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## Mitotic Transcriptional Activation: Clearance of Actively Engaged Pol II Via Transcriptional Elongation Control in Mitosis

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### Summary

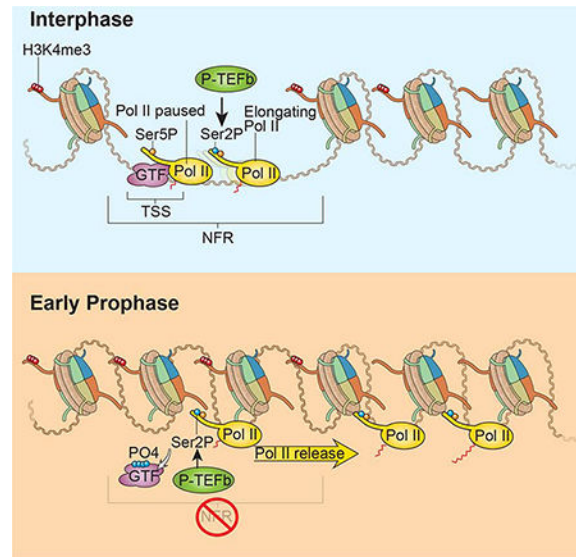
Although it is established that some general transcription factors are inactivated at mitosis, many details of Mitotic Inhibition of Transcription (MIT) and its underlying mechanisms are largely unknown. We have identified Mitotic Transcriptional Activation (MTA) as a key regulatory step to control transcription in mitosis for genes with transcriptionally engaged RNA Polymerase II (Pol II) to activate and transcribe until the end of the gene to clear Pol II from mitotic chromatin, followed by global impairment of transcription reinitiation through MIT. Global nascent RNA sequencing and RNA fluorescence in situ hybridization demonstrate the existence of transcriptionally engaged Pol II in early mitosis. Both genetic and chemical inhibition of P-TEFb in mitosis lead to delays in the progression of cell division. Together, our study reveals a mechanism for MTA and MIT whereby transcriptionally engaged Pol II can progress into productive elongation and finish transcription to allow proper cellular division.

### Graphical abstract

How transcription is shut down as cells begin to condense chromosomes during mitosis is poorly understood. Liang et al. report the requirement of Mitotic Transcriptional Activation by P-TEFb to release paused Pol II as a prerequisite for this process, and ultimately for proper cell cycle progression.

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## Introduction

In mitotically dividing cells, replicated chromatin needs to be separated into two daughter cells. It has long been recognized that mitotic chromosomes are highly compacted, physically excluding the transcription machinery and leading to transcriptional repression (Parsons and Spencer, 1997; Prescott and Bender, 1962). Numerous studies have suggested that most transcription factors are displaced from mitotic chromosomes (Gottesfeld and Forbes, 1997; Martinez-Balbas et al., 1995), while others remain as mitotic bookmarks for subsequent rapid gene activation (Kadauke and Blobel, 2013; Kadauke et al., 2012). However, a fundamental unanswered question is how the transition from active transcription to transcriptional repression at the beginning of mitosis is achieved. Mitotic Inhibition of Transcription (MIT) could be as the result of phosphorylation of the general transcription factors TFIID and TFIIB, which can prevent transcription reinitiation (Akoulitchev and Reinberg, 1998; Long et al., 1998; Segil et al., 1996). However, the DNA:RNA:Pol II complex is extremely stable, raising the question of the fate of Pol II that is already initiated as cells enter mitosis (Gottesfeld and Forbes, 1997; Henriques et al., 2013).

The transcription cycle for RNA Pol II begins with the initiation of transcription through the binding of general transcription factors and RNA polymerase II (Pol II). Their recruitment depends on the eviction of nucleosomes around the Transcription Start Site (TSS) to generate a Nucleosome-free Region (NFR) (Orphanides et al., 1996). In metazoans, initiation of Pol II is typically followed by transient promoter-proximal pausing 20-60 nt downstream of the TSS (Fuda et al., 2009). This pausing involves DRB-sensitivity inducing factor (DSIF), negative elongation factor (NELF) and the serine 5 phosphorylated (Ser5P) C-terminal Domain (CTD) form of the Pol II largest subunit RPB1 (Adelman and Lis, 2012; Jonkers and Lis, 2015; Kwak et al., 2013)(Smith and Shilatifard 2013). The paused Pol II elongation complex is proposed to serve as a temporal window for the recruitment of additional transcription factors and to allow well-regulated gene expression (Boettiger and Levine, 2009; Gilchrist et al., 2010; Henriques et al., 2013)(Smith and Shilatifard 2013). The

transition from stalling to productive elongation requires the recruitment of Cyclin-dependent Kinase 9 (CDK9) containing Positive Transcription Elongation Factor  $\beta$  (P-TEFb) (Marshall et al., 1996), which phosphorylates DSIF, NELF, and the RPB1 CTD tail at serine 2, leading to the removal of NELF and switching DSIF from a negative to a positive elongation factor (Lin et al., 2010; Peterlin and Price, 2006)(Smith and Shilatifard 2013). Accordingly, blocking of PTEFb kinase activity with the CDK9 inhibitor flavopiridol inhibits release of paused Pol II into productive elongation (Rahl et al., 2010), but has no effect on elongating Pol II that is already in gene bodies (Jonkers et al., 2014).

The extremely stable nature of engaged, promoter-proximal paused Pol II has been demonstrated both *in vitro* (Kireeva et al., 2000; Wuarin and Schibler, 1994) and *in vivo* (Chen et al., 2015; Henriques et al., 2013; Jonkers et al., 2014). Since metaphase chromosomes lack Pol II complexes, any preexisting paused and elongating Pol II complexes must be removed at an early phase of mitosis, such as prophase or prometaphase. However, detailed mechanisms for Pol II removal in early mitosis remain largely unknown. Studies by Price and colleagues (Jiang et al., 2004) demonstrated that the transcription termination factor 2 (TTF2) could mediate premature termination of Pol II complexes. Gdown1, which has dual functions in inhibiting Pol II termination by counteracting TTF2 and by blocking elongation stimulation by TFIIF (Cheng et al., 2012; Jishage et al., 2012), is predominately inactivated by phosphorylation in mitosis (Guo et al., 2014). Gdown1 phosphorylation decreases the inhibitory effects toward TTF2 and TFIIF (Guo et al., 2014). Therefore, transcriptionally engaged Pol II complexes may be terminated by increased TTF2 activity in mitosis. However, Pol II has been shown to be active at the kinetochore, producing centromeric satellite transcripts during mitosis (Chan et al., 2012), arguing against a general termination mechanism at mitosis. Alternatively, preexisting paused Pol II may transition to productive elongation, and these and other elongating Pol II could continue transcription to the natural Transcription End Site (TES).

In this study, we find a P-TEFb-dependent Mitotic Transcriptional Activation (MTA) for the release of paused Pol II into productive elongation in cells arrested at prophase/prometaphase. Using nascent RNA sequencing, we also demonstrate the existence of nascent transcription throughout gene bodies at the beginning of mitosis, suggesting that as cells enter mitosis, genes with transcriptionally engaged Pol II can continue transcription until the 3'-end of the gene. RNA fluorescence in situ hybridization (FISH) of individual genes *HSPA8* and *ACTG1* confirms the existence of nascent transcription. Chemical and mutational inhibition of MTA through P-TEFb's kinase activity delays cell cycle progression, indicating functional consequences of blocking MTA at early mitosis.

## Results

### Genome-wide analysis of Mitotic Inhibition of Transcription in synchronized cells

To study MIT in mitosis, we performed histone H3K4 trimethylation (H3K4me3) and TFIIB ChIP-Seq in asynchronous and synchronized HeLa cells. We arrested human HeLa cells with a double thymidine block followed by nocodazole treatment and shake-off (Figure 1A) (Whitfield et al., 2002). This method results in a highly pure population of early mitotic cells (prophase/prometaphase) (Feng et al., 2011) as assessed by anti-histone H3 phosphorylated-

serine 10 (phH3S10) (Figure S1A), 5-ethynyl-2'-deoxyuridine (EdU), and 7-Aminoactinomycin D (7-AAD) staining (Figure 1B). ChIP-Seq of H3K4me3 and TFIIB demonstrates that H3K4me3 is still present on the mitotic chromosomes while TFIIB occupancy is dramatically reduced (Figures 1C and 1D), although bulk TFIIB protein levels are not decreased during early mitosis (Figure S1A). Genome-wide analyses demonstrate that a shift of H3K4me3 occupancy to the NFRs, and loss of TFIIB at these regions in mitotic cells (Figures 1E and 1F). Loss of NFRs was confirmed by micrococcal nuclease digestion and qPCR for Heat Shock 70kDa Protein 8 (*HSPA8*) and gamma actin (*ACTG1*) genes (Figures 1G and 1H). Loss of the NFR was even observed for the *PLK1* gene, which a prior study suggested was maintained in mitosis (Kelly et al., 2010) (Figure S1B and S1C). Our studies are also consistent with recent studies demonstrating broad reductions in DNase I hypersensitive sites in mitotically arrest cells (Hsiung et al., 2015; Kadauke and Blobel, 2013). Together, these results provide genome-wide evidence that general transcription factors are detached from mitotic chromatin, and that transcription reinitiation is generally impaired during early mitosis through MIT.

### The P-TEFb inhibitor flavopiridol blocks Mitotic Transcriptional Activation at the onset of mitosis

To determine the fate of Pol II that was transcriptionally engaged at the onset of mitosis, nocodazole-arrested cells were treated with the P-TEFb inhibitor flavopiridol for 1 hour before harvesting, in order to block the transition from promoter-proximal pausing to productive elongation (Figure 1A). Flavopiridol treatment does not affect the purity of the harvested mitotic cells as determined by phH3S10 and DNA content analysis (Figure S2A). Compared to asynchronous cells, the bulk level of Ser5P Pol II is normally decreased in mitosis but is somewhat restored by flavopiridol treatment, while total Pol II is unchanged (Figure S2B). We therefore performed ChIP-Seq with a Pol II antibody directed against the N-terminus of RBP1 (N20) in flavopiridol-treated asynchronous and mitotic cells. Genome browser track examples of *ACTG1* and *HSPA8* genes show that flavopiridol results in an increase in Pol II upstream of the TSS and a dramatic decrease of Pol II occupancy in gene bodies in asynchronous cells (Figures 2A and 2B). These findings are consistent with previous findings that flavopiridol specifically inhibits the release of promoter-proximal paused Pol II (Rahl et al., 2010) but not elongating Pol II (Jonkers et al., 2014; Rahl et al., 2010).

In synchronized mitotic cells, Pol II levels are extremely low at *HSPA8* and *ACTG1* promoters and gene bodies (Figures 2A and 2B), consistent with a general inhibition of transcription initiation. However, pretreatment with flavopiridol results in a Pol II binding profile similar to flavopiridol-treated asynchronous cells (Figures 2A and 2B). Heatmap analysis of Pol II occupancy demonstrates that flavopiridol induces a global retention of Pol II at the promoter regions in early mitosis (Figures 2C). As seen with asynchronous cells, flavopiridol also increases the pausing index in mitotic cells (Fig 2D). Pretreatment with flavopiridol can result in our ability to detect promoter-proximal paused Pol II because during the flavopiridol treatment there are still a proportion of cells that have not yet rounded up, and are therefore at an earlier stage where most Pol II has not yet been cleared from the promoter-proximal region. Together, our data indicate that the clearance of Pol II

from promoter-proximal via MTA at the onset of mitosis requires the kinase activity of P-TEFb.

### Presence of Pol II and transcription factors at G2/M transition

To profile Pol II occupancy at an earlier stage, we arrested HeLa cells with the CDK1 inhibitor RO-3306, which led to the arrest of most cells (more than 90%) at the G2/M transition (Vassilev et al., 2006) (Figure 3A). The arrested cells were used for total Pol II and Ser5P Pol II ChIP-Seq. Comparable occupancies of Pol II and Ser5P Pol II are observed at the G2/M transition and in asynchronous cells (Figures 3B, 3C and S3A). Furthermore, G2/M cells still preserve the NFR and TFIIB occupancy as seen in asynchronous cells (Figures 3D and S3B-D). These data support the idea that early mitosis is a temporal window for Pol II clearance through elongation in a P-TEFb-dependent process.

### Nascent RNA transcription in early mitotic cells

In addition to our studies of Pol II occupancy and the pattern of chromatin and its modifications, we wanted to further characterize the genome-wide existence of continued transcription itself at early mitosis. Therefore, we isolated chromatin from asynchronous and nocodazole-arrested mitotic cells as described in the experimental procedures and in Figure S4A (Khodor et al., 2011). Western blotting with Pol II N20 antibody shows that there are lower levels of Pol II associated with mitotic chromatin compared to asynchronous chromatin, but the Pol II found on mitotic chromatin is hyperphosphorylated on Serine 2 of the CTD, indicative of elongating Pol II (Figures S4B and S4C).

We isolated nascent RNA from the mitotic and asynchronous chromatin and depleted ribosomal RNA and any contaminating polyadenylated mRNA. We observed that the nascent RNA transcripts from mitotic cells are on average shorter than the RNA transcripts from asynchronous cells (Figure 4A). Although yields per cell of nascent RNA are less for arrested mitotic cells, the same amount of nascent RNA (500 ng) from arrested and asynchronous cells was used for library preparation and Illumina sequencing. Nascent transcripts at genes such as Septin 2 (*SEPT2*), *ACTG1* and *HSPA8* are relatively enriched in early mitosis (Figures 4B, 4C and 4D, blue box). However, for some genes such as *SEPT2*, the short antisense transcripts (Pelechano and Steinmetz, 2013) found at the promoter are dramatically decreased in mitosis (Figure 4B, green box). We also observed that the enhancer RNA transcripts (Natoli and Andrau, 2012) (Figures 4C and 4D, yellow boxes) are also decreased in early mitosis. These profiles of nascent RNA transcripts are consistent with the loss of transcriptional reinitiation during mitosis.

The nascent RNA isolated from mitotic cells has some unique features consistent with the shorter average RNA length of mitotic nascent RNA. For example, genes expressed in mitotic cells have enhanced exon marking as compared to asynchronous cells, indicating higher rates of RNA processing (Figures 4B, 4C and 4D, blue boxes). Higher splicing rates of mitotic nascent transcripts could result from the presence of similar levels of the splicing machinery in asynchronous and mitotic cells but fewer nascent transcripts for them to process during early mitosis. Nascent transcript sequencing also shows that some genes have

more reads towards the 3' end of genes in mitotic cells than asynchronous cells (Figure 4B, blue box).

We performed genome-wide data analysis to identify high-confidence genes for which nascent transcripts are enriched in mitotic chromatin compared to asynchronous chromatin ( $\log_2(\text{fold change}) > 1$ ,  $\log_2(\text{r.p.m.}) > 4$  and  $p < 1\text{E-}10$ ). We identified 870 high-confidence enriched genes, and for comparison 542 underrepresented genes in early mitosis ( $\log_2(\text{fold change}) < -1$ ,  $\log_2(\text{r.p.m.}) > 4$  and  $p < 1\text{E-}10$ ) (Figure 4E, Supplementary Tables 1 and 2). Interestingly, some of these enriched genes have a bias of RNA-Seq reads toward the 3' end (Figure 4F). This 3' bias could reflect higher ratios of decapping factors and 5'-3' exonucleases to nascent transcripts in mitotic cells, analogous to the enhanced splicing of introns observed for nascent transcripts in early mitotic cells. Genes whose expression is enriched in mitotic compared to asynchronous cells are significantly shorter than genes with unchanged expression or significantly lower expression in mitotic cells (Fig. S4D).

Comparing Pol II occupancy at these genes in asynchronous cells demonstrates that the genes with higher expression in mitotic cells generally also have higher levels of Pol II in asynchronous cells (Fig S4E). Gene ontology (GO) analysis of mitotically enriched genes finds some enrichment for genes involved in translation, protein folding, mRNA catabolic process and nucleotide binding. High expression levels correlate with shorter genes (Castillo-Davis et al., 2002), all of these characteristics are consistent with the nascent transcription on mitotic chromatin representing highly expressed genes that are finishing transcription as cells enter mitosis. To test if the nascent transcription in early mitosis was due to defects specific to nocodazole treatment, we arrested HeLa cells with S-Trityl-L-cysteine (STLC), which is an Eg-5 inhibitor that blocks mitotic progression without affecting microtubule dynamics (Skoufias et al., 2006). Nascent transcripts from STLC-arrested HeLa cells display similar features as those seen in nocodazole-arrested mitotic cells (Figures S4F and S4G). To determine if the nascent transcription was unique to HeLa cells, we arrested HCT116 cells in early mitosis with a double thymidine block and nocodazole treatment. Nascent transcripts from HCT116 mitotic cells have similar properties as seen in nocodazole and STLC-treated HeLa cells (Figures S4F and S4G).

### Flavopiridol blocks nascent transcription in individual prophase cells

To visualize nascent transcription in individual mitotic cells, we designed Stellaris RNA fluorescence in situ hybridization (FISH) probes (Raj et al., 2008) against two enriched genes, *ACTG1* and *HSPA8*. Each probe set consists of 48 different fluorescently labeled (Quasar 670 Dye) oligonucleotides that are biased toward the 3' end of the target gene (Figure S5A). The multiple labeled oligonucleotides independently hybridize along the target transcript to collectively generate a bright signal at the site of transcription. Simultaneous RNA FISH and immunofluorescence with a pH3S10 antibody was performed in HeLa cells with or without flavopiridol treatment for 1 hour. In interphase cells, we could detect very bright FISH spots (1-5) with *ACTG1* and *HSPA8* probes in the nucleus (Figures 5A and 5B). These spots are much stronger than cytoplasmic RNA transcripts, indicating that they likely represent sites of multiple nascent transcripts. These bright spots are also easily detected in early prophase cells marked by high levels of H3S10 phosphorylation (Figures 5A and 5B). Furthermore, 3D reconstructions confirm that these



transcripts are nuclear (Supplementary Movies 1 and 2). The nascent transcription signal dramatically decreases or disappears once the chromatin is fully condensed, suggesting that Pol II has been fully cleared from these genes by the end of prophase (Figures 5A and 5B).

Treatment of cells with flavopiridol inhibits the nascent transcription in both interphase and prophase cells, indicating that the prophase nascent transcription is canonical Pol II transcription (Figures 5A and 5B). To determine if the prophase nascent transcription was specific to HeLa cells, we also performed RNA FISH for *ACTG1* in the diploid human fibroblast cell line BJ-5ta. We detected one to two spots of nascent transcripts in BJ-5ta prophase cells, and the nascent transcription was abolished by flavopiridol treatment as well (Figure S5B). Quantification of the FISH studies demonstrates that most prophase cells have nascent transcripts (Figure 5C).

### A requirement for Mitotic Transcriptional Activation (MTA) for cycle progression

We also investigated the consequences of flavopiridol treatment on further cell cycle progression after release from the nocodazole block. Since long treatments with nocodazole can adversely affect the kinetics of cell cycle progression, HeLa cells were arrested with a double thymidine block and released for 8 hours before nocodazole treatment. After 4 hours in nocodazole, the mitotic cells were collected every 30 minutes by mitotic shake off for a total of 6 hours nocodazole treatment. The mitotic cells were released from nocodazole for 90 min in the presence of DMSO or flavopiridol (Figure 6A). Flow cytometry analysis with propidium iodide staining demonstrates that flavopiridol substantially decreases the G0/G1 population in a dose-dependent manner (Figure 6B).

To exclude the potential off-target of flavopiridol on other cyclin-dependent kinases needed for progression through the cell cycle, we generated PP1 analog sensitive HEK293 cells (Blethrow et al., 2008; Lopez et al., 2014). First, we established a tetracycline-inducible Flag-CDK9 mutant (F103G) stable cell line in Flp-In T-REx 293 cells. We used an shRNA targeting the 3'-UTR of CDK9 mRNA to knockdown endogenous CDK9. Knockdown of endogenous CDK9 and overexpression of mutant Flag-CDK9 was confirmed by western blotting (Figure 6C). We also generated a wild-type Flag-CDK9 stable cell line as a negative control (Figure 6D). Since 293 cells are not very sensitive to nocodazole arrest, control or PP1 analog-sensitive cells were arrested with RO-3306 for 24 hours. Cells were released into early prophase for 15 min before treatment with DMSO or 1-NA-PP1 for 150 min. FACS analysis demonstrates that treatment with 1-NA-PP1 resulted in a decreased G0/G1 population in a dose-dependent manner (Figure 6E). As a control, 1-NA-PP1 did not affect wild-type Flag-CDK9 cells (Figures 6F).

We also examined the effects of flavopiridol on chromosome segregation during mitosis. We used a low concentration of flavopiridol (250 nM) to treat the RO-3306-arrested G2/M cells. 1 hour after RO-3306 washout, we crosslinked the cells and visualized the chromosomes with DAPI staining. Flavopiridol treatment increases the incidence of chromosome segregation defects (Figure S6A-B), including a possible lagging chromosomes, anaphase bridges, and misaligned chromosomes (off plate) in metaphase cells ( $p=0.0008$  for anaphase defects,  $p=1.3E-6$  for off plate metaphase). Collectively, these results indicate that Mitotic

Inhibition of Transcription (MIT) and Mitotic Transcriptional Activation (MTA) through P-TEFb-mediated Pol II clearance may be necessary for the execution of proper cell division.

## Discussion

Here, we report that paused Pol II and elongation complexes are enriched on the promoters and gene bodies at the G2/M border and that the majority of Pol II complexes are cleared from mitotic chromatin at the beginning of mitosis through Mitotic Transcriptional Activation (Figures 2 and 3). Therefore, the early stages of mitosis, presumably prophase, function as a temporal window for MTA for Pol II clearance from mitotic chromatin. Inhibition of P-TEFb by flavopiridol pretreatment in mitotic cells results in a global retention of Pol II at the promoter regions. Based on the cell cycle progression defects upon inhibition of P-TEFb with flavopiridol in HeLa cells or inhibition of an analog-sensitive CDK9 in 293T cells, this mitotic transcriptional clearance is likely to be essential.

Evidence for ongoing transcription as cells enter mitosis can be found in prior studies. Shermoen and O'Farrell (Shermoen and O'Farrell, 1991) proposed that the 78 kilobase long *ubx* gene may use premature termination to abort Pol II in pre-cellular blastoderm *Drosophila* embryos, as the time needed to be transcribed by Pol II was longer than the cell cycle. By performing in situ hybridization, *ubx* nascent transcripts were seen to decrease in prophase and disappear later in mitosis; similar to what we observe in our FISH studies of prophase arrested HeLa cells. More recent studies (Bothma et al., 2014; Garcia et al., 2013; Lucas et al., 2013) in precellular blastoderm *Drosophila* embryos with the real-time nascent RNA detection system (MS2 system) demonstrated that *hb* and *eve* genes have intermittent bursts of transcription between mitotic cycles 10-12. The bursts are synchronous and occur just before cells enter mitosis. Transcript levels of *hb* and *eve* decrease in prophase and are gone by metaphase. The *hb* and *eve* genes were previously shown to pre-loaded with Pol II at this pre-MBT stage (Chen et al., 2013), suggesting that the intermittent bursts in nascent transcription may be generated by the release of engaged Pol II followed by Pol II clearance.

The transcription termination factor TTF2 was previously proposed to mediate clearance of Pol II (Guo et al., 2014; Jiang et al., 2004). Since TTF2 can terminate Pol II complexes regardless of the phosphorylation state in vitro, it was assumed that TTF2 was involved in the premature termination of promoter-proximal paused Pol II. Based on our studies, we propose that paused Pol II must first be released by P-TEFb before the Pol II can be terminated by TTF2. Furthermore, the observed 3' bias in nascent RNA-Seq is consistent with decapping and 5'-3' exonucleases aiding in the termination of Pol II by TTF2 (Brannan et al., 2012). Since nascent mitotic transcripts also exhibit enhanced exon marking, it is possible that the overall lower level of transcription in mitosis results in more RNA processing factors being available for splicing and termination.

As cells enter mitosis, TTF2 levels are upregulated and TTF2 is transported into the nucleus (Jiang et al., 2004). Although knockdown of TTF2 leads to mitotic defects, potential roles for TTF2 in DNA repair could not be ruled out, as DNA damage could have accumulated during the RNAi treatment (Jiang et al., 2004). Our finding that the inhibition of P-TEFb delays mitotic cell division and increases the incidence of chromosome segregation defects,



is consistent with proper mitotic progression requiring TTF2's termination activity, albeit downstream of Pol II release from the paused state. Given that protein translation is globally inactivated by phosphorylation at mitosis (Pyronnet et al., 2001), and that the nascent transcripts show a 3' end bias in mitosis, the mitotic nascent transcripts are less likely to affect cell division and chromosome segregation through a requirement to be translated into functional proteins. However, we cannot exclude the possibility that these mitotic nascent transcripts may affect chromosome segregation and cell division through other unknown mechanisms. This includes the possibility that inhibition of CDK9 kinase activity may affect the Pol II-dependent Shugoshin loading at centromeres (Chan et al., 2012; Liu et al., 2015).

Based on our findings, we propose a model as shown in Figure 6G. In interphase cells, Pol II is generally paused at the promoter regions and P-TEFb is required for productive Pol II elongation. At the onset of mitosis, Mitotic Activation of Transcription and Mitotic Inhibition of Transcription set in with the release of paused Pol II, the loss of NFRs and displacement of general transcription factors. Genes with actively engaged Pol II can continue transcription until the end of the gene, effectively clearing Pol II complexes from mitotic chromatin. Clearance of Pol II complexes could facilitate cell cycle progression and prevent the rapid re-activation of most genes after mitosis, which might reset the subsequent daughter cells into a basal state. Pol II clearance would ensure a requirement for ongoing cell signaling to maintain a previous expression profile upon mitotic exit. Therefore, understanding the regulatory mechanisms by which MTA and MIT regulate activation of release of paused Pol II and its clearance and inhibition of transcriptional initiation at the onset of mitosis has broad implications for studies of cell proliferation and differentiation during development. Expression of specific genes during mitosis through MTA should also be considered and further molecular studies are required for a detailed understanding of this process.

## Experimental Procedures

### Cell Lines and Cell Culture

HeLa and HCT116 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Flag-CDK9 and PP1 analogy sensitive Flag-CDK9-AS (F103G) stable cell lines were generated by insertion of pCDNA5 cDNA plasmids into 293 Flp-in-TRex cells. The human foreskin fibroblast cell line, BJ-5ta (ATCC® CRL4001™) was a gift from Rong Li (Stowers Institute for Medical Research) and maintained in a 4:1 mixture of DMEM and Medium 199 containing 10% FBS. Details of cell cycle synchronization and drug treatment are included in Supplemental Experimental Procedures.

### Antibodies

Histone H3, H3K4me1, H3K4me2, H3K4me3 and CDK9 polyclonal antibodies were generated in our lab. The Supplemental Experimental Procedures contains details for the commercial antibodies used in our study, including Pol II (N20), Pol II Ser5P (3E8), Ser2P (3E10), Ser7P (4E12), alpha tubulin, phospho-Histone 3 serine 10 (phH3S10), TFIIB (C18) and secondary antibodies.

## Flow Cytometry

Asynchronous and arrested cells were fixed and stained with anti-phospho-Histone 3 and 7-AAD or propidium iodide (PI). To label the S phase cells, asynchronous and arrested cells were pulsed with EdU and stained with Click-iT plus kit (Life Technologies). For mitotic cell release, the cells were stained with PI for cell cycle analysis. See Supplemental Experimental Procedures for details.

## ChIP-Seq Analysis

$5 \times 10^7$  cells were used for ChIP assay according to a published protocol (Lee et al., 2006). Libraries were prepared with Illumina's ChIP-Seq sample prep kit for the next-generation sequencing. ChIP-Seq reads were aligned to the human genome (UCSC hg19). Alignments were processed by Bowtie version 1.0.0 (Langmead et al., 2009), allowing only uniquely mapping reads with up to two mismatches within the entire length of the read. Details of ChIP-Seq data analysis are included in Supplemental Experimental Procedures.

## Chromatin Isolation and Nascent RNA-Seq

Chromatin fraction from HeLa or HCT116 cells were purified as described (Khodor et al., 2011) and dissolved in TRIzol reagent for RNA extraction. The extracted RNA was poly-A depleted with Oligo(dT) magnetic beads (Invitrogen) and treated with DNase I (NEB) to get rid of the mRNA and genomic DNA contamination. After Ribo-zero treatment, library preparation was performed with TruSeq Stranded Total RNA with Ribo-Zero Gold kit. Nascent RNA-Seq reads were aligned to the human genome (UCSC hg19) using the Tophat aligner. See Supplemental Experimental Procedures for details.

## Nucleosome Occupancy Assay

MNase digestions and mononucleosomal DNA purification were performed as previously described (Cui and Zhao, 2012). QPCR was performed with different sets of primers around the promoter regions. The primer sets were normalized with HeLa genomic DNA as different primer sets may have different amplification efficiencies. See Supplemental Experimental Procedures for details.

## Simultaneous Stellaris FISH and Immunofluorescence

The Simultaneous Stellaris FISH and Immunofluorescence were performed as described (Raj et al., 2008). The cells were incubated with specific Stellaris FISH probes and anti-phospho-Histone 3 Serine 10 antibody. After wash, the cells were stained with DAPI. With a  $100\times$  1.4 NA Plan-apochromatic objective, images were acquired with a CSU-X1 Yokogawa disc on a Perkin-Elmer Ultraview system. Signal was detected with a C9100 Hamamatsu Photonics EM-CCD. To characterize of chromosome segregation defects, synchronized HeLa cells were released for 90 min and stained with anti-alpha tubulin antibody and DAPI. See Supplemental Experimental Procedures for details.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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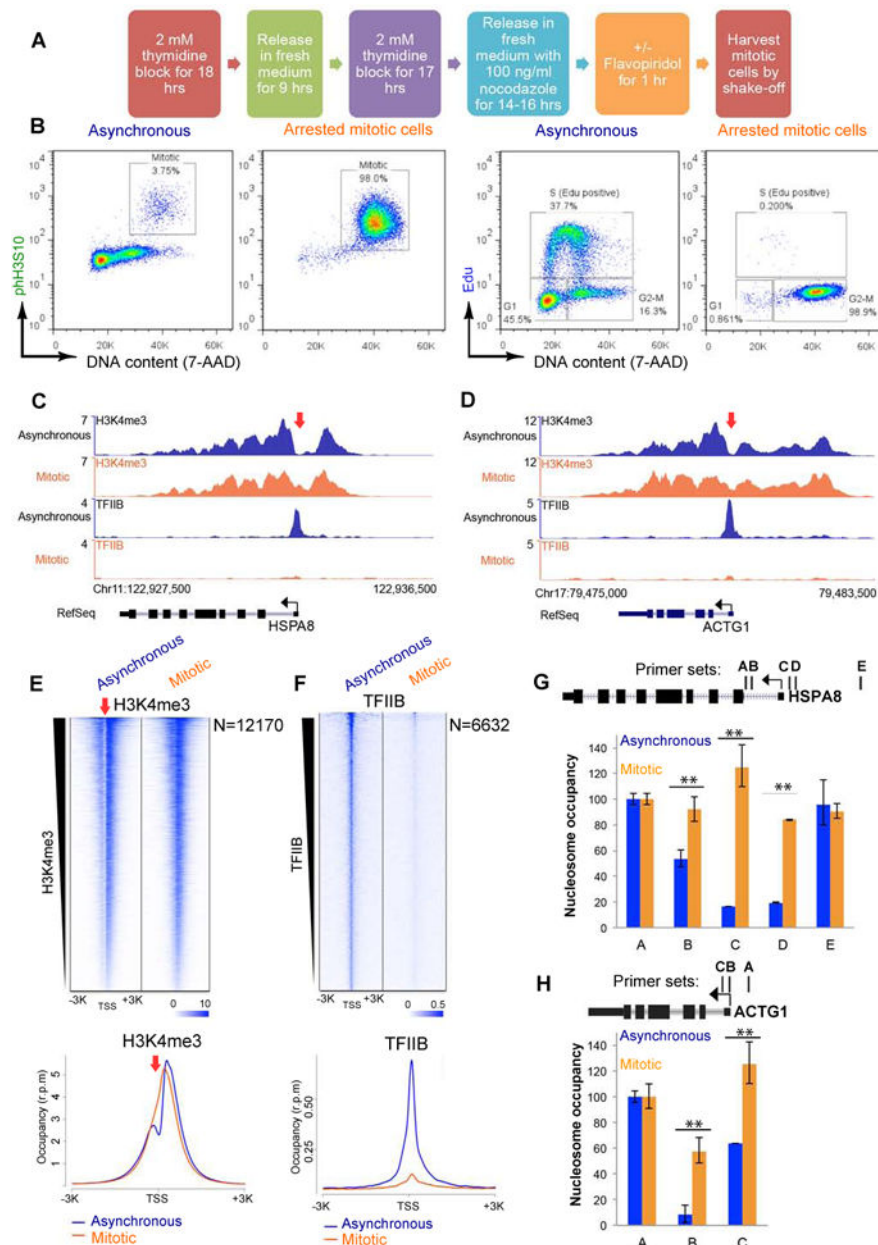
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**Highlights**

1. Mitotic Inhibition of Transcription occurs in early mitosis.
2. P-TEFb is required for Mitotic Transcriptional Activation and release of paused Pol II.
3. Nascent RNA-Seq and RNA FISH reveal active transcription at the onset of mitosis.
4. Inhibition of Mitotic Transcriptional Activation delays cell cycle progression.

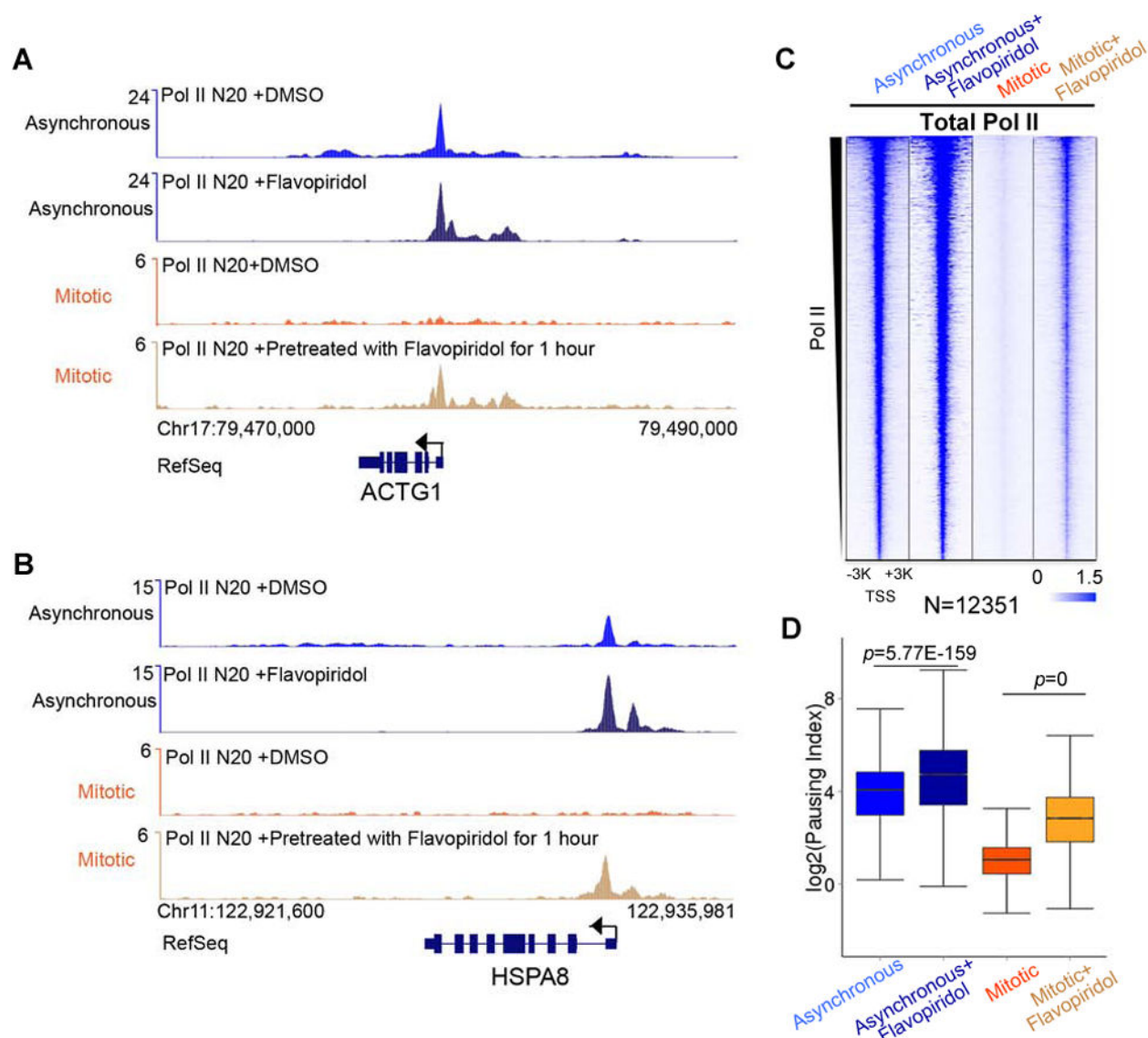




**Figure 1. Genome-wide analysis of Mitotic Inhibition of Transcription in early mitosis as revealed by histone H3K4me3 and TFIIB ChIP-Seq**

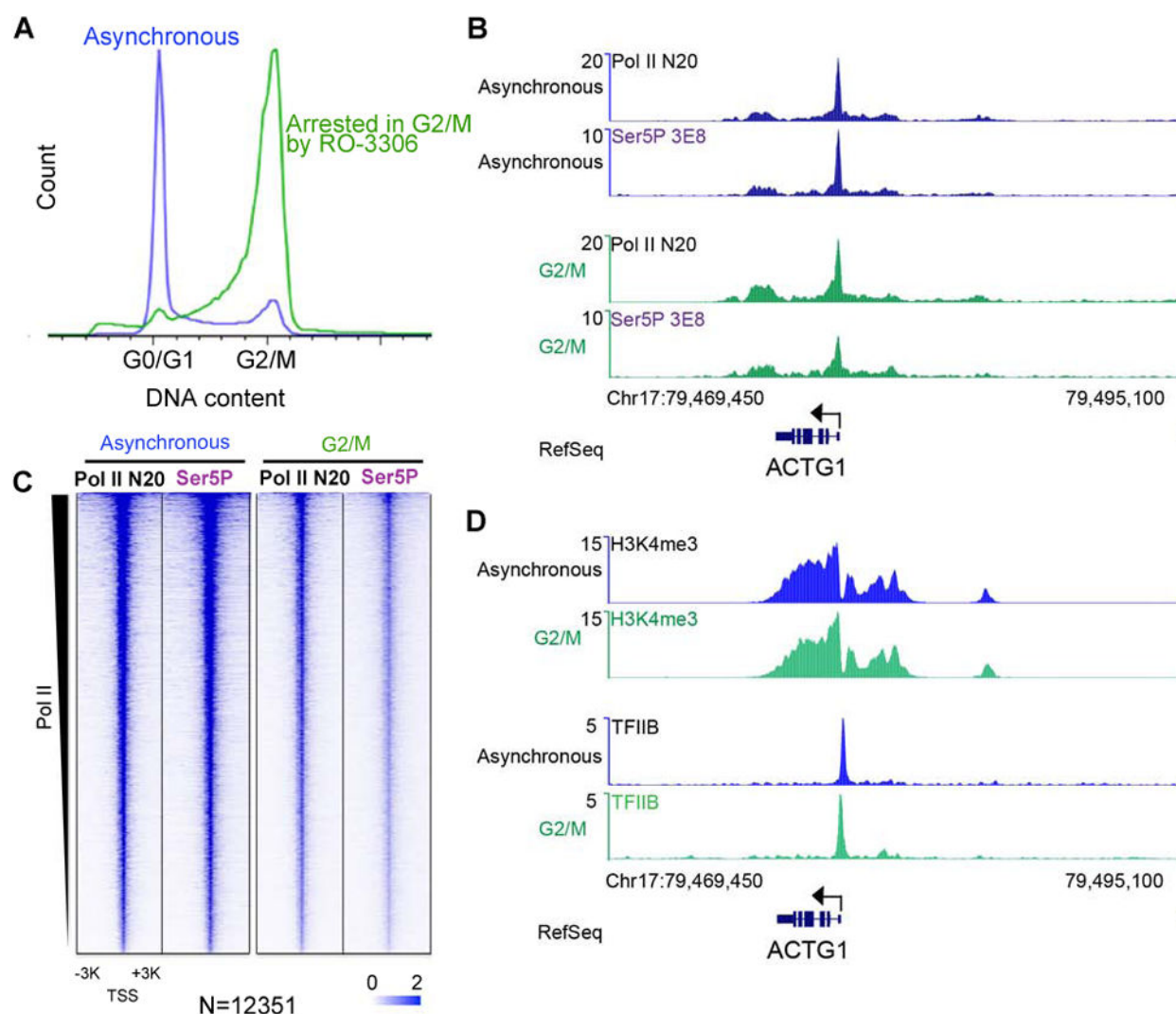
(A) Schematic of thymidine and nocodazole treatments used to obtain early mitotic cells (prophase/prometaphase). (B) Left panels, flow cytometry analysis of asynchronous and nocodazole-arrested HeLa cells with anti-phH3S10 antibody and 7-Aminoactinomycin D (7-AAD) staining (DNA content). Right panels, asynchronous and arrested HeLa cells were also pulsed with EdU for 1 hour and labeled with Click-iT (Life Technologies). Gates for G1, S and G2/M phase cells are indicated. (C-D) The nucleosome free regions (NFRs) at transcription start sites (TSSs) observed in asynchronous cells are occupied by nucleosomes bearing H3K4me3 in mitotic cells. General transcription factor TFIIB occupancy is also dramatically reduced in synchronized HeLa mitotic cells. *HSPA8* (C) and *ACTG1* (D) genes

are shown as examples with the red arrows indicating the NFR and the TFIIB occupied regions. (E) Upper panel, heat map representation of H3K4me3 occupancy around the promoter regions of protein coding and long non-coding RNA genes within a  $\pm 3$  kb window. 12,170 genes with H3K4me3 are ordered by H3K4me3 occupancy and centered at the TSS. Bottom panel, average gene representation of H3K4me3 signals for the same 12,170 genes shows that the NFR seen in asynchronous cells is lost in mitotic cells. Compared to asynchronous cells, the peak of H3K4me3 shifts toward upstream in mitotic cells (representative example of two biological replicates). (F) Upper panel, TFIIB occupancy at 6,632 genes is globally decreased in early mitosis. TFIIB enrichment is shown within 3 kb of TSS and ordered according to the TFIIB occupancy in asynchronous cells. Bottom panel, metagene analysis of TFIIB occupancy at the promoter regions in HeLa mitotic cells for the same 6,632 genes (representative example of two biological replicates). Red arrows indicate the locations of the NFR and the TFIIB occupied regions. (G-H) Mnase-qPCR analysis of nucleosome occupancy at the promoter regions of *HSPA8* and *ACTG1*. Asynchronous and mitotic cells were fixed and digested with micrococcal nuclease (Cui and Zhao, 2012) and isolated DNA was used for qPCR with primers sets flanking the promoter regions. The primer sets were normalized using genomic DNA to control for any difference in amplification efficiency. The data are represented as Mean $\pm$ S.D. (n=3, nucleosome occupancy with primer A sets were normalized to 100%). \*\*,  $p < 0.005$  (unpaired two-sample t-test). See also Figure S1.



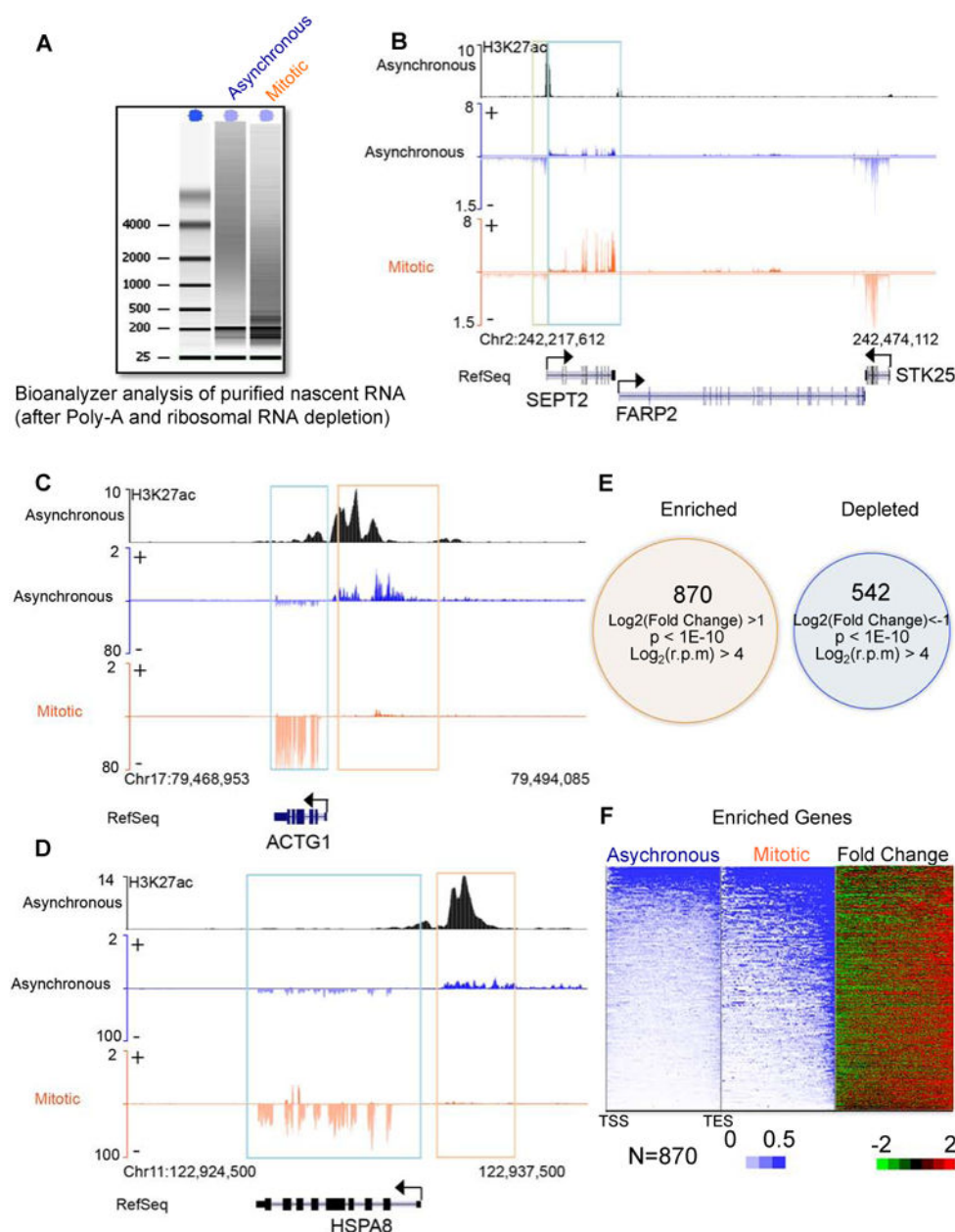
**Figure 2. The P-TEFb inhibitor flavopiridol prevents Mitotic Transcriptional Activation by inhibition of the release of promoter-proximal Pol II in early mitosis**

(A-B) Genome browser track examples of total Pol II (N20, against RPB1 N-terminal) at *ACTG1* and *HSPA8* loci in asynchronous and nocodazole-arrested HeLa cells. The cells are pre-treated with 1  $\mu$ M flavopiridol or DMSO for 1 hour before harvesting. (C) Heatmap analysis of Pol II coverage changes after flavopiridol treatment in asynchronous and mitotic cells as labeled on the top. Genes are sorted by Pol II enrichment in asynchronous cells (representative of two biological replicates). (D) Comparison of pausing indexes in the DMSO or flavopiridol treated asynchronous and mitotic HeLa cells. The pausing indexes were calculated as the ratio of Pol II reads at the TSS  $\pm 250$  bp over the reads in the gene body. See also Figure S2.



**Figure 3. Pol II and transcriptional factors at G2/M transition**

(A) DNA content analysis of asynchronous and RO-3306-arrested G2/M cells (Vassilev et al., 2006) by propidium iodide staining. (B) Distribution of total Pol II and Ser5P (clone 3E8) Pol II on *ACTG1* gene in asynchronous and CDK1 inhibitor RO-3306-arrested cells at G2/M transition (Vassilev et al., 2006). (C) Total Pol II and Ser5P Pol II binding profiles in asynchronous and G2/M transition cells are shown within 3 kb of the center of TSS. Genes are sorted by total Pol II enrichment in asynchronous cells (representative of two biological replicates). (D) Distribution of H3K4me3 and TFIIB on *ACTG1* promoter at G2/M transition. See also Figure S3.



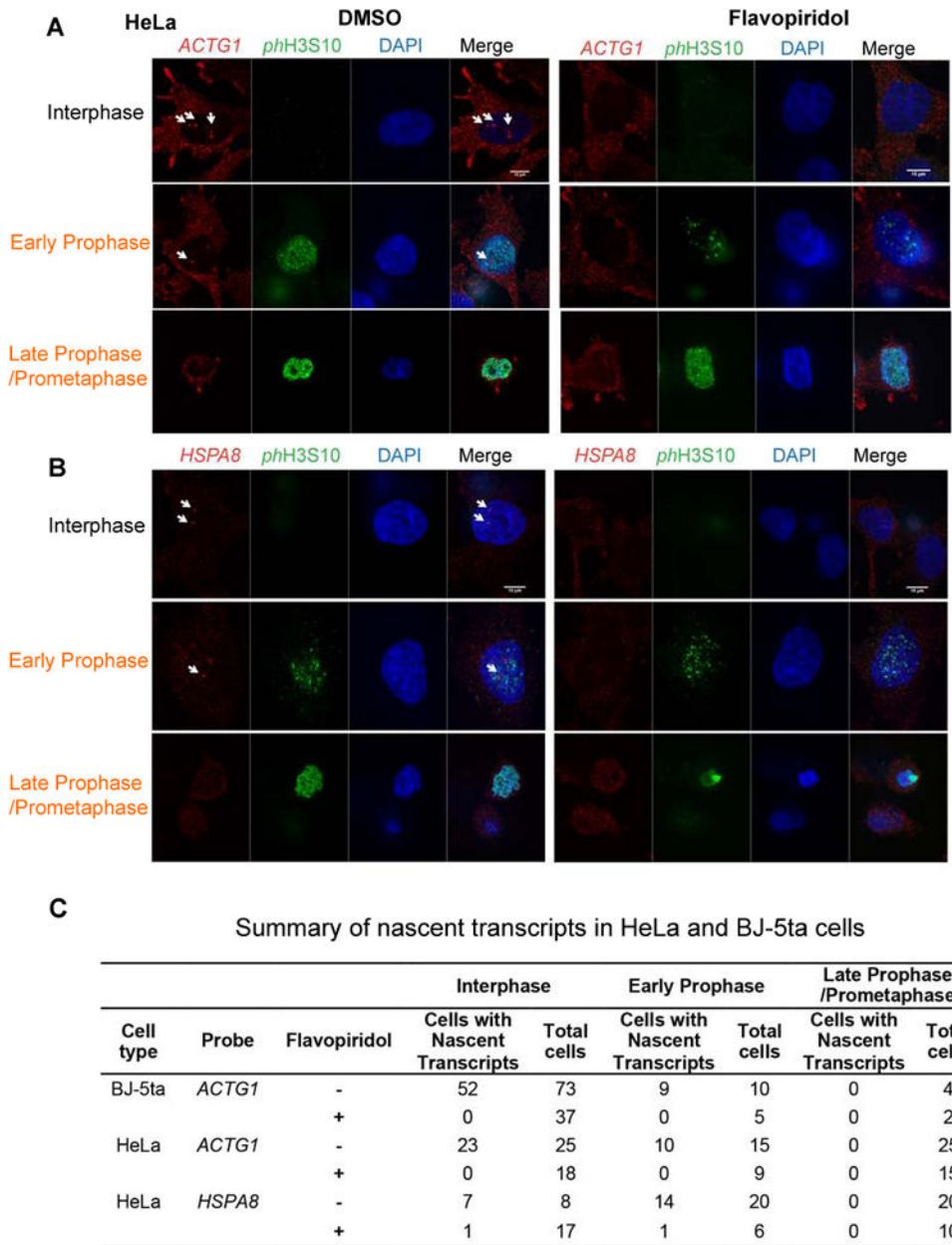
**Figure 4. Nascent RNA sequencing of transcripts in early mitotic cells**

(A) Bioanalyzer analysis of nascent RNA isolated from asynchronous and nocodazole-arrested HeLa mitotic cells. Chromatin associated RNA is depleted of rRNA and polyA+ RNA. The nascent RNA transcripts from mitotic cells are on average shorter than the RNA transcripts from asynchronous cells. (B) Genome browser tracks of nascent RNA sequencing at the *SEPT2* gene and adjacent region in asynchronous and mitotic cells. *SEPT2* (as highlighted in blue box) shows enriched nascent RNA transcripts. The short antisense transcripts found at *SEPT2* promoter are lost in mitosis (green box). (C and D) Track examples of nascent transcripts at the *ACTG1* and *HSPA8* genes. *ACTG1* and *HSPA8* (blue box) have enriched nascent RNA transcripts. H3K27ac is used to mark the active enhancers (ChIP-Seq data from ENCODE, GEO accession: GSE29611). The enhancer RNA transcripts

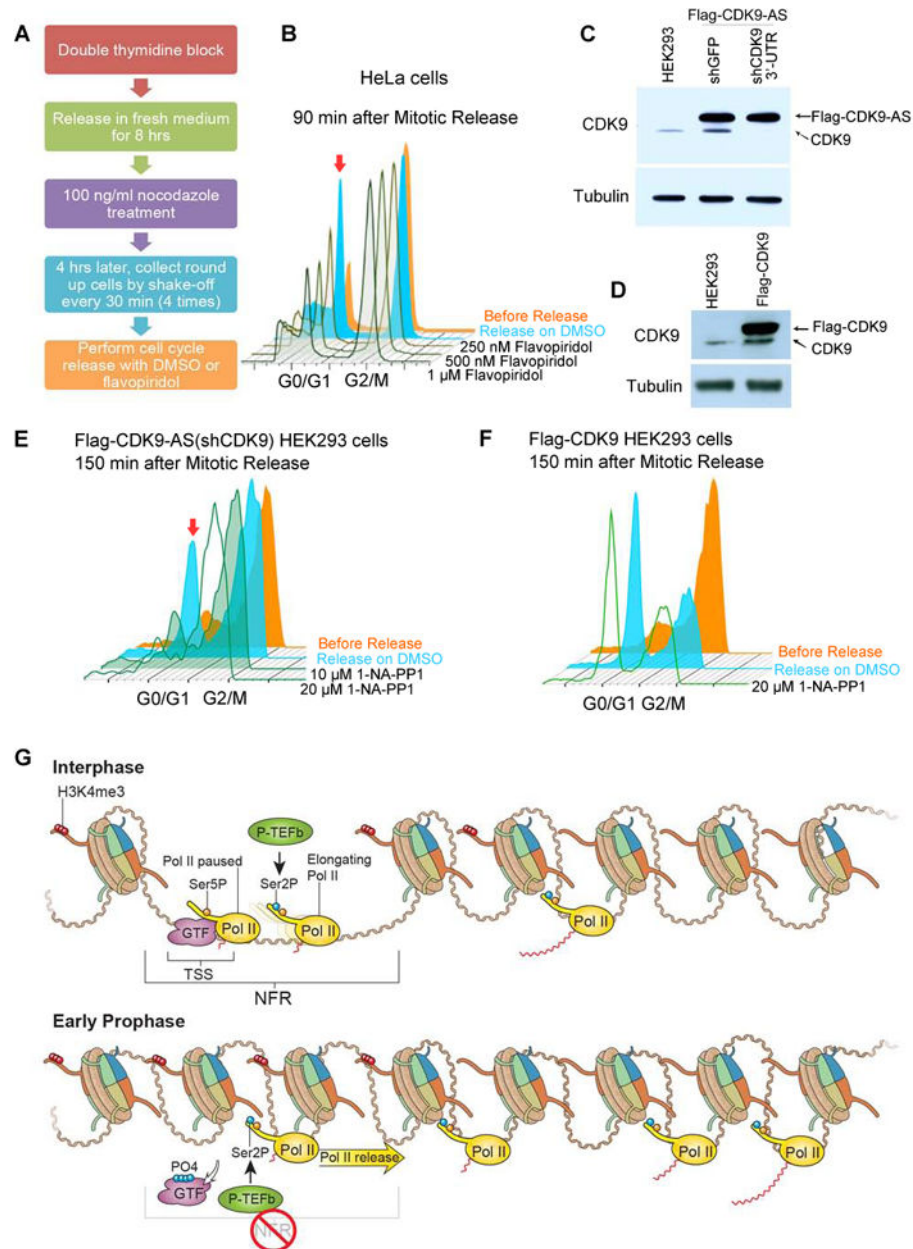


found at the putative enhancers are lost in HeLa mitotic cells (yellow box). (E) Identification of enriched and depleted transcripts in early mitosis relative to asynchronous cells. Mitosis-enriched transcripts are defined as having an increase of greater than 2 fold and  $p < 1E-10$ . Only those protein coding and long non-coding RNA transcripts with  $\log_2$  (r.p.m) (reads per million) more than 4 were considered. Depleted transcripts decrease more than 50% in mitosis and  $p < 1E-10$ . P values were calculated using edgeR glmLRT test (generalized linear models likelihood ratio test) with Benjamini–Hochberg FDR adjustment. (F) Reads coverage analysis of enriched genes in early mitosis. Some enriched genes in early mitosis exhibit a 3'-bias in nascent RNA-Seq reads (representative of two biological replicates). TSS, transcription start site. TES, transcriptional end site. See also Figure S4.





**Figure 5. Flavopiridol inhibits nascent transcription in individual prophase cells**  
(A-B) Fluorescence in situ hybridization (FISH) of *ACTG1* and *HSPA8* in HeLa cells in the presence of DMSO or flavopiridol. Simultaneous RNA FISH and immunofluorescence were performed with Stellaris probes (Raj et al., 2008) for *ACTG1* and *HSPA8* (red). Anti-phH3S10 antibody (green) was used to specifically stain mitotic cells. The nascent transcripts are indicated with white arrowheads (Images are representative captures from at least three biological replicates). Scale bars: 10  $\mu$ m. (C) Summary of RNA FISH data in HeLa and BJ-5ta cells with or without flavopiridol treatment. Most prophase cells have nascent transcripts. Cells were considered to be active for nascent transcription if there was a nuclear spot that was at least three times brighter than the apparent intensity of the single cytosolic transcripts. See also Figure S5.



**Figure 6. A requirement for Mitotic Transcriptional Activation on cell cycle progression**  
 (A) Schematic of thymidine and nocodazole treatments used to obtain early mitotic cells for cell cycle release. (B) HeLa mitotic cells are released in the presence of flavopiridol or DMSO for 90 min and stained with propidium iodide. Defects in progressing to G0/G1 (red arrow) are observed at the indicated flavopiridol concentrations (representative experiment of three biological replicates is shown). (C) Western analysis of CDK9 in control HEK293, and in cells expressing Flag-tagged CDK9 bearing a mutation rendering it ATP-analog sensitive (Flag-CDK9-AS). In the Flag-CDK9-AS cells, the endogenous CDK9 (lower band) was reduced with an shRNA targeting the 3'-UTR of CDK9 compared to a non-targeting shRNA (shGFP). (D) Western blotting of CDK9 in cells expressing Flag-tagged wild-type CDK9. (E) Flag-CDK9-AS cells were blocked by RO-3306 for 24 hours and released with

fresh medium for 15 min. DMSO or the PP1 analog 1-NA-PP1 was added to the released cells for another 150 min. The released cells were stained with propidium iodide (representative experiment of three biological replicates is shown). (F) 1-NA-PP1 does not affect mitotic cell cycle release in wild-type Flag-CDK9 HEK293 cells treatments (representative of three biological replicates). (G) Model for how Pol II is cleared from chromatin during early mitosis. Top panel, in interphase cells, Pol II is generally paused at the promoter regions and P-TEFb is required for productive Pol II elongation. At the onset of mitosis (bottom panel), P-TEFb stimulates the release of paused Pol II to clear the promoter-proximal region through active elongation. NFR, nucleosome free region. See also Figure S6.

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