Supplementary Materials for

Mitotic Transcription and Waves of Gene Reactivation During Mitotic Exit

Katherine C. Palozola, Greg Donahue, Hong Liu, Gregory R. Grant, Justin S. Becker, Allison Cote, Hongtao Yu, Arjun Raj, and Kenneth S. Zaret

correspondence to: zaret@upenn.edu

This PDF file includes:

Table S1. Aligned and unique reads.

Table S2. Control #1 alignment.

Table S3. Control #2 alignment.

Table S4. Spike-in scalars.

Table S9. Mitotically-enriched genes do not represent another cell cycle stage.

Other Supplementary Materials for this manuscript includes the following:

Table S5. Mitotically-expressed transcripts.

Table S6. Maximum non-mitotic FPMs from publicly-available liver RNA-Seq studies.

Table S7. Mitotically-enriched transcripts.

Table S8. Complete Gene Ontology enrichments of mitotically-enriched genes.

Table S10. 40 min transcripts.

Table S11. 80 min transcripts.

Table S12. 105 min transcripts.

Table S13. 165 min transcripts.

Table S14. 300 min transcripts.

Table S15. Async. transcripts.

Table S16. Complete Gene Ontology enrichments of 40’ genes.

Table S17. Complete Gene Ontology enrichments of 80’ genes.

Table S18. Complete Gene Ontology enrichments of 105’ genes.

Table S19. Complete Gene Ontology enrichments of 165’ genes.

Table S20. Complete Gene Ontology enrichments of 300’ genes.

Table S21. Complete Gene Ontology enrichments of async. Genes.

Table S22. Complete Gene Ontology enrichments of longest 40 and 80’ genes.

Table S23. Spike-in normalized eRNA FPMs for mitotic and async.

Table S24. Intergenic eRNA FPMs.

Materials and Methods

Cell Culture

HUH7 human hepatoma cells were cultured in DMEM High Glucose with L-glutamine (Invitrogen, 11965), 10% FBS (HyClone), and 1% penicillin-streptomycin (Invitrogen) at 37˚C in 5% CO_2. For mitotic arrest experiments, the cells were synchronized at the G1/S transition by culturing for 24 hr in complete DMEM supplemented with 2 mM thymidine (Sigma, T1895). The thymidine media was removed and cells were washed once with PBS. The cells were then cultured for 2 hr in complete DMEM to complete synchronization. The media was then supplemented with nocodazole (Sigma, M1404) for a final concentration of 0.06 ug/mL, and incubated at 37˚C for 16 hours. Mitotic cells were manually collected by shake-off. To release the cells from arrest, mitotic cells were washed twice with PBS and re-plated in complete DMEM.

H3S10p Immunofluorescence

HUH7 cells were fixed in 4% PFA for 30 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 30 minutes at room temperature, blocked in 10% FBS/0.1% Triton X-100 for 3 hours at room temperature, and incubated with 1 ug/mL primary antibody (Abcam ab5176) in 10% FBS/0.1% Triton X-100 over night at 4˚C. A Nikon inverted widefield microscope was used to image the fixed cells at 30X magnification.

Quantification of Mitotic Arrest

Immediately following shake-off, a fraction of the mitotic cells was removed, washed once with PBS and fixed in 4% paraformaldehyde at room temperature and stained with DAPI. A Nikon inverted widefield microscope was used to image the fixed cells at 20X magnification,

and the number of mitotic (highly compact chromatin) and non-mitotic cells were manually quantified based on chromatin morphology. A minimum of 250 cells per replicate were counted for EU-labeling. The average mitotic index of was 97% for the cells that had spike-ins added after EU-labeling for mitotic versus asynchronous comparison, and 94.5% for the cells that were subsequently labeled with EU during exit. Mitotic cells were similarly quantified (blinded) at each time point during mitotic exit to assess their ability to re-enter G1 following nocodazole arrest.

Custom Spike-In Control Sequences

We designed and generated our own biotinylated spike-in controls to normalize between mitotic (0') and async. nascent RNA populations since commercial spike-ins are not biotinylated and thus would not be pulled down in our assay. Two endogenous loci were chosen (control #1 = hg19:chr7:110258409-110258798 and control #2 = hg19:chr13:107098286-107098585) that we found not to be transcribed in HUH7 cells, unique in the genome, and amplified linearly as described below. These sequences were PCR-amplified from HUH7 genomic DNA, sanger-sequenced to confirm their sequence, cloned into a TOPO vector, and transcribed *in vitro* (Ambion) with biotin-UTP.

EU Pulse-Labeling of HUH7 Cells and EU-RNA-Seq Library Construction

Cells were pulse labeled with 0.5 mM EU in standard growth media for 40 minutes at 37˚C (*11*). The media was removed and the cells were collected with TRIzol Reagent (Ambion). Total RNA was isolated using miRNeasy (Qiagen). Biotin-azide was then conjugated to EU with the Click-iT Nascent RNA Capture Kit (Invitrogen). Both custom biotinylated , spike-in control RNAs were then added to 1.7 ug of each biotinylated sample (5e-5 ug of control #1 and 5e-4 ug of control #2). Biotin-EU-RNAs and spike-in controls, where applicable, were pulled down from

total RNA using streptavidin-coated magnetic beads (Invitrogen), and used for stranded, ribo-depleted cDNA library generation directly off the beads (Ovation Human FFPE RNA-Seq Multiplex System).

Sequencing and Alignment

The EU-RNA-Seq libraries were sequenced on the Illumina NextGen500. Reads were trimmed to 35 nucleotides, extended to a total length of 300, and aligned to human genome build hg19 using Bowtie2 2.2.9 TopHat "very sensitive" option in end-to-end mode (*31*). Bed files were then reduced to unique, non-PCR duplicated fragments with a custom script. Read alignment data for EU-RNA-Seq libraries are presented in table S1; read alignment data for spike-in control reads are presented in tables S2 and S3.

Transcription Rate Quantification

Reads present in the "NoEU" negative labeling control were subtracted on a base pair basis for all transcripts in each sample. The samples were scalar normalized and the fragments per million fragments mapped per kilobase of transcript model (FPKM) was calculated for all transcripts for each sample. To quantify over nascent transcripts, introns were included in the transcript model, unlike a traditional FPKM that maps fragments per million per kilobase of exons.

Global Normalization

For the mitotic and asynchronous replicates that received spike-ins, sequencing reads were aligned to the individual control loci. Total reads aligned to each locus were normalized to sequencing depth to produce each library's spike-in scalar (table S4). All FPKMs in the mitotic and asynchronous libraries were then multiplied by their spike-in scalar prior to further analysis.

Modeling Interphase Contamination

All RNA-Seq data sets for human liver cell lines were downloaded from the Short Fragments Archive (SRA) (*32*) giving 222 samples. This includes approximately 20 cells lines across a heterogenous range of conditions and treatments, and no samples were excluded. We aligned 5M fragments from each sample to the human genome build hg19 with STAR v2.4.2a (*33*).We did the same for the asynchronous and mitotic arrest (0') EU-labeled triplicates. The FPM (Fragments Per Megaread) of gene *G* is calculated as the number of fragments mapping to (any transcript of) gene *G*, divided by the number of millions of aligned fragments out of the 5M total (*34*). For gene *G* denote this value by F_G. This normalized quantity is thus comparable across samples and studies.

RT-qPCR

Cells were treated with either DMSO or 5 uM triptolide for 1 hour at 37˚C prior to RNA harvest with TRIzol reagent (Ambion). Primary transcripts were detected using intron/exon primer pairs, with the expception of GAPDH which was detected with exonic primers. Sequences are available upon request.

Transcription Run-On of Intact Mitotic Cells with FITC-UTP

As previously described (*22*) , the chromosome spreads were firstly treated with 0.2 % Triton X-100 and then FITC-UTP (+/- α-amanitin) was added onto slides. The slides were then incubated at room temperature for 20 minutes to allow transcription to incorporate FITC-CTP into newly synthesized RNA. The cells were then fixed with 4% paraformaldehyde and stained with DAPI. Fluorescence was observed with a microscope. Fluorescence intensities of DNA and RNA on chromosome arms were converted into numerical values by Image J. After background subtraction, the mean intensities of DNA and RNA for each cell were obtained. The values of

RNA intensity were normalized to DAPI intensity to obtain relative intensity. A two-tailed t-test was performed in Excel between control and α-amanitin-treated cells.

RNA FISH

Hybridizations were carried out as previously described (*35*). Briefly, asynchronous HUH7 cells were grown on 18 mm #1 cover slips, fixed in 3.7% formaldehyde for 10 min at room temperature, and permeabilized in 70% ethanol overnight (minimum) at 4˚C. The coverslips were then incubated in wash buffer (2X SSC, 10% formamide) for 1~2 minutes at room temperature. Custom probes against introns and exons of *KLF4, ATF3,* and *ELF3* were designed using LGC Biosearch Technologies' Stellaris® RNA FISH Probe Designer. Probes were prepared as per manufacturer's recommendation. Hybridization of 1:100 probes in hybridization buffer (dextran sulfate, formamide, SSC) was carried out at 37˚C overnight. Cells were then stained with DAPI and imaged on a widefield fluorescent microscope at 100X magnification. Maximum projections were compiled with ImageJ, and intron and exons signals were quantified as previously described (*35*). Images were false colored and mereged in ImageJ for identification of intron/exon colocalization events. A one-sample t-test was performed in R for colocalization events in each category.

Identification of Transcripts First Increased at Each Time Point During Mitotic Release

For all transcripts that had an FPKM of at least 19 in the asynchronous population, we pulled out those that had an FPKM at least 1.5 fold higher than the FPKM in mitosis (0') for the first time. That is, if the FPKM is 1.5 fold higher at 80' min than at 0', but less than 1.5 fold higher at 40' than at 0' then that transcript was called an 80' transcript.

Comparison to Murine Erythroblasts

The study by Hsiung *et al* (*17*) used principle component analysis to determine that approximately half of the genes expressed in their murine cells line spiked at 90 minutes of mitotic exit. Projection of all genes onto the first principle component indicates the similarity between a given gene and the 90 minute spike. These values were plotted for homologs that first increase in our HUH7 cells after 80 minutes or were only expressed in the asynchronous cells. A Wilcoxon rank-sum (with continuity correction) test was performed in R between the PC1adj values of the mouse orthologs of our 80' transcripts and the PC1adj values of the 90' transcripts.

Gene Ontology Enrichment

Unique genes corresponding to the transcripts found to first increase at a specific time point were used to perform GO analysis for cellular component, molecular function, and biological process categories on DAVID (*24, 25*), relative to all transcripts with an FPKM of at least 19 in the asynchronous population and ≥1.5 fold higher than in mitosis (i.e. expressed in asynchronous cells). Significant categories were filtered for those that were at least 1.3 fold enriched over background with the FDR controlled at 10%.

eRNA Expression with Putative Targets

The 292,429 enhancers from Leung et al. (*29*) were mapped to hg19 and measured for RNA FPKM during mitosis and mitotic exit. Enhancers were defined as -200 to +200 bp around the central nucleotide position and were associated to genes by the nearest TSS and classified by the time of first 1.5 fold increase of their putative regulatory targets. Only intergenic enhancers, those falling into the region 1kb to 100kb upstream of their target gene, without overlapping other genes, were considered. Because high EU-RNA-Seq background was detected at SINE elements, all tags overlapping annotated SINE elements were removed. The FPKMs of both + and – strand non-SINE nascent EU-RNA-Seq tags were then averaged at the enhancer locus.

To rule out enhancers inactive in asynchronous cells, the enhancers were filtered to remove those with an FPKM ≤ 0.1 in the asynchronous state. To eliminate enhancers which occur in regions of broad background enrichment, for each enhancer two additional intergenic 1kb windows 1.2 kb away from the enhancer centers were measured for EU-RNA-Seq signal. Each enhancer was tested to see whether its fold over background at the activation time was greater than 2X (the background was the maximum of both strands on both regions). Enhancers failing this filter (or without a matching background region) were rejected.

For each activation group, the filtered list of intergenic enhancers is box-plotted over the mitotic exit time-course. The p-value of the time point of activation vs t=0 (blue) or vs the previous time point (red) was computed by a Wilcoxon rank sum test.

eRNA Expression in Mitosis and Asynchronous HUH7 Cells

Enhancers were filtered as described above. FPM scores reflect the average of both strands at a 400 bp window surrounding the enhancer center. eRNA scores were adjusted using the spike-in scalar normalization, and SINE overlapping RNA-seq tags were excluded. The p-value between spike-in normalized mitotic and asynchronous eRNAs was computed by a Wilcoxon rank sum test.

Identification of eRNAs First Increased at Each Time Point

For all eRNAs that had an FPKM of at least 0.1 in the asynchronous population, we pulled out those that had an FPKM at least 1.5 fold higher than the FPKM in mitosis (0') for the first time. That is, if the FPKM is 1.5 fold higher at 80' min than at 0', but less than 1.5 fold higher at 40' than at 0' then that eRNA was called an 80' eRNA.

Statistics

The Wilcoxon rank sum test was used for figures 1D, 2M, 4B-C, S3C, S9C, S10B-C, S10E, and S11A. The Pearson correlation coefficient was computed between all replicates for experiment 2 in figure S3D. The Spearman rank correlation coefficient was computed between all spike-in normalized replicates for experiment 1 in figure S5D. A two-tailed t-test was used to compute all p-values in figures S6A-B and S6U. A one-sided t-test was used to compute all p-values in figure S7D. A Kruskall Wallis test was used to compute the p-value in figures S9A. A hypergeometric test for enrichment was used to calculate the p-values in table S9 and figures S10A and S10F.

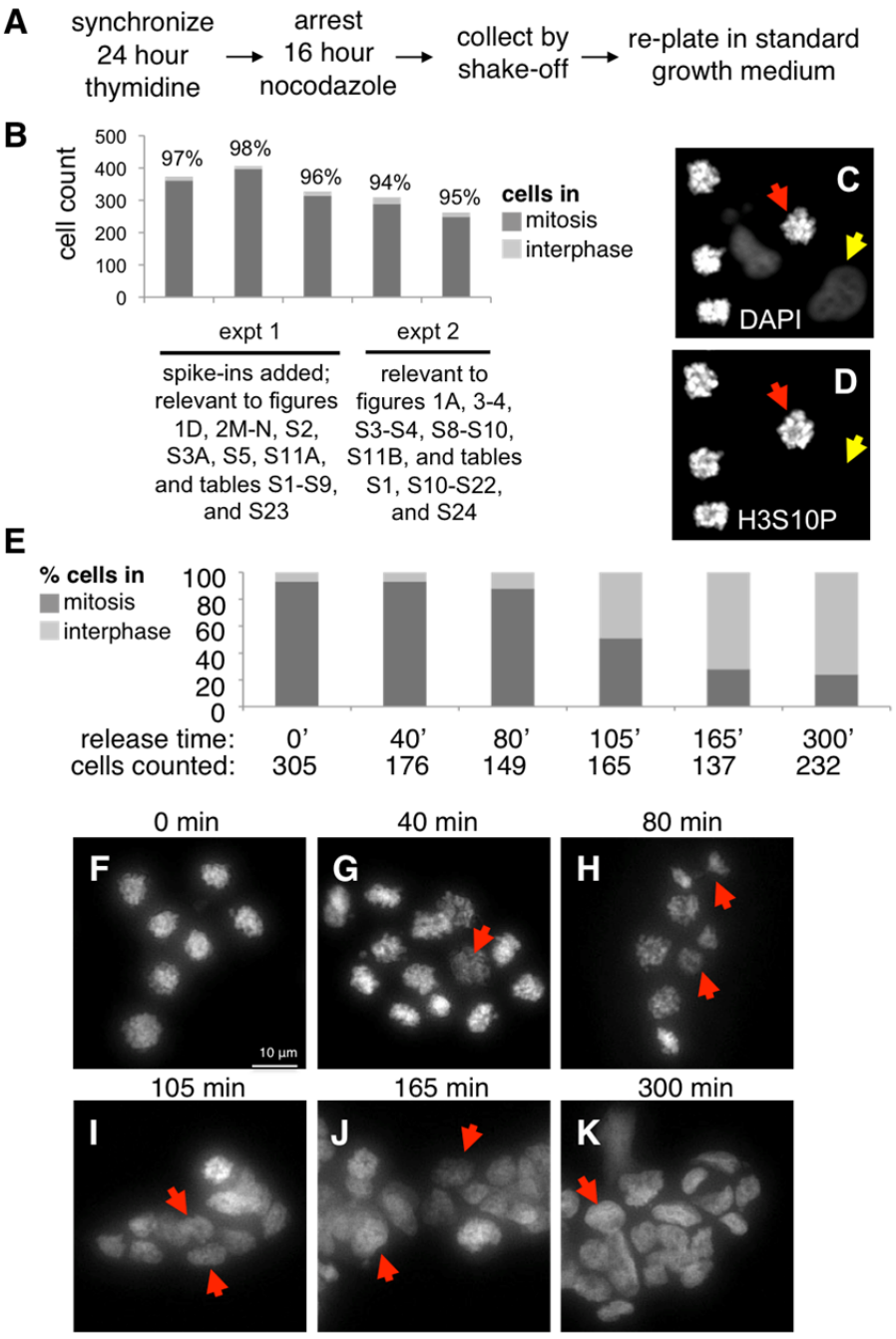


Fig. S1. Quantification of mitotic index and successful cell cycle re-entry after mitotic release of HUH7 hepatoma cells. **(A)** Synchronization, arrest, and release protocol. **(B)** The mitotic index of replicate shake-offs for each experiment; error bars = standard error of the mean, n = 3 replicates for experiment 1, n = 2 replicates for experiment 2; relevant data panels for each experiment are indicated. **(C)** DAPI staining of mitotic and interphase cells. **(D)** H3S10p staining

of mitotic cells indicates that the compact chromatin morphology is representative of mitosis; red arrows indicate condensed mitotic chromatin, yellow arrows indicate decondensed interphase nuclei. **(E)** Sample-blinded quantification of cells that have condensed and de-condensed chromatin after nocodazole wash-out and a representative bright field image of each time point. **(F)-(K)**. Red arrows indicate cells exited from metaphase.

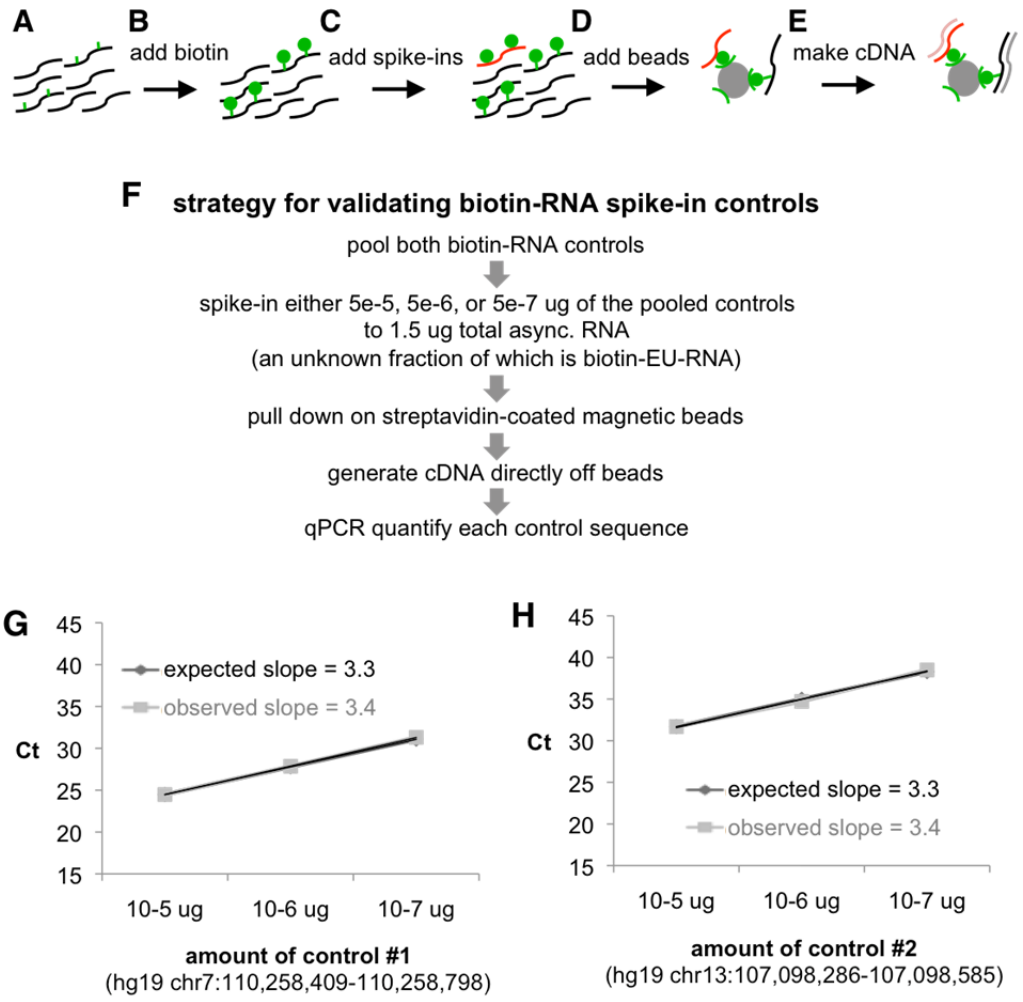


Fig. S2. The custom biotin-RNA control sequences retain their proportionality when pulled down and converted to cDNA. **(A)** Total RNA (black), including those labeled with EU (green), was recovered, **(B)** conjugated to biotin-azide (green circle), **(C)** biotin-RNA control sequences were spiked-in at known quantities (red), **(D)** biotinylated RNAs were isolated with streptavidin-coated magnetic beads (gray circle), and **(E)** converted to cDNA libraries. **(F)** Experimental approach to validating controls. Ct values following RT-qPCR of **(G)** control #1 or **(H)** control #2, when added at the indicated levels to 1.5 ug of async. biotin-EU-RNA and pulled down with streptavidin-coated magnetic beads**;** expected values are based on linear pull down and cDNA conversion.

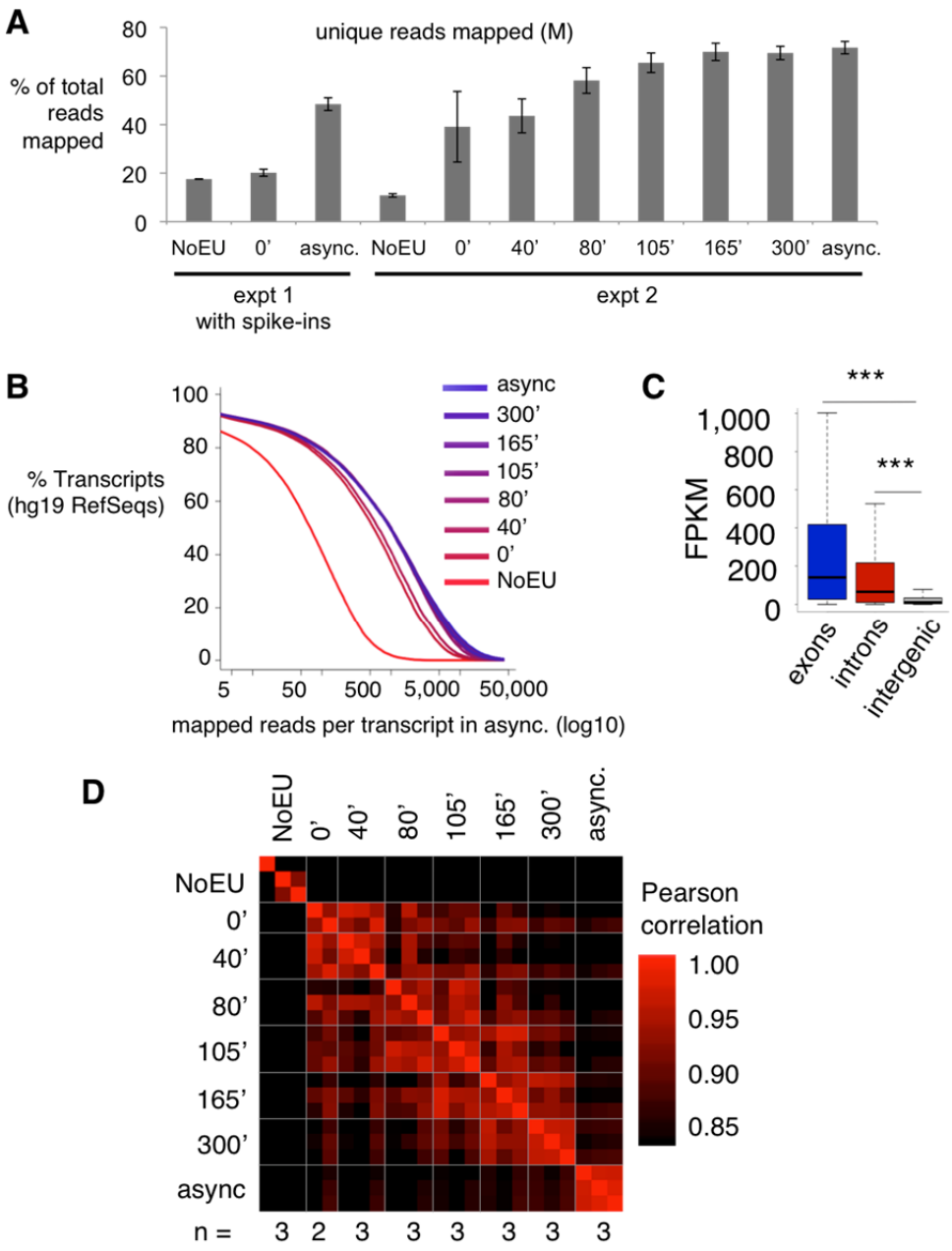


Fig. S3. EU-RNA-Seq robustly maps nascent transcripts. **(A)** Average reads mapped (PCR-duplicates removed) for each time point. **(B)** Transcript coverage by tag count. (**C)** Read coverage over exons, introns, and intergenic regions (TSS-1kb) over all transcripts in async. cells; bar = mean, whiskers = quartiles, $p < 0.001$. **(D)** Pearson correlation coefficient of the FPKM of each transcript in a given replicate versus all others; n = library replicates.

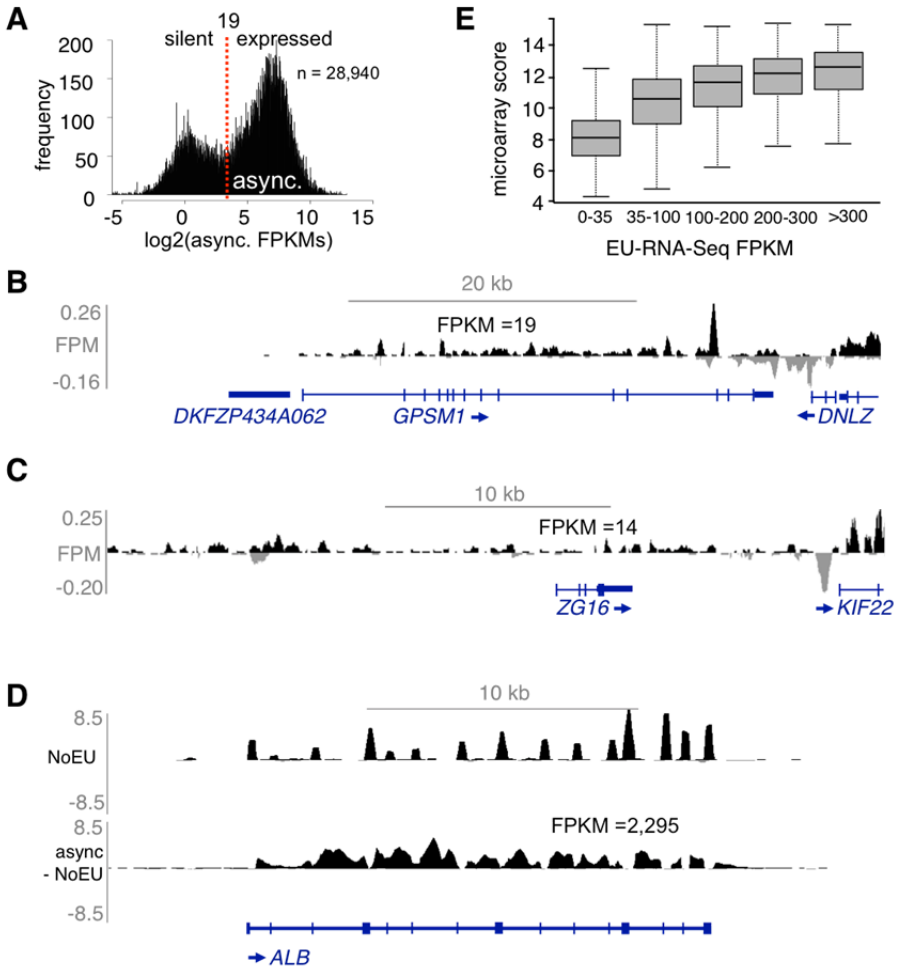


Fig. S4. Nascent transcripts are reliably detected above FPKM 19. **(A)** Log2 histogram of spike-in-normalized FPKMs for all transcripts in asynchronous cells; y-axis = number of transcripts, x-axis = log2(FPKM) values, n = transcripts above cutoff. **(B)** Browser shot of *GPSM1*, a transcript with an FPKM of 19 in async. **(C)** Browser shot of *ZG16*, a transcript with an FPKM of 14 in async. **(D)** Upper track; browser view of a highly-abundant mRNA that is non-specifically pulled down during EU-RNA-Seq. Lower track, same gene displayed after NoEU subtraction. **(E)** Microarray expression score of 27,000 transcripts grouped by their NoEU-normalized FPKM in EU-RNA-seq for asynchronous HUH7, whiskers = upper and lower quartiles.

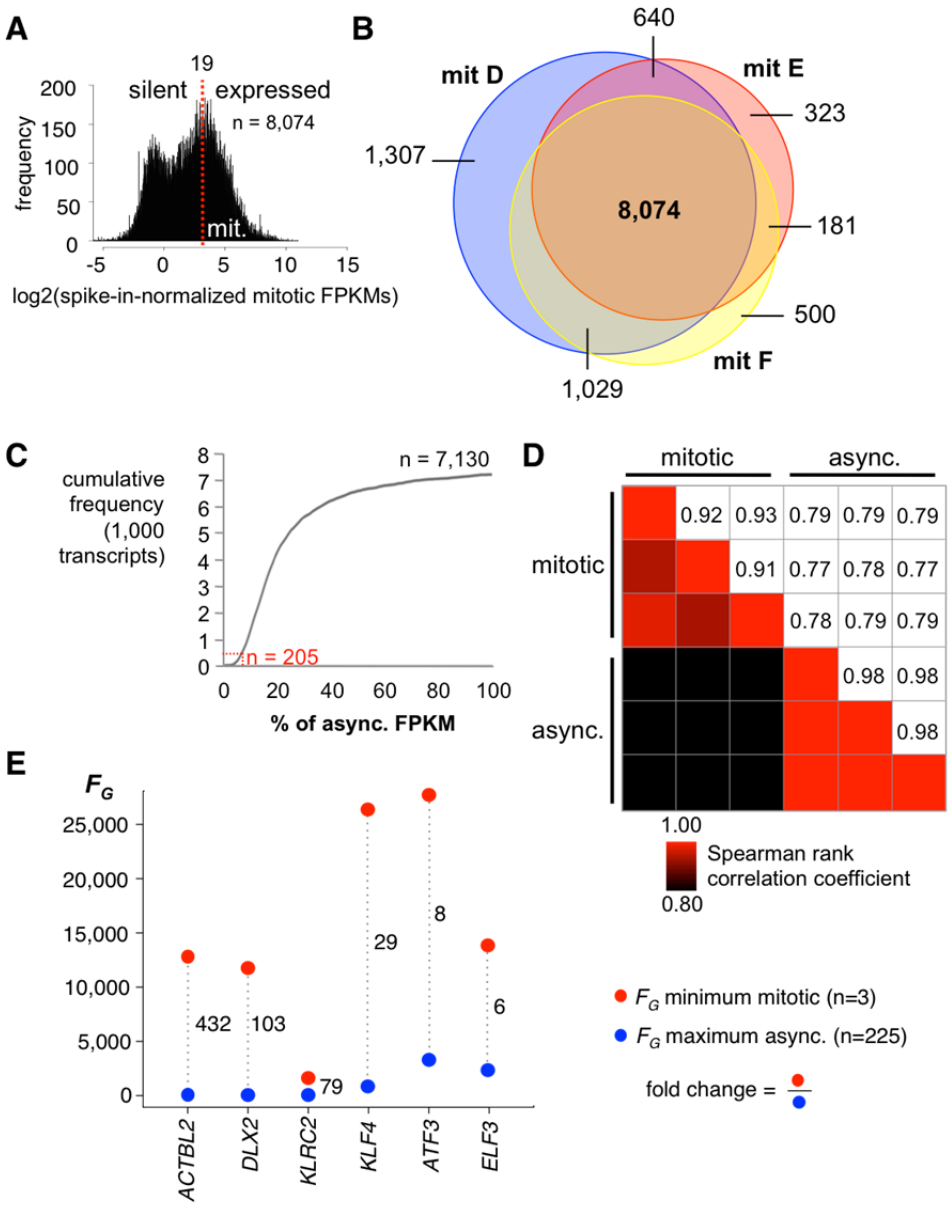


Fig. S5. Global mitotic transcription in HUH7 cells is not the results of contaminating interphase cells. **(A)** Log2 histogram of spike-in-normalized FPKMs for all transcripts in mitotic cells; y-axis = number of transcripts, x-axis = log2(FPKM) values, n = transcripts above cutoff. **(B)** Overlap of 12,054 mitotically-expressed transcripts as they occur in each spike-in-normalized mitotic replicate. **(C)** The cumulative frequency of genes expressed in mitosis as a percentage of their async. expression level, up to 100% (n=7,335 of 8,074 mitotically-expressed transcripts).

(D) Spearman rank correlation coefficient between replicates for all transcripts with an FPKM ≥ 19 in mitosis (0') after spike-in normalization. **(E)** Examples of genes whose minimum expression level (F_G = FPM of a given gene) in the mitotic samples (n=3 EU-RNA-Seq replicates) is higher than the maximum expression level (F_G) among our async. EU-RNA-Seq triplicates, as well as 222 adult human RNA-Seq studies (n=225 non-mitotic studies), when co-aligned and normalized to sequencing depth; the numbers indicate the fold difference between the mitotic and non-mitotic F_G values; i.e. how many times higher the gene would have to be expressed in our mitotic samples than the maximum non-mitotic expression level seen, if the signal was due to contaminating interphase cells.

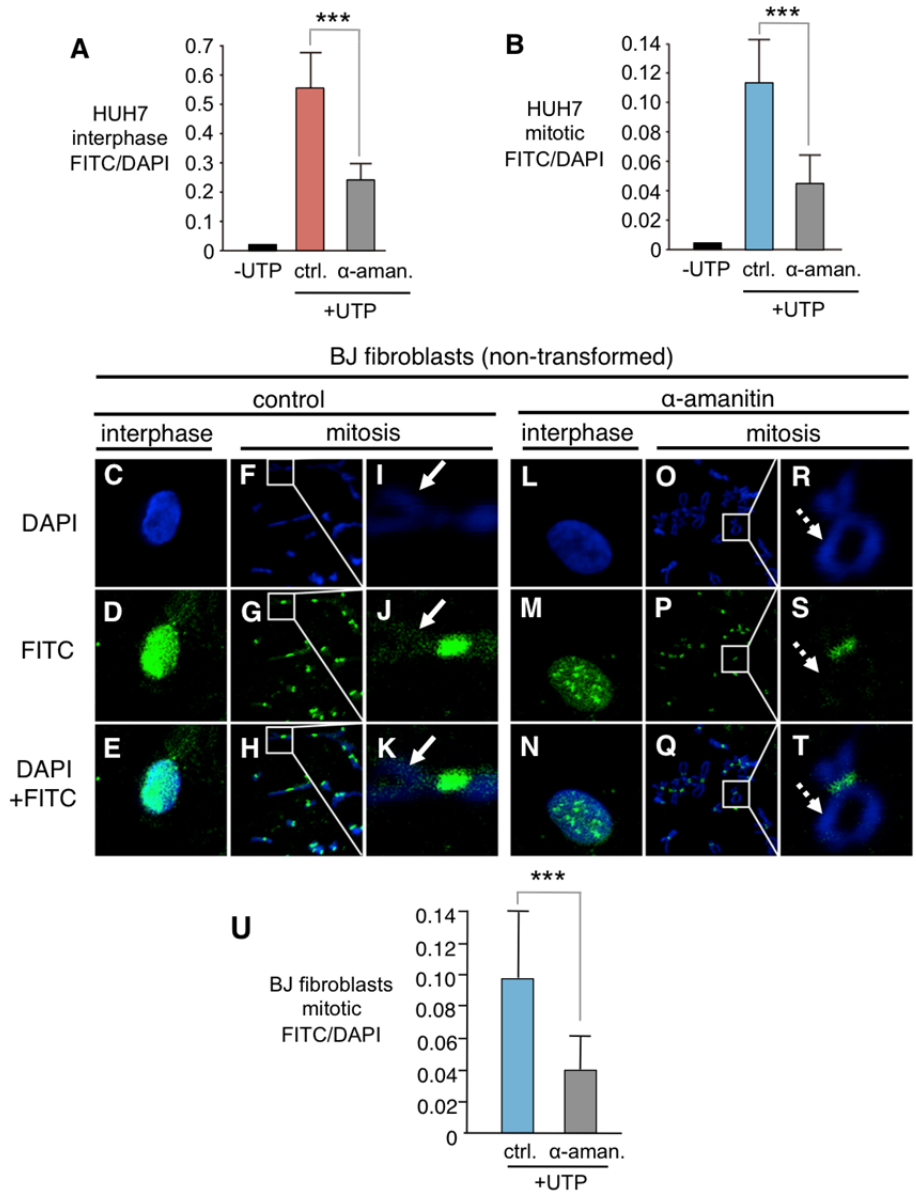
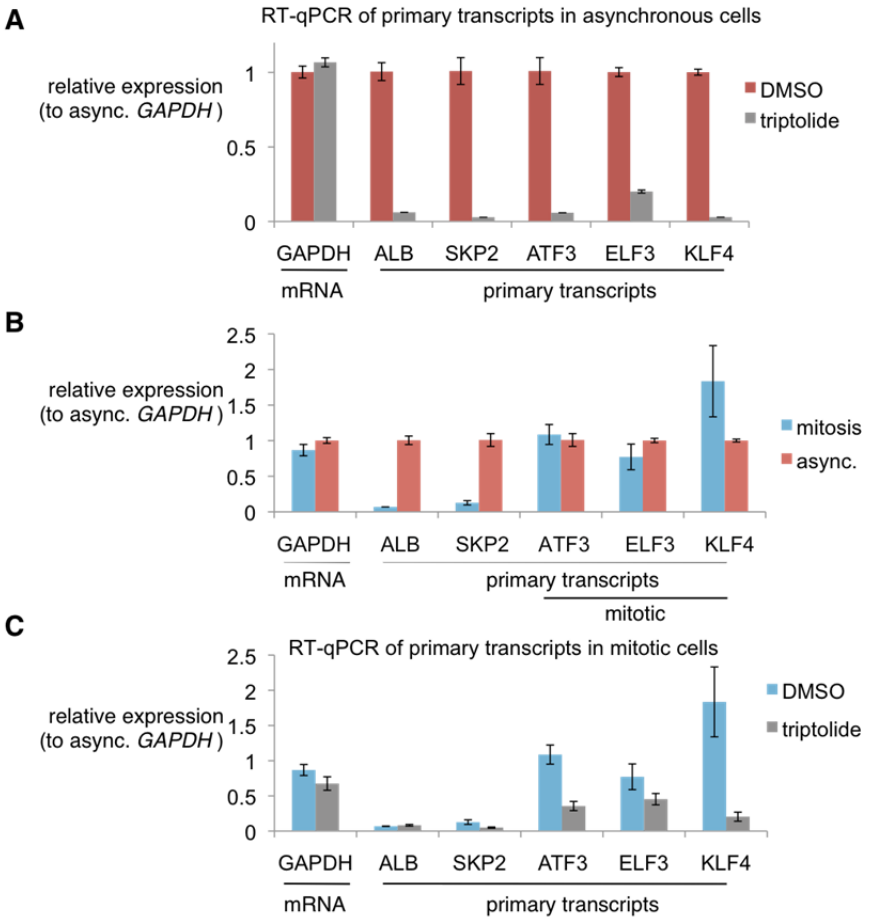


Fig. S6. Mitotic transcription occurs in HUH7 hepatoma and nontransformed BJ fibroblast cells. **(A)** Quantification of FITC-UTP signal over DAPI, with and without UTP or α-amanitin in interphase HUH7 cells; n = 8 nuclei for each condition, error bars = standard error of the mean, $p < 0.001$. **(B)** Quantification of FITC-UTP signal over DAPI, with and without UTP or α-amanitin in metaphase HUH7 cells; n = 40 chromosome arms (5 per cell) for each condition, error bars = standard error of the mean, $p < 0.001$. Interphase **(C-E)** or mitotic **(F-K)** BJ fibroblast cells labeled with FITC-UTP; white box magnified in **(I-K).** Interphase **(L-N)** or mitotic **(O-T)** cells

treated with α-amanitin and labeled with FITC-UTP; white box magnified in **(R-T). (U)** Quantification of FITC-UTP signal over DAPI, with and without α-amanitin in metaphase BJ fibroblasts; n = 40 chromosome arms (5 per cell) for each condition, error bars = standard error of the mean, $p < 0.001$.



D

	stage	cells with ≥1 colocalization	total cells	p-value
KLF4	mitosis	6	43	0.027
	interphase	44	134	2.98e-07
ATF3	mitosis	6	49	0.011
	interphase	24	110	9.94e-07
ELF3	mitosis	10	55	0.002
	interphase	49	105	3.55e-12

Fig. S7. Nascent transcript quantification in mitotic cells. **(A)** Async. cells were treated with either DMSO or triptolide; error bars = standard error of the mean for 3 replicates. **(B)** Relative expression in mitotic and async. cells; error bars = standard error of the mean for 3 replicates. **(C)** Mitotic cells were treated with either DMSO or triptolide; error bars = standard error of the mean for 3 replicates. **(D)** Quantification of chromatin-associated colocalization events per cell.

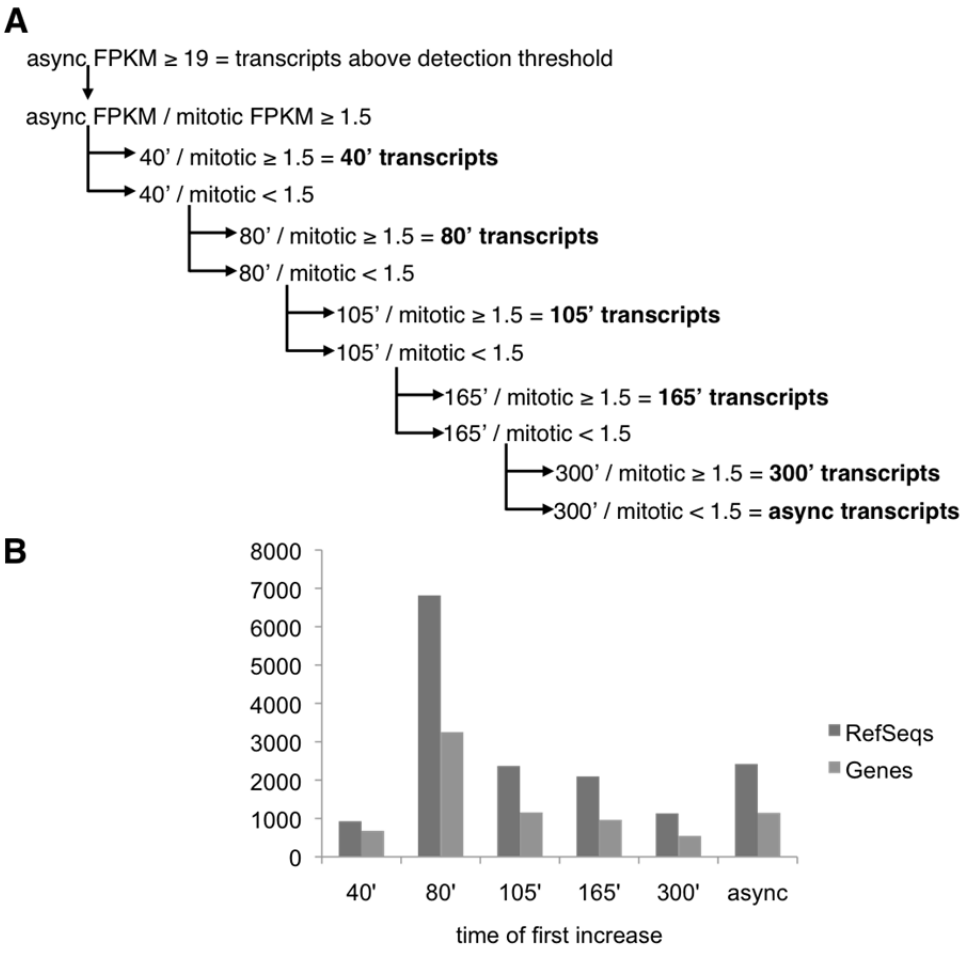


Fig. S8. Identification of transcripts by time of first increase over mitosis (0'). **(A)** Filtering for transcripts that first increase at each time point. **(B)** Quantification of transcripts coming up at each time point.

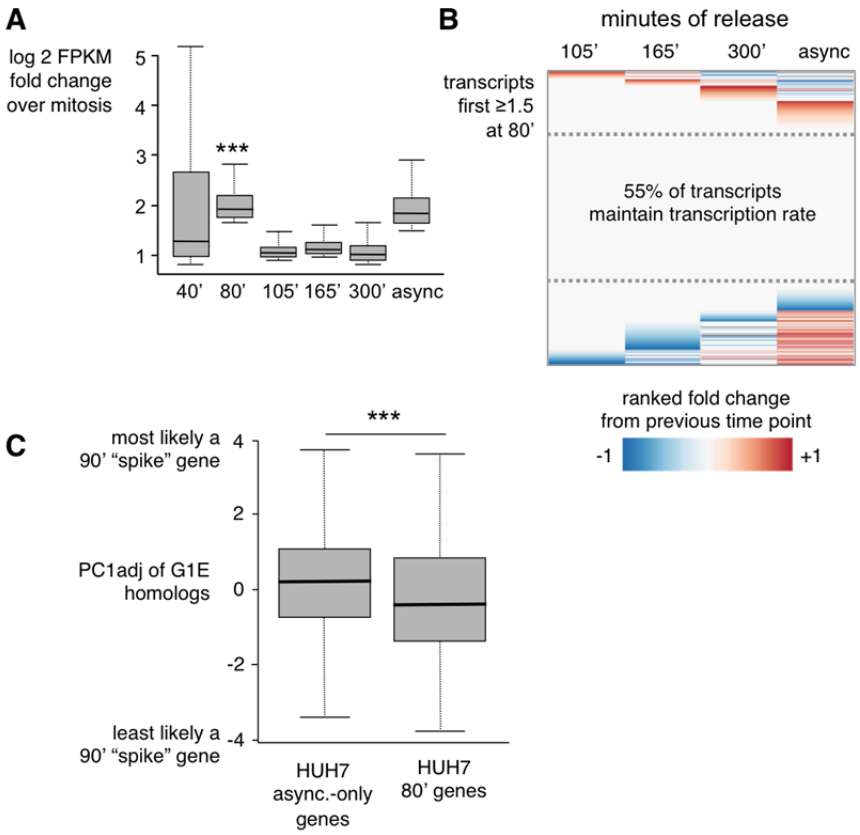


Fig. S9. The 80 min burst is similar to the spike seen at 90 min in murine erythroblasts. **(A)** Amplitude of log2 fold change for transcripts first increased at each time point; bar = mean, whiskers = quartiles, $p<0.001$, n = top 400 transcripts by fold change. **(B)** Ranked fold change from previous time point for each transcript that was first activated at 80' in HUH7 cells. **(C)** The projection onto the first principle component of homologs to murine erythroblast genes that first increase at 80' or are only in the asynchronous HUH7 cells; bar = mean, whiskers = quartiles, $p < 0.001$.

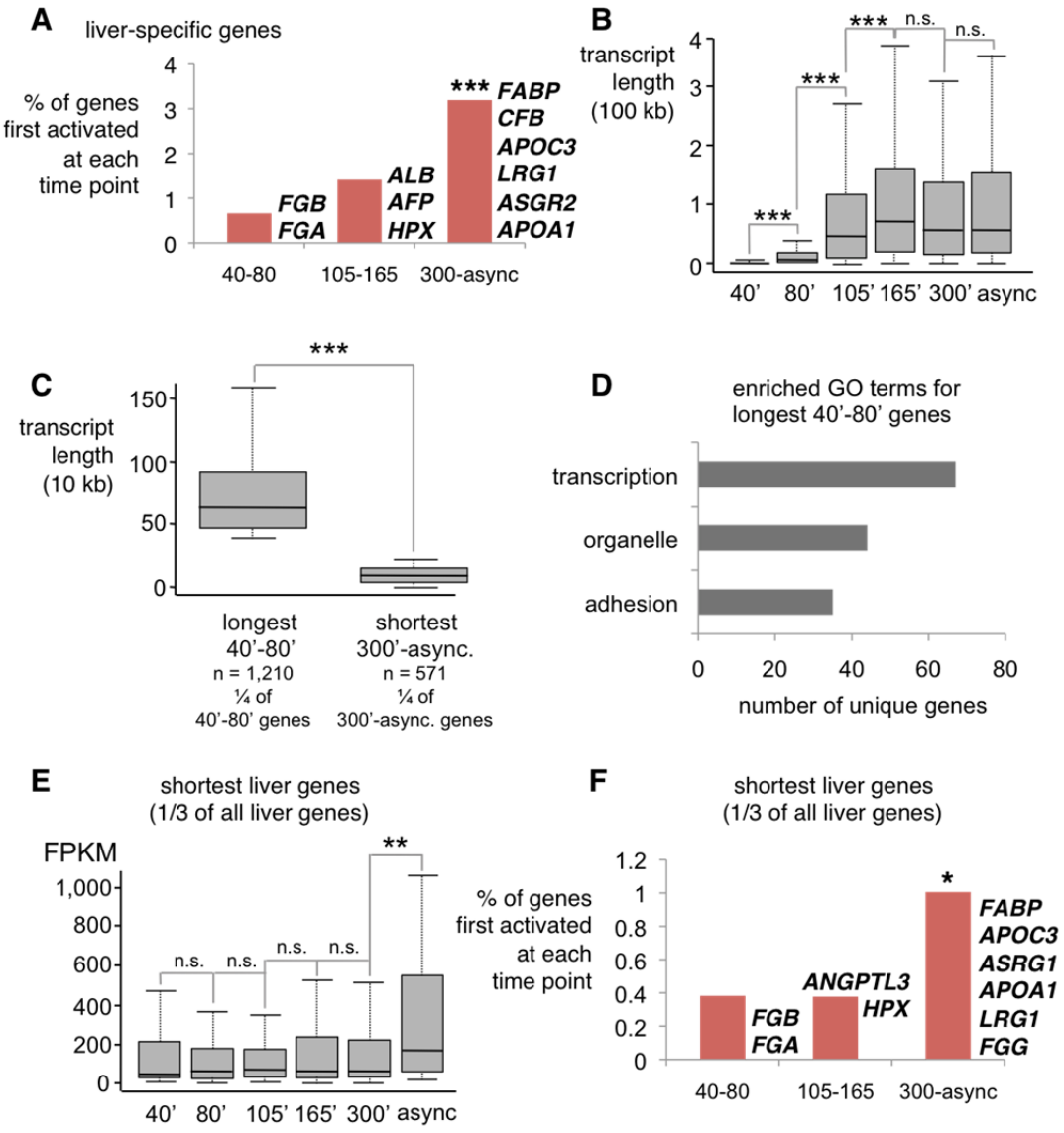


Fig. S10. Genes involved in basic cell functions are prioritized over cell type-specific genes, independent of transcript length. **(A)** Proportion of the liver-specific genes that are expressed in HUH7 cells and come up in each category; representative genes are indicated, p = 0.002. **(B)** Length of transcripts that first increase at the indicated time points; bar = mean, whiskers = quartiles, p<0.001, n = top 400 transcripts by fold change. **(C)** Length of longest (upper quartile) 40'-80' genes and shortest (lower quartile) 300'-async. genes; bar = mean, whiskers = quartiles, p<0.001. **(D)** Representative GO categories for the longest (upper quartile) 40-80' genes. **(E)**

FPKMs of the shortest third of liver-specific genes at all time points; bar = mean, whiskers = quartiles, $p < 0.01$, n=149. **(F)** Proportion of the shortest third of liver-specific genes, that are expressed in HUH7 cells and come up in each category; representative genes are indicated, $p = 0.04$, n=50.

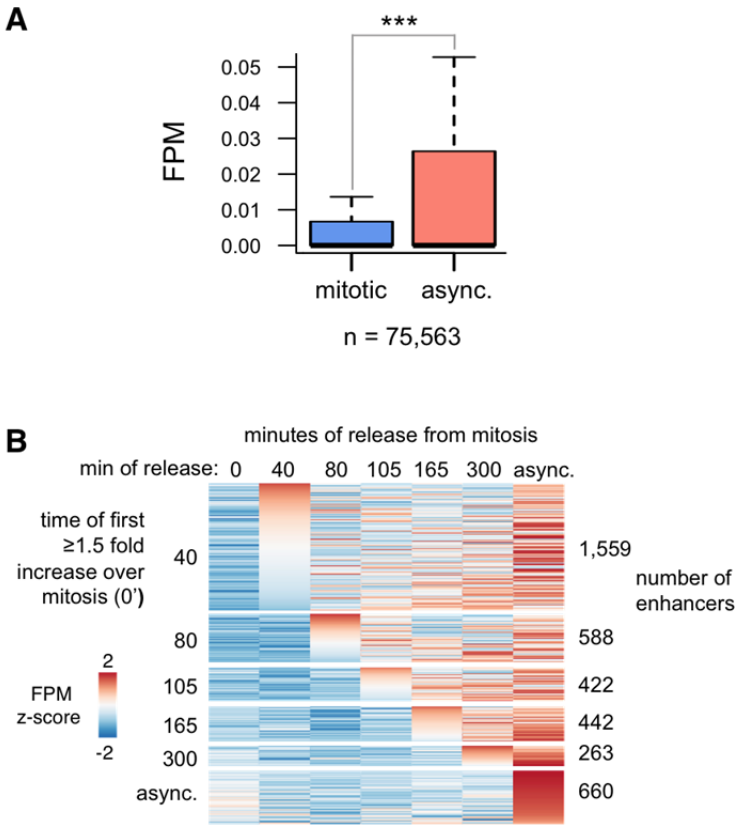


Fig. S11. Dynamics of eRNA expression during mitotic exit. **(A)** Mitotic and async. FPMs of all intergenic enhancers detected in async. HUH7 cells; bar = mean, whiskers = quartiles, n=75,563, $p<0.001$. **(B)** Z-score of eRNAs (rows) that first increase $\geq$1.5 fold over mitosis at each time point (columns).

Table S1. hg19 Read Alignment for All Replicates.

	sample	reads mapped	unique reads mapped	% unique
expt 1	mitotic D	55,701,154	12,206,269	21.91
with spike-ins	mitotic E	67,256,034	14,283,543	21.24
	mitotic F	65,939,315	11,421,202	17.32
	async. D	77,000,901	39,275,347	51.01
	async. E	74,217,968	37,885,317	51.05
	async. F	83,076,849	35,901,940	43.22
	NoEU D	82,506,504	14,377,407	17.43
	NoEU E	74,576,940	13,166,001	17.65
expt 2	mitotic A	68,234,896	27,073,185	39.68
	mitotic B	117,334,773	15,998,843	13.64
	mitotic C	48,474,467	31,021,487	64.00
	40' A	80,314,006	28,997,931	36.11
	40' B	65,871,959	24,450,535	37.12
	40' C	55,212,231	31,758,132	57.52
	80' A	77,150,021	45,652,578	59.17
	80' B	56,311,863	27,352,822	48.57
	80' C	56,077,423	37,444,954	66.77
	105' A	59,383,990	39,846,242	67.10
	105' B	65,445,101	37,845,826	57.83
	105' C	49,695,448	35,502,507	71.44
	165' A	64,855,877	48,918,739	75.43
	165' B	56,458,158	35,732,674	63.29
	165' C	63,152,860	44,927,278	71.14
	300' A	70,540,752	48,757,474	69.12
	300' B	57,138,674	37,100,634	64.93
	300' C	49,853,577	37,090,935	74.40
	async. A	80,493,213	55,909,443	69.46
	async. B	62,372,008	42,905,105	68.79
	async. C	51,533,721	39,565,970	76.78
	NoEU A	20,205,165	2,263,937	11.20
	NoEU B	72,340,450	6,906,360	9.55
	NoEU C	53,188,902	6,275,858	11.80

Table S2. Spike-in control #1 alignment data.

5e-4 ug control #1 (hg19 coordinates: chr7:110258409-110258798)

Library ID	reads aligned to genome	reads per million (RPM)	reads aligned to control locus	RPM-normalized reads aligned to control locus
NoEU D	69,519,012	0.0144	2,224	31.9912
NoEU E	62,926,590	0.0159	2,521	40.0626
mit. D	47,089,339	0.0212	850	18.0508
mit. E	56,296,832	0.0178	781	13.8729
mit. F	55,434,245	0.0180	957	17.2637
async. D	63,367,256	0.0158	199	3.1404
async. E	60,669,456	0.0165	187	3.0823
async. F	67,509,870	0.0148	129	1.9108

The number of reads aligned to control locus #1 were normalized for sequencing depth in each replicate that received spike-ins.

Table S3. Spike-in control #2 alignment data.

5e-4 ug control #2 (hg19 coordinates: chr13:107098286-107098585)

Library ID	reads aligned to genome	reads per million (RPM)	reads aligned to control locus	RPM-normalized reads aligned to control locus
NoEU D	69,519,012	0.0144	22,286	320.5742
NoEU E	62,926,590	0.0159	27,182	431.9637
mit. D	47,089,339	0.0212	7,030	149.2907
mit. E	56,296,832	0.0178	10,520	186.8666
mit. F	55,434,245	0.0180	9,026	162.8235
async. D	63,367,256	0.0158	2,104	33.7714
async. E	60,669,456	0.0165	1,742	28.7130
async. F	67,509,870	0.0148	1,607	23.8039

The number of reads aligned to control locus #1 were normalized for sequencing depth in each replicate that received spike-ins.

Table S4. Spike-in scalars.

Library ID	control avg	spike-in scalar
mit. D	16.49	5.90
mit. E	16.28	5.83
mit. F	16.77	6.00
async. D	3.26	1.17
async. E	2.98	1.07
async. F	2.15	0.77

The average of RPM-normalized reads mapped for 5e-5 ug of control #1 and 1/10 of the RPM-normalized reads mapped for 5e-4 ug of control #2 was calculated for each library. This value pwas then divided by the average for the async. libraries to give the spike-in scalar.

Table S9. Mitotically-enriched genes do not represent another cell cycle stage.

hESC FUCCI Cluster	Peak Expression	Secondary Expression	Mitotically-Enriched (EU-RNA-Seq)	Enrichment	p-value
1	Late G1	S		0.00	1
2	G2	S	*HSPA1A*	0.01	1
3	S	Late G1	*TOR2A, F2RL2, MTRNR2L8*	0.08	1
4	S	G2		0.00	1
5	Early G1	Late G1	*PGAM4*	0.02	1
6	Late G1	Early G1	*LOC643387, DLX2*	0.03	1
7	G2	Early G1	*CDK2AP2*	0.02	1
8	S	Early G1		0.00	1
9	Late G1	G2		0.00	1
10	G2	Late G1		0.00	1

Genes in each cell cycle phase cluster *(23)* were compared to those that are enriched in mitosis by EU-RNA-Seq following spike-in normalization; p = 1.