Supporting online material for

RNA Pol II CTD Phosphorylated on Threonine 4 Is Required for Histone mRNA 3' end Processing

This PDF file includes:

Supplementary Online Material

Materials and Methods

Cell culture and cloning

DT40 cells and derivatives were cultured in RPMI1640 medium containing 10% FBS and 1% chicken serum at 37^{o}C with 5% CO_2.

Targeting vectors: The genomic PCR product containing chicken Rpb1 exon 11, intron 11, and exon 12 (11F2/12R) was inserted into pBlueScript KS by EcoRV to build pKS-11F2/12R. A second genomic PCR product containing chicken Rpb1 intron 9, and exon 10 was inserted into pBlueScript KS by EcoRV to build pKS-9/10R4. Next, a hygromycin- or zeocin-resistance gene driven by the chicken β-actin promoter was inserted into pKS-11F2/12R to obtain pKS-hygromycin-11F2/12R and pKS-zeocin-11F2/12R. The pKS-9/10R4 was then cut by HindIII (filled in by Klenow) and EagI. The fragment containing intron 9 and exon 10 from pKS-9/10R4 was inserted upstream of the β-actin promoter of pKS-hygromycin-11F2/12R and pKS-zeocin-11F2/12R to create pKS-9/10R4-hygromycin-11F2/12R and pKS-9/10R4-zeocin-11F2/12R, which were later linearized by XhoI for electroporation.

HA-tagged Rpb1: The pTRE-HA (Clontech), modified by mutating the ccatg sequence after the HA-tag to cc, was used to construct conditionally expressed Rpb1. Human Rpb1 was cloned into pTRE-HA. The TRE-HA-Rpb1 (TRE promoter, HA tag, and Rpb1) fragment was excised and cloned into pPGK-HisD (a gift from Tsuyoshi Kashima), which contains a histidinol-resistance gene under the PGK promoter. pTRE-HA-Rpb1-HisD was linearized by RsrII for electroporation.

The pTRE-puro-CMV-tTA plasmid: The tTA fragment from ptTA (a gift from T. Kashima) was used to replace the tetR-SP1 fragment of the tTA-SP1 plasmid (a gift from A. Kornblihtt) to obtain CMV-tTA. The puromycin-resistance gene was cloned into pTRE2 (a gift from T. Kashima), which contains the TRE promoter and GH poly-A signal. A fragment containing TRE, puromycin-resistance gene, and GH poly-A signal was then inserted into the CMV-tTA plasmid to obtain pTRE-puro-CMV-tTA, which was later linearized by HindIII for electroporation.

Rpb1 derivatives with mutant CTDs: The chicken beta-actin promoter from pAZeo plasmid (a gift from T. Kashima) was ligated with a FLAG-tag sequence from p3XFLAG-CMV7 (SIGMA). The ligated fragment of beta-actin and FLAG was inserted into pBlueScript. The Neomycin resistance gene from pNeo (*1*) was then inserted into the plasmid to obtain Actin-Flag-Neo. The human *RPB1* body without the CTD was inserted between the FLAG tag and Neomycin resistance gene. Various CTD fragments obtained by PCR or by ligating oligos to form concatemers (*2*) were inserted directly 3' to the *RPB1* body. For electroporation, plasmids were linearized with NotI. All constructs were verified by DNA sequencing. Primer and oligo sequences are available upon request.

Construction of Rpb1 conditional knock-out DT40 cells

Procedures for establishing *Rpb1* knock-out cell lines were essentially as described (*1, 3*). The first *RPB1* allele was disrupted using the pKS-9/10R4-hygromycin-11F2/12R targeting vector. A plasmid encoding HA-tagged human Rpb1 under the control of the tet-repressible CMV promoter was used to rescue the Rpb1 knock-out cells. After pTRE-puro-CMV-tTA and pTRE-HA-Rpb1-HisD were introduced into the heterozygous *Rpb1*

knock-out cells, the second *RPB1* allele was disrupted with pKS-9/10R4-zeocin-11F2/12R. Southern blotting and PCR were performed to confirm disruption of the *Rpb1* alleles.

Southern blotting

Ten ug of genomic DNA was digested with BamH1, separated on an 0.7% agarose gel, and processed for southern blotting as described (*1*). Probes specific to exon 9 of chicken Rpb1 were made using Rediprime kit (Amersham). Hybridization was carried out at 65^{o}C overnight. Membranes were washed under high stringency.

Complementation assays and construction of stable cell lines

About 10^7 cells were harvested, and washed once in cold PBS, then resuspended in 0.5 ml cold PBS and transferred to a cuvette (0.4 cm, Bio-Rad). About 15 ug of linearized DNA (in 50-100 ul PBS) were added, and the cuvette was incubated on ice for 10 min. After electroporation (550 V, 25u F with a Bio-Rad Gene Pulser II), cells were chilled on ice for 10 min, and allowed to recover in 20 ml of medium (without antibiotics). After 24 hours, cells were resuspended in 70 ml of medium containing appropriate antibiotics, plated into four 96-well plates (about 150 ul per well), and selected for 6-9 days. Surviving clones were picked and analyzed by western blotting. Concentration of antibiotics for selection: G418 (neomycin) 1.5 mg/ml, puromycin 0.25 ug/ml, hygromycin B 1.5 mg/ml, histidinol 0.5 mg/ml, zeocin 0.5 mg/ml, and tetracycline 1 ug/ml.

In vivo labeling of nascent RNA

$4x10^6$ cells were incubated with 200 uCi of ^{3}H-uridine (1 mCi/ml) for 30 min. Total RNA was extracted using Trizol (Invitrogen) and dissolved in 10 mM Tris-HCl (pH 8). For poly(A) selection, each RNA sample was divided into 3 equal parts to control for possible variations during selection. Selections were performed using oligo(dT) magnetic beads (Novagen), following procedures provided by the supplier. Unbound and eluted RNAs were collected for scintillation counting. Average counts from the three aliquots from each RNA sample were calculated and considered to be one independent value.

Nuclear run-on

$2x10^7$ cells were harvested, washed in cold PBS, and processed for nuclear run on as described (*4*) with the following modifications. Nuclear pellets were washed once in ice-cold NP-40-free lysis buffer and resuspended in 50 ul of ice-cold storage buffer. Nuclei were used immediately or were quick frozen on dry ice and stored at -80^{o}C. For one reaction, 25 ul nuclei were mixed with 25 ul 2X reaction buffer (10 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, 1 mM ATP, CTP, and GTP, 0.01 mM UTP, 100 uCi of α-^{32}P UTP (800 Ci/mmol) and incubated at 30^{o}C for 30 min. RNA was extracted and purified using Trizol (Invitrogen). To facilitate RNA precipitation, one tenth volume of 3 M NaOAc and glycogen (~1 ug) were added prior to addition of isopropanol. RNA was dissolved in 20 ul formamide for slot blotting.

Slot blotting

5 ug samples of synthesized single-strand DNA oligo in 200 ul of 10X SSC were loaded onto Nytran N (0.2 um, moderately charged) filter. Membranes were washed with 400 ul 10X SSC, and DNA was cross-linked to the membrane by UV irradiation. Prior to hybridization, membranes were boiled in 0.1X SSC, 0.5% SDS for 5 min. RNAs from nuclear run-ons were denatured at 85^{o}C for 5 min. About 1-5x10^{6} cpm of labeled RNA was used in 500 ul hybridization buffer (1 mM EDTA, 0.5 M NaPO4 pH 7.2, 7% SDS). Hybridization was carried out at 65^{o}C overnight. Membranes were washed first with 1X SSC, 0.1% SDS at room temperature for 5 min, then with 1X SSC, 0.1% SDS at 45^{o}C for 15 min twice. DNA oligo sequences are available upon request.

RT-qPCR

RNA was extracted using Trizol (Invitrogen) and further treated with DNase I. Reverse transcription was performed using Maxima Reverse Transcriptase (Fermentas) and random hexamer or oligo-dT primers. cDNA was further diluted and analyzed by qPCR (ABI StepOnePlus). Cyber green PCR reagents for qPCR were from Fermentas. To analyze the relative mRNA level, the 2-ΔΔCt method was used (*5*). The target amplification efficiency of primers was determined, and all primers had amplification efficiencies >0.95. Primer sequences will be provided upon request.

Immunoprecipitation

About 3x10^{7} cells were collected, and washed with PBS. Then, 1 ml cold RIPA (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.5% NP-40, 0.25% sodium deoxycholate, 50 mM NaF, 10 mM β-glycerolphosphate) buffer and 10 ul of protease inhibitors (140

ug/mL Pepstatin A, 35 ug/mL Leupeptin, and 170 ug/mL Aprotinin in 90% ethanol) was added. After brief sonication, samples were rotated at 4^{o}C for 15 min. Debris was centrifuged at 12,000 g, 4^{o}C, for 15 min, and the supernatant was removed to a new tube. 50 ul of the lysate were kept for input control, and the rest of the extract was incubated with 20 ul of pre-washed protein G Sepharose and 1-4 ug of antibody. Samples were rotated at 4^{o}C for 2 hr, and beads were washed with cold RIPA buffer for 5 min three times, and then were resuspended in 100 ul of 1X SDS sample buffer for western blotting.

Chromatin immunoprecipitation assay (ChIP)

Cells were grown in the presence of tet (1ug/ml) for 30 hr to 70% confluence (2-3x10^{6}/ml), cross-linked with 1% formaldehyde for 10 min, and processed for ChIP as described (*6*) with the following modifications. Samples were sonicated until the length of chromatin fragments was in a range of 100-400 bp. Immunoprecipitation was carried out at 4^{o}C for 4 hr or overnight. Lysates of equal number of cells (1-2x10^{7}), 1-4 ug antibody, and 20 ul Sepharose G (GE Healthcare) were used per IP. After IP, beads were washed sequentially with WB1 (150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8), 0.1% SDS, 1% Triton X-100), WB2 (500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8), 0.1% SDS, 1% Triton X-100), WB3 (250 mM LiCl, 1 mM EDTA, 50 mM Hepes-KOH (pH 7.5), 0.7% sodium deoxycholate, 1% NP-40), and with TE-plus (1X TE, 50 mM NaCl), 5 min for each wash. DNA was eluted with 100 ul of elution buffer (50 mM Tris-HCl pH 8, 2 mM EDTA, 1%SDS, 200 ug/ml RNase A) at 65^{o}C for 4 hr or overnight. A PCR purification kit (QIAGEN) was used to purify DNA for analysis by qPCR (ABI StepOnePlus). Fold occupancy was calculated as described (*7*). The background signal

was determined from an internal region of the 18S rRNA gene. Primer sequences will be provided upon request. ChIP was performed using antibodies against Rpb1 (N20; Santa Cruz), CPSF100 and SLBP (a gift from W. Marzluff).

Western blotting

Cell lysates were resolved in composite SDS-PAGE with two resolving layers, 6% and 12%. Western blotting was performed using standard protocols with antibodies against CTD (8WG16; Covance), actin (Sigma), CPSF73 (Bethyl Laboratories), CPSF100, Flag (M2) (Sigma), GAPDH (Sigma), histone H3 (Abcam), HA (Covance), Lsm11 (a gift from Z. Dominski), mouse IgG (Sigma), mouse IgG (IRDye 800; Li-Cor), rabbit IgG (Sigma), rabbit IgG (IRDye 680; Li-Cor), rat IgG (IRDye 680; Li-Cor), Rpb1 (N20; Santa Cruz), phospho-ser2 CTD heptad (3E10; Millipore), phospho-thr4 CTD heptad (Novus #NBP1-49546), SLBP (a gift from W. Marzluff), and symplekin (Bethyl Laboratories). Western blots were quantified using ImageJ.

Supplemental Figure Legends

Figure S1. Construction of DT40-Rpb1 cells. (A) Diagrams depict four exons, 9~12, of chicken Rpb1, and the targeting vectors designed to delete the two *Rpb1* alleles. A region encompassing exon 10, intron 10, and exon 11 of Rpb1 was disrupted by targeting vectors carrying hygromycin-resistance gene ($Hygro^r$) or zeocin-resistance gene (Zeo^r). The vertical bars represent BamH1 digestion sites, and the thick line underneath exon 9, the probe used in Southern blotting. The two alleles of *Rpb1* were disrupted sequentially. Before the second allele was disrupted, a tet repressible HA-tagged human Rpb1 was

introduced. (B) Genomic DNA samples were digested with BamH1, and analyzed by Southern blotting using exon 9 as a probe.

Figure S2. The CTD is highly conserved in vertebrates. Human and zebra fish CTDs both consist of 52 repeats and are 97% identical. Variant residues occur mostly at ser 7 positions. Likewise, the *X. laevis* and human CTDs are 97% identical, and the *A. carolinensis* (lizard) CTD is 100% identical to the human CTD. Although the sequence of the chicken genome was reported in 2004 (*8*), the *RPB1* sequence remains incomplete. The available partial chicken *RPB1* sequence, which lacks regions that encode 60 N terminal amino acids and 220 amino acids at the C terminus, is 97% identical to human, and the first 22 CTD repeats are 100% identical between chicken and human.

Figure S3. Expression of HA-tagged Rpb1. Western blot of lysates prepared from DT40, DT40 *rpb1(+/-)* and DT40-Rpb1 cells, treated with tet as indicated and for the times shown. Blots were probed with anti-HA and anti-actin antibodies.

Figure S4. Egr1 mRNA splicing and 3'-end processing. 26r and T4V cells were treated with tet for 40 hr, and egr1 expression was induced by ionomycin and PMA for 20 min. The unspliced, spliced, uncleaved, and total egr1 mRNA levels were determined by qPCR. The ratios of unspliced/spliced and uncleaved/total were calculated and are plotted relative to values obtained with 26r. N=3. Error bars indicate standard deviation.

Figure S5. 3' end cleavage efficiency of several polyadenylated mRNAs. RNA from 26r and T4V cells, treated with tet for 40 hr, was isolated and analyzed by RT-qPCR using primers to detect uncleaved and total mRNA. The ratios of uncleaved/total were calculated and are plotted relative to values obtained with 26r. Actb: beta-actin, Rps11: ribosomal protein S11, Rpl32: ribosomal protein L32. Rplp1: ribosomal protein, large, P1. N=3. Error bars indicate standard deviation.

Figure S6. Western blot analysis of select 3' processing factors. T4V and 26r cells were treated with tet for 30 hr. Cell lysates were prepared and analyzed by Western blotting using antibodies against the indicated proteins. Actin and GAPDH serve as loading controls.

Figure S7. Nuclear run-on analysis. (A) Cells were treated with tet for 24 hr and nuclei isolated. Following incubation with ^{32}P-UTP for 30 min, RNA was purified and analyzed by slot blot containing DNA oligos corresponding to the indicated genes (Top). NC1, NC2: negative controls (anti-sense U1 and U2). Signals from each gene were normalized to 18S, and plotted (Bottom). (B) Cells were treated with tet for 40 hours and analyzed as in (A). N=3. Error bars display standard deviation.

Figure S8. Dot blot analysis of P-thr4 antibody specificity. Indicated amounts of heptad peptides with or without phosphorylation on thr, and of two oligo-peptides, with phosphorylation on ser 5 (SYSPTS(p)PSYS), or on both ser 2 and ser 5 (SYS(p)PTS(p)PSYS), were spotted on membrane. Blot was probed with P-thr4 antibody.

Figure S9. DRB sensitivity of thr 4 phosphorylation. Wild-type DT40 cells were treated with tet for 24 hr and the indicated concentrations of DRB were added and cells grown for an additional 6 hr. Cell lysates were analyzed by Western blotting.

Figure S10. Flavopiridol inhibits expression of histone mRNAs. 26r cells were treated with tet for 24 hr and grown for an additional 16 hr with or without flavopiridol (300 nM). RNA was isolated and mRNA levels determined by RT-qPCR. Values relative to the untreated samples were plotted. N=3. Error bars indicate standard deviation.

References

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2. M. L. West, J. L. Croden, Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. *Genetics* **140**, 1223 (1995).
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5. K. Livak, Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402 (2001).
6. M. M. Chong *et al.*, Epigenetic propagation of CD4 expression is established by the Cd4 proximal enhancer in helper T cells. *Genes & Development* **24**, 659 (Apr 1, 2010).
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8. I. C. G. S. Consortium, Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **432**, 695 (9 2004).

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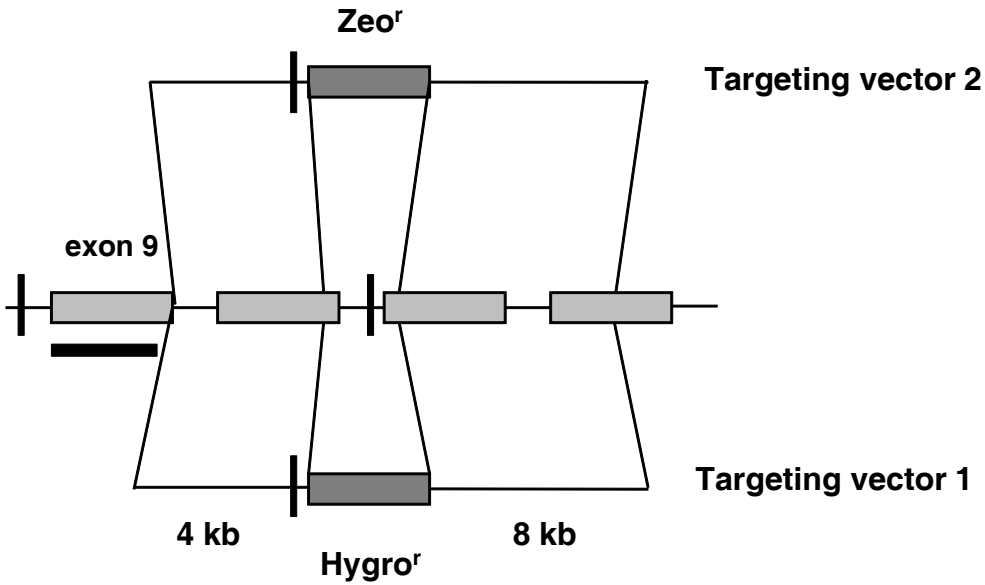
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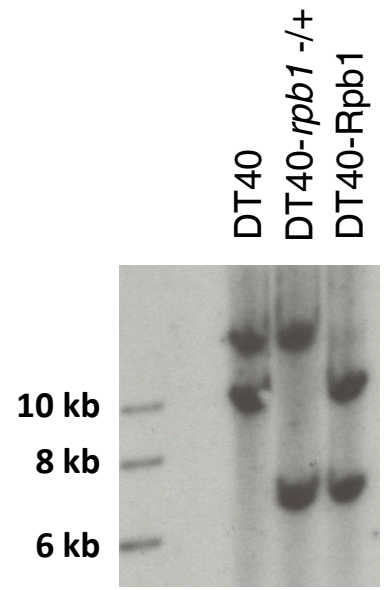
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DT40-Rpb1
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8 kb
6 kb

Figure S1

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zebra_fish      YSPTSPAYEPRSPGGGYTPQSPGYSPTSPSYSPTSPSYSPTSPNYSPTSPSYSPTSPSYS 60
                ************** ******.************************************

human           PTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSP 119
zebra_fish      PTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSPSYSPTSP 120
                ************************************************************

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                **********************************.************************

human           TSPNYTPTSPNYSPTSPSYSPTSPSYSPTSPSYSPSSPRYTPQSPTYTPSSPSYSPSSPS 239
zebra_fish      TSPSYSPTSPNYTPTSPNYSPTSPSYSPTSPSYSPSSPRYTPQSPTYTPSSPSYSPSSPS 240
                ***.*:******:****.****************************************

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zebra_fish      YSPTSPKYTPTSPSYSPSSPEYTPTSPKYSPTSPKYSPTSPKYSPTSPTYSPTTPKYSPT 300
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human           SPTYSPTSPVYTPTSPKYSPTSPTYSPTSPKYSPTSPTYSPTSPKGSTYSPTSPGYSPTS 359
zebra_fish      SPTYSPTSPTYTPTSPKYSPTSPTYSPTSPKYSPTSPTYSPTSPKGSTYSPTSPGYSPTS 360
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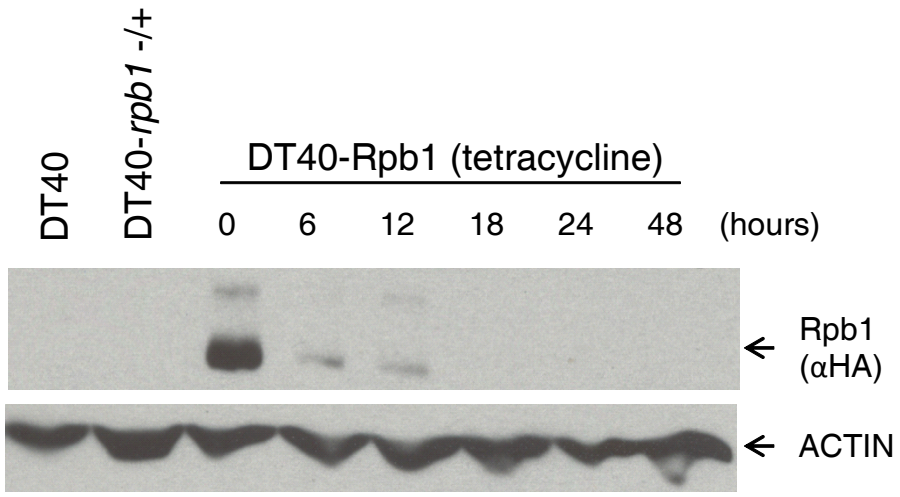
Figure S2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12
18
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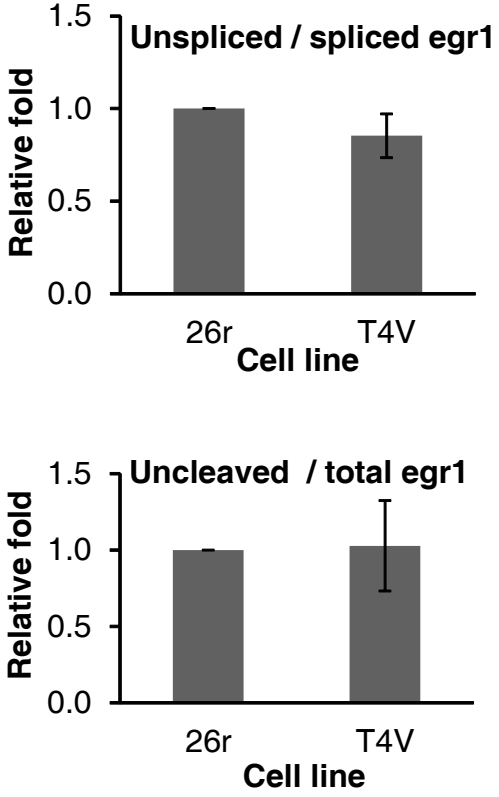
48
(hours)
Rpb1
(αHA)
ACTIN

Figure S3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0.5
0.0
26r
T4V
Cell line
Uncleaved / total egr1
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1.0
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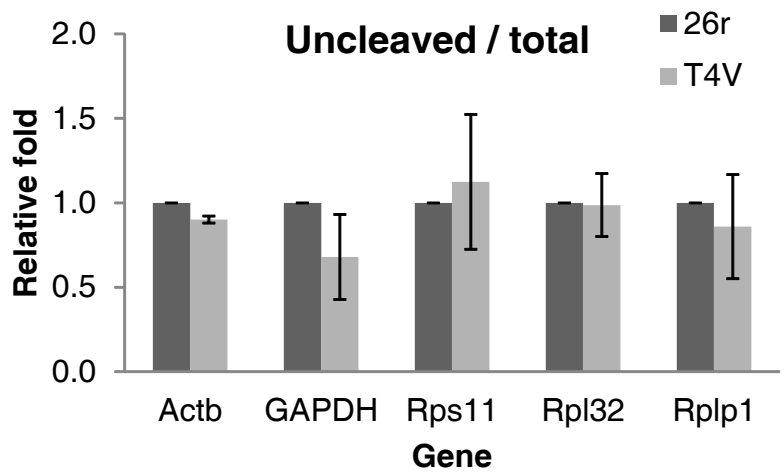
0.0
26r
T4V
Cell line

Figure S4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T4V
Relative fold
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Gene

Figure S5

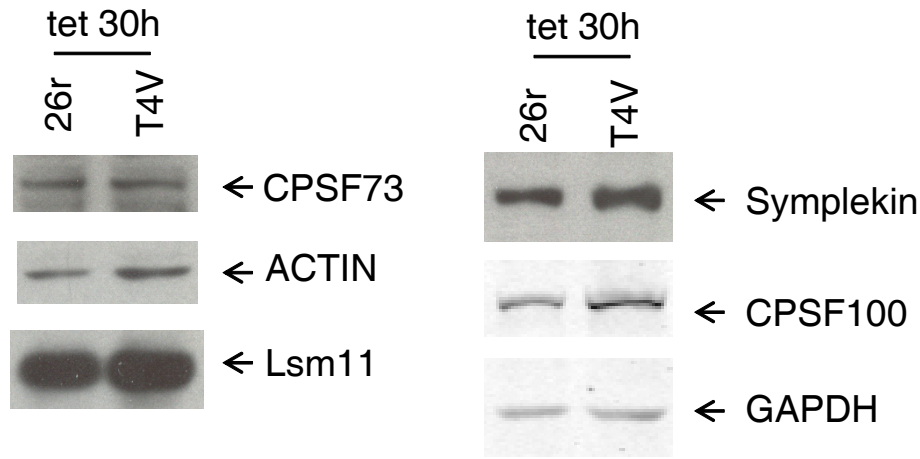
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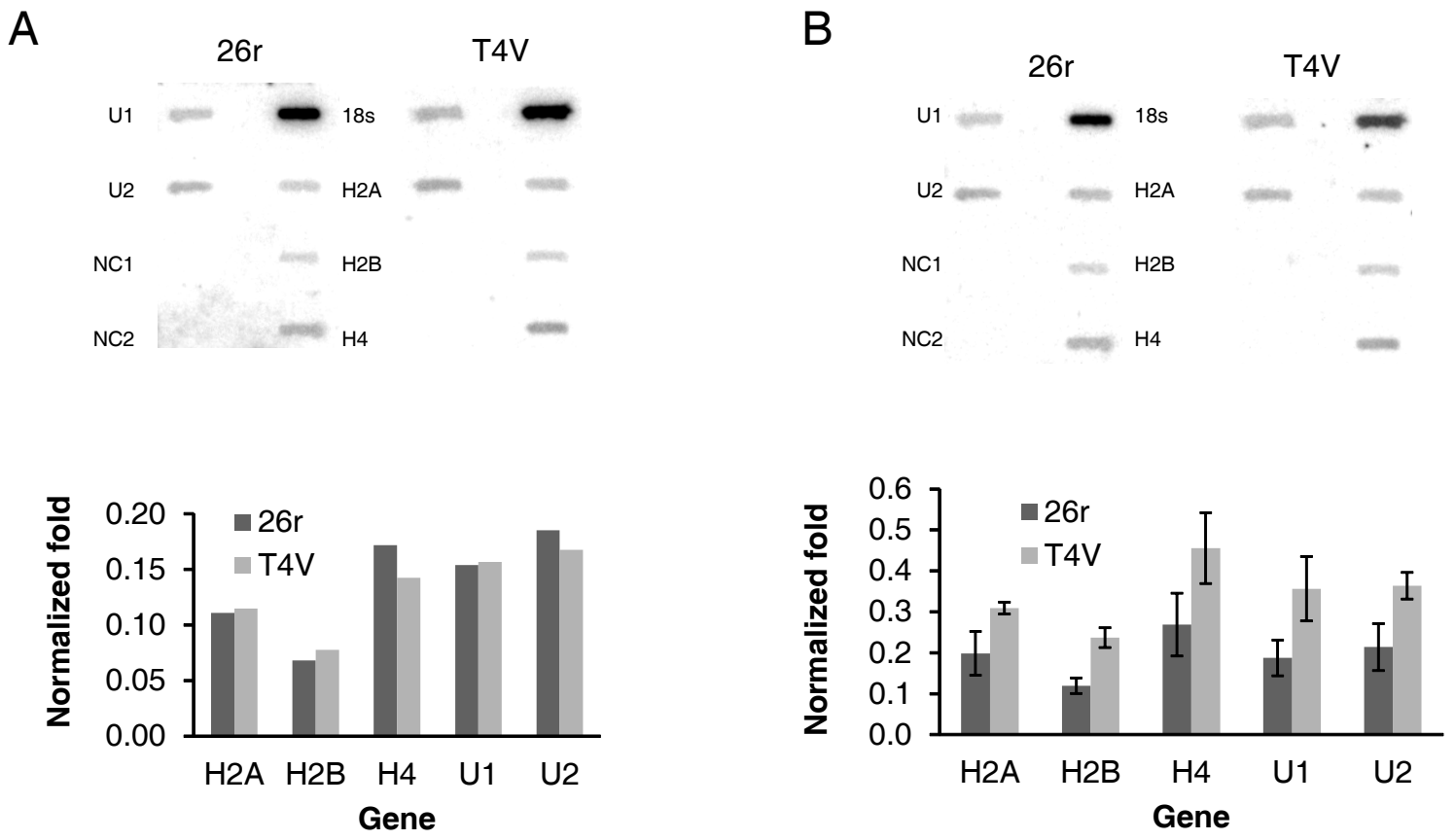
Figure S6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0.05
0.00
26r
T4V
H2A
H2B
H4
U1
U2
Gene
B
26r
T4V
U1
18s
U2
H2A
NC1
H2B
NC2
H4
Normalized fold
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0.5
0.4
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T4V
H2A
H2B
H4
U1
U2
Gene

Figure S7

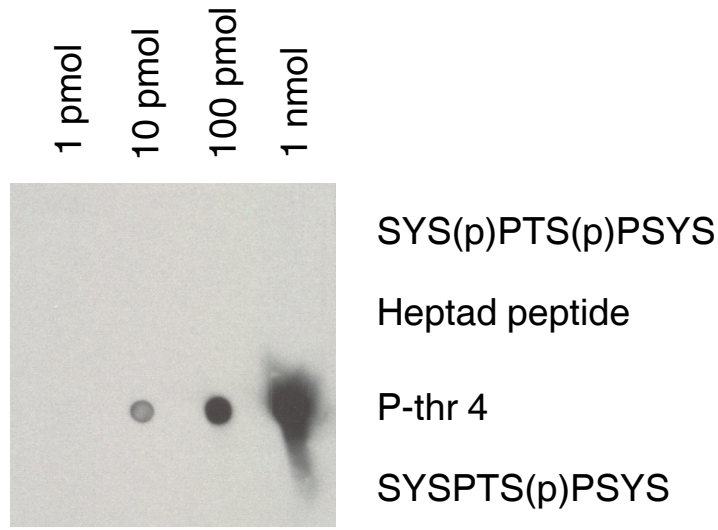
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Figure S8

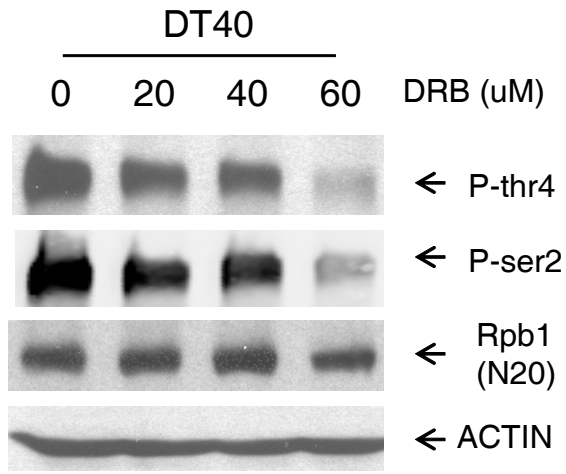
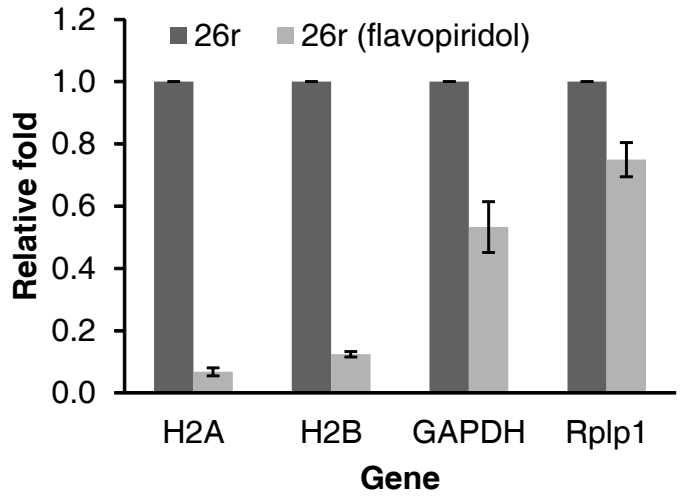


Figure S9


1.2
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Relative fold
26r
26r (flavopiridol)
H2A
H2B
GAPDH
Rplp1
Gene

Figure S10