

Figure S1. Related to Figure 1

(A) A construct encoding a full length RPB1 tagged with a fluorescent protein and auxin-degron was first stably transfected into an HEK293 cell line expressing a DOX-inducible OstIR1 from the AAVS1 locus (Natsume et al., 2016). Dox and auxin (DA)-sensitive mKO2-expressing cells (non-fluorescent cells sorted in bulk after DA treatment) were then transfected with a plasmid expressing a guide RNA targeting selectively the endogenous RPB1 gene and a Cas9-GFP construct. Cells positive for both mKO2 and GFP were finally sorted as single clones. 146 clones were first screened for substantial cell death after prolonged treatment with DA, indicating inactivation of all endogenous copies of RPB1 as shown in **(B)** for a clone depending (#19, green) or not (clone #10, red) on the tagged RPB1 for survival. The data also show efficient depletion (loss of mKO2 fluorescence level) of the tagged construct in the presence of auxin with massive cell death (#19) or no effect on cell viability (#10). 47 clones (32%) passed this first round of selection and the absence of endogenous RPB1 was confirmed by immunoblot using RIPA extracts prepared from isolated nuclei as shown in **(C)** for a few representative clones. E indicates the position of the endogenous RPB1 and T the position of the tagged RPB1. **(D)** Protocol used for the preparation of NUN nuclear and pellet protein extracts as well as nascent RNA isolation. **(E)** Ratio of different RNA levels in total RNA versus chromatin-bound fraction in RPB1-expressing (Ctl) or RPB1-depleted (DA) cells for clones #7, #12 and #19. Pre-tRNAs, however, are likely not processed co-transcriptionally and are expected to be released from the polymerase before excision of leader and trailer sequences (Arimbasseri, 2018). **(F)** Left panel, EtBr staining of a denaturing polyacrylamide gel loaded with RNAs prepared from isolated nuclei (Nuclear RNA), NUN-extracted chromatin pellets (Nascent RNA) and increasing amount of total RNA (Total). Right panel, tRNA-iMet Northern blot showing complete depletion of mature tRNAs in the nascent RNA fraction. Note that 0.5 μ g Nascent RNA corresponds to 12.5 μ g of total RNA. Scales bars represent 100 μ m and apply to all micrographs within the same panel.

Figure S2. Related to Figure 2 and 5

(A) RPB1 immunoblots of RIPA nuclear extracts and pellets for clone #7 and 19. **(B)** RPB1 and Flag immunoblots of nuclear extracts (NE) or chromatin pellets (P) prepared, respectively, from HeLa cells expressing endogenous RPB1 (Endo) and a Flag-tagged RPB1 lacking the entire CTD (Δ CTD) or DA-treated cells. The truncated RPB1 is present only on the chromatin-bound (pellet) fraction in DA-treated cells as seen in **(A)**. **(C)** Immunoblots and corresponding Coomassie-stained membranes of NUN extracts and pellets from untreated, Dox-treated or DA-treated cells probed for expression of different polymerase subunits. The physical isolation method used efficiently separates transcribing Pol III from Pol III containing a phosphorylated RPC53 (slower migrating bands in nuclear extracts) that is presumably inactive (Lee et al., 2015). **(D)** Quantification of Pol I and III expression levels in nuclear extract (NE) and chromatin pellets (NP) shown in **(C)**. Data represent the average \pm SD. **(E)** Protocol used for time course analysis of RNA expression in serum-deprived cells following serum stimulation (also for **Figure 5G-I**). **(F)** Left, Phase contrast and fluorescence microscopy images of clone #7 cultured in the presence or absence of serum prior to RPB1 depletion. Right, immunoblot analysis of RPB1 and RPC53 in chromatin pellets for all three clones cultured in these conditions. **(G)** Time course analysis of relative mRNA levels of selected immediate early genes in serum-starved RPB1-expressing (Ctl) and RPB1-depleted (DA) cells prior (Time 0) or after stimulation with serum as shown in **(E)**. Data represent the average \pm SD. * indicates statistically significant differences (T-test; $p < 0.05$). **(H)** Histogram of all protein-coding serum-inducible genes in Control (Ctl) and DA-treated cells revealed in a microarray analysis of pools of RNAs prepared from all three clones showing complete abolishment of the serum induction (the remaining enhancement observed in DA-treated cells corresponds to the fraction of cells that do not respond to the DA-treatment, see **Figure 1E**). Scales bars represent 100 μ m and apply to all micrographs within the same panel.

Figure S3. Related to Figure 3

(A) Schematic representation of the experimental approach and expected effects of DRB versus Pol II degradation on tRNA gene transcription. Local RNA Pol II observed at some tRNA loci could impact downstream tRNA transcription via multiple mechanisms, including transcriptional interference as shown in **Figure 3A**. Depletion of Pol II is expected to reveal whether Pol II does play a role in Pol III regulation, but not whether the effect is mediated by Pol II transcriptional activity or simply by Pol II occupancy. To address this question functionally, a comparison between the effects of DRB, a CDK9 inhibitor that blocks pause-release and hence productive transcription, and the RPB1-degron should allow investigation of each possibility. The upper part of the panel indicates that DRB is expected to stabilize all Pol II enzymes near their initiation sites, thereby leading to a specific loss of Pol II transcription, but not Pol II occupancy (locally paused Pol II), and revealing all Pol III-transcribed tRNA genes sensitive to transcriptional interference by Pol II. The lower part of the panel indicates that, in contrast, Pol II degradation by DA treatment is expected to remove Pol II at a Pol II-regulated tRNA locus (left), unless the tRNA gene is located downstream of a stably paused Pol II gene (right) – in which case DA treatment leads to an actively transcribing Pol IIB that will eventually transcribe through the downstream tRNA gene and thereby compensate for the loss of local Pol II. Hence, analysis of nascent-RNA in cells treated with DRB versus DA will allow (i) identification of the Pol III-transcribed genes whose transcription is altered by Pol II, (ii) determination of whether or not regulation of Pol III involves Pol II transcription and, at the same time, (iii) confirmation that Pol II transcription indeed does interfere with Pol III function when a truncated, transcribing Pol IIB generated at an upstream Pol II locus still maintains repression of a tRNA gene that, in contrast, is induced by DRB. **(B)** Venn diagram showing the number of tRNA genes affected >1.5-fold with a FDR <0.05 in DRB-treated wild-type HEK293 cells and DA-treated clones #7 and #19. Numbers in gray indicate numbers of tRNA genes affected by overlapping Pol II transcripts (and hence not considered in following analyses). **(C)** Nascent-RNA profiles of tRNA genes appearing up- or down-regulated because of

changes in overlapping Pol II transcripts (CTD-less Pol II read-through or *bona fide* Pol II genes). Control conditions for clone #7 and #19 are shown in blue, DA-treated profiles in red.

Figure S4. Related to Figure 4

(A) Immunoblots and corresponding Ponceau-stained membranes of NUN extracts and chromatin pellets prepared from RPB1-expressing (Ctl) or RPB1-depleted (DA) clone #7 cells pretreated with DMSO, LDC or Trip for 1 hr prior to auxin addition. IIO, IIA and IIB indicate the positions, respectively, of phosphorylated-, unphosphorylated- and truncated forms of RPB1. **(B)** Relative pre-tRNA expression levels in all three clones normalized to DMSO Ctl in RPB1-expressing (Ctl) or RPB1-depleted (DA) cells pre-treated with LDC or DMSO. **(C)** DA/Ctl ratios for data presented in **(B)** as well as for some mRNAs. **(D)** Same as **(B)** but with cells pre-treated with Triptolide and DMSO. **(E)** Same as **(C)** but for data shown in **(D)**. **(F)** Relative expression levels of different types of Pol III-transcribed RNAs (other than tRNAs) in RPB1-expressing (Ctl) or RPB1-depleted (DA) cells for all 3 clones normalized to their levels in Ctl condition. Data shows levels in total (black bars) or metabolically labelled (white bars) RNAs. **(G)** Relative levels of mature and precursor pre-Thr tRNA in total RNA prepared from RPB1-expressing (Ctl) and RPB1-depleted (DA) cells for all three clones. The histograms represent the average expression. **(H)** Same as **(G)** but for EU-labelled RNAs. This shows that despite the fact that the primers used to measure the mature form also hybridize to the precursor form, the contribution of the labelled precursor is negligible (1/28 of the mature tRNA level). **(I)** Decay curve fits used to calculate the stability of pre-Thr tRNA in RPB1-expressing (Ctl) and RPB1-depleted (DA) cells for all three clones treated with ActD to block transcription. The data are shown as % of the levels in cells prior to ActD addition (Time 0). **(J)** Half-life of precursor tRNAs in RPB1-expressing (Ctl) or RPB1-depleted (DA) cells for all three clones. **(K)** DA/Ctl ratios of RNA expression levels in RPB1-expressing (Ctl) or RPB1-depleted (DA) cells for all 3 clones pre-treated 15 min with cycloheximide (CHX) or DMSO. Data in **(B)-(F)** and **(I)** to **(K)** represent the average +/- SD. * indicates statistically significant differences, ns non-significant differences (T-test; p<0.05).

Figure S5. Related to Figure 5

(A) Protocol for siRNA-based knock-down prior to RPB1 depletion. **(B)** Fluorescence microscopy pictures showing the effects of MYC knock-down on RPB1 depletion in DA cells. **(C)** Relative mRNA expression levels normalized to siCtl-treated cells for all 3 clones transfected with a non-targeting (siCtl) or MYC-targeting pool of siRNAs (siMyc) prior to RPB1 depletion. **(D)** Protocol for rapamycin (Rapa) treatments prior to or after RPB1-depletion. **(E)** DA/Ctl ratios for data presented in **(Figure 5F)** for pre-tRNAs. **(F)** Time course analysis of relative pre-tRNAs levels in serum-depleted RPB1-expressing (Ctl) or RPB1-depleted (DA) cells stimulated with 20% FCS after RPB1 depletion. Red triangles emphasize the faster response of DA-treated cells during the first 30 min of serum stimulation. Data in **(C)-(F)** represent the average \pm SD. * indicates statistically significant differences (T-test; $p < 0.05$). Scales bars represent 100 μ m and apply to all micrographs within the same panel.

Figure S6. Related to Figure 6

(A) RNA expression levels and **(B)** Pol II Occupancy at different loci in IMR90 cells treated with DRB or DMSO. NR (Negative Region) specifies the background levels (dashed line). Data in **(A)** and **(B)** represent the average \pm SD. * indicates statistically significant differences (T-test; $p < 0.05$). **(C)** UCSC browser snapshot of the tRNA-Thr-CGT-2-1 locus displaying ENCODE ChIP-seq data for common histone modifications found at promoters and regulatory regions as well as all available data for Pol II, its associated general transcription factors and the sequence-specific transcription factors immunoprecipitated in the vicinity of this locus, suggesting the presence of a local Pol II promoter near this tRNA locus. **(D)** Microscopy picture showing transfection efficiencies (EGFP-positive cells) of HCT116 cells co-transfected with plasmids and EGFP and a Δ N-TCF4 constructs. **(E)** Immunoblot and corresponding Coomassie-stain membrane of total extracts prepared from HCT116 cells transfected cells probed with anti-MYC antibody to evaluate expression of the myc-tagged Δ N-TCF4 construct. Scales bars represent 100 μ m and apply to all micrographs within the same panel.

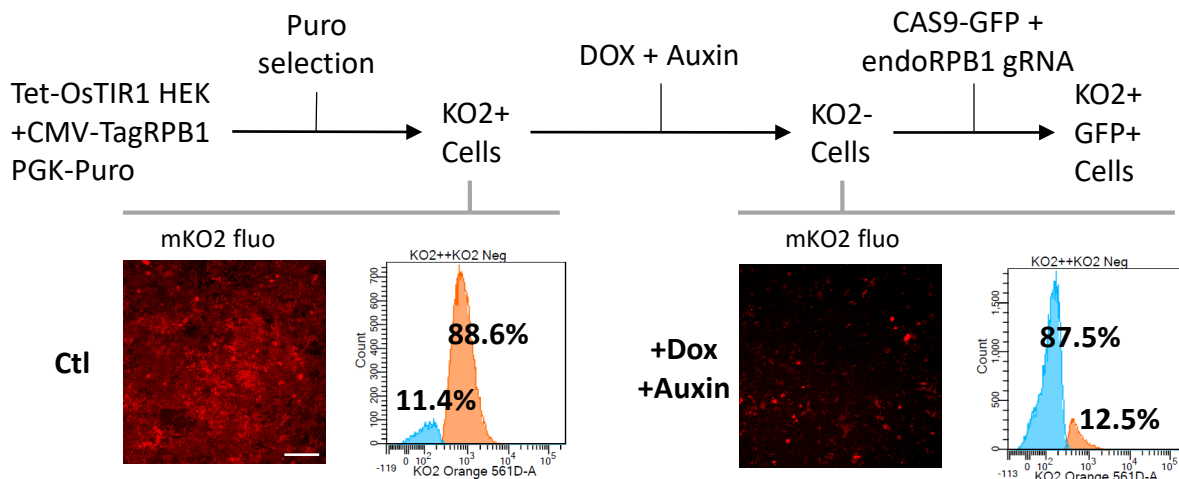
Figure S7. Related to Figure 7

Histogram showing the proportion (and numbers) of tRNA genes (grouped by anticodon) induced by

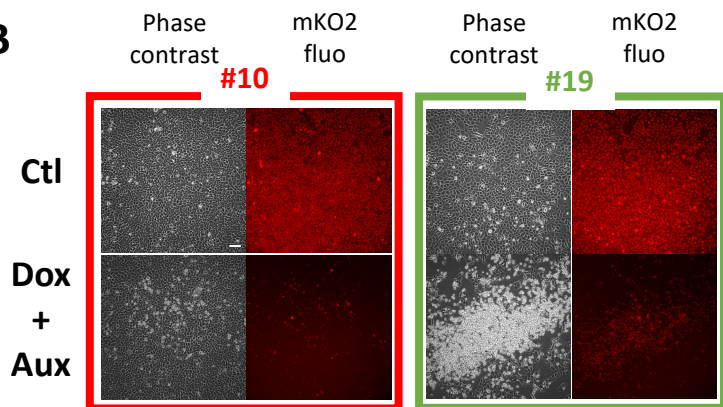
DRB. See Discussion for details.

Figure S1

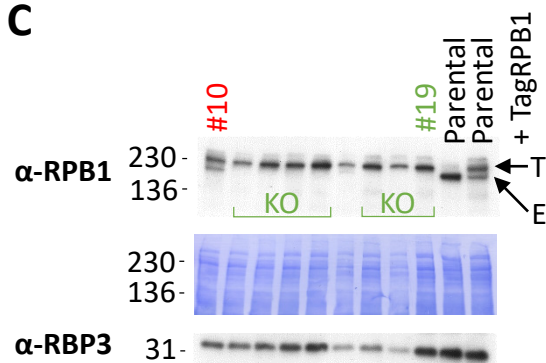
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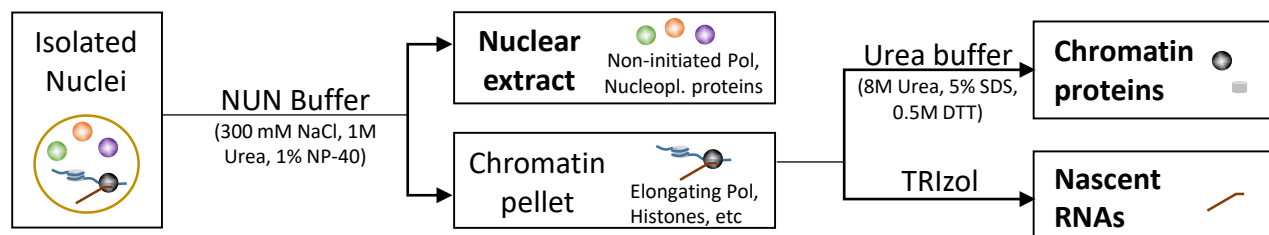
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C

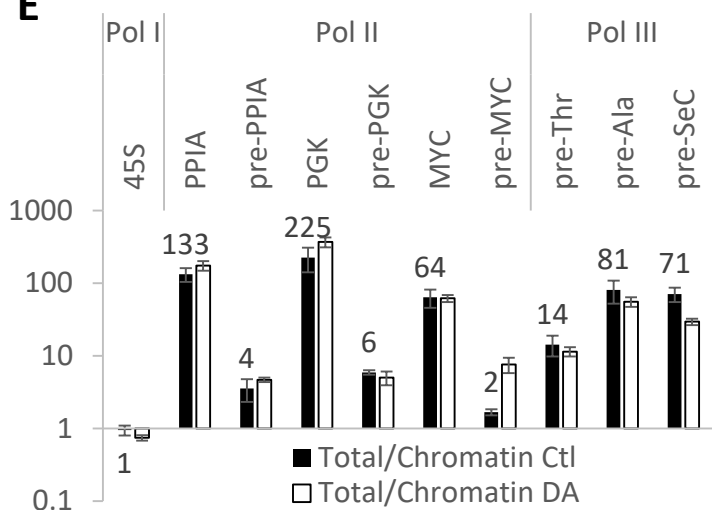


D



E

Total / Chromatin ratio



F

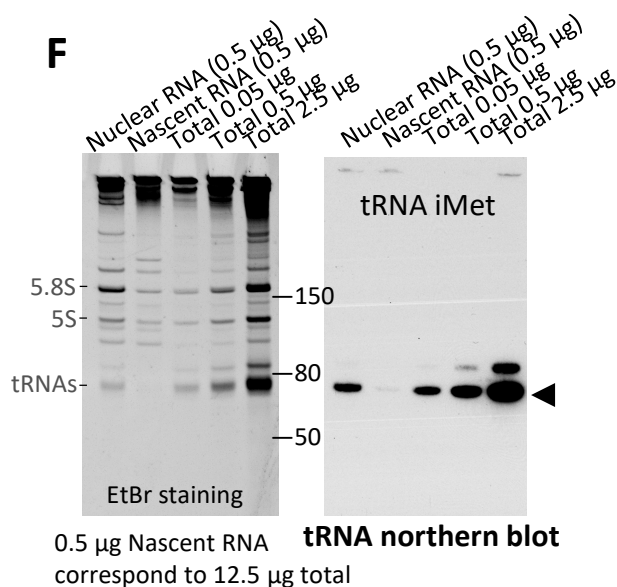
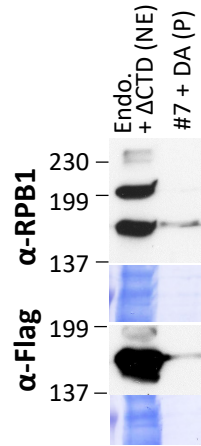


Figure S2

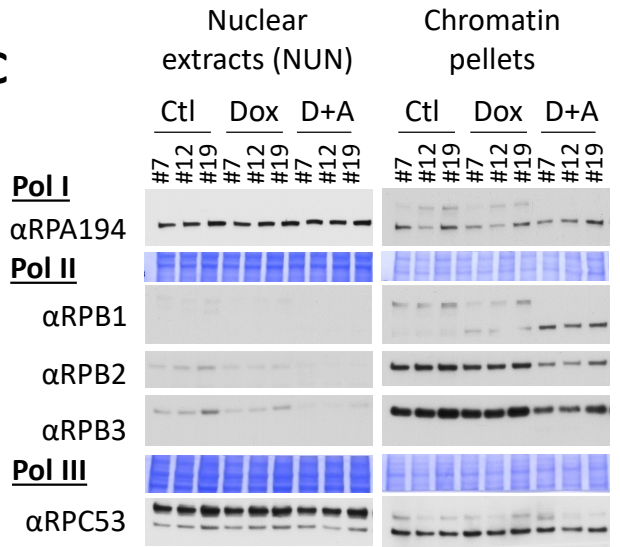
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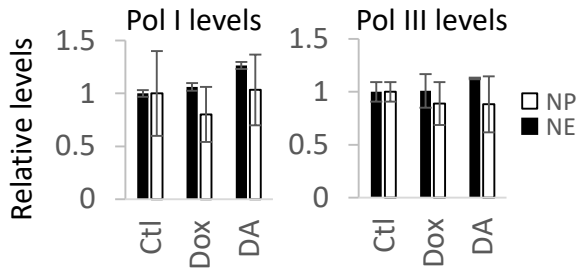
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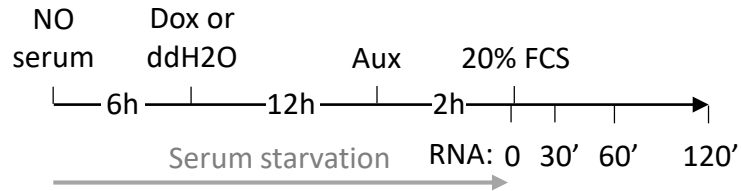
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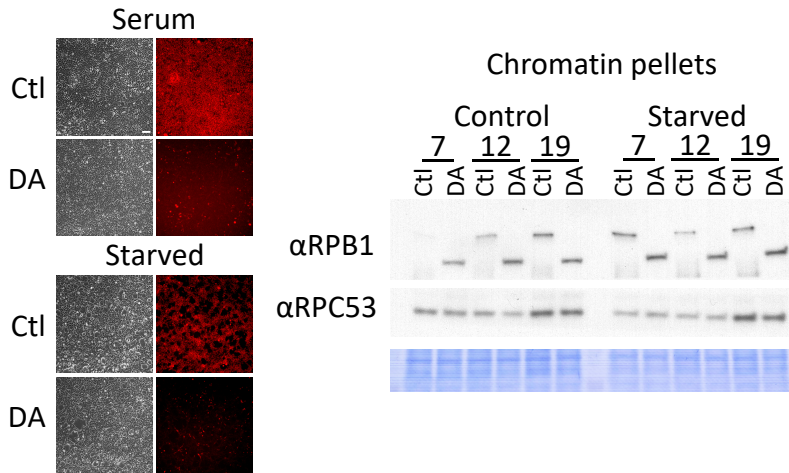
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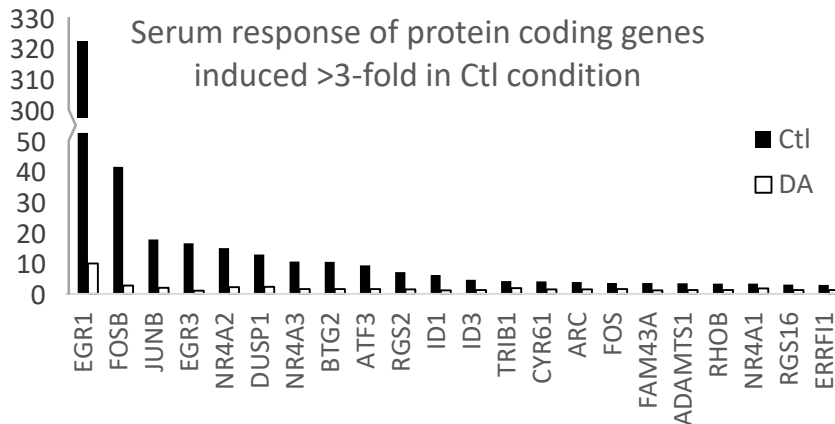
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H



G

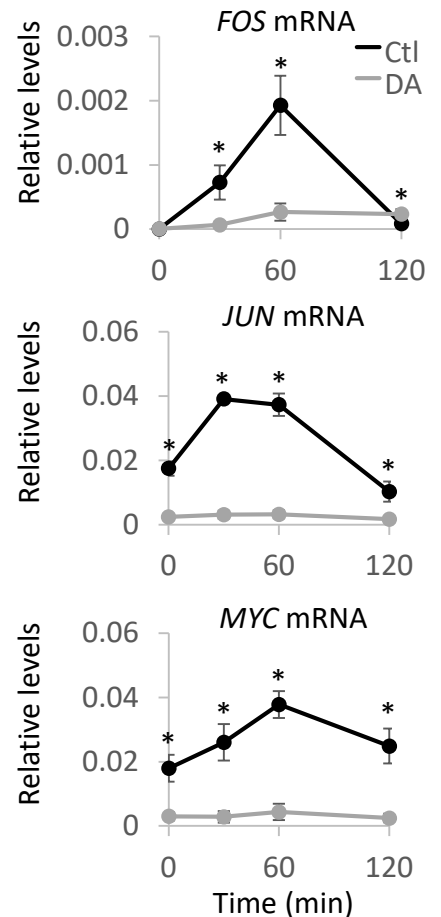
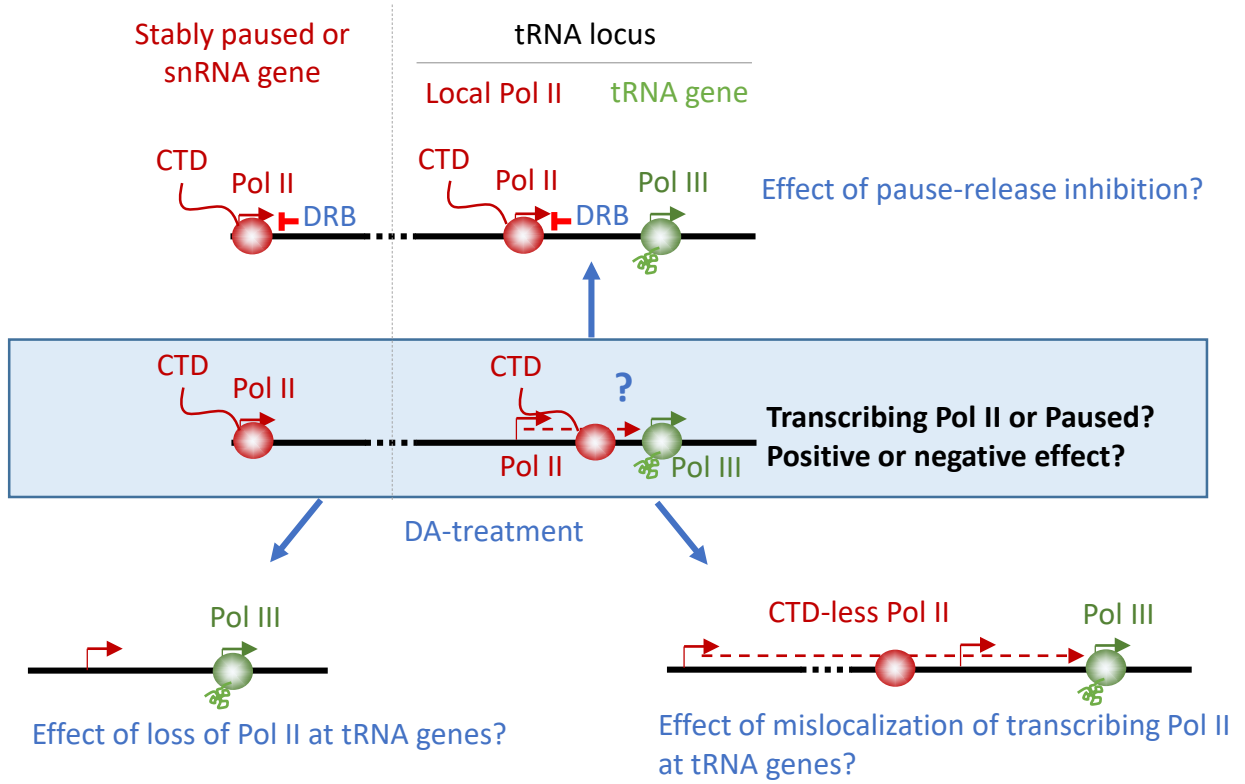
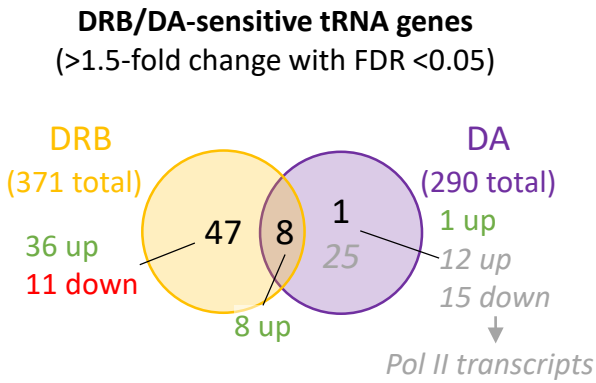


Figure S3

A



B



C

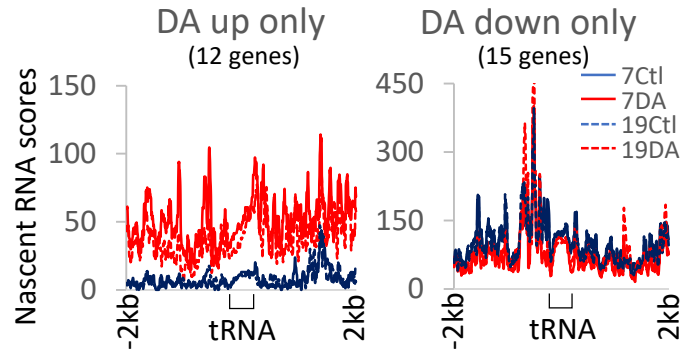
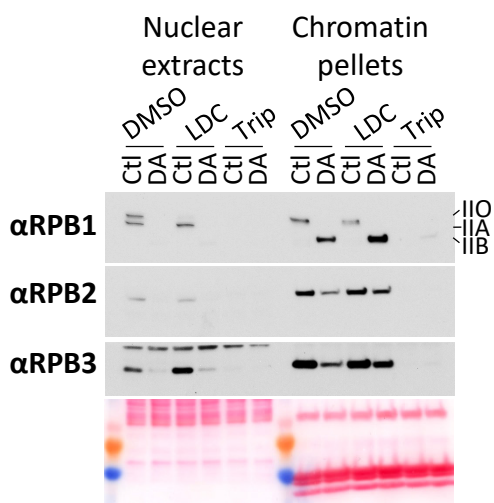
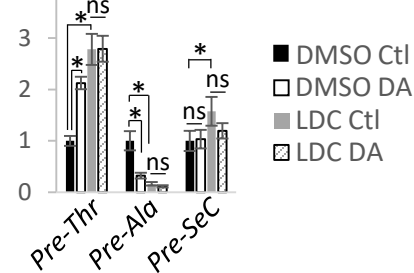


Figure S4

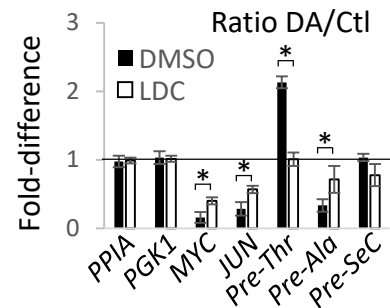
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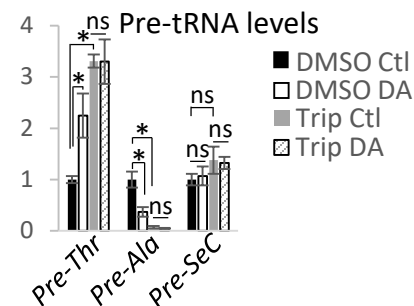
B Pre-tRNA levels



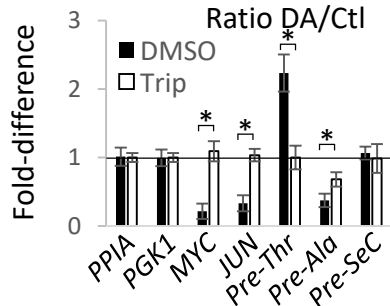
C Ratio DA/Ctl



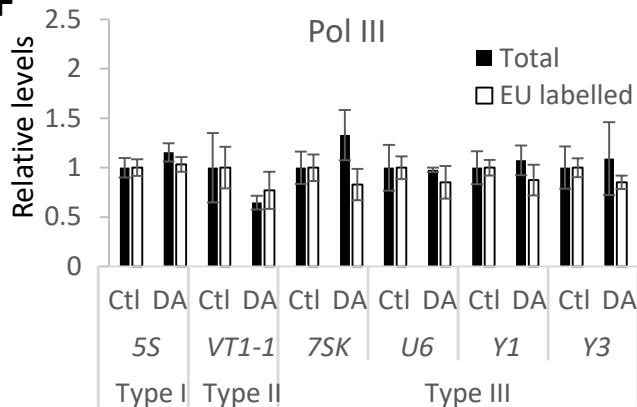
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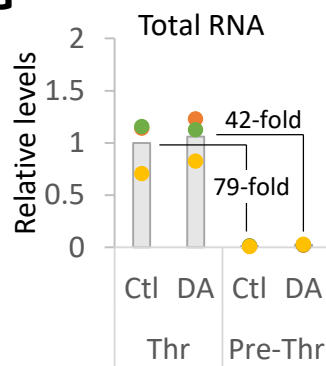
E Ratio DA/Ctl



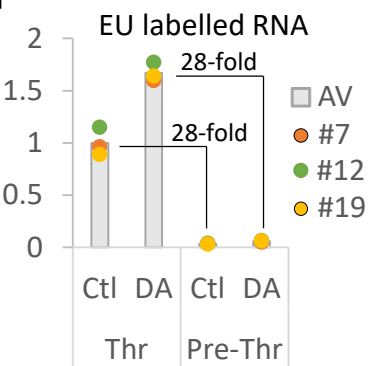
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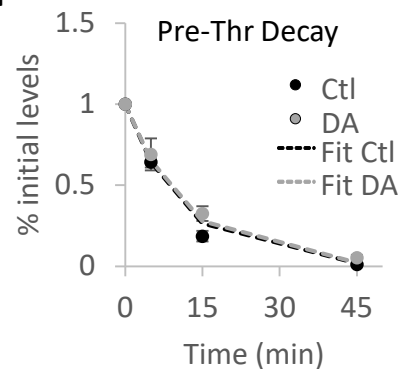
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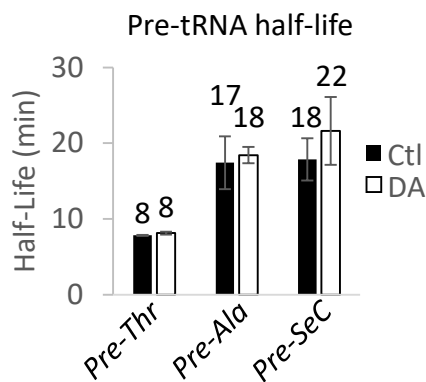
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I



J



K

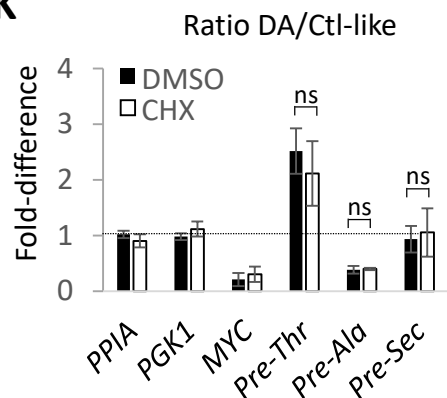


Figure S5

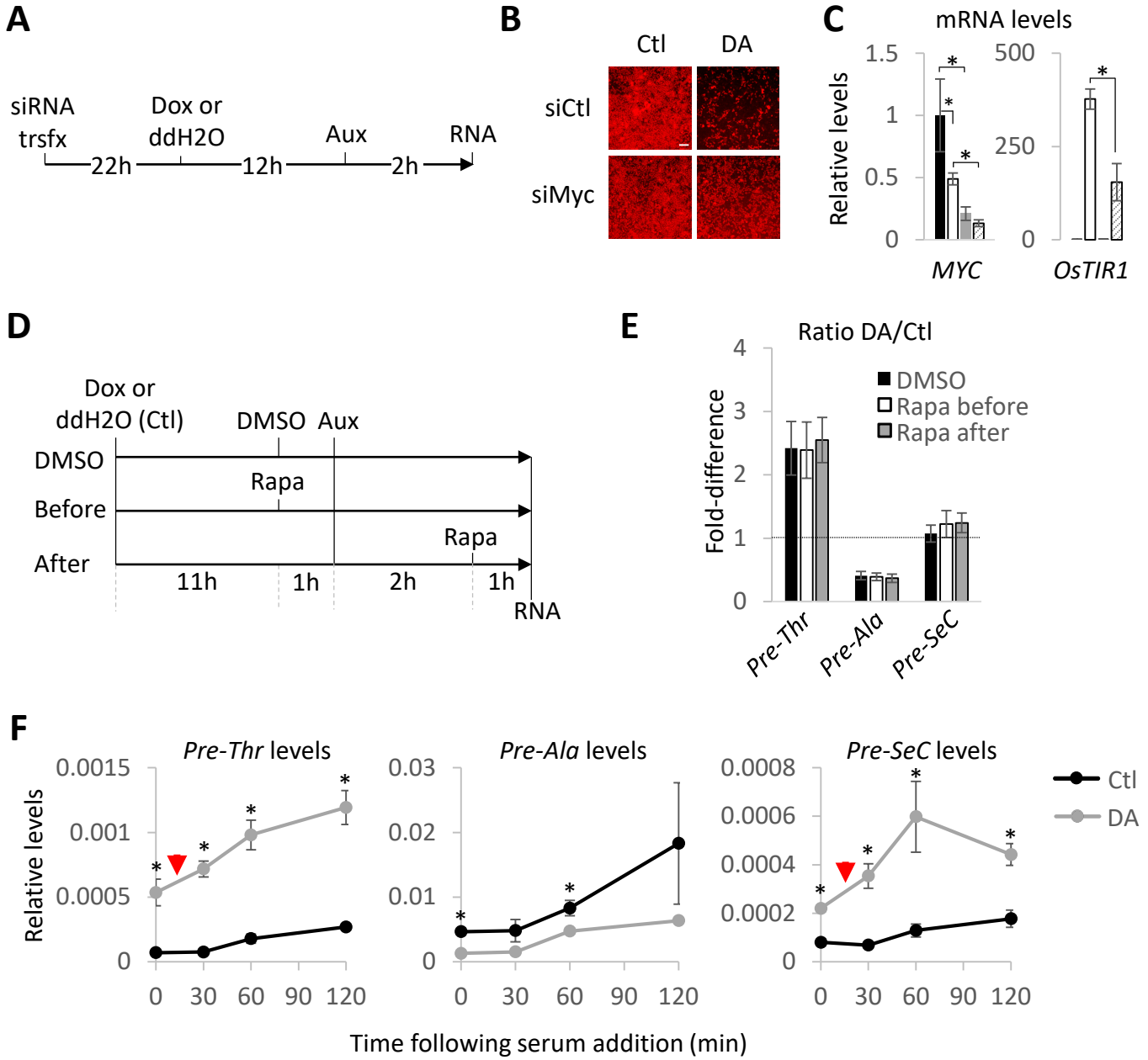


Figure S6

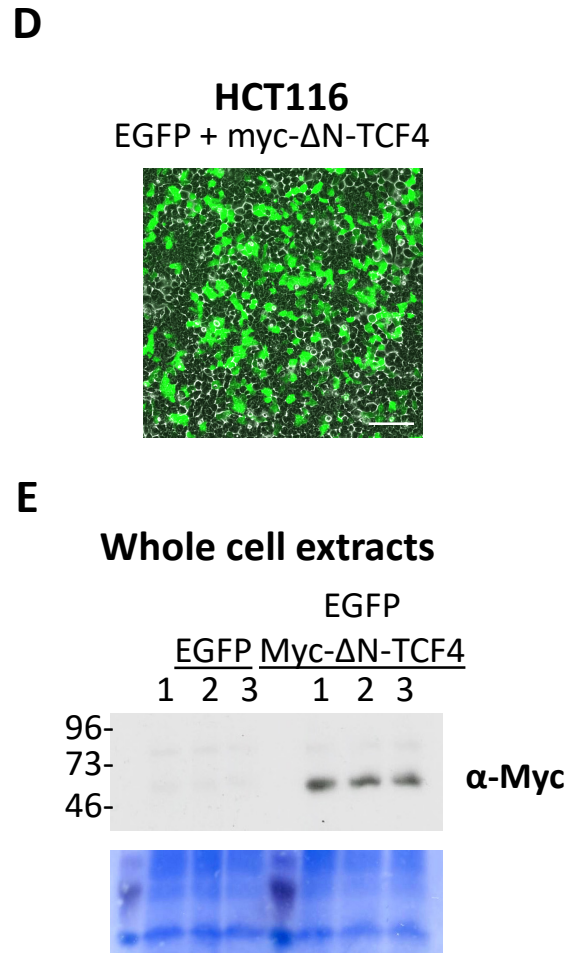
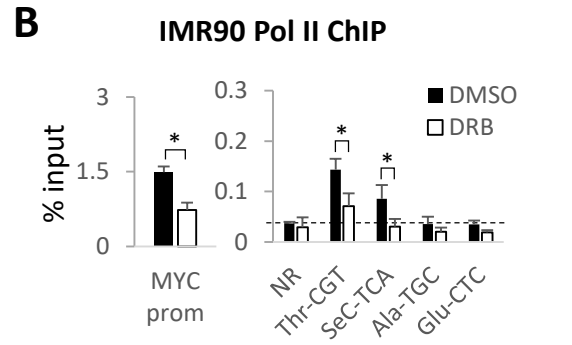
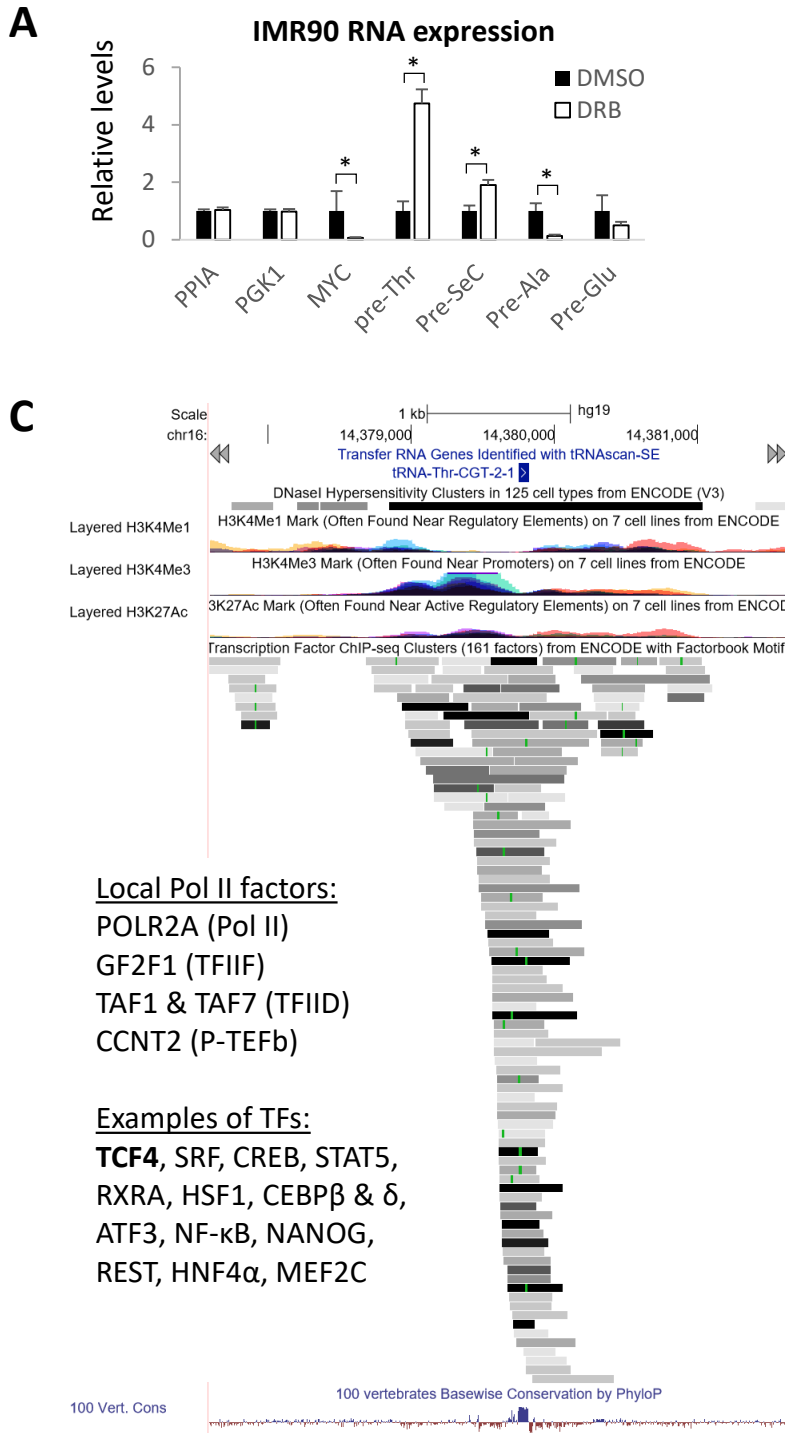


Figure S7

% expressed tRNA isodecoders upregulated in DRB (FDR <0.05)

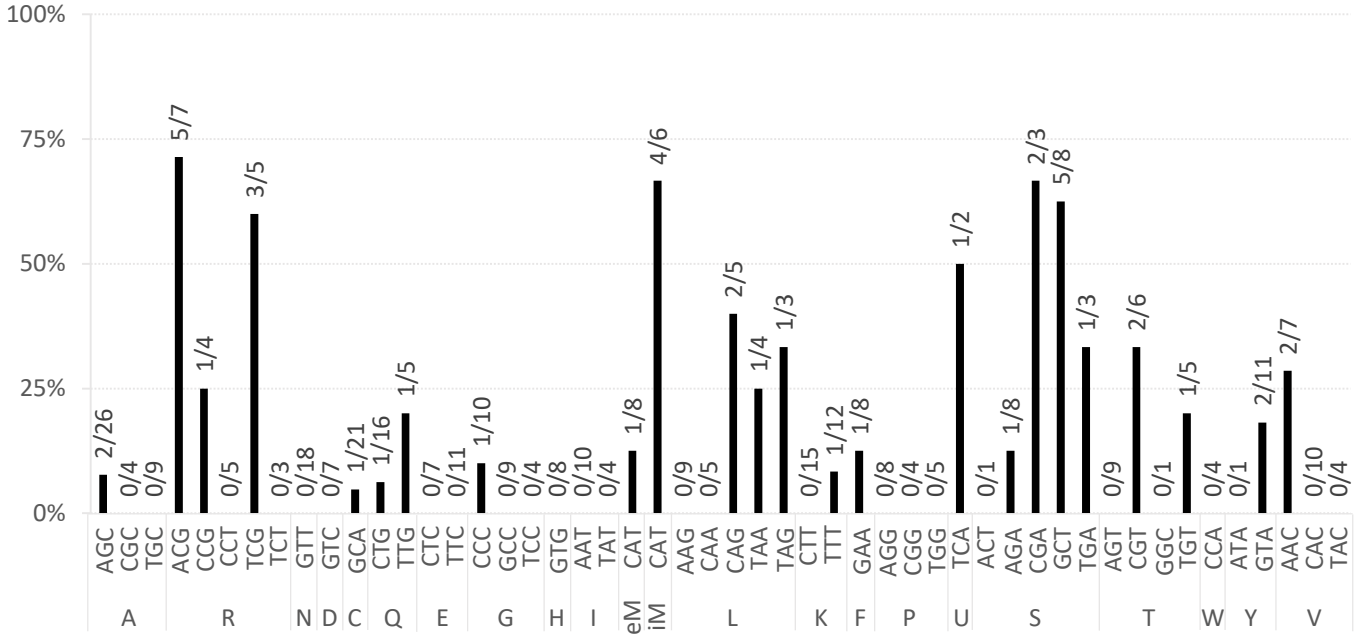


Table S1. Oligonucleotides, Probes, siRNAs and gBlock sequences. Related to STAR Methods section

qPCR primers	Sequences	Source	Identifier
q45Sfw	GAACGGTGGTGTGTCGTTCC	IDT	N/A
q45Srv	TCTCGTCTCACTCAAACCGC	IDT	N/A
q18Sfw	CTTTCGATGGTAGTCGCCGT	IDT	N/A
q18Srv	CCTTGGATGTGGTAGCCGTT	IDT	N/A
qFOSfw	CAAGCGGAGACAGACCAACT	IDT	N/A
qFOSrv	AGTCAGATCAAGGGAAGCCA	IDT	N/A
qJUNfw	TCCAAGTGCCGAAAAAGGAAG	IDT	N/A
qJUNrv	CGAGTTCTGAGCTTTCAAGGT	IDT	N/A
qGAPDHfw	GAAGGTGAAGGTCGGAGTCA	IDT	N/A
qGAPDHrv	TGAGGTCAATGAAGGGGTCA	IDT	N/A
qpreTBPfw	TTAGCAGCAGCCAGCCTAAC	IDT	N/A
qprePPIAfw	GAGGCTGCTTGTGGTGGTT	IDT	N/A
qPPIAfw	CATCTGCACTGCCAAGACTG	IDT	N/A
qPPIArv	GGCCTCCACAATATTCATGC	IDT	N/A
qprePGK1fw	TGGAGCCATCACATTTTCTGT	IDT	N/A
qPGK1fw	AGGGAAAAGATGCTTCTGGG	IDT	N/A
qPGK1rv	TGGAAAGTGAAGCTCGGAAA	IDT	N/A
qMYCfw	TTTCGGGTAGTGAAAACCA	IDT	N/A
qMYCrv	CACCGAGTCGTAGTCGAGGT	IDT	N/A
qpreMYCfw	TAActCAAGACTGCCTCCCG	IDT	N/A
qTIR1fw	ACCTGAGCTATGCACCAACC	IDT	N/A
qTIR1rv	CCACAGTCGCTGGAGTTTCA	IDT	N/A
MAF1rv	ATTCACCACCCAGCTAAGGC	IDT	N/A
MAF1fw	CCTGATTGCCACGCTCAATG	IDT	N/A
5S_fw	GGCCATACCACCTGAACGC	IDT	N/A
5S_rv	CAGCACCCGGTATTCCCAGG	IDT	N/A
U6-fw	GCTTCGGCAGCACATATACT	IDT	N/A
U6-rv	GGAACGCTTCACGAATTTGC	IDT	N/A
7SK_fw	CGATCTGGTTGCGACATCTG	IDT	N/A
7SK_rv	CGTTCTCTACAAATGGAC	IDT	N/A
VT1-fw	GGCTTTAGCTCAGCGGTTAC	IDT	N/A
VT1-rv	GGTCTCGAACAACCCAGACA	IDT	N/A
fwY1	GGTCCGAAGGTAGTGAGTTATC	IDT	N/A
revY1	ACTAGTCAAGTGCAAGTAGGAG	IDT	N/A
hufwY3	CCGAGTGCAAGTGGTGGTTTAC	IDT	N/A
revY3	GTCAAGTGAAGCAGTGGGAG	IDT	N/A
tRNA-i-met_fw	CTGGGCCATAACCCAGAG	IDT	N/A
tRNA-i-met_rv	TGGTAGCAGAGGATGGTTTC	IDT	N/A
tRNA-e-met_fw	CTCGTTAGCGCAGTAGGTAGC	IDT	N/A

tRNA-e-met_rv	GGATCGAACTCACGACCTTC	IDT	N/A
SeCTCA1-1-fw	CCGGATGATCCTCAGTGGTC	IDT	N/A
SeCTCA1-1-rv	GGAATTGAACCACTCTGTCG	IDT	N/A
Pre-SeCTCA1-1-fw	AGCTGTCTAGCGACAGAGTG	IDT	N/A
Pre-SeCTCA1-1-rv	GTTACTACCGCCCGAAAGGT	IDT	N/A
ThrCGT2-1-fw	CGGTGGCCAAGTGGTAAGG	IDT	N/A
ThrCGT2-1-rv	GTTCGAACCCGTGATCTTCG	IDT	N/A
Pre-ThrCGT2-1-fw	AAGGCGTCGGTCTCGTAAAC	IDT	N/A
Pre-ThrCGT2-1-rv	CACCCTATAGCTGAAGGTTGGA	IDT	N/A
AlaTGC7-1-fw	AGCTCAGTGGTAGAGCGCA	IDT	N/A
AlaTGC7-1-rv	GGGGATCGAACCGAGGC	IDT	N/A
Pre-AlaTGC7-1-fw	GTATGAGGCCTCGGTTTCGAT	IDT	N/A
Pre-AlaTGC7-1-rv	CTGCCAGAGGGAAACCATCA	IDT	N/A
ArgTCT1-1-fw	GGCTCCGTGGCGCAATG	IDT	N/A
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PreGluCTC-fw	TCCCTGACCGGGAATCGAAC	IDT	N/A
PreGluCTC-RV	GCCCTGAAGCCGCCTCTC	IDT	N/A
MYCprom_fw	AGGGATCGCGCTGAGTATAA	IDT	N/A
MYCprom_rv	TGCCTCTCGCTGGAATTACT	IDT	N/A
NR_fw	TCCATCAAGGCCATTTTCCTTCAGT	IDT	N/A
NR_rev	TGAGATCCCCATCAGATCCTGG	IDT	N/A
Northern Probe and Unfolder			
iMetProbe	/5Biosg/TTATGGGCCAGCAGCCTTC	IDT	N/A
iMetUnfolder	TAGCAGAGGATGGTTTCGATCCATCGACCTCTGGG	IDT	N/A
ThrProbe	GATCTTCGGTTTACGAGACCGAC	IDT	N/A
PreThrProbe	GTCTCCACCCTATAGCTGAAGGTTGGATATC	IDT	N/A
ThrUnfolder	GCCTTACCACTTGGCCACCGCGCC	IDT	N/A
AlaProbe	GGCCTCATAATGCAAAGCATGC	IDT	N/A
PreAlaProb	GGTCTGTGCTTGAGAACTGCCAGAGGGAAACCATCACT	IDT	N/A
AlaUnolder	GCTTACCACTGAGCTACACCCCC	IDT	N/A
CRISPR / guide RNA primers			
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gRPB1rv	aaacCCatgCACGGGGGTGGCCCC	IDT	N/A
gAAVS1fw	CACCGGGGCCACTAGGGACAGGAT	IDT	N/A
gAAVS1rv	AAACATCCTGTCCCTAGTGGCCCC	IDT	N/A
Cloning (In-fusion) primers			
2Ainfusion1	TCCACTAGTCCAGTGTGGTGGTACTACAAGGACGACG	IDT	N/A
2Ainfusion2	TGTAATCCATGTTCTCCTCGTCACTGTCATCC	IDT	N/A
Tag2Ainfusion1	CGAGGAGAACATGGATTACAAGGATGACGATGAC	IDT	N/A
Tag2Ainfusion2	CACTGTGCTGGATATCTGCATTAGGAATGAGCTACTGCATC TTC	IDT	N/A

siRNAs siGENOME Human MYC SMARTpool		Dharmacon	M-003282-07-0005
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	ggaacuaugaccucgacua		
	gaacacacaacgucuugga		
	cuaccaggcugcgcaaa		
GeneBlock Tag-degron-mKO2		IDT	N/A
atggattacaaggatgacgatgacaagGAGAAGAGTGCTTGTCTAAAGATCCAGCCAAACCTCCGGCCAAGGCACAAGTTGTGGG ATGGCCACCGGTGAGATCATACCGGAAGAACGTGATGGTTTCTGCCAAAAATCAAGCGGTGGCCCGGAGGCGGCGGCG TTCGTGAAGGTATCAATGGACGGAGCACCGTACTTGAGGAAAATCGATTTGAGGATGTATAAAGGTAAGCCTATCCCTAAC CCTCTCCTCGGTCTCGATTCTACGGTGAGTGTGATTAACCAGAGATGAAGATGAGGTACTACATGGACGGCTCCGTCAAT GGGCATGAGTTCACAATTGAAGGTGAAGGCACAGGCAGACCTTACGAGGGACATCAAGAGATGACACTACGCGTCACAAT GGCCGAGGGCGGGCCAATGCCTTTCGCGTTTGACTTAGTGTACACGTGTTCTGTTACGGCCACAGAGTATTTACTAAATAT CCAGAAGAGATACCAGACTATTTCAAACAAGCATTTCTGAAGGCCTGTCATGGGAAAGGTCGTTGGAGTTCGAAGATGG TGGGTCCGCTTCAGTCAGTGCGCATATAAGCCTTAGAGGAAACACCTTCTACCACAAATCCAAATTTACTGGGGTTAACTTT CCTGCCGATGGTCTATCATGCAAACCAAAGTGTGATTGGGAGCCATCAACCGAGAAAATTACTGCCAGCGACGGAGTT CTGAAGGGTGTGTTACGATGTACCTAAACTTGAAGGAGGCGGCAATCACAAATGCCAAATGAAGACTACTTACAAGGC GGCAAAGAGATTCTTGAAATGCCAGGAGACCATTACATCGGCCATCGCCTCGTCAGGAAAACCGAAGGCAACATTACTG AGCAGGTAGAAGATGCAGTAGCTCATTCTAA			