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Supplemental Information

Rapid Genome-wide Recruitment of RNA Polymerase II Drives Transcription, Splicing, and Translation Events during T Cell Responses

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Figure S1

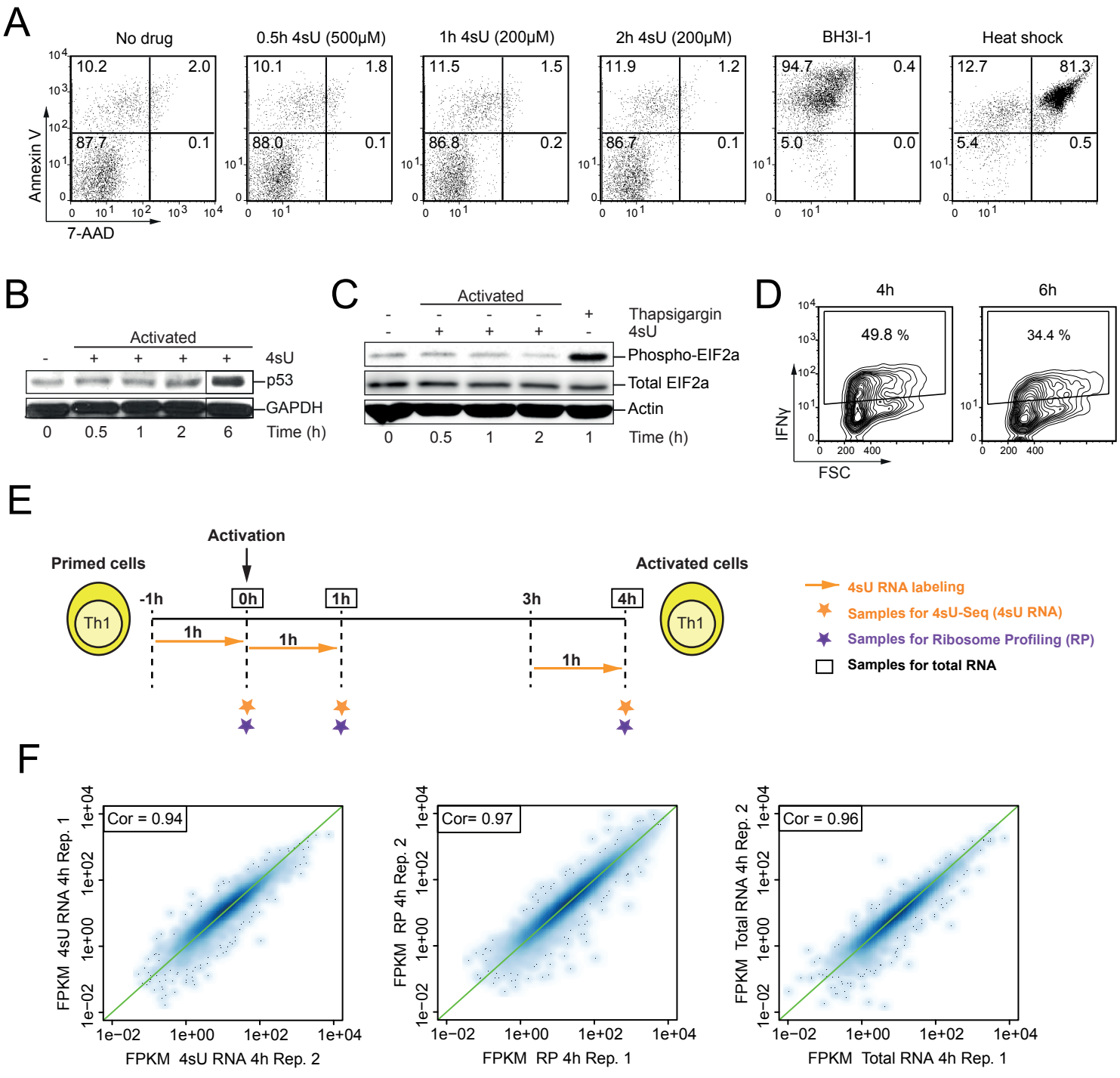


Figure S1. Optimal 4sU-labeling conditions and comparison to the biological repeat. Related to Figure 1.

(A) Detection of cell apoptosis by FACS analysis. In vitro generated Th0 cells were treated with different concentrations of 4sU (indicated in brackets) for 0.5h, 1h and 2h, respectively. BH3I-1 treatment was used to induce apoptosis determined by Annexin V, whereas heat shock (5min at 95°C) was used to induce cell death determined by 7-AAD.

(B) Western blot analysis of p53 of 4sU treated and activated cells. Samples were labeled with 200µM 4sU for the indicated time of activation, except the 0.5h time point that was labeled with 500µM 4sU.

(C) Western blot analysis of phospho-EIF2a and total EIF2a in activated Th1 cells with the same labeling conditions as in (B). Thapsigargin was used as positive control.

(D) FACS analysis of IFNγ at 4h and 6h upon activation of Th1 cells. The percentage of IFNγ producing cells is indicated for both time points.

(E) Experimental setup of the biological repeat: Th1 cells were activated at t=0h. 4sU-labeling was carried out in 1h intervals (indicated by orange arrows) followed by sequencing (indicated by orange asterisks). Ribosome profiling (RP) (indicated by purple asterisks) and sequencing of total RNA (indicated by black boxes) were performed at indicated time points.

(F) Scatter plots comparing expression values (FPKM) between newly transcribed RNA (4sU RNA), translated RNA (RP) and total RNA at 4h of activation for the two replicates, respectively. The green line indicates equal FPKM values and rank correlation is indicated in each plot.

Figure S2

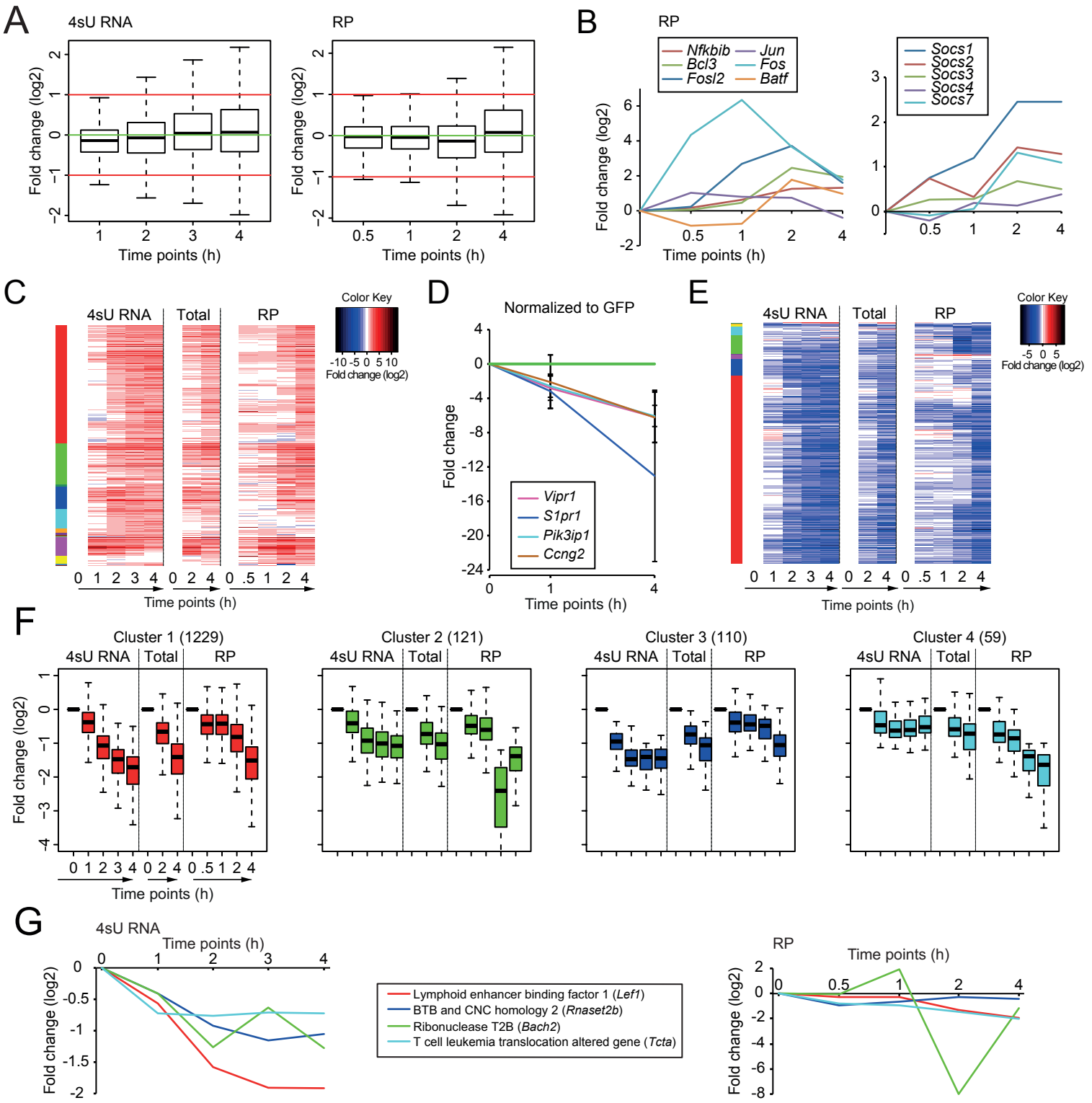


Figure S2. Transcriptional and translational regulation of housekeeping genes, defined clusters and selected genes. Related to Figure 1.

(A) Distribution of fold changes (log2) of housekeeping genes before normalization to ~3400 housekeeping genes (taken from Eisenberg and Levanon, 2013) is illustrated using boxplots for all time points of newly transcribed (4sU) RNA and translated RNA (RP). The green line indicates no regulation and the red lines 2-fold up- or downregulation.

(B) Fold changes (log2) over time in translation (RP) for selected negative regulators of immune response.

(C) Heatmap of fold changes (log2) in *de novo* transcription (4sU RNA), translation (RP) and total RNA at indicated time points upon activation for all upregulated genes. The color of the vertical bar indicates the different clusters.

(D) Fold changes of selected genes that were defined as downregulated based on the RNA-Seq data were determined by qRT-PCR at 1h and 4h upon activation. Samples are normalized to spiked-in GFP plasmids (indicated by the green line). The mean and standard deviation (indicated by error bars) of three individual experiments are shown.

(E) Heatmap of fold changes (log2) in *de novo* transcription (4sU RNA), translation (RP) and total RNA at indicated time points upon activation for all downregulated genes. The color of the vertical bar indicates the different clusters.

(F) Hierarchical clustering was performed for all downregulated genes based on fold changes (log2) in *de novo* transcription (4sU RNA) and translation (RP) at 1h, 2h and 4h upon activation. Boxplots illustrating the distribution of fold changes (log2) for the four largest downregulated clusters are shown and the number of genes in each cluster is indicated in brackets.

(G) Fold changes (log2) of representative examples of newly transcribed (4sU RNA) and translated (RP) genes for each cluster are depicted over time. Colors are the same as for the respective cluster in (F).

Figure S3

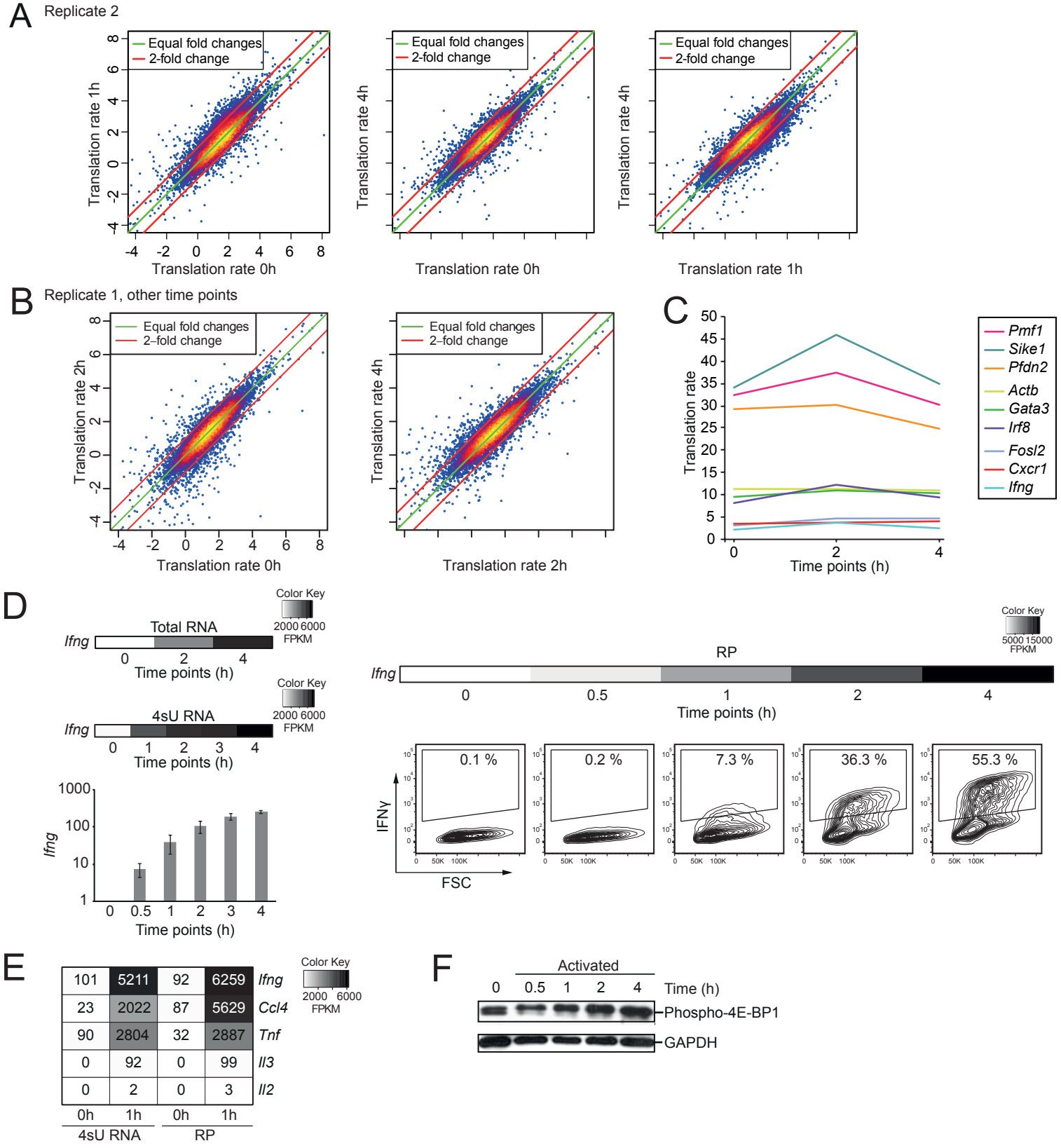


Figure S3. Transcriptional and translational regulation are coupled globally. Related to Figure 2.

(A) Scatter plots comparing translation rates determined from the repeat replicate between different time points. Translation rates were calculated as the ratio of translated RNA (FPKM in RP) to total RNA (FPKM in total RNA). The green line indicates equal fold changes and the red lines 2-fold changes.

(B) Scatter plots comparing translation rates determined from the first replicate between different time points. Translation rates were calculated as described in Figure S3A. The green line indicates equal fold changes and the red lines 2-fold changes.

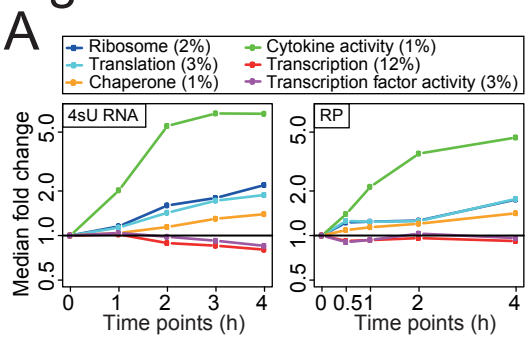
(C) Changes in translation rates are shown for selected genes over time.

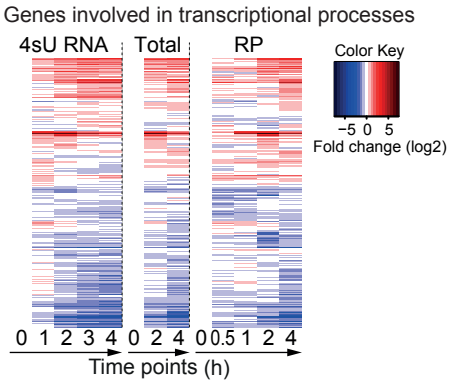
(D) Top left: Expression value (FPKM) of Ifng in sequencing data (total and newly transcribed RNA (4sU RNA)); bottom left: qRT-PCR results for Ifng (normalized to HPRT). The mean and standard deviation (error bars) of three independent experiments is represented. Top right: Expression value of Ifng (FPKM) in ribosome profiling for each time point. Bottom right: Representative FACS plots for IFNγ protein. The percentage of IFNγ producing cells is indicated above for each time point.

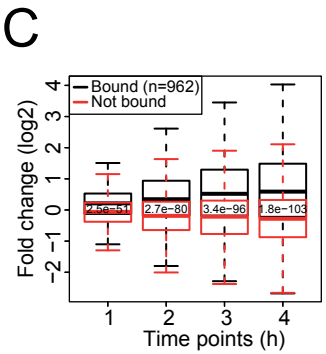
(E) Heatmap of expression values (FPKM) for selected immunological genes. Expression values are indicated for non-activated and 1h activated Th1 cells for de novo transcription (4sU RNA) and translation (RP), respectively.

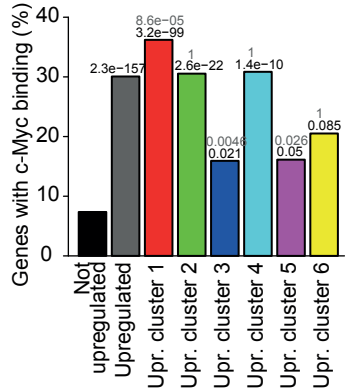
(F) Western blot analysis with antibodies against phosphorylated version of 4E-BP1 in activated Th1 cells over time. GAPDH was used as loading control.

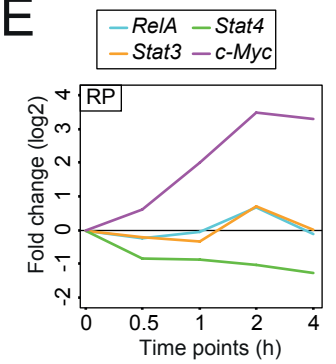
Figure S4

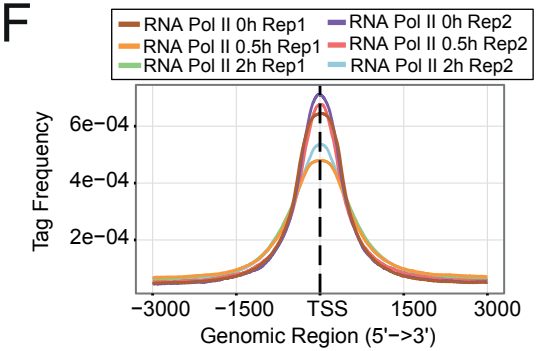


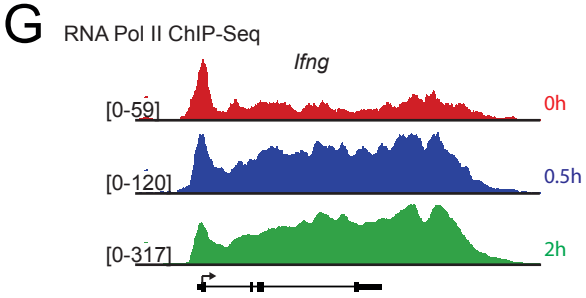


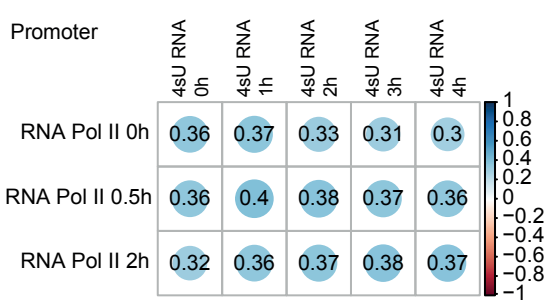


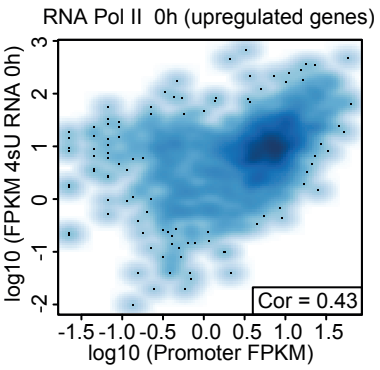


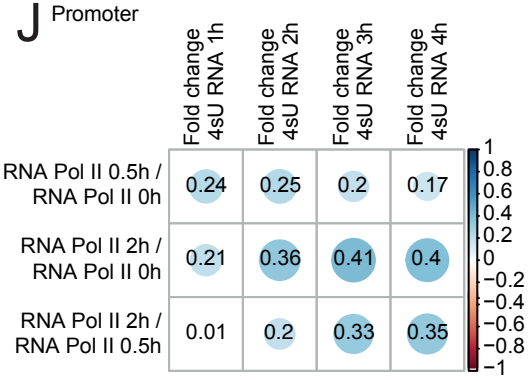


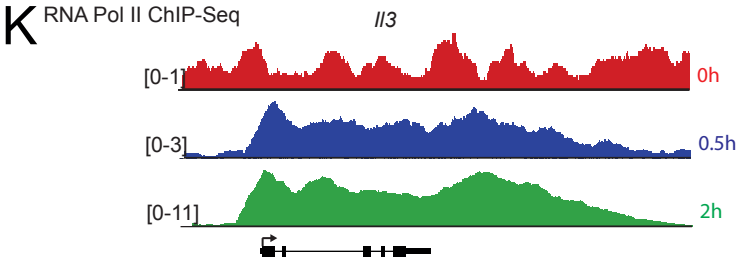












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	RNA Pol II ChIP-Seq (FPKM)			Fold change RNA Pol II Promoter/ RNA Pol II Gene		4sU RNA (FPKM)				
	0h	0.5h	2h	0.5h / 0h	2h / 0h	0h	1h	2h	3h	4h
Csf2	1.15	2.95	9.10	0.56	0.59	2.09	58.19	116.13	193.99	224.02
Lif	1.26	0.70	0.57	0.28	0.31	1.43	22.32	17.38	28.05	34.90
Kdm6b	1.51	3.26	4.08	0.33	0.50	2.23	28.27	26.29	11.97	9.30
Krtcap2	1.54	1.39	1.19	0.60	0.52	20.97	16.33	28.72	33.62	46.73
Pprc1	1.60	1.56	1.53	0.52	0.55	6.33	13.46	21.14	29.30	30.71
Rrp9	2.14	2.03	2.00	0.47	0.32	5.89	9.96	32.01	58.81	68.34
Stk32c	2.30	1.48	1.38	0.57	0.57	2.43	4.84	9.07	8.44	8.29
Rps18	2.89	3.45	3.78	0.55	0.65	17.17	40.27	36.40	49.95	56.52
Nr4a1	3.00	60.40	34.73	0.50	0.64	12.02	1266.59	693.98	189.65	118.63
Agpat3	3.25	4.51	3.48	0.61	0.63	17.69	23.62	30.32	53.90	63.02
Cd82	3.34	5.48	7.86	0.44	0.47	21.79	71.70	217.07	173.61	145.78
Acy1	3.36	4.98	6.35	0.42	0.55	1.85	1.80	2.63	4.92	5.96
Fam69b	3.59	3.90	3.73	0.36	0.34	0.73	0.51	0.48	1.65	2.83
Egr1	3.79	14.89	6.50	0.15	0.37	1.86	187.94	27.12	12.91	10.49
Rpl18	4.29	3.65	3.02	0.36	0.33	49.27	81.40	107.18	126.61	161.11
Snrpa	5.85	9.46	9.76	0.67	0.70	18.98	23.85	31.53	38.33	41.10
Csrnp1	5.87	15.31	10.33	0.65	0.68	35.17	269.57	245.64	183.07	153.00
Lonp1	5.89	10.69	10.01	0.62	0.56	17.26	27.13	27.50	31.82	35.16
Sdf4	8.90	17.37	14.48	0.67	0.46	45.93	85.08	126.82	97.86	95.13
Lcp2	9.69	30.17	28.99	0.56	0.61	98.35	184.35	349.35	252.76	211.13
Rpl26	11.09	27.53	26.22	0.69	0.66	6.14	16.82	15.16	19.82	25.47
Ifng	14.22	118.48	142.27	0.51	0.52	101.01	5210.68	6908.32	7227.41	7580.33
Gnb2l1	14.63	28.31	33.51	0.68	0.68	75.74	122.32	170.20	213.45	263.90
Stip1	15.19	30.92	25.85	0.60	0.67	43.71	59.20	102.78	95.68	105.54
Irf4	19.10	60.79	81.98	0.61	0.61	127.00	395.44	519.58	737.37	570.12
Npm1	24.04	57.22	50.84	0.68	0.63	27.85	51.58	87.70	126.90	155.83
Pkm	24.11	69.55	49.63	0.65	0.60	219.46	371.21	529.79	455.75	473.39
Rpsa	26.53	56.53	42.24	0.65	0.64	104.06	217.82	274.62	344.06	484.43
Irf8	51.97	120.13	85.37	0.65	0.67	18.83	89.55	230.96	173.11	128.45

Figure S4. Regulation by transcription factors and rapid recruitment of RNA Pol II. Related to Figure 3.

(A) Median fold changes in newly transcribed (4sU) RNA and translated RNA (RP) over time compared to non-regulated Th1 cells are shown for selected GO terms and SP-PIR keywords. Numbers in brackets indicate which percentage of analyzed genes is annotated with this GO term or keyword.

(B) Heatmap of fold changes (log2) for genes annotated with a function in transcription.

(C) Comparison of fold changes (log2) in *de novo* transcription for genes with c-Myc binding sites (black) within their promoters compared to genes without c-Myc binding sites (red) at each time point (top 1000 binding sites identified in $CD8^+$ T cells by ChIP-Seq (Chou et al., 2014)). Boxplots illustrate the distribution of fold changes (log2) and p-values (Wilcox test, Bonferroni multiple testing correction) indicate the significance of differences in the distributions.

(D) Percentage of c-Myc binding sites (as determined in (C)) within promoters of upregulated genes in general (gray bar) and within all identified clusters of upregulated genes (as defined in Figure 1 D). P-values comparing the percentage of c-Myc binding to genes not upregulated (black) and upregulated (gray) were obtained using Fischer's exact test (Bonferroni multiple testing correction).

(E) Fold change (log 2) of transcription factors determined by EnrichR (see Figure 3A).

(F) Frequency of peaks identified with MACS2 around the transcription start site (TSS). Frequency was determined by calculating for each position the number of identified peaks including this position divided by the sum over all positions.

(G and K) Mapped sequencing reads (RNA Pol II ChIP-Seq) for *Ifng* from the biological replicate (G) and for *Il3* from the first replicate (K) at all time points. The range of read counts (y-axis) is depicted in brackets. Gene annotation is indicated below, with exons depicted as boxes (smaller boxes = untranslated regions) and introns as lines.

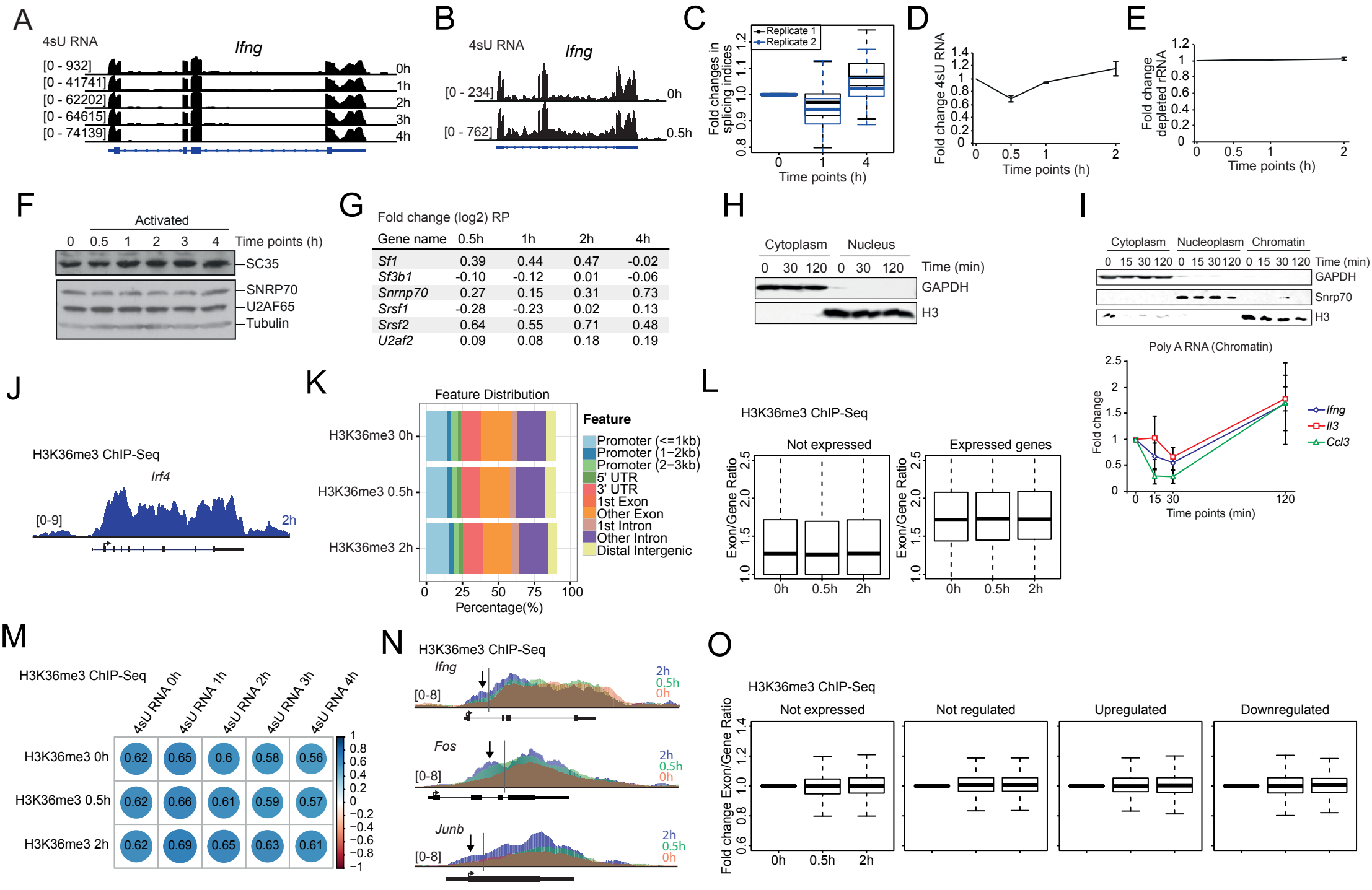
(H) Rank correlation of promoter RNA Pol II abundance to 4sU RNA FPKM levels at each time point.

(I) Scatter plot comparing RNA Pol II abundance at the promoter (FPKM) at 0h with 4sU FPKM values at 0h. Rank correlation is indicated.

(J) Rank correlation between fold changes in promoter RNA Pol II abundance between each pair of time points and fold changes in 4sU RNA compared to non-activated cells for the biological replicate.

(L) 29 genes with a significant RNA Pol II signal at the promoter at 0h (FPKM >1), a >2-fold change in 4sU RNA at 4h and at least 30% reduction in promoter to gene body ratio of RNA Pol II.

Figure S5


A
4sU RNA
Ifng
[0 - 932]
[0 - 41741]
[0 - 62202]
[0 - 64615]
[0 - 74139]
0h
1h
2h
3h
4h
B
4sU RNA
Ifng
[0 - 234]
[0 - 762]
0h
0.5h
C
Replicate 1
Replicate 2
Fold changes in splicing indices
Time points (h)
D
Fold change 4sU RNA
Time points (h)
E
Fold change depleted rRNA
Time points (h)
F
Activated
0 0.5 1 2 3 4 Time points (h)
SC35
SNRP70
U2AF65
Tubulin
G
Fold change (log2) RP
Gene name 0.5h 1h 2h 4h
Sf1 0.39 0.44 0.47 -0.02
Sf3b1 -0.10 -0.12 0.01 -0.06
Snrnp70 0.27 0.15 0.31 0.73
Srsf1 -0.28 -0.23 0.02 0.13
Srsf2 0.64 0.55 0.71 0.48
U2af2 0.09 0.08 0.18 0.19
H
Cytoplasm Nucleus
0 30 120 0 30 120 Time (min)
GAPDH
H3
I
Cytoplasm Nucleoplasm Chromatin
0 15 30 120 0 15 30120 0 15 30 120 Time (min)
GAPDH
Snrp70
H3
Poly A RNA (Chromatin)
Fold change
Ifng
Il3
Ccl3
Time points (min)
J
H3K36me3 ChIP-Seq
Irf4
[0-9]
2h
K
Feature Distribution
H3K36me3 0h
H3K36me3 0.5h
H3K36me3 2h
Feature
Promoter (<=1kb)
Promoter (1−2kb)
Promoter (2−3kb)
5' UTR
3' UTR
1st Exon
Other Exon
1st Intron
Other Intron
Distal Intergenic
Percentage(%)
L
H3K36me3 ChIP-Seq
Not expressed
Expressed genes
Exon/Gene Ratio
0h 0.5h 2h
M
H3K36me3 ChIP-Seq
4sU RNA 0h
4sU RNA 1h
4sU RNA 2h
4sU RNA 3h
4sU RNA 4h
H3K36me3 0h 0.62 0.65 0.6 0.58 0.56
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N
H3K36me3 ChIP-Seq
Ifng
Fos
Junb
[0-8]
2h
0.5h
0h
O
H3K36me3 ChIP-Seq
Not expressed
Not regulated
Upregulated
Downregulated
Fold change Exon/Gene Ratio
0h 0.5h 2h

Figure S5. Fluctuations in splicing rates and H3K36me3 correlations. Related to Figure 4 and 5.

(A) Mapped sequencing reads (4sU RNA) for *Ifng* at each time point. The range of read counts (y-axis) is depicted in square brackets. Gene annotation is indicated below, with exons depicted as boxes (smaller boxes = untranslated regions) and introns as lines.

(B) Mapped sequencing reads (4sU RNA) for *Ifng* using 0.5h labeling in non-activated (0h) cells and 0.5h after activation.

(C) Fold changes in splicing indices compared to 0h were evaluated for 1h and 4h after activation for both replicates and the distributions are represented as boxplots (black = replicate 1, blue = replicate 2).

(D) Equal numbers of *in vitro* generated Th1 cells were activated for the indicated time points and 4sU-labeling was performed for the last 0.5h of each time point. Fold changes in 4sU RNA compared to 0h were calculated (newly transcribed RNA was quantified via Qubit Fluorometer and for each time point related to total biotinylated RNA after precipitation). The mean and standard deviation (error bars) are illustrated for two independent experiments.

(E) Fold changes in depleted rRNA (measured via Qubit Fluorometer) compared to 0h for 4sU RNA shown in Figure S6D. The mean and standard deviation (error bars) are illustrated for two independent experiments.

(F) Western blot analysis with antibodies recognizing different splicing factors in activated Th1 cells at indicated time points. Tubulin was used as loading control.

(G) Fold changes (log2) for selected splicing factors in translated RNA (RP) at all time points compared to non-activated cells.

(H) Confirmation of clean cytoplasm (GAPDH) versus nucleus (Histon 3) fractionation for the different time points of Figure 4 D by Western blot analysis.

(I) Western blot confirmation of clean cytoplasm (GAPDH) versus nucleoplasm (Snrp70) and chromatin (Histon 3) fractionation. Fold change of exon/intron ratio (qRT-PCR with primers for spliced and unspliced RNA as shown in Figure 4 C) from Figure 4 E including the 15 minutes time point for the chromatin associated polyadenylated RNA. Mean and standard deviation (error bars) are shown for five independent experiments.

(J) Mapped sequencing reads (H3K36me3 ChIP-Seq) for *Irf4* at 2h after activation. The range of read counts (y-axis) is depicted in square brackets. Gene annotation is indicated below, with exons depicted as boxes (smaller boxes = untranslated regions) and introns as lines.

(K) Distribution of H3K36me3 over genomic regions (promoters, exons, introns, UTRs and intergenic/distal) for all time points. Percentage of H3K36me3 for each type of region is indicated in different colors.

(L) Boxplot indicating exon per gene ratio of H3K36me3 for not expressed and expressed genes at all time points.

(M) Rank correlation between H3K36me3 signal intensity on the gene body and newly transcribed (4sU) RNA FPKM values for each time point.

(N) Mapped sequencing reads (H3K36me3 ChIP-Seq) for *Ifng*, *Fos* and *Junb* at 0h, 0.5h and 2h. The range of read counts (y-axis) is depicted in square brackets. Gene annotation is indicated below, with exons depicted as boxes (smaller boxes = untranslated regions) and introns as lines. The arrows mark the increasing number of reads near the 5' end.

(O) Boxplot analysis of fold changes in the exons per gene ratio for the different time points. Genes were grouped according to their type of regulation.

Supplemental Experimental Procedures

CD4 T cell isolation and differentiation

Peripheral $CD4^+$ T cells were isolated with CD4 Dynabeads (Invitrogen) and kept in RPMI medium (supplemented with 10% (vol/vol) FCS, β-mercaptoethanol (0.05 mM, Gibco), penicillin-streptomycin (100 U/ml, Gibco), Sodium Pyruvate (1 mM, Lonza), Non-Essential Amino Acids (1x, Gibco), MEM Vitamin Solution (1x, Gibco), Glutamax (1x, Gibco) and HEPES pH 7.2 (10 mM, Gibco)). Naïve $CD4^+$ T cells were stimulated for 36–48h under Th1 conditions (on surfaces coated with goat anti–hamster immunoglobulin G (55397, MP Biomedicals)) with soluble anti-CD3 (0.1 µg/ml; 145-2C11) and anti-CD28 (1 µg/ml; 37N), anti-interleukin 4 (anti-IL-4, 10 µg/ml; IIB11), all produced and purified in-house (Kremmer, E.) and recombinant IL-12 (10 ng/ml, R&D Systems). Stimulation of naïve $CD4^+$ T cells under Th0 conditions was done with soluble anti-CD3 (0.1 µg/ml) and anti-CD28 (1 µg/ml) on surfaces coated with goat anti-hamster.

After being stimulated for at least 36h cell populations were expanded in RPMI medium (as described above) supplemented with Proleukin S (200 IE/ml, IL2-analog, Novartis) for additional 2 or 3 days. One day before activation, cells were cultured in RPMI medium (as described above), but without Proleukin S. Th1 cells were activated with plate-bound goat anti–hamster immunoglobulin G or plate-bound anti-CD3 (5 µg/ml) and/or soluble anti-CD3 (1 µg/ml) and anti-CD28 (2.5 µg/ml).

Western blotting and Immunoprecipitation

For Western blot the cells were washed once with phosphate-buffered saline (PBS), resuspended in RIPA lysis buffer (20 mM Tris HCl pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 1% NP40, 0.1% SDS, 0.5% Sodium Deoxycholate) with freshly added DTT (1 mM), Proteinase (Complete, Roche) and if required Phosphatase Inhibitor (PhosStop, Roche), incubated for 15min on ice and centrifuged at 10.000 rpm.

For Immunoprecipitation up to 1.6 x 10^8 cells were lysed in 1.6 ml Meister lysis buffer (20 mM Tris HCl pH 7.5, 0.25% NP40, 150 mM NaCl, 1.5 mM $MgCl_2$) freshly complemented with DTT (1 mM), EDTA (1 mM), Proteinase and Phosphatase Inhibitor, incubated for 10min on ice and quick-frozen in liquid nitrogen before centrifugation at 10.000 rpm. 30 µl Protein-G beads (Dynabeads Protein G, 10004D) were pre-coupled with 5 µg antibodies (anti-U2AF65, sc-53942 or anti-Cyclin T1, sc-8128) in PBS and 0.05 % Tween (1h, RT) and further equilibrated in Meister lysis buffer. Coupled beads were added to lysed cells and incubated for 3h at 4°C. After repeated washing with Meister lysis buffer, proteins were eluted with 20 µl SDS Lämmli loading dye (2x) for Western blot analysis.

The following antibodies were used: anti-RNA Pol II (phospho S2) (ab24758), anti-Rpb1 (Pol3.3, M-G1, kindly provided by D. Eick) (Chapman et al., 2007), anti-RNA Pol II (phospho S5) (3E8, kindly provided by D. Eick) (Chapman et al., 2007), anti-U2AF65 (MC3) (sc-53942), anti-SNRP70 (ab83306), anti-SC35 (BD Bioscience 556363), anti-p53 (abcam, ab26), anti-Phospho 4E-BP1 (Thr37/46) (Cell Signaling 2855), anti-Phospho EIF2a (Ser51) (9721), anti-Cyclin T1 (sc-8128), anti-CDK9 (sc-8338), anti-Phospho CDK9 (Thr-186) (Cell Signaling 2549S), anti-Tubulin (sc-23948), anti-GAPDH (1A7, generated in house/ E.Kremmer) and anti-Actin (Chemicon, MAB1501R).

Subcellular Fractionation and RNA Isolation

40-60 x 10^6 activated Th1 cells were washed once in ice cold PBS in 15 ml conical tubes (250 rpm, 6 min, 4°C).

The cell pellets were resuspended in 250 µl cold cytoplasmic lysis buffer (0.05% NP-40, 10mM Tris pH 7.5, 150 mM NaCl) using wide orifice tips and incubated on ice for 5 min. The lysate was layered on top of 1 ml cold sucrose buffer (10 mM Tris pH7.5, 150 mM NaCl, 24% sucrose), and centrifuged in microfuge tubes at 10.000 rpm for 2 min at 4°C. 10% of the supernatant volume (= Cytoplasmic fraction) was used for immunoblot analysis.

The nuclear pellet was gently resuspended into 250 µl cold glycerol buffer (20 mM Tris pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 50% glycerol, 0.85 mM DTT) using wide orifice tips. An additional 250 µl of cold nuclei lysis buffer (20 mM HEPES pH 7.6, 7.5 mM MgCl2, 0.2 mM EDTA, 0.3M NaCl, 1M urea, 1% NP-40, 1 mM DTT) was added to the samples, followed by a pulsed vortexing and incubation on ice for 1 min. Samples were then spun in microfuge tubes for 2 min at 14.000 rpm and at 4°C. The supernatant from this spin represented the nucleoplasmic fraction, and 10% of supernatant was kept for immunoblot analysis.

50 µl of cold PBS was added to the remaining chromatin pellet and vortexed. The chromatin pellet was vigorously vortexed to fully resuspend. 5 µl of the PBS supernatant was collected for immunoblot analysis as above, and 500 µl QIAzol Lysis Reagent (Qiagen) was added to the pellet. After vigorous vortexing to resuspend the chromatin, chromatin-associated RNA was extracted by adding 100 µl chloroform and incubated at room temperature for 5 min. The chromatin samples were then centrifuged in microfuge tubes for 15 min at 13.000 rpm at 4°C. The resulting upper aqueous layer was then added to 3.5x volumes QIAGEN RLT buffer.

RNA purification from RLT-dissolved samples was performed according to the QIAGEN RNA cleanup (74204) protocol.

cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen, 205311) with genomic whipeout. For nuclear mRNA random hexamer and oligo d(T) primer were used and for chromatin associated RNA only oligo d(T) primer were used. Complete removal of genomic DNA was confirmed with primer pairs for not expressed genes. Amount of introns was normalized to exons (Δ CT-Method) per gene at each time point and fold change was calculated.

4sU-labeling, 4sU-Seq and Ribosome Profiling

Samples for ribosome profiling were prepared according to manufacturers instructions (Illumina, TruSeq Ribo Profile, Mammalian) except that we used the whole amount of RNA for both gel purifications steps. Overnight gel extraction was used at every stage of the procedure. At least 60 x 10^6 Th1 cells were used for each time point. Depletion of rRNA was performed with Ribo-Zero Gold rRNA Removal Kit (Illumina, Human/Mouse/Rat) and RNA purification was done with RNA clean and concentrator kit according to the manufacurer's advice (Zymo Research).

Metabolic labeling of newly transcribed RNA with 4sU (Carbosynth, NT0618690), RNA isolation and biotinylation was performed as described before (Radle et al., 2013) with minor changes. 500 µM 4sU was used for 0.5h of labeling and 200 µM 4sU was used for 1h labeling. In step 5 we eluted the newly transcribed RNA into 400 µl Agencourt RNAClean XP Beads (Beckman Coulter) and continued with the recovery following the manufacturers instructions. RNA was eluted in 11 µl nuclease-free H_2O and quantified using Qubit 2.0 Fluorometer.

For both total and 4sU RNA samples, library preparation and rRNA depletion was performed using the TruSeq Stranded Total RNA Library Prep Kit (Illumina) starting with 400 ng RNA as input for each sample. Only 11 cycles were used for PCR amplification to minimize PCR bias.

Amplified cDNA libraries were further purified using Agencourt RNAClean XP Beads (Beckman Coulter) and quality control of biotinylated RNA and cDNA libraries were performed using Agilent Bioanalyzer with RNA 6000Nano Reagents (Agilent Technologies, 5067-1511) or High Sensitivity DNA Reagents (Agilent Technologies, 5067-4626). Barcoded libraries were sequenced on a HiSeq 2500 (Illumina) with paired-end, 100 bp reads.

ChIP-Seq

$30x10^6$ T cells were crosslinked with 1% Formaldehyde (10min, RT) and crosslinking reaction was stopped by the addition of glycine to a final concentration of 0.125 M. Chromatin immunoprecipitation (ChIP) assays were performed as described (DeKoter et al., 2002) with minor changes: After sonification for 25 cycles (Bioruptor; 30s on, 30s off at high) chromatin was diluted to 1 ml total volume in nuclei-lysis buffer with protease inhibitors and added to 3 ml ChIP IP buffer (0.01% SDS; 1.1% Triton X-100;1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.1; 16.7 mM NaCl) with protease inhibitors. 10 µg antibody (anti-RNA Pol II, 8WG16, ab817; anti-Histon H3K36me3, ab9050) were coupled to 80 µl Dynabeads Protein G (10004D) for at least 1h at RT and further blocked with sonicated salmon sperm DNA for 30min before washing. Beads and diluted chromatin were incubated at 4°C over night and washed with 1 ml of the following buffers each at RT for 5min with rotation: Buffer I (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20mM Tris-HCL pH 8.1; 150 mM NaCl), Buffer II (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCL pH 8.1; 500 mM NaCl), Buffer III (0.25 M LiCl; 1% NP-40; 1 mM EDTA; 10 mM Tris-HCl, pH 8.1), 2x TE pH 8.0. DNA purification was done according to (Blecher-Gonen et al., 2013).

Library preparation was performed from 2 ng of ChIP DNA using the Kapa Hyper Prep Kit with PCR library amplification (KK8504, KapaBiosystems) according to the manufactures protocol. Shortly, ChIP DNA was end-repaired and A-tailed before adapter ligation. After adapter ligation, the ChIP DNA was purified with Agencourt AMPure XP beads (A63880, Beckman Coulter) and size-selected for 360-600 bp using the Pippin Prep System from Saga Science. The size-selected library is amplified by PCR using the Kapa Hyper Prep Kit (KK8504, KapaBiosystems) and purified terminally with Agencourt AMPure XP beads.

Bioinformatics and statistical analysis

Sequencing quality was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/) and sequencing adapters were trimmed using cutadapt (Martin, 2011). Reads were mapped against the mm10 mouse reference genome and rRNA sequences using ContextMap version 2.5.2 (Bonfert et al., 2015). For 4sU and total RNA, reads were mapped in paired-end mode. For ribosome-associated mRNA (RP), the sequence of the ~30nt ribosome-protected mRNA sequence was determined first as the consensus sequence from both mates of a read pair and then mapped in single-end mode.

To calculate FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped) values, fragment counts per gene were calculated using the featureCounts program from the subread package version 1.4.6-p3 (Liao et al., 2014). For 4sU and total RNA, fragment counts were determined for all exons. For ribosome-associated mRNA, only fragments mapped to the coding sequence were counted. FPKM values were additionally normalized by the median fold changes of ~3400 housekeeping genes (Eisenberg and Levanon, 2013). Only genes with an FPKM >1 in at least one sample for 4sU-Seq, total RNA-Seq, and ribosome profiling were included in the analysis.

ChIP-Seq reads were aligned to the mouse mm10 reference genome using BWA-MEM version 0.7.13 (Li and

Durbin, 2009) and PCR duplicates removed using Picard Tools version 1.119 (https://broadinstitute.github.io/picard/). For visualization, mapped reads were converted to bedGraph using the HOMER software suite version 4.8.3 (Heinz et al., 2010) and visualized in the UCSC genome browser (Kent et al., 2002). Peaks were called over input using MACS2 version 2.1.0 (Zhang et al., 2008) with an FDR threshold of 0.05. RNA Pol II promoter and gene body FPKM was determined as for RNA-Seq by counting reads with featureCounts on the promoter (TSS ± 500nt) or the gene body (including exons and introns) and normalizing to promoter/gene length and sequencing depth.

Functional enrichment analysis was performed using the DAVID webserver (Huang da et al., 2009). Enrichment analysis for experimentally-determined transcription factor binding was performed using EnrichR (Chen et al., 2013). ChIP-Seq data for c-Myc in CD8+ T cells (Chou et al., 2014) was downloaded from Gene Expression Omnibus (GEO, Sample GSM1400431) and analyzed using the ChIPseeker Bioconductor package (Yu et al., 2015).

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Position	1h	2h	3h	4h
1	Il3	Il3	Il3	Il3
2	Egr3	Ccl1	Cul1	Il24
3	Ccl3	Ccl3	Ccl1	Lad1
4	Egr2	Ccl4	Lad1	Ccl1
5	Nr4a1	Crabp2	Il24	Il13
6	Fos	Il13	Ccl3	Ccl3
7	Egr1	Nr4a3	Il13	Csf2
8	Ccl4	Ifng	Csf2	Ccl4
9	Nr4a2	Nr4a1	Ifng	Ifng
10	Fosb	Tnfsf14	Ccl4	Hbegf
11	Tnfsf14	Xcl1	Hbegf	Adora2b
12	Nr4a3	Csf2	Adora2b	6430571L13Rik
13	Ifng	Rgs16	Rgs16	Rgs16
14	Il21	Adora2b	Il2	Il2
15	Il10	Egr3	Crabp2	Crabp2
16	Xcl1	Il2	6430571L13Rik	Atf3
17	Tnf	Hbegf	Tnfsf14	Lta
18	Il13	Nr4a2	Crtam	Prss35
19	Csf2	Il10	Tnfsf11	Tnfsf14
20	Ccl1	6430571L13Rik	Atf3	Tnfsf11

Table S2. Related to Figure 1. Most upregulated genes (4sU RNA)
Table shows most upregulated genes in newly transcribed (4sU) RNA. *Ifng* is highlighted in red color.

Position	0.5h	1h	2h	4h
1	Rps23	Il3	Il3	Il3
2	Cycs	Csf2	Il2	Csf2
3	Atp5g1	Ccl3	Csf2	Il2
4	Rpl26	Tnf	Ccl1	Rpl10
5	Rpl10	Nr4a2	Ccl3	Ccl1
6	Ahcy	Fosb	Ccl4	Ccl3
7	Idi1	Fos	Xcl1	Lad1
8	Fos	Ifng	Il17a	Il4
9	Il3	Nr4a1	Nutf2	Ifng
10	Tnf	Ccl4	Ifng	Xcl1
11	Il17a	Egr2	Mrpl23	Il17a
12	Gad2	Il10	Il4	Crabp2
13	Egr1	Il21	Nr4a3	Il13
14	Il10	Timm8a1	Crabp2	Nutf2
15	Prss35	Egr1	Il13	Il24
16	Fosb	Tnfsf14	Il10	Ccl4
17	Ifng	Egr3	Nr4a2	Gad2
18	Kremen2	Il17a	Rgs16	Rpl30
19	Egr2	Nr4a3	Tnf	Hbegf
20	Csf2	Xcl1	Hbegf	Rgs16

Table S2. Related to Figure 1. Most upregulated genes (RP)
Table shows most upregulated genes in translated (RP) RNA. *Ifng* is highlighted in red color.