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Acknowledgements. We thank K. Maruthainer for sequencing; A. Kuroiwa for chicken clones; L. Ariza-McNaughton for help with *in situ* hybridization; B. Graves for the gift of Ets-1 protein; Z. Webster and A. Hewett for animal husbandry; and all other members of R.K.'s laboratory for advice and encouragement. This work was supported by fellowships from EMBO and HFSP (M.M.) by the MRC and an HFSP network grant (R.K.); by grants from the Hong Kong RGC (C.-T.K. and M.H.S.); and by a fellowship from the ACS (S.C.) and a grant from the NIH (S.B.); G.S.B. is an assistant investigator of the HHMI.

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Sex lethal controls dosage compensation in *Drosophila* by a non-splicing mechanism

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Dosage compensation in *Drosophila* requires the *male-specific lethal* (*msl*) proteins (MSL) to make gene expression from the single male X chromosome equivalent to that from both female X chromosomes[1,2]. Expression of *msl2* is repressed post-transcriptionally by Sex lethal (SXL), a female-specific RNA-binding protein that regulates alternative splicing in the sex-determination hierarchy. Although *msl2* RNA is alternatively spliced in males and females, this does not alter its coding potential and splicing is not required for male-specific expression of MSL2 protein. Instead, our results suggest that the association of SXL protein with multiple sites in the 5′ and 3′ untranslated regions of the *msl2* transcript represses its translation in females. Thus, this well characterized alternative splicing factor regulates at least one target transcript by a distinct mechanism.

Male-specific lethal-2 (*msl2*) encodes a key male-limited regulator of dosage compensation in *Drosophila*. The MSL proteins function as a multisubunit complex bound to hundreds of sites on the male X chromosome, where they are postulated to recruit the MOF (males absent on the first) histone acetyltransferase[3]. Histone acetylation is thought to play a major role in remodelling the chromatin architecture of the male X chromosome, allowing a doubling in transcription[2]. The MSL complex is essential for male development and is normally absent from females. Inappropriate expression of high levels of MSL complex is toxic to females because of hyperexpression of both X chromosomes[4]. This critical binary switch is regulated by the *Sex lethal* (*Sxl*) gene[5,6] and its primary target in this dosage compensation pathway is the *msl2* transcript[4,7,8]. SXL is a female-specific RNA-binding protein that regulates the sex-specific splicing of its own transcript as well as that of *transformer* in the sex determination hierarchy[9,10]. Therefore, it was reasonable to presume that SXL might regulate *msl2* using a similar mechanism.

The *msl2* gene produces two transcripts that differ by an intron of 133 nucleotides in the 5′ untranslated region (UTR) (Fig. 1)[4,7,8]. Most female transcripts retain the intron, whereas most male transcripts remove it (Fig. 1b)[7]. Consensus binding sites for SXL protein (AU_7 or U_{8+})[11] are located adjacent to both 5′ and 3′ splice junctions (Fig. 1c)[4,7,8], and female cells that are mutant for *Sxl* splice the 5′ intron[8] and derepress *msl2* translation[4]. However, unlike the previously described cases of SXL regulation, this sex-specific intron is located 279 nucleotides before the AUG start codon and does not affect the open reading frame. In addition to the size difference, *msl2* RNA is less abundant in females than males[7], but this difference is not sufficient to account for the absence of MSL2 protein in females. Instead, it has been proposed that *msl2* is repressed at the level of translation in females[4,7,8]. We have explored two models for sex-specific regulation of *msl2* expression by *Sxl*. In the first model, some feature of the *msl2* intron, such as RNA secondary structure or a non-productive initiation codon, results in translational inhibition. If so, male viability would require removal of the intron so that MSL2 translation could occur. SXL would play an indirect role by blocking removal of the intron from female transcripts. An alternative model is that translational inhibition results from direct association of SXL protein with multiple poly(U) sites within the 5′ and 3′ UTRs of the mature transcript in females. According to this model, there is no obvious reason to splice out the intron in males because they lack the SXL repressor protein.

To distinguish between these models, we constructed a series of wild-type and mutant *msl2* transgenes (Fig. 1) and introduced them into flies lacking a functional *msl2* gene. We used western blots and the more sensitive fluorescent immunolocalization assay on polytene chromosomes to measure MSL2 levels *in vivo*. All constructs carry an 11-nucleotide *Bgl*II linker inserted 22 nucleotides before the AUG start codon. This eliminates the possibility that any MSL2 protein might arise from translation initiation upstream in the long 5′ UTR and also allows endogenous and transgenic *msl2* RNAs to be distinguished. The wild-type control transgene, M2NG11, is properly regulated such that MSL2 protein is made in males but not females (Fig. 2b, d), and its transcript is properly spliced in each sex (Fig. 3a,b, lanes 3, 4). The second transgene, SM53, carries mutations in both 5′ and 3′ splice junctions of the sex-specific intron (Fig. 1b, c). Reverse-transcribed polymerase chain reaction (RT-PCR) analysis of *msl2* RNA derived from this construct shows that both males and females produce identical transcripts retaining the intron in the 5′ UTR (Fig. 3b, lanes 5, 6). This 'female-like' *msl2* RNA is efficiently translated in males, as shown by (1) rescue of *msl2* males, (2) wild-type pattern of MSL2 protein distribution on the transgenic male X (Fig. 2f), and (3) similar levels of MSL2 protein in SM53 and M2NG11 transgenic males when assayed by western blots (Fig. 2s). These results demonstrate that the intron within the 5′ UTR does not contain sequences that inherently block translation. Besides the intron, the SM53 transcript carries a long 5′ UTR with several short opening reading frames starting with AUG. Such structures can strongly inhibit cap-dependent translation initiation in many organisms[12,13], but are commonly encountered in *Drosophila*[14]. The ability of males to translate the SM53 *msl2* RNA shows that this 5′ structure is insufficient to block translation at the authentic AUG in *Drosophila*. By contrast, the same SM53 transcript produces no protein in females that contain the SXL repressor (Fig. 2h). Thus, SXL is able to regulate SM53 *msl2* translation properly in the absence of alternative splicing. We conclude that SXL does not require the presence of splice junctions to be able to recognize its target.

The next *msl2* transgene tested, SXB1, contains the same splice junction mutations as SM53, with additional changes in the first poly(U) segment postulated to bind SXL protein, located just inside the male intron (Fig. 1b, c). This mutant transgene functions properly in males. Western analysis failed to detect any MSL2 protein in females, but examination of polytene chromosomes

revealed that translation is weakly derepressed (Fig. 2j). Mutations in the second poly(U) segment (SXB2) also allow modest levels of translation in females (Fig. 2l). This demonstrates that complete repression in females requires a full set of SXL binding sites. Transgenes mutant for both poly(U) segments in the 5′ UTR (SXB1-2) show a greater derepression in females (Fig. 2n), which is high enough to be detected by western blots (Fig. 2s).

The *msl2* transcript contains four poly(U) stretches in the 3′ UTR in addition to the two sites found within the male-specific intron in the 5′ UTR (Fig. 1). Removal of the 3′ UTR was previously shown to cause weak derepression in females[8]. We replaced the 3′ UTR with the polyadenylation region of simian virus 40 (SV40) in pMM2SV, and also observed weak derepression in females (Fig. 2p). When all of the poly ($U_{>7}$) stretches are mutated or deleted in the NOPU transgene, high MSL2 levels are observed in females, indicating similar translational efficiency in males and females (Fig. 2r). Females carrying one copy of NOPU contain about 30% of the MSL2 protein found in their transgenic brothers (Fig. 2s). This amount of MSL2 is insufficient to kill females, possibly because they have twice as much X chromatin to act upon and also because MSL1 is downregulated in females[15,16]. We therefore constructed females carrying two copies of the NOPU transgene and one copy of a transgene overexpressing MSL1 (K. Chang and M.I.K., unpublished). Such females have delayed development because of inappropriate dosage compensation (data not shown).

We next examined whether the reduced levels of *msl2* RNA in females are a consequence of SXL action. Quantitative nuclease protection assays revealed that wild-type females contain only about 20% as much *msl2* RNA as males (Fig. 3c,d). However, female *msl2* transcripts lacking SXL-binding sites made by the NOPU transgene accumulate to about 70% of male levels when normalized to *rp49* transcripts (Fig. 3d). This suggests that translated *msl2* RNA is more stable than transcripts repressed by SXL, although we have not excluded the possibility that mutating the poly(U) clusters somehow stimulates transcription preferentially in females.

To investigate whether SXL repression of *msl2* could be direct, we tested SXL protein for binding to *msl2* transcripts *in vitro*. Figure 4a shows that two shifted bands are evident when SXL protein is incubated with a 246-nucleotide region of the *msl2* 5′ UTR containing both poly(U) stretches. Similarly, two shifted bands are also observed with a poly(U)-containing region of the *msl2* 3′ UTR. Overall binding is stronger to the 5′ UTR than the 3′ UTR, on the basis of competition experiments with cold *Sxl* RNA containing a U_9AU_8 sequence (data not shown). Figure 4b compares the SXL binding ability of a 340-nucleotide region of the *msl2* 5′ UTR (M2NG11) with the mutations used in the *in vivo* analysis. The unaltered RNA shows two shifted bands, and mutations in the splice junctions of the male intron (SM53) do not affect SXL binding. However, consistent with the derepression seen *in vivo*, mutating individual poly(U) segments reduces SXL binding *in vitro*. Altering the U_{11} run (SXB1) causes a modest reduction, altering the U_{16} run (SXB2) causes a significant reduction, and transcripts with mutations in both poly(U) runs (SXB1-2) fail to bind SXL at the concentrations tested.

Translational control is emerging as a widely used regulatory mechanism in higher organisms. The expression of several *Drosophila* and *Caenorhabditis. elegans* proteins required for embryonic pattern formation, sex determination, and germ-cell

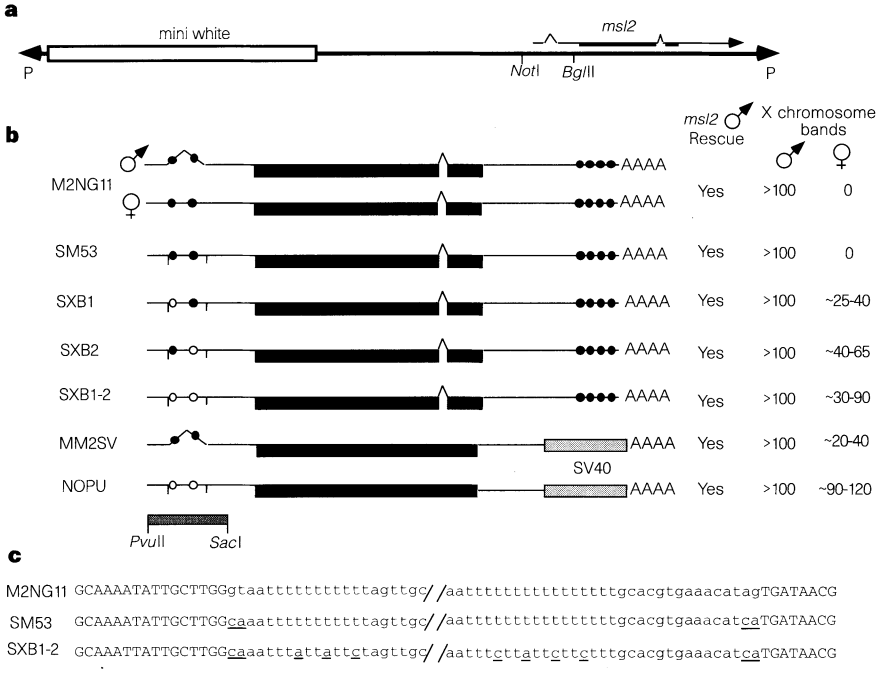

Figure 1 Summary of *in vivo* analysis of *msl2* regulation. **a**, All transgenes carried a complete *msl2* gene (6 kb) cloned into pCasPeR (ref. 21). *Cla*I and *Xma*I sites at the 5′ end of *msl2* were converted with oligomer adapters to *Not*I and *Bgl*II sites, respectively, to provide unique cloning sites. **b**, M2NG11 caries the wild-type *msl2* gene. Females retain the 5′ intron, whereas males splice it out of most transcripts. The coding region is shown as a thick bar and the 5′ and 3′ UTRs as thin lines. Consensus SXL binding sites are black circles[11]. Mutated sites are white circles. SM53, SXB1, SXB2, SXB1-2 and NOPU all carry mutations in both splice junctions of the male-specific intron shown as short vertical bars. SXB1 also has three mutations in the first poly(U) stretch; SXB2 has four nucleotide changes in the second poly(U) stretch; SXB1-2 and NOPU have both 5′ poly(U) stretches mutated. MM2SV has a wild-type 5′ UTR, but the poly(U) clusters in the 3′ UTR have been replaced by a segment of the SV40 genome containing a polyadenylation signal. NOPU carries the 5′ end of SXB1-2 and the 3′ end of MM2SV such that it lacks all poly(U) clusters. Each transgene was assayed for complementation of *msl2* mutant male lethality. In each case, the desired class of males was recovered in numbers equal to their *msl2* sisters, demonstrating complete rescue with several independent lines. The presence of multiple MSL2 bands on the female X chromosomes indicates a failure to repress *msl2* translation (Fig. 2). The shaded box at the bottom is the 340 bp *Pvu*II–*Sac*I fragment used to produce *in vitro msl2* transcripts for SXL gel shift experiments (Fig. 4). **c**, The sequences of the relevant portions of the *msl2* 5′ UTR are shown with exon sequence in upper case and male intron sequence in lower case. The mutated nucleotides are underlined.

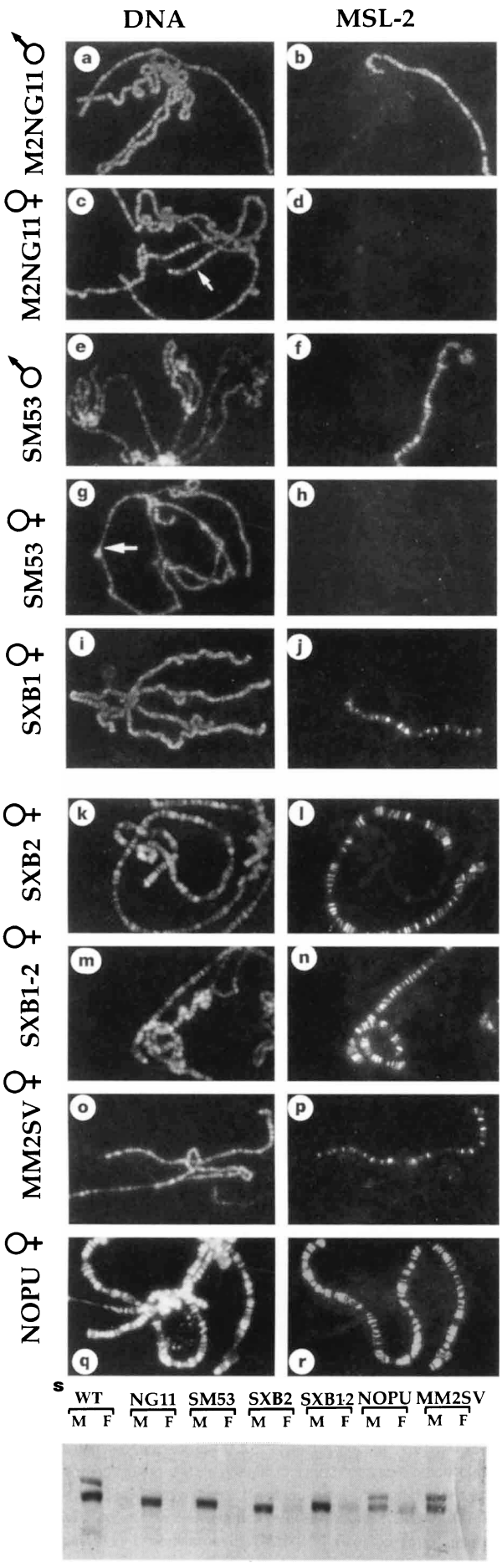

Figure 2 MSL2 expression in transgenics. Salivary gland chromosomes were stained for DNA (left) and MSL2 (right). All larvae were *w*; *msl2 cn*, so the MSL2 protein detected must be derived from the single copy of the [w^+ $msl2^+$] transgene on either the X or 3rd chromosome. **a**, **b**, M2NG11 wild-type control male; **c**, **d**, M2NG11 wild-type control female; **e**, **f**, SM53 splice-mutant male; **g**, **h**, SM53 splice-mutant female; **i**, **j**, SXB1 poly(U) mutant female; **k**, **l**, SXB2 poly(U) mutant female; **m**, **n**, SXB1-2 poly(U) double-mutant female; **o**, **p**, MM2SV 3′ UTR replacement female (carried two copies of transgene); **q**, **r**, NOPU females. The arrows in **c** and **g** indicate the female X chromosomes that fail to stain with MSL2 antibodies. **s**, Western blot to MSL2 protein in males (M) and females (F) of the same transgenic lines. The upper protein band seen in some lanes appears sporadically on western blots and is of unknown significance.

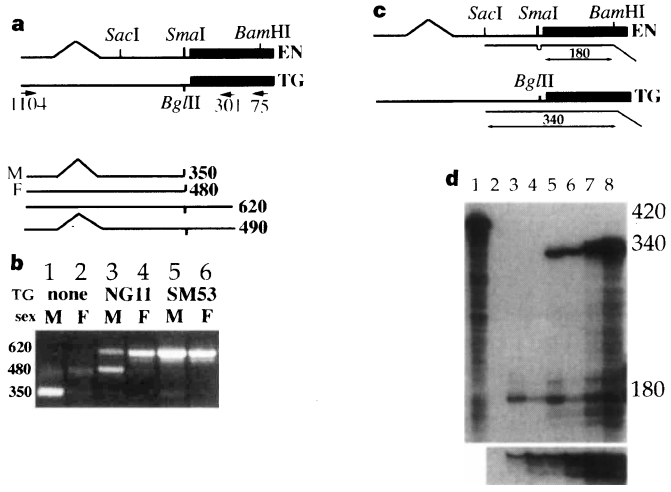

Figure 3 Analysis of transgenic *msl2* RNA. **a**, The 5′ structures of endogenous (EN) and transgenic (TG) *msl2* RNA are shown along with the strategy for RT-PCR amplification. Thin lines are 5′ UTR and thick lines are coding sequences. A *Sma*I site preceding the *msl2* start codon has been replaced with a *Bgl*II adapter in transgenes. Oligo number 75 was used for reverse transcription and PCR was carried out with oligos 1104 and 301. Following *Sma*I digestion, the endogenous male and female PCR products are 350 and 480 bp long, respectively. The transgenic products are not cut by *Sma*I, so spliced and unspliced molecules are 490 and 620 bp long, respectively. **b**, The endogenous *msl2* RNA from non-transgenic wild-type males (lane 1) and females (lane 2) produce predominantly spliced (350 bp) or unspliced (480 bp) PCR products, respectively. Animals carrying the control M2NG11 transgene with wild-type splice junctions show male-specific splicing of the transgenic transcript. The spliced M2NG11 male transgene product is 490 bp (lane 3) and the unspliced female transgene product is 620 bp (lane 4). Animals carrying the SM53 *msl2* splice mutant transgene produce the same unspliced product (620 bp) both in males (lane 5) and in females (lane 6). The 350-bp band seen in lane 5 is the product of the endogenous spliced male transcript. The endogenous transcript in the *msl2* host fly stock (lanes 3–6) is reverse-transcribed less efficiently than the transgenic product because primer 75 spans the proline 51 codon mutated in the $msl2^1$ allele[7]. **c**, Nuclease protection assay of *msl2* RNA levels in wild-type and transgenic animals. The labelled riboprobe is shown below the transcript. The first 80 nt of the riboprobe (bent line) are derived from the pBlueScript vector and do not form base pairs with *msl2* RNA. The remainder is fully collinear with the transgenic sequence, but the *Bgl*II sequence does not form base pairs with endogenous *msl2* transcript. The endogenous *msl2* transcript (EN) protects a 180-nucleotide (nt) segment of the probe from nuclease digestion and a 340-nt fragment is protected by the transgenic RNA (TG). **d**, Results of the nuclease protection assay. Lane 1, starting probe; lane 2, nuclease digestion with no fly RNA; lane 3, with 1 μg wild-type male poly(A)$^+$ RNA; lane 4, with 1 μg wild-type female poly(A)$^+$ RNA; lane 5, with 1 μg NOPU male poly(A)$^+$ RNA; lane 6, with 1 μg NOPU female poly(A)$^+$ RNA; lane 7, with 3 μg NOPU female poly(A)$^+$ RNA; lane 8, with 10 μg NOPU female poly(A)$^+$ RNA. The lower panel shows the same RNA samples protecting a *rp49* probe, except that only 5% as much fly RNA was used in each assay and the exposure time was 7% as long as the upper panel. Endogenous female *msl2* RNA is about 500-fold less abundant than *rp49*.

formation are controlled at the level of translation by repressors binding to specific sequences in the 3′ UTRs of their target transcripts[17]. The iron-dependent translational repressor binds to a stem–loop structure in the 5′ UTR of its target transcripts[18]. We have shown that complete repression of *msl2* requires that SXL bind both the 5′ and 3′ UTRs. This may be important in light of a recent report that the 5′ and 3′ ends of transcripts are in close contact as messenger RNAs are loaded onto ribosomes[19]. The exact mechanism by which SXL represses translation of *msl2* RNA is not clear, as we have been unsuccessful in visualizing the spatial distribution of the low-abundance *msl2* RNA to see whether females sequester the transcript in the nucleus. Our results clearly demonstrate that the 5′ UTR strongly inhibits translation, but only in the presence of SXL repressor. Although this provides a compelling reason for females to retain the intron, it is puzzling why males, who lack SXL protein, splice this sequence from their *msl2* transcripts. Perhaps this sequence provides a subtle regulatory function not detected by our assays.

The finding that partial repression of *msl2* transcripts occurs if only a subset of poly(U) clusters is present lends support to our proposal that SXL mediates a second dosage compensation pathway in females[4,20]. We proposed that SXL directly binds poly(U) clusters found in the 3′ UTRs of a subset of X-linked transcripts such as *runt* to halve their translation. Partial repression by SXL is also consistent with the suggestion that MSL1 expression may be modulated through poly(U) clusters in the 3′ UTR[15,16]. Our results demonstrate that SXL has a function distinct from mRNA splicing regulation, and suggest that SXL can repress translation of transcripts to varying degrees, based on the number and location of binding sites in the target RNA. □

Methods

MSL2 measurements in transgenic animals. Mutations were constructed by PCR with mutant primers[23], sequenced and substituted as *Not*I–*Bgl*II fragments into M2NG11. Transgenic animals were constructed[24] using the w^+ marker in the pCasPeR vector[21]. Insertions were mapped to chromosomes, crossed into a *w*; *msl2*[1] *cn* background, assayed for rescue of mutant males, and examined by genomic Southern blots to determine which lines carried only a single copy of the transgene. Polytene chromosomes were prepared[25] from transgenic *msl2* larvae carrying a single copy of the transgene and stained with Hoechst 33258 and affinity-purified rabbit polyclonal antibodies to MSL2[4]. Four independent insertion sites were stained for each construct. Western blots were carried out as described[16] using protein from one adult per lane. MSL2 was visualized with an affinity-purified polyclonal rabbit anti-MSL2 and an alkaline phosphatase-coupled secondary antibody.

RNA analysis. RT-PCR analysis was as described[4] using Oligo number 75 (5′-AGTAGGGATCCACCAGC) and SuperScript II (Difco BRL) at 42 °C to reverse-transcribe 3 μg DNase I-treated poly(A)$^+$ mRNA. Oligo number 75 pairs perfectly with the wild-type *msl2* sequence, but has a 1-bp mismatch with the mutant *msl2*[1] sequence present in all transgenic lines, such that the endogenous PCR product is under-represented. PCR amplification was carried out using primers 1104 (5′-GGTCACACCTATGCCGCACTGCAGCTAG) and 301 (5′-GCCGTAGCTCGCCGAGCCCGGAGTTCAG) for 30 cycles annealing at 65 °C. The samples were cut with *Sma*I and size-fractionated on 1.0% agarose. Nuclease protection assays were done with the HybSpeed RPA kit (Ambion) according to the supplier's instructions. The *msl2* antisense riboprobe was made from a template carrying the SXB1-2 sequence, extending from the *Bam*HI to *Sac*I sites, +147 to − 188 relative to the start codon using T7 RNA polymerase.

SXL gel shift assay. 5′ UTR RNA was transcribed by T7 RNA polymerase from an *Ssp*I to *Sac*I fragment subcloned into pBluescript, linearized with *Sac*I and made blunt-ended by T4 DNA polymerase. 3′ UTR RNA was transcribed by T3 RNA polymerase from an *Eco*RV to 3′ end subclone, linearized at the vector *Bam*HI site. Plasmids containing wild-type or mutant 5′ UTRs were linearized with *Sac*I, blunt-ended with T4 DNA polymerase, and transcribed by T7 RNA polymerase. Production of GST-SXL and RNA gel shifts have been as described previously[22], except that electrophoresis was at 200 volts for 4 h at room temperature.

Received 17 February; accepted 11 March 1997.

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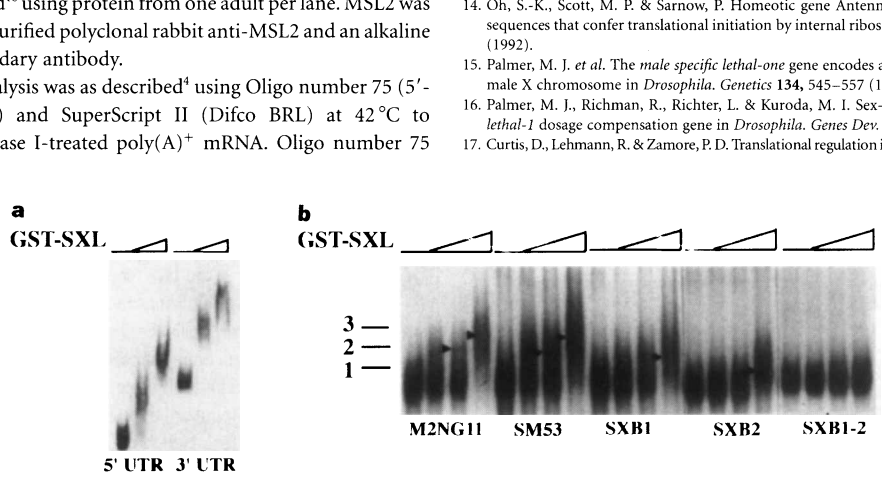

Figure 4 SXL binding to *msl2* RNA is reduced by mutations in the poly(U) stretches. **a**, Band-shift assays testing GST-SXL binding to a 246-nt region of the wild-type 5′ UTR and a ~600-nt region of the wild-type 3′ UTR. The 5′ UTR RNA contains three potential binding sites, as the U16 stretch should comprise a double site to which two SXL molecules could bind cooperatively[22]. The 3′ UTR RNA contains at least four separate binding sites. The first lane in each set contains RNA without SXL protein. **b**, Band-shift assays on the wild-type and mutant RNA substrates shown in Fig. 1. For each 340-nt RNA substrate, the first lane lacks GST-SXL protein; in lanes 2 to 4, the protein concentration is increased in 1.5-fold increments. Numbers indicate three RNA–protein complexes of different sizes, which hypothetically contain 1, 2 or 3 molecules of SXL protein on a single RNA. M2NG11 and SM53 form complexes 2 and 3; SXB1, with an altered single site, forms complex 2; SXB2, with an altered double site, forms only complex 1; and SXB1-2, with no poly(U) stretches, forms no complex.

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Acknowledgements. We thank M. J. Palmer, K. Chang and K. Copps for critical reading of the manuscript and K. Chang for the *msl1*$^{+}$ transgenic fly stock. This work was supported by grants from the NIH and NSF. M.I.K. is an Associate investigator of the Howard Hughes Medical Institute.

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Homotypic vacuolar fusion mediated by t- and v-SNAREs

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Membrane fusion is necessary both in the eukaryotic secretory pathway and for the inheritance of organelles during the cell cycle. In the secretory pathway, heterotypic fusion takes place between small transport vesicles and organelles. It requires *N*-ethylmaleimide-sensitive fusion protein (NSF/Sec18p), soluble NSF attachment proteins (SNAPs/Sec17p) and SNAP receptors (SNAREs). SNAREs are integral membrane proteins (v-SNAREs on vesicles, t-SNAREs on the target organelles) and are thought to provide specificity to the fusion process[1–5]. It has been suggested that Sec17p and Sec18p bind to v-SNARE/t-SNARE complexes and mediate the membrane fusion event[1–3]. Homotypic fusion of yeast vacuoles also requires Sec17p and Sec18p (ref. 6), but *in vitro* they are needed only to 'prime' the vacuoles, not for subsequent docking or fusion[7,8]. It has been unclear whether these reactions involve SNAREs that are similar to those previously identified in heterotypic fusion systems and, hence, whether the actions of Sec18p/NSF and Sec17p/αSNAP in these systems can be compared. Here we identify typical v- and t-SNAREs on the yeast vacuolar membrane. Although both are normally present, vacuoles containing only the v-SNARE can fuse with those containing only the t-SNARE. Vacuoles containing neither SNARE cannot fuse with those containing both, demonstrating that docking is mediated by cognate SNAREs on the two organelle membranes. Even when t- and v-SNAREs are on separate membranes, Sec17p and Sec18p act at the priming stage. Their action is not required at the point of assembly of the SNARE complex, nor for the fusion event itself.

We surveyed the yeast genome database for proteins related to the syntaxin family of t-SNAREs and the VAMP/synaptobrevin family of v-SNAREs[9,10], and the candidates included the open reading frames (ORFs) YOR106w (*VAM3*) and YLR093c (*NYV1*). Both have typical SNARE features, including a carboxy-terminal membrane anchor preceded by a region that has the potential to form an α-helical coiled-coil. The coiled-coil region of YOR106w shows between 40 and 18% identity to the yeast t-SNAREs Pep12p (ref. 11), Sed5p (ref. 4), Sso1p (ref. 9) and Ufe1p (ref. 12), whereas the corresponding region of YLR093c is 41% identical to Snc1p, a v-SNARE involved in transport to the plasma membrane[10] (Fig. 1a). Immunofluorescence microscopy of the functional, epitope-tagged proteins expressed in yeast showed that each colocalizes with a subunit of the vacuolar ATPase and hence is located on the vacuolar membranes (Fig. 1b). Furthermore, co-immunoprecipitation of the two proteins could be observed, which suggests that they are capable of interacting with each other (data not shown).

Deletion of YOR106w was not lethal, but resulted in abnormal vacuoles. In the mutant, endocytosis of the membrane dye FM4-64 (ref. 13) led to labelling of numerous small organelles (Fig. 2a), as did staining with antibodies specific for the vacuolar ATPase (not shown). The mutant cells did not secrete significant amounts of the vacuolar protein carboxypeptidase Y (CPY)[14] but they contained unusually high levels of CPY precursors (Fig. 2b). This phenotype is very similar to that of *ypt7* (ref. 15), a Rab-like GTPase required for vacuolar fusion[16], and of the *vam* mutants[17]. While this work was in progress, a GenBank entry identified YOR106w as *VAM3* (Y. Wada, Y. Ohsumi and A. Hirata, accession number U57827). Purification of the organelles[18] from the *vam3* deletion strain demonstrated that they contain roughly normal amounts of mature CPY and the other

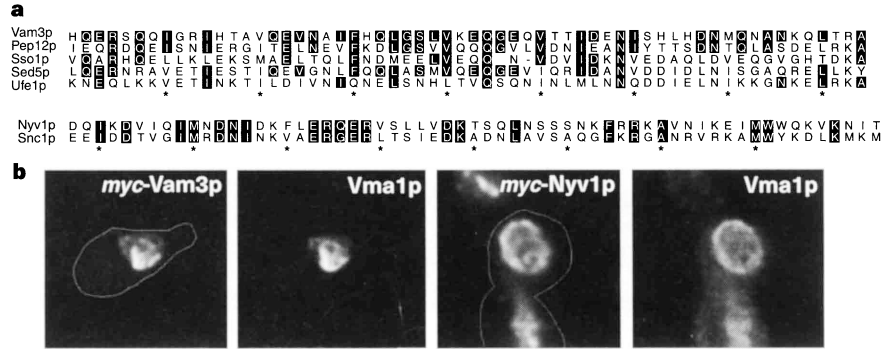

Figure 1 *VAM3* and *NYV1* encode SNARE-like proteins located in the vacuole. **a**, Sequences of potential coiled-coil domains from Vam3p and Nyv1p. Residues 193–253 of Vam3p are aligned with other t-SNAREs: residues 198–258 of Pep12p, 252–312 of Sed5p, 192–252 of Sso1p and 258–318 of Ufe1p. Residues 171–231 of Nyv1p are aligned with residues 34–94 of the v-SNARE Snc1p. Heptad repeats of hydrophobic amino acids are indicated (*). Residues showing identity with Vam3p or Nyv1p are highlighted. **b**, Left- and right-hand images correspond to double labelling of cells containing Myc-tagged Vam3p or Nyv1p, with antibodies against the Myc-epitope and against the vacuolar membrane protein Vma1p, visualized by confocal microscopy. Only the vacuoles are visible at this exposure, but the outlines of the cells have been traced in the left-hand panels.