looked at the distribution of SCF protein on polytene chromosomes in a *mof* mutant background.

Compared with wild type (Fig. 3B), the many faint signals of SCF normally observed on the male X chromosome were significantly reduced in the *mof* mutant background (Fig. 3N,O). By contrast, the prominent SCF bands on the X chromosome and autosomes were not affected. These results strongly suggest that MOF activity is necessary for the male-specific pattern of localization of SCF on the male X chromosome.

Expression of X-linked genes is reduced in males deficient in SCF function

Dosage compensation in *Drosophila* is achieved by an approximately twofold upregulation of X-linked genes specifically in males (Hamada et al., 2005; Straub et al., 2005). If SCF is truly involved in dosage compensation, the reduction of SCF function should change or abolish the upregulation of X-linked genes. To test this, we examined the effects of SCF RNAi on the expression levels

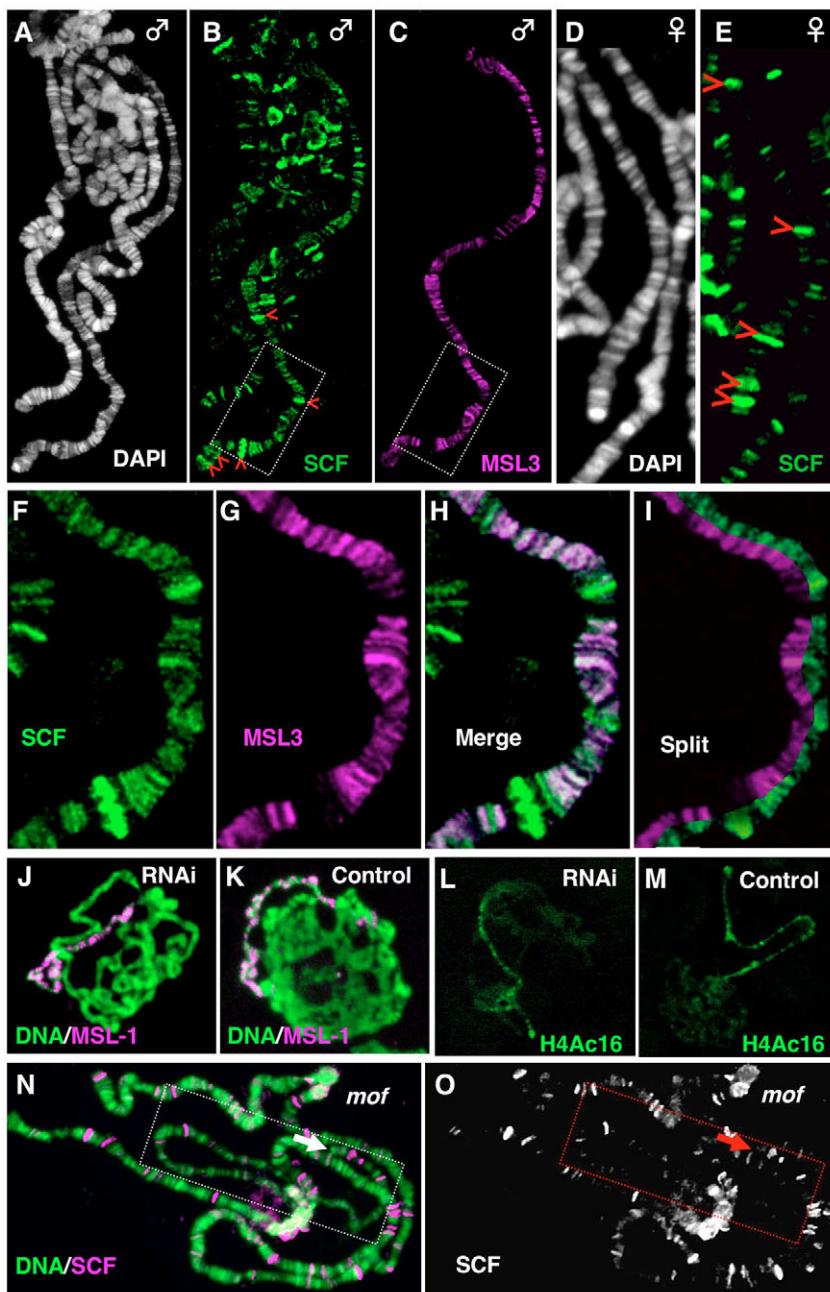


Fig. 3. Localization of SCF along the dosage-compensated male X chromosome. (A-E) Extra SCF signals are associated with the male X chromosome when compared with autosomes or the female X chromosomes. Polytene chromosomes prepared from males (A-C) or females (D,E) were stained with antibodies or DAPI as indicated. Arrowheads indicate examples of the intensely stained SCF bands, which are identical in males and females. Note that the 'milky way'-like signals between the intense bands are specific to the male X chromosome. (F-I) Most of the faint signals (but not the intense bands) of SCF colocalize with the MSL complex. Boxed regions in B and C are enlarged in F and G, and merged and split images are shown in H and I, respectively. (J-M) MSL-1 localization (J,K) and acetylation of histone H4K16 (L,M) along the male X chromosome are not affected by knockdown of SCF (J,L). To generate control individuals (carrying only *GAL4*) or RNAi-induced individuals (carrying both *GAL4* and *UAS-IRscf*), the *Act5C-GAL4* driver was crossed to *yw* or *yw; [UAS-IRscf]* (line 2-1) at 25°C. Polytene chromosomes from RNAi (J,L) or control (K,M) third-instar larvae were stained with DAPI (green in J,K) and anti-MSL-1 antibody (magenta in J,K), or with anti-acetylated H4K16 antibody (L,M). (N,O) The presence of SCF on the male X chromosome is reduced in a *mof*¹ mutant background. Polytene chromosomes were labeled with DAPI (green in N) and anti-SCF antibody (O; magenta in N). The tip of the X chromosome is marked by an arrow, and the X chromosome is boxed.

of X-linked genes using quantitative RT-PCR (Fig. 4). Six X-linked genes were examined: *Pgd*, *Sgs-4*, *Zw*, *Broad-Complex (BR-C)*, *Gs2* and *mRpl16*. Each of these genes has been shown to be dosage compensated in an MSL complex-dependent manner (Belote and Lucchesi, 1980; Smith et al., 2001; Chiang and Kurnit, 2003; Hamada et al., 2005; Straub et al., 2005). The levels of transcripts from these genes, normalized to the value of $\beta 1$ -tubulin as an internal standard (Bhadra et al., 1999), were compared between individuals deficient in SCF function by RNAi and control individuals for both sexes.

We found that the expression levels of all six X-linked genes were significantly reduced in males but not in females deficient in the SCF function (Fig. 4, X chromosome). By contrast, the autosomal control genes *Rp49*, *Dspt4*, *blue* and *trio* were not affected by the knockdown of SCF in either sex (Fig. 4, Autosome). The extent of male-specific reduction in X-linked gene expression upon SCF RNAi was similar to that observed for MSL-2 RNAi (Hamada et al., 2005; Straub et al., 2005). These results indicate that SCF is involved in the final step of the dosage compensation; that is, hypertranscription of the male X chromosome

Overexpression of SCF causes a male X chromosome defect

In the larval salivary gland, overexpression of MSL-1 and MSL-2 causes aberrant male X chromosome morphology (Oh et al., 2003). The defective morphology is similar to that observed for *Iswi* or *nurf301* loss-of-function mutants in that, in all cases, the male X chromosome appears to be much less compact than the autosomes or female X chromosomes (Deuring et al., 2000; Corona et al., 2002; Badenhorst et al., 2002), suggesting an unusual chromatin organization. Interestingly, we found that the male X chromosome is similarly affected in individuals overexpressing SCF from the *Hsp83-SCF* transgene (Fig. 5A-F). The male X chromosome, identified by anti-MSL-1 staining, was strikingly different in its appearance to the *yw* control, exhibiting a bloated appearance and a

partial loss of its banding pattern. By contrast, the morphology of autosomes and the female X chromosomes appeared normal. To see whether the morphological defect is accompanied by an increased amount of SCF on the chromosome, we performed anti-SCF staining in this transgenic line. Compared with the endogenous level, a strong staining was observed along the aberrant male X chromosome (Fig. 5E,F). However, the disrupted banding pattern of the male X chromosome prevented us from determining whether the staining pattern of SCF or MSLs was altered. Immunoblot analyses confirmed that SCF was indeed overexpressed in the *Hsp83-SCF* transgenic line, and revealed that the bloated appearance of the male X chromosome was not due to reduced expression of ISWI (Fig. 6). The defect was specific to overexpression of SCF, as we were unable to observe it upon overexpression of unrelated chromatin proteins, such as the GAGA factor, SPT16, SSRP1 and SPT6 (M.N. and S.H., unpublished). Taken together, the results suggest that SCF promotes opening of the chromatin structure.

To further understand the functional relationship between SCF and ISWI, we carried out a genetic study. The male X chromosome defect associated with overexpression of SCF was suppressed by simultaneous overexpression of ISWI (Fig. 5G), suggesting that SCF counteracts the ISWI function. Consistent with the observation that MOF activity is necessary for the proper loading of SCF on the male X chromosome, the overexpression phenotype of SCF was also suppressed by the loss-of-function mutation of *mof* (Fig. 5H) that abolishes acetylation of H4K16 (Fig. 5I). From these data, we conclude that SCF plays a role in the alteration of chromatin structure to accomplish hyperactivation of the male X chromosome.

DISCUSSION

Male-specific effects of SCF knockdown

The results reported here show that SCF has an important role in the *Drosophila* dosage-compensation mechanism. Reduction of SCF function via RNAi leads to male lethality and a male-specific decrease in the expression of X-linked genes.

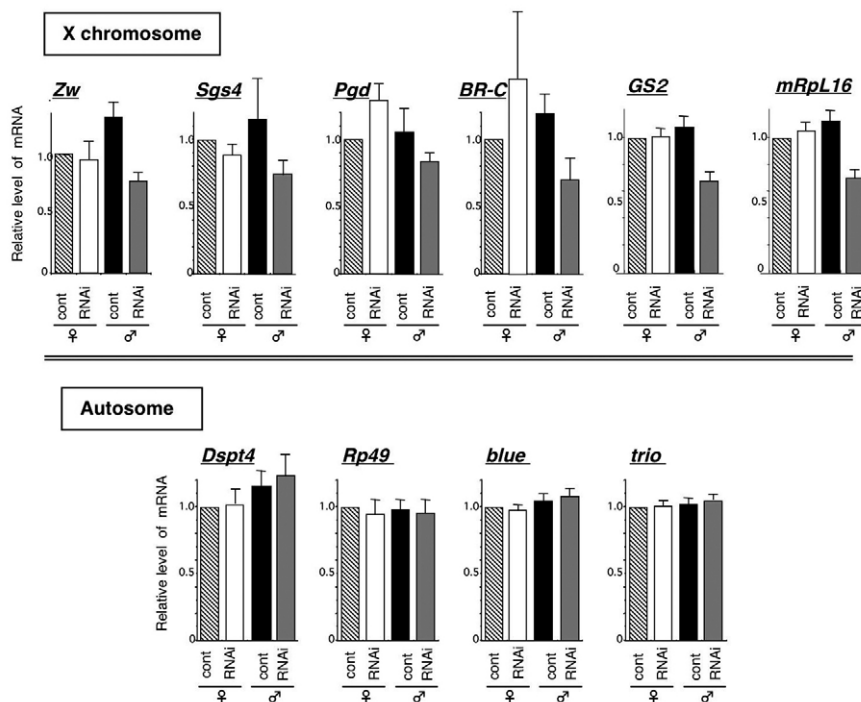


Fig. 4. Expression levels of X-linked genes are reduced in males deficient in SCF function. To generate control individuals (carrying only *GAL4*) or RNAi-induced individuals (carrying both *GAL4* and *UAS-IRscf*), the *Act5C-GAL4* driver was crossed to *yw* or *yw*; [*UAS-IRscf*] (line 2-1) at 25°C. The effect of SCF reduction on the expression levels of the X-linked genes (*Zw*, *Sgs4*, *Pgd*, *BR-C*, *Gs2* and *mRpl16*) and the autosomal genes (*Dspt4*, *Rp49*, *blue* and *trio*) was examined by quantitative RT-PCR. The level of transcripts from each gene was normalized to an internal standard ($\beta 1$ -tubulin) and then individuals deficient in SCF function via RNAi were compared with control individuals. Relative level of mRNA means the ratio of values relative to the value of the control female; error bars represent s.d. of at least three different samples.

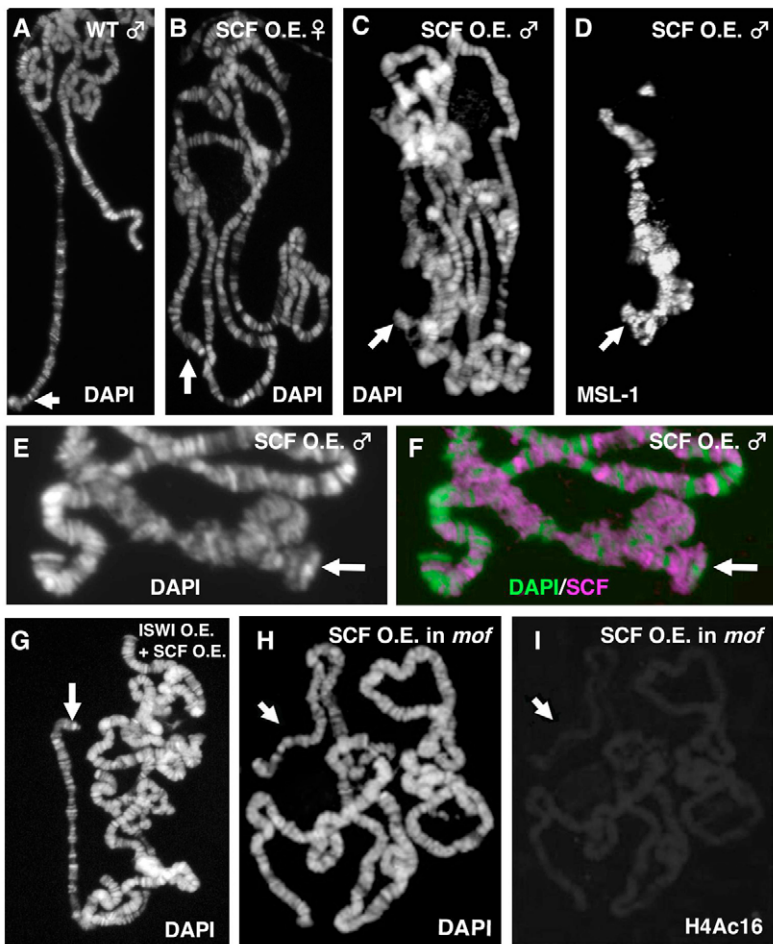


Fig. 5. Overexpression of SCF results in abnormal morphology of the male X chromosome but not of autosomes or the female X chromosomes. (A) Polytene chromosomes prepared from an *yw* control male were labeled with DAPI. (B) Chromosomes prepared from a female of the transgenic SCF-overexpression line were labeled with DAPI. (C-F) Chromosomes prepared from males of the transgenic SCF-overexpression line were labeled with DAPI (C,E; green in F) and anti-MSL-1 (D) or anti-SCF (magenta in F) antibody. (G,H) The male X chromosome appears normal in the line overexpressing both SCF and ISWI (G), and upon overexpression of SCF in the *mof* mutant background (H). (I) Acetylation of H4K16 was not detectable on polytene chromosomes in *mof*¹. The chromosomes shown in I were labeled with anti-acetylated H4K16 antibody. Arrows mark the X chromosome.

Furthermore, overexpression of SCF causes a male-specific morphological defect in the X chromosome. These findings were unexpected because SCF localizes to many puffs and interbands on polytene chromosomes, suggesting a global role for this protein in active chromatin (Kobayashi et al., 1998). Immunostaining of polytene chromosomes revealed that SCF is distributed along the male X chromosome in the form of many faint signals that are weaker in intensity than the discrete and prominent signals on autosomes or female X chromosomes (Fig. 3). Most of the faint signals of SCF colocalize with the MSL complex. Analysis using a *mof* mutant showed that the histone acetyltransferase activity is necessary for the proper loading of SCF on the male X chromosome. It is most likely that the MSL complex per se, and/or its function, including the acetyltransferase activity of MOF, creates many low-affinity sites that promote SCF association on the male X chromosome. This idea is supported by the weak interactions between SCF and the MSL components observed in co-immunoprecipitation assays using embryonic nuclear extracts (H.F. and S.H., unpublished). Owing to its relatively weak affinity for the male X chromosome, we speculate that the threshold for maintaining SCF function along this chromosome is higher than that for maintaining SCF function at the other genomic sites. Hence, males are thought to be more sensitive than females to a reduction in SCF levels. However, we cannot exclude the formal possibility that the relatively weak staining might be caused by other factors, such as differences in epitope availability in each context, or loss of the antigen upon fixation for reasons unrelated to true affinity.

Possible role of SCF in the hypertranscription of the male X chromosome

The present findings support the previously proposed model in which SCF is responsible for transcriptional activation via alteration of chromatin structure (Kobayashi et al., 1998), and further extend the model by suggesting underlying mechanisms. It has been proposed that the generation of superhelical torsion in DNA by chromatin remodeling activities could affect the formation of higher order chromatin structure (Havas et al., 2000). In addition, recent single-molecule analyses of chromatin remodeling reactions have revealed that the SWI/SNF-type complexes introduce negative supercoils into DNA, whereas the ISWI-type remodelers generate positive supercoils as reaction intermediates (Lia et al., 2006) (T. Owen-Hughes, personal communication). Therefore, the negative supercoiling activity of the SCF-topoisomerase II complex might function synergistically with the SWI/SNF-type complexes and counteract ISWI-type remodeling factors. This idea is supported by our finding that the bloated appearance of the male X chromosome in the SCF-overexpressing line is similar to that observed in the loss-of-function of *Iswi*, and is suppressed by overexpression of ISWI. Moreover, acetylation of H4K16 has been shown to inhibit ISWI activity (Corona et al., 2002). These data suggest that SCF function and acetylation of H4K16 cooperate to form and/or maintain transcriptionally active open chromatin on the male X chromosome by counteracting ISWI action.

Another possible mechanism of SCF action is facilitation of transcription elongation on chromatin. The X-linked dosage compensated genes *Pgd* and *Zw* are known to be more highly

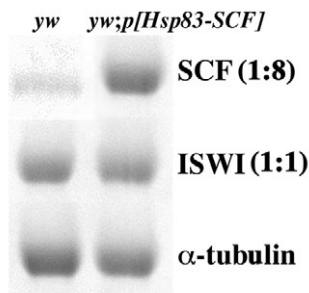


Fig. 6. Immunoblotting analyses. Proteins from the indicated lines were resolved by SDS-PAGE, blotted onto a membrane, and probed with antibodies against SCF, ISWI or α -tubulin (loading control). Numbers in parenthesis indicate the ratio of the band intensity of *yw*; *p[Hsp83-SCF]* to that of *yw*, normalized to the ratio of the α -tubulin control.

acetylated in the coding region than in the promoter region (Smith et al., 2001). This suggests that the dosage-compensation machinery might function in the transcription elongation step. As transcription proceeds, a positively supercoiled domain should be formed in front of the RNA polymerase, and a negatively supercoiled domain should be generated behind it (Liu and Wang, 1987). Transcription-coupled supercoiling has been documented using a yeast mutant defective in topoisomerases (Giaever and Wang, 1988). Recently, Matsumoto and Hirose observed the same effect on *Drosophila* polytene chromosomes, even in the presence of active topoisomerases (Matsumoto and Hirose, 2004). The generation of negative supercoils by the action of SCF may facilitate smooth tracking of the polymerase and the reassembly of nucleosomes after the polymerase has passed. These processes might also be coupled with replacement of histone H3 by its variant H3.3 during transcription, culminating in maintenance of the active state through cell division (Henikoff et al., 2004; Mito et al., 2005).

We observed a bloated appearance of the male X chromosome upon overexpression of SCF (Fig. 5). Nevertheless, overexpression of SCF did not significantly affect the male to female ratio in the adult, nor expression of X-linked genes in males (data not shown). Thus, there seem to be at least two separable steps in the upregulation of X-linked genes: first, the alteration of higher order chromatin structure; and second, fine-tuning of the expression levels of X-linked genes. SCF may be directly involved in the first step, but not in the second step. Whether SCF is required for the formation of an active chromatin structure, or for its maintenance (or both), remains to be clarified.

In summary, we found that SCF affects dosage compensation in *Drosophila*, and showed that SCF plays an important role in the alteration in chromatin structure required to execute hyperactivation of the male X chromosome. Further investigation to clarify the relationships between supercoiling activity and the topological status of the active chromosomal domain may provide new insights into the mechanisms of chromosome-wide gene regulation.

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