P-TEFb regulation of transcription termination factor Xrn2 revealed by a chemical genetic screen for Cdk9 substrates

Miriam Sansó¹, Rebecca S. Levin², Jesse J. Lipp², Vivien Ya-Fan Wang³, Ann Katrin Greifenberg⁴, Elizabeth M. Quezada¹, Akbar Ali⁵, Animesh Ghosh⁵, Stéphane Larochelle¹, Tariq M. Rana⁵,⁶, Matthias Geyer⁴, Liang Tong³, Kevan M. Shokat² and Robert P. Fisher¹*  

¹Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, 10029-6574, USA  
²Department of Cellular and Molecular Pharmacology and Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA, 94143, USA  
³Department of Biological Sciences, Columbia University, New York, NY, 10027, USA  
⁴Department of Structural Immunology, Institute of Innate Immunity, University of Bonn 53127, Bonn, Germany  
⁵Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA  
⁶Department of Pediatrics, University of California San Diego School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093, USA  

*corresponding author: robert.fisher@mssm.edu
Supplemental Materials and Methods

Plasmids
Insert to generate pET15b-Xrn2 was obtained by PCR amplification from HCT116 cDNA.

Protein purification
DSIF protein purification was performed as previously described (Yamaguchi et al., 1999).

Kinase assays
Various kinase complexes, in indicated amounts, were incubated with 1 µg of substrate in 10 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl₂ and 1 mM ATP for unlabeled kinase assays or 100 µM unlabeled ATP and γ-³²P-ATP for radioactive kinase assays. Reactions were incubated 15 min at 25°C for Spt5 and GST-CTD, or 1 hr for Xrn2 substrate. Reactions were stopped by boiling in SDS, and products were visualized by SDS-PAGE followed by immunoblot or autoradiography and quantified with a phosphorimager.

Gene ontology term enrichment
Gene ontology analysis was performed using GOrilla software for biological function (Eden et al., 2009).

ChIP-seq data analysis
Wiggle files for Xm2 ChIP and input DNA (Brannan et al., Mol Cell 2012) were downloaded from GEO (Series: GSE36185) and analyzed in Cistrome (Liu et al., 2011)
using SitePro function. Average profiles were generated over human UCSC Known Genes (hg18) transcription start (txStart) and stop (txEnd) sites using a span of 2000 bp at a profiling resolution of 50 bp and considering the +/- direction.

**Supplemental References**

Eden, E., Navon, R., Steinfeld, I., Lipson, D., and Yakhini, Z. (2009). GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 10, 48.

Liu, T., Ortiz, J.A., Taing, L., Meyer, C.A., Lee, B., Zhang, Y., Shin, H., Wong, S.S., Ma, J., Lei, Y., et al. (2011). Cistrome: an integrative platform for transcriptional regulation studies. Genome Biol 12, R83.

Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. Cell 97, 41-51.
Supplemental Figures and Legends

Supplemental Figure 1. Identification of AS kinase substrates. (A) Cdk9<sup>AS</sup> uses N<sup>6</sup>(phenethyl)-ATP<sub>γ</sub>S with high efficiency. Phosphorylation of 1 µg GST-CTD by 150 ng Cdk9<sup>WT</sup> or Cdk9<sup>AS</sup> with indicated N<sup>6</sup>-modified analogs of ATP<sub>γ</sub>S (B) Cdk1/cyclin B target sequence, generated from previously published data. (C) Venn diagram showing overlapping target proteins (upper diagram) or peptides (lower diagram) from Cdk9<sup>AS</sup>/cyclin T1 analysis, compared with prior analysis of Cdk1<sup>AS</sup>/cyclin B or Cdk2<sup>AS</sup>/cyclin A. (D) Gene Ontology (GO) term enrichment of identified S/T-P targets of Cdk9/cyclin T1. Analysis was done with GOrilla and compared to the entire human proteome as background.
Supplemental Figure 2. Validation of Xrn2 as a CDK substrate. (A) Immunoblot of 1 µg purified His-Xrn2, phosphorylated or not by 150 ng Cdk9/cyclinT1, probed with Xrn2-T439 phosphospecific antibody. The kinase alone was probed as control. (B) Coomassie blue stained gel of all purified CDK complexes used in the manuscript. (C) Quantification of Cdk7 and Cdk9 kinase activity towards GST-CTD. Phosphorylation signals were visualized by autoradiography (lower panel) and quantified (top panel). (D) Phosphorylation in vitro of 1 µg His-Xrn2∆C incubated with 150 ng activated Cdk7/cyclin H/Mat1 (K7), Cdk9/cyclin T1 (K9), Cdk12/cyclin K (K12) or Cdk13/cyclin K (K13) complexes; no kinase treatment was the control (-). Top panel: autoradiograph; bottom panel: Coomassie blue stained gel. (E) Phosphorylation in vitro of 1 µg His-Xrn2∆C; either wild-type (WT) or mutants T439A (TA), T439D (TD) E203G (EG), or Xrn2∆C lacking the linker domain (-L), incubated with 150 ng activated Cdk7/cyclin H/Mat1 or Cdk9/cyclin T1. No kinase treatment and no substrate (-) were controls. Top panel: autoradiograph; bottom panel: Coomassie blue stained gel.
**Supplemental Figure 3.** Phosphorylation of Xrn2 occurs on chromatin. (A) Immunoblot analysis of soluble and chromatin-associated protein fractions after 4-hr treatment with 1 µM FP or 2-FP-FP or DMSO as negative control. Pan Protein Phosphatase 1 (PP1) is loading control for both fractions. (B) Cdk7<sup>As</sup> inhibition by treatment of CDK7<sup>As/As</sup> cells with 10 µM 3-MB-PP1 for 4 hr does not alter Xrn2-T439 phosphorylation in vivo, detected by immunoblotting. Wild-type (WT) HCT116 cells were used as control.
Supplemental Figure 4. Phosphospecific Spt5 antibody validation. (A) Schematic representation of Spt5 protein: acidic domain, five regions of homology to KOW domains in prokaryotic NusG, NusG N-terminal motif (NGN) and two C-terminal repeat (CTR) regions are indicated, as are Cdk9 target residues Ser666 and Thr806 revealed in our analysis. (B) Coomassie blue stained gel of purified human DSIF complex. (C) Spt5-S666 and Spt5-T806 phosphospecific antibodies were tested against Spt5 from purified DSIF complex after incubation with active Cdk9/cyclinT1 or mock treatment. CDK complex alone was used as control.
Supplemental Figure 5. ChIP controls for Xrn2 and Xrn2-T439-P. (A) Xrn2 binding profile on MYC in shXrn2 cells compared to shEmpty cells as control; minimal residual binding of the protein validates the knockdown. (B) Xrn2-T439 phosphorylation profile of MYC in shXrn2 cells compared to shEmpty cells as control; minimal residual binding validates the specificity of the antibody for ChIP. Bars show average of n=3 and +S.E.M. (C) Average profile of Xrn2 binding centered on the TSS (left panel) or the TTS (right panel). Analysis was performed with published ChIP-seq data (Brannan et al. 2012). (D) Ratio of Pol II-Ser2P to total Pol II ChIP signals calculated from data in figures 4D and 4E.
Supplemental Figure 6. Effect of Cdk7 inhibition on Xrn2 recruitment and phosphorylation on chromatin. (A) At top is a schematic diagram of MYC gene with positions of TSS (horizontal arrow), PAS (vertical arrow) and primers used for ChIP. Plots below show ChIP profiles of Xrn2, Xrn2-T439-P, total Pol II, Pol II Ser2-P and ratio of Xrn2-T439-P to total Xrn2 ChIP signals, as indicated, in wild-type (WT) or CDK7ass/ass HCT116 cells treated with DMSO or 10 μM 3-MB-PP1 for 1 or 4 hr (n = 2 + SEM). (B) Same as in (A) for CCNB1 gene.
Supplemental Figure 7. (A) Xrn2 activity is enhanced by treatment with Cdk9. Comparison of exonucleolytic activity of 50 nM full-length Xrn2 with or without pre-incubation with 50 nM Cdk9/cyclin T1 for 1 or 2 hr, as indicated. 500 nM RNA:DNA substrate (30-nt RNA hybridized to 17-nt DNA) was analyzed at 30°C in (A-C) (n=2, +SEM). (B) Xrn2 stimulation by Cdk9 depends on ATP. Comparison of exonucleolytic activity of 50 nM full length Xrn2 pre-incubated in the presence or absence of 1 mM ATP, with or without 10 nM Cdk9/cyclin T1 for 1 hr (n=2, +S.E.M.). (C) Xrn2 stimulation by Cdk9 is abolished by pre-incubation with FP. 50 nM full length Xrn2 was pre-incubated with 10 nM Cdk9/cyclin T1, previously treated with 500 nM FP or mock-treated with DMSO, for 15 min. Xrn2 pre-incubated with FP was used as control (n=2, +SEM). (D) Slopes derived from interval of linear product accumulation with respect to time from figure 5A. (E) Gel-based exonuclease assay of mock control and 250 nM Xrn2ΔC –L on 500 nM 30-nt ssRNA substrate. (F) Exonuclease assay on 30-nt RNA hybridized to 17-nt DNA substrate, performed as in (E).
**Supplemental Figure 8.** Readthrough transcription due to CDK inhibition or Xrn2 depletion. (A) Readthrough transcription is increased by treatment with 500 nM FP or 2-FP-FP for 1 or 4 hr. Readthrough transcription was measured using primers diagrammed schematically in Fig. 6A, and is expressed as fold-change relative to DMSO-treated cells. (B) Amplification of short fragments downstream of the CCNB1 PAS. Total RNA was converted into cDNA with a mixture of random hexamers and oligo(dT), followed by PCR amplification of ~100-bp fragments, as indicated schematically. In cells treated with DMSO, cDNA is not detectable >500 nt downstream of the polyadenylation site (F2-R2), whereas in cells treated with 500 nM FP for 4 hr, transcripts were detected ~2750 nt downstream (F5-R5). The last exon of CCNB1 mRNA was amplified as control for total transcription level, and $\beta$-actin was used as a cDNA loading control. (C) Amplification of continuous readthrough products downstream of the CCNB1 PAS. Experiment was performed as in (A), using primer pairs shown schematically. Genomic DNA (gDNA) amplification was performed as control for primer efficiency. Treatment with 500 nM FP or inhibition of Cdk7 with 10 μM 3-MB-PP1 for 4 hr caused continuous readthrough up to 2.7 kb beyond the most downstream signal detected in DMSO-treated cells. A similar effect is produced by Xrn2 depletion.
Supplemental Table 1. Complete list of peptides thiophosphorylated by Cdk9\textsuperscript{cyclinT1} and identified by mass spectrometry after covalent capture and release.

Supplemental Table 2. Primer sequences used in this study.

| MYC  | Forward     | Reverse       |
|------|-------------|---------------|
| 1 (-200) | GTAGTTAATTCCATGGGCGCTCTTACT | GGGCAGGCGGACACCTCTA |
| 2 (+1)   | GAGGGAGCTGCGCTGAGGA | TCTGCCCTTCGCTGGAATTAC |
| 3 (+190) | GCCGATTCCAGGAAACTTT | TCCTTGCTCGGTTGGTGAAG |
| 4 (+35)  | GTCCTAACAGCTATTAGCTTCTTAGTA | CACCTTCACAGGATAGGAACATTTG |
| 5 (+155) | AAGCTATTTTTTTCTTTTTAAATTTGTTTATT | GGCTCAATTGATATATTTTCGACGATTATTTA |
| 6 (+6028)| TCCCTATTAGATGAGATGAGGCAAA | CTGGGCGATGAGAGCTGCT |
| 7 (+7028)| ATCCGGAAGAGGTTGATGCTGAGATAC | CACTCTCTCTCTTCTCTCGAGGCTT |
| 8 (+11382)| TGAAGGATTTGAGGCTCTGGAATA | TCCTTCTGACAGGCTCTTGAAG |

| FRAT2 | Forward   | Reverse      |
|-------|-----------|--------------|
| +1502 | GCTCACCTGAGCGCTTTTGCT | CAACAGGCGCTTTCTGAGG |
| +2517 | GAGGGTTTTTCTACCTGGA | CCAAGGCGTATTCACCAGAC |

| CCNB1 | Forward     | Reverse       |
|-------|-------------|---------------|
| 1 (-239) | TTGACTCTTCTGAGACTGTGG | AAAGGGTTACCGGGGCGAGAGT |
| 2 (+7874) | ATCCCTTGGGAACCCATTCT | GCCAGGCTTAGGCTCAGATTTA |
| 3 (+7874) | TTGGTAGGTAAGAGATGCTG | GTCACCAATTCTCGAGGAGGTAC |
| 4 (+11180) | GCGACTCTTTTTGTTTTGTTT | GTGGGATATTGAAGAAGCCTT |
| 5 (+12071) | GAGCAGTTTGGCTCCATGTTGG | TGTTGCTGACGCGCTCTTAAAC |
| 6 (+13656) | CCAGATGAGGATCTGGTGGT | TTGGCACCCTGCTGATGTAAG |
| 7 (+14171) | ACTGCTGCAAGGAGCGATC | CAGTGGCTCAACCCTGGAATC |
| +10518 | GTCGAAAGCAAGATACGCCCAC | CACCTTTGCCACACGGCTTG |
| +12454 | GATTTCAAGTGGTGGCGAGAT | AGATCATGCCACTGCTC |
| F0    | GTCAAGGAAAGTCTGGCCAC | |
| F1    | AGCTATGATATTCCAAAACCTTTC | |
| F2    | GTCAACATAGTATGAGATGCC | |
| F3    | GCTTAGAACACCGACTCCAG | |
| F4    | GCTTGGCCCTACTGAAATATTC | |
| F5    | CTCACCTAGTTGTTGCAAGGCT | |
| R0    | CACCTTTGCACAGCCTTGG | |
| R1    | CCATATGTGATGCTCTCCAGG | |
| R2    | TACTCAACAAACATCTGATGG | |
| R3    | TATGCTATCTGGTTCATACAA | |
| R4    | GTGTTGATGATACGCCCATATA | |
| R5    | AACTGATGTCACCTGGATAG | |

| XRN2 | Forward     | Reverse       |
|------|-------------|---------------|
| T498A | CTCTGTAATGCAGTTTATATGCTTCTCATG | GAACCCAAAGCAGTGAGGAGcTAATATTC |
| T493D | CACTCACTGATGATATATGATCTTCTCA | CACTAGGAG |
| T493D | CACTCACTGATGATATATGATCTTCTCA | CACTAGGAG |
| E203G | GCTAGTGCGCTCTGTGTGGAGAGAAC | GATTTTATGTTTCTCTcCACCGAGGCA |
|     | ATAAAATC | CTAGC |

Fluorescently labeled probes for exonuclease assays:

- RNA 30 nt: 5' - 5'Phos/ACUCACUCACUCACCA------------------------------/3'FAM/-3'
- RNA 19 nt: 5' - 5'Phos/CACCA------------------------------/3'FAM/-3'
- DNA 17 nt: 5' - 5'55 TAMRA/55 TAMRA/3'FAM/-3'
- RNA 17 nt: 5' - 5'55 TAMRA/GGUUUUUUUUUUUUGG-3'