

Supplementary Online Material

Suppressor analysis – the suppressor rescues poly(A) switching

SXL has an early function (formation of egg chamber) and a late function (progression of the meiotic cycle) during oogenesis. The female-sterile Sxl^{f4} mutation disrupts oogenesis at an early step, leading to proliferating, undifferentiated cells. A dominant suppressor of female-sterile Sxl^{f4} mutation $Su(Sxl^{f4})46$, which allows egg chamber formation and restores oogenesis/fertility but not meiotic recombination, was identified previously (Bopp et al., 1999). Therefore, as an additional control, we analyzed the suppressor by Northern analysis. The suppressor rescued the female-specific poly(A) switching of the $e(r)$ transcript (Fig. S1, lane 3 versus lane 2). Although a molecular mechanism for suppression is not fully understood, based on what we know about the suppressor (see details below), these results are consistent with our model that SXL regulates $e(r)$ in the female germline.

The *bag-of-marbles* (*bam*) gene is a potential suspect for the suppressor. This suspected link also correlates well with the chromosomal location of the suppressor and *bam*; both map to 3-[85]. Furthermore, the suppression activity is *bam* dose dependent; for example, removal of one dose of *bam* substantially reduced $Su(Sxl^{f4})46$ -mediated suppression in Sxl^{f4} females, whereas removal of one copy of *orb* had no effect; the suppressor does not alter the coding region of *bam*, but *cis*-regulatory mutations have not been ruled out (D. Bopp, personal communication). Previously, Bopp *et al.* showed that *bam* ovaries are mildly affected for the female-specific splicing of the *Sxl* transcript, and are severely affected for subcellular localization of the SXL protein, *i.e.*, high cytoplasmic SXL due to lack of subcellular redistribution (Bopp et al., 1993). Both the

Sxl^{f4}/Sxl^{f4} mutant and the *bam* mutant show mislocalization of the SXL protein. Taken together, these results suggest that *bam* acts directly or indirectly by controlling the synthesis and/or subcellular localization of the SXL protein in the female germline. Furthermore, in Sxl^{f4} ovaries, where SXL remains cytoplasmic (no nuclear transition is observed), the domain of BAM-C expression is greatly expanded. In suppressed ovaries, a nuclear redistribution of SXL is seen and the domain of BAM-C expression is confined to the first 2-4 cell stages of cystocyte proliferation. Thus, *Su(Sxl-fs)46* “**must**” act upstream of the signal that directs the redistribution of SXL and the confinement of BAM-C expression (D. Bopp, personal communication). As a corollary, it is not surprising that the suppressor therefore restores the SXL-dependent poly(A) switching of *e(r)*. Our findings provide further insight into the basis for suppression, and *e(r)* offers a valuable molecular handle to define mysterious SXL-dependent events in the female germline.

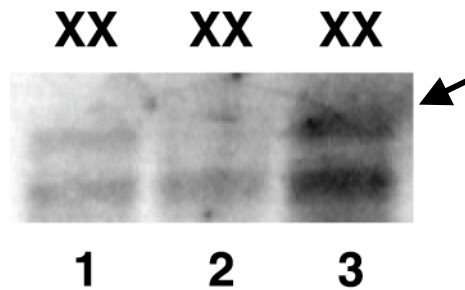


Fig. S1. Suppressor of the female-sterile Sxl^{f4} mutation restores *er-fs* expression. Lane 1: $Sxl^{f4}/+$, Lane 2: Sxl^{f4}/Sxl^{f4} , Lane 3: $Sxl^{f4}/Sxl^{f4}; Su(Sxl^{fs})46^{46}/+$.

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Supplementary Online Material

RNAi - loss of *e(r)* is embryonic lethal

The *e(r)* gene is present in many organisms, including the fruitfly, *Xenopus*, mouse, human, and zebrafish (Gelsthorpe et al., 1997), and is highly conserved (76-100% amino acid identity), suggesting that it performs an important function. However, a biological function(s) of *e(r)* or its sex-specific transcripts remains poorly defined. The function of *e(r)* may be related to transcriptional regulation because the *Xenopus e(r)* homolog (XERH) has been shown to repress the transcription of reporter constructs in certain cultured cells (Pogge von Strandmann et al., 2001). The *Drosophila e(r)* is possibly regulated in a cell-cycle dependent manner (Gelsthorpe et al., 1997), and genetically interacts with the *rudimentary (r)* gene; mutations in the *r* gene result in a rudimentary wing phenotype and female sterility (Wojcik et al., 1994). A known *P*-element insertion in *e(r)* has no phenotype by itself most likely because it is not a null (Wojcik et al., 1994).

We tested whether *e(r)* plays an important role in *Drosophila* by using the double-stranded RNA-interference (RNAi) approach, which is widely used for the degradation of cognate transcripts (Kennerdell and Carthew, 1998). We injected either buffer or double-stranded RNAs corresponding to the *e(r)*, *Sxl*, or *msl2* transcripts into *Drosophila* embryos at the syncytial blastoderm stage, and analyzed their effect on development. RNAi for *e(r)* using the entire 3' UTR (Proximal (P) + Distal (D)), the coding sequence plus 296 nucleotides of 3'UTR (*e(r)*-CDS + 3'UTR), or the proximal portion of 3'UTR (P) all resulted in embryonic lethality of both sexes (Table 1); all three should degrade both *e(r)-fs* and *e(r)-nss* transcripts. Developing embryos progressed to embryonic stage 14 based on gut morphology, and showed proper segmentation (data not shown). The

short female-specific 3'UTR portion of *e(r)* (D) was inactive for RNAi in embryos (data not shown). Mock RNAi (injection of buffer) had no effect on development. As controls for specificity, RNAi for *Sxl* and *msl2*, which are essential only in females and males, respectively (Schutt and Nothiger, 2000), produced expected sex-specific lethality (Table 1). Furthermore, our observation on *e(r)* is consistent with results from genomic-wide inactivation of nematode genes with RNAi, which shows that loss of the *e(r)* transcript results in embryonic lethality in *C. elegans*, although it was not further characterized (Gonczy et al., 2000). Our results suggest that *e(r)* performs a vital function for both sexes in *Drosophila* embryos.

RNAi protocol

Double-stranded RNA was transcribed with T7 RNA polymerase, and a 1-3 μ M dsRNA solution was microinjected into embryos (Kennerdell and Carthew, 1998). The number of male and female flies surviving to adulthood was scored for each injection.

Table 1. RNAi for *e(r)* is embryonic lethal. The data is an average of three independent experiments. * Approximately 300 embryos in triplicates were injected for each set.

Table 1

dsRNA injected	Surviving Adults		
	Male (%)	Female (%)	Total*
Buffer	52 \pm 2	48 \pm 2	222
<i>e(r)</i> 3'UTR (<i>nss+ fs</i>)	0	0	0
<i>e(r)-nss</i> 3'UTR	0	0	0
<i>e(r)-CDS + 3'UTR</i>	0	0	0
<i>Sxl</i>	99 \pm 1	1 \pm 1	94
<i>msl2</i>	10 \pm 6	90 \pm 6	100

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Supplementary Online Material

Plasmids, DNA templates, and primers

EGFP reporter constructs

The 6.2 kb *Sal* I *e(r)* genomic DNA fragment (Wojcik et al., 1994) was cloned in the *Xho* I site of the pCaSpeR4 vector. The 2.1 kb *Xba* I fragment at the 3' end of *e(r)* was excised (pCaSpeR4-5'*e(r)*) and replaced by an EGFP reporter containing various portions of the 3' UTR of *e(r)* described below. In addition, the excised 3' 2.1 kb *Xba* I fragment of *e(r)* was cloned into pBlueScriptSK (+) vector (BS-3'*e(r)*), and was used for oligonucleotide directed mutagenesis to generate mutant1 (primers U and V), mutant2 (primers A and AH), and D₁ (primers W and X). D₁₂₃ was generated by cloning the PCR fragment (primers R and Y) into the *Bam* HI site of D₁. The PCR fragments containing wt, mutant 1, and mutant2 (primers S and Q), and D and D₁ (primers R and Q) sequences from relevant templates were cloned into the *Bgl* II site of pEGFP-c2. The PCR fragments for the P portion of DP (primers S and T) and the D' portion of DD', D₁D' and D₁₂₃D' (primers R and Q) were cloned into the *Bam* HI site of the relevant pEGFP-c2 constructs. For wt, mutant 1, and mutant2, the *Eco* 47 III/*Sma* I fragment and for DP, DD', D₁D' and D₁₂₃D' the *Eco* 47 III/*Xba* I (Klenow filled-in) DNA fragments from the relevant pEGFP-c2 constructs were cloned into the *Xba* I (Klenow filled-in) site of the pCaSpeR4- 5'*e(r)* plasmid.

Transcription templates

Templates for transcription were generated by PCR amplification as follows: for RNA-protein cross-linking substrates wt and mutant2 (primers E and F) using BS-3'*e(r)*

template; for RNA affinity selection substrates w (primers AF and B) and c (primers AF and AG) using BS-3'*e(r)* template; and for RNAi substrates *e(r)* CDS+3'UTR (primers J and K), *e(r)* 3'UTR (primers G and H), *e(r)-nss* 3'UTR (primers G and I), *e(r)-fs* 3'UTR (primers E and H) using BS-3'*e(r)* template, *Sxl* (primers L and M) using *Sxl* cDNA (Valcarcel et al., 1993) and *msl2* (primers N and O) using *msl2* cDNA (Kelley et al., 1995).

EGFP translation reporter constructs

The 3' splice site from adenovirus major late (MINX) DNA was PCR amplified using primers Z and AA and cloned into the *Eco* RI/*Bam* HI sites of pEGFP-N1 vector (pMINX-EGFP-N1). The 3'UTR DNA from the *Adh* and *K10* genes was removed from vectors pRmHA3 (Gebauer et al., 1998) and UASp (Rorth, 1998) with *Xba* I (Klenow filled-in) and *Xba* I/ *Pst* I (T4 DNA polymerase blunt-end) respectively, and cloned into the pMINX-EGFP-N1 *Xba* I/ *Not* I sites (Klenow filled-in). For the P (primers AB and AC), D (primers AD and AE) and P+D (primers AB and AE) constructs, the 3'UTR of *e(r)* was PCR amplified and cloned into the *Not* I site of pEGFP-N1. The *Xba* I (Klenow filled-in) DNA fragments from all of the MINX-EGFP-N1 3' UTR plasmids were cloned into the *Xba* I site (Klenow filled-in) of the pCaSpeR4- 5' *e(r)* plasmid.

Primers

A, 5' TTCGTGCGGCAGCCACAGGTGA 3'
B, 5' AAGTTCAAATGATAAAGGGGGAG 3'
C, 5' GGAATTCATGGCAGATAAGGCGCAGG 3'
D, 5' GGAATTCGGTCCCTGAAGCAGCTGCT 3'
E, 5' GTAATACGACTCACTATAGGGATCCAAGTGTGTGTGTT 3'
F, 5' TCCTGCACACTTGCAAATTTGCCATT 3'
G, 5' GTAATACGACTCACTATAGGGACGGCGATGCGACAAG 3'
H, 5' GTAATACGACTCACTATAGGGCTCAGTGTGTCCTGATGTTAG T 3'

I, 5' GTAATACGACTCACTATAGGGTGTATATACAGTATATATATATA 3'
 J, 5' GTAATACGACTCACTATAGGGACCATGTTCGCACACCATCCTA 3'
 K, 5' GTAATACGACTCACTATAGGGCGATCTAGTCGGTAGCAG 3'
 L, 5' GTAATACGACTCACTATAGGGCGTGGATTTGGGATGT 3'
 M, 5' GTAATACGACTCACTATAGGGTCCAATGGCTCTGAATA 3'
 N, 5' GTAATACGACTCACTATAGGGCCCAGACGGCATACTTGA 3'
 O, 5' GTAATACGACTCACTATAGGGCATGTCCTTGAGGAACG 3'
 P, 5' GAAGATCTAGGGACGGCGATGCGACA 3'
 Q, 5' GAAGATCTGCTTAAAACAAGCCGTTAAAACG 3'
 R, 5' GAAGATCTG TGC GGC AGC CAC AGG TGA 3'
 S, 5' GAAGATCTTGCAACATAACGCAATGCAAAA 3'
 T, 5' GAAGATCTCACCTGTGGCTGCCGCAC 3'
 U, 5' GAATTCACACAACACCGGCAGCCACAGGTGACGG 3'
 V, 5' GTGTGGTGTGTGTCTAAAACAGTTGTCAGTTGGGTGTATATAC 3'
 W, 5' TGTTTCAATGTTTTTTTTGTGGATCCAACTACTGATTCCCCCCCCTC 3'
 X, 5' CACAAAACACACACAACAATATGTTTATATATGCTCAGTG 3'
 Y, 5' GAAGATCTGCCGTGTGTGCTAGCTTAAGT 3'
 Z, 5' GGAATTCCTCTAGAGCTGCACGTCTAGGGCGC 3'
 AA 5' CCTCGTGGTAGCTTGGGCTG 3'
 AB 5' ATAAGAATGCGGCCGCGGGACGGCGATGCGACAAG 3'
 AC 5' ATAAGAATGCGGCCGCTCACCTGTGGCTGCCGCAC 3'
 AD 5' ATAAGAATGCGGCCGCGACAACACTGTGTGTGTTTTTGTGTG 3'
 AE 5' ATAAGAATGCGGCCGCGCTTAAAACAAGCCGTTAAAACG 3'
 AF 5' GTAATACGACTCACTATAGGGATAAACTGTATAACTTAGAAATGG 3'
 AG 5' GAAGATCTGTTGTCAGTTGGGTGTATATACA 3'
 AH 5' TTCAACACACACAGTTGTCAGTTGG 3'

RNA-protein UV cross-linking assay and immunoprecipitation

The RNA-protein UV cross-linking assay was performed as previously described (Singh et al., 1995; Valcarcel et al., 1993). For immunoprecipitation (IP), anti-hsCstF64 antibodies were crosslinked to protein A beads. For mock IP, pre-immune serum was used. The crosslinked RNA-protein samples were added to a 50% slurry of antibody/beads in IP buffer (50mM Tris pH 7.4, 50mM NaCl, 0.5% NP40, 10µg/ml leupeptin, 4 µg/ml pepstatin, 1 mM PMSF), mixed at room temperature for 1.5 hours, and washed four times in IP buffer. Beads were resuspended in 1X Laemmli buffer, boiled

for 5 minutes, and pelleted. The supernatant was separated on an SDS-PAGE gel. The gel was dried, exposed to a phosphorimager plate, and scanned.

RNA affinity selection and Western analysis

RNA was synthesized from relevant templates using T7 RNA polymerase, immobilized on adipic acid dihydrazine agarose beads as described (Caputi et al., 1999), and incubated with nuclear extract (Dignam et al., 1983) prepared from untransfected S2 cells, from S2 cells transfected with HA-CstF-64, or from HeLa cells in 50mM Tris pH 7.4, 5 mM EDTA, 5 mM DTT, 250mM NaCl, 0.45 $\mu\text{g}/\mu\text{l}$ BSA for 40 minutes at room temperature. Beads were washed four times with 20mM Hepes pH 8.0, 20% glycerol, 100mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 4 mM MgCl_2 , and boiled for 5 minutes in 1X Laemmli buffer. The supernatant was separated on an SDS-PAGE gel, transferred to a PVDF membrane, and probed with anti-HA (12CA5 antibody 1:5000), anti-hsCstF64 (1:100 rabbit serum), or anti-hsCstF77 (1:1000 antibody). An appropriate secondary antibody coupled to HRP was used, and visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunostaining

Immunostaining was carried out on isolated ovaries, as described (Mohr et al., 2001). Polyclonal rabbit anti-GFP antisera (CloneTech 8363-2) was used as a primary antibody at a dilution of 1:1000 and Alexa 594 (Molecular Probes A-11037) goat anti-rabbit antisera was used as a secondary antibody at a dilution of 1:500.

References

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