SUPPLEMENTAL FIGURE 3. Analysis of Y-box protein 2 (YBX2) binding to the *Prm1* translation control element (TCE) with UV-crosslinking assays.

The findings described below use UV-crosslinking RNA binding assays to demonstrate that YBX2 binds the *Prm1* TCE. These findings also reveal that the detection of complexes containing YBX2 and its Y-box recognition sequence (YRS) can be blocked by a previously unrecognized artifact of RNase T1.

The 17 nt *Prm1* TCE, GAACAAUGCCACCUGUC, has been claimed on the basis of studies of mutations in transgenic mice to be “necessary and sufficient” for *Prm1* mRNA translational repression in round spermatids (Zhong *et al*. 2001). The identification of the *Prm1* TCE merits serious consideration because the experimental approach, transgenic mice, can accurately reveal the functions of *cis*-elements in spermatids. Unfortunately, the TCE has received little attention by students of translational regulation in spermatids because the factor that binds the TCE has not been identified.

To search for RNA-binding proteins that bind the *Prm1* TCE, we used an established UV-crosslinking protocol that detects sequence-specific binding in crude tissue extracts (Walker *et al*. 1998). Our protocol has been described in detail previously and is used here without modification (Chowdhury & Kleene 2012). The protocol includes an initial treatment with RNase T1 that decreases non-specific background by degrading probe that is not protected by a RBP, followed by the addition of heparin, which competes with electrostatic binding of basic amino acids to the phosphodiester RNA backbone. Finally, amino acids and bases that are in direct contact are covalently cross-linked with ultraviolet light, and SDS-PAGE separates

crosslinked complexes according to the combined molecular weights of the protein and RNase T1 digestion fragment.

The sequences of the probes are listed in the legend to Supplementary Figure 2. The probes in lanes 1-3 and 5 display two closely spaced complexes, a strong upper complex and a much weaker lower complex. This distinctive doublet is consistently detected with RNA-EMSAs and non-denaturing electrophoresis, UV-crosslinking and SDS-PAGE, and Northwestern blots using crude testis extracts (Kwon *et al*. 1993, Fajardo *et al*. 1994, Giorgini *et al*. 2001, 2002, Davies *et al*. 2000, Chowdhury & Kleene 2012). The presence of YBX2 in the major complex has been established with gel mobility super shifts, immunoprecipitation of native and UV-crosslinked complexes, and northwestern and western blots (Kwon *et al*. 1993, Davies *et al*. 2000, Giorgini *et al*. 2002). The apparent MW of YBX2 measured with SDS PAGE, ~52 kDa, is larger than its true MW, 38.0 kDa, due to anomalous mobility of Y-box proteins (Fajardo *et al*. 1994, Kwon *et al*. 1993, Davies *et al*. 2000, Skabkin *et al*. 2006).

The probes that form complexes in Lanes 1-3 and 5 contain YRSs that have been demonstrated to bind YBX2 avidly by studies of point mutations in three laboratories: Lane 1, UCCAUCA; Lanes 2 and 3, AACAUCU; and Lane 5, UCCACCU (Bouvet *et al*. 1995, Giorgini *et al*. 2001, Chowdhury & Kleene 2012). The mobility of the complexes vary slightly in accord with the predicted number of bases (enclosed in brackets in the legend to Supplemental Fig. 1) in the cross-linked RNA fragments created by cleavage with RNase T1 after G-residues flanking the YRS in each probe.

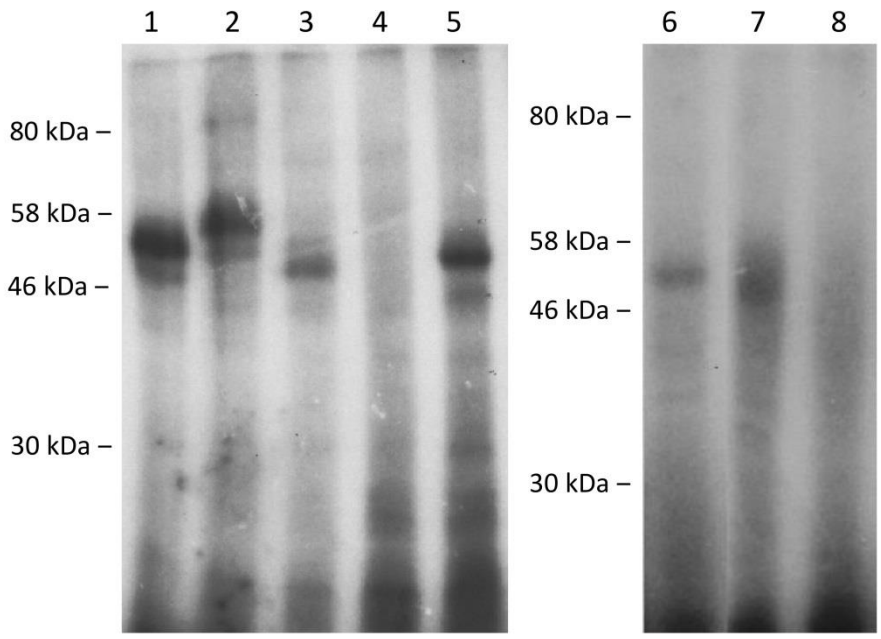
UV-crosslinking assay did not detect complexes with the *Prm1* TCE (Lane 4) as reported previously (Fajardo *et al*. 1994). The absence of the band with the TCE is

notable because the TCE contains an element, GCCACCU, that differs at one base from three elements that bind YBX2 avidly, UCCACCU (Lane 5), ACCACCU and CCCACCU (Chowdhury & Kleene 2012). We hypothesized accordingly that the failure of YBX2 to bind GCCACCU might be an artifact of RNase T1, an enzyme that digests after G-residues. This hypothesis is also supported by evidence that rabbit YBX1 dramatically increases degradation by RNase T1 (Evdokimova *et al*. 1995) and YBX2 binds YRSs with a G in the first position in RNA-binding assays without RNase T1 (Bouvet *et al*. 1995, Giorgini *et al*. 2001).

This line of reasoning predicts that binding of YBX2 to the TCE should be detectable with a protocol in which the probe is treated with heparin and UV-crosslinked before RNase T1 digestion. As predicted, this protocol detects a complex with the *Prm1* TCE (Lane 7) that is similar in intensity to, and slightly smaller and more diffuse than that formed with UCCAUCA (Lane 6). These differences can be explained by the smaller size of the RNase T1 fragment, 7 nt vs. 16 nt, and partial protection of the crosslinked TCE-YRS from RNase T1 digestion. The complex with the *Prm1* TCE is abrogated by a GCCACCU→GCACGAU mutation (Lane 8). This mutation has been previously demonstrated to drastically reduce binding of YBX2 to UCCAUCA *in vitro* and relieves translational repression by UCCAUCA in transgenic mice (Giorgini *et al*. 2001). Due to high background, the small weak complex was not detected. We suggest that RNase T1 diminishes YBX2 binding to GCCACCU by decreasing the YRS below a critical length, because the cold shock domain, the critical YBX2 domain for YRS binding (Bouvet *et al*. 1995), binds more strongly to 7 nt ssRNAs than it does to 6 nt ssRNAs (Mayr *et al*. 2012). Studies of multiple and single point mutations using

RNA-EMSAs, filter binding assays, yeast three assays and UV-crosslinking assays in three laboratories define the YBX2 YRS as a 7 nt element, [ACGU][AC]CA[UC]C[ACU], in which most, but not all, permutations of alternative bases at the degenerate sites (bracketed) bind strongly (Bouvet *et al*. 1995, Giorgini *et al*. 2001, Chowdhury & Kleene 2012,). Importantly, Fig. 4, lanes 2 and 3, using RNA-affinity chromatography without RNase T1 and mass spectrometry sequencing demonstrate that YBX2 binds GCCACCU in the *Prm1* TCE. The possibility that YBX2 binds YRSs containing G at other positions can be examined in the absence of RNase T1 with recombinant YBX2 or ammonium sulfate fractionated testis extract (Schlicker *et al*. 1997). It is also likely that YBX2 binds non-cognate YRSs that are not included in the degenerate YRS, because complexes of cold shock domains and ssRNA involve two flexible molecules which can assemble in different configurations (Clery *et al*. 2013).

These findings have two implications in our studies of translational repression of the *Smcp* mRNA. First, YBX2 appears to be the elusive factor that binds the *Prm1* TCE, the critical element for repression of the *Prm1* mRNA in round spermatids (Zhong *et al*. 2001). The inference that YBX2 represses *Prm1* and *Smcp* mRNA translation is supported by findings that the *Ybx2*-null spermatids prematurely translate both mRNAs (Fig. 5). Second, the large number of mutations in the *Smcp* 3'UTR that we have analyzed with UV-crosslinking and RNase T1 likely failed to detect binding to YRSs that contain G.



Supp Figure 2. UV-crosslinking analysis of YRSs in the *Prm1*, *Smcp* and *Tnp1* 3'UTRs. [^{32}P]-CTP labeled RNA probes were incubated with total testis cytoplasmic extracts, and sequence-specific complexes were formed through the sequential use of *E.coli* tRNA, RNaseT1 and heparin. The complexes were UV cross-linked, resolved by SDS-PAGE, and visualized by autoradiography. The samples in Lanes 6-8 were treated with heparin and UV-cross linked before digestion with RNase T1. YRSs are highlighted yellow, canonical and non-canonical poly(A) signals are highlighted grey, and mutated bases are bold underlined. The number of nucleotides in the RNase T1 digestion fragment containing the YRS is enclosed in brackets.

Lane 1, *Prm1* 3' UTR wild-type YRS [16 nt]

AGAUGCACAGAAUAGCAAGUCCAUCAAAACUCCUG;

Lane 2, *Tnp1* 3'UTR [29 nt]

GAAUUCCCCCAACAUCUCAAUAACAUUUUGAAAACAAAUAAAAUUGUGA;

Lane 3, *Smcp* wild type YRS [10 nt]

GAAGGUAGAAAAGGAUAGAAACAUCUUGUCUAGUGAUCCUGACAUUUAGAU;

Lane 4, *Prm1* wild-type TCE [7 nt] GAACAAUGCCACCUGUCAAUAAAU;

Lane 5, *Prm1* TCE with G→U mutation in YRS [14 nt]

GAACAAUUCCACCUGUCAAUAAAU;

Lane 6, *Prm1*, 3' UTR wild type YRS [16 nt]

AGAUGCACAGAAUAGCAAGUCCAUCAAAACUCCUG;

Lane 7, *Prm1* wild type TCE [7 nt] GAACAAUGCCACCUGUCAAUAAAU;

Lane 8, mutated *Prm1* TCE, GAACAAUGCACGAUGUCAAUAAAU.

REFERENCES

Bouvet P, Matsumoto K & AP Wolffe 1995 Sequence-specific RNA recognition by the Xenopus Y-box proteins. An essential role for the cold shock domain. *J Biol Chem* **270** 28297-28303.

Chowdhury TA & Kleene KC 2012 Identification of potential regulatory elements in the 5' and 3' UTRs of 12 translationally regulated mRNAs in mammalian spermatids by comparative genomics. *J Androl* **33** 244-256.

Clery A, Boudet J & Allain FH 2013 Single-stranded nucleic acid recognition: is there a code after all? *Structure* **21** 4-6.

Davies HG, Giorgini F, Fajardo MA & Braun RE 2000 A sequence-specific RNA binding complex expressed in murine germ cells contains MSY2 and MSY4. *Dev Biol* **221** 87-100.

Evdokimova VM, Wei CL, Sitikov AS, Simonenko PN, Lazarev OA, Vasilenko KS, Ustinov VA, Hershey JW & Ovchinnikov LP 1995 The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. *J Biol Chem.* **270** 3186-3192.

Fajardo MA, Butner KA, Lee K & Braun RE 1994 Germ cell-specific proteins interact with the 3' untranslated regions of Prm-1 and Prm-2 mRNA. *Dev Biol* **166** 643-653.

Giorgini F, Davies HG & Braun RE 2001 MSY2 and MSY4 bind a conserved sequence in the 3' untranslated region of protamine 1 mRNA in vitro and in vivo. *Mol Cell Biol* **21** 7010-7019.

Giorgini F, Davies HG & Braun RE 2002 Translational repression by MSY4 inhibits spermatid differentiation in mice. *Development* **129** 3669-3679.

Kwon YK, Murray MT & Hecht NB 1993 Proteins homologous to the Xenopus germ cell-specific RNA-binding proteins p54/p56 are temporally expressed in mouse male germ cells. *Dev Biol* **158** 99-100.

Mayr F, Schutz A, Doge N & Heinemann U 2012 The Lin28 cold-shock domain remodels pre-let-7 microRNA. *Nucleic Acids Res* **40** 7492-7506.

Schlicker M, Reim K, Schluter G & Engel W 1997 Specific binding of a 47-kilodalton protein to the 3' untranslated region of rat transition protein 2 messenger ribonucleic acid. *Biol Reprod* **56** 697-706.

Skabkin MA, Liabin DN & Ovchinnikov LP 2006 Nonspecific and specific interaction of Y-box binding protein 1 (YB-1) with mRNA and posttranscriptional regulation of protein synthesis in animal cells. *Mol Biol (Mosk)* **40** 620-633.

Walker J, de Melo Neto O & Standart N 1998 Gel retardation and UV-crosslinking assays to detect specific RNA-protein interactions in the 5' or 3' UTRs of translationally regulated mRNAs. *Methods Mol Biol* **77** 365-378.

Zhong J, Peters AH, Kafer K & Braun RE 2001 A highly conserved sequence essential for translational repression of the protamine 1 messenger rna in murine spermatids. *Biol Reprod* **64** 1784-1789.