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Investigation into the Role of Zn72D and Belle in the Regulation of Drosophila Dosage Compensation

by

Kathleen A. Worringer

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by
Kathleen A. Worringer

This thesis is dedicated to my parents, whose love and support always encouraged the work described in these pages.

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The text of Chapter I of this thesis is reprinted with permission as it appears in *Molecular and Cellular Biology* (2007) Dec 27(24): 8760-8769, Epub: Oct 8 2007 (with some additional figures). The coauthor listed in this publication directed and supervised the research that forms the basis for the thesis.

The mass spectrometry described in Chapter II and in the Appendix was performed by Feixia Chu.

Abstract

Investigation into the Role of Zn72D and Belle in the Regulation of *Drosophila* Dosage Compensation

The Male Specific Lethal (MSL) complex of proteins is enriched on the single X chromosome in male *Drosophila melanogaster*, resulting in a twofold enrichment of gene expression from the X chromosome in order to equalize expression with females, which have two X chromosomes. In Chapter I, we show that the zinc finger protein Zn72D is required for proper splicing of the *maleless* (*mle*) transcript, which encodes one of the proteins in the MSL complex. In addition, we found that Zn72D colocalizes with elongating RNA Polymerase, suggesting it has a broader role in regulating gene expression outside of its role in dosage compensation. In Chapter II, we identify proteins that interact with Zn72D and find it interacts with several proteins involved in RNA metabolism. Co-knockdown of Zn72D and one of the proteins that interacts with Zn72D, the DEAD box helicase Belle, resulted in partial restoration of *mle* splicing and 70% restoration of MLE protein levels, suggesting that Zn72D and Belle regulate translation of MLE. These results implicate Zn72D as a protein that links mRNA splicing to localization and translation.

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General Introduction

Proper cellular gene expression is very important: too much or too little expression of any gene can be detrimental to a cell. Having chromosome-wide dysregulation of gene expression is generally not compatible with life. One example of this is Down Syndrome, which results from trisomy of chromosome 21. Having three copies of chromosome 21 is only tolerated because chromosome 21 is small and therefore has fewer genes than larger chromosomes (Gardiner and Davisson, 2000; Kahlem et al., 2004). Organisms that determine their sex by sex chromosomes have a chromosomal imbalance between the sexes: females have two large X chromosomes and males one X and one small Y chromosome. In order to balance gene expression between the sexes, these organisms undergo a process called dosage compensation. Early in development, female mammals inactivate gene expression on one of their two X chromosomes in order to equalize expression with males, which have on X chromosome (Lucchesi et al., 2005). *Caenorhabditis elegans* hermaphrodites dowregulate gene expression from both of their two X chromosomes by half in order to equalize gene expression with males, with one X (Meyer, 2000). *Drosophila melanogaster* males upregulate gene expression twofold from their single X chromosome in order to equalize expression with females, which have two X chromosomes (Lucchesi et al., 2005).

Although dosage compensation in mammals and *Drosophila* takes different paths toward the same result (silencing versus upregulation of gene expression), one striking feature they have in common is the use of noncoding RNA in a complex of proteins to carry out the process of dosage compensation. In mammals, *Xist* RNA spreads from the chromosome from which it is transcribed to coat the chromosome and silence gene

expression. *Xist* RNA recruits a number of repressive epigenetic marks to the inactive X, including DNA methylation, histone methylation, and the histone variant macroH2A (reviewed in (Cohen et al., 2005). *Xist* RNA is required for the recruitment of the Eed-Ezh2-Suz12 polycomb group complex that trimethylates lysine K27 on histone H3 during initiation of X chromosome inactivation (de la Cruz et al., 2005; Plath et al., 2003).

In *Drosophila*, the Male Specific Lethal (MSL) complex of proteins, MSL1, MSL2, MSL3, Maleless (MLE), and Males Absent on the First (MOF), and noncoding RNAs *roX*1 and *roX*2 coat the X chromosome and upregulate gene expression. MOF is a histone acetyltranferase and acetylates histone H4 lysine 16, a transcriptionally activating modification. The Lucchesi lab recently demonstrated that the ATPase activity of MLE is sufficient for its role in transcriptional enhancement while the helicase activity of MLE is required for the spreading of the MSL complex along the length of the X chromosome (Morra et al., 2007). The MSL proteins were identified through *in vivo* mutagenesis screens designed to identify genes that caused lethality solely in males, thereby missing genes that have a broader role in regulating gene expression, in addition to their function in dosage compensation. *Drosophila* dosage compensation is also studied in cell culture. The MSL complex of proteins and the noncoding RNA *roX*2 is enriched on the active X chromosome in the Schneider S2 cell line, a male *Drosophila* cell line derived from embryos. S2 cells are an ideal model system with which to study the effects of removing one gene from the system, due to the ease with which one can knock down gene expression by RNA interference (RNAi).

There are several unanswered questions in the field of *Drosophila* dosage compensation. First, how is the MSL complex targeted to and assembled specifically on

the X chromosome? Second, how does the MSL complex spread to coat the X chromosome from its initial ~35-100 chromatin entry sites to coat hundreds of sites on the X chromosome? And finally, how are transcription and translation of components of the MSL complex regulated? It is known that in females, SXL inhibits translation of MSL2, but little is known about regulation of gene expression of the other MSL proteins (which unlike MSL2, are also expressed in females). We thought it was possible that proteins involved in these processes might serve more general roles and therefore may be required for survival of both male and female flies.

To begin to address the aforementioned types of questions, we performed an RNAi screen in S2 cells and looked for factors that, when knocked down, resulted in loss of the MSL complex from the X chromosome. In Chapter I, I report the results of this screen, identifying *Zn72D* and *fumble* as candidate genes from the screen. (Additional candidates that affected expression of the reporter gene are identified in the Appendix.) Upon knockdown of *zn72d* and *fumble*, the MSL complex on no longer coats the X chromosome. Zn72D is a putative RNA binding, zinc finger protein that I demonstrated is required for proper splicing of the *mle* transcript in both males and females. I also found that Zn72D colocalizes with elongating RNA Polymerase II, implicating it as a more general factor having a role in cellular RNA metabolism. In Chapter II, I show that Zn72D coimmunoprecipitates with several proteins involved with RNA metabolism, including proteins involved in splicing, transport/nucleocytoplasmic shuttling of RNAs, RNA binding, translation, and RNA interference. One of the proteins that co-IPs with Zn72D is Belle (Bel). I found that co-knockdown of *bel* and *zn72d* restores localization of the MSL complex to the X chromosome. Productive splicing of the *mle* transcript is

partially restored and MLE protein levels are restored to ~70% of wild-type levels, suggesting that in wild-type cells, the *mle* transcript is not being translated efficiently. This implicates Zn72D and Bel as being involved in regulating translation and places Zn72D as a factor linking splicing to translation. Finally, in the Concluding Remarks section, I suggest some future directions for this work.

Chapter I

The Zinc Finger Protein Zn72D Promotes Productive Splicing of the *maleless* Transcript

I. Abstract

In organisms with sex chromosomes, dosage compensation equalizes gene expression between the sexes. In male *Drosophila*, the male-specific lethal (MSL) complex of proteins and two noncoding *roX* RNAs coats the X chromosome, resulting in twofold transcriptional upregulation to equalize gene expression with females. It is not well understood how MSL complex enrichment on the X chromosome is regulated. We performed an RNAi screen to identify new factors required for dosage compensation. Using a *Drosophila* S2 cell line in which GFP-tagged MSL2 localizes to the X, we assayed ~7,200 knockdowns for their effects on GFP-MSL2 distribution. One factor identified is the zinc finger protein Zn72D. In its absence, the MSL complex no longer coats the X chromosome. We demonstrate that Zn72D is required for productive splicing of the transcript for the MSL protein Maleless, explaining the dosage compensation defect. However, Zn72D is required for viability of both sexes, indicating its functions are not sex-specific. Consistent with this, Zn72D colocalizes with elongating RNA Polymerase II, implicating it as a more general factor involved in RNA metabolism.

II. Introduction

Transcription is a highly regulated process, ensuring the production of appropriate levels of gene products to direct cellular proliferation and differentiation programs. Regulation occurs at every step, from initiation and elongation of transcription and splicing of pre-mRNAs, to export and translation of the mature transcripts. One specific example of regulation at the level of transcription elongation is dosage compensation. Organisms in which males and females are distinguished by sex chromosomes undergo

dosage compensation to achieve equal gene expression between the sexes. Female mammals (XX) inactivate one of their two X chromosomes in order to equalize gene expression with males (XY), which have only one X chromosome (Lucchesi et al., 2005). Recent studies indicate there is also upregulation of gene expression from the single X in males and single active X in females to provide proper diploid gene expression (Gupta et al., 2006; Nguyen and Disteche, 2006). In *C.elegans*, hermaphrodites (XX) downregulate transcription from both X chromosomes by half to match expression in males (XO) (Meyer, 2000). In *Drosophila*, X-linked gene expression is upregulated twofold on the single X chromosome in males (XY) to equalize expression with females (XX) (Lucchesi et al., 2005). Dosage compensation in both mammals and *Drosophila* requires noncoding RNA-containing protein complexes (Lucchesi et al., 2005). In male *Drosophila*, the male-specific lethal (MSL) complex (also known as the dosage compensation complex) is localized to the single X chromosome, where it directs upregulation of gene expression (reviewed in (Lucchesi et al., 2005; Taipale and Akhtar, 2005).

The MSL complex is composed of five proteins, MSL1, MSL2, MSL3, MLE (Maleless), and MOF (Males absent on the first), and two noncoding RNAs, *roX*1 and *roX*2. All MSL proteins and at least one *roX* RNA must be expressed for localization of the entire MSL complex to the X chromosome and for proper X-linked gene expression. *msl2* mRNA is translated exclusively in male cells, ensuring sex-specific deployment of the MSL complex (Beckmann et al., 2005; Gebauer et al., 2003; Kelley et al., 1997). MSL2 stabilizes MSL1, and these two proteins, in the absence of any one of the other MSL proteins, bind to approximately 35 sites along the length of the X chromosome

(Kelley et al., 1999; Lyman et al., 1997; Meller et al., 2000). When MSL3, MLE, MOF and at least one of the *roX* RNAs are also expressed, the complete complex forms and localizes to additional sites on the X chromosome. This was originally proposed to be the result of spreading of the complex from the ~35 sites to additional sites along the X (Kelley et al., 1999; Lyman et al., 1997; Meller et al., 2000), and more recently proposed to be the result of the complex binding first to ~35 high affinity sites and then to additional lower affinity sites (Dahlsveen et al., 2006; Demakova et al., 2003; Fagegaltier and Baker, 2004; Gilfillan et al., 2007; Oh et al., 2004). MOF, a histone acetyltransferase, hyperacetylates histone H4 at lysine 16, resulting in the twofold increase in expression of X-linked genes (Akhtar and Becker, 2000; Smith et al., 2000). The localization and function of each component of the complex is highly regulated. For example, the acetyltransferase activity of MOF and the helicase and/or ATPase function of MLE are both required for the complex to associate with the X chromosome in regions beyond the chromatin entry sites (Gu et al., 2000). MOF and MLE, as well as MSL3, require RNA for their localization to the X chromosome, and in turn the *roX* RNAs are stabilized by the localization of the MSL complex to the X chromosome (Akhtar et al., 2000; Buscaino et al., 2003; Meller et al., 2000; Richter et al., 1996). In addition, transcription of *roX* RNAs is controlled by the MSL proteins (Bai et al., 2004; Lee et al., 2004; Rattner and Meller, 2004). Together, these data uncover a series of interactions between components of the MSL complex that are required to ensure its correct localization and activity.

While translational regulation of *msl2* by Sex-lethal and the regulation of *roX* transcription by the MSL proteins is well documented, the factors that regulate

expression of the remaining components of the MSL complex remain largely uncharacterized. We carried out an RNAi screen to identify novel factors involved in the localization of the MSL complex to the X chromosome. In this screen we identified the zinc finger protein Zn72D as a new protein required for MSL complex localization and dosage compensation. Zn72D is essential for development in both males and females. It is not enriched on the X chromosome in males, but rather colocalizes with elongating RNA Polymerase II, suggesting it may have a more general role in RNA metabolism. We found that Zn72D is required to promote the production of the correctly spliced form of *mle* mRNA, thus elucidating its role in dosage compensation.

III. Materials and methods

Cell culture and Generation of stable cell lines

S2 cells and Kc cells were grown in Schneider's media plus 10% fetal bovine serum, penicillin and streptomycin. Cells were maintained according to the Invitrogen *Drosophila* Expression System Protocol. GFP-MSL-2 line was created by cloning a PCR product of MSL-2 into the Not1/Age1 sites in pAC5.1/V5His-B, which carries emeraldGFP as an EcoRI-NotI fragment (gift from Renny Feldman and Pat O'Farrell). pAFH-Zn72D (HA-Zn72D), pAM-MLE (Myc-MLE), and pAM-MLE2intWT (2 intron-containing construct) were created using the Invitrogen Gateway system. 20μg GFP-MSL2 plus 1μg pCoHygro (Invitrogen) was transfected into S2 cells using the protocol described in the Invitrogen *Drosophila* Expression System Protocol and selected with 300μg/mL Hygromycin-B. All other plasmids (20μg each) were transfected with 1μg pCoBlast (Invitrogen) and cells were selected with 15μg/mL Blasticidin S HCl.

RNAi screen

The RNAi library was constructed as previously described (Foley and O'Farrell, 2004) and additional information about this library can be found at http://rnai.ucsf.edu. DsRNAs were added to S2 cells (~15μg/mL) in 50% conditioned/50% fresh Schneider's media in a 96-well format. Four days later, cells were transferred to glass-bottom imaging plates (Greiner Bio-One) coated with conconavalin A (0.5 mg/mL) and allowed to settle for one hour. Cells were then fixed in 1% formaldehyde in 1xPBS for 10 minutes, washed three times with 1xPBS, and fluoromount-G (SouthernBiotech) was added. A visual screen was performed to assay for loss of the GFP-MSL2-coated X chromosome using a Nikon TE-200 inverted microscope. All dsRNAs produced for subsequent RNAi experiments were produced as previously described (Foley and O'Farrell, 2004). The following primers were used to make the dsRNAs:
msl1, RNAi1.1-msl1: GGGCGGGTAATTACCTTTTGGAATTGGA,
RNAi1.2-msl1: GGGCGGGTGGTGGACTGATGGTTGGCTA;
mle (in RNAi library), left: GGGCGGGTTTATGGCTTCGTACTCTAGCACC,
right: GGGCGGGTAAGTTAAGCCAGTTGTCAACGC;
an alternative *mle* dsRNA, *mle*RNAi#2F: GGGCGGGTCCCAAAAATCGCCAGCGG,
*mle*RNAi#2R: GGGCGGGTCGCGAATGTTGTTCGTCTGC;
and the *Zn72D*3'UTR, *Zn72D*3'UTR(s):
GGGCGGGTGCGGCGAGAATAGGTTATATAC,
*Zn72D*3'UTR(as): GGGCGGGTCCGCTTCGTTCTAGTATTTGTG.

Immunofluorescence

S2 cells were allowed to settle on concanavalin A treated coverslips for one hour, fixed in 3.7% formadehyde/0.1% Triton X-100 in 1xPBS for 5 minutes, washed 2 times in 0.1% Triton X-100, blocked for 30 minutes in 0.1% Triton X-100/5% goat serum/1xPBS, and primary antibodies added overnight at 4 degrees. Polytene chromosomes were prepared following the online protocol: http://www.epigenome-noe.net/researchtools/protocol.php?protid=1. Polytene spreads were stained for 1 hour at 37 degrees for MSL-1 and MLE staining, and at 4 degrees overnight for Pol II stained chromosomes. The antibodies were used at the following dilutions: rabbit anti-MOF at 1:500, rabbit anti-MSL-1 at 1:200, guinea pig anti-MLE at 1:200 (gifts from J. Lucchesi), rabbit anti-GFP (ab290-50, Abcam) at 1:750, mouse anti-GFP (JL-8, Clontech) at 1:500, mouse anti-HA (HA.11, Covance) at 1:200, Pol II antibodies H5 at H14 both at 1:50, and mouse anti-c-Myc (sc-40, Santa Cruz) at 1:500. Coverslips were washed 2 times in 0.1% Triton X-100, blocked for 5 min, and secondary antibodies (all from Vector labs, used at 1:200, except for anti-IgM which was from Jackson Immunologicals and used at 1:1000) were applied for 45 minutes at 37 degrees followed by DAPI staining for 5 minutes. All samples were visualized with an Olympus BX60 microscope, and images were collected with a Hamamatsu ORCA-ER digital camera using Openlab 4.0.1 software and assembled with Adobe Photoshop 7.0. Levels were adjusted to enhance contrast.

Western Blotting

S2 cells +/- dsRNA treatment for 6 days were counted, spun down, and lysed in 1x Sample buffer (2x sample buffer: 8.3% glycerol, 1.25% SDS, 0.1M Tris-HCl pH 6.7,

0.083mg/mL bromophenol blue, 50μL/mL 2-mercaptoethanol) at 5x10^4 cells/μL. Samples were boiled for 5 minutes and spun down at 14,000rpm at 4 degrees for 20 minutes. 15μL lysate was loaded per lane of a 4-15% Tris-HCl gradient gel. For western blotting of larvae, 3 third instar larvae equivalents in 2x sample buffer were loaded per lane (larvae were homogenized in an eppendorf tube, boiled, and spun down to remove debris). Gels were transferred to nitrocellulose, blocked with 1% nonfat dry milk/0.05% Tween/1xPBS and probed overnight at 4 degrees with guinea pig anti-MLE antibody at a 1:500 dilution, mouse anti-γ-tubulin at 1:1000 (GTU-88, Sigma), mouse anti-HP1 at 1:2000 (C1A9, Developmental Studies Hybridoma Bank), or chicken anti-Zn72D serum (1:200). The Zn72D antibody was created against a Zn72D peptide, TYREHLEGQKHKKREASL. Donkey anti-guinea pig Cy3 (1:1000), donkey anti-mouse Cy3 (1:500), and donkey anti-chicken Cy3 (1:500) (Jackson ImmunoResearch) were used as a secondary antibodies and detected using a Typhoon 9400 instrument and quantitated using Quantity One software (Bio-Rad). MLE levels were normalized to HP1 or γ-tubulin.

Flies

The *43S2* fly line was a gift from Helena Richardson and its generation is described in Brumby et al. (Brumby et al., 2004). The mutation was identified by sequencing of PCR products along the mutant *Zn72D* gene. The GFP-Zn72D transgene was generated by using the Invitrogen Gateway system to clone Zn72D into pTGW. This construct was used to create transgenic flies (BestGene, Inc.). pTGW-Zn72D flies were

crossed to w118 hsGal4 (III) flies (gift from Pat O'Farrell), and third instar larvae were heat shocked for 1 hour to induce GFP-Zn72D expression.

qRT-PCR

Cells were treated with dsRNAs for 5-6 days (as described above for S2 cells, and Kc cells were incubated with dsRNA in serum-free media for 1 hour and then 2 times the volume of complete Schneider's medium was added). Cells were Trizol extracted, treated with DnaseI (Worthington), phenol-chloroform extracted, and RNA samples were quantitated and 2.5μg RNA was put into a 50μL RT reaction. cDNA was diluted 3-fold and 2.5μL was used in 20μL quantitative-PCR assays using SYBR Master Mix (Applied Biosystems) and qPCR was done on an ABI 7300 instrument. Each sample was normalized to *rp49* and to the sample untreated with dsRNA.

Quantitative PCR Primers

The following are the primers used for quantitative PCR:

msl1: QF1: GAACAGGGCACACAAACGA, QR1: CCCCTGGGAAGTGCATTC;

msl2: QF1: GCATCCTTTGGTGCTTGTTC, QR2: GCTGCCCTGGAAGATATTGAA;

msl3: QF2: TGGCAAGCGAAAGGAAAA, QR2: GCCCCGGTTTCCCTTTAA;

mle (exons2-3; red primers in Fig. 6): QF1: CGGAACACGCTAGGAGCTTT, QR1: TGAGCGCCGGCACAT;

mle (exons3-4; blue primers in Fig. 6): F1: GATGAGGTGATTAAGGGTTTGG, R3: GAGGAATCTATACGGCTTAAG;

mle (black primer in Fig. 6): QF1adj: TGGGCCCGGAACACGC;

mof: QF3: CAGGGAGACGGTCATCACA, QR3: CGGGATTTTCGCTTATATCGA;

Zn72D: QF2: CGATGATAATCTGGACGATTCG, QR2:

CGCCTACTGGCTTAATGTTGTC;

*roX*2: QF1: TTGCGCCTATGACAATCCTAA, QR1: GGCCATCCGAGCTACCTAAA;

mRpL16: QF2: TCAACACAGCCGGTCTTAAGTAT, QR2:

GGCTGCTCCACATTCTGGTA;

rp49: QF3: GCCGTAATTGTCGTTTTTGG, QR3: CGAACAGCGCACGGACTA;

arm, CG14804, and RpII140: (Straub et al., 2005).

Northern Blotting

Northern blots were performed using a NorthernMax Kit (Ambion) and BrightStar-Plus membrane (Ambion). 10μg of RNA was loaded per lane from S2 cells treated +/- Zn72D dsRNA for 6 days. Northerns were probed with a labeled DNA probe that was antisense to the full-length MLE cDNA, and exposed to a phosphor screen overnight.

IV. Results

An RNAi screen identifies the zinc-finger protein Zn72D as required for MSL complex localization

To identify new factors involved in the regulation of dosage compensation in *Drosophila*, we performed an RNAi screen in a male *Drosophila* S2 cell line expressing GFP-tagged MSL2, which localizes to the X chromosome (Fig. 1A). Using an RNAi library that consists of 7,216 dsRNAs targeting genes conserved between *Drosophila*, *C. elegans*, and mammals (Foley and O'Farrell, 2004), we assayed for loss of GFP-MSL2 on

the X chromosome. In addition to identifying the known components of the MSL complex that were represented in the library, we identified two new candidates for regulation of dosage compensation, Zn72D and Fumble. Upon knockdown of *Zn72D* or *fumble*, GFP-MSL2 and MOF were no longer enriched on the X chromosome (Fig. 1A and Fig. 2A), consistent with disruption of the MSL complex rather than an effect on expression of the MSL-GFP transgene.

Fumble is homologous to the mammalian pantothenate kinase, an enzyme required for acetyl-CoA synthesis (Robishaw and Neely, 1985). When S2 cells were treated with the histone deacetylase inhibitor TSA, levels of acetylation on histone H4 lysine 16 increased. When *fumble* was knocked down before treatment with TSA, there was no similar increase in the level of acetylation on histone H4 lysine 16, consistent with disruption of acetyl-CoA production (Fig. 3). This implied that Fumble indirectly regulates MSL complex formation, as acetylation activity of MOF is required for MSL complex localization (Gu et al., 2000). We focused our studies on the second candidate, Zn72D. When *Zn72D* is knocked down in wild-type S2 cells, MLE, MSL1 and *roX*2 were no longer enriched on the X chromosome (Fig. 1B and Fig. 2B), confirming that Zn72D is necessary for the correct localization of the MSL complex. Zn72D has three C2H2 zinc finger domains of the type that are found in the U1C snRNP protein, and a DZF domain, which is a domain found in some proteins containing a C2H2 zinc finger or double strand RNA binding domains (Fig. 1C).

Zn72D is required *in vivo* for MSL complex localization and dosage compensation

Next we asked whether Zn72D was required for proper MSL complex localization to the X chromosome in male flies. In the line *43S2* (Brumby et al., 2004), a single base pair insertion causes a frame shift prior to the first zinc finger domain of Zn72D (Fig. 1C). Polytene chromosomes isolated from third instar larvae were stained with antibodies to MSL1 and MLE. Wild-type males showed the complete banding pattern of MSL complex on the X chromosome, while Zn72D mutant males had significantly fewer bands (Fig. 4). These data demonstrate that Zn72D is required for the localization of the MSL complex to the X chromosome *in vivo*.

To determine if Zn72D is required for proper dosage compensation, we performed quantitative reverse transcription PCR (qRT-PCR) to assess steady-state levels of mRNA from X-linked genes when either *mle* or *Zn72D* was knocked down in S2 cells or in female Kc167 cells. We assayed three X-linked genes, *arm*, *CG14804*, and *mRpL16*, which are regulated by the MSL complex in male cells (Hamada et al., 2005; Straub et al., 2005). Levels were normalized to the autosomal gene *rp49*. When *mle* was knocked down in S2 cells, we observed the expected decrease in X-linked gene expression and no effect on expression of the autosomal gene *RpII140* (Fig. 5A). When *Zn72D* was knocked down in S2 cells, there was a similar decrease in X-linked gene expression (Fig. 5A). qRT-PCR performed on male Zn72D-/- larvae also showed a decrease in levels of *arm* and *CG14804* compared to wild-type larvae; levels of *mRpL16* were not affected in Zn72D-/- larvae (Fig. 6A). Upon knockdown of *mle* or *Zn72D* in Kc167 cells, there was no change in expression levels of *arm*, *CG14804*, or *mRpL16*, indicating that, like MLE, Zn72D regulates X-linked gene expression solely in males (Fig. 5B). However, all

Zn72D mutants pupate but fail to hatch (Brumby et al., 2004 and data not shown), indicating Zn72D is required in females as well as males. In contrast, mutations in the MSL complex proteins result in lethality at the larval or pupal stage of development only in males (Belote and Lucchesi, 1980; Fukunaga et al., 1975; Hilfiker et al., 1997). This suggests Zn72D has a broader function outside of its role in dosage compensation.

Zn72D is associated with transcriptional puffs

In order to gain insight into the role of Zn72D, we determined its cellular distribution. We expressed HA-tagged Zn72D in S2 cells and performed immunofluorescence with an anti-HA antibody. Zn72D was localized predominantly in the nucleus (Fig. 7A). Unlike the MSL complex, Zn72D was not enriched on the X chromosome, consistent with a function for Zn72D outside of its role in dosage compensation. To determine if Zn72D was associated with chromosomes, we generated transgenic flies that express Zn72D fused to GFP under control of the Gal4 UAS. The GFP-Zn72D transgene encodes a functional Zn72D protein, as it rescues MSL complex localization in S2 cells when endogenous Zn72D is knocked down with dsRNA targeting the 3'UTR, not present in the transgene (Fig. 8). The GFP-Zn72D transgenic flies were crossed to flies expressing heat-shock inducible Gal4. Polytene chromosomes from heat-shocked third instar larvae were isolated and stained with an anti-GFP antibody. GFP-Zn72D was localized to all chromosomes in both sexes with no notable enrichment on the X chromosome. Instead, Zn72D appeared to be enriched on transcriptional puffs (Fig. 7B), which was confirmed by colocalization of GFP-Zn72D and elongating RNA Polymerase II phosphorylated on serine 2 of the carboxyl-terminal domain (Pol IIo-Ser2)

(Fig. 7C and see Fig. 9 for Pol IIo-Ser5). Endogenous Zn72D also localizes to chromatin under normal and heat shock conditions (Fig. 8). This pattern of distribution suggests that Zn72D may have a role in regulating transcription, RNA splicing, processing, or transport, as factors that regulate these processes are all present on transcriptional puffs (Andrulis et al., 2002; Daneholt, 2001; Kim et al., 1992; Sass and Pederson, 1984; Soop et al., 2003; Weeks et al., 1993).

Zn72D is required for normal levels of *mle* RNA and protein

To determine if Zn72D functions in dosage compensation through regulation of transcription or stability of one or more of the MSL complex mRNAs, we used qRT-PCR to analyze mRNA levels of the components of the MSL complex when either *Zn72D*, or *mle* as a control, was knocked down in S2 cells. Upon *mle* knockdown, the levels of *mle* mRNA and protein were decreased (Fig. 10A and 10C), *msl3* levels remained unchanged, and *msl1* and *msl2* mRNAs increased slightly (Fig. 10A). When *Zn72D* was knocked down (Fig. 5A and 5C), the levels of *mle* mRNA were reduced about 16-fold, while *msl1*, *msl2*, and *msl3* mRNA levels were unaltered (Fig. 10A). *mof* and *roX*2 levels decreased slightly when *Zn72D* or *mle* was knocked down, which was expected since *mof* is X-linked and *roX*2 is destabilized in the absence of a functional MSL complex (Fig. 10A; (Meller et al., 2000). Additionally, MLE regulates transcription of *roX*2 (Bai et al., 2004; Lee et al., 2004); therefore the decrease in *mle* levels when *Zn72D* is knocked down likely contributes to reduced levels of *roX*2. Supplementing the result in S2 cells, *mle* and *roX*2 transcripts were lower in male Zn72D-/- third instar larvae compared to wild-type larvae (Fig. 6B).

MLE is expressed in both males and females. If the role of Zn72D is to regulate mle RNA levels, we expected that the level of mle mRNA would decrease upon Zn72D knockdown in females as well as males. To test this, the level of mle transcripts was assayed when Zn72D was knocked down in the female Kc167 cell line. The level of mle mRNA did decrease, indicating that Zn72D is required for mle expression in both sexes (Fig. 10B). Therefore, in males lacking Zn72D, the drop in mle mRNA levels might in turn affect MSL complex localization due to a decrease in the amount of MLE protein. This was indeed the case: the levels of MLE protein were reduced to less than one-fifth of the wild-type levels when Zn72D was knocked down in S2 cells and in Zn72D mutant larvae (Fig. 10C). MLE protein levels did not decrease when msl1 was knocked down, indicating that MLE protein is relatively stable even when the MSL complex does not form. Together, these results suggest that Zn72D is required for MLE expression by affecting the amount of mle mRNA that is produced. This function may be either direct or indirect, as we were unable to determine, by RNA immunoprecipitation, if Zn72D binds directly to mle RNA.

Zn72D promotes productive splicing of *mle* transcripts

As we attempted to determine the fate of the *mle* mRNA, it became clear that the entire *mle* transcript was not being turned over to the degree indicated in our original qRT-PCR experiments. Other regions of the *mle* transcript did not show the same dramatic decrease as the region assayed in the original qRT-PCR experiment (Fig. 11B). *mle* is alternatively spliced at the exon 2-exon 3 splice junction (Kuroda et al., 1991). One splice isoform produces an mRNA that directs production of full length MLE

(Isoform 1, Fig. 11A). The other isoform employs a downstream splice donor, resulting in production of a 342 nucleotide longer mRNA containing several in-frame stop codons (Isoform 2, Fig. 11A). Isoform 2 would direct translation of a truncated MLE protein of 226 amino acids, instead of the full-length protein of 1,293 amino acids. The primers we used to quantify *mle* mRNA levels detected only isoform 1, as the forward primer spans the exon 2-exon 3 junction (Fig. 11A). To determine if loss of Zn72D affected abundance of both isoforms, we examined the exon2-exon3 junction by using primers that flank the intron and therefore amplify both isoforms plus unspliced RNA. In Zn72D-/- larvae and in Zn72D knockdown cells, the amount of isoform 1 decreased while the amount of isoform 2 increased (Fig. 11C and data not shown), suggesting that Zn72D regulates the relative amounts of the two *mle* isoforms rather then the absolute amount of *mle* mRNA. We performed Northern blots as a second assay to measure the relative amounts of these two *mle* splice isoforms. Northern blotting indicated the presence of at least 3 *mle* transcripts (Fig. 11D), consistent with 2 poly(A) sites which are approximately 350 nucleotides apart (Kuroda et al., 1991) and the two splice sites that are also about 350 nucleotides apart. Identity of these transcripts was confirmed by using a probe that hybridizes to the region between the two poly(A) sites (data not shown). Upon *Zn72D* knockdown, the transcripts that can be attributed to splice isoform 1 decreased in abundance, while those that can be attributed to isoform 2 increased in abundance (Fig. 11D), indicating that Zn72D promotes splicing of *mle* such that the full-length MLE protein is produced.

If the role of Zn72D in dosage compensation is to promote the productive splicing pattern of *mle*, then when an *mle* transcript that lacks introns is expressed, it should

circumvent the requirement for Zn72D. To test this, we created a stable S2 cell line expressing the *mle* cDNA sequence tagged with Myc. Overexpressed Myc-MLE localized throughout the nucleus (Fig. 12A), consistent with the previous observation that overexpressed MLE localizes to all chromosomes in flies (Richter et al., 1996). MSL1 was still localized to the X chromosome in these Myc-MLE overexpressing cells, indicating that the overexpressed protein did not interfere with MSL1 localization. When *mle* was knocked down in these cells, Myc-MLE expression was reduced and MSL1 no longer appeared in a pattern consistent with enrichment on the X chromosome, indicating that even when MLE is overexpressed, formation of the MSL complex is still sensitive to changes in amounts of MLE. When *Zn72D* was knocked down, Myc-MLE levels did not decrease and MSL1 localization was unaltered, indicating that expression of an *mle* cDNA rescues the MSL localization defect observed when *Zn72D* is knocked down (Fig. 12A).

We next added back the first two introns to the Myc-*mle* cDNA, expressed it in S2 cells, and knocked down either *mle* or *Zn72D*. Upon *mle* knockdown, Myc-MLE levels were decreased and MSL1 was no longer localized on the X chromosome (Fig. 12B). In contrast to the Myc-*mle* cDNA, the Myc-*mle* transgene containing introns 1 and 2 was subject to Zn72D regulation: when *Zn72D* was knocked down, the intron-containing transgene did not rescue MSL1 localization (Fig. 12B). The level of protein expressed from the Myc-*mle* cDNA did not change significantly upon *Zn72D* knockdown, while the level of the full-length protein expressed from the intron containing-transgene decreased in the absence of Zn72D (Fig. 12B). In the intron-containing transgene line, an additional protein, of the approximate molecular weight

predicted for a Myc-tagged protein that would be produced from isoform 2, was detected using Myc and MLE antibodies (Fig. 12C and data not shown). It is possible that this shorter product may account for the cytosolic Myc-tagged protein that was specific to the *mle* transgenic line that contained introns (Fig. 12B). While the observed increase in isoform 2 upon Zn72D knockdown led us to anticipate an increase in the abundance of the shorter protein product, none was observed. If the smaller Myc-MLE protein is unstable, then steady state levels of the protein may not accurately reflect the amount of isoform 2. We were unable to detect a truncated MLE protein upon Zn72D knockdown in wild-type cells (data not shown), suggesting that the shorter protein produced from isoform 2 may be very unstable. In combination, our data are consistent with a role for Zn72D in promoting usage of the correct splice site at intron 2 of *mle* mRNA.

V. Discussion

In an RNAi screen for proteins that regulate fly dosage compensation, we identified the zinc-finger containing protein Zn72D. Zn27D mediates its role in dosage compensation by promoting proper splicing of the *mle* transcript, thus affecting MLE protein levels and the localization of the MSL complex to the X chromosome. Zn72D, either directly or indirectly, promotes productive splicing of *mle* in male and female cells, and likely regulates additional targets, as both male and female Zn72D mutants pupate but do not hatch. Zn72D is enriched on transcriptional puffs and colocalizes with elongating Pol IIo on polytene chromosomes, consistent with a general role in splicing regulation.

Zn72D, a zinc finger splicing factor

Zn72D contains three zinc fingers that are similar to the zinc finger in the U1 small nuclear ribonucleoprotein C (U1C). U1C binds the 5' splice site (5'ss) in a sequence specific manner (Du and Rosbash, 2002), and mutations in the zinc finger of U1C bypass the need for the DExH/D box helicase Prp28 to unwind the 5'ss and U1 snRNA base pairing (Chen et al., 2001), suggesting that U1C stabilizes the 5'ss-U1 snRNA commitment complex. The similarity between the zinc fingers of Zn72D and U1C suggests the possibility that, like U1C, Zn72D might promote splicing through regulation of commitment complex formation between the 5'ss in pre-mRNAs and U1 snRNA. Alternatively, Zn72D may recognize a 5'ss and inhibit its use in the splicing reaction, forcing use of an alternative splice site. We were unable to detect an interaction between Zn72D and *mle* RNA, suggesting that Zn72D might instead play a more indirect role in 5'ss selection during *mle* splicing.

***mle* RNA is not subject to NMD**

The splice isoform that is upregulated upon knockdown of Zn72D includes several in-frame premature translation termination codons (PTCs). PTCs can signal nonsense-mediated mRNA decay (NMD). The PTC-containing *mle* transcript was easily detected, both by Northern and RT-PCR. In addition, this transcript was readily detected in cytoplasmic extracts by RT-PCR (data not shown), indicating that it is exported from the nucleus. When we overexpressed the intron-containing MLE transgene, a smaller Myc-tagged MLE protein product was expressed in addition to the full-length tagged MLE, suggesting that a protein can be produced from the PTC-containing spliced

transcript. The increase in levels of the PTC-containing *mle* transcript upon Zn72D knockdown is unlikely to be a consequence of disruption of NMD, since there was a compensatory decrease in the other *mle* splice isoform and no increase in overall *mle* levels. This suggests that *mle* transcripts are not significantly affected by NMD, and that Zn72D is required for productive splicing of *mle*. It is not yet clear how premature termination codon-containing transcripts are recognized as aberrant and targeted for NMD in *Drosophila*. Unlike in mammals and yeast, where these transcripts are recognized based on the presence of exon-junction complexes on the transcript downstream from the premature termination codon, *Drosophila* mRNAs do not require the exon-junction complexes for NMD (Gatfield et al., 2003). As the PTC-containing *mle* transcript apparently lacks the *Drosophila* NMD signal, *mle* may be a useful transcript to investigate the NMD pathway in flies, as one can ask why the PTC-containing *mle* transcript avoids the fate of other transcripts that contain PTCs.

Does Zn72D link splicing to localization and translation?

The closest mammalian homologue of Zn72D is ZFR; these proteins are 42% identical and 55% similar, and both contain the same domain structure, with three zinc fingers followed by a DZF domain. ZFR was shown to be chromosome-associated in meiosis, and it is required for normal development of the mouse embryo; homozygous *Zfr* mutants have gastrulation defects and die between 8 and 9 days of gestation (Meagher and Braun, 2001; Meagher et al., 1999). Human ZFR was identified in a screen for Staufen2-interating proteins and was implicated in nucleo-cytoplasmic shuttling of Staufen2 (Elvira et al., 2006). Staufen1 and Staufen2 are double-strand RNA binding

proteins that are implicated in the transport of mRNAs from the nucleus to cytoplasmic RNA granules (reviewed in (Kiebler et al., 2005; Miki et al., 2005), which are clusters of ribosomes, translation factors, and mRNAs presumed to be incompetent for translation as they lack eIF4E, 4G, and tRNAs (Krichevsky and Kosik, 2001). Like its human counterpart, *Drosophila* Staufen also plays a role in RNA localization: it is required for localization of the *oskar* mRNP complex to the posterior and *bicoid* mRNA to the anterior of the oocyte (reviewed in (Johnstone and Lasko, 2001).

If Zn72D, like ZFR, is involved in localizing mRNP complexes within cells, this suggests it may be involved in linking splicing and mRNA localization. There is a precedent for the connection between splicing and RNA localization: splicing at a specific exon-exon junction of *oskar* is necessary for the proper localization of the *oskar* mRNA to the posterior (Hachet and Ephrussi, 2004), and members of the exon-junction complex are also necessary for its localization (Hachet and Ephrussi, 2001; Le Hir et al., 2001). Zn72D contains 2 putative nuclear export signals and a nuclear localization sequence, suggesting Zn72D might participate in nucleo-cytoplasmic shuttling events. We did not observe any affect on localization of the dosage compensation complex upon RNAi-mediated knockdown of *staufen* (data not shown), so any potential homologous role of Zn72D in shuttling is likely mediated by other proteins.

Acknowledgements

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VI. Figure Legends

Figure 1. Zn72D is required for localization of the MSL complex to the X chromosome in *Drosophila* S2 cells. (A) S2 cells expressing GFP-MSL2 (green) were either left untreated (top) or treated with *Zn72D* dsRNA (bottom). Cells were stained with anti-MOF (red) and DAPI (blue) to delineate the nuclei. (B) Wild-type S2 cells either untreated (top) or treated with *Zn72D* dsRNA (bottom), and stained with anti-MSL1 (green) and anti-MLE (red). (C) Diagram of the domains in the Zn72D protein, identified in a Blast search. Zn72D has three C2H2 zinc fingers, a DZF domain, two putative nuclear export sequences (NES), and a nuclear localization sequence (NLS). The asterisk indicates the approximate position of the frame shift mutation present in the *Zn72D* mutant flies.

Figure 2. (A) Upon knockdown of *fumble*, the MSL complex no longer coats the X chromosome. GFP-MSL2 (green), anti-MOF (red), and DAPI (blue) staining are indicated. (B) Upon knockdown of Zn72D, the noncoding RNA *roX*2, the only *roX* RNA that is expressed in S2 cells, no longer localizes to the X chromosome. Methods: FISH for *roX*2 RNA using a double-stranded cy3 (red) labeled probe to *roX*2 cDNA was performed, and cells were DAPI stained. *roX*2 cDNA (gift from H. Amrein) using the Bioprime DNA labeling system (Invitrogen) and substituting Cy3-dCTP (Amersham). The reaction was purified over an S-200 MicroSpin column (Amersham) and precipitated with 100ug tRNA. Probe was resuspended in 360uL ddH20, and 40uL 3M sodium acetate and 1 mL ethanol were added for storage at –20. 100uL of the labeled probe in ethanol was used to prepare the probe mix, as described in Panning, B. (2004) X-

inactivation in mouse ES cells: histone modifications and FISH. *Methods Enzymol*. 376:419-28. S2 cells were centrifuged onto slides using a Cytospin3 (Shandon) and treated with cytoskeletal buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES, pH 6.8) for 30s, cytoskeletal buffer + 0.5% Triton X-100 for 30s, again with cytoskeletal buffer for 30s, and fixed in 4% paraformaldehyde in 1xPBS for 10 minutes at 25 degrees. Slides were dehydrated through a 70-80-90-100% ethanol series and probe was added under a coverslip in a humid chamber overnight at 37 degrees. All washes were done at 39 degrees: 3 washes in 2xSSC/50% formamide for 2 min, 3 washes in 2xSSC for 2 min, one wash in 1xSSC+DAPI for 5 min, 2 washes in 1xSSC for 2 min. Coverslips were added with Vectashield (Vector labs).

Figure 3. As mutations in MOF that disrupt its ability to bind acetyl-coA disrupt the X chromosomal localization of the MSL complex, we speculated that knockdown of *fumble* may indirectly result in loss of localization of the MSL complex to the X due to a drop in acetyl-coA levels. When S2 cells are treated with the histone deacetylase inhibitor TSA, levels of histone H4 acetylated on lysine 16 (H4K16ac) increased (compare "none" to "none+TSA"). However, upon *fumble* knockdown, levels of H4K16ac decreased and did not subsequently increase upon treatment with the histone deacetylase inhibitor TSA (compare "*fumble*" to "*fumble*+TSA"), consistent with a possible role for Fumble in production of acetyl-CoA. As it is already known that acetylation of H4K16 is required for localization of the MSL complex, the role of Fumble in dosage compensation was not investigated further.

Figure 4. Male *Drosophila* require Zn72D for the complete localization of the MSL complex across the X chromosome. Immunofluorescence was performed on polytene chromosomes isolated from male third instar larvae to detect MSL1 (top row, green) and MLE (second row, red) on the X chromosome in a wild-type (left column) and *Zn72D*-/- larvae (center and right columns). DAPI (third row, blue) stains all chromosomes. The bottom row contains the merged images of MSL1, MLE, and DAPI.

Figure 5. Zn72D is necessary for proper X-linked gene expression in males but not females. (A) Quantitative RT-PCR for the X-linked genes *arm*, *CG14804*, and *mRpL16* and the autosomal gene *RpII140* was done after S2 cells were either untreated (gray bars), treated with *mle* dsRNA (white bars), or treated with *Zn72D* dsRNA (black bars). Samples were normalized first to *rp49* and then to the untreated sample, setting untreated to 1. The bar represents the average of two independent experiments, with qPCR performed in triplicate, and error was determined using standard error propagation methods. (B) Female Kc cells were treated with dsRNAs and qRT-PCR was performed as described in (A).

Figure 6. Quantitative RT-PCR on wild-type and Zn72D-/- mutant third instar larvae shows similar results to knockdowns in S2 cells. (A). qRT-PCR for the X-linked genes *arm*, *CG14804*, and *mRpL16* from wild-type larvae (gray bars) or Zn72D-/- larvae (black bars). (B) qRT-PCR for *mle* and *roX*2 in wild-type (gray bars) and Zn72D-/- (black bars) larvae.

Figure 7. Zn72D is nuclear, enriched at transcriptional puffs, and colocalizes with elongating RNA Pol II. (A) S2 cells expressing HA-tagged Zn72D stained with anti-HA (red) and DAPI (blue). (B) *Drosophila* larvae were heat shocked to induce Gal4 to activate GFP-Zn72D expression, and polytene chromosomes were isolated and stained with anti-GFP (green) and DAPI (blue). (C) Polytene chromosomes from GFP-Zn72D induced larvae were stained with anti-GFP (green) and anti-PolIIoSer2 (red) and DAPI (blue).

Figure 8. GFP-Zn72D (green) and the elongating form of Pol II that is phosphorylated at serine 2 of the carboxyl-terminal domain (CTD) colocalize on the polytene chromosomes (Fig. 4C), while Pol II phosphorylated at serine 5 of the CTD (red) appear mostly at adjacent regions (compare Fig. 4C to this figure). Arrowheads indicate examples of Pol IIoSer5 adjacent but not overlapping GFP-Zn72D.

Figure 9. (A) The GFP-Zn72D transgene is functional, as it can substitute for endogenous Zn72D. Gal4-expressing S2 cells were transfected with the pTGW-Zn72D and a stable cell line was selected. In untreated cells, MSL1 localizes to the X chromosome in all cells and GFP-Zn72D is predominantly in the nucleus in transfected cells. Upon knockdown of mle (middle), MSL1 is no longer enriched on the X chromosomes in all cells. When endogenous Zn72D is knocked down using a dsRNA in the 3'UTR not present in the transgene (bottom), MSL1 localizes to the X chromosome only in cells expressing the GFP-Zn72D transgene. Cells not expressing the transgene

(indicated with arrowheads) lose MSL1 from the X. (B and C) Endogenous Zn72D localizes to chromosomes and to heat shock puffs as does GFP-Zn72D. (B) Zn72D (red) localizes to chromosomes under non-heat shock conditions. (C) Zn72D (green) becomes enriched on heat shock puffs and colocalizes there with Pol IIoSer2 (red).

Figure 10. Zn72D is required for proper *mle* mRNA and protein levels. (A) S2 cells were treated with either (left) *mle* or (right) *Zn72D* dsRNA and qRT-PCR was performed, as described in the legend to Figure 3 and the Materials and methods, to assay the transcript levels of *msl1* (orange), *msl2* (mauve), *msl3* (green), *mof* (yellow), *mle* (red), *roX*2 (light purple), and *Zn72D* (blue). Samples were normalized first to *rp49* and then to the untreated sample, setting untreated to 1. (B) Kc167 cells were treated with (left) *mle* or (right) *Zn72D* dsRNA as above and the levels of *mle* (red) and *Zn72D* (blue) transcripts were assayed by qRT-PCR. (C) Western blots of (left and middle) extracts from S2 cells that had been treated with either no dsRNA, *msl1*, *mle*, or *Zn72D* dsRNAs or (right) extracts from wild-type and *Zn72D*-/- third instar larvae. Levels of MLE and Zn72D were normalized to the amount of γ-tubulin or HP1.

Figure 11. Zn72D promotes productive splicing at the second intron of the *mle* transcript. (A) (Top) Depicted is the *mle* gene, which contains 5 exons (gray boxes) and 4 introns (black or green horizontal lines). Poly(A)-1 and -2 are depicted as red and black vertical lines, respectively. (Bottom) Four *mle* transcript isoforms differ in retention of part of intron 2 (Isoforms 1 and 2 (I1, I2)) and usage of the upstream or downstream poly(A) sites (P1, P2). The partially retained intron is in green. (B) qRT-PCR in two

regions of the *mle* transcript was performed +/- *Zn72D* knockdown. Primers detect the *mle* transcript at the exon 2-exon 3 junction (red; these are the primers used previously in Fig. 5) and the exon 3-exon4 junction (blue). The location of these primer sets along the length of *mle* is depicted in Fig. 6A as red and blue arrows, respectively. The forward primer in the red primer set spans the exon2-3 junction and this primer set specifically amplifies I1. (C) RT-PCR on wild-type and Zn72D-/- third instar larvae, using primers that flank intron 2 of the *mle* transcript, shown as a black arrow pointing to the right and a red arrow pointing to the left in part A. Three transcripts were amplified: the unspliced RNA; I1, which decreases in Zn72D mutants; and I2, which increases in Zn72D mutants. (D) Northern analysis with RNA collected from S2 cells either treated or untreated with *Zn72D* dsRNA. For untreated S2 cells, the top band corresponds to isoform I2,P2; the middle band corresponds to both isoforms I1,P2 and I2,P1; and lower band corresponds to isoform I1,P1. Upon Zn72D knockdown, only I2 isoforms remain.

Figure 12. An *mle* cDNA transgene rescues Zn72D knockdown but an intron-containing *mle* transgene does not. (A) S2 cells expressing Myc-tagged MLE cDNA were either untreated (top), or treated with *mle* (middle) or *Zn72D* dsRNAs (bottom), and stained with anti-MSL1 (green) and anti-Myc (red). White arrowheads (bottom) indicate cells that express Myc-MLE. (B) S2 cells expressing a Myc-tagged MLE transgene that contains the first two introns of *mle* were either untreated (top) treated with *mle* (middle), or *Zn72D* dsRNAs (bottom), and stained with anti-MSL1 (green) and anti-Myc (red). (C) Western blots of S2 cells expressing transgenic Myc-MLE from the cDNA construct (left 3 lanes) or Myc-MLE containing 2 introns (right 3 lanes) and untreated or treated with

mle or *Zn72D* dsRNAs, as indicated. Levels of full-length (indicated above the gel) and truncated (indicated below the gel) Myc-MLE were normalized to γ-tubulin and the sample untreated with dsRNA.

Figure 1

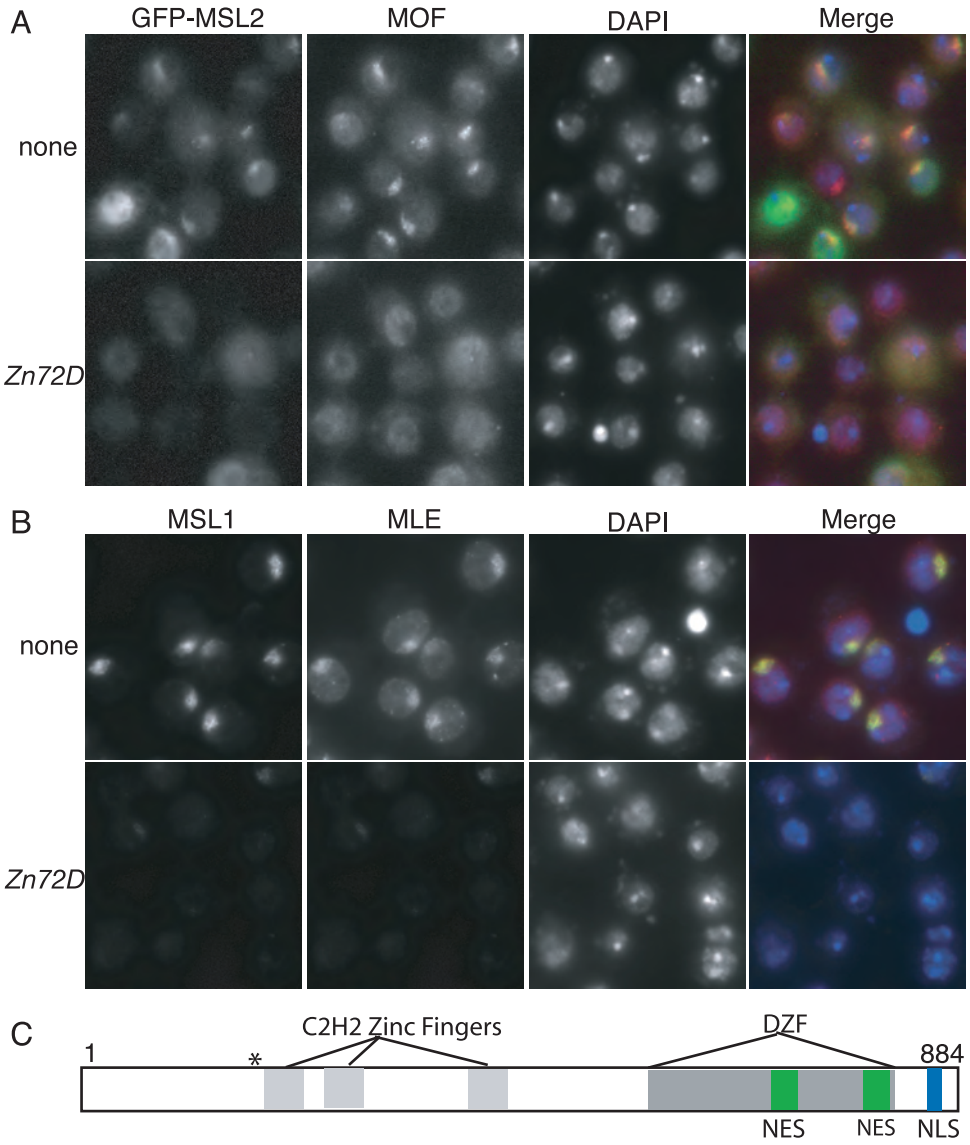
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Figure 2

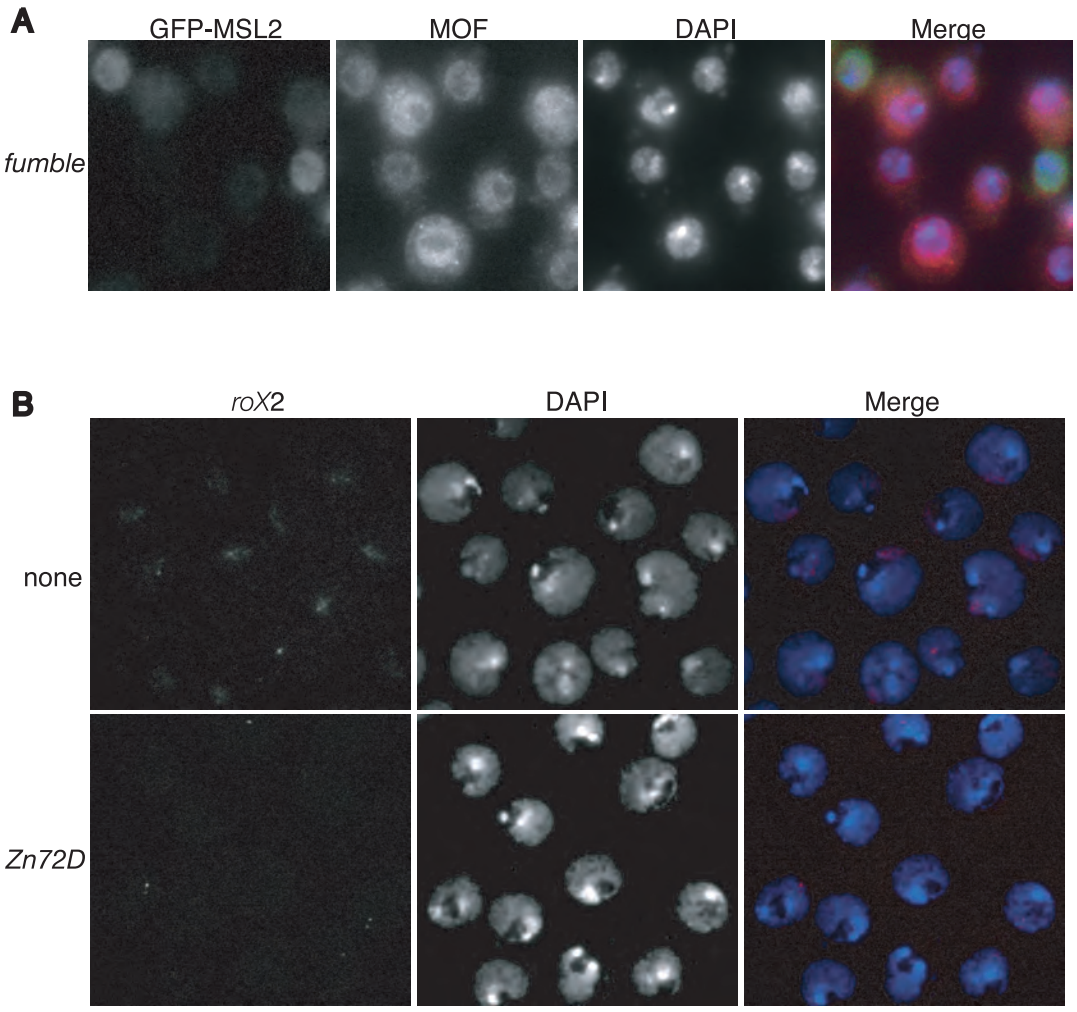


Figure 3

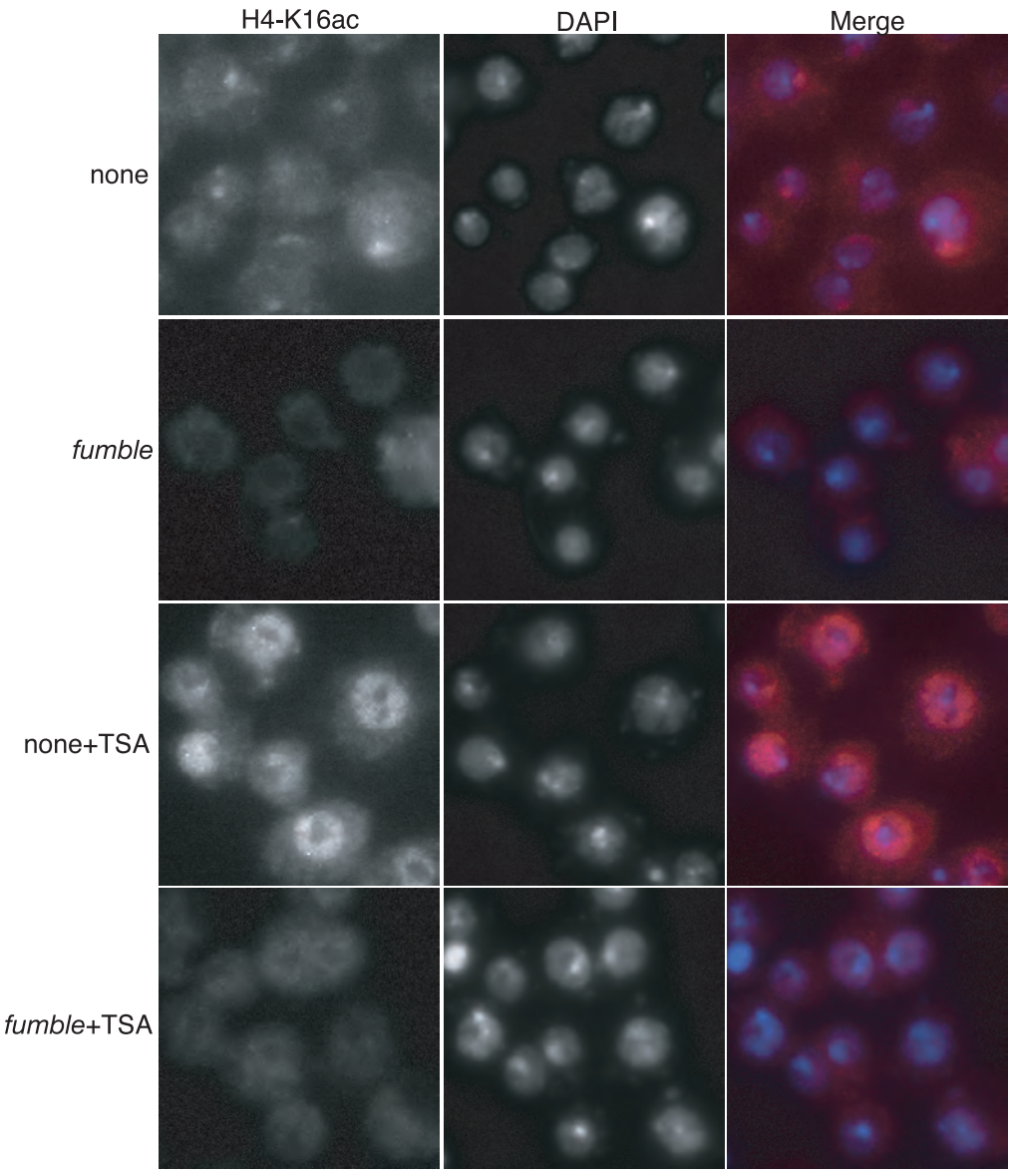


Figure 4

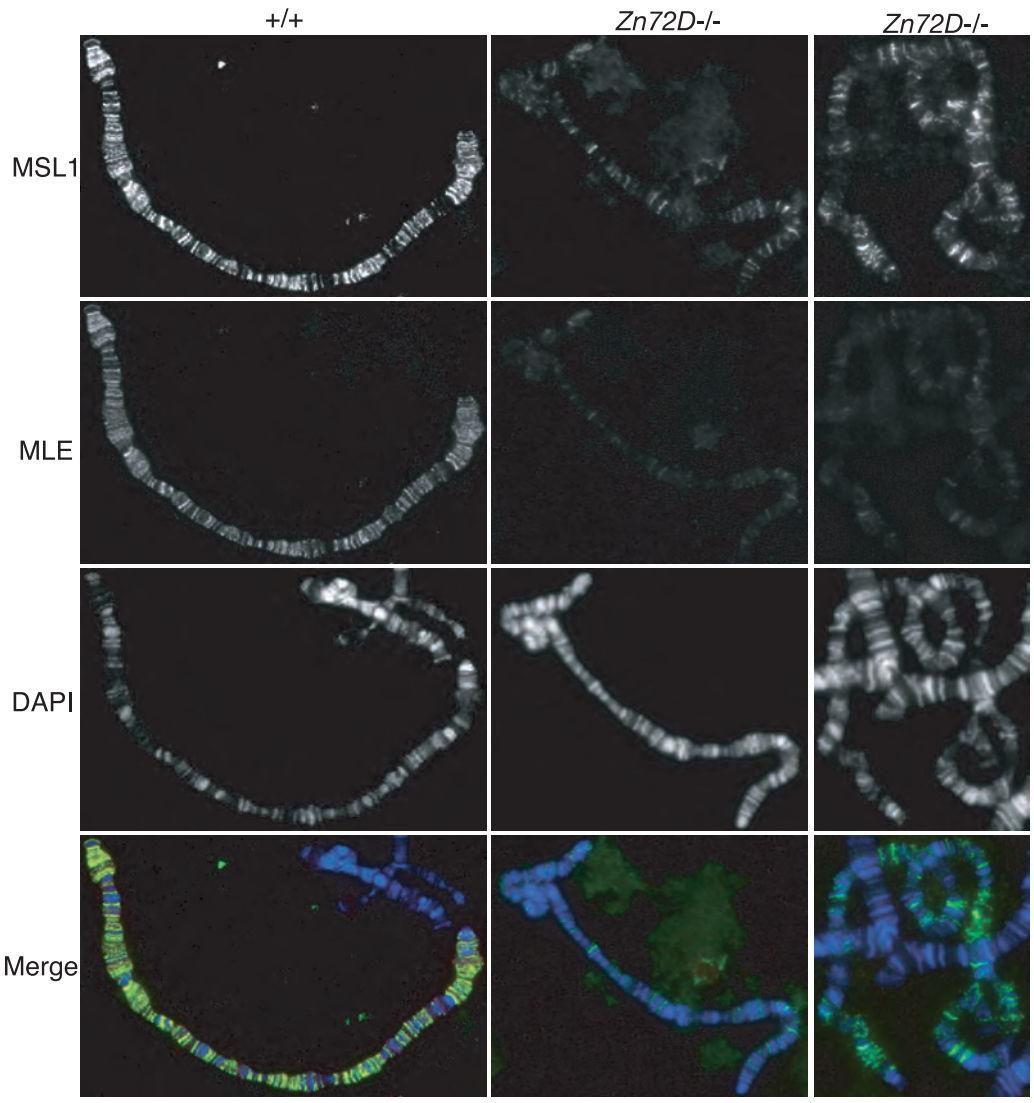
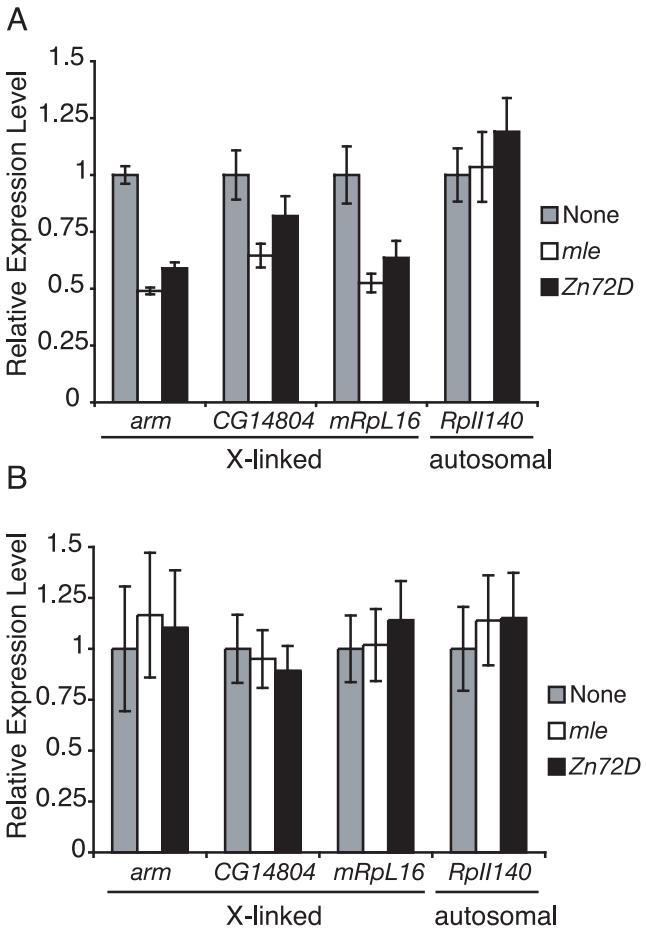


Figure 5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autosomal
None
mle
Zn72D
B
Relative Expression Level
1.5
1.25
1
0.75
0.5
0.25
0
arm
CG14804
mRpL16
RpII140
X-linked
autosomal
None
mle
Zn72D

Figure 6

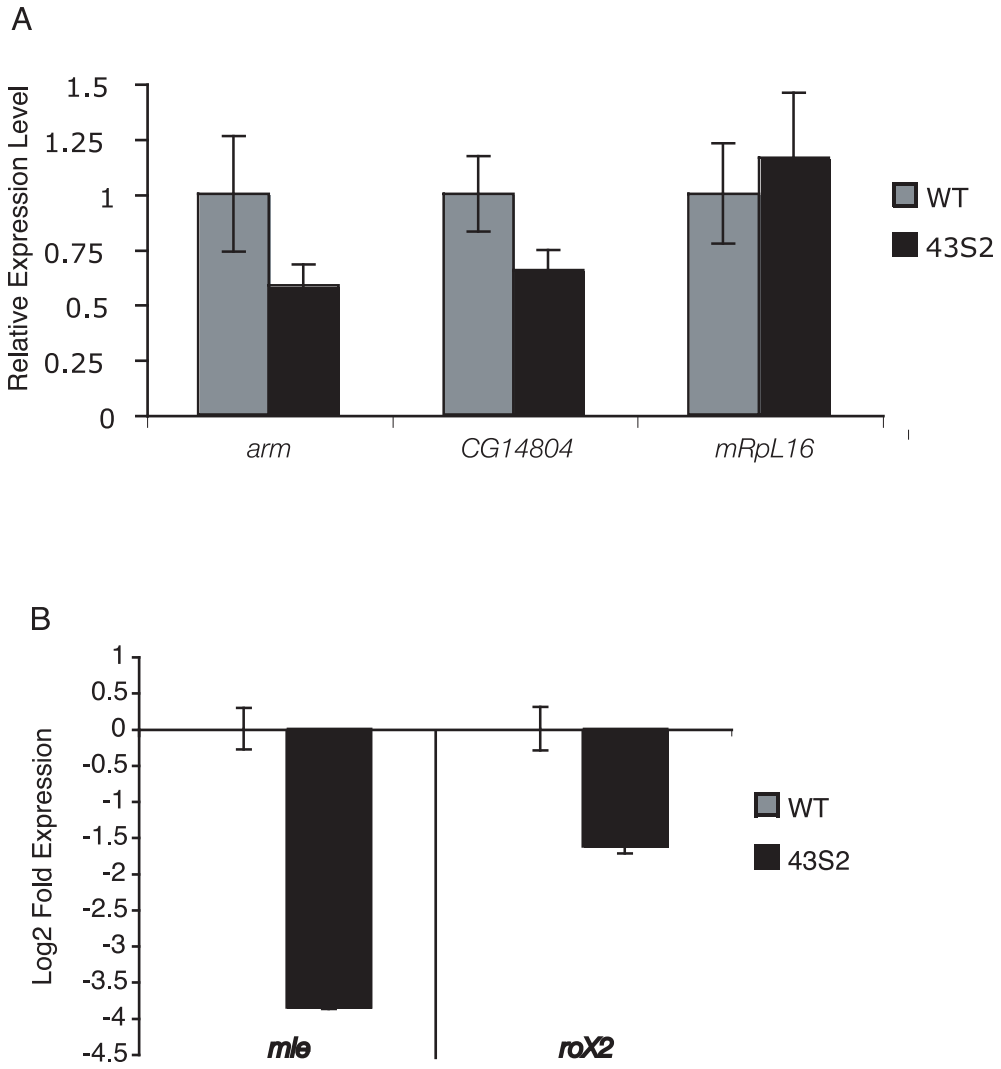

A
Relative Expression Level
1.5
1.25
1
0.75
0.5
0.25
0
arm
CG14804
mRpL16
WT
43S2
B
Log2 Fold Expression
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
mle
roX2
WT
43S2

Figure 7

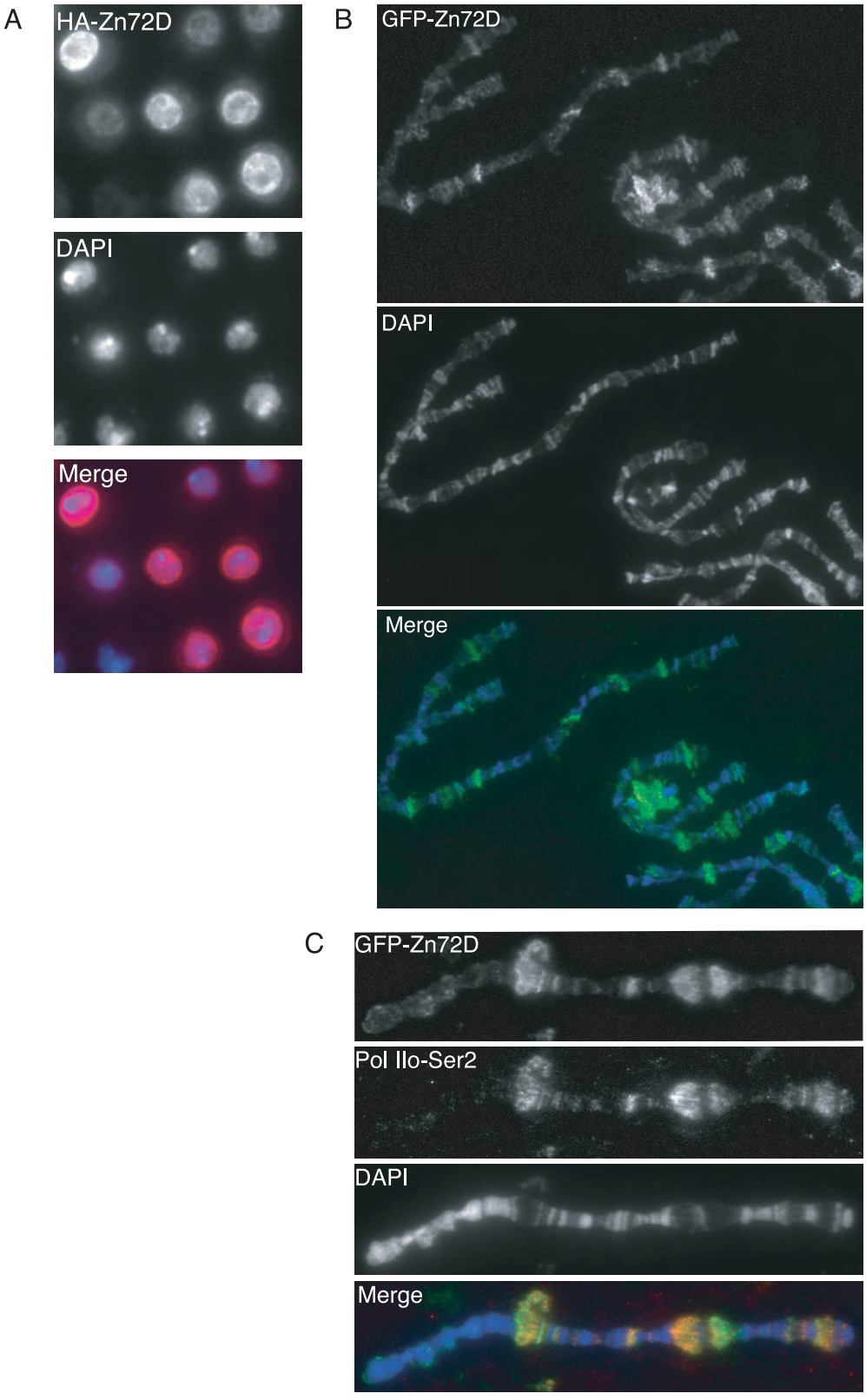

A
HA-Zn72D
DAPI
Merge
B
GFP-Zn72D
DAPI
Merge
C
GFP-Zn72D
Pol IIo-Ser2
DAPI
Merge

Figure 8

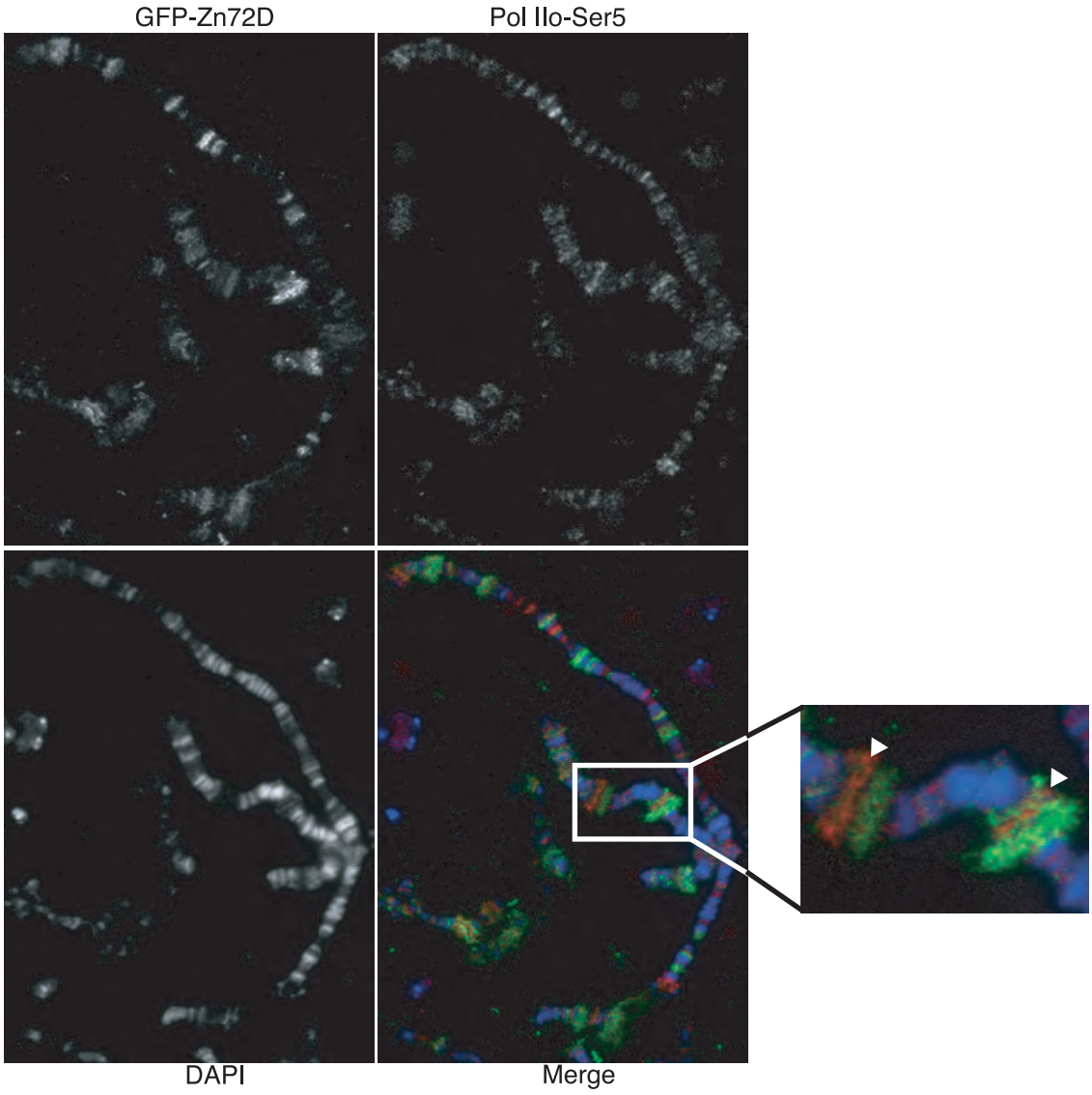


Figure 9

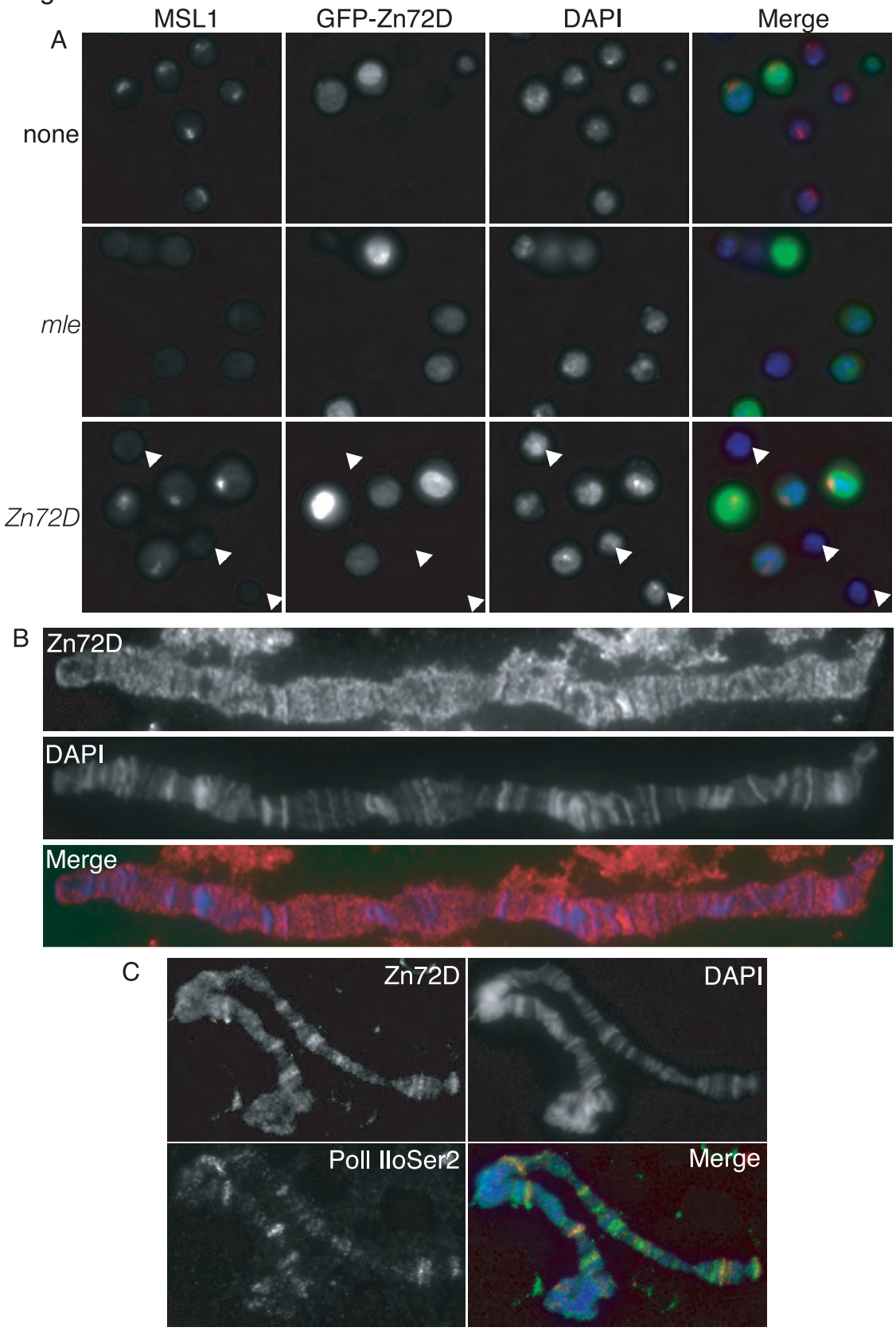

MSL1
GFP-Zn72D
DAPI
Merge
A
none
mle
Zn72D
B
Zn72D
DAPI
Merge
C
Zn72D
DAPI
PolI IIoSer2
Merge

Figure 10

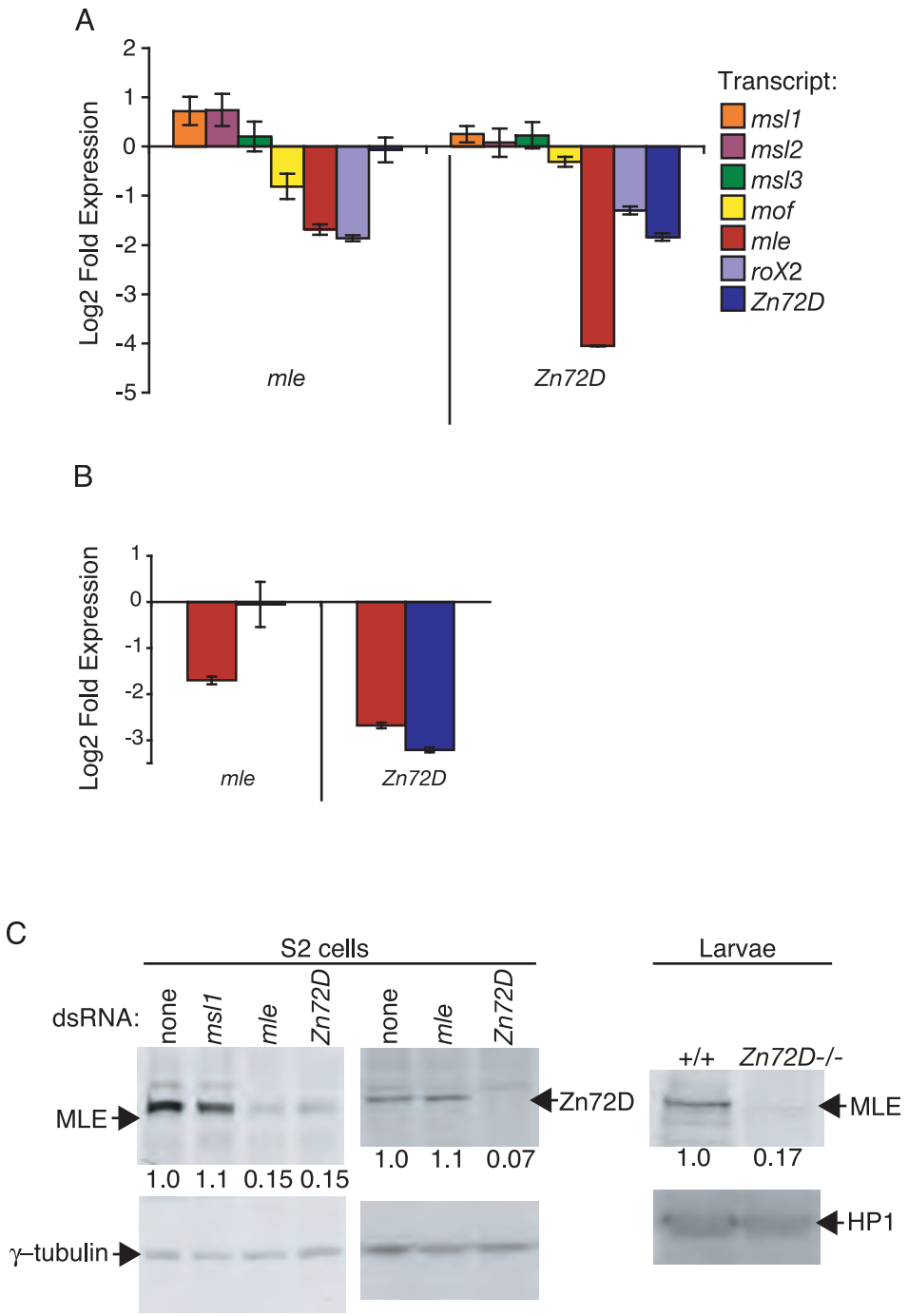
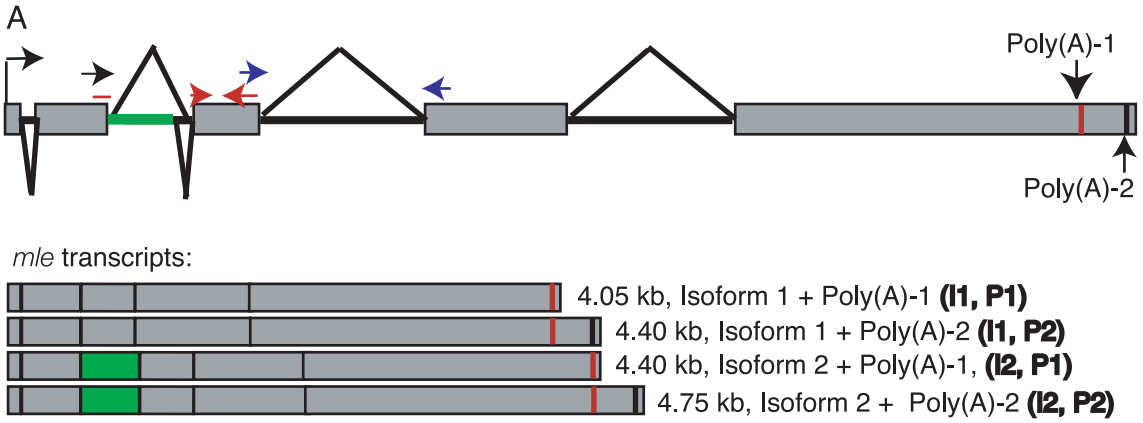
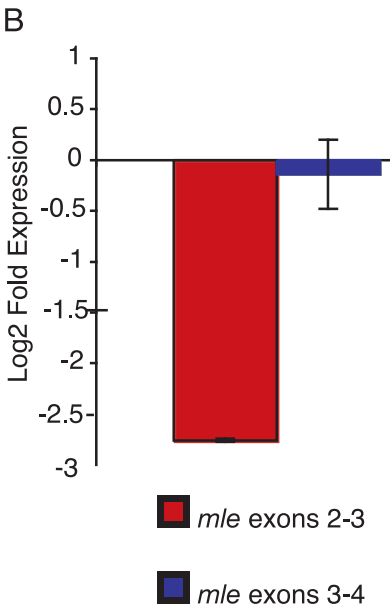
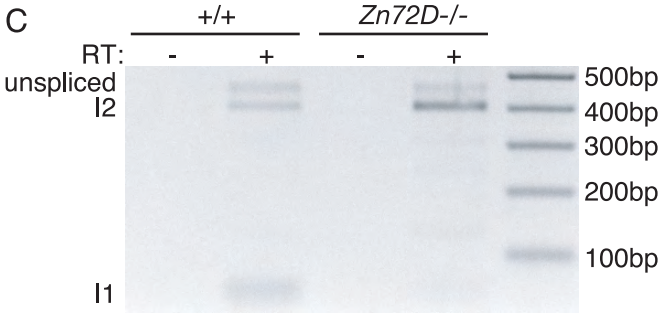

A
Log2 Fold Expression
2
1
0
-1
-2
-3
-4
-5
mle
Zn72D
Transcript:
msl1
msl2
msl3
mof
mle
roX2
Zn72D
B
Log2 Fold Expression
1
0
-1
-2
-3
mle
Zn72D
C
S2 cells
Larvae
dsRNA:
none
msl1
mle
Zn72D
none
mle
Zn72D
+/+
Zn72D-/-
MLE
Zn72D
MLE
1.0 1.1 0.15 0.15
1.0 1.1 0.07
1.0 0.17
γ–tubulin
HP1

Figure 11


A
Poly(A)-1
Poly(A)-2
mle transcripts:
4.05 kb, Isoform 1 + Poly(A)-1 (I1, P1)
4.40 kb, Isoform 1 + Poly(A)-2 (I1, P2)
4.40 kb, Isoform 2 + Poly(A)-1, (I2, P1)
4.75 kb, Isoform 2 + Poly(A)-2 (I2, P2)


B
Log2 Fold Expression
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
mle exons 2-3
mle exons 3-4


C
+/+
Zn72D-/-
RT: - + - +
unspliced I2
I1
500bp
400bp
300bp
200bp
100bp

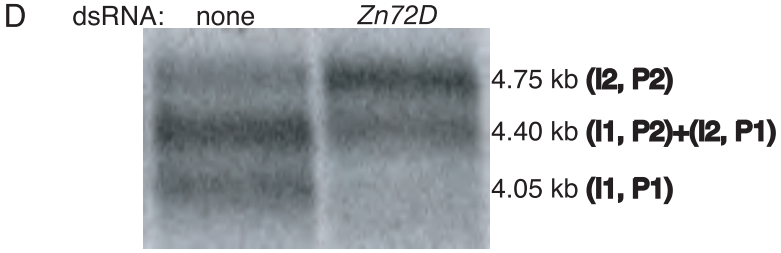

D
dsRNA: none
Zn72D
4.75 kb (I2, P2)
4.40 kb (I1, P2)+(I2, P1)
4.05 kb (I1, P1)

Figure 12

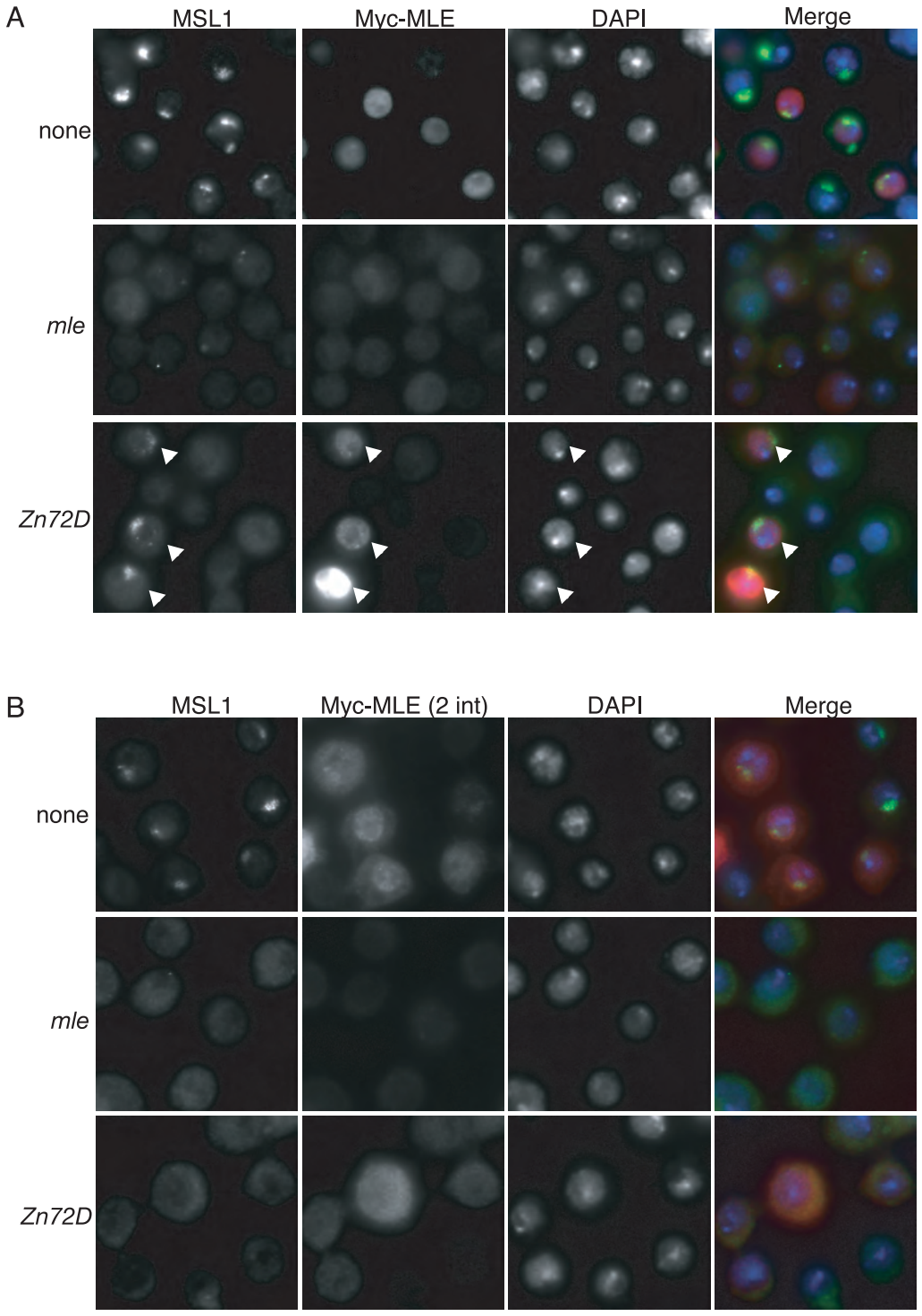
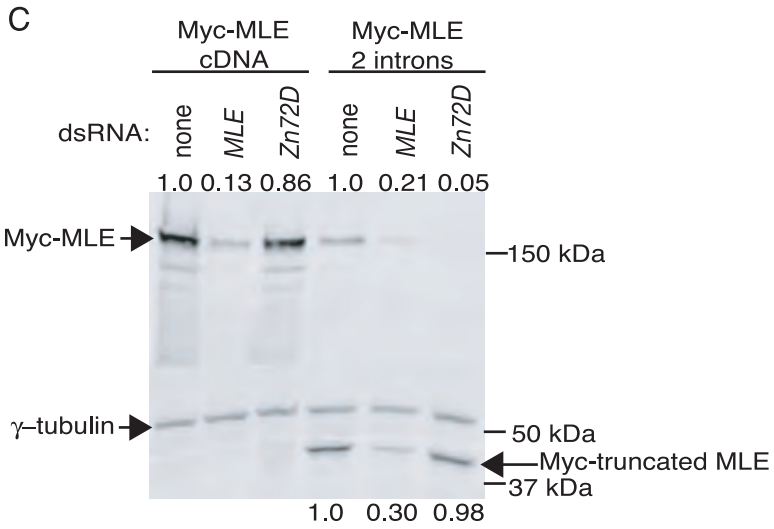

A
MSL1
Myc-MLE
DAPI
Merge
none
mle
Zn72D
B
MSL1
Myc-MLE (2 int)
DAPI
Merge
none
mle
Zn72D

Figure 12 continued



Chapter II

Zn72D and Belle Inhibit Translation of the *maleless* Transcript

I. Abstract

While in the nucleus, mRNAs may acquire information to target them to particular regions of the cytoplasm for translation. One mechanism through which to acquire this targeting information is through the protein complexes deposited on the mRNAs during splicing. The *Drosophila* zinc finger protein Zn72D is required for productive splicing of the *maleless* (*mle*) transcript, which encodes for the MLE protein, a component of the Male Specific Lethal (MSL) complex of proteins enriched on the X chromosome in male flies. We have identified proteins that interact with Zn72D, including the DEAD box helicase Belle (Bel). Co-knockdown of the Zn72D and Bel proteins restores the MSL complex localization to the X chromosome that is lost when only Zn72D is knocked down. Loss of Zn72D and Bel restores the level of MLE protein from less productively spliced *mle* transcript compared to wild-type cells, suggesting that Zn72D and Bel regulate translation of the *mle* transcript. Zn72D is therefore implicated as a factor linking splicing to translation.

II. Introduction

One way to target proteins to particular regions of a cell where they are needed is through intracellular localization of mRNAs. This has been well demonstrated in the *Drosophila melanogaster* oocyte, where the anterior-posterior and dorsal-ventral body axes are patterned by the localization of the *bicoid*, *nanos*, *gurken*, and *oskar* transcripts (reviewed in Johnstone and Lasko, 2001). mRNAs may acquire information about cytoplasmic destination in the nucleus. For example, for the *oskar* transcript it has been shown that splicing of the first exon-exon junction is required for proper localization of

the transcript to the posterior end of the oocyte where it is translated (Hachet and Ephrussi, 2004). The exon junction complex components Y14 and Mago nashi are also necessary for the correct cytoplasmic localization of the *oskar* transcript (Hachet and Ephrussi, 2001) (Mohr et al., 2001) (Newmark and Boswell, 1994).

Belle (Bel) is a DEAD box helicase that colocalizes with the translational regulator, DEAD box helicase Vasa at the oocyte posterior, thus implicating Bel having a role in regulating local translation (Johnstone et al., 2005). Bel is homologous to and can functionally substitute for the Ded1p protein in yeast, which is required for translation (Chuang et al., 1997; Johnstone et al., 2005). Bel is also homologous to DDX3 in humans, which has been implicated in numerous cellular processes. DDX3 is a nucleocytoplasmic shuttling factor, binds the nuclear export factor CRM1, and is a component of neuronal cytoplasmic transport granules, implicating it in mRNA transport (Kanai et al., 2004; Yedavalli et al., 2004). It has also been isolated with a functional spliceosome and was shown to associate specifically with spliced mRNP complexes (Merz et al., 2007; Zhou et al., 2002). Recently, DDX3 was shown to interact with eIF4E and inhibit cap-dependent translation (Shih et al., 2007).

We previously demonstrated that the *Drosophila* zinc finger protein Zn72D is required for proper splicing of the *maleless* (*mle*) transcript. The MLE protein is an essential component of the Male Specific Lethal (MSL) complex of proteins that is enriched on the single X chromosome in male flies and upregulates X-linked gene expression twofold to equalize gene expression between males and females, with two X chromosomes (Lucchesi et al., 2005). In the absence of Zn72D, the *mle* transcript preferentially retains part of the second intron, which contains in-frame stop codons.

This results in production of *mle* transcripts that do not code for functional MLE protein, and therefore the MSL complex does not localize to the X chromosome (Worringer and Panning, 2007). Zn72D is predominantly in the nucleus and colocalizes with elongating RNA Polymerase II on polytene chromosomes, but there is also some Zn72D in the cytoplasm. The human homologue of Zn72D, ZFR, is also mainly in the nucleus but a subset of it is found in the cytoplasm in neuronal granules that contain the Staufen2, a protein involved in mRNA transport and localization. ZFR interacts with and is required for the cytoplasmic localization of the Staufen2^{62} isoform (Elvira et al., 2006). As granules are involved in translational regulation and localization of mRNAs, these data suggest that Zn72D and ZFR may have a role in linking splicing, translation, and cytoplasmic localization of mRNAs.

Here we identify Bel among several proteins that interact with Zn72D. In addition we find that co-knockdown of *zn72d* and *bel* rescues localization of the MSL complex to the X chromosome. The level of MLE protein is restored to ~70% of wild-type levels in the double knockdown even though the level of productively spliced *mle* transcripts is still four-fold lower than in wild-type cells, indicating that loss of Zn72D and Bel proteins relieves a translational block to MLE protein production.

III. Materials and Methods

Cell Culture and Generation of Stable Cell Lines

S2 cells were grown in Schneider's media plus 10% fetal bovine serum, penicillin and streptomycin. Cells were maintained according to the Invitrogen *Drosophila* Expression System Protocol. S2 cell lines expressing GFP-MSL2 and HA-Zn72D were

described previously (Worringer and Panning, 2007). pAM-CG5641 (Myc-CG5641) was cloned using the Invitrogen Gateway system. 20μg of the plasmid plus 1μg pCoBlast (Invitrogen) was transfected into S2 cells using the protocol described in the Invitrogen *Drosophila* Expression System Protocol and selected with 15μg/mL Blasticidin S HCl.

Co-immunoprecipitation

S2 cells from a 10 cm dish were washed with 1xPBS and lysed in lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 0.1% TritonX-100, supplemented with protease inhibitors and RNasin [Promega]). For Co-IPs that were performed in the presence of RNase A, RNAsin was left out of the buffer and RNAse A was added at a concentration of 0.1mg/mL after sonication. The lysate was sonicated three times, 10 seconds each (on ice for one minute in between pulses) on setting 3, constant duty cycle on a Branson sonicator. The extract was clarified by centrifugation at 14,000 rpm for 15 min. at 4 degrees. At least 600ug extract was added to the 10 μL of Dynal Dynabeads (Invitrogen), precaptured with the appropriate antibody. Beads and extracts were rotated overnight at 4 degrees and then washed 3 times with lysis buffer. Proteins were boiled off the beads in 1x sample buffer. (2x sample buffer: 8.3% glycerol, 1.25% SDS, 0.1M Tris-HCl pH 6.7, 0.083mg/mL bromophenol blue, 50μL/mL 2-mercaptoethanol.) For large scale IPs for mass spectrometry, 45mg of clarified S2 cell extract was added to 250 μL protein G Dynal Dynabeads preincubated with 25 μL HA.11 antibody (Covance). The proteins boiled off the beads were loaded on a 7.8% SDS-PAGE gel and which was later stained with G-250 coomassie blue.

On-line Capillary LC-MS and LC-MS-MS Analysis

Affinity-purified Zn72D-containing samples were separated by SDS-PAGE gel, in-gel digested and analyzed by LC-MS and LC-MS-MS as described previously (Chu et al., 2006). Briefly, 1μl aliquot of the digestion mixture was injected into an Ultimate capillary LC system via a FAMOS Autosampler (LC Packings, Sunnyvale, CA), and separated by a 75 μm × 15 cm reverse-phase capillary column at a flow rate of ~330 nl/min. The HPLC eluent was connected directly to the micro-ion electrospray source of a QSTAR Pulsar QqTOF mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA). Typical performance characteristics were > 8000 resolution with 30 ppm mass measurement accuracy in both MS and CID mode. LC-MS data were acquired in an information-dependent acquisition mode, cycling between 1-s MS acquisition followed by 3-s low energy CID data acquisition. The centroided peak lists of the CID spectra were searched against the National Center for Biotechnology Information (NCBI) *Drosophila melanogaster* protein database using Batch-Tag, a program in the in-house version of the University of California San Francisco ProteinProspector package. The CID spectra were further inspected manually. Protein hits with more than two confident MSMS spectra are reported in the table.

Western blotting

S2 cells +/- dsRNA treatment for 6 days were counted, spun down, and lysed in 1x Sample buffer (2x sample buffer: 8.3% glycerol, 1.25% SDS, 0.1M Tris-HCl pH 6.7, 0.083mg/mL bromophenol blue, 50μL/mL 2-mercaptoethanol) at 5x10^4 cells/μL. Samples were boiled for 5 minutes and spun down at 14,000rpm at 4 degrees for 20

minutes. 15μL lysate was loaded per lane of a 7.8% gel. Gels were transferred to nitrocellulose, blocked with 1% nonfat dry milk/0.05% Tween/1xPBS and probed overnight at 4 degrees with mouse anti-HA antibody at a 1:2000 dilution (HA.11, Covance), guinea pig anti-MLE antibody at 1:500 (gift from John Lucchesi), mouse anti-γ-tubulin at 1:1000 (GTU-88, Sigma), rabbit anti-Belle at 1:2500 (gift from Paul Lasko), and chicken anti-Zn72D serum at 1:200 (Worringer and Panning, 2007). Donkey anti-guinea pig Cy3 (1:1000), anti-mouse Cy3 (1:500), anti-rabbit (1:1000), and anti-chicken (1:500) (Jackson ImmunoResearch), were used as a secondary antibodies and detected using a Typhoon 9400 instrument and quantitated using Quantity One software (Bio-Rad). MLE levels were normalized to γ-tubulin.

RNAi

dsRNAs were added to S2 cells (~15μg/mL) in 50% conditioned/50% fresh Schneider's media. The following primers were used to produce PCR products which were used in T7 in-vitro transcription reactions to produce RNA. *mle*(s): GGGCGGGTTTATGGCTTCGTACTCTAGCACC, *mle*(as): GGGCGGGTAAGTTAAGCCAGTTGTCAACGC; *belle(s):* GGGCGGGTGTCTGGACTTGAATGGCGGC, *belle*(as) GGGCGGGTCGTTGTAGTTGTCCTCGAAACGTC*; Zn72D*(s): GGGCGGGTGTTGAACTTACAATCGCACAGC, *Zn72D*(s): GGGCGGGTACTGGTATCAGCGTAAGATGGG; or *Zn72D*3'UTR(s): GGGCGGGTGCGGCGAGAATAGGTTATATAC, *Zn72D*3'UTR(as): GGGCGGGTCCGCTTCGTTCTAGTATTTGTG.

qRT-PCR

qRT-PCR was performed as described previously (Worringer and Panning, 2007). The primers used are described below:

mle QF1: CGGAACACGCTAGGAGCTTT, QR1: TGAGCGCCGGCACAT; *mRpL16* QF2: TCAACACAGCCGGTCTTAAGTAT, QR2: GGCTGCTCCACATTCTGGTA; *arm*-F GCTGCTGAACGATGAGGATCA, *arm*-R: CCAAAGCGGCTACCATCTGA, *CG14804*-F: CTGAGCACAAGACGGCAGAG, *CG14804*-R: GAGGGTCACGTTCACCTTGC, *RpII140*-F: CACAATGGCGGCGGTT, *RpII140*-R: ACGCAGATGTTCAGGCAGAGT (Straub et al., 2005).

Northern blotting

Northern blots were performed with a NorthernMax Kit (Ambion) and BrightStar-Plus membrane (Ambion). 10µg of RNA from S2 cells treated +/- Zn72D dsRNA for 6 days was loaded per lane. Blots were probed with a labeled DNA probe antisense to the full-length MLE cDNA, and exposed to a phosphor screen overnight.

Sucrose Gradient Fractionation

3x10^6 S2 cells were seeded in 10 mL media in a 10 cm dish and either treated or not with dsRNA against *zn72d* and *bel*. Four days later, cells were removed from the dish and cycloheximide was added to the media to a final concentration of 90µg/mL and the cells were rocked at room temperature for 10 minutes. Cells were then spun down at 2000rpm for 5 min. at room temp and washed with 5 mL cold PBS (-Magnesium,-Calcium) in the presence of 90µg/mL cycloheximide and placed on ice for 5 min. Cells

were spun down and then washed with 5 mL cold PBS (+Magnesium, +Calcium) and spun down again. Cells were resuspended in 1 mL cold PBS (+Magnesium, +Calcium) and spun down. Cells were resuspended on ice in 150 μL cold Reticulocyte Standard Buffer (RSB: 10mM NaCl, 10 M Tris-HCl pH 7.4, 15 mM $MgCl_2$) containing 100μg/uL heparin. 18μL of lysis buffer (10% Triton X-100, 10% deoxycholate in RSB) was added and then samples were mixed for 3 seconds on a vortex. Samples were incubated for 5 min. on ice, and then mixed again for 3 seconds before centrifugation at 10,000 rpm for 5 min. at 4 degrees. The supernatant was diluted with 168 μL polysomal buffer (25 mM Tris-HCl pH 7.5, 10mM MgCl2, 25 mM NaCl, 0.05% Triton X-100, 0.14 M sucrose, 500 ug heparin/mL). Samples were loaded onto 5-60% sucrose gradients. The gradients were centrifuged at 37,000 rpm for 2.5 hours at 4 degrees in a Beckman SW40 Ti swing out rotor and fractionated on an ISCO gradient fractionator. Fractions were Trizol extracted, isopropanol precipitated, and then further purified over Rneasy columns (Qiagen).

IV. Results

Identification of proteins that interact with Zn72D

We performed mass spectrometry to identify proteins that co-immunoprecipitate with HA-tagged Zn72D in *Drosophila* S2 cells in order to gain further insight into the role of Zn72D. Proteins that co-IP with anti-HA antibody in S2 cells expressing HA-Zn72D but not in wild-type S2 cells are identified in Figure 1A and Table 1. Zn72D interacts with Bel, EF1α48D, Fragile X Mental Retardation Protein (FMR1), Hrp59, insulin growth factor II mRNA-binding protein (IMP), Argonaute 2, Poly A Binding

Table 1. Identification of Zn72D-interacting proteins by Mass Spectrometry

Annotation Symbol	Name	number of peptides	% coverage
CG7349	Argonaute 2	6	7%
CG5215	HA-Zn72D	44	43%
CG5787	CG5787	6	5%
CG4147	Belle	4	5%
CG6203	FMR1	2	3%
CG4147	Hsc70-3	28	41%
CG4264	Hsc70-4	16	26%
CG14648	CG14648	8	14%
CG9393	Hrp59	2	3%
CG5119	PABP	11	25%
CG1691	IMP	2	3%
CG5641	CG5641	21	49%
CG8280	EF1α48D	6	12%
CG5641	CG5641	25	55%
CG5502	RpL4	3	6%
CG7434	RpL22	3	16%
CG6779	RpS3	7	23%
CG2168	RpS3A	6	20%
CG17489	RpL5	4	18%
CG11276	RpS4	18	64%
CG1263	RpL8	4	17%
CG10944	RpS6	3	10%

Protein (PABP), and several ribosomal proteins, all of which have been shown to be involved or implicated in one or more aspects of RNA metabolism, including splicing, transport/nucleocytoplasmic shuttling of RNAs, RNA binding, translation, and RNA interference (Condeelis, 1995; Costa et al., 2005; Geng and Macdonald, 2006; Hammond et al., 2001; Hase et al., 2006; Hovemann et al., 1988; Ishizuka et al., 2002; Johnstone et al., 2005; Kiesler et al., 2005; Mangus et al., 2003; Xu et al., 2004). In addition, three proteins of unknown function co-IP with Zn72D, CG5787, CG14648, and CG5641. We confirmed that three proteins, CG5641, FMR1, and Bel, co-IP with Zn72D by western blotting (Fig1B and 1C). CG5641 has the DZF domain, which is a domain found in Zn72D and is also found in some other C2H2 zinc finger-containing or dsRNA binding proteins. As the interaction between Zn72D and Bel proved to be interesting, we performed co-IPs between these proteins in the presence or absence of RNAse A to determine if the interaction required RNA (Fig 1C). Bel and Zn72D co-IP whether or not RNAse A was added to the extract, indicating that the interaction between Zn72D and Bel is independent of RNA.

We have previously shown that Zn72D is mostly but not entirely nuclear (Worringer and Panning, 2007), similar to the Zn72D homologue ZFR: a subset of ZFR was shown to colocalize in Staufen2 containing granules in the cytoplasm of neurons (Elvira et al., 2006). A portion of HA-tagged Zn72D was also found in the cytoplasm with Belle, as observed by coimmunostaining with antibodies to the HA tag and Belle (Fig 2A). It appears that we had predominantly identified proteins that were likely to interact with Zn72D in the cytoplasm; there may be additional factors that interact with Zn72D in the nucleus that we did not identify here. One additional localization pattern of

interest among five of the proteins, Zn72D, Bel, FMR1, CG5641, and Hrp59, is that they display a common pattern of localization during stages 13-16 of embryonic development consistent with a role in the central nervous system development (Tomancak et al., 2007; Van Emden et al., 2006; Wan et al., 2000).

Co-knockdown of *zn72d* and *bel* restores MSL complex localization to the X chromosome and X-linked gene expression

We have previously shown that Zn72D promotes productive splicing of the *mle* transcript. In the absence of Zn72D, a downstream splice site in the second intron of *mle* is preferentially utilized, resulting in partial retention of the intron (Worringer and Panning, 2007). Therefore, the role of Zn72D in regulating the MSL complex localization to X chromosome in male flies and male cells is through its promotion of proper splicing of *mle*. We knocked down each protein that co-IPs with Zn72D and determined that none are required for localization of the MSL complex as is Zn72D (data not shown). However, we also tested knockdown of each protein that co-IPs with Zn72D in combination with *zn72d* knockdown and found that for one co-knockdown, *zn72d* and *bel*, MSL complex localization to the X chromosome was restored (Fig 2B). This suggests a simple model in which the role of Zn72D is to inhibit Bel from blocking some aspect of MSL complex localization, and in the absence Bel, Zn72D is no longer required.

As Bel has been implicated as being involved in the RNAi pathway downstream of dsRNA uptake (Ulvila et al., 2006), we wanted to determine if the phenotype observed was solely due to a failure to completely knockdown *zn72d* upon co-knockdown *zn72d*

and *bel*. To address this issue, we performed western blots using antibodies directed against Zn72D and Bel when *zn72d*, *bel*, or both was knocked down (Fig 2C). Zn72D appeared to be equivalently knocked down in both the *zn72d* single and *zn72d+bel* knockdown, indicating that knocking down *bel* is not interfering with knockdown of *zn72d*. The level of Bel protein also decreased upon knockdown of *bel* and in the co-knockdown. Another way Bel could have been functioning was through Crm1, since the Bel human homologue DDX3 has been shown to bind to CRM1 and undergo nucleocytoplasmic shuttling (Yedavalli et al., 2004). We performed co-knockdown of *Drosophila crm1* and *zn72d*, but this did not mimic the *bel+zn72d* co-knockdown phenotype of restoration of the MSL complex localization to the X chromosome (data not shown).

When both *zn72d* and *bel* are knocked down, the MSL complex is on the X chromosome, in contrast to knockdown of *zn72d* by itself. Previously we showed that Zn72D is required for proper X-linked gene expression in males (Worringer and Panning, 2007). To determine if, in addition to restoration of the MSL complex to the X chromosome upon co-knockdown of *zn72d* and *bel*, X-linked genes are also being expressed akin to normal, we performed quantitative reverse transcription PCR (qRT-PCR) for three X-linked transcripts and one autosomal transcript as a control. S2 cells were either untreated or treated with dsRNAs against *mle*, *zn72d*, *bel*, or *zn72d+bel*. The level of X-linked transcripts *arm*, *CG14804*, and *mRpL16* were decreased by about twofold upon knockdown of *mle* or *zn72d* (Fig. 2D). Knockdown of *bel* had little to no affect on X-linked gene expression (Fig 2D). Co-knockdown of *zn72d* and *bel* restored X-linked gene expression of *CG14804* and *RpL16* and partially rescued expression of

arm (Fig 2D). Therefore, in addition to restoring MSL complex localization to the X chromosome, knocking down both *zn72d* and *bel* also restores proper X-linked gene expression.

Co-knockdown of *zn72D* and *bel* only partially rescues the *mle* splicing

If MSL complex localization is restored to the X chromosome when *zn72d* and *bel* are knocked down together, we expected that productive splicing of the *mle* transcript would be restored. To address this, qRT-PCR was performed using a primer set that specifically amplifies the productively spliced transcript (Worringer and Panning, 2007). As a control, spliced *mle* transcript decreases upon knockdown of *mle* (Fig. 3B). The level of spliced *mle* transcript was decreased about 16-fold upon Zn72D knockdown compared to wild-type cells, as expected (Fig. 3B). The level of spliced *mle* transcript was barely affected by *bel* knockdown and decreased about four-fold upon *zn72d+bel* co-knockdown (Fig. 3B). The co-knockdown result indicates that there is some recovery of splicing of the *mle* transcript upon co-knockdown, compared to knockdown of Zn72D alone. This was confirmed by Northern blot analysis, where the intensity of bands corresponding to productively spliced *mle* transcript is more intense in the co-knockdown than in the Zn72D single knockdown; however, there is still an abundance of *mle* transcripts that retain part of the intron (Fig 3A and 3C). These results rule out the simple model whereby Zn72D inhibits Bel from inhibiting productive splicing of *mle*.

Zn72D and Bel likely inhibit translation from the *mle* transcript

Since co-knockdown of *zn72d* and *bel* results in restoration of MSL complex localization to the X chromosome, we expected that more MLE protein was being produced compared to the single *zn72d* knockdown. Quantitative western blots for MLE protein were performed on S2 cell extracts in which *mle*, *zn72d*, *bel* or *zn72d+bel* had been knocked down. In the absence of *zn72d*, levels of MLE protein decreased as observed previously (Fig 3D and Worringer and Panning, 2007). Upon knockdown of *bel*, MLE protein levels appeared unaffected. When *zn72d* and *bel* were knocked down together, MLE protein levels were restored to 70% of wild-type levels. This 70% of MLE protein is produced from 4-fold less productively spliced *mle* mRNA compared to wild-type cells. Therefore in wild-type cells, ~75% of the *mle* transcript must only produce about 30% of MLE protein. As high levels of protein are produced from a comparably small amount of *mle* transcript in the absence of *zn72d* and *bel*, this suggests that perhaps in wild-type cells the abundant *mle* transcript is not translated efficiently. Upon co-knockdown of *zn72d* and *bel*, there may be a release from translational inhibition, and in spite of the decreased levels of productively spliced *mle* transcript, increased levels of MLE protein are produced.

One way to explain poor translational efficiency of a particular mRNA transcript would be if was not associated with polysomes. To test if *mle* transcripts were associated with polysomes, we performed sucrose gradients with S2 cell extracts in the presence or absence of *zn72d* and *bel* dsRNA treatment. RT-PCR using primers specific for the productively spliced *mle* transcripts was performed on each fraction collected from the gradient. Unexpectedly, in both wild-type S2 cells and when *zn72d* and *bel* are knocked

down, the majority of productively spliced mle transcripts are found on polysomes. That spliced *mle* transcripts are found mostly on polysomes in wild-type S2 cells suggests that translational inhibition due to presence of Zn72D and Bel must occur downstream from translation initiation.

V. Discussion

We have identified several proteins involved in RNA metabolism that interact with Zn72D. Co-knockdown of the Zn72D-interacting protein Bel and Zn72D rescues the loss of the MSL complex and decreased X-linked gene expression observed in the absence of Zn72D. *zn72d+bel* co-RNAi minimally restores proper *mle* splicing but restores MLE protein levels to 70% of wild-type levels, implicating Zn72D and Bel as involved in involved in regulating translation of MLE. Since knockdown of Bel by itself has no affect on MLE protein levels, it seems likely that Bel needs to be recruited to *mle* transcripts by Zn72D and that these two proteins work together to repress production of MLE protein.

Post-Initiation Regulation of Translation

The *mle* transcript was unexpectedly found predominantly on polysomes in wild-type S2 cells, suggesting that Zn72D and Bel may act downstream of initiation of translation in order to block translation of MLE protein. This is not unlike what has been observed for the *oskar* transcript in oocytes, in which *oskar* mRNA is found on polysomes even when Oksar protein is not accumulating before *oskar* is localized to the posterior of the oocyte (Braat et al., 2004). In addition, a similar phenomenon has been

documented for the *nanos* transcript in the developing *Drosophila* embryo. *nanos* is expressed throughout the embryo but only about 4% is localized to the posterior end where it is translated (Bergsten and Gavis, 1999) and the rest is translationally repressed (Gavis and Lehmann, 1994). Over 50% of *nanos* transcripts were found on polysomes, suggesting that the mechanism by which *nanos* is translationally repressed occurs downstream from initiation (Clark et al., 2000). However, a recent study contradicted this claim (Qin et al., 2007) so it is unclear if the *nanos* transcript is found on polysomes yet translationally repressed post-initiation like oskar and as we propose for *mle*.

Regulation of MLE levels by Zn72D and Bel

MLE localizes to all chromosomes and throughout the nucleus when overexpressed (Richter et al., 1996; Worringer and Panning, 2007). The general affinity of MLE for RNA can be detrimental to the development to the fly since heat shock over-expression of transgenic MLE protein can result in male and female lethality (Richter et al., 1996). It is possible that translational repression by Zn72D and Belle is one mechanism by which levels of MLE protein are kept under control such that too much MLE protein is not produced. Overexpression of a transgenic MLE cDNA in S2 cells results in a large production of MLE protein; however, inclusion of the first two introns in the same transgene results in a reduction of the amount of MLE protein produced from the transgene (data not shown and Worringer and Panning, 2007). This suggests that perhaps recruitment of Zn72D to the *mle* transcript has the effect of not only productively splicing the transcript but also targeting it for translational regulation.

Zn72D is required for the productive splicing of *mle*, and during the process of splicing, it may bind the mRNA and remain associated with it and transport it to the cytoplasm for translation. Once in the cytoplasm, Zn72D may encounter Bel and these two proteins may act together to repress translation from the *mle* transcript. Alternatively, Bel may initiate its interaction with Zn72D in the nucleus, as it may be loaded onto the *mle* mRNA in the nucleus as part of the exon junction complex, as has been proposed for its homolog DDX3 (Merz et al., 2007). In either case, we suggest that Zn72D is a splicing factor loaded onto mRNAs in the nucleus that then targets these mRNAs for localized, regulated translation in the cytoplasm.

Acknowledgements

We thank Davide Ruggero, Tony Gaba, and Christina Spevak for assistance with sucrose gradient fractionation and polysome analysis. We appreciate Tony Shermoen for his continual support with the RNAi library. We thank Brian Margolin for help with statistical analysis. We greatly appreciate the generosity of Paul Lasko for providing the Belle antibody and John Lucchesi for providing the MLE antibody.

VI. Figure Legends

Figure 13. Identification of proteins that co-immunoprecipitate with Zn72D. (A) Co-IPs were performed with an anti-HA antibody in wild-type (wt) S2 cells and in S2 cells expressing HA-Zn72D. Proteins that co-IP with HA-Zn72D were identified by mass spectrometry. (B) (Top) HA-Zn72D co-IPs with Myc-CG5641 when cells expressing HA-Zn72D were transiently transfected with a vector expressing Myc-CG5641 (right 2 lanes) and co-IPs were performed using an anti-Myc antibody. Left 2 lanes are controls in which no Myc-CG5641 was transfected. (Bottom) HA-Zn72D co-IPs with FMR1 when co-IPs were performed using an anti-FMR1 antibody (left 2 lanes). In the right 2 lanes, IPs were performed in cells not expressing HA-Zn72D. (C) The interaction between Zn72D and Belle is RNA-independent. Co-IPs using anti-HA antibody were performed in the absence (lane 3) or presence (lane 6) of RNase A. Lane 9 is a control IP performed with an anti-HA antibody in S2 cells not expressing HA-Zn72D. The ethidium bromide stained gel to the right shows equivalent amounts of input extract loaded per lane, either untreated or treated with RNase A. For parts B and C, "in" indicates 2% of the input into each IP and "FT" indicates 2% of flow-through from each IP.

Figure 14. Co-knockdown of Bel and Zn72D restores wild-type MSL localization to the X chromosome and X-linked gene expression. (A) Zn72D and Bel are both present in the cytoplasm. Cells were stained with antibodies to HA (red), Bel (green), and DAPI (blue) to delineate the nucleus. The nucleolar staining in the anti-Bel image is the result of antibody cross-reactivity, as when *bel* is knocked down, the nucleolar staining remains

while the cytoplasmic Bel staining decreases (data not shown). (B) GFP-MSL2 expressing S2 cells were either untreated or treated with *zn72d, bel*, or *zn72d+bel* dsRNAs. (C) Western blots with anti-Zn72D antibody (top), anti-Bel antibody (bottom), or anti-γ-tubulin as a loading control, demonstrating knockdown of Zn72D and Bel proteins. (D) qRT-PCR for the X-linked genes *arm*, *CG14804*, and *mRpL16* and the autosomal gene *RpII140* was done after S2 cells were untreated (gray bars), or treated with dsRNAs to knockdown *mle* (white), *zn72d* (black), *bel* (blue), or *zn72d+bel* (red). Samples were normalized first to *rp49* and then to the untreated sample, setting untreated to 1. The error bars represent the average of three independent experiments, with qPCR performed in triplicate, and error was determined using standard error propagation methods.

Figure 15. Co-knockdown of *zn72d* and *bel* partially restores productively spliced *mle* mRNA levels and restores MLE protein levels to 70% of normal levels. (A) Depicted is the *mle* gene, which contains 5 exons (white boxes) and 4 introns (black horizontal lines). Poly(A)-1 and -2 are depicted as gray vertical lines within the fifth exon. (Bottom) Four *mle* transcript isoforms differ in retention of part of intron 2 (Isoforms I1 and I2) and usage of the upstream or downstream poly(A) sites (P1 and P2). The partially retained intron is shown as a thicker horizontal black line. (B) S2 cells were untreated or treated with *mle*, *Zn72D*, *bel*, or *zn72d+bel* dsRNAs and qRT-PCR was performed with primers to the *mle* transcript that specifically amplify isoform 1. Samples were normalized first to *rp49* and then to the untreated sample, setting untreated to 1. The error bars represent the average of four independent experiments, with qPCR performed in triplicate, and error

was determined using standard error propagation methods. (C) Northern analysis with RNA collected from S2 cells untreated or treated with dsRNAs to *zn72d*, *bel*, or *zn72d+bel*. The top band corresponds to isoform (I2,P2); the middle band corresponds to both isoforms (I1,P2) and (I2,P1); and lower band corresponds to isoform (I1,P1). In the absence of *zn72d*, only I2 isoforms remain. Upon *zn72d+bel* knockdown, there are predominantly I2 isoforms but a subtle increase in I1 isoforms compared to *zn72d* knockdown. (D) One representative western blot (from three independent experiments) for MLE protein upon knockdown of *mle*, *zn72d*, *bel*, or *zn72d+bel*. MLE levels were quantitated by normalizing to the amount of γ-tubulin and then averaging between the numbers obtained from running identical samples on two gels. (E) On the top are the sucrose gradient fractionation polysome profiles for wild-type S2 cell lysates and S2 cells co-knocked down with dsRNAs against *zn72d* and *bel*. RT-PCR was performed on each fraction from the sucrose gradient using the primers that specifically amplify isoform I1.

Figure 13

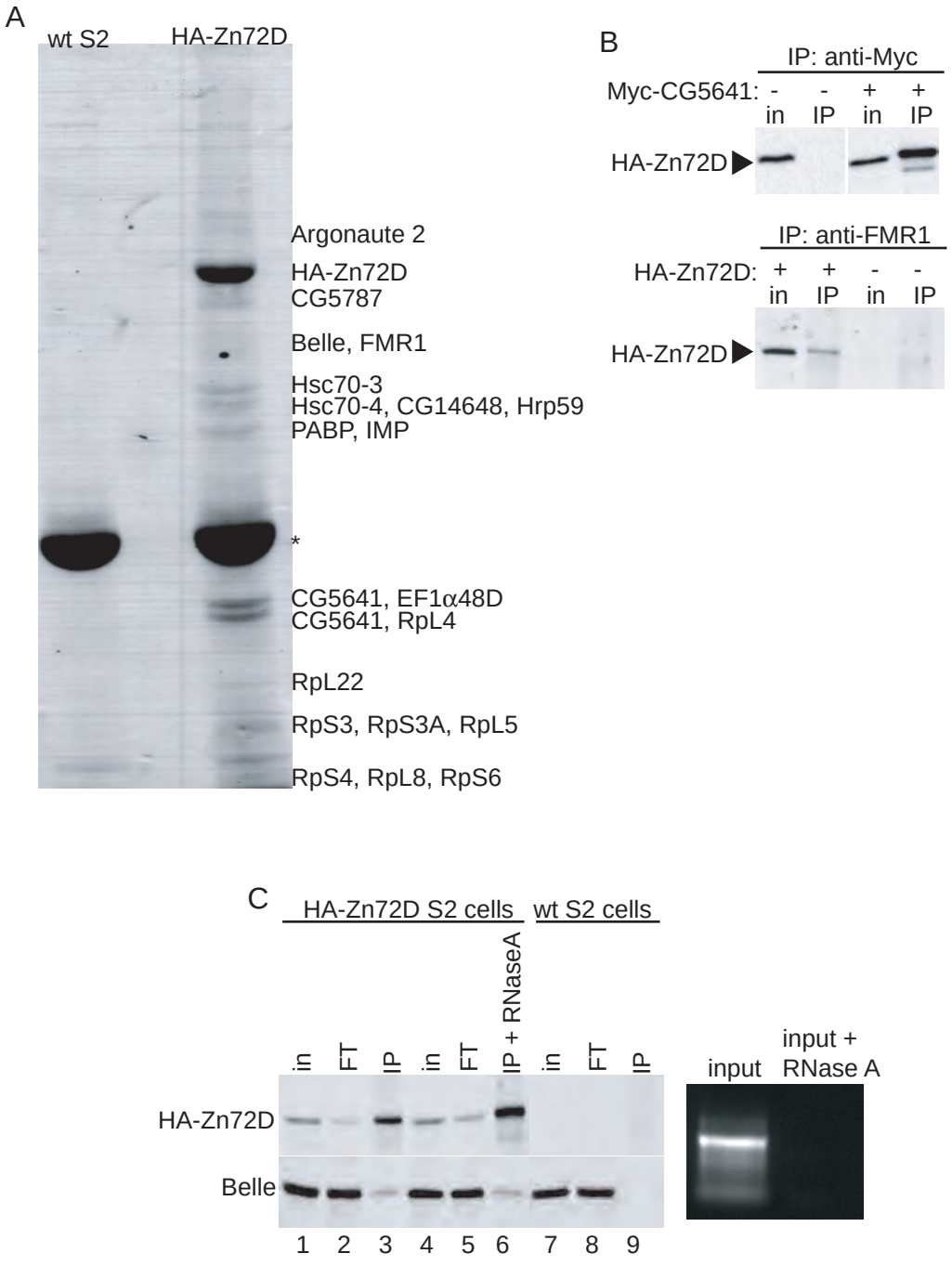

A
wt S2
HA-Zn72D
Argonaute 2
HA-Zn72D
CG5787
Belle, FMR1
Hsc70-3
Hsc70-4, CG14648, Hrp59
PABP, IMP
*
CG5641, EF1α48D
CG5641, RpL4
RpL22
RpS3, RpS3A, RpL5
RpS4, RpL8, RpS6
B
IP: anti-Myc
Myc-CG5641: - - + +
in IP in IP
HA-Zn72D
IP: anti-FMR1
HA-Zn72D: + + - -
in IP in IP
HA-Zn72D
C
HA-Zn72D S2 cells
wt S2 cells
in
FT
IP
in
FT
IP + RNaseA
in
FT
IP
HA-Zn72D
Belle
1 2 3 4 5 6 7 8 9
input
input +
RNase A

Figure 14

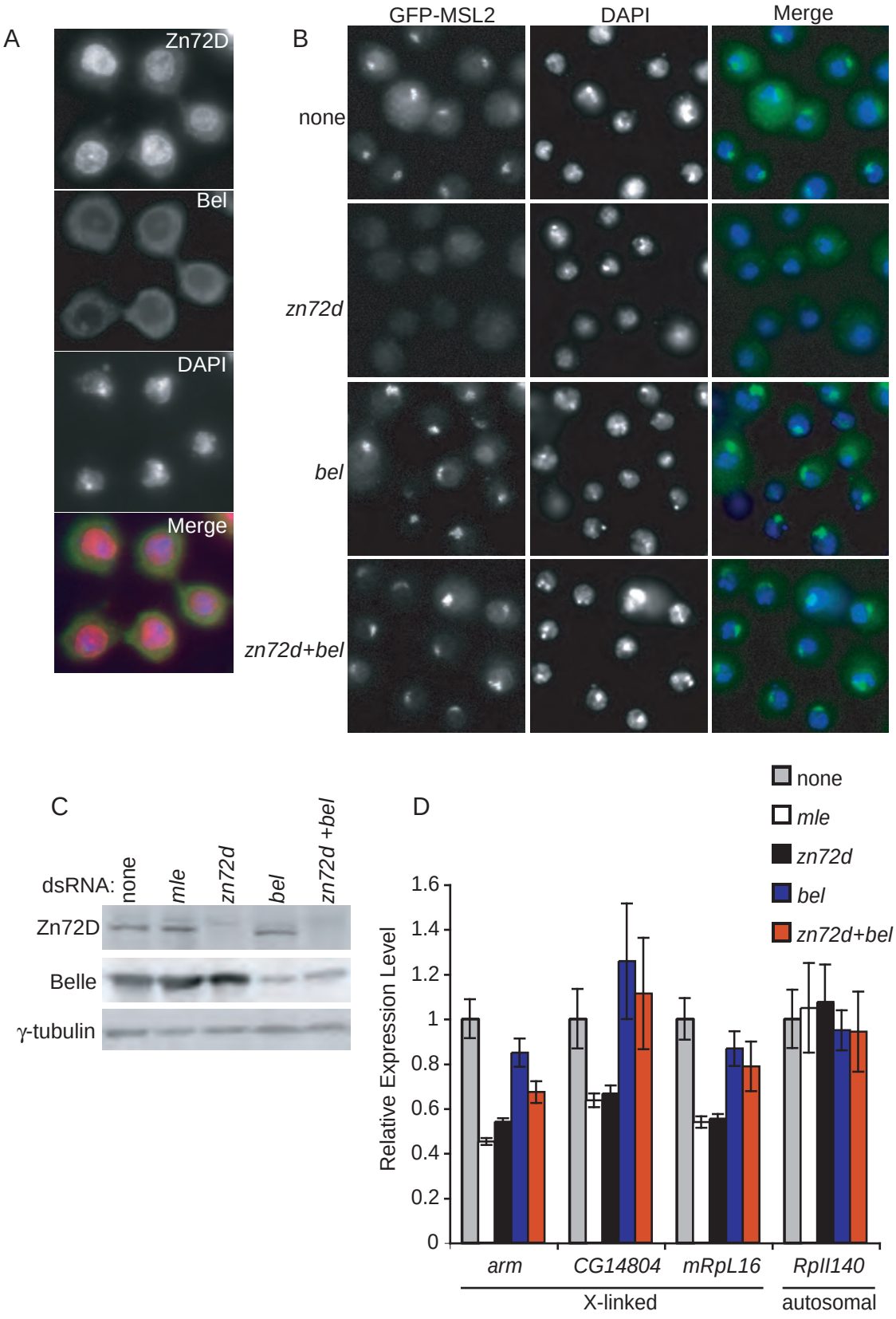

A
Zn72D
Bel
DAPI
Merge
B
GFP-MSL2
DAPI
Merge
none
zn72d
bel
zn72d+bel
C
dsRNA:
none
mle
zn72d
bel
zn72d +bel
Zn72D
Belle
γ-tubulin
D
none
mle
zn72d
bel
zn72d+bel
Relative Expression Level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
arm
CG14804
mRpL16
RpII140
X-linked
autosomal

Figure 15

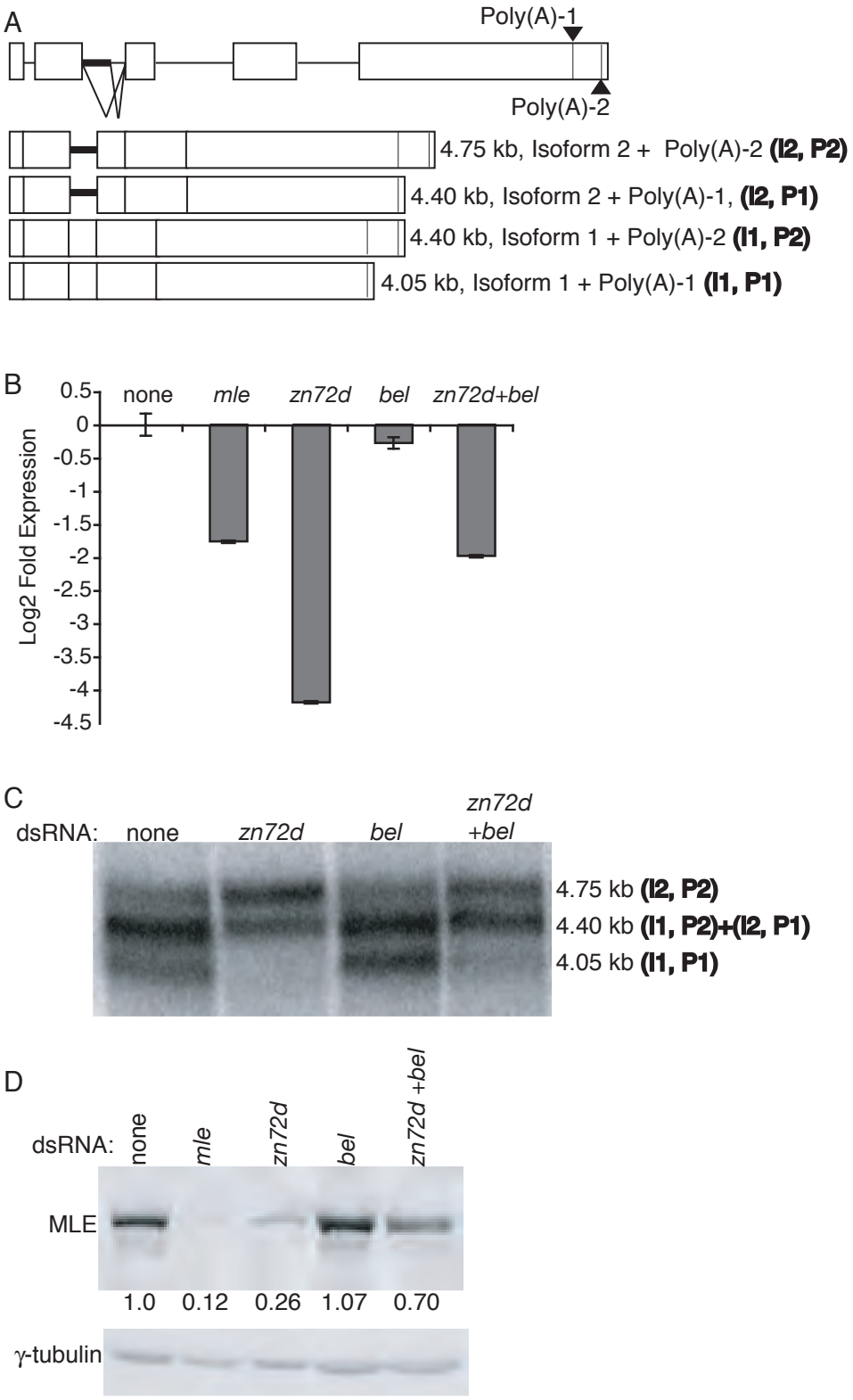
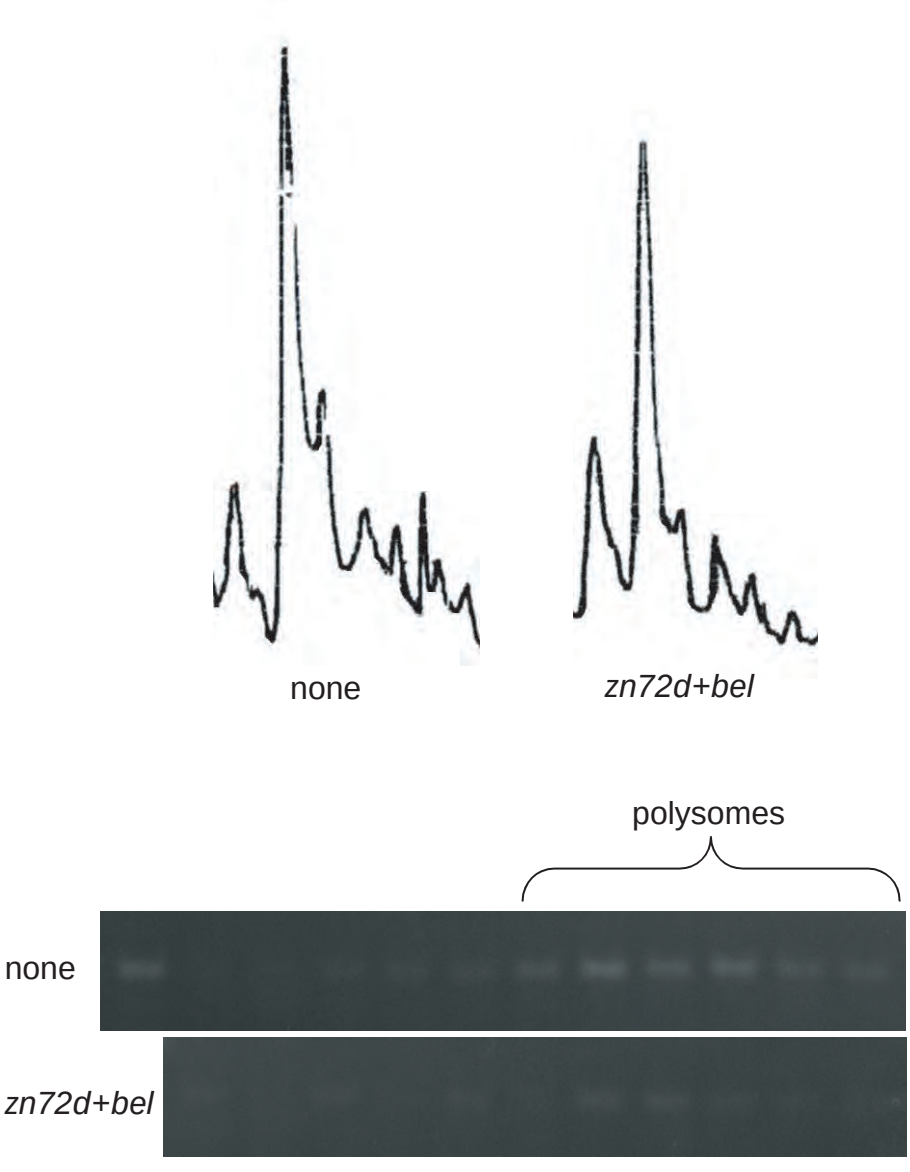

A
Poly(A)-1
Poly(A)-2
4.75 kb, Isoform 2 + Poly(A)-2 (I2, P2)
4.40 kb, Isoform 2 + Poly(A)-1, (I2, P1)
4.40 kb, Isoform 1 + Poly(A)-2 (I1, P2)
4.05 kb, Isoform 1 + Poly(A)-1 (I1, P1)
B
Log2 Fold Expression
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
none
mle
zn72d
bel
zn72d+bel
C
dsRNA:
none
zn72d
bel
zn72d +bel
4.75 kb (I2, P2)
4.40 kb (I1, P2)+(I2, P1)
4.05 kb (I1, P1)
D
dsRNA:
none
mle
zn72d
bel
zn72d +bel
MLE
1.0
0.12
0.26
1.07
0.70
γ-tubulin

Figure 15E



Concluding Remarks

Why regulate *mle* at the level of splicing and translation?

It is unclear why the *maleless* transcript is regulated at both the level of splicing and at the level of translation in the manner that it is: why use Zn72D to productively splice the majority of the mRNA only to inhibit translation from the majority of the same mRNA? One reason to have an abundance of transcript stalled on polysomes would be such that a cell could immediately resume translation of the protein if conditions changed such that the protein was needed. It is unclear when the MLE protein is needed during development or even in which tissues it is expressed. (It is also unclear why the cell would need to target translation of MLE to a particular region of the cytoplasm, given that it is a nuclear protein.) Perhaps MLE is expressed more efficiently in some tissues and less efficiently in others – in those that express Belle and Zn72D for instance. Performing *in situ* hybridizations for *mle* transcripts on *Drosophila* embryos would be a good first step to determining where *mle* is expressed during development. Just like for the *oskar* and *nanos* transcripts, the mechanism by which translational repression on polysomes has yet to be worked out.

To show that the regulation at the level of splicing and translation is important for the *mle* transcript, it would be necessary to remove the second intron from the endogenous *mle* gene. The prediction based on my work is that removal of the second intron would result in overproduction of MLE protein, as the transcript would no longer be targeted for translational control by Zn72D and Bel. Overproduction of MLE protein may result in fly lethality or other intermediate phenotypes due to MLE binding RNA throughout the nucleus.

A broader role for Zn72D and Belle in regulating gene expression

It is likely that Zn72D plays a much broader role in regulating gene expression outside of its role in dosage compensation. In order to identify new target genes, we have begun a collaboration with the UCSF Sandler Center Functional Genomics Core Facility. I have knocked down expression of *zn72d* in S2 cells and prepared RNA samples that will be amplified and hybridized to an Agilent array, designed based on the *Drosophila* Genome Resource Center DGRC-2 oligo array. We added probes in the region of the second intron of the *mle* transcript that is affected by *zn72d* knockdown, and we added two probes at the 3' end of the *zn72d* transcript for controls for *zn72d* knockdown.

Ideally we will identify new genes that Zn72D regulates by alternative splicing in S2 cells. To follow up on the genes identified in the microarray, we will perform RT-PCR across splice junctions on the candidate genes to determine if splicing has been affected as a result of knockdown of *zn72d*. In addition, we can test if *zn72d+bel* co-knockdown rescues splicing and/or protein levels to determine if the co-knockdown phenotype we observe for *mle* is specific for *mle* or a more general phenomenon of how Zn72D and Bel function together in the cell.

Very preliminary observations by post docs in Yuh Nung Jan's and Graeme Davis's lab suggested that there may be a phenotype in the Zn72D-/- larvae that looked like a defect in the neuromuscular junction or defect with the synaptic boutons. *In situ* hybridizations for Zn72D transcripts in embryos show a pattern indicating that Zn72D may have a role in the development of the central nervous system (Tomancak et al., 2007; Van Emden et al., 2006). This is consistent with the defects observed in the nervous system seen in Zn72D mutant larvae. It will be interesting to examine the results

of the microarray to see if there are genes involved in regulating nervous system development whose expression is dysregulated upon knockdown of *zn72d*. The *bel* transcript was more universally expressed but was also expressed in the nervous system, so it is possible that Bel and Zn72D may co-regulate translation of nervous system proteins.

Does ZFR play a role in splicing?

The human homologue of Zn72D, ZFR, has been shown to colocalize in Staufen2-containing neuronal granules, interact with Staufen2, and be required for Staufen2's localization. The Staufen proteins, which are encoded by two genes in humans, are RNA binding proteins that have been shown to be nucleocytoplasmic shuttling proteins involved in mRNA transport in neurons (reviewed in Miki et al., 2005). It is possible that ZFR, like Zn72D, is involved in splicing transcripts and then targeting them for localized translation. To address this, a similar microarray experiment could be performed in either human or mouse cells as we have performed in *Drosophila* S2 cells: knockdown ZFR and compare wild-type cells to the knockdown cells to determine which transcripts have had their levels affected by knockdown of ZFR. For each candidate gene which has its levels affected, examine splicing by doing RT-PCR using primers across each splice junction to determine which splice junction is affected by knock down of ZFR. If no splice junctions are differentially affected, it is possible that ZFR does not affect splicing but rather affects some other aspect of RNA metabolism of the effected genes.

Model for Zn72D and Bel in their role in linking splicing, localization of mRNAs, and translation

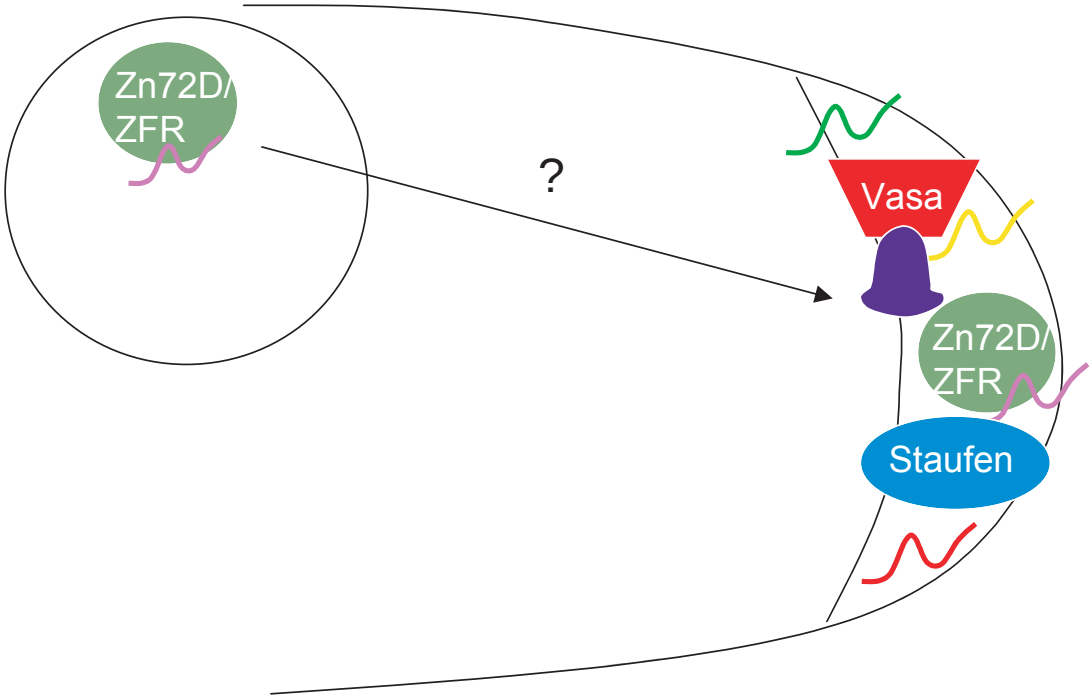
We identified Zn72D through an RNAi screen for proteins involved in dosage compensation in *Drosophila* and found that it is required for productive splicing of the *maleless* transcript. We screened Zn72D-interacting proteins that were identified by mass spectrometry and found that co-knockdown of Zn72D and one Zn72D-interacting protein, Bel, resulted in restoration of MSL complex localization to the X chromosome. Zn72D and Bel co-knockdown restored 70% of MLE protein from 25% of spliced *mle* transcript, suggesting that Zn72D and Bel inhibit translation in wild-type cells.

Bel has been shown by the Lasko lab to colocalize with Vasa, a translational activator, in the *Drosophila* oocyte posterior (Johnstone et al., 2005). The *Drosophila* Staufen protein also localizes to the oocyte posterior. As the human homologue of Zn72D ZFR has been shown to interact with Staufen 2, this leads to the intriguing possibility that perhaps Zn72D is also localized to the oocyte posterior. It is possible that Zn72D and Bel may link splicing to mRNA localization and translation. Zn72D may be loaded onto mRNAs in the nucleus as they are spliced and shuttle them to particular regions of the cytoplasm, for example the posterior end of the oocyte, for localized translation (Fig. 16). The *oskar* transcript is inhibited from being expressed until it is properly localized at the posterior end of the oocyte; thus this may be the function of the translational inhibitory role for Zn72D: to inhibit translation until the mRNAs with which it associates are properly localized within the cytoplasm.

Figure Legend

Figure 16. Model for how Zn72D may link mRNA splicing to localization and translation. Zn72D splices transcripts in the nucleus and remains associated with these transcripts and shuttles them to specific regions of the cytoplasm for localized translation. In this example, Zn72D takes mRNAs to the posterior end of the oocyte where translation can occur. The Zn72D homologue ZFR has been shown to interact with Staufen2, whose *Drosophila* homologue is Staufen, which localizes in the oocyte posterior. Belle (drawn bell shaped in the figure) colocalizes with Vasa in the oocyte posterior.

Figure 16



Appendix

RNAi Screen Candidates

In the RNAi screen in MSL2-GFP expressing S2 cells, we pulled out several candidates that were not confirmed in wild-type S2 cells. Presumably knockdown of these genes affected expression of the MSL2-GFP transgene. In particular, knockdown of Argonaute 2 and Dicer 2 was striking: upregulation of MSL2-GFP such that GFP fills the nucleus. As transgenes insert into the genome bidirectionally and produce dsRNAs that continually knockdown expression of the transgene, it is likely that knocking down Ago2 and Dcr2 results in overproduction of transgenic proteins, in this case MSL2-GFP.

Table 2. Initial Candidates from the RNAi Screen in MSL2-GFP expressing S2 cells

Gene Knocked Down	Phenotype in GFP-MSL2 S2 Cells	Phenotype in WT S2 Cells
Zn72d	complex not on the X	complex not on the X
fumble	complex not on the X	complex not on the X
cycT	complex not on the X	complex on the X
nup358	complex not on the X	complex on the X
spt6	complex not on the X	complex on the X
CG11990	complex not on the X	complex on the X
Ago2	GFP-MSL2 fills the nucleus	no effect
Dcr2	GFP-MSL2 fills the nucleus	no effect
sin	GFP-MSL2 fills the nucleus	no effect

Overexpression of Zn72D in S2 cells increases levels of *mle* transcripts

Knockdown of Zn72D in S2 cells results in a decrease in properly spliced *mle* transcripts and an in increase in transcripts that retain part of intron 2. We wanted to see if the opposite was also true: if overexpression of Zn72D could cause an increase in productively spliced *mle* transcripts, at the expense of the transcripts that contain the intron. I performed Northern blots on S2 cell extracts from wild type S2 cells and S2 cells that overexpress HA-Zn72D, probing with an *mle* cDNA probe. Inconsistent with

our hypothesis, I observed a slight increase in levels of all *mle* transcripts: approximately 1.5-fold (Fig. 17). It is unclear if overexpression of Zn72D is promoting transcription of *mle* or stabilization of *mle* transcripts and whether this is a function of Zn72D in addition to its role in splicing.

Knockdown of Zn72D does not affect deposition of RNA Pol II at the *mle* gene

Zn72D colocalizes with elongating Pol IIo-Ser2. Prior to knowing that Zn72D affects splicing of the mle transcript at intron 2, I performed chromatin immunoprecipitation (ChIP) at the *mle* gene with antibodies to RNA Polymerase II +/- Zn72D knockdown to determine if RNA Pol II deposition on the gene was affected in the absence of Zn72D (Fig. 18).

ChIP

S2 cells were treated with or without Zn72D dsRNA for 6 days (initially seeded at 5.5x10^6 cells/mL in 10 mLs of 50% conditioned/50% fresh Schneiders media). Two 10cm dishes of each sample (+/- dsRNA treatment) were harvested in conical tubes and formaldehyde was added to a final concentration of 1% and cells were rocked for 10 minutes at room temperature. Glycine was added to a final concentration of 0.25M, and cells were rocked for 5 min and then spun down at 1.5K for 5 min. Cells were washed in 1xPBS twice and then cell pellets were frozen in liquid nitrogen and stored at –80 degrees. Pellets were resuspended in Chromatin-IP Lysis buffer (50mM Hepes-KOH pH 8.0, 1mM EDTA, 140mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) and nutated for 10 minutes at 4 degrees. Crude nuclei were collected by spinning at 600g 5

min at RT. Pellets were resuspended 10 minutes in wash buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA, 0.5 mM EGTA, 200 mM NaCl) and nutated for 10 minutes at 4 degrees and then centrifuged at 600g for 5 min at RT. Pellets were resuspended in 2 mL1xRIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and sonicated on a Branson Sonicator on power setting 5 on 100% duty 6 times 20 seconds each. Lysates were clarified by centrifugation at 14,000rpm for 10 minutes at 4 degrees two times. 600 uL lysate was added to each IP and 20% (120 uL) was saved at 4 degrees. 10 uL of the H14 (Pol IIo-Ser5) and H5 (PoIIo-Ser2) antibodies were used per IP and 6 uL of the 8WG16 (Pol IIa) was used per IP. The lysate and antibodies were added to the Dynabeads at the same time and IPs were carried out overnight. Three washes were done: 1)1xRIPA 140 mM NaCl, 2) 1xRIPA 500 mM NaCl, 3) 1xRIPA 140 mM NaCl. All washes were done for 5 minutes at room temperature. 200 uL of proteinase K buffer plus 100ug/mL proteinase K was added to the beads overnight at 65 degrees to proteinase K treat and reverse crosslinks at the same time. The beads were then phenol chloroform extracted, ethanol precipitated (with 20ug glycogen as a carrier) and the pellets were resuspended in 100uL 10mM Tris pH 8.0. Quantitative PCR was performed immediately, as samples degrade over time.

CG5641 colocalizes with FMR1 in S2 cell processes

CG5641, an unknown protein that has the DZF domain found in Zn72D, was the protein that interacts most strongly with Zn72D, as judged by the intensity of the band of CG5641 protein that co-IPs with Zn72D. Myc-tagged CG5641 expressed in S2 cells

localizes to both the cytoplasm and the nucleus (Fig. 19A). However, when Myc-CG5641 is co-expressed in cells also overexpressing HA-Zn72D, the localization of Myc-CG5641 appears very similar to the pattern of Zn72D in that it remains mostly nuclear (Fig. 19B), although there is some CG5641 in the processes that form upon treatment of S2 cells with cytochalasin D (data not shown).

Ling et al. demonstrated that FMR1 is present in processes that form in S2 cells when the are plated on concanavalin A in the presence of cytochalasin D (Ling et al., 2004), and FMR1 coprecipitates with Zn72D, we decided to ask if CG5641, Zn72D, and Bel are also in these granules in the processes in S2 cells. Bel and Vasa have been shown colocalize in the *Drosophila* oocyte; here we show that Vasa colocalizes in granules in S2 cell processes with FMR1 (Fig. 20A). CG5641 is strongly enriched in the granules in S2 cell processes; most cells expressing Myc-CG5641 have it in the processes. It colocalizes with FMR1 (Fig 20B) and sometimes with Vasa (Fig. 20C). Even though Bel and Vasa colocalize in the *Drosophila* oocyte, Bel is rarely found in S2 cell processes. One example of a cell where Bel was found in a process to colocalize with FMR1 is shown in Fig. 20D. HA-Zn72D and GFP-Zn72D were also very rarely found in S2 cell processes, indicating that Zn72D generally is not found in granules in S2 cells (data not shown). The significance of the localization pattern of Zn72D, Bel, CG5641, and FMR1 in granules in S2 cells is unclear since the processes are artificially induced. Further study is necessary to determine if CG5641 and Zn72D may have a role in regulating translation in the *Drosophila* oocyte or in neuronal granules.

Identification of proteins that interact with Belle by mass spectrometry

In order to gain insight into the cellular role of Belle, we HA tagged Bel, expressed it in S2 cells, and performed a large scale co-IP. Mass spectrometry analysis, performed by Feixia Chu, revealed that Bel Co-IPs with many ribosomal proteins and proteins involved in translation, splicing, and mRNA binding (Fig. 21). Most of the proteins that co-IP'd with Zn72D also co-IP'd with Bel, supporting the Zn72D analysis. In addition, this data lends support to the idea that Bel plays a role in translation and/or splicing.

The ATPase and helicase activities of Bel are not required for its role in MSL complex localization.

Since Bel is a member of the DEAD box helicase family of proteins, we decided to test if its canonical ATPase and helicase domains were required for its role in MSL complex localization. To do this, point mutations to disrupt these motifs were introduced into Bel. The DEAD box (in motif II, also known as the Walker B motif) was mutated to DAAD to decrease or abolish ATPase and helicase activity without affecting RNA binding activity (Cordin et al., 2006), creating the BelΔDEAD mutant. Another mutation was introduced into the SAT motif (motif III), creating the BelΔSAT mutant. Mutations in the SAT motif have been shown to result in loss of helicase activity while having only subtle effects on RNA unwinding and ATP binding and hydrolysis (Cordin et al., 2006). In wild-type S2 cells, knockdown of *zn72d* results in loss of MLE from the X chromosome. *bel* knockdown has no effect on MSL complex localization, and

knockdown of *zn72d+bel* restores MLE to the X chromosome (Fig. 22). In cells expressing the HA-tagged Bel cDNA, co-knockdown of *zn72d* and the endogenous *bel* (by targeting the dsRNA against the 5'UTR which is not present in the *bel* cDNA transgene) mimics the *zn72d* single knockdown phenotype, since overexpressing the *bel* cDNA rescues knocking down endogenous *bel*. Cells expressing the BelΔDEAD and are co-knocked down for zn72d and endogenous bel also mimic the single zn72d knockdown, indicating that the BelΔDEAD mutant can function as wild-type Bel for its role in MSL complex localization (Fig. 22). The ATPase and helicase functions of Bel are not required for the role of Bel in inhibiting translation of the *mle* transcript. Overexpression of the BelΔSAT mutant with knockdown of zn72d+bel results in a subtle enrichment of MLE protein on the X chromosome in some cells (Fig. 22); the meaning of this result is unclear in light of the BelΔDEAD mutant which should also disrupt helicase activity.

We also mutated a critical lysine residue in motif I (Walker A motif) that destroys ATPase activity of DEAD box helicases; however overexpression and selection of S2 cells expressing the BelΔK345A transgene resulted in massive cell death of all cells, suggesting overexpression of this mutant is lethal (data not shown).

Figure Legends

Figure 17. Overexpression of Zn72D results in an increase in levels of *mle* transcripts. Northern blots on wild-type S2 cells or S2 cells overexpressing Zn72D were performed. Quantitation was performed using Bio-Rad Quantity One Software.

Figure 18. Knockdown of Zn72D does not influence transcription across the *mle* locus. S2 cells +/- knockdown of Zn27D were harvested after 6 days of RNAi. ChIP was performed using three antibodies to RNA Pol II, Pol IIa, Pol IIo-Ser5, and Pol IIo-Ser2. Primers at the 5' and 3' end of the mle gene were used for quantitative PCR. Three independent ChIP experiments were performed and error was determined using standard error propagation methods.

Figure 19. The intracellular localization pattern of CG5641. (A) S2 cells expressing Myc-CG5641 were grown in the presence of cytochalasin D for 24 hours on coverslips treated with concanavalin A to induce processes. Immunofluorescence using an anti-Myc antibody (Abcam ab9106, 1:500 dilution) was performed. (B). S2 cells expressing both Myc-CG5641 and HA-Zn72D were grown as described in (A) and immunofluorescence using anti-Myc and anti-HA antibodies was performed. Data in this figure were acquired at the Nikon Imaging Center on the spinning disk confocal microscope.

Figure 20. CG5641, FMR1, Vasa, and Bel can be found in granules in processes in S2 cells. Immunofluorescence was performed on S2 cells treated as in Figure 18. (A) Antibodies directed against FMR1 (red; Developmental Studies Hybridoma Bank, diluted

1:10) and Vasa (green; Developmental Studies Hybridoma Bank, diluted 1:10), (B) FMR1 (red) and Myc (green), (C) Vasa (red) and Myc (green), and (D) Bel (red) and Vasa (green) indicate that these proteins can be found in granules in processes in S2 cells. Data in this figure were acquired at the Nikon Imaging Center on the spinning disk confocal microscope.

Figure 21. Identification of proteins that coimmunoprecipitate with Belle. CoIPs with HA-Belle expressing S2 cells (pAFH-Belle cloned using the Invitrogen Gateway system and stable cell line selected by cotrasfection with pCoBlast) were performed as described in Materials and Methods in Chapter II for HA-Zn72D. Proteins in boxes are those that also co-IP'd with HA-Zn72D.

Figure 22. The ATPase and helicase functions of Bel are not required for its role in MSL complex localization to the X chromosome. S2 cells were treated for 6 days with either no dsRNA or with dsRNAs against *zn72d*, the 5'UTR of *bel*, or *zn72d*+5'UTR of *bel*. Left column: Immunofluorescence in wild-type S2 cells using anti-Bel (green) and anti-MLE (red) antibodies. All other columns: Immunofluorescence in S2 cells expressing HA-tagged Bel transgenes using anti-HA (green) and anti-Mle (red) antibodies. Cell nuclei were stained with DAPI.

Figure 17

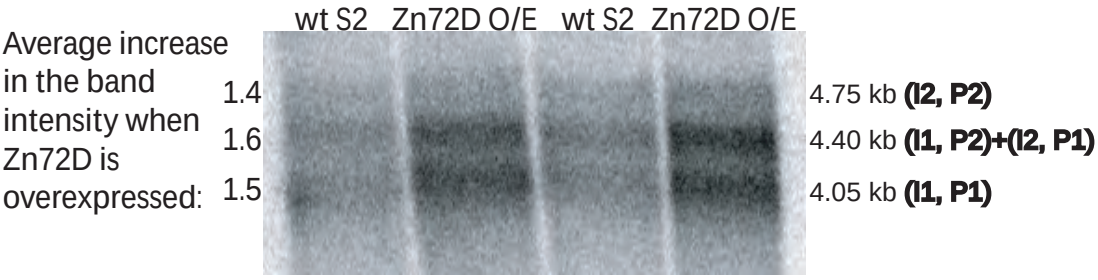

wt S2
Zn72D O/E
wt S2
Zn72D O/E
Average increase in the band intensity when Zn72D is overexpressed:
1.4
1.6
1.5
4.75 kb (I2, P2)
4.40 kb (I1, P2)+(I2, P1)
4.05 kb (I1, P1)

Figure 18

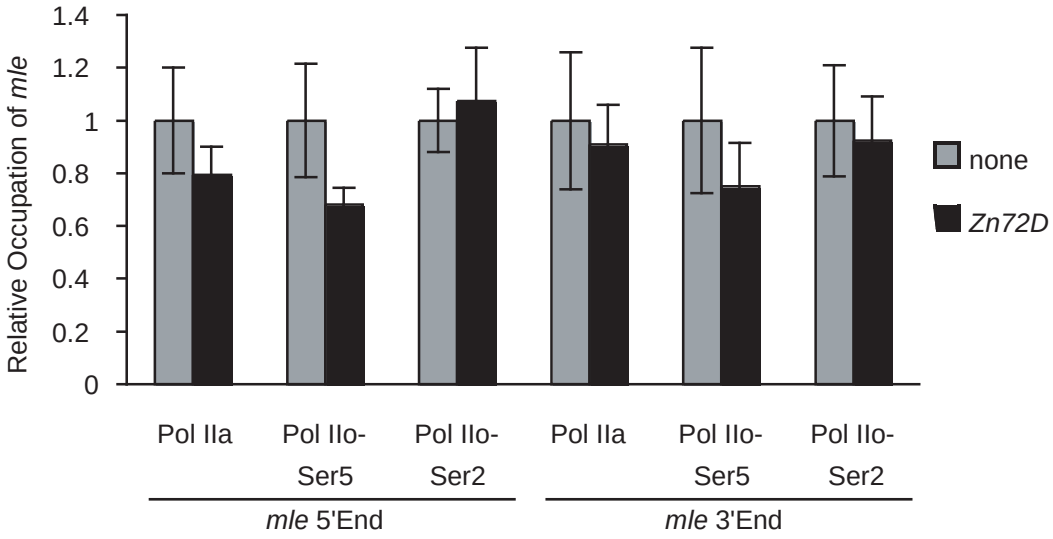

Relative Occupation of *mle*
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pol IIa
Pol IIo-Ser5
Pol IIo-Ser2
Pol IIa
Pol IIo-Ser5
Pol IIo-Ser2
mle 5'End
mle 3'End
none
Zn72D

Figure 19

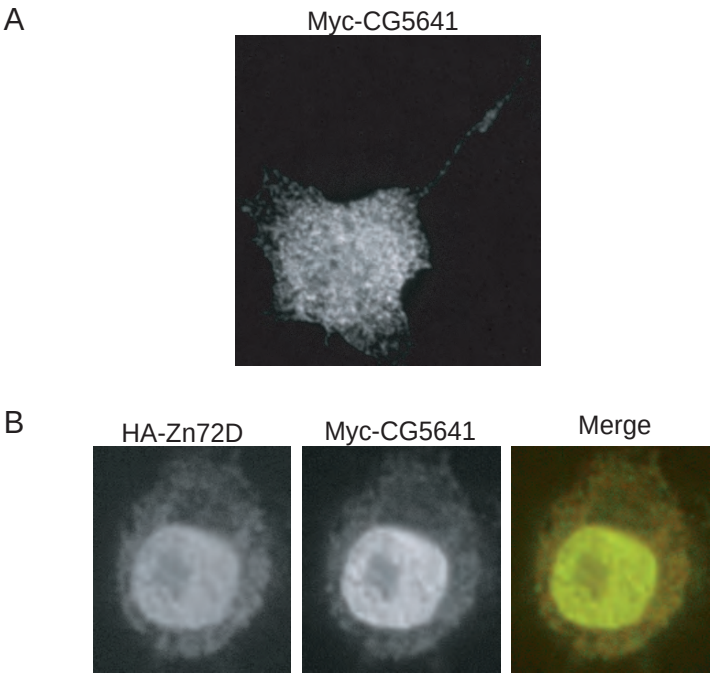


Figure 20

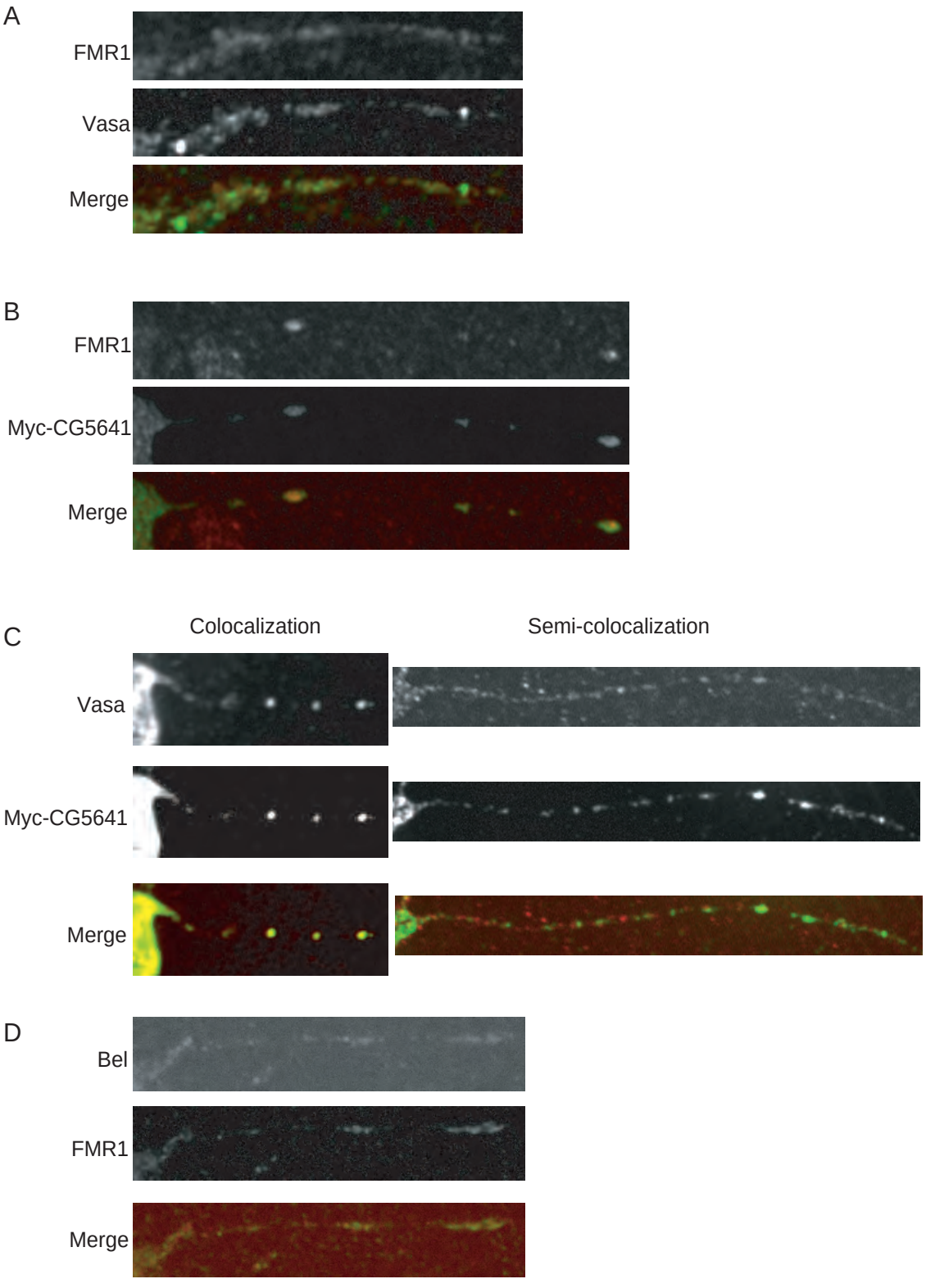

A
FMR1
Vasa
Merge
B
FMR1
Myc-CG5641
Merge
C
Colocalization
Semi-colocalization
Vasa
Myc-CG5641
Merge
D
Bel
FMR1
Merge

Figure 21

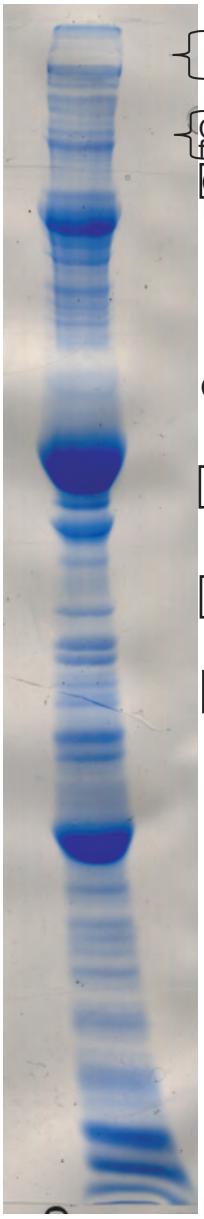
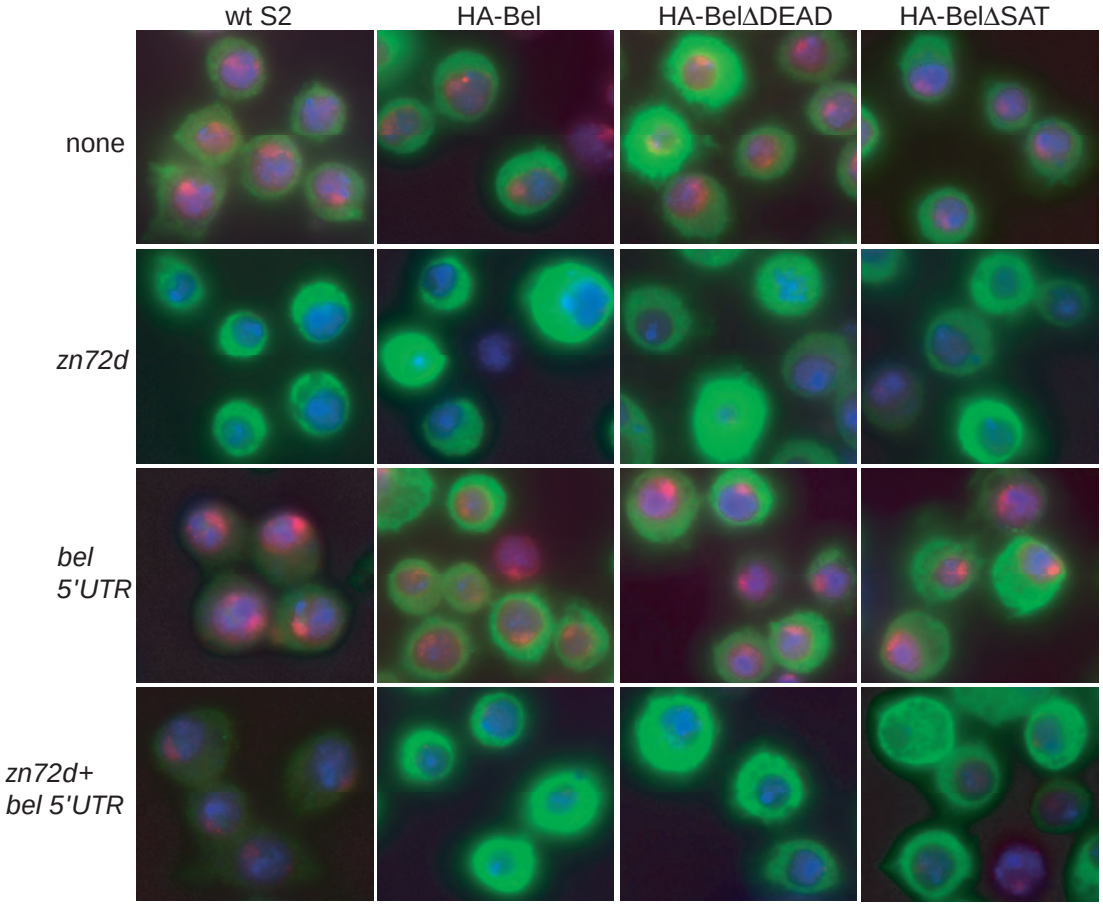

HA-Belle
Myosin heavy chain, non-muscle (Zipper/Myosin II), eIF4G, CG31022,
CG33130, La-related protein, slender lobes
DNA topoisomerase 2, lingerer, Atx-2, CG5728
CG6995, eIF3-S10, CG5792, Argonaute 2, αCop, Upf1, MLE, CG7065,
flightless-I, jaguar
CG5787, CG5726, eIF3-S8, Kinesin-like protein Klp10A
HA-Belle, Zinc finger protein on ecdysone
nonA, spenito, FMR1, ncd, EF-2, HSP82, α-adaptin, CG10077,
Hsc70-4, PABP, Hsc70-3, belphegor, Hrp59, Lamin
CG9641, ATP-dependent RNA helicase p62
RpL4, Actin-42A, prod, EF1α48D, Hnrnp27C, EIF4A
Actin-5C, FK506-bp1
Lark, Actin-42A, CG12233, CG7903
RpL22, eIF-3β, Borealin
Histone H1, fibrillarin, CG10630, CG12030, stubarista,
RpL23A, Rbp2, CG10540, RpL5
RpS3, Aly, RpL5, xl6
RpL6, RpS6, SF2, RpL8
RpL7, RpS4, CG11522, sesB
RpL13A, Ran, RpL18, RpS4
RpS7, RpS9, RpL17, RpL18A, RpL11, RpL13A, TCTP, RpL34b
RpL24, RpL35A, RpL34a
RpL14, RpL21, RpS11, RpL26
RpS18, RpS13, RpL28, RpL27A, RpS15, RpL35, rp49 (RpL32)
Histone H3, RpL36
Histone H2B
HHistone H2A, Histone H4

Figure 22


wt S2
HA-Bel
HA-BelΔDEAD
HA-BelΔSAT
none
zn72d
bel 5'UTR
zn72d+ bel 5'UTR
faint MLE staining

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