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Supplemental Data

A Genome-Wide RNA Interference Screen Reveals that Variant Histones Are Necessary for Replication-Dependent Histone Pre-mRNA Processing

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Supplemental Experimental Procedures

GFP Reporter Construction

5'UTR and amino acids 1-62 was amplified by PCR using forward primer 5' GGCCGAATTCCGACAAAAAC CCGAGAGAGTAC 3' and reverse primer 5' GGCCGGTACCTTAGGCAGCTTG CGGATTAGAAGC 3' and subcloned into pEGFPN1 (Clontech, Palo Alto CA) using EcoRI and KpnI. The 3' end of the H3 gene starting immediately after the stop codon and continuing until 18 nt. downstream of the HDE was amplified using forward primer 5' GGCCGGTACCACTTGCAGAT AAAGCGCTAGCG 3' and reverse primer 5' GGCCGGATCCTTGTTATAAATAG TCGGCAACA GAAAATTTTTTCTC 3' followed by ligation to the 5' product using KpnI and BamHI. The resulting construct contained the H3 promoter, amino acids 1-62 of histone H3 ORF, and H3 3' end containing a portion of the downstream intergenic region upstream of an EGFP ORF. Note that the H3 ORF is in frame with GFP and that we created a single mutation (U to A) within the HDE in order to disrupt a stop codon (red box in Fig. S1). The OpIE2 promoter was removed from pIZ-V5/His (Invitrogen, Carlsbad CA) vector by inverse PCR using forward primer 5' GGCCGCTAGCACAG CATCTGTTCGAATTTA 3' and reverse primer 5' GGCCGCTAGCAGACAT GATAAGATACATTGATGA 3' followed by digestion with NheI and religation. The reporter was then subcloned into the promoterless pIZ vector using forward primer 5' GGCCGAATTCCGACAAAAACCCGAGAGAG TAC 3' and reverse primer 5' GGCCTCTAGATTACTTGTACAGCTCGTCCAT GCC 3' followed by digestion with EcoRI and XbaI.

Immunofluorescence of Polytene Nuclei and Brains

Salivary glands from early wandering 3rd instar larvae were dissected into 1X PBS containing 0.1% Triton-X-100. Glands were fixed for 2 minutes in 3.7% paraformaldehyde in 1X PBS-TritonX, and then for 2 minutes in 3.7% paraformaldehyde/50% acetic acid. Glands were immediately squashed and gently

tapped under a coverslip onto a polylysine Q coated slide. Slides were washed 3X in PBS containing 0.1% Tween20. Slides were incubated in the following primary antibodies diluted in PBS-Tween20 overnight at 37 degrees: HP1 (mouse C1A9 1:100 from Developmental Studies Hybridoma Bank, rabbit Lsm11 1:200 generously provided by Dr. Joe Gall (Carnegie Institute)). Slides were washed 3X in PBS-Tween20, and incubated at room temperature for 1 hour in αmCy3 (1:500) or αrCy5 (1:500) (Jackson Immunolabs). Slides were washed 1X in PBS-Tween20 and stained with DAPI (1mg/ml) 1:1000 for 1 minute. Slides were washed in the dark 3X in PBS-Tween20 and mounted in Fluoromount medium. Brains from wandering 3rd instar larvae were dissected into 0.1% TritonX in PBS. They were then fixed in 4.5% formaldehyde in 0.1%TritonX in PBS for 22 minutes. Brains were washed 3X in 0.1%TritonX in PBS and subsequently treated for 1 hour in 1% TritonX in PBS. Samples were blocked for 1 hour at RT in 5% Normal Goat Serum in0.1% TritonX in PBS. After blocking, samples were washed 3X in 0.1% TritonX PBS. Samples were incubated in primary antibodies overnight at 37 degrees (rα Lsm11 (1:1000), mαMPM-2 (1:1000) (Upstate). Slides were washed 3X in 0.1% TritonX in PBS, and incubated at room temperature for 1 hour in αmCy3 (1:500) or αrCy5 (1:500). Slides were washed 3X i0.1% TritonX in PBS and mounted with Fluoromount media.

RT-PCR Analysis

Dmel-2 cells were treated with dsRNA for three consecutive days and then allowed to grow for two more days. The cells were then harvested and total RNA was isolated using Trizol reagent (Invitrogen). 4 μg of RNA was used for a reverse transcription reaction using random hexamers as primers according to manufacturer's protocols (MMLV, Invitrogen). Drosophila PTB was amplified using standard PCR techniques using primers F 5'GTTTCTTTAAAGTGGGTAGCGACC 3' and R 5'TGGTGGCCAGGTCCTGATTGTC 3' at 35 cycles (55^{o}C annealing temp) and resolved on a 3% agarose gel using ethidium staining to visualize PCR products. Analysis of the CPSFs and CstFs were done using the following primer sets and cycling conditions: CPSF30 primers F 5'GCATCCGCACTTCGAGCTGCCC 3' and R 5' TTGACGAAGCCGGGACCATGC 3' using 63^{o}C as an annealing temp. and 32 cycles. CPSF73 primers F 5'GTATGATGCAGTCGGGATTGTCGC 3' and R 5' TGCTCGCCGTGGACGAGCACGAC 3' using 63^{o}C as an annealing temp and 28 cycles. CPSF100 primers F 5' AGAGAGCAGTTCCGAGTCCGAGG 3' and R 5' AACTCATAGCCTGTCGCATCCGC 3' at an annealing temp or 60^{o}C and 28 cycles. CPSF160 primers F 5'TGTCGGTAATCTCGGACAGCAGC 3' and R 5' CCTTTCACTGTGAATAGTCCTG 3' using 49^{o}C as an annealing temp. and 40 cycles. CstF50 primers F 5' CCCGGAACCACATTCCTACGAAACC 3' and R 5' TCGCGCGAGGCGGAGGCCAGGA 3' using 55^{o}C as an annealing temp and 37 cycles. CstF 64 primers F 5' GGCCAATGTCCATCCGAACGATATCG 3' and R 5' TGCTGAGGACCAGGTCCTGCCC3' using 61^{o}C as an annealing temp and 36 cycles. CstF77 primers F 5' ACGAGTCGCTAGTTAATGTGTTTCC 3' and R 5' CTGAACGAGTGTAGATCCATGCCG 3' using 55^{o}C as an annealing temp and 36 cycles. Symplekin primers F 5' CCTTCAGTCGAGAGCCGCCAATGC 3' and R 5' AGTTGCGTGGCAGAGGCTTGGTG 3' using 63^{o}C as an annealing temp and 36 cycles. Fip1 primers F 5' GAGGAACCCTTCTTCCACGAGCC 3' and R 5'

GTTGGGTGGCATAATGCCGCGCAA 3' using 55^{o}C as an annealing temp and 36 cycles.

To the analyze the expression of the domino gene, 2μg of total RNA from Dmel-2 cells was incubated with 200ng of random hexamer primers (GE Healthcare Bio-Sciences AB) at 95^{o}C for 1 minute and cooled to room temperature. Reverse transcription reactions were done in a total of volume of 20 μl according to manufacturer's protocols (Invitrogen). 2 μl of each RT reaction was used as template for a PCR reaction using the cycling conditions: 95ºC/30sec, 57.5ºC/30sec, and 72ºC/40sec for a total of 32 cycles. The domino B isoform was amplified using the primer set: F 5' GCCAAAGC TGCCGAAGAAAGAAG 3' and R 5' CACCACTGACTGC TGCTGATGAG '3. The domino A isoform was amplified using the primer set: F 5'ATCAGCTCAAGCCCT GGCTGCGG 3', and R 5' CCAGCGGTTG GACCCGCAATACTT 3'. All RT-PCR products were sequenced to confirm identity and in all cases there were no products in the absence of RT.

Western Blot Analysis

Dmel-2 cells were lysed in a buffer containing 50mM Tris-HCl (pH 8.3), 0.1% NP-40, and 50mM NaCl for 30' on ice. Lysates were separated with SDS-PAGE using standard techniques and were then transferred to immobilon-PVDF membrane (Biorad, Hercules CA) and probed with either a 1:1000 dilution of an α-GFP JL8 monoclonal antibody (Clontech), a 1:1000 dilution of α-dSLBP antibody (*10*), a 1:1000 dilution of α-Lsm11 antibody, or a 1:1000 dilution of α-Lsm10 antibody (*24*). Blots were then probed using a secondary HRP-conjugated antibody and developed using chemiluminescence. Sufficient material could be obtained for Western blotting using cells from 6 wells of a 384 well plate.

Northern Blot

RNA was isolated from wandering third instar *H2Av* mutant larvae of the genotype w^{1118}; +/+; $H2Av^{810}/H2Av^{810}$ (identified using a TM6B balancer) using TRIzol Reagent (Gibco/Invitrogen). 2 μg of total RNA from each sample was resolved by formaldehyde agarose gel electrophoresis, and probed with probes to either the histone H3 or histone H2a coding regions.

S1 Nuclease Protection Assay

5 μg of total RNA from S2 cells was analyzed essentially as described previously (Godfrey et al. 2006). Plasmid DNA containing the *H2a* gene was digested with BspE I and the 3' end labeled with ^{32}P-dCTP. The labeled probe was then digested with HindIII and 650 nt labeled fragment containing the 3' end of the H2a gene was purified by agarose gel electrophoresis.

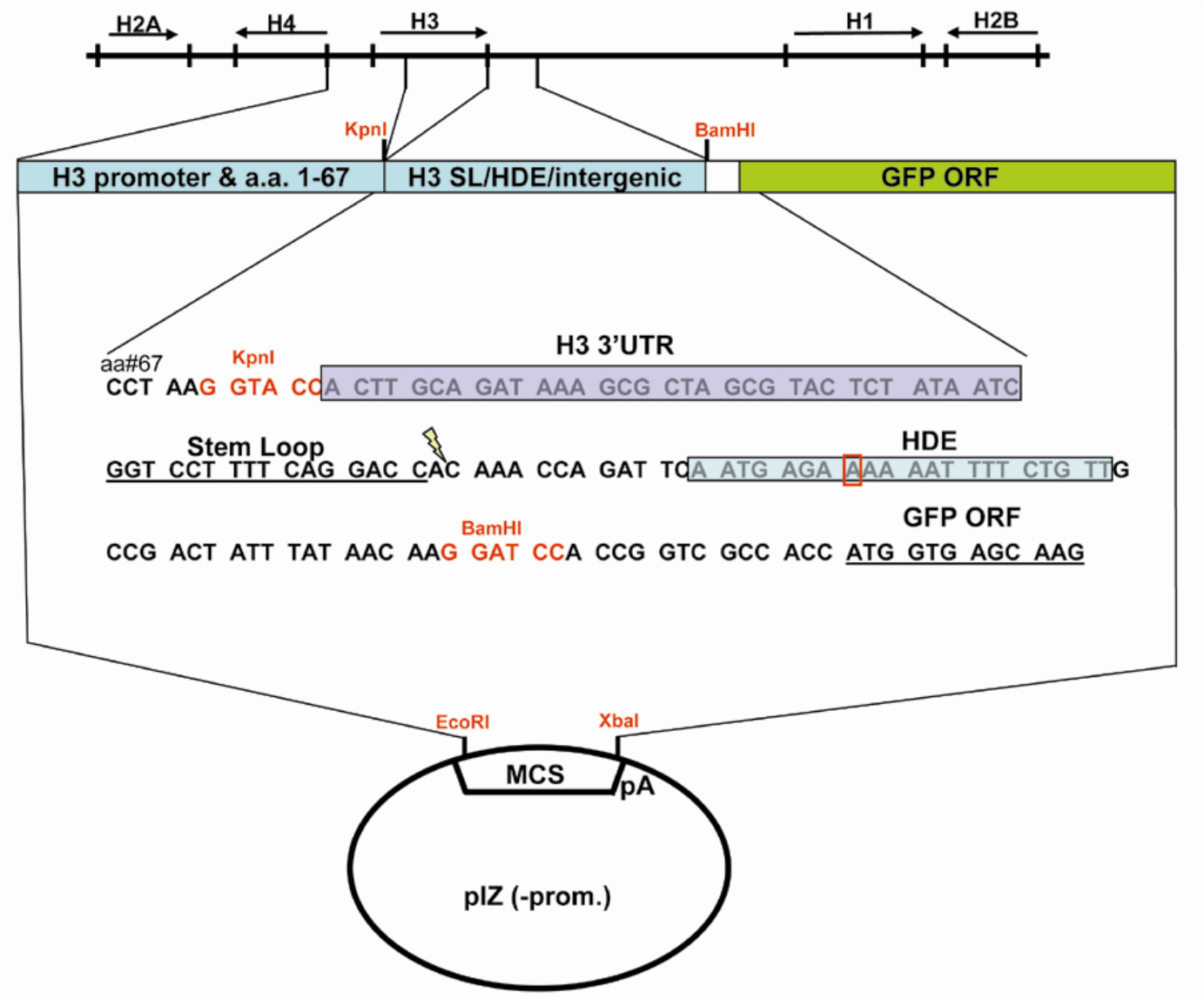

Figure S1. Schematic of GFP Reporter Used in Genome-Wide RNAi Screen

Note the lightening bolt symbol denotes the cleavage site of the H3 pre-mRNA.

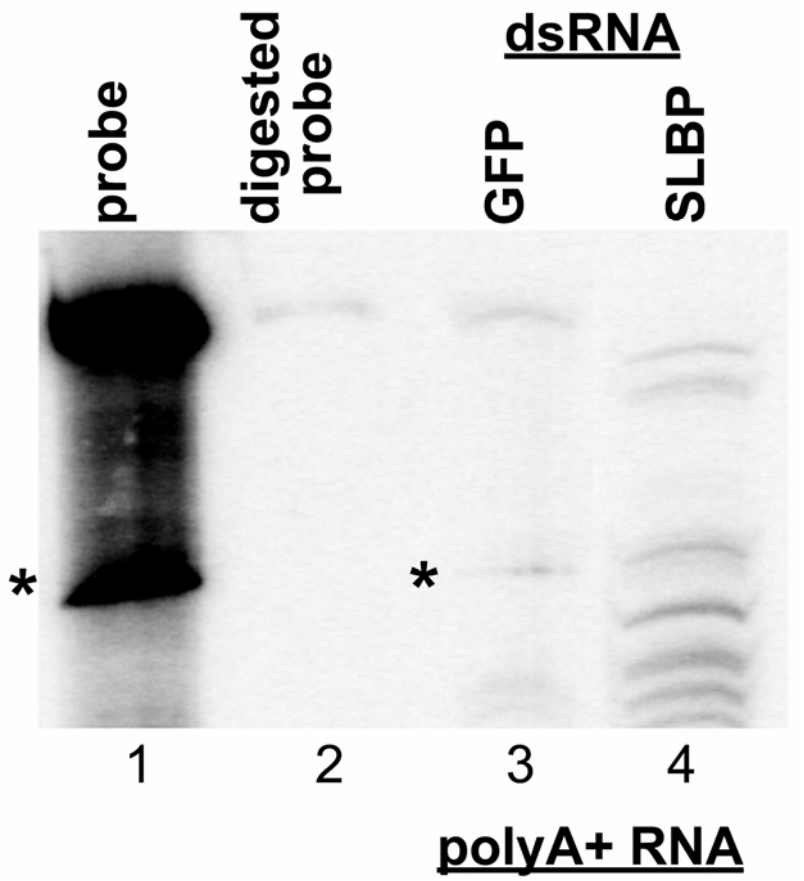

Figure S2. Endogenous Histone mRNA Is Polyadenylated in Response to SLBP Depletion

S1 nuclease protection assay of RNA from Dmel-2 cells treated with the indicated dsRNA that has been purified using oligo-dT agarose. The asterisk indicates a degradation product of the probe preparation and not a misprocessed histone mRNA.

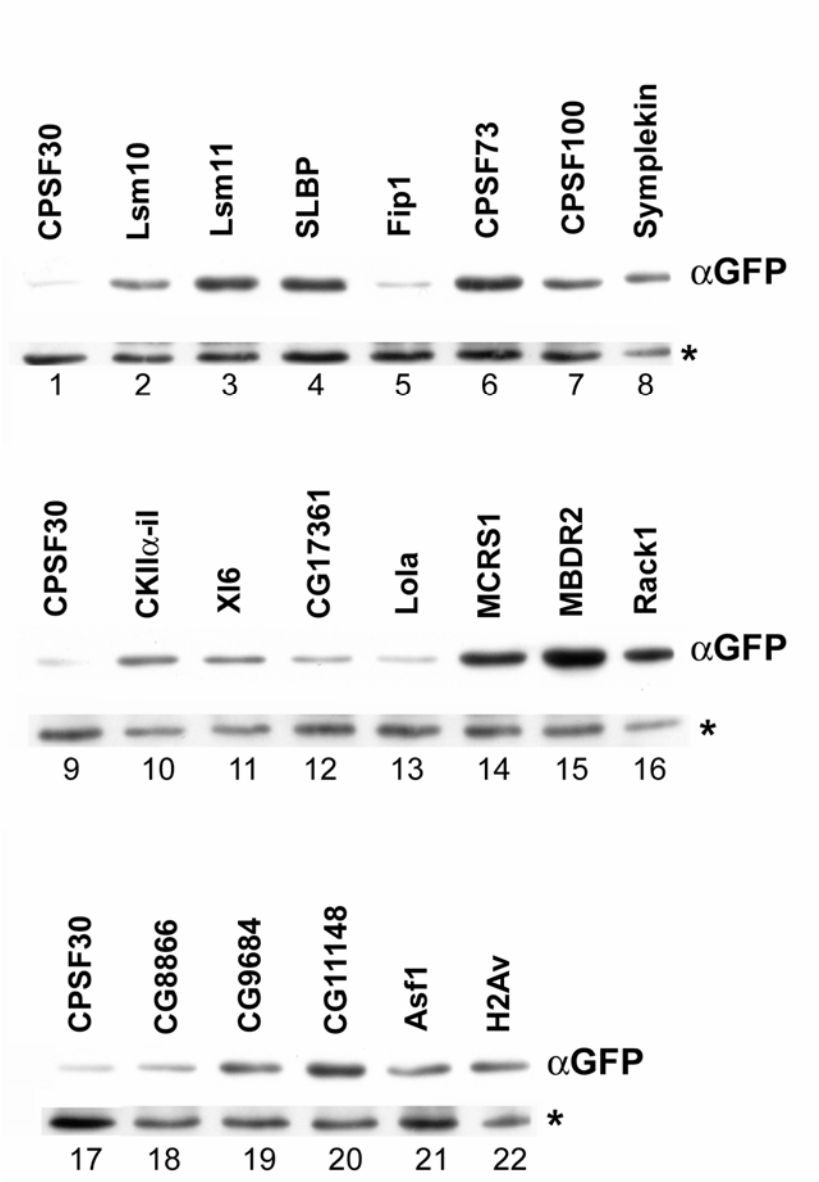

Figure S3. Western Blot Analysis of Positive Hits from the Genome-Wide dsRNA Screen

Lysates from Dmel-2 cells treated with dsRNA targeting genes identified in the screen were analyzed by Western Blot. Lysates were probed with α-GFP antibodies to detect the amount of readthrough from the reporter. The asterisk indicates a background band that served as a loading control.

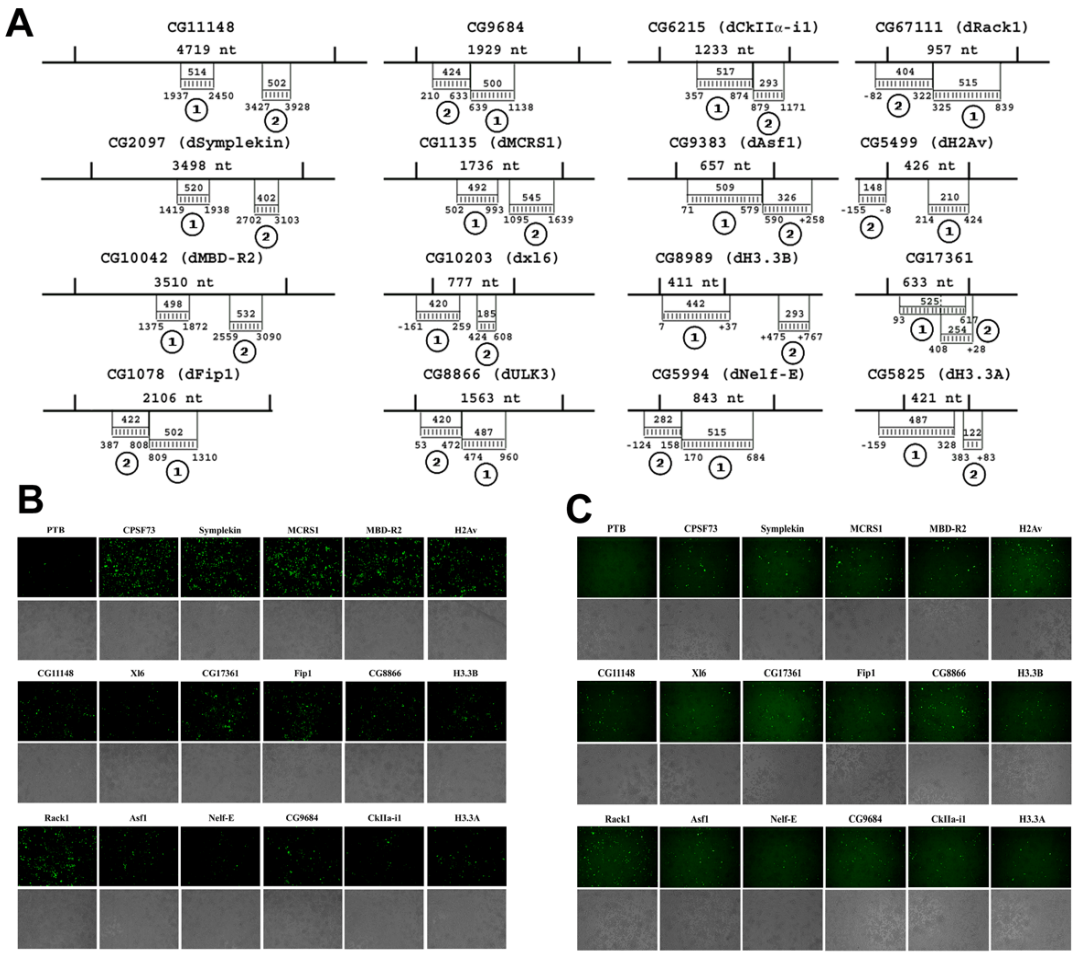

Figure S4. Primary and Secondary dsRNA Target Analysis of 16 Genes Identified in the Genome-Wide Screen

(A) Schematic of nucleotide positions of all first and second site targeting with dsRNA. The first targeting sites (labeled 1) were chosen by the DSRC and we designed the second sites to not overlap with the first, with the exception of CG17361 (due to its small size).
(B) Fluorescence microscopy of first site targeting relative to negative control dsRNA (PTB) and positive control dsRNA (CPSF73). The lower panels are brightfield images.
(C) Fluorescence microscopy of second site targeting relative to negative control dsRNA (PTB) and positive control dsRNA (CPSF73). Note that second site targeting was performed in serum-dependent S2 cells, and the amount of fluorescence in the CPSF73 control (with site 1 oligonucleotide) was lower than in panel B.

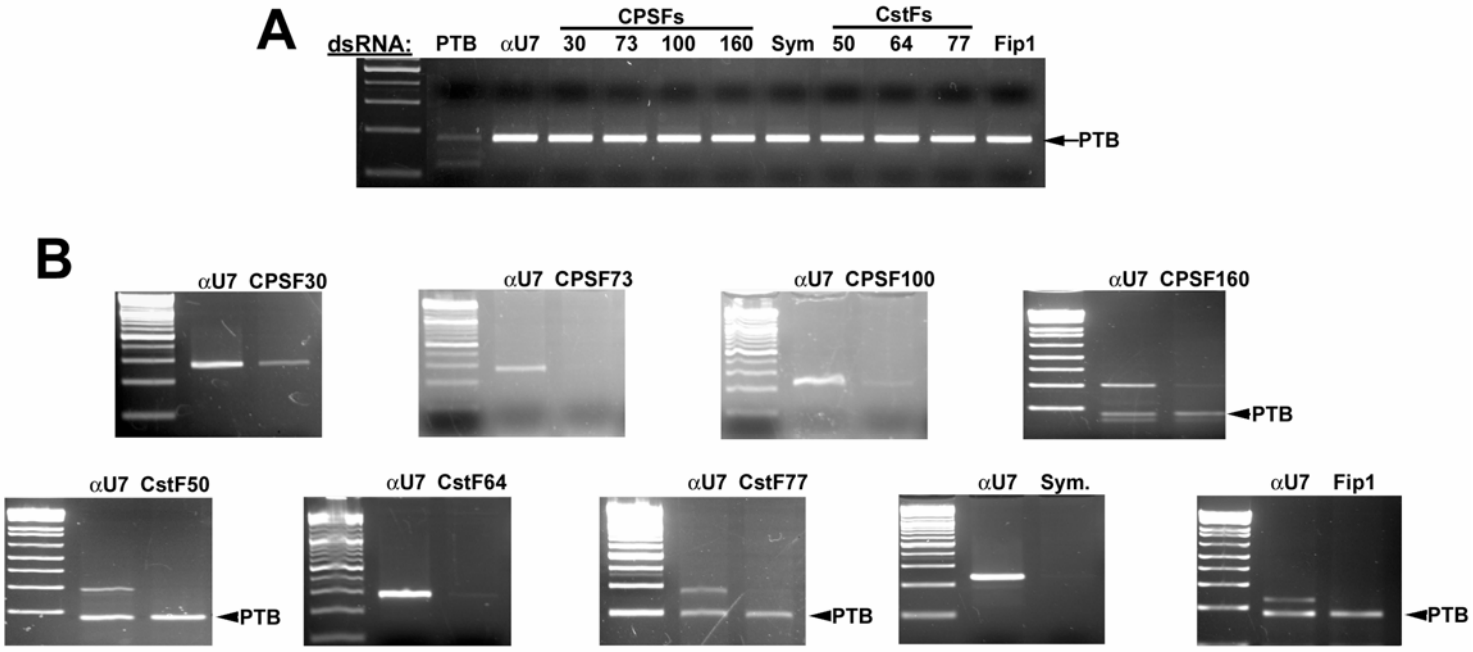

Figure S5. RT-PCR Analysis of RNA Isolated from Dmel-2 Cells Treated with Various dsRNAs

(A) Total RNA was isolated from Dmel-2 cells treated with dsRNA targeting the labeled genes or when treated with a 2'-O-CH3 oligonucleotide targeting U7snRNA. RNA was subject to reverse transcription using random primers and the levels of PTB control mRNA were determined using PTB specific primers in a PCR reaction. PTB mRNA was equal in all samples with the exception of the RNA isolated from Dmel-2 cells treated with PTB dsRNA.
(B) The same RT reaction in panel A was further subjected to RT-PCR analysis of individual mRNAs that were being targeted using dsRNA. In each case the levels of mRNA is compared between RNAi treatment and control treatment. Note that not all the RT-PCR conditions were compatible to allow for the simultaneous amplification of targeted mRNA and PTB mRNA.

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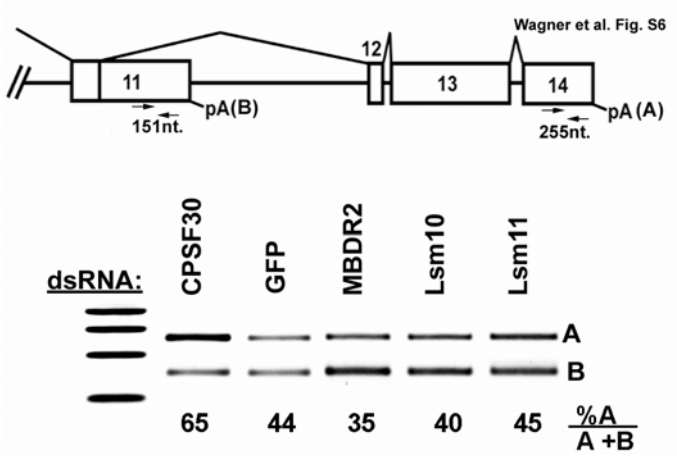

Figure S6. Reduced Expression of Histone Pre-mRNA Processing Factors Does Not Enhance Usage of the domino Distal Polyadenylation Site

Total RNA from Dmel-2 cells treated with dsRNA targeting the indicated genes was subject to RT-PCR analysis using 4 primers, targeting either the distal polyadenylation site (A form) or the proximal polyadenylation site (B form). PCR products were resolved on 3.5% agarose gel. The dsRNA targeting CPSF30 is the positive control for readthrough relative to the negative control using GFP dsRNA.

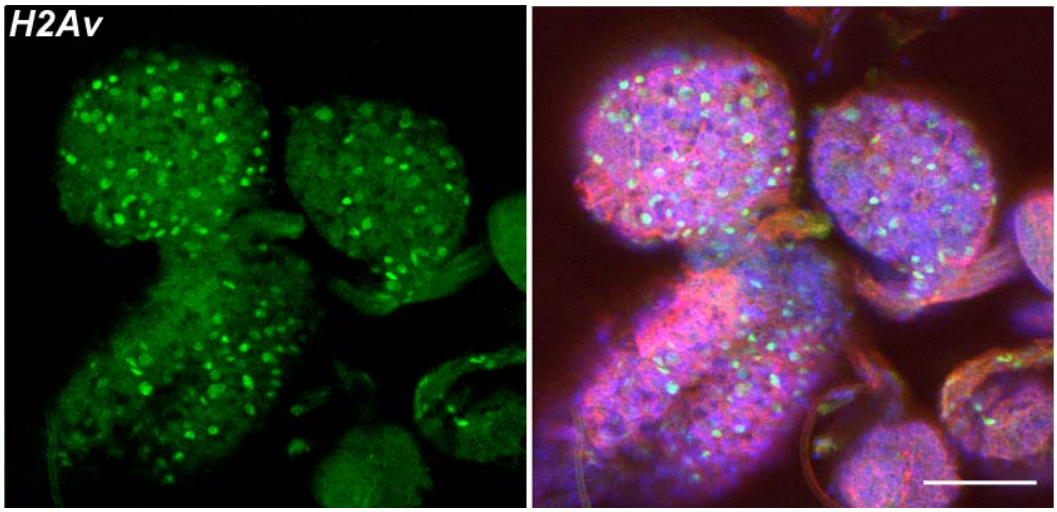

Figure S7.

Confocal images of third instar larval brains from *H2Av* null mutant flies containing the reporter stained as in Fig. 1C (DAPI (blue), GFP (green), phosphotyrosine (red)). Scale bar = 50 μm.

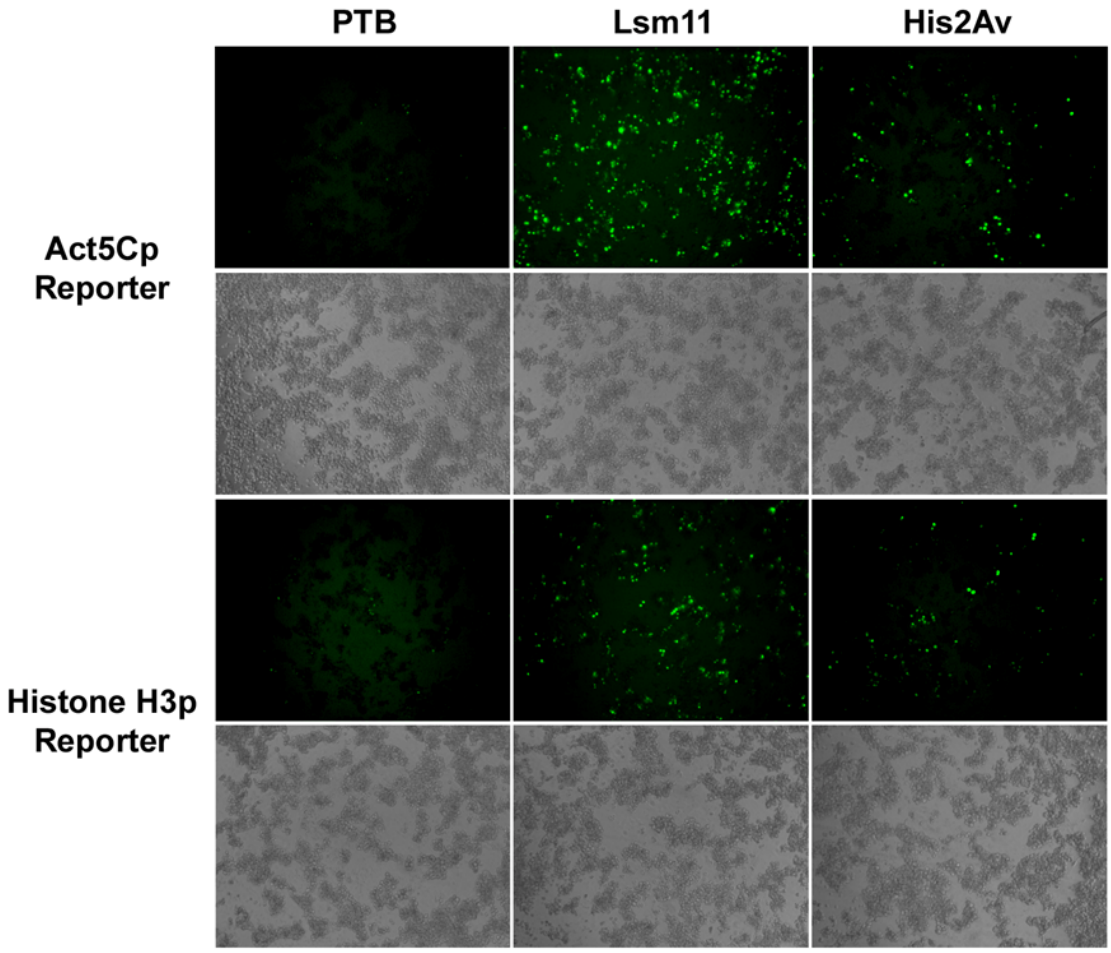

Figure S8. RNAi-Mediated Depletion of H2Av Results in Misprocessing of Histone Pre-mRNA in a Promoter-Independent Fashion

Dmel-2 cells were treated with either PTB dsRNA (negative control), Lsm11 dsRNA (positive control), or with dsRNA targeting H2Av. Cells were transfected with either GFP reporter driven by the histone H3 promoter or by the actin promoter and visualized for GFP expression (upper panels) or by brightfield.

Table S1. Localization of Transiently Transfected, myc-Tagged Proteins in Dmel-2 Cells

Gene	Localization
SLBP	Nuc. stronger than cyt.
Fip1	Nuclear
MBDR2	Nuclear
CG17361	throughout cell
CPSF30	throughout cell
CstF50	cyt. stronger than nuc.
Symplekin	HLB/nuclear
Lsm10	HLB/cyt.
Lsm 11	HLB
CPSF 73	throughout cell
CPSF 100	throughout cell
MCRS1	nucl./ rare HLB

In all cases the Histone Locus Body (HLB) was marked by staining Dme-2 cells with α-Lsm11 antibody as in Fig. 4D.