Engineered Covalent Inactivation of TFIIH-Kinase Reveals an Elongation Checkpoint and Results in Widespread mRNA Stabilization

Highlights

- A general approach for covalent chemical inhibition of kinases in vivo
- Targeted inhibition of Kin28 reveals an elusive elongation checkpoint in yeast
- Varying impact on promoter escape and transition to productive elongation
- Stabilization of existing mRNA buffers/masks reduction in nascent transcripts

In Brief

Rodriguez-Molina et al. describe a general strategy for irreversible inhibition of kinases in vivo. Inhibition of Kin28/CDK7 reduces nascent transcription, increases stability of existing mRNA, and reveals an underappreciated role for Kin28 in priming Pol II for productive transcription elongation.
Engineered Covalent Inactivation of TFIIH-Kinase Reveals an Elongation Checkpoint and Results in Widespread mRNA Stabilization

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SUMMARY

During transcription initiation, the TFIIH-kinase Kin28/Cdk7 marks RNA polymerase II (Pol II) by phosphorylating the C-terminal domain (CTD) of its largest subunit. Here we describe a structure-guided chemical approach to covalently and specifically inactivate Kin28 kinase activity in vivo. This method of irreversible inactivation recapitulates both the lethal phenotype and the key molecular signatures that result from genetically disrupting Kin28 function in vivo. Inactivating Kin28 impacts promoter release to differing degrees and reveals a “checkpoint” during the transition to productive elongation. While promoter-proximal pausing is not observed in budding yeast, inhibition of Kin28 attenuates elongation-licensing signals, resulting in Pol II accumulation at the +2 nucleosome and reduced transition to productive elongation. Furthermore, upon inhibition, global stabilization of mRNA masks different degrees of reduction in nascent transcription. This study resolves longstanding controversies on the role of Kin28 in transcription and provides a rational approach to irreversibly inhibit other kinases in vivo.

INTRODUCTION

The transition of Pol II from a promoter-bound pre-initiation complex (PIC) to active transcription initiation is gated by TFIIH (Egly and Coin, 2011). The kinase subunit (Kin28 in S. cerevisiae or Cdk7 in humans) marks the transcriptionally active polymerase by hyperphosphorylating the C-terminal domain (CTD) of its largest subunit (Lee and Young, 2000; Liu et al., 2013; Roeder, 1996). The CTD is comprised of a repeating heptapeptide (Y1S2P3R4S5P6E7) that is phosphorylated at positions 5 and 7 by Kin28/Cdk7 (Akhtar et al., 2009; Glover-Cutter et al., 2009; Kim et al., 2009). Hyperphosphorylation of the CTD by Kin28/Cdk7 is thought to play an essential role in releasing Pol II from the promoter-bound PIC. Kin28/Cdk7-mediated phosphorylation of the CTD also creates a scaffold to recruit factors that act on nascent transcripts, the underlying chromatin, and the CTD itself. Promoter-proximal marks on Ser5 and Ser7 are also thought to “prime” the CTD for subsequent modifications that coordinate sequential association of different cellular machines that facilitate transcription (Buraworski, 2009; Corden, 2013; Eick and Geyer, 2013; Perales and Bentley, 2009; Phatnani and Greenleaf, 2006; Zhang et al., 2012). Facilitating promoter release was thought to be the primary function of Kin28/Cdk7, because certain temperature-sensitive (ts) alleles of Kin28 severely disable transcription initiation at non-permissive temperatures (Cismowski et al., 1995; Valay et al., 1995). Genetic studies further highlighted the essential nature of this kinase, as deletion of Kin28 or substitution with a catalytically inactive form resulted in loss of cellular viability (Keogh et al., 2002). More recently, a small molecule inhibitor that conjugates to a cysteine residue outside the kinase domain of human Cdk7 was shown to inhibit kinase activity and result in cytotoxicity in cancer cell lines (Kwiatkowski et al., 2014). These observations suggest that blocking Kin28/Cdk7 from hyperphosphorylating the CTD effectively blocks promoter release and disables transcription initiation by Pol II.

In contrast, a catalytically impaired kin28 (T17D) mutant showed defective placement of the m7G cap on nascent transcripts but only had a minor impact on mRNA synthesis (Rodriguez et al., 2000). Non-covalent inhibition of an analog-sensitive allele of Kin28 (kin28as) with purine analogs demonstrated that loss of Kin28-dependent CTD hyperphosphorylation did not efficiently block promoter release in vivo (Kanin et al., 2007; Liu et al., 2004). Using this chemical-genetic approach, subsequent studies confirmed the observation that Kin28/Cdk7 kinase activity was not essential for promoter release or Pol-II-dependent RNA synthesis (Akhtar et al., 2009; Bataille et al., 2012; Glover-Cutter et al., 2009; Hong et al., 2009; Kim et al., 2010a; Lee et al., 2005; Tietjen et al., 2010; Viladevall et al., 2009; Wong et al., 2014). Depleting Kin28 from the nucleus using the in vivo “anchor-away” approach in S. cerevisiae also did not abrogate transcription initiation (Wong et al., 2014). Similarly, attenuating Cdk7 function in mammalian cells did not block transcription initiation (Akoulitch et al., 1995; Glover-Cutter et al., 2009; Helenius et al., 2011; Larochelle et al., 2012). Furthermore, chemical inhibition of Cdk7 with THZ1 did not dramatically impact...
transcription initiation and promoter release (Nilson et al., 2015). In sum, these observations suggest that Kin28/CDK7 functions to prime the CTD for subsequent binding of factors that coordinate the transcription cycle.

Despite significant inhibition of kinase activity with non-covalent inhibitors, the lack of cellular lethality led to the possibility that activity of other promoter-bound kinases, such as Cdc28 (Chymkowitch et al., 2012) or Srb10 (Tietjen et al., 2010), or residual activity due to reversible inhibition of Kin28as permitted low levels of CTD phosphorylation to persist. Minimal CTD phosphorylation may suffice for promoter release and productive RNA synthesis. To resolve these discrepancies and definitively address the critical role of Kin28 activity in RNA synthesis, we developed a targeted covalent inhibition approach that robustly inactivates Kin28 in vivo. We recapitulate the lethal phenotype of a catalytically inactive kin28 allele. Using our “irreversibly sensitized” Kin28 allele (kin28is), we observe that covalent inhibition impacts promoter escape to differing degrees and impairs transition to productive elongation, revealing a rate-limiting “checkpoint” coincident with the +2 nucleosome. Promoter-proximal pausing of Pol II, a hallmark of the metazoan transcription cycle, is not observed by chromatin immunoprecipitation (ChIP) in budding yeast (Adelman and Lis, 2012). However, upon irreversible inhibition of Kin28is, a clear enrichment of Pol II is observed within a transition window where promoter-proximal factors are exchanged for elongation and 3' end processing factors (Görnemann et al., 2005; Hossain et al., 2013; Lidschreiber et al., 2013; Pokholok et al., 2002). Consistent with a potential defect in early transcription elongation, a catalytically “slow” version of Pol II is particularly sensitive to any degree of Kin28 inhibition, whereas a “fast” transcribing Pol II is more resistant (Malagon et al., 2006). Unexpectedly, we observe a global decrease in nascent transcription along with a stabilization of extant mRNA levels. Taken together, our results reconcile long-standing controversies and reveal a role for Kin28 in facilitating promoter release, enhancing the transition to productive elongation, and priming the CTD for downstream stages of the transcription cycle. Moreover, given the highly conserved ATP-binding site across kinases, our strategy for structure-guided covalent inhibition of Kin28 readily lends itself to targeted irreversible inhibition of other kinases in vivo.

RESULTS

Design of an Irreversibly Sensitized Allele of Kin28
To definitively dissect the role of Kin28, we used structure-guided protein design to generate an “irreversibly sensitized” version of Kin28 (kin28is) that can be selectively and covalently inhibited by an ATP analog bearing a thiol-reactive chloromethylketone moiety (CMK). The approach was inspired by the use of CMK to inhibit Cdc5, which has a naturally occurring solvent-exposed cysteine within its ATP-binding pocket. CMK docks into an enlarged ATP-binding pocket of Cdc5 (cdc5as) and becomes covalently conjugated to the reactive cysteine, thereby irreversibly inhibiting kinase function (Cohen et al., 2005; Snead et al., 2007). We reasoned that rational engineering of Kin28 to possess an enlarged ATP-binding pocket and a reactive Cys in the structurally identical position to Cdc5 would render it sensitive to CMK. We created a mutant allele of Kin28 that contains two substitutions within its ATP-binding pocket: (1) a smaller gatekeeper residue (L83G) to accommodate the para-methylphenyl group of CMK and (2) a solvent-exposed Cys positioned to react with CMK in place of a conserved Val (V21C) (Figure 1A). Our design predicts that CMK would meet the two selectivity filters by docking into the enlarged active site and irreversibly conjugating to Kin28is, thereby preventing engagement of cellular ATP. In contrast, non-covalent inhibitors equilibrate with the kinase as a function of on/off rates, inhibitor concentrations, and cellular ATP levels (Figure 1B). In other words, CMK would irreversibly silence the catalytic activity of the kinase.

Covalent Inhibition of Kin28is Irreversibly Arrests Cell Growth
kin28is cells grown in the presence of CMK display striking sensitivity (growth rate, as measured by doubling time, is halved at 40 nM CMK) and growth arrest on solid media as well as in liquid cultures (Figures 1C and S1). In the presence of 1-NA-PP1 (a non-covalent reversible inhibitor), kin28as and kin28is cells display partial decrease in growth rates and, over time, attain growth densities comparable to uninhibited cells. Importantly, cells with Kin28 bearing only the gatekeeper mutation (L83G) or the V21C mutation are not sensitive to CMK (Figures 1C, S1B, and S1C). Given the strict requirement for two selectivity filters, the ATP-binding sites of endogenous yeast kinases would not permit covalent conjugation by CMK. Consistent with this expectation, CMK does not perturb the transcriptome in wild-type cells or in cells bearing the kin28as allele (details below).

To determine whether the CMK-rendered growth arrest was irreversible, we treated cells with 1-NA-PP1 or CMK for 24 hr, followed by cell harvest, extensive washes with fresh media, and further growth for 24 hr in media lacking either inhibitor. Wild-type (WT) and kin28as cells were able to recover from inhibition after drug washout, whereas kin28is cells treated with CMK were unable to recover (Figure 1D). Together, these data suggest our covalent inhibition strategy is specific, irreversible, and able to recapitulate the growth-arrest phenotype manifested by a catalytically inactive kin28 allele. Although we have been cognizant of partial kinase inhibition by reversible inhibitors, others may not realize this caveat (Bataille et al., 2012).

Irreversible Inhibition of Kin28is Drastically Reduces Cellular Ser5-P Levels
Promoter-proximal phospho-Ser5 (Ser5-P) and phospho-Ser7 (Ser7-P) marks are placed primarily by Kin28 (Akhtar et al., 2009). Inhibition of Kin28 with reversible ligands, however, does not result in bulk decrease in Ser5-P or Ser7-P marks (Akhtar et al., 2009; Chymkowitch et al., 2012; Liu et al., 2004). To assess the effect of covalent inhibition of Kin28is on bulk levels of phospho-CTD marks, we prepared whole-cell extracts from exponentially growing cells treated with the DMSO solvent control (“Untreated”), the reversible inhibitor 1-NA-PP1, or the irreversible inhibitor CMK. Western blots show a striking decrease in Ser5-P marks in cells treated with CMK (p < 0.05, Student’s t test) (Figure 2). Partial loss of bulk Ser7-P marks is consistent with the role of Bur1 (CDK9 in humans) in placing Ser7-P marks...
Ser2-P, phosphorylated tyrosine-1 (Tyr1-P), and phosphorylated threonine-4 (Thr4-P) levels do not appear to change appreciably, suggesting that these are not major targets of Kin28 in cells. Importantly, treatment of WT cells does not affect phospho-CTD levels in line with the requirement of an “is”-sensitized kinase for CMK to inhibit kinase activity.

Together, these data further highlight the selectivity and effectiveness of our covalent inhibition approach.

Genome-wide Ablation of Promoter-Proximal Ser5-P and Ser7-P Marks

To determine the impact of covalent inhibition of Kin28 on Pol II distribution, we performed ChIP with antibodies against the tandem affinity purification (TAP) tag (Rpb3-TAP) or phospho-CTD marks. Covalent inhibition of Kin28 caused a global decrease in Ser5-P marks and a precipitous decrease in promoter-proximal Ser7-P marks (Figures 3A and 3B). The loss of Ser5-P and Ser7-P marks were more pronounced when cells were treated with CMK compared to 1-NA-PP1 at the same concentration (Figure 3B). We validated these decreases in Ser5-P and Ser7-P marks near active gene promoters by ChIP-qPCR at ADH1 (Figure S3A). Residual Ser5-P signal within genes, as well as remaining Ser7-P signal near promoters and within genes, is consistent with the activity of Bur1 on CTD at these regions (Tietjen et al., 2010).

Phosphorylation of the Pol II CTD by Kin28 is thought to release the CTD from its interaction with the Mediator Head module (Robinson et al., 2012). Covalent inhibition of Kin28, followed by ChIP-chip analysis of the Mediator Head module subunit, Med8, reveals a shift in Med8 occupancy to 42 bp, rather than its typical location at 116 bp with respect to the transcription start site (TSS) (Figure S3B), consistent with previous studies (Jeronimo and Robert, 2014; Wong et al., 2014). While we do not observe a significant difference in Pol II occupancy at genes regulated by TFIID or SAGA complexes (Figures S7A and S7B), nascent transcription is slightly more affected at TFIID-dependent rather than SAGA-dependent genes (Figure S7C). Kin28 inhibition is also reported to alter start site selection at TFIID-targeted promoters in vitro (Murakami et al., 2015). However, under
Covalent inhibition conditions, no such shift was apparent in vivo (Figure S7D). The sum of these results highlights the specific and potent effects of covalent inhibition of Kin28is with CMK previously not attained by reversible inhibition strategies.

**Depleted Pol II Occupancy at 3’ Ends of Transcribed Regions**

Covalent inhibition of Kin28is revealed a reproducible Pol II decrease toward the 3’ ends of protein-coding genes (pc-genes) (Figure 3). The graded decrease in Pol II levels is inversely correlated with gene length (Figure 3C, Pearson correlation −0.509, p = 8.6 × 10⁻⁴⁰ versus mean p = 0.501 from 10⁴ randomized iterations of the data), suggesting a length-dependent mechanism by which Pol II is depleted from chromatin in the absence of Kin28 activity. To parse out different patterns of Pol II perturbation, we performed k-means clustering of changes in Pol II occupancy at pc-genes with robust levels of Pol II occupancy (top 10%). Visual inspection of the clusters revealed six distinct patterns of Pol II change. For 71% of these genes, Pol II shows depletion at the cleavage and polyadenylation site (CPS) (elongation coefficient), which does not necessarily correlate with Pol II changes at the TSS (promoter escape coefficient) (Figure 3D and Table S1). However, the ratio of Pol II at the CPS versus the TSS (Pol II travel index) invariably shows Pol II has a reduced travel index within most of the clusters identified (Figure 3E and Table S1). These data suggest Kin28 impacts Pol II promoter escape to different degrees at different promoters and unexpectedly plays an important role in Pol II elongation.

**Global Decrease in Nascent RNA Synthesis and Concomitant Stabilization of mRNA**

To determine the role of Kin28 activity on RNA synthesis, we performed comparative dynamic transcriptome analysis (Sun et al., 2012). The newly synthesized transcripts were pulse labeled with 4-thiouracil, which is incorporated into growing RNA chains. The thiolated transcripts were biotinylated and purified using streptavidin-coated magnetic beads (Nascent, Tables S2 and S4). In parallel, we purified Total RNA (Total, Table S3) as a measure of steady-state transcript levels. To account for any global defects in transcription upon inhibition of Kin28 activity, we spiked in S. pombe cells for which RNA was simultaneously prepared and sequenced (6:1 S. cerevisiae:S. pombe cellular ratio). We fit local linear regressions of gene expression across replicates from S. pombe, which were used to normalize S. cerevisiae Nascent and Total RNA sequencing (RNA-seq) data across replicates and treatments. Thus, we circumvent potentially misleading normalization approaches that rely on a handful of model genes that do not reflect the full range of transcriptome perturbations in cells (Hong et al., 2009). Thus, if Kin28 inhibition were to cause global reduction in transcription, it would be reliably detected by our analyses. We have consistently used spike in controls to account for global changes in the yeast transcriptome (Karin et al., 2007), contrary to assertions which claim otherwise (Hong et al., 2009).

Unexpectedly, the data revealed that Total RNA levels remain generally unchanged, whereas Nascent RNA levels show nearly 4-fold decrease upon covalent inhibition of Kin28is (Figures 4A–4C and Table S5). To determine the number of genes that were significantly downregulated upon covalent inhibition of Kin28is, we employed edgeR and DESeq, two widely used statistical packages. Although both generally agreed on the effect of Kin28is inhibition on Total RNA (edgeR: 283 downregulated genes, DESeq: 643 downregulated genes, with ≥2-fold decrease, 5% FDR), we found edgeR and DESeq differed in their analysis of the Nascent RNA data. Analysis of the raw Nascent RNA data clearly shows a global decrease in abundance upon covalent inhibition of Kin28is. However, DESeq, but not edgeR, preserved this initial observation (edgeR: 166 versus DESeq: 4,321, with ≥2-fold decrease, 5% FDR, out of 5,390 pc-genes) (Figure 4S). Indeed, in simulation studies, edgeR and other commonly used analysis methods performed poorly when there was global reduction in transcript abundance (Soneson and Delorenzi, 2013). We conclude that covalent inhibition of Kin28is leads to downregulation of the majority of the Nascent RNA fraction.

For both Total and Nascent RNA fractions, irreversible inhibition of Kin28is with CMK causes the largest overall change in
RNA abundance (Figures S5A–S5D). Treatment of WT cells with either 1-NA-PP1 or CMK did not result in any significant changes in Total or Nascent RNA levels (Figure S5B). Likewise, non-cova-

lent inhibition of Kin28is or Kin28as with 1-NA-PP1 did not result in significant changes in RNA levels, consistent with previous reports (Figures S5B–S5D) (Kanin et al., 2007). These results reconcile previous discrepancies and indicate that different transcriptional processes depend on different degrees of CTD phosphorylation, with capping requiring maximal levels of phosphorylation, whereas promoter escape and transcription elongation can occur at low levels of Kin28-dependent CTD phosphorylation.

Highly Repressed Genes Reveal a Checkpoint Prior to Productive Elongation

Among the genes with the most pronounced decrease in Pol II travel index, we observed genes with Pol II accumulation 100–200 nt downstream of the TSS (Figure 4D). Likewise, Pol II profiles at the genes most severely affected in their Total and Nascent pools of RNA revealed accumulation of Pol II, which
coincides with the +2 nucleosome and the transition from initiation to elongation (Figures 5A and 5C) (Albert et al., 2007; Görnemann et al., 2005; Hossain et al., 2013; Kim et al., 2010a; Lidschreiber et al., 2013; Pokholok et al., 2002). This Pol II accumulation is consistent with a checkpoint at the onset of productive elongation after successful transcription initiation and promoter escape. The Pol II buildup also coincides with the region of maximal Spt5 recruitment, which occurs upstream of maximal Bur1 and Paf1 occupancy (Figures 5B and 5D) (Mayer et al., 2010; Segal et al., 2006). Phosphorylation of Spt5 and Pol II by Bur1 contributes to efficient recruitment of the Paf1 complex, which is a critical step for efficient transcription elongation (Liu et al., 2009; Qiu et al., 2012; Yu et al., 2015). To further define the nature of the defect in the transition to elongation, we examined the levels of Spt5 and phosphorylated Spt5 (Spt5-P) at two representative genes (Figures 5E and 5F). The data show that inhibiting Kin28 significantly reduces the level of Spt5-P, indicative of compromised Bur1 function.

Figure 4. Inhibition of Kin28 Leads to Stabilization of mRNA Abundance and Reduction in Nascent Transcripts
(A and B) Coverage of RNA-seq reads at the indicated genes for the (A) Total and (B) Nascent fractions of RNA from untreated (black line) and CMK-treated (orange fill) kin28Δ cells.
(C) Scatterplot of expression changes in the Total (x axis) and Nascent (y axis) fraction plotted for each pc-gene. The distributions of expression changes are plotted as histograms, adjacent. Differentially expressed genes with greater than 2-fold decrease, as called by edgeR, are boxed in red.
(D) Pol II traces at DIP5 before (line) and after (filled) inhibition of Kin28Δ (see also Figure S5 and Table S5).
Impact of Pol II Alleles with Different Elongation Rates

To test whether a functional link between Kin28 activity and the rate of Pol II elongation exists, we examined the impact of Kin28 inhibition in strains bearing Pol II alleles with either fast or slow elongation rates. We first replaced WT Kin28 with the *kin28is* allele in the anchor-away system (Haruki et al., 2008). In this

Figure 5. Kin28 Plays a Crucial Role in Priming Pol II for Elongation

(A) Difference in Rpb3, Ser5-P, Ser7-P, and Ser2-P occupancy after inhibition of Kin28is with 2 μM CMK at genes most affected by loss of Kin28 function (i.e., genes boxed in Figure 4C) and sorted by gene length. Following a decrease in Ser5-P and Ser7-P marks, Rpb3 ChIP signal accumulates on average 177 bp downstream of the TSS (top blue bar, see Supplemental Experimental Procedures) and coincides with the +2 nucleosome (bottom panel) (see also Figure S6).

(B) Occupancy of factors recruited during initiation and elongation (Mayer et al., 2010).

(C) Average occupancy traces for Pol II and nucleosome data shown in (A).

(D) Average occupancy traces for Pol II and data shown in (B). Vertical dotted line denotes TSS. Blue highlighted regions denote the site of Pol II stalling.

(E and F) ChIP-qPCR of Rpb3 (blue), Spt5 (orange), and Spt5-P (maroon) at the indicated regions (5', mid, and 3') of *CDC60* (E) and *RPS4B* (F). Average fold changes (n = 3–6) relative to DMSO control are plotted. *p < 0.05, one sample two-sided Student’s t test.
system, rapamycin-induced heterodimerization of an Rpb1-FRB fusion with RPL13A-FKBP12 actively exports Rpb1 from the nucleus due to the normal nucleo-cytoplasmic trafficking of RPL13A (Figure 6A). Expression of Rpb1 variants in this system permits the replacement of endogenous WT Pol II with Pol II variants with reduced/slow (rpb1-N488D) or enhanced/fast (rpb1-E1103G) elongation rates (Malagon et al., 2006) (Figure 6). The slow Pol II variant is thought to hinder initiation or early elongation and should therefore exacerbate the effects of irreversible inactivation of Kin28 and show a negative genetic interaction with sub-optimal levels of Kin28 activity. Consistent with a shared function in elongation, kin28is cells grown in otherwise sub-lethal concentrations of CMK (0.2 μM) displayed a synthetic lethal phenotype when combined with the slow Pol II variant (Figure 6B). Reciprocally, we predicted the fast Pol II variant would be less sensitive to Kin28 inhibition. In further agreement, the fast variant of Pol II did not display the synthetic lethal phenotype when Kin28 activity was dialled down (Figure 6B).  

DISCUSSION

To resolve the long-standing discrepancies on the function of Kin28/Cdk7, we rationally designed a sensitized variant of Kin28 (kin28is) that can be inhibited specifically and irreversibly in vivo by CMK. To achieve targeted inhibition, CMK requires the simultaneous presence of two selectivity filters, a small gatekeeper residue and a precisely positioned cysteine in the ATP-binding site. Conveniently, none of the endogenous kinases in budding yeast have both sequence elements, enabling selective inhibition of the doubly mutated kin28is allele.

We find that Kin28 plays a crucial role at multiple stages of Pol II transcription and that when the kinase is chemically inhibited in vivo, the impact on transcription is masked by increased stability of mature mRNA. Furthermore, we find Kin28 enhances transcription elongation by priming the CTD and enabling the engagement of complexes that help overcome an “elongation checkpoint” well after promoter release and transcription initiation (Figure 7). Thus, by irreversibly inhibiting Kin28 with a covalent ligand, we have effectively dialled down Kin28 activity past a physiologically relevant threshold previously unattained with non-covalent inhibitors. By achieving more complete inactivation, our study has revealed the essential role of Kin28 catalytic activity at different stages of the transcription cycle.

We conclude that differential levels of kinase inhibition reveal distinct transcriptional processes that display differing degrees of dependence on CTD phosphorylation states. Promoter-proximal recruitment of Ceg1 and 5’ capping of nascent pre-mRNAs is highly sensitive to partial Kin28 inhibition and partial reduction of CTD phosphorylation. The heightened sensitivity of Ceg1 for any perturbation in Kin28 activity may arise in part from the kinetic competition between transcribing Pol II and 5’ surveillance factors. This competition may restrict the essential function of the capping enzyme to a narrow window of opportunity immediately following initiation. Thus, capping defects are readily detectable when using non-covalent inhibition strategies against Kin28 (Kanin et al., 2007). On the other hand, further reduction in Kin28-dependent Ser5-P and Ser7-P levels diminishes the formation of elongation-competent complexes. Upon irreversible inhibition of Kin28, the position of Pol II buildup coincides with proposed “checkpoints” that are thought to coordinate 5’ capping of nascent transcripts with productive elongation and splicing (Görmemann et al., 2005; Hossain et al., 2013; Lidschreiber et al., 2013; Pokholok et al., 2002). This checkpoint...
was not detected by previous genome-wide chromatin immuno-precipitation studies of Pol II but is readily evident upon covalent Kin28 inhibition.

Consistent with a role for Kin28 in productive elongation, we find that upon Kin28 inactivation, Pol II abundance at the 3' end of genes correlates negatively with gene length. Interestingly, a slow-transcribing Pol II (rpb1-N488D) shows a synthetic lethal interaction with Kin28, suggesting a functional link between Pol II elongation rates and Kin28 activity. In line with a role for Kin28 in early transcription elongation, recruitment of the cap binding complex is required for efficient recruitment of Bur1 and Ctk1, proper levels of Ser2-P marks, and efficient elongation (Görnemann et al., 2005; Hossain et al., 2013; Lidschreiber et al., 2013; Qiu et al., 2009; Viladevall et al., 2009). Fast Pol II, on the other hand, is proposed to display transcription rates that may bypass elongation checkpoints and compromise co-transcriptional processing (Hazelbaker et al., 2013; Jimeno-González et al., 2014). Our observations suggest a critical role for Kin28 in priming Pol II for elongation competency by facilitating timely recruitment of elongation and pre-mRNA processing factors and protecting the elongation complex from capping surveillance factors that would remove Pol II molecules carrying uncapped pre-mRNAs (Chang et al., 2012). Our findings are also consistent with a model wherein Kin28 activity primes Pol II and Spt5 for downstream phosphorylation by Bur1. These modifications recruit elongation complexes required to overcome a transition checkpoint imposed by the +2 nucleosome. This “kinetic” checkpoint contrasts with the stable promoter-proximal Pol II pause in mammalian systems that occurs within 100 bp of the TSS and is mediated by the Negative Elongation Factor (NELF) and DSIF (Spt4/5). While NELF does not exist in yeast and the kinetic checkpoint is detected downstream of the mammalian pause site, both mechanisms present a rate-limiting step prior to transition into productive transcription elongation.

It is important to note that Nascent RNA synthesis decreases upon covalent inhibition of Kin28is but is not dramatically impaired upon partial inhibition with a reversible inhibitor. Unexpectedly, steady-state RNA levels remain mostly unaffected for an extended period after covalent inhibition of Kin28is, reflecting increased stabilization of the cellular mRNA pool in response to defective transcription by Pol II. This dichotomous response to Kin28 inhibition supports a built-in safeguard against defective Pol II transcription (Haimovich et al., 2013; Pelechano et al., 2009; Sun et al., 2013). Our findings help reconcile initial observations that suggested an essential role for Kin28 in transcription and more recent findings showing that mRNA levels remain unaffected when Kin28 activity is compromised by certain mutations (Keogh et al., 2002; Komarnitsky et al., 2000) or blocked with reversible ligands (Akhtar et al., 2009; Bataille et al., 2012; Hong et al., 2009; Kanin et al., 2007; Kim et al., 2010a, Liu et al., 2004). Pol II occupancy is not affected to the same degree as Nascent RNA upon covalent inhibition of Kin28. This is
reminiscent of reports wherein inhibition of general transcription using the rpb1-1 allele shut down RNA synthesis within 5–15 min after switching to non-permissive temperatures (Nonet et al., 1987), yet Pol II remained robustly associated with chromatin for an additional hour (Kim et al., 2010b; Zanton and Pugh, 2006). Similarly, under certain nutrient conditions, Pol II association with transcribed regions increases subtly even though the rate of Nascent RNA synthesis is dramatically reduced (Pelechano et al., 2009). Therefore, although RNA synthesis is reduced, remaining Pol II occupancy may reflect a combination of slowly transcribing, backtracking, or actively engaged Pol II. Taken together, our results converge on multiple roles for Kin28/Cdk7 in regulating post-initiation stages of the transcriptional cycle and reconcile long-standing controversies.

Furthermore, unlike kinase-specific inhibitors that require exhaustive and cost-prohibitive small molecule screens and may have off-target effects due to non-specific interaction with related kinases (Kwiatkowski et al., 2014), our approach has the potential to be broadly applied to other kinases. Our results clearly demonstrate that the active site of a kinase can be rationally sensitized to irreversible inhibition and that CMK does not perturb cellular growth rates, CTD phosphorylation patterns, or the transcriptome in wild-type cells. Importantly, our strategy faithfully recapitulates the phenotype of a catalytically inactive kin28 allele and achieves a greater degree of inhibition than reversible ligands. These outcomes are likely to extend to other kinases as our structurally and bioinformatically guided engineering of Kin28 can be readily applied to confer “irreversible sensitization” to thiol-reactive ATP analogs. Thus, the strategy that we describe here may serve as a resource for the broader scientific community studying kinases, as well as ATP-binding proteins and enzymes that are amenable to rationally designed chemical-genetic retrofitting.

EXPERIMENTAL PROCEDURES

Kin28is Allele Design and Isolation

The gatekeeper (L80) and conserved valine (V21) residues of Kin28 were identified by aligning the primary sequence of Kin28 to that of Cdc5, which has a naturally occurring solvent-exposed Cys in place of the conserved Val. The endogenous Kin28 gene was substituted with its kin28is counterpart by two-step allele replacement into an Rpb3-TAP (Open Biosystems) or HHY170 background. After recombination, loss of URA3 was screened on SC+SFOA and potential kin28is colonies were screened by growing them on rich media (YPD) or media containing CMK (YPD+CMK). Colonies that displayed a growth defect on YPD+CMK, but not on YPD, were selected and genotyped. CMK can be purchased from Bakul Pharma.

Extracts and Antibodies for Western Blotting

Whole-cell extracts were prepared from exponentially growing cells (see Supplemental Experimental Procedures), resolved by SDS-PAGE, and immunoblotted using anti-phosphorylated CTD antibodies (3E8, 3E10, 4D12, 3D12, 1G7, and 6G7) were gifts from Dirk Eick, anti-Ser5-P (Covance H14), anti-TAP (Pierce 26613). Western blots were probed with anti-Rpb3 for Rpb3-TAP or Anchor-Away Assays

HHY170 and HYH170 kin28is were transformed with plasmids bearing WT (pL-rpb1), slow (pL-rpb1-N488D), or fast (pL-rpb1-E1103G) variants of RNA Polymerase II. Cells were grown in synthetic complete media without tryptophan (SC-Trp) overnight, and cultures were serially diluted and plated on SC-Trp with or without rapamycin (1 μg/mL), 0.2 μM or 2 μM CMK, or respective CMK + rapamycin combinations.

ACCESSION NUMBERS

The accession number for the RNA-seq and ChIP microarray data reported in this paper is GEO: GSE83417.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.06.036.

AUTHOR CONTRIBUTIONS

J.B.R.-M., S.C.T., J.T., and A.Z.A. conceived the project, designed experiments, and wrote the paper. J.B.R.-M. designed the kin28is allele, performed ChIP-chip experiments and inhibitor wash-out experiments, designed genetic interaction assays, carried out growth curves, and analyzed the data. S.C.T. performed RNA-seq, cDTA-seq, growth curves, inhibitor titration, ChIP-qPCR, and immunoblots, and analyzed the data. S.P.S. carried out genetic interaction assays and ChIP-qPCR.

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REFERENCES


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