SUPPLEMENTARY DATA:

Cyclin D1 overexpression induces global transcriptional downregulation in lymphoid neoplasms

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SUPPLEMENTARY DATA INDEX:

Supplemental Material and Methods

Cyclin D1 ChIP

Fifty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125mM of glycine and washed twice with cold PBS. Cells were lysed according to the manufacturer's guidelines and chromatin was fragmented with the Covaris S220 instrument for 20 min (5% duty factor, 135w intensity, 200 cycles per burst) using the truChIP™ HighCell Chromatin Shearing Kit with Non-ionic Shearing Buffer (Covaris). Immunoprecipitation was performed with anti-cyclin D1 antibody or control IgG. Sheared chromatin was clarified by 10 mins of maximum speed centrifugation at 4°C. Sheared DNA was immunoprecipitated overnight at 4ªC with 20 µg of anti-cyclin D1 antibody or equivalent amount of control IgG. Antibody complexes were recovered with 80µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) through co-incubation for 90 min at 4°C. After intensive washes in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer (1% Nonidet 40, 0.25M LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM TrisHCl pH=8.0, 1mM EDTA), bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M NaHCO3). Chromatin was decrosslinked and purified with for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse and Proteinase K before purification. AgencourtAMPure beads (Beckman Coulter) were used to purify DNA according to the manufacturer's guidelines. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency. Equal volumes of eluted chipped

DNA were amplified using SYBR Green PCR Master Mix (Applied Biosystems) under the manufacturer's guidelines. Specific primers were designed for the peaks obtained in the ChIP-seq analysis (Supplemental Table S3). Primers for negative regions were also designed to test the specificity of the enrichment.

Pol II ChIP

Twenty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125Mm of glycine and washed twice with cold PBS. Cytoplasmic membranes were lysed in Pol II lysis buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl pH=8) with protease inhibitors (Thermo Fisher Scientific) for five minuts incubation on ice and nuclei were pelleted by centrifugation (2000g, 5min, 4°C). Pol II lysis buffer was added to obtain a concentration of 25 million nuclei/mL and sonicated with a Bioruptor (Diagenode) for 18 mins (Cycles: 30''on/30''off, High Power, 4°C). In order to precipitate the SDS, sonicates were incubated on ice for 30-60 mins. Following 15 mins 4ºC centrifugation at maximum speed, the supernatants were diluted with ChIP buffer between 8-10 times. ChIP buffer was added to ensure that all samples had the same final volume. 30µg of total chromatin were immunoprecipitated overnight at 4ºC with 20 µg of anti-Pol II antibody or equivalent amount of control IgG. Antibody complexes were recovered after 90 mins of co-incubation with 50µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) at 4°C. Beads were washed for five mins at 4ºC in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer (1% Nonidet 40, 0.25M LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM TrisHCl pH=8.0, 1mM EDTA).

Bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M NaHCO3). Chromatin was decrosslinked for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse A (Sigma Aldrich) and Proteinase K before purification. Samples were purified using the phenol-chloroform method. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency.

RNA Sequencing

Total RNA was isolated using Trizol (Zymo Research). Preparation of RNA-seq libraries was carried out using the TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's standard protocol. Briefly, mRNA molecules were purified from 800ng of total RNA using poly-T oligo attached magnetic beads. Following purification, mRNA was fragmented using divalent cations at 94ºC and copied into first strand cDNA using reverse transcriptase and random primers. Second strand cDNA synthesis was performed using DNA Polymerase I and RNase H. The cDNA fragments were end repaired and a single ‘A’ base overhang was added before adapter ligation. The products were then purified and amplified by PCR to generate the final cDNA library. The libraries were 75 bp paired-end sequenced at ~ 80 million reads per library with Illumina technology.

Immunoprecipitation and Western blot

Protein extracts preparation and western blot analysis were performed as previously described (Palomero et al, 2014). The primary antibodies and the working dilutions are described in Supplemental Table 5. For endogenous CDK9 and Cyclin D1 immunoprecipitation, approximately 15-20 million exponentially growing cells were

washed in PBS RT (300g, 5 mins) twice. Protein extracts were done in immunoprecipitation buffer (0.5% Nonidet-40, 150nM NaCl, 10mM KCl, 1.5mM MgCL, 10mM Tris-HCl pH=8.0) with protease inhibitors (Thermo Fisher Scientific). Total protein was quantified and 1.5mg of total protein was used per co-immunoprecipitation and incubated overnight at 4°C with gently mixing with 7.5µg of CDK9, cyclin D1 antibody or IgG Mouse control antibody in a final volume of 350µL of Co-IP buffer. Then, for each sample 50µL of protein G-magnetic beads (Invitrogen) were equilibrated by washing twice with co-IP buffer. Beads were finally resuspended in 25µL of co-IP buffer and added to co-IPS. After 90 mins incubation, supernatants were recovered (Unbound fractions) and beads were washed twice for 5 mins with gentle rotating at 4°C with co-IP buffer. To elute, 40µL of Laemmli buffer (x2.5) without DTT were added to beads and heated at 70°C for 10 mins. A second elution was performed with 40µL of sample buffer (x2.5) and 5µL of DTT 1M. Finally, 5µL of DTT 1M were added to first elution and both elutions were boiled as usual for western blotting. Anti-Mouse light chain antibody-HRP conjugate (Millipore) was used to detect primary antibodies. Flag immunoprecipiation of CDK9-Flag complexes was performed using ANTI-FLAG® M2 Affinity Gel ((Sigma-Aldrich) following manufacturer's guidelines.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

A) Western blot analysis of cyclin D1 in MCL cell lines. α-Tubulin was used as loading control. B) Linear correlation between cyclin D1 protein amount and the number of ChIP-seq peaks inMCL cell lines. Cyclin D1 intensity was corrected by α-Tubulin. Pearson correlation is shown. C) Genome browser views displaying the ChIP-seq tag density of the entire length of four representative cyclin D1 target genes in GRANTA-519 cell line.

Supplemental Figure 2

A) Profile of cyclin D1 occupancy around the TSS according to gene expression in UPN-1, JeKo-1 and GRANTA-519 cell lines. Genes were divided in ten groups based on their expression levels (from higher to lower expression). The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group. B) Western blot analysis of cyclin D1 expression in Z-138 MCL cell line (b), JVM13 transduced with empty-vector (JVM13-Ctrl) and JVM13 transduced with constitutive HA tagged Cyclin D1-T286A (JVM13-cD1^{T286A}) (a). β-Actin was used as loading control. C) Linear correlation between cyclin D1 protein amount and the number of ChIP-seqpeaks in four MCL cell lines and JVM13-cD1^{T286A} cells.D-E) Profile of cyclin D1 occupancy around the TSS in JVM13-cD1^{T286A} cells. Genes were divided in ten groups regarding gene expression in JVM13-cD1^{T286A} cells (D) or JVM13 control cells (E) from higher to lower expression. The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group.

Supplemental Figure 3

A) Kernel distribution of normalized cyclin D1 tag density showing a unimodal distribution in MCL cell lines. B) Heatmap showing the cyclin D1 ChIP-seq tag density within gene intervals of Z-138 and MYC ChIP-seq tag density in GM12878. Each row represents an interval of +/− 2kb around the summit part of the peak in Z-138. Regions are sorted by the number of cyclin D1 tags in Z-138. C) Tag density of cyclin D1 peaks in Z-138 with or without MYC co-localization in GM12878 cells. Data is shown as mean ± SEM. ****P*<2.2e-16,Student's t-test,Holm Bonferroni correction.

Supplemental Figure 4

A) RNA quantification by pyronin Y staining in JVM13 inducible cell lines. Only cells in G2/M phase were analyzed. Data isshown relative to the Ctrl (mean +/- SEM,n=3). **P*<0.05,Student's t-test. B) RNA quantification by Nanodrop corresponding to total RNA extracted from one million cells in JVM2 inducible cell lines. Results are shown relative to the Ctrl (mean +/- SEM,n=9).C)RNA quantification by pyronin Y staining in JVM2 inducible cell lines. Only cells in G1 phase were analyzed. Pyronin Y mean signal is shown relative to the Ctrl (mean +/- SEM,n=3). **P*<0.05,Student's t-test. D)RNA quantification by pyronin Y staining in cyclin D1-depleted GRANTA-519 cells. Only cells in G2/M phase were analyzed. Data is shown relative to the Ctrl (mean +/- SEM,n=4). ****P*<0.001,Student's t-test.Holm Bonferroni correction for multiple comparisons was applied to panels A-D.E) Western blot showing cyclin D1 expression in different MCL cell lines and cyclin D1 overexpressing cell models. Endogenous cyclin D1 (b) or overexpressed-tagged cyclin D1 (a) are shown. α-Tubulin was used as loading control. F) Western blot showing cyclin D1 in different multiple myeloma cell lines. α-Tubulin was used as loading control. G) Correlation between cyclin D1 protein

levels measured by western-blot and the total RNA content per cell quantified by pyronin staining in primary MCL cases. Pyronin signals represented are the mean of two technical replicates of G1 cell fraction. Cyclin D1 intensity was corrected by β-Actin.

Supplemental Figure 5

Representative images of EU staining (red) for the evaluation of nascent RNA synthesis after cyclin D1 induction for 2 days in JVM-13 cells (A) or following cyclin D1 depletion in GRANTA-519 cells (B). DAPI (blue) was used to create the nuclear masks for quantification. Scale bars, 10 μm.

Supplemental Figure 6

A) Percentage of TSG and non-TSG genes with cyclin D1 peaks in their promoter regions (-5Kb from TSS) that were found expressed by the Cancer Panel in JVM13-cD1^{T286A} (P=0.038, χ^2 test). B) Percentage of TSG and non-TSG genes with peaks in the four MCL cell lines(P= P<3.3E-9, χ^2 test). C) Proportion of TSG genes distributed among the four quartiles corresponding to the gene expression levels in primary MCL cases. The group of highly expressed genes shows enrichment in TSGs. Color indicates percentage of TSG in each quartile.

Supplemental Figure 7

A) Pol II occupancy on three representative cyclin D1-bound genes. Pol II (8GW16) profiles are shown in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cyclin D1 binding pattern in JVM13-D1^{T286A} is also represented. B) Correlation between normalized Pol II ChIP-seq tag density at promoters in JVM13-Ctrl and JVM13-

D1^{T286A}. Promoters were sorted into 50 equal-size groups based on ChIP-seq tag densities in JVM13-Ctrl. Dashed line marks diagonal. C) Western blot showing total Pol II (IIo and IIa isoforms), Pol II (8WG16) and cyclin D1 in 14 primary MCL cases. MCL cases with cyclin D1 expression above the mean were considered as high cyclin D1 (red) and samples below the mean as low cyclin D1 (green). β-Actin was used as loading control

Supplemental Figure 8

A) Co-immunoprecipitation experiment in JeKo-1 using antibodies against cyclin D1 and control IgG. Immunoprecipitated proteins were analyzed by western blot analysis by blotting with cyclin D1 and Pol II antibody. Input at 1% was loaded as a control.B) Endogenous CDK9 co-immunoprecipitation experiment in Z-138 and Jeko-1 (C) using antibodies against CDK9 (B) and control IgG. Immunoprecipitated proteins were analyzed by Western blot with cyclin D1 and CDK9 antibodies. Input at 1% was loaded as a control.

SUPPLEMENTAL TABLES

Supplemental Table 1. Enrichment of cyclin D1 ChIP regions in different genomic features (%)

Genomic feature	GRANTA-519	Jeko-1	UPN-1	Z-138	Genomicbackground
Promoter	26.0	26.7	24.8	18.9	3.5
Exon	4.0	5.2	3.5	3.5	1.9
Intron	23.9	24.0	26.2	28.6	42.4
Intergenic	46.1	44.1	45.5	49.0	52.2

Supplemental Table 2. Enrichment of cyclin D1 ChIP regions with tag density higher that the mean in each cell line in different genomic features (%)

Genomic feature	GRANTA-519	Jeko-1	UPN-1	Z-138	Genomicbackground
Promoter	56	56	49	42	3.5
Exon	5	6	5	5	1.9
Intron	12	12	15	19	42.4
Intergenic	27	26	31	34	52.2

Supplemental Table 3. Annotation clusters with Enrichment Score>5 in CycD1-bound regions.

Annotation_Cluster1	Enrichment Score: 11.575770882907724		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006412~translation	6.11E-13	3.04E-16
KEGG_PATHWAY	hsa03010:Ribosome	3.21E-08	2.60E-11
GOTERM_BP_FAT	GO:0006414~translational elongation	4.35E-06	2.36E-09

Annotation Cluster2	Enrichment Score: 11.362884382236716		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0007049~cell cycle	2.65E-12	1.49E-15
GOTERM_BP_FAT	GO:0000278~mitotic cell cycle	4.68E-11	2.54E-14
GOTERM_BP_FAT	GO:0022402~cell cycle process	9.85E-11	5.35E-14
GOTERM_BP_FAT	GO:0022403~cell cycle phase	7.09E-10	3.85E-13
GOTERM_BP_FAT	GO:0000087~M phase of mitotic cell cycle	2.40E-08	1.30E-11
GOTERM_BP_FAT	GO:0051301~cell division	2.08E-07	1.12E-10
GOTERM_BP_FAT	GO:0007067~mitosis	2.80E-07	1.52E-10
GOTERM_BP_FAT	GO:0000280~nuclear division	2.80E-07	1.52E-10
GOTERM_BP_FAT	GO:0000279~M phase	5.17E-07	2.80E-10
GOTERM_BP_FAT	GO:0048285~organelle fission	5.76E-07	3.10E-10

Annotation Cluster3	Enrichment Score: 8.546639796201333		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006396~RNA processing	4.11E-13	2.68E-16
GOTERM_BP_FAT	GO:0016071~mRNA metabolic process	5.33E-07	2.89E-10
GOTERM_BP_FAT	GO:0008380~RNA splicing	5.64E-07	3.06E-10
GOTERM_BP_FAT	GO:0006397~mRNA processing	2.86E-05	1.55E-08
GOTERM_BP_FAT	GO:0000398~nuclear mRNA splicing, via spliceosome	2.71E-04	1.47E-07
GOTERM_BP_FAT	GO:0000377~RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	2.71E-04	1.47E-07
GOTERM_BP_FAT	GO:0000375~RNA splicing, via transesterification reactions	2.71E-04	1.47E-07
KEGG_PATHWAY	hsa03040:Spliceosome	0.00439914	3.56E-06

Annotation Cluster4	Enrichment Score: 8.190837507398893		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	8.06E-07	4.37E-10
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	4.40E-06	2.39E-09
GOTERM_BP_FAT	GO:0006281~DNA repair	2.22E-05	1.20E-08
GOTERM_BP_FAT	GO:0033554~cellular response to stress	2.52E-04	1.36E-07

Annotation Cluster5	Enrichment Score: 7.557183124723839		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0022613~ribonucleoprotein complex biogenesis	9.06E-10	2.50E-13
GOTERM_BP_FAT	GO:0042254~ribosome biogenesis	2.10E-06	1.14E-09
GOTERM_BP_FAT	GO:0034470~ncRNA processing	1.28E-04	6.93E-08

GOTERM_BP_FAT	GO:0034660~ncRNA metabolic process	2.35E-04	1.27E-07
GOTERM_BP_FAT	GO:0006364~rRNA processing	0.01140151	6.19E-06
GOTERM_BP_FAT	GO:0016072~rRNA metabolic process	0.02713958	1.47E-05

Annotation Cluster6 Enrichment Score: 6.433945684537975

Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0044265~cellular macromolecule catabolic process	6.96E-06	3.78E-09
GOTERM_BP_FAT	GO:0043632~modification-dependent macromolecule catabolic process	8.36E-05	4.54E-08
GOTERM_BP_FAT	GO:0019941~modification-dependent protein catabolic process	8.36E-05	4.54E-08
GOTERM_BP_FAT	GO:0006511~ubiquitin-dependent protein catabolic process	1.03E-04	5.61E-08
GOTERM_BP_FAT	GO:0009057~macromolecule catabolic process	1.59E-04	8.61E-08
GOTERM_BP_FAT	GO:0051603~proteolysis involved in cellular protein catabolic process	2.78E-04	1.50E-07
GOTERM_BP_FAT	GO:0044257~cellular protein catabolic process	3.65E-04	1.98E-07
GOTERM_BP_FAT	GO:0030163~protein catabolic process	0.00107267	5.82E-07
GOTERM_BP_FAT	GO:0006508~proteolysis	97.8774912	1.88E-06

Annotation Cluster7 Enrichment Score: 5.754700439254545

Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0051276~chromosome organization	1.15E-05	6.26E-09
GOTERM_BP_FAT	GO:0006325~chromatin organization	0.0408486	2.21E-05
GOTERM_BP_FAT	GO:0016568~chromatin modification	0.07203701	3.91E-05

Supplemental Table 4. Primers for cyclin D1 ChIP-seq validation and shSequences

Primers		
Gene	***Position***	***Sequence***
XPC	Forward	TTTAAGGAGGTCGCTCGAAG
XPC	Reverse	GGCCATTTTTCCTGAGTCTG
RFC3	Forward	TAGCCTTTCCGTCCAAAATC
RFC3	Reverse	GGCCTACGCTTGAAAATCC
CCNT1	Forward	CCGAGTTAACAGCCAATATGC
CCNT1	Reverse	GTTCTCGCGGGAAGATACAC
CDC5L	Forward	CTTTGGCCAGAGTGGTTTG
CDC5L	Reverse	GATATTGGGTGGCTGAAAGG
POLE	Forward	CGCTCCTCAGAGACATGGA
POLE	Reverse	CAAATTTCTCCCCTGAAGCA
TIPIN	Forward	CTCACCTCACGCAGAAAACA
TIPIN	Reverse	CCCAGGAGTTCCCGAGTATC
MRE11A	Forward	GCAGGATCCGTGAAAAGAA
MRE11A	Reverse	AGAGCCGAACTGGACTTGAA
RPL4	Forward	CCAAACCACTCCTATTCCCT
RPL4	Reverse	TAGCCAACTCGTAATAAGACCA
Chr12negativeregion	Forward	CCATTGTAGGAGCCAAATCC
Chr12negativeregion	Reverse	ATTGAACACCAGCTCCCAAC

sh_Sequences		
Identifier	***Reference***	***Sequence***
shCycD1 #1	(TRCN0000295873)	CCGGCCACAGATGTGAAGTTCATTTCTCGAGAAATGA ACTTCACATCTGTGGTTTTTG
shCycD1 #2	(TRCN0000295874)	CCGGACAACTTCCTGTCCTACTACCCTCGAGGGTAGTA GGACAGGAAGTTGTTTTTTG

Supplemental Table 5. Antibodies

Primary antibodies

Reference	Antigen detected	Company	W.B. Dilution	ChIP	Co- IP
sc-8396	Cyclin D1	Santa Cruz	1:1000	X	X
sc-20044	Cyclin D1	Santa Cruz	1:1000		
sc-753	Cyclin D1	Santa Cruz	1:1000		
13499	Phospho-Rpb1 CTD (Ser2) (E1Z3G)	Cell signalling	1:500		
sc-899	Pol II (N-20)	Santa Cruz	1:100		
sc-55492	Pol II (F-12)	Santa Cruz	1:500		
920101	RNA Polymerase II(8WG16)	Biolegend	1:500	X	
sc-13130	CDK9 (C-20)	Santa Cruz	1:1000		X
CP06-100UG	Alpha-Tubulin	Oncogene	1:10000		
A5341-100UL	Beta-actin	Sigma	1:5000		

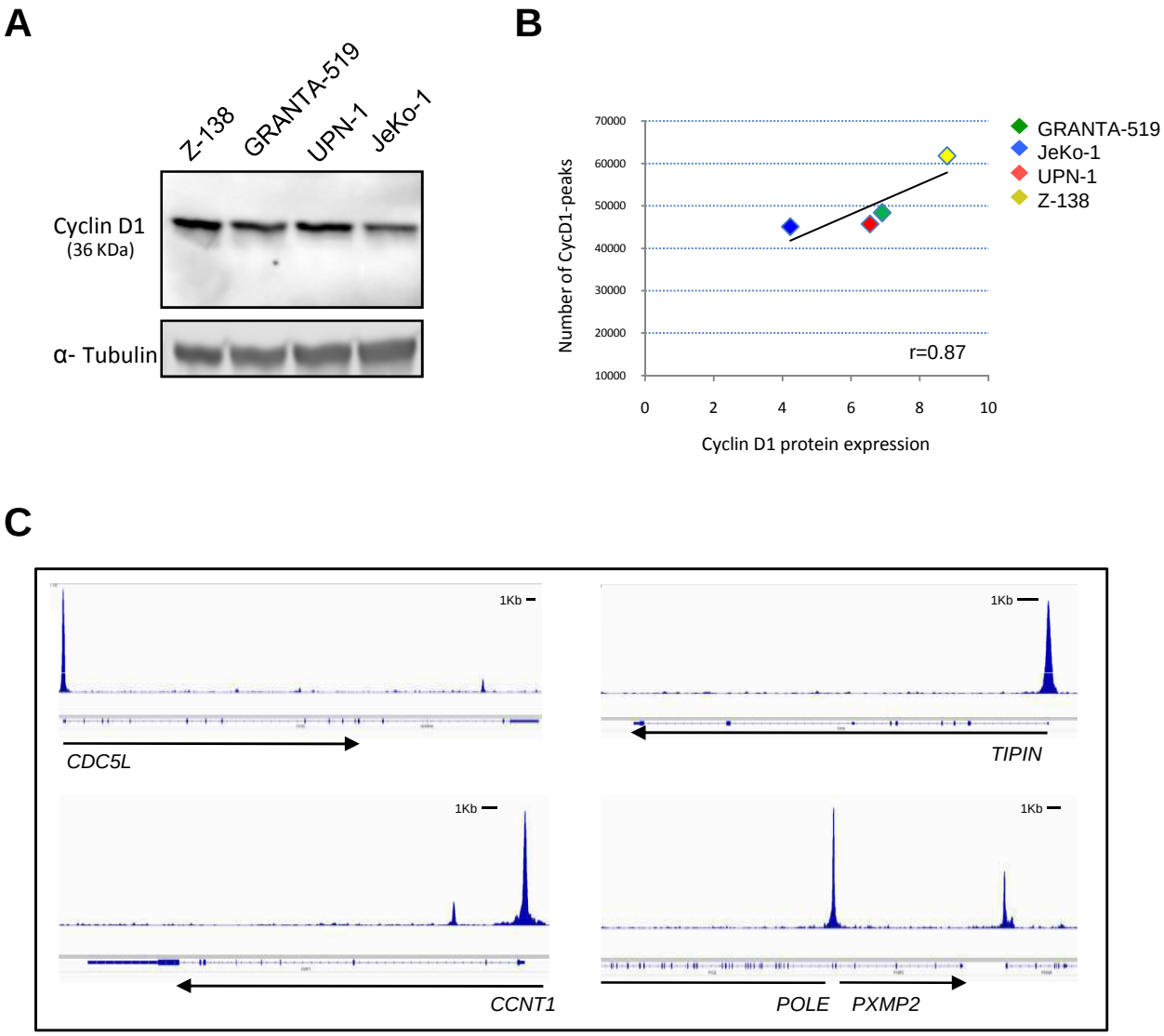
Secondaryantibodies

Reference	Antigen detected	Company	W.B. Dilution
P0217	Anti-rabbit (HRP conjugated)	DAKO	1:3000
P0260	Anti-mouse (HRP conjugated)	DAKO	1:3000
AP200P	Anti-Mouse light chain antibody- HRP	Millipore	1:1000
7074	Anti-rabbit IgG, HRP-linked	Cell signalling	1:1000

ChIP/IP Control antibodies

Reference	Antigen detected	Company
sc-2025	Normal mouse IgG	Santa Cruz

Supplemental Figure 1.



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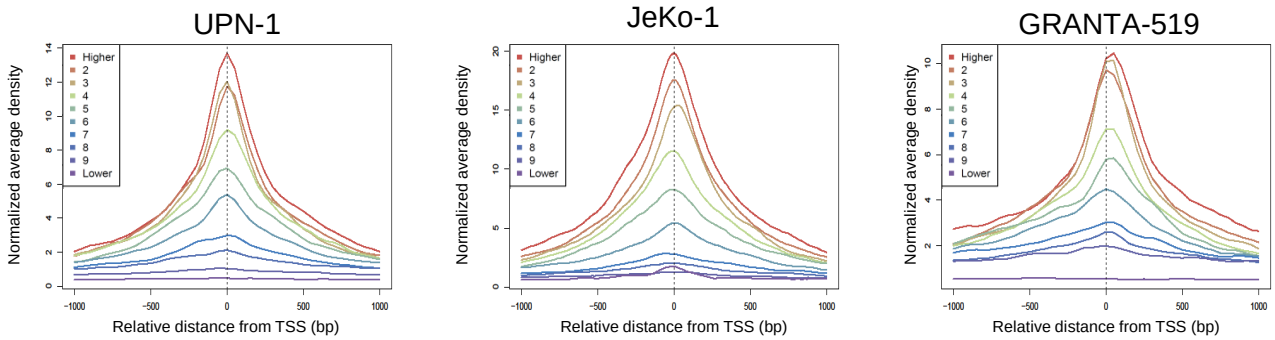

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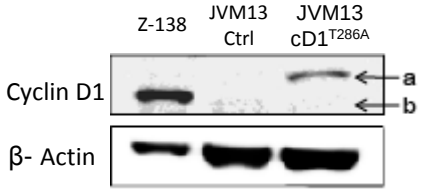

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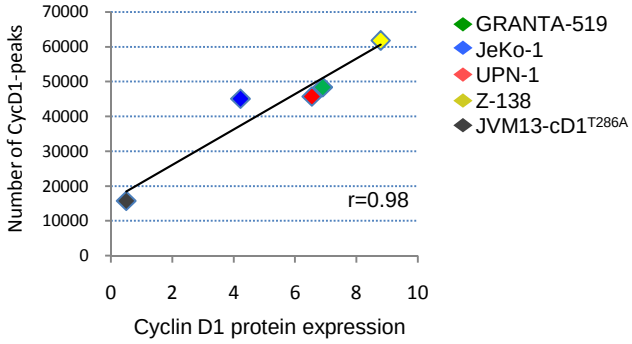

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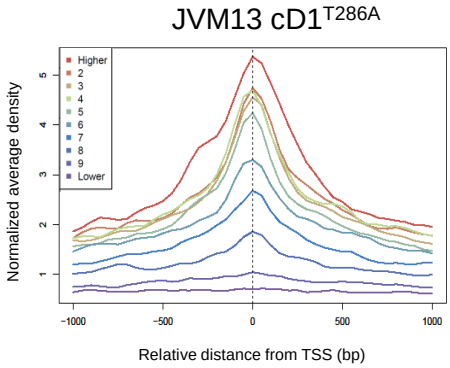

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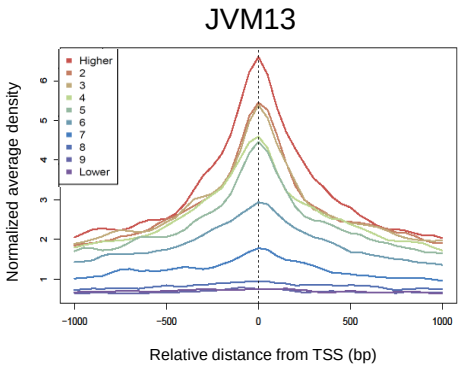

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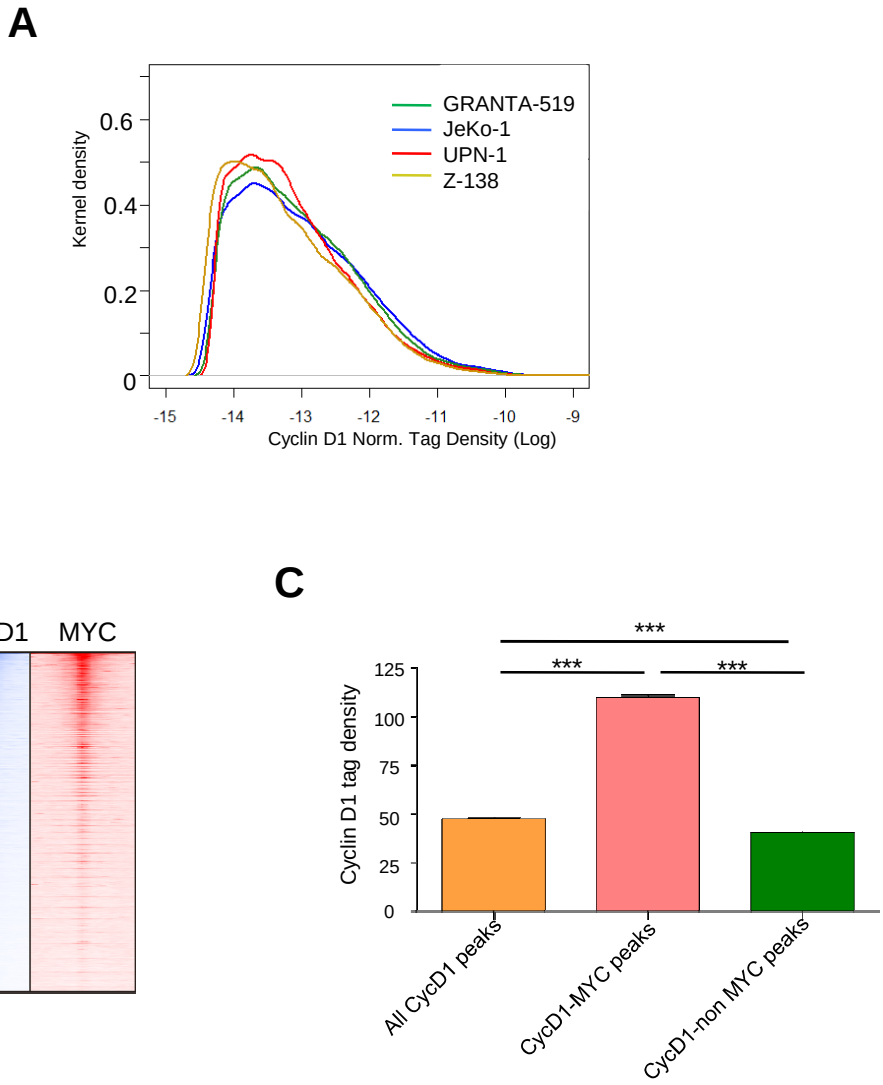

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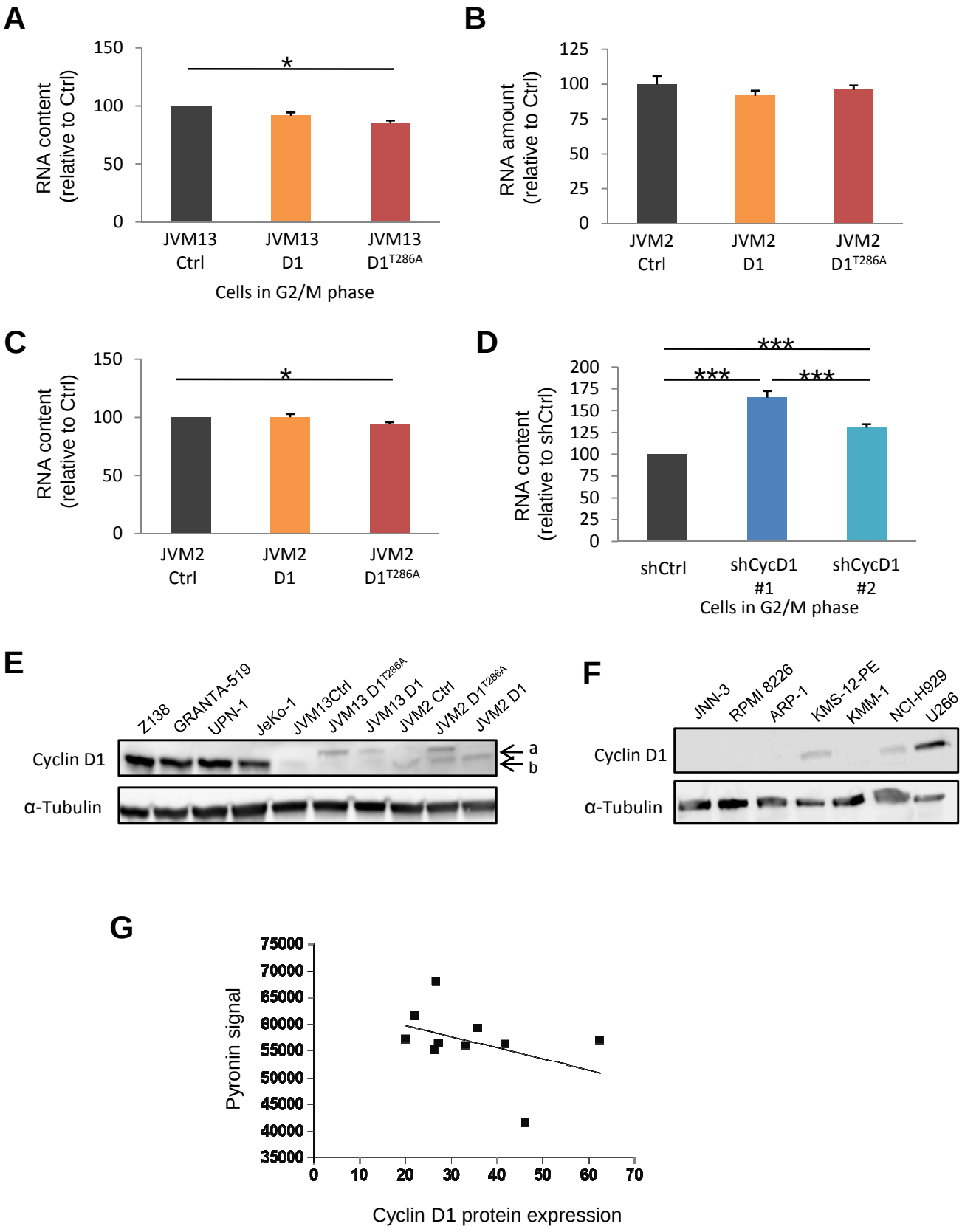

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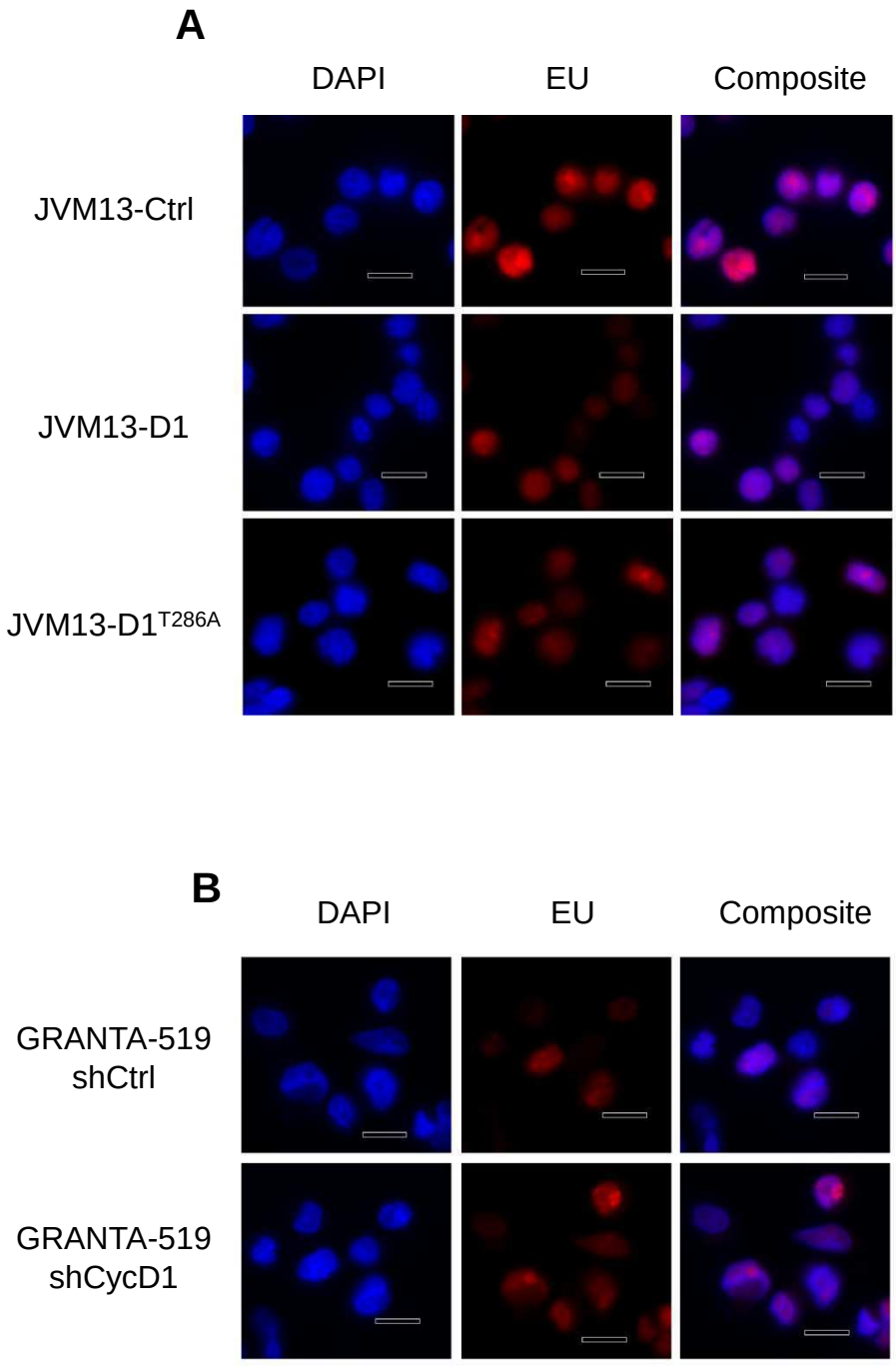

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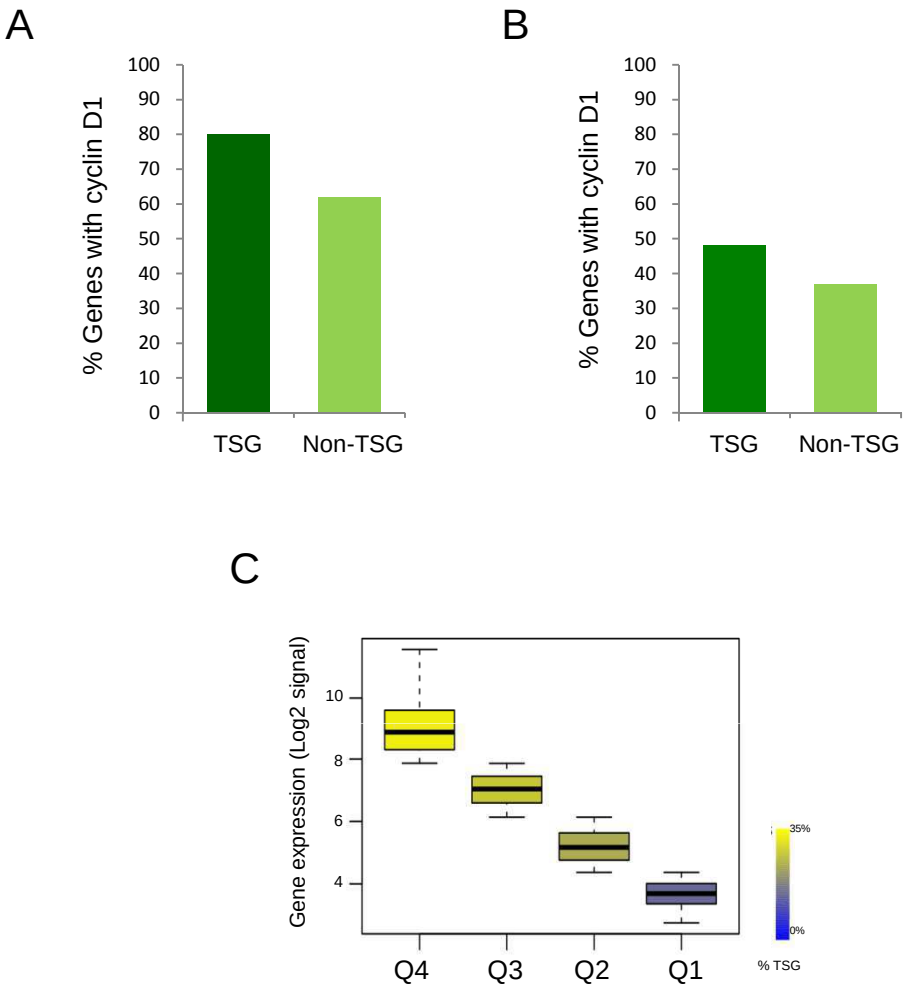

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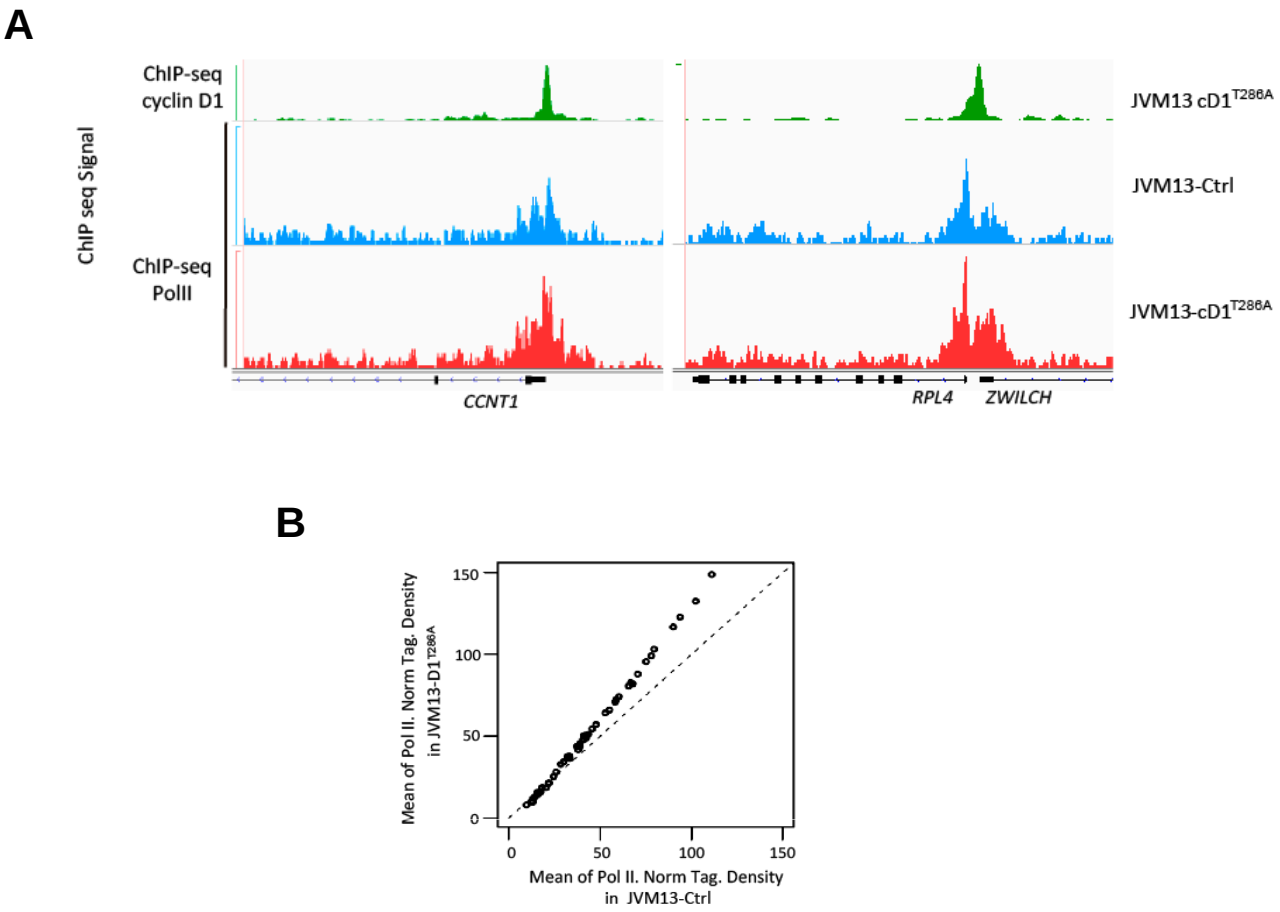

Supplemental Figure 8.