CDK12 loss in cancer cells affects DNA damage response genes through premature cleavage and polyadenylation

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Cyclin-dependent kinase 12 (CDK12) modulates transcription elongation by phosphorylating the carboxy-terminal domain of RNA polymerase II and selectively affects the expression of genes involved in the DNA damage response (DDR) and mRNA processing. Yet, the mechanisms underlying such selectivity remain unclear. Here we show that CDK12 inhibition in cancer cells lacking CDK12 mutations results in gene length-dependent elongation defects, inducing premature cleavage and polyadenylation (PCPA) and loss of expression of long (>45 kb) genes, a substantial proportion of which participate in the DDR. This early termination phenotype correlates with an increased number of intronic polyadenylation sites, a feature especially prominent among DDR genes. Phosphoproteomic analysis indicated that CDK12 directly phosphorylates pre-mRNA processing factors, including those regulating PCPA. These results support a model in which DDR genes are uniquely susceptible to CDK12 inhibition primarily due to their relatively longer lengths and lower ratios of U1 snRNP binding to intronic polyadenylation sites.
Eukaryotic gene transcription is facilitated by the orchestrated action of transcriptional cyclin-dependent kinases (CDKs) and associated pre-mRNA processing factors. Transcriptional CDKs phosphorylate the carboxy-terminal domain (CTD) of RNA Polymerase II (Pol II) which serves as a platform for the recruitment of factors controlling transcriptional and post-transcriptional events. During transcription initiation, CDK7, a subunit of TFIIH, phosphorylates serine 5 of the CTD; subsequently, the release of paused Pol II and the transition to elongation is mediated by CDK9, a subunit of pTEFb, which phosphorylates the CTD at serine 2. Studies in yeast and metazoans have shown that another transcriptional kinase, CDK12, together with its associating partner, cyclin K, modiﬁes the latter, the addition of an ABCB1 drug efflux pump inhibitor (tariquidar) was necessary to overcome high expression of this protein and subsequent inhibitor efflux [20,21] (Supplementary Fig. 1f). Despite the role of CDK12 in transcription elongation, THZ531 induced variable dose- and time-dependent decreases in Pol II Ser2 phosphorylation, with minimal effects on Ser5 or 7 phosphorylation (Fig. 1b). However, we observed striking downregulation of termination-associated Pol II threonine (Thr)22,23 phosphorylation, indicating that distal elongation was affected (Fig. 1b). Together, these results indicate that THZ531, by binding to CDK12/13, induces cytotoxicity in NB cells through effects on transcription elongation.

**CDK12 inhibition preferentially affects DDR genes.** CDK12 inhibition has been shown to affect the expression of genes involved in the DDR. To determine whether similar effects are produced by our selective inhibitor in NB cells, we analyzed the gene expression profiles of cells treated with and without THZ531 for 6 h, a time point at which there were little or no confounding effects due to cell cycle changes (Supplementary Fig. 1e). Unlike the effects seen with THZ116, predominantly an inhibitor of CDK7 with some activity against CDK12/13 [24], we failed to observe a complete and global transcriptional shutdown in THZ531-treated NB cells; instead, only 57.4% of the transcripts were downregulated (n = 10,707), with 0.35% (n = 66) upregulated [false discovery rate (FDR) < 0.05] (Supplementary Fig. 2a; Supplementary Data 1). Consistent with earlier studies [14,15], THZ531 led to significant downregulation of both transcription-associated and DDR genes (Fig. 1c, Supplementary Fig. 2c, d). To determine whether these effects were due to inhibition of CDK12 or 13, we depleted the expression of each kinase individually in NB cells, and in keeping with prior studies [5,10], observed selective downregulation of DDR genes with CDK12 but not CDK13 knockdown (KD) (Supplementary Fig. 2e). Additionally, the expression of DDR genes was not signiﬁcantly affected in Kelly E9R THZ531-resistant cells, further implicating the selective role of CDK12 in regulating the DDR (Fig. 1d). Consistent with these observations, THZ531 also led to increased DNA damage with elevated γ-H2AX levels (Fig. 1e, Supplementary Fig. 2f) and decreased radiation-induced RAD51 foci, indicating defects in DNA repair (Fig. 1f). Thus, our findings indicate that DDR genes are selectively affected by THZ531 and that such regulation is driven predominantly by CDK12.

**CDK12/13 inhibition leads to an elongation defect.** The transcriptional effects of CDK12/13 inhibition with THZ531, including downregulation of the steady-state expression of DDR genes, occurred as early as 6 h. post-treatment and independently of cell cycle changes (Supplementary Fig. 1e). This result, plus the fact that CDK12 and 13 have been implicated in pre-mRNA processing [10,26,27] where observable changes are likely to occur within minutes or hours, indicated that further analysis of steady-
Fig. 1 CDK12/13 inhibition results in selective cytotoxicity in NB cells and affects transcription elongation. 

a Dose-response curves for human NB cells treated with increasing concentrations of THZ531 for 72 h. Kelly E9R cells, which express a homozygous mutation at the Cys1039 THZ531-binding site in CDK12/20 (see Methods) were also included. Fibroblast cells (NIH-3T3, IMR-90, BJ) were used as controls. Cytotoxicity is reported as percent cell viability relative to DMSO-treated cells. Data represent mean ± SD; n = 3. 

b Western blot analysis of Pol II phosphorylation in NB cells treated with THZ531 or DMSO at the indicated concentrations for the indicated times. 

c Waterfall plot of fold-change in gene expression in IMR-32 NB cells treated with THZ531, 400 nM for 6 h; selected DDR genes are highlighted. 

d qRT-PCR analysis of the indicated DDR gene expression in Kelly WT (left) cells and Kelly E9R (right), treated with THZ531 or DMSO at the indicated concentrations for 6 h. Data are normalized to GAPDH and compared to the DMSO control. 

e Flow cytometry analysis of γ-H2AX staining in Kelly NB cells treated with 400 nM THZ531 for the indicated time points (left). Gating was performed as shown in the left panel. Numbers indicate the percentages of living cells that stained positive for γ-H2AX. Quantification of staining (right). 

f Immunofluorescence staining of RAD51 focus formation in Kelly NB cells treated with THZ531 (400 nM) or DMSO for 24 h prior to exposure to gamma radiation (IR, 8 Gy). Nuclei are stained with DAPI (scale bar, 10 μM). Quantification of staining (right) of RAD51+ cells (>5 RAD51 foci per cell). Throughout the figure, error bars indicate mean values ± SD of three independent experiments, **p < 0.01, ***p < 0.001; two-tailed Student’s t-test.
state RNA would not be sufficient to fully characterize alterations in the tightly coupled Pol II transcription and RNA processing or discriminate between early and late effects due to CDK12 perturbation. Hence, we used transient transcriptome sequencing (TT-seq), a modification of the 4-thiouridine (4sU)-pulse labeling method, with spike-in controls for normalization of input RNA amount to identify the immediate changes in nascent RNA production in cells exposed to THZ531 for 30 min and 2 h. The data showed adequate exonic and intronic coverage, including that of 5′ upstream and 3′ downstream regions outside annotated
transcripts (mean 59% of reads to introns, 35% to exons and 6% to flanking regions), indicating that nascent RNAs, including a large proportion of preprocessed RNAs were captured (Supplementary Fig. 3a). Altogether, we detected 12,260 protein coding, 4,809 long non-coding and 3,816 short non-coding genes (transcripts per million > 2). At 30 min post-treatment, several immediate-early response gene transcripts were induced, thus confirming the ability of TT-seq to detect early changes in

Fig. 3 CDK12 inhibition leads to PCPA of long genes. a Average metagene profiles of normalized poly(A) 3′-seq reads at the transcription end sites (TES) (−1 to +4 kb) of all long genes (>64.5 kb) (left), and short genes (RD histone genes) (right). b Average metagene profiles of normalized poly(A) 3′-seq reads over gene bodies and extending −2 to +2 kb of all detected genes in cells treated with THZ531 400 nM for 2 and 6 h. Sense and antisense reads are depicted by solid and dashed lines, respectively. c Histograms showing the genomic distributions and rankings of the top 5000 poly(A) 3′-seq peaks in DMSO- and THZ531-treated cells (400 nM, 6 h). The poly(A) 3′ peaks were binned according to the depicted genomic regions and their intensities (x-axis). d Bar plot indicating the number of protein-coding genes that underwent premature cleavage and polyadenylation (PCPA) with THZ531. The expanded window on the right shows the genomic distribution of the identified intronic poly(A) sites. e Average metagene profiles of normalized poly(A) 3′-seq reads at the TSS (−1 to +10 kb) for all detected genes in Kelly WT (left) and Kelly E9R (right) cells. Changes (insets) in read density between DMSO- and THZ531 (200 nM, 6 h)-treated Kelly WT (p = 5.8e−41) and Kelly E9R (p = 0.11) cells; comparisons between groups by Wilcoxon rank-sum test. The center line indicates the median for each data set. f Density plot of odds-ratios of poly(A) site usage (intronic vs 3′ UTR) for genes in Kelly WT and E9R cells (p = 0, Kolmogorov-Smirnov test)
transcription, but this effect was not sustained at 2 h (Supplementary Fig. 3b; Supplementary Data 2). Instead, the changes in nascent transcription were more pronounced at 2 h, leading us to focus our analyses on this time point. DDR genes were on average more downregulated compared to other genes (Supplementary Data 3; Supplementary Fig. 3c, d), consistent with our gene expression profiling of steady-state RNA, which had demonstrated downregulation of these genes after a 6-h treatment with THZ531 (Fig. 1c and Supplementary Fig. 2a–c). We validated this result by measuring nascent RNA expression along the BRCA1 gene by qRT-PCR, observing a gradual decline in expression from the 5′ to the 3′ end of the gene following THZ531 treatment (Supplementary Fig. 3e). Gene ontology (GO) enrichment analysis of the top 400 most downregulated genes also revealed genes associated with transcription and mRNA processing (Supplementary Fig. 3f).

To further elucidate the effect of CDK12/13 inhibition on RNA synthesis, we first analyzed the changes in nascent RNA expression over gene bodies. Average meta-gene analysis of protein-coding genes and all classes of long noncoding RNAs demonstrated a pronounced increase of TT-seq signals both upstream and downstream of the transcription start sites (TSS) (Fig. 2a). Since increased Pol II pausing has recently been shown to inhibit new transcription initiation,49,50 this result led us to ask whether pausing was affected by CDK12/13 inhibition by calculating the change in nascent transcript read density over regions flanking the TSS (−500 to 1000 bp) following THZ531 treatment. This analysis showed a gradual increase in TT-seq reads, with peak signal accumulation occurring 1000 bp downstream of the TSS—well beyond known Pol II pausing sites (20–100 bp)31 (Fig. 2b, Supplementary Fig. 4a). Moreover, the rate at which the change in TT-seq signals occurred following THZ531 treatment, calculated by comparing the difference in accumulation between consecutive 50 bp bins, continued to increase up to 250 bp beyond the TSS, after which a decrease was seen (Fig. 2b). Together, these observations suggest that THZ531 treatment does not delay Pol II pause release; in fact, in keeping with the recently proposed model29,30, pause release may even be increased, which in turn would account for the observed increase in initiation. The finding that upstream antisense RNAs (which are short-lived and do not undergo extensive processing) were also increased at the TSS (Fig. 2a) supports this notion. After the initial 5′ increase in read density, a rapid loss of reads from the 5′-ends to the 3′-ends of genes was seen (Fig. 2a), with a net average loss of read density of around 6 kb 3′ of the TSS (Supplementary Fig. 4a). Together, these findings point to an elongation defect upon CDK12/13 inhibition.

THZ531 induces a gene length-dependent elongation defect. Because of the wide range in gene lengths throughout the human genome (<1 kb to >1 Mb) and prior reports that CDK12 preferentially regulates the expression of long genes,6 we next determined whether this variable had any effect on the elongation defect seen with the CDK12/13 inhibitor. Notably, there was a significant correlation between gene length and downregulation of gene expression: the longer the gene, the more likely it was to be downregulated (Fig. 2c). To define this relationship further, we divided the downregulated genes into 4 quartiles based on the distribution of gene lengths [short (<9.9 kb), medium-short (9.9–26.4 kb), medium-long (26.4–64.5 kb) and long (>64.5 kb)]. As shown in Fig. 2d, the long genes consistently had the most pronounced elongation defect and, concomitantly, the greatest transcriptional downregulation. When we restricted our differential gene expression analysis to protein-coding genes and nascent RNA reads that fell within exonic regions, and compared the results against these unbiased gene length groups, we observed that 362 (7%) of 5110 longer genes (202 long and 160 medium-long) were downregulated, while only 111 (2%) of 4895 shorter genes (14 medium-short and 97 short) were upregulated (adjusted p < 0.05; log2 fold change <−1). GO analysis of these genes showed that the top categories comprised DDR genes (Fig. 2e). This length-dependent elongation defect was not observed for long or short noncoding RNAs, although global downregulation of the latter was observed (Supplementary Fig. 4b). In contrast to longer genes, we observed that short or very-short (<3.4 kb) genes were upregulated or transcribed normally and showed significant 3′ UTR increase and extension (Supplementary Fig. 4c). This subgroup was enriched for replication-dependent (RD) histone genes (n = 72) (Fig. 2f) which do not normally rely on polyadenylation for transcription termination but on stem-loop binding22,23, a process regulated by Pol II Thr4P that was profoundly decreased following THZ531 treatment (Supplementary Fig. 4d, 1b). This effect (validated by qRT-PCR of total and nascent RNA) was also prominent in the amplified and over-expressed MYCN oncogene (6.4 kb long) and explained its lack of downregulation in MYCN-amplified cells treated with THZ531 (Supplementary Fig 4e, f). Together, these results suggest that CDK12/13 inhibition with THZ531 leads to an elongation defect which predominantly involves genes within the longer length categories, with normal or increased expression of shorter genes.

CDK12/13 inhibition leads to PCPA. The gradual decrease in nascent RNA reads from the 5′ to the 3′ ends of long genes with THZ531 treatment implied a possible termination defect. To pursue this notion, we performed global poly(A) 3′-sequencing of cells treated with THZ531. The majority (69%) of the identified poly(A) peaks were associated with known upstream polyadenylation site (PAS) motifs, the most abundant being the canonical AATAAA motif (Supplementary Fig. 5a). In agreement with their nascent RNA expression profiles (Fig. 2e, f), transcripts associated with long genes showed a loss of annotated terminal or 3′ poly(A) sites (Fig. 5a, left), while short transcripts, such as those of the histone processing genes and MYCN, terminated at distal unannotated poly(A) sites (Fig. 5a, right, Supplementary Fig. 4e). These findings suggest a transcription termination defect following CDK12/13 inhibition, with differential effects based on gene length.

Given the decrease in annotated terminal poly(A) sites, we attributed the genome-wide elongation and termination defects in THZ531-treated cells to premature cleavage and polyadenylation (PCPA). Transcription of most protein-coding genes is terminated at the 3′ ends of genes through cleavage and polyadenylation, whereas premature transcription termination such as PCPA occurs within a short distance from the TSS and results in the production of truncated transcripts34–36. We therefore analyzed the distribution of poly(A) 3′-seq reads for all protein-coding genes across the genome, observing a time-dependent increase in polyadenylated sites at the 5′ proximal ends of genes following CDK12/13 inhibition (Fig. 3b). Further study of the poly(A) sites that were differentially utilized between THZ531- and DMSO-treated cells focused on those that showed at least a 2-fold change (Supplementary Fig. 5b). This approach revealed strikingly different 3′-peak distributions in THZ531-treated compared with DMSO-treated cells, with significant enrichment in intronic regions (60% vs. 36%) and an almost complete absence of reads at annotated transcription end sites (TES) (4% in THZ531-treated vs. 20% in DMSO-treated cells; Supplementary Fig. 5c), an effect that was most prominent for the top 5000 differential poly(A) peaks between the two samples (Fig. 3c). Together, these findings indicate that CDK12/13 inhibition in NB cells causes generalized
PCPA with the use of cryptic intronic polyadenylation sites. Interestingly, the THZ531-induced effect at the nascent RNA level was computationally inferred as occurring as early as 2 h post-treatment, with PCPA apparent in 809 (7%) of the 11,902 protein-coding genes containing at least one intron (Fig. 3d). Poly(A) 3′-seq data showed that more than half of these genes underwent early termination in the first two introns/exons (59%, 476/809) and almost three-quarters in the first four introns/exons (73%, 587/809) (Fig. 3d). Integrative analysis of TT-seq and poly(A) 3′-seq data at the 5′ proximal regions (−1 to +1 kb of TSS) revealed that the aberrant accumulation of 5′ proximal TT-seq reads coincided with the peaks of proximal 3′ poly(A) usage, implying that most transcripts were terminated early at the beginning of productive elongation (Supplementary Fig. 5d). This conclusion was further supported by the inverse correlation between nascent reads along the 5′ proximal regions and the usage of proximal poly(A) sites in THZ531-treated cells, suggesting a high probability of proximal poly(A) site usage that gradually diminishes when elongation is terminated due to PCPA.

The THZ531-induced termination defect is due to CDK12 loss. We next asked whether the observed effects on termination through PCPA could be assigned specifically to CDK12 or 13 by genetic depletion (shRNA KD) followed by poly(A) 3′-sequencing. CDK12-depleted cells displayed the highest and most significant increase in poly(A) 3′-sequencing reads at the 5′ proximal ends of genes compared to control shRNA-expressing cells (Supplementary Fig 5e). Although depletion of CDK13 also resulted in an increase in 5′ proximal reads, this effect was significantly lower than that seen with CDK12 depletion (Supplementary Fig 5e). Only CDK12-depleted cells showed an increased usage of intronic poly(A) sites; this phenomenon was not evident in CDK13-depleted cells (Supplementary Fig 5f). Importantly, THZ531 treatment in Kelly E9R cells with the THZ531-binding site mutation did not display any increase in 5′ proximal reads (Fig. 3e) or in intronic poly(A) site usage compared to wild-type Kelly cells (Fig. 3f), suggesting that targeting of CDK13 alone was not sufficient to induce the PCPA defect. The gene length-dependent decrease in nascent RNA expression observed following THZ531 treatment (Fig. 2c) was also noted in cells with CDK12 shRNA depletion, but not in cells with CDK13 shRNA KD or in E9R cells treated with THZ531 (Supplementary Fig. 5g). Together, these results further identify PCPA as the main defect resulting from THZ531 treatment, an outcome that is mediated primarily by its targeting of CDK12.

CDK12/13 inhibition induces minimal splicing alterations. Because previous studies point to a role for CDK12 in splicing regulation, we determined whether aberrant splicing could explain the elongation defect seen with THZ531 treatment.

Fig. 4 CDK12/13 inhibition results in minimal splicing alterations. a Diagrammatic representation (left) and bar plot of splicing events (right) observed in TT-seq analysis of NB cells treated with THZ531 (400 nM) for 2 h. b Scatterplot of intron retention index (IR index) vs. the ratio of exon and intron lengths in log2 scale. Genes with an IR index >1 or ≤-1 display intron retention and loss respectively (adjusted p < 0.05, Fisher’s exact test). c Box plot illustrating the length distributions of genes that display intron loss or retention. The center line indicates the median for each data set. d Density plots illustrating the contributions of the proximal (first intron/exon) and distal (last intron/exon) gene regions in calculation of the IR index. Comparison of IR index distribution between proximal and distal intron/exon pairs (p = 0, Kolmogorov-Smirnov test)
Fig. 5 Gene length and a lower U1/PAS ratio predispose DDR genes to PCPA. 

(a) GO enrichment analysis of the 809 genes that underwent PCPA (FDR < 0.01) based on TT-seq analysis of cells treated with THZ531 (400 nM for 2 h). 

(b) Box plots and bar plots showing the distribution and numbers of PCPA and DDR genes in the different gene-length categories established in Fig. 2d (**p < 0.01, Fisher’s exact test). The center line indicates the median for each data set. 

(c) TT-seq and poly(A) 3′-seq tracks at the BLM DDR gene locus depicting the loss of annotated terminal polyadenylation signal and the presence of early termination due to PCPA in cells treated with THZ531 as in (a). 

(d) Number of intronic poly(A) sites as a function of transcript length. A polynomial regression curve is plotted for all genes (black) and DDR genes only (red) (p = 1.7e−13, predicted vs. observed, Wilcoxon rank-sum test).

(e) Box plots comparing the indicated determinants of PCPA in all genes vs. PCPA genes only and the proportion of DDR genes within the latter subset (see figure for p and d values; Wilcoxon rank-sum test & Cohen’s d effect-size, respectively). The black and red center lines indicate the median of all PCPA and DDR genes respectively.

(f) Cumulative fraction plot showing the change in expression of PCPA (p = 2.2e−16, Kolmogorov-Smirnov test) and DDR (p = 1.9e−14, Kolmogorov-Smirnov test) transcripts relative to other transcripts following THZ531 treatment as in (a).
Analysis of the nascent transcriptomic data showed that in general, there was a paucity of significantly altered splicing events following THZ531 treatment. The largest proportion of splicing defects comprised intron retention (13.4%), followed by alternative 5′ and 3′ splicing (4.7% and 4.8% respectively), while skipped and mutually exclusive exons were rarely observed (Fig. 4a). To further investigate intron retention, we calculated the intron retention (IR) index (log2 ratio of intron vs. exon TT-seq signal coverage differences between THZ531- and DMSO-treated cells; see Methods), and noted overall intron loss (642 of 11,155 protein-coding genes, 5.7%) together with a low exon/intron length ratio (IR < 1) (Fig. 4b) in genes that were downregulated by THZ531, in fact, suggestive of increased splicing efficiency. Importantly, this effect was seen primarily at long genes. Short
genes, on the other hand, were characterized by intron retention (156 of 11,155 genes, 1.3%) and a high exon/intron length ratio (IR > 1, adjusted \( p < 0.05 \)) (Fig. 4c). We reasoned that the apparent increased splicing efficiency in long genes was likely not due to a more efficient spliceosome, but rather, a secondary effect of the severe elongation defect seen within these genes (Fig. 2a, d). To pursue this hypothesis, we calculated the individual IR indices for the combination of the first exon/intron and last exon/intron length-ratios of the long genes that displayed intron loss, observing a greater intron loss for the last exon/intron compared to that of the first exon/intron (Fig. 4d). These results suggest that the lack of intron coverage at the 3' end in longer genes was likely due to defective elongation together with the reduced formation of such long transcripts following THZ531 treatment.

**Gene length and the U1 snRNP/PAS ratio influence PCPA.**

Genes that underwent THZ531-induced PCPA were significantly longer than genes that did not undergo this change, as might be expected from the elongation defect in the long gene group (>64.5 kb; Fig. 2d, Supplementary Fig. 6a). Importantly, the group of long genes that underwent PCPA was specifically enriched for DDR genes, such as **BARD1** and **BLM**, with respective lengths of 84 and 98 kb (Fig. 5a–c, Supplementary Fig. 6b). We validated this finding through 3' RACE of the **BARD1** transcript in THZ531-treated cells (Supplementary Fig. 6c). Interestingly, we noted that DDR genes undergoing PCPA as a result of CDK12 inhibition had a statistically higher number of intronic poly(A) sites relative to other genes of similar length (Fig. 5d), indicating that gene length alone does not fully explain the specific vulnerability of this subset of genes to early termination. Hence, to assess the relative contribution of gene length to the early termination phenotype observed after THZ531 treatment, we tested other determinants known to influence co-transcriptional processing\(^{36,39,40}\). Apart from longer gene length, we noted that a longer first intron, a larger number of introns, higher gene expression, lower GC content and a lower U1 snRNP/PAS ratio were also associated with early termination due to PCPA, with the latter two features emerging as the most significant based on effect size (Fig. 5d, e).

The U1 snRNP complex prevents premature termination through recognition and inhibition of cryptic poly(A) sites\(^{33-37,41}\). Indeed, Oh et al.\(^{37}\) demonstrated that direct depletion of U1 in HeLa cells using morpholinod KD results in decreased expression of long genes. We observed a significant overlap between genes that underwent PCPA in this data set and those that were similarly affected by THZ531 treatment, even though they represent two different cancer cell types and were studied at different time points after perturbation of different targets – U1 at 4 and 8 h\(^{37}\) and CDK12 at 2 h (this study) (Supplementary Fig. 7a; Supplementary Data 4). This finding is supported by the significantly increased usage of intrinsic poly(A) sites in DDR genes, even when compared with the genome-wide increase that was observed following THZ531 treatment in wild-type Kelly NB cells (Supplementary Fig. 7b, left; Fig. 3f). Importantly, no such change was seen in Kelly E9R THZ531-resistant cells (Supplementary Fig. 7b, right). In addition, genes that showed increased intronic poly(A) site usage following THZ531 exposure were enriched for GO categories associated with DNA damage (Supplementary Fig. 7c), and their expression was significantly reduced in WT compared to E9R cells expressing the Cys1039 mutation (Supplementary Fig. 7d, e). In conclusion, these observations indicate that CDK12 inhibition leads to premature termination that depends on gene length and the U1 snRNP/PAS ratio and may provide an explanation for the selective effects of this transcriptional kinase on DDR gene expression (Fig. 5f).

**CDK12/13 phosphorylates RNA processing proteins.**

Our results demonstrate the effect of CDK12 inhibition on transcription elongation and identify PCPA as a potential explanation for this selectivity. Given that the transcriptional activity of Pol II and processing of nascent transcripts occur simultaneously\(^2\), we hypothesized that the CDK12 and/or 13 kinases may regulate the phosphorylation of targets other than the Pol II CTD, and could contribute to cotranscriptional RNA processing. To address this question, we performed phosphoproteomics analyses of cells treated with and without THZ531 using stable isotope labeling with amino acids in cell culture (SILAC). This study revealed a ≥2-fold increase of 88 phosphopeptides and a similar decrease in 129 sites (\( p < 0.1 \); Student's t-test; Fig. 6a, Supplementary Data 5). The majority of phosphorylation sites that decreased in abundance upon THZ531 treatment occurred at serine or threonine residues, usually with a proline in the +1 position—the minimal consensus recognition site for all CDKs\(^{42}\) (Supplementary Fig. 8a). Protein interaction network analysis of all identified substrates clustered into two groups, the larger of which contained phosphorylated proteins centered on Pol II, while the other consisted of phosphorylated proteins that interact directly with CDK12 (Supplementary Fig. 8b). Interestingly, proteins encoded by DDR genes were not significantly represented in this analysis, suggesting that CDK12 may not directly regulate DDR protein phosphorylation. GO analysis of candidate CDK12 substrates that were significantly decreased in abundance after THZ531 treatment revealed mRNA processing factors as the top category, accounting for more than 50% of the identified phosphopeptides (Fig. 6b). Interestingly, one of the top mRNA processing factors was the small nuclear ribonucleoprotein SNRNP70, which associates with U1 as part of the U1 snRNP complex\(^{43}\) (Fig. 6a). Other top phosphoproteins that were affected by CDK12/13 inhibition included the PRP19 complex protein\(^{44,45}\), CDC5L with roles in RNA splicing and genomic stability and SF3B1, a...
component of the splicing machinery that is involved in pre-mRNA splicing. We confirmed the phosphorylation of these candidates using ^32P-labeled ATP in vitro kinase assays using GST-tagged substrates together with CDK12/Cyclin and CDK13/Cyclin (Supplementary Fig. 8c). Similar to CDK12, CDK13 phosphorylated the substrate proteins in a time-dependent manner (Fig. 6c). Of note, CDK12-mediated phosphorylation resulted in a higher rate of ^32P incorporation for CDC5L and SF3B1, suggesting that CDK12 phosphorylates more sites in these substrates than CDK13 (Fig. 6c). Additionally, control experiments without the addition of either kinase revealed that phosphorylation of CDC5L and SF3B1 was significantly below that measured in the presence of the active kinases. Next, we repeated the kinase assays after pre-treatment of the CDK/cyclin complex with THZ531, noting reduced phosphorylation of the CDC5L and SF3B1 substrate proteins with increasing concentrations of the inhibitor (Fig. 6d). Finally, to identify the exact sites phosphorylated by CDK12/Cyclin in the in vitro kinase assays, we performed peptide mass fingerprint analyses of the recombinant protein substrates, which confirmed the following phosphorylation sites identified in the SILAC analysis: CDC5L (pT396), SF3B1 (pT326), (Supplementary Fig. 8d, Supplementary Data 6). Together, these results suggest that both CDK12 and 13 phosphorylate pre-mRNA processing factors that could affect their recruitment to Pol II.

**Discussion**

In this study, we took advantage of the selectivity and irreversibility of a covalent inhibitor of CDK12/13 to dissect the early alterations in cotranscriptional RNA processing in NB cells. Using nascent RNA and poly(A) 3′-sequencing, we demonstrate that such inhibition leads to a gene length-dependent elongation defect associated with early termination through PCPA (Fig. 6e). Especially vulnerable to this defect were long genes with a lower ratio of U1 snRNP binding to poly(A) sites, which include many of those involved in the DDR. Conversely, short genes showed an increased likelihood of intron retention and 3′ UTR extension or, as in the case of the non-polyadenylated replication-dependent histone genes, the generation of polyadenylated transcripts. We further identified CDK12 as the predominant kinase mediating the transcriptional effects of THZ531 in treated cells. NB cells harboring a point mutation at the CDK12 Cys1039 binding site of the transcriptional effects of THZ531, noting reduced phosphorylation of the CDC5L and SF3B1, component of the U1 snRNP complex, was identified as a potential phosphorylation substrate of CDK12/13 in our study; hence, it is quite possible that its decreased phosphorylation could partly account for the increased usage of alternate polyadenylation sites in DDR and other long genes. This could also explain why in contrast to findings in other studies implicating CDK12 in splicing regulation, CDK12 inhibition did not lead to major splicing alterations, most likely because transcription was terminated well before it reached the 3′ splice sites.

As shown schematically in Fig. 6e, we propose that CDK12 inhibition leads to an increased probability of using cryptic intronic poly(A) sites and undergoing PCPA, possibly due to a slowing of productive Pol II elongation. As such, long genes with low U1 snRNP/PAS ratios, such as DDR genes, are especially vulnerable to this loss, yielding an aborted elongation phenotype, manifested at the 3′ ends of these genes. Most importantly, our analysis demonstrates that CDK12 by itself lacks any intrinsic preference for DDR genes; instead, the structural properties of the gene target determine its sensitivity to CDK12 inhibition, and many DDR genes possess the requisite features. Not only was gene length a significant contributor to the PCPA phenotype, but the DDR genes significantly affected by CDK12 inhibition harbored more intronic poly(A) sites than expected based on their longer gene lengths. DDR genes that evaded PCPA were those with genetic determinants that did not favor this process—such as shorter lengths, a short first intron and decreased numbers of introns. Future work is needed to resolve why so many genes involved in DNA repair have this genetic composition compared to the genome-wide background.

In conclusion, by inducing an RNA Pol II elongation defect and subsequent usage of proximal poly(A) sites that led to premature cleavage and polyadenylation of long DDR genes, we were able to clarify the mechanism by which THZ531 selectively abolishes the DDR in NB cells, which are highly dependent on adequate DNA repair function for their survival. Dubbury et al. recently examined the later effects of CDK12 depletion on total RNA expression in mouse ES cells, showing that CDK12 suppresses intronic polyadenylation as a mode of DDR gene regulation. The authors found that this mechanism is preserved in ovarian and prostate tumors with CDK12 loss-of-function mutations or deletions. Our findings augment those of the Dubbury study by (i) linking the unique susceptibility of DDR genes to CDK12 inhibition with their relatively longer lengths, lower GC content and lower ratios of U1 snRNP binding sites to intronic polyadenylation sites, and (ii) showing that these transcriptional effects occur at the nascent RNA level as early as 2 h after CDK12 loss. Thus, effects attributed to CDK12 loss do not
appear to be restricted to cancers with loss-of-function mutations, but encompass those with severe underlying DNA damage, such as NB. Moreover, as recently demonstrated in a subset of prostate cancers with CDK12 loss-of-function mutations35, the PCPA, as well as intron retention, observed with CDK12 inhibition could facilitate the formation of neoantigens that might be exploited to improve immune therapies or to develop personalized cancer vaccines34. The extent to which these observations apply to other genomically unstable cancers lacking CDK12 loss-of-function mutations will be pivotal in generating molecular rationales for the therapeutic targeting of CDK12 across a broad cross-section of vulnerable tumors.

**Methods**

**Cell culture.** Human neuroblastoma (NB) cells (Kelly, IMR-32, IMR-5, LAN-1, LAN-5, NGP, SK-N-AS, SH-SY5Y, CHLA-20, CHLA-15, and SK-N-FI) were obtained from the Children’s Oncology Group cell line bank and genotyped at the DFCI Core Facility. The cell lines were authenticated through STR analyses. The Kelly E9R NB cell line harbors a single point mutation in CDK12 at the cysteine 12–20 °C. This mutation was acquired spontaneously in Kelly NB cells upon exposure to escalating doses of CDK12 inhibitor, E9 over the course of few months as previously reported30. Human lung (IMR-90) and skin fibroblasts (BJ) were kindly provided by Dr. Richard Gregory (Boston Children’s Hospital). NIH3T3 cells were purchased from the American Type Culture Collection (ATCC). The cell lines were authenticated through STR analyses. The NIH3T3 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS. IMR-90, BJ, and NIH3T3 cells were grown in DME (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were routinely tested for mycoplasma.

**Compounds.** THZ531 was prepared by Dr. Nathanael Gray’s laboratory4. 

**Cell viability assay.** Cells were plated in 96-well plates at a seeding density of 4×10^4 cells/well. After 24 h, cell cultures were transfected with increasing concentrations of THZ531 (10 nM to 10 µM), DMSO solvent without compound served as a negative control. 72 h incubation, cells were analyzed for viability using Glo Luminescence Cell Viability Assay (Promega) according to the manufacturer’s instructions. All proliferation assays were performed in biological triplicates and error bars represent mean ± SD. Drug concentrations that inhibited 50% of cell growth (IC_{50}) were determined using a nonlinear regression curve fit using GraphPad Prism 6 software.

**Fluorescence-activated cell sorting analysis (FACS).** For cell cycle and DNA damage analysis, cells were treated with DMSO or THZ531, 400 nM. After 2.5 h, cells were trypsinized and fixed in ice-cold 70% ethanol overnight at −20 °C. After washing with ice-cold phosphate-buffered saline (PBS), cells were incubated in PBS-0.5% Tween supplemented with 50% ACN/2 M lactic acid and twice with 50% ACN/0.1% TFA. Phospho-peptides were dissolved in 50% acetonitrile (ACN; Honeywell)/2 M lactic acid (Lee Biosolutions), incubated with 1.25 mg TiO2 microspheres (GL Sciences) per 1 mg of protein. After 2 h, light-labeled cells were incubated in THZ531 (400 nM) for 45 min and then washed with 0.05% Tween-20 and 5% BSA (PBS-Tween-BSA) for 1 h to block nonspecific binding. Cells were then incubated overnight at 4 °C with anti-RAD51 primary antibody in PBS-Tween-BSA, extensively washed and incubated for 45 min with AlexaFluor 488-conjugated secondary antibody and counterstained with DAPI. Images were acquired on a Zeiss AXIO Imager Z1 fluorescence microscope using a x63 immersion objective, equipped with AxioVision software. Nuclei with >5 RAD51 foci were considered positive and 100 nuclei per condition were analyzed.

**Target engagement assay.** Cells were treated with THZ531 or DMSO for 6 h at the indicated doses. Subsequently, total cell lysates were prepared as for western blotting. To IP CDK12 and CDK13, 1 mg and 4 mg, respectively of total protein was incubated with 1 µM of biotin-THZ531 at 4 °C overnight. Subsequently, lysates were incubated with streptavidin agarose (30 µl) for 2 h at 4 °C. Agarose beads were washed 3x with cell lysis buffer and boiled for 10 min in 2x gel loading buffer. Proteins were resolved by WB. Fifty microgram of total protein was used as a loading control.

**Stable isoform labeling by amino acids in cell culture (SILAC).** IMR-32 and Kelly cells were grown in arginine- and lysine-free RPMI with 10% dialyzed FBS supplemented with either 1\(^{13}\)C\(_6\), 1\(^5\)N\(_2\) lysine (100 mg/ml) or 1\(^{13}\)C\(_6\), 1\(^5\)N\(_4\) arginine (100 mg/l) (Cambridge Isotope Laboratories, Inc.) (heavy population) or identical concentrations of isotope normally labeled lysine and arginine (light population) for at least six cell doublings. Heavy-labeled cells were incubated in THZ531 (400 nM) for 2 h and light-labeled cells were incubated in DMSO solvent as a control. After inhibitor treatment, cells were collected by trypsinization and counted. Equal numbers of heavy and light cells were mixed, washed twice in PBS, snap-frozen, and stored at −80 °C prior to lysis.

**Phosphopeptide purification.** Phosphopeptide enrichment was performed using titanium dioxide microspheres as previously described35. Briefly, lyophilized phosphopeptides were dissolved in 50% acetonitrile (ACN; Honeywell)/2 M lactic acid (Lee Biosolutions), incubated with 1.25 mg TiO2 microspheres (GL Sciences) per 1 mg pericellular lysate and vortexed at 75% power for 1 h. Microspheres were washed twice with 50% ACN/2 M lactic acid and twice with 50% ACN/0.1% TFA. Phosphopeptides were eluted with 50 mM K2HPO\(_4\), Sigma pH 10 (adjusted with ammonium hydroxide; Sigma). Formic acid (EMD) to 90% was added to obtain a final concentration of 1.7%. The acidified phosphopeptides were desalted using a C18 solid-phase extraction (SPE) cartridge and the eluate was vacuum centrifuged to dryness.

**Offline HPLC pre-fractionation.** Approximately 120 µg phosphopeptides were resuspended in 0.1% TFA (trifluoroacetic acid) and ion-exchanged using an anion exchange chromatography as previously described36. The 48 collected fractions were reduced to 16 by combining every 16th fraction, vacuum centrifuged to dryness and stored at −80 °C prior to analysis by LC-MS/MS.

**LC-MS/MS analysis.** LC-MS/MS analysis was performed on an Orbitrap Fusion Trubrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a EASY-nL 1000 ultra-high pressure liquid chromatograph (ThermoFisher Scientific, Wallingford, MA). Phosphopeptides were dissolved in loading buffer (5% methanol (Fisher)/1.5 % formic acid) and injected directly onto an in-house pulled polymer coated fritless fused silica analytical resolving column (40 cm length, 100 µm inner diameter; PolyMicro) packed with ReproSil, C18 AQ 1.9 µm 120 Å pore polymer coated fritless fused silica analytical resolving column (40 cm length, 100 µm inner diameter; PolyMicro) packed with ReproSil, C18 AQ 1.9 µm 120 Å pore

**Data analysis.** Phosphopeptides were quantified with Progenesis software (Becton Dickinson). A minimum of 50,000 events was counted per sample and used for further analysis. Data were analyzed using Flowjo software.

**shRNA Knockdown.** pLKO.1 plasmids containing shRNA sequences targeting CDK12 (sh1#: TRCN0000001795; sh2# TRCN000001972), CDK13 (sh1#: TRCN000000701; sh2# TRCN000000704) and GFP were obtained from the RNAi Consortium of the Broad Institute (Broad Institute, Cambridge, MA), knocked down as previously described34,35. Briefly, cells were transfected into HEK293T cells with helper plasmids: pCMV-ΔR8.91 and pMD2.G-VSV-G for virus production. Cells were then transduced with virus, followed by puromycin selection for two days.

**Western Blotting.** Cells were collected by trypsinization and lysed at 4 °C in NP40 buffer (Invitrogen) supplemented with complete protease inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche) and PMSF (1 mM). Protein concentrations were determined with the Biorad DC protein assay kit (Bio-Rad). Whole cell protein lysates were resolved on 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). After blocking nonspecific binding sites for 1 h using 5% dry milk (Sigma) in Tris-buffered saline (TBS) supplemented with 0.2% Tween-20 (TBS-T), membranes were incubated overnight with primary antibody at 4 °C. Chemiluminescent detection was performed with the appropriate secondary antibodies and developed using Genemate Blue ultra-autoradiography film (VWR). Uncovered versions of all western blots can be found in Supplementary Fig. 9.
Peptide spectral matching and bioinformatics. Raw data were searched using COMET\(^\text{TM}\) against a target-decoy version of the human (Homo sapiens) proteome sequence database (Uniprot; downloaded 2013: 202,241 total proteins) with a precursor mass tolerance of ±0.10 Da and requiring fully tryptic peptides with up to 3 missed cleavages, carboxymethyl cysteine as a fixed modification and oxidized methionine as a variable modification. For SILAC experiments, the additional masses of lysine and arginine isoïne labels were searched as variable modifications. Protein phosphorylation (both serine, threonine and tyrosine) were searched with up to 4 variable modifications per peptide, and were localized using the phosphoRS algorithm\(^\text{88}\). The resulting peptide spectral matches were filtered to <1% false discovery rate (FDR) by defining thresholds of decoy hit frequencies at particular mass measurement accuracy (measured in parts per million from theoretical), Xcorr and delta-Xcorr (ΔCn) values.

Antibodies. The following antibodies were used: RNP11 CTD SS (Bethyl cat# A300-655A, 1:10,000); RNP11 CTD 5S (Bethyl cat# A300-655A, 1:10,000); RNP11 (Santa Cruz cat# sc-899, 1:1000); RNP11 CTD7 (Millipore cat# 041570, 1:10,000); RNP11 CTD Thr4 (Active Motif cat# 63161, 1:1000) cleaved PARP (Cell Signaling cat# 9541, 1:1000); β-Actin (Cell Signaling cat# 4967, 1:4000); CDK12 (Cell Signaling cat# 11973 s, 1:1000); CDK13 (BD Transduction cat# A30-458A, 1:1000); CDK13 antibody (1:1000) used for WB in Supplementary Fig. 2e was kindly provided by Dr. Arne Greenleaf; γ-H2AX (Cell Signaling cat# 5718, 1:2000); RAD51 (GeneText cat# GTX70230, 1:1000), BRCA1 (Cell signaling cat# 9010S, 1:500); BARD1 (Santa Cruz Biotechnology cat# sc11438, 1:500), Alexa-488 (Molecular Probes cat#A11008, 1:500).

RT-PCR. Total RNA was isolated from the RNNeasy Mini kit (QIAGEN). One µg of purified RNA was reverse transcribed using Superscript III First-Strand (Invitrogen) with random hexamer primers following the manufacturer’s protocol. Quantitative PCR was carried out using the QuantFast SYBR Green PCR kit (Qiagen) and analyzed on an Applied Biosystems StepOne Real-Time PCR System (Life Technologies). Each individual biological sample was qPCR-amplified in technical triplicate and normalized to GAPDH as an internal control. Relative quantification was calculated according to the ΔΔCt relative quantification method. Error bars indicate ± SD of three replicates. Primers sequences are available on request.

RNA extraction and synthetic RNA spike-in for gene expression analysis. Cells were treated with 400 nM of THZ331 or with DMSO for 6 h. Cell numbers were determined prior to lysis and RNA extraction. Biological duplicates (5 million cells per replicate) were collected and homogenized in 1 ml of TRIzol Reagent (Invitrogen) and purified using the mirVANA miRNA isolation kit (Ambion) following the manufacturer’s instructions. Total RNA was treated with DNA-free™ DNase I (Ambion), spike-in with ERCC RNA Spike-In Mix (Ambion), and analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies) for integrity. RNA was hybridized to Affymetrix GeneChip® PrimeView Human Gene Expression arrays (Affymetrix).

Transient transcriptome sequencing. Cells were treated with DMSO or 400 nM of THZ331 for 30 min and 2 h. Cells were labeled in media for 10 min with 500 µM 4-thiouridine (4sU, Sigma-Aldrich). RNA extraction was performed with TRIzol (Ambion) following the manufacturers’ instructions. Total RNA was treated with DNAse I (Invitrogen). Subsequently, the purified RNA was fragmented on a BioRuptor Next Gen (Diagenode) at high power for one cycle of 30/30° ON/OFF. Fragmented samples were subjected to labeled RNA purification as previously described\(^\text{88}\). Labeled fragmented RNA was spike-in with ERCC RNA Spike-In Mix (Ambion) and analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies) for integrity. Sequencing libraries were prepared with the RNA-seq library kit (TruSeq Stranded Total RNA RiboZero Gold, Illumina) as per the manufacturers’ instructions. All samples were sequenced on a HiSeq 2500 sequencing.

Poly(A) 3’-sequencing data processing. For each sample, paired-end 75 bp reads were obtained and mapped to the human genome (GRCh38) and ERCC spike-in sequences. An average of 50 M (~90%) read pairs were mapped with STAR (version STAR_2.5.1b_modified) with default parameters. Only high quality and properly paired reads were retained for further analysis using samtools (v1.3.1) with parameters “-q 7 and –fr 7,” normalized for sequencing depth variation, individual spike-in reads were counted with samtools tools. This was used as input to calculate a sample-specific size factor with estimateSizeFactorsForMatrix (DESeq2). To create strand-specific sample coverage profiles in 100 bp bins, we used bamCoverage (DeepTools v2.5.4) with previously calculated size factors and parameters “–scale-factor=normalizerUsingRPKM --filterRNAstrand=+” and the following parameters “-outFilterType BySJout --outFilterMultimapNmax 20 20 minlength 20 qtrim 20 /30 qtrim /30 qtrim /30 qtrim /30 100 --nomodel --extsize 100 --shift 0”. To create strand-specific sample coverage profiles in 50 bp bins, we used bamCoverage (DeepTools v2.5.4) with parameters “–normalizeUsingRPKM --filterRNAstrand=+” and the following parameters “-outFilterType BySJout --outFilterMultimapNmax 20 20 minlength 20 qtrim 20 /30 qtrim /30 qtrim /30 qtrim /30 50 --nomodel --extsize 100 --shift 0”. Genome-wide correlation of biological replicates was calculated using Spearman’s rank coefficient and visualized using scatterplots and heatmaps. These results showed high reproducibility for each condition and hence, for all analyses except differential expression and transcript usage, replicates were merged using samtools merge and processed again as described for the individual replicates.

Peptide mass fingerprinting. GST-tagged proteins were resolved on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue. Protein bands were cut from the SDS-PAGE gel and submitted for mass spectrometry analysis to the Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, Gottingen, Germany.

3’ RACE PCR. Cells were treated with 400 nM of THZ331 or with DMSO for 6 h. RNA extraction was performed with TRIZol (Ambion) following the manufacturers’ instructions. Total RNA was treated with DNase I (Invitrogen). Subsequently, total RNA was poly(A) selected using oligo-dT dyneads (Invitrogen). RNA was reverse transcribed using 3’-RACE adapter oligonucleotide (FirstChoice RLM-RACE Kit; Life Technologies). Nested PCR was performed using Phusion High Fidelity DNA Polymerase (New England Biolabs). PCR products were resolved on a 1.3% agarose gel, purified using a gel extraction kit (Qiagen), and sequenced. PCR primer sequences available upon request.

Gene expression analysis. Microarray data were analyzed using a custom CDF file (GPL16043) that contained the mapping information of the ERCC probes used in the spike-in RNAs. The arrays were normalized according to previously described protocols\(^\text{85}\). Briefly, all chip data were imported in R (version 3.0.1) using the affy package\(^\text{62}\), converted into expression values using the expresso command, normalized to take into account the different numbers of cells and spike-ins used in the different experiments and renormalized using loss regression fitted to the spike-in probes. Sets of differentially expressed genes were obtained using the limma package\(^\text{86}\) and a false discovery rate (FDR) of 0.05. Statistical comparisons of distributions of fold changes were done using the Mann–Whitney U-test.

TT-sequencing data processing. For each sample, paired-end 75 bp reads were obtained and mapped to the human genome (GRCh38) and ERCC spike-in sequences. An average of 50 M (~90%) read pairs were mapped with STAR (version STAR_2.5.1b_modified) with default parameters. Only high quality and properly paired reads were retained for further analysis using samtools (v1.3.1) with parameters “-q 7 and –fr 7,” normalized for sequencing depth variation, individual spike-in reads were counted with samtools tools. This was used as input to calculate a sample-specific size factor with estimateSizeFactorsForMatrix (DESeq2). To create strand-specific sample coverage profiles in 100 bp bins, we used bamCoverage (DeepTools v2.5.4) with previously calculated size factors and parameters “–scale-factor=normalizerUsingRPKM --filterRNAstrand=+” and the following parameters “-outFilterType BySJout --outFilterMultimapNmax 20 20 minlength 20 qtrim 20 /30 qtrim /30 qtrim /30 qtrim /30 100 --nomodel --extsize 100 --shift 0”. Genome-wide correlation of biological replicates was calculated using Spearman’s rank coefficient and visualized using scatterplots and heatmaps. These results showed high reproducibility for each condition and hence, for all analyses except differential expression and transcript usage, replicates were merged using samtools merge and processed again as described for the individual replicates.

In vitro kinase assay. Recombinant CDK12/CycK complex was prepared from baculovirus infected Sf9 cells as described\(^\text{60}\). Substrate proteins (CatF64 (aa 509-577), CDC5L (aa 370-505), SPT6H (aa 1434-1544) and SF3B1 (aa113-462) were expressed as GST-fusion proteins in E. coli and purified to homogeneity. Radioactive kinase reactions were performed with 0.2 µM CDK12/CycK or CDK13/CycK and 100 nM each of substrate protein, and 1 nM ATP at 30 °C for 30 min in kinase buffer as described\(^\text{89}\). Reactions were spotted onto P81 Whatman paper squares, washed three times and radioactivity counted on a Beckman Scintillation Counter (Beckman-Coulter) for 1 min. Measurements were performed in triplicate and are represented as mean ± SD.
identify if these genes as a whole were more downregulated, 1000 random and equal-sized gene sets were generated and a distribution of the average level of expression change was plotted and compared to that of the initial DDR gene set to calculate a z-score.

Gene biotype and size selection. Gene biotypes assigned by Gencode were simplified in a two-step manner. First, genes with only gene biotypes with a minimum of 20 members were considered. Next gene lengths of all detected non-coding genes (i.e., excluding protein-coding) were clustered using kmeans in two groups (short vs. long non-coding genes). Together, this resulted in three groups selected on biotype and gene length: (1) protein-coding genes (2) long non-coding genes (lincRNA, antisense_RNA, processed_transcript, sense_intronic, transcribed_unitary_pseudogene, TEC (to be experimentally confirmed), transcribed_processed_pseudogene, transcribed_unprocessed_pseudogene, unprocessed_pseudogene), and (3) other non-coding genes (snRNA, scaRNA, snoRNA, Mt_tRNA, misc_RNA, processed_pseudogene, RNA). Protein-coding genes were further stratified into 4 length classes based on the quartiles of length distribution, i.e. long (>64.5 kb), medium-long (26.4–64.5 kb), medium-short (9.9–26.4 kb) and short (<9.9 kb). The latter group was further divided into three equal groups based on the 0.33, 0.66, and 1 quantiles of the only short gene length distribution, resulting in short (9.9–6.4 kb), very short (6.4–3.4 kb), and ultra-short (<3.4 kb) groups. Simple linear regression and Spearman correlation coefficient between log2 scaled length and log2 fold-change of exonic reads for genes was performed in R.

Metagene profiles. A gene metaprofile was created by dividing each gene (from TSS to TES) into 50 equally sized bins; 2 kb upstream and downstream flanking regions were binned in bins of 100 bp. Bedgraph files with normalized reads from TT-seq or poly(A) 3′-seq were used to calculate read density (RPM/bp) across those bins and subsequently summarized for all genes. To create a TSS or TES metaprofile, we followed an analogous approach to the downstream flanking regions and summarized bins of 50 bp. To compare TT-seq and poly(A) 3′-seq profiles, calculated read densities were rescaled between 1 and 100.

Inference of proximal RNA polymerase dynamics. Transcription dynamics were calculated in 50 bp bins in the 500 bp upstream and 10 kb downstream flanking regions of the TSS. Change in read accumulation was calculated by normalized TT-seq read subtraction (THZ531 – DMSO) and the first derivative was computed to obtain the rate of accumulation change. To overlay both calculations, the rate of change was rescaled to the minimum and maximum of the change values and a loss smoothing curve was then fitted for visualization purposes.

Correlation of transcript length and 3′ expression changes. To identify differentially expressed genes based on 3′ poly(A)-sequencing, all counts for 3′ UTR-associated polyadenylation sites were summarized per gene. This data matrix was log2 normalized and used to identify differential expression and fold-changes with the limma package in R. Correlation between fold-changes and transcript length was performed on the highest expressed transcript for each gene in the control condition. A generalized additive model (GAM) smoothing curve was fitted to each treatment to observe global changes and for visualization purposes.

PCPA analysis. Treatment-induced PCPA for protein-coding genes was calculated as in Oh et al.37 with minor modifications. To determine whether a gene exhibits a coverage profile expected with PCPA, two scores were calculated. First, for each gene, we calculated an exon-score to determine if there was an increased loss of reads at the last exon compared to the first exon with THZ531 treatment: last exon [log2 (THZ531_2h/DMSO)] – first exon [log2 (THZ531_2h/DMSO)]. Next, an iQ-score to determine if there was an increased number of reads in the first quarter (iQ1) of the region between a gene’s first 5′ splice site and the last 3′ splice site and the last quarter (iQ4) was calculated for each gene in the THZ531-treated samples: log2(iQ1/iQ4). Genes were considered to undergo THZ531-induced PCPA with an exon-score <1 and an iQ-score >1.

Intronic polyadenylation usage. For each transcript (TPM >1) the reads of all intronic and 3′ UTR-associated poly(A) sites were summarized. To compare the change and usage of intronic versus 3′ UTR-associated poly(A) sites between different treatments, an odds ratio (OR) was calculated for each treatment sample but excluding any poly(A) genes only. A Wilcoxon Rank Sum test was used to determine if the difference between predicted values for DDR genes between the two models [prediction DDR – prediction all genes] was significantly different.

Correlation of transcript length and number of intronic polyadenylation sites. To identify the relationship between the number of identified polyadenylation sites and transcript length, a polynomial regression curve (y = poly(x,2)) was fitted for all genes or DDR genes only. A Wilcoxon Rank Sum test was used to determine if the difference between predicted values for DDR genes between the two models [prediction DDR – prediction all genes] was significantly different.
Supplementary Figs. 1 available from the corresponding author upon request. Data underlying Figs. 1, 6 and 2147 was performed as previously described34 with modifications. In brief, position weight matrices (PWMs) for 5‘ and 3‘ splice sites were created using all introns that contain the established 5‘ GT and 3‘ AG sequence. For the 5‘ and 3‘ splice sites (ss) 9 (–2 bps bp of 5‘ ss) and 15 bp (–14 bp of 3‘ ss) respectively were used. Next, introns without and with intronic polyadenylation sites (intronic poly(A) > 0) were scored for both the 5‘ and 3‘ PWM for their respective splice sites. A combined score for each intron was computed by summarizing the scores of the 5‘ and 3‘ splice site. The Wilcoxon Rank Sum test and Cohen’s d effect size were used to determine biologically meaningful differences between introns with and without an intronic polyadenylation.

Enrichment analysis. Gene ontology enrichment for selected gene sets was performed using the enrichr package in R. The Enrichr score29 is the combined score of the adjusted p-value and the z-score using the Fisher’s exact test. Enrichment of individual gene sets was considered significant if the adjusted p-value < 0.01, unless stated otherwise. The Fisher’s exact test was used to determine significant overlap between other publicly available datasets.

Genomic visualization. To visualize coverage tracks a custom build visualization tool was used (github.com/RubD/GeTrackViz2).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Microarray, TT-seq, Poly(A) 3‘seq datasets have been deposited in the Gene Expression Omnibus (GEO), accession numberGSE113314. The SILAC dataset has been deposited in the ProteomeXchange Consortium, accession numberPXD009533. All other data are available from the corresponding author upon request. Data underlying Figs. 1, 6 and Supplementary Figs. 1–4 are provided as a Source Data file.

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References

1. Buratowski, S. The CTD code. Nat. Struct. Biol. 10, 679–680 (2003).
2. Bentley, D. L. Coupling mRNA processing with transcription in time and space. Nat. Rev. Genet. 15, 163–175 (2014).
3. He, C. K. & Shuman, S. Distinct roles for CDT Set-2 and Set-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol. Cell 3, 405–411 (1999).
4. Ramanathan, Y. et al. Three RNA polymerase II carboxyl-terminal domain protein families display distinct substrate preferences. J. Biol. Chem. 276, 10913–10920 (2001).
5. Bartkowiak, B. et al. CDK12 is a transcription elongation-associated CDT kinase, the metazoan ortholog of yeast Ctk1. Genes Dev. 24, 2303–2316 (2010).
6. Blazek, D. et al. The Cyclin K/CDk12 complex maintains genomic stability via emerging roles in metazoans. Nat. Rev. Genet. 13, 720–731 (2012).
7. Tian, B., Pan, Z. & Lee, J. Y. Widespread mRNA polyadenylation events in introns indicates dynamic interplay between polyadenylation and splicing. Genome Res. 17, 156–165 (2007).
8. Gao, Y. et al. Overcoming resistance to the THZ series of covalent transcriptional CDK inhibitors. Cell Chem. Biol. 25, 135–142 e135 (2018).
9. Martin, C. et al. The molecular interaction of the high affinity viral repressor X9576 with P-glycoprotein. Br. J. Pharmacol. 128, 403–411 (1999).
10. Harlen, K. M. et al. Comprehensive RNA polymerase II interactions reveal distinct and varied roles for each phospho-CTD residue. Cell Rep. 15, 2147–2158 (2016).
11. Hsin, J. P., Sheth, A. & Manley, J. L. RNAPII CTD phosphorylated on threonine-4 is required for histone mRNA 3‘ end processing. Science 334, 683–686 (2011).
12. Kwiatkowski, N. et al. Targeting transcription regulation in cancer with a CDK7 inhibitor (7T02) (2015).
13. Prakash, R., Zhang, Y., Feng, W. & Jasin, M. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. Cold Spring Harb. Perspect. Biol. 7, a016600 (2015).
14. Oh, J. M. et al. U1 snRNP transcription initiation. Elife 6, e29736 (2017).
15. Adelman, K. & Liu, J. T. Promoter- proximal pausing of RNA polymerase II: new emerging roles in metazoans. Nature 473, 73–80 (2012).
16. Harris, M. E. et al. Regulation of histone mRNA in the unperturbed cell cycle: evidence supporting control at two posttranscriptional steps. Mol. Cell. Biol. 11, 2416–2424 (1991).
17. Dominis, Z. & Marzluff, W. F. Formation of the 3‘ end of histone mRNA. Gene 239, 1–14 (1999).
18. Tian, B., Pan, Z. & Lee, J. Y. Widespread mRNA polyadenylation events in introns indicates dynamic interplay between polyadenylation and splicing. Genome Res. 17, 156–165 (2007).
19. Kaida, D. et al. U1 snRNP protects pre-mRNAs from premature cleavage and polyadenylation. Nature 468, 664–668 (2010).
20. Berg, M. G. et al. U1 snRNP determines mRNA length and regulates isoform expression. Cell 150, 53–64 (2012).
21. Oh, J. M. et al. U1 snRNP transcription regulates a size-function–stratified human genome. Nat. Struct. Mol. Biol. 24, 993–999 (2017).
22. Chen, H. H., Wang, Y. C. & Fann, M. J. Identification and characterization of the CDK12/cyclin L1 complex involved in alternative splicing regulation. Mol. Cell. Biol. 26, 2736–2745 (2006).
23. Heyn, P., Kalinka, A. T., Tomancak, P. & Neugebauer, K. M. Introns and gene expression: cellular contribution to splicing regulation, and evolutionary consequences. Bioessays 37, 148–154 (2015).
24. Zhang, J., Kuo, C. C. & Chen, L. GC content around splice sites affects splicing through pre-mRNA secondary structures. BMC Genom. 12, 90 (2011).
25. Almada, A. E., Wu, X., Kriz, A. J., Burge, C. & Sharp, P. A. Promoter directionality is controlled by U1 snRNP and polyadenylation signals. Nature 499, 360–363 (2013).
26. Nigg, E. A. Cellular substrates of p34cdc2 and its companion cyclin-dependent kinases. Trends Cell Biol. 3, 296–301 (1993).
27. Spritz, R. A. et al. Human U1-70K snRNP protein: cDNA cloning, chromosomal localization, expression, alternative splicing and RNA-binding. J. Biol. Chem. 268, 1045–1051 (1993).
28. Grote, M. et al. Molecular architecture of the human Prp19/CDC5L complex. Mol. Cell. Biol. 30, 2105–2119 (2010).
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Author contributions

M.K. designed and carried out molecular, cellular and genomic experiments. R.D. performed bioinformatics analyses. A.V.G. performed the SILAC experiments under the supervision of S.A.G. S.D. performed the in vitro kinase assays under the supervision of M.G. D.S.D. analyzed the microarray expression data. Y.G. established Kelly E3R cells and performed target engagement experiments with CDK13. N.K. provided protocols and assistance with target engagement studies. M.P. provided technical support with generation of CDK12/13 shRNA knockdowns. B.S. performed 3′ RACE PCR and provided technical support. E.C. performed the initial cell viability assays of THZ531. H.H. generated the consensus sequences from the SILAC data. O.R.D. performed viability assays under the supervision of M.K. T.H.Z. generated THZ531. A.L.G. provided CDK13 antibody and advice. G.-C.Y. supervised the bioinformatics analyses. N.S.G. and R.A.Y. provided feedback on study design and experimental results. M.K., R.D., and R.E.G. wrote the manuscript. R.E.G. conceived the project and supervised the research. All authors discussed the results and commented on the manuscript.

Additional information

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Competing interests: N.S.G. is a founder and equity holder of Syros Pharmaceuticals, C4 Therapeutics, Petra Pharma, Gatekeeper Pharmaceuticals and Soltego. R.A.Y. is a founder and shareholder of Syros Pharmaceuticals, Camp4 Therapeutics, Omega Therapeutics and Dewpoint Therapeutics. R.E.G. is on the SAB of Global Gene Corp. The remaining authors declare no competing interests.

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