Chromosomes and Gene Expression

Human promoter directionality is determined by transcriptional initiation and the opposing activities of INTS11 and CDK9

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Abstract

RNA polymerase II (RNAPII) transcription initiates bidirectionally at many human protein-coding genes. Sense transcription usually dominates and leads to messenger RNA production, whereas antisense transcription rapidly terminates. The basis for this directionality is not fully understood. Here, we show that sense transcriptional initiation is more efficient than in the antisense direction, which establishes initial directionality. After transcription begins, the opposing functions of Integrator (INTS11) and cyclin-dependent kinase 9 (CDK9) maintain directionality. Specifically, INTS11 terminates antisense transcription, whereas sense transcription is protected from INTS11-dependent attenuation by CDK9 activity. Strikingly, INTS11 attenuates transcription in both directions upon CDK9 inhibition, and the engineered recruitment of CDK9 desensitises transcription to INTS11. Therefore, the preferential initiation of sense transcription and the opposing activities of CDK9 and INTS11 explain mammalian promoter directionality.

eLife assessment

The **important** study uses a new experimental method to provide **compelling** evidence on how sense- and anti-sense transcription is differentially regulated. The method described here can generally be used to study the alterations in transcription. This paper will be of interest to scientists working in the gene regulation community.

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Introduction

In humans, most protein-coding genes initiate transcription bidirectionally. Usually, only sense transcription efficiently elongates and leads to mRNA synthesis. Antisense transcription terminates within a few kilobases (kb), and the short non-coding (nc)RNA is degraded[1]. Similar asymmetry frequently occurs in unicellular eukaryotes (e.g., budding yeast) and at some plant

promoters[2,3]. Thus, promoter directionality is a broadly observed phenomenon. Interestingly, bidirectionality is the ground state of promoters and directionality is acquired over evolutionary time[4]. This is proposed to be through a combination of DNA sequences and proteins that favour the directional initiation and elongation of transcription.

The present explanation for the directionality of mammalian RNAPII promoters involves the arrangement of U1 snRNA binding sites and polyadenylation signals (PASs). U1 snRNA promotes RNAPII elongation through protein-coding genes by binding to RNA and preventing early termination[5–7]. RNA-bound U1 prevents early termination by inhibiting PASs and antagonising other attenuation mechanisms, which include the PP1 regulator, PNUTS, and the Restrictor complex[8,9]. In contrast, U1 binding sites are rarer in short antisense transcripts, which are often rich in PAS sequences that are proposed to promote transcriptional termination[10]. This model predicts that polyadenylation factors control a large fraction of antisense transcriptional termination.

The multi-subunit Integrator complex also regulates promoter-proximal transcription[11–15]. The Integrator complex comprises the backbone, arm/tail, phosphatase, and endonuclease modules[11]. Its endoribonuclease is INTS11, and its phosphatase activity is mediated by INTS6 and protein phosphatase 2A (PP2A). INTS11 endonuclease broadly affects promoter-proximal transcriptional attenuation whereas INTS phosphatase is proposed to regulate the escape of RNAPII into elongation[16,17]. INTS6/PP2A phosphatase functionally antagonises CDK9, which is vital for RNAPII promoter escape and productive elongation[18]. By this model, CDK9 activity promotes elongation across protein-coding genes and INTS6/PP2A opposes it.

We tested the prediction that PAS factors control transcription directionality by terminating antisense transcription, but this is not usually the case. Instead, we find that promoter directionality is often conferred by preferential initiation in the sense direction and is thereafter maintained by INTS11 and CDK9. The termination of antisense transcription is constitutively INTS11-dependent, whereas sense transcription is hypersensitive to INTS11 only when CDK9 activity is simultaneously inhibited. We hypothesise that CDK9 activity protects sense transcription from attenuation by INTS11 and that reduced CDK9 activity in the antisense direction exposes RNAPII to INTS11-dependent termination.

Results

Polyadenylation factor depletion does not increase or extend antisense transcription

The current explanation of mammalian promoter directionality invokes early PAS-dependent termination of antisense transcription[10]. This is partly based on the direct detection of polyadenylated antisense transcripts, which provides evidence that some of their transcriptional termination is PAS-dependent. However, there are non-polyadenylated antisense RNAs that might be attenuated in other ways[19]. To test the contribution of PAS-dependent termination toward antisense transcriptional termination and promoter directionality we tagged *RBBP6* with the dTAG degron. Because RBBP6 is required to activate the PAS cleavage machinery[20,21], its depletion should inhibit any PAS-dependent transcriptional termination. Three homozygous *dTAG-RBBP6* clones were isolated (**Supplemental Figure 1A**) and tagged RBBP6 was efficiently depleted after exposure to the dTAGv-1 degrader (**Figure 1A**). To test the contribution of RBBP6 to nascent transcription, we used POINT (Polymerase Intact Nascent Transcript)-seq[22], which maps full-length RNA extracted from immunoprecipitated RNAPII.

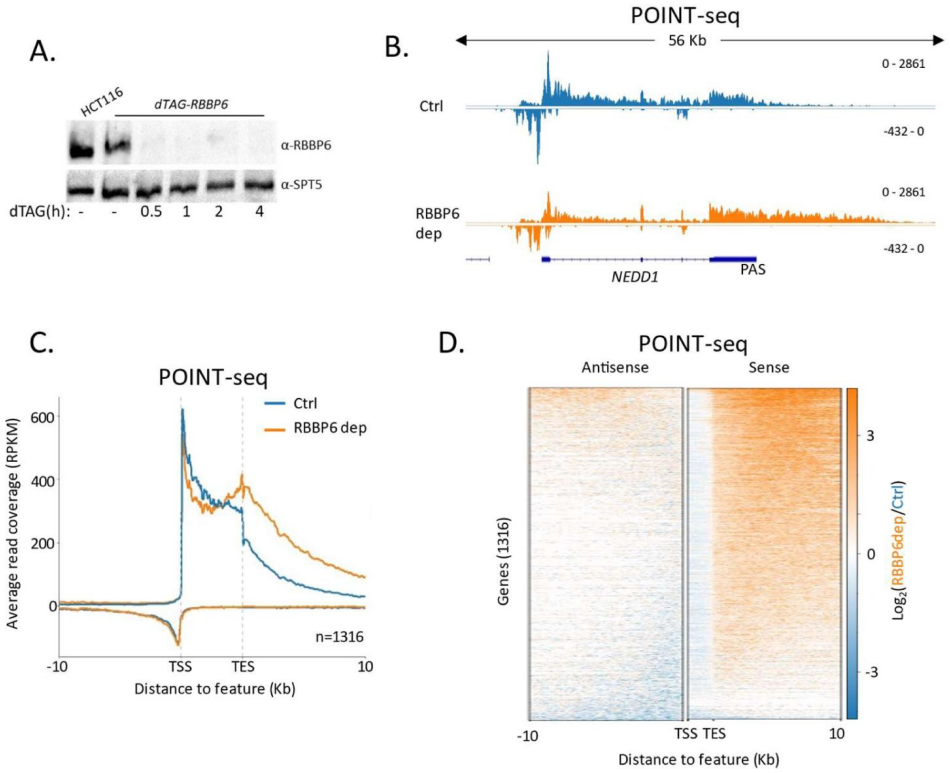

FIGURE 1

RBBP6 loss disrupts PAS-dependent termination of sense transcription.

A. Western blot demonstrating the depletion of dTAG-RBBP6 over a time course of dTAGv-1 addition. SPT5 serves as a loading control.

B. Genome browser track of *NEDD1* in POINT-seq data from *dTAG-RBBP6* cells treated or not (2hr) with dTAGv-1. RBBP6 depletion induces a termination defect in the protein-coding direction (downstream of the indicated PAS) but not the upstream antisense direction. The y-axis shows Reads Per Kilobase per Million mapped reads (RPKM).

C. Metaplot of POINT-seq data from *dTAG-RBBP6* cells treated (RBBP6 dep) or not (Ctrl) (2hr) with dTAGv-1. This shows 1316 protein-coding genes selected as separated from any expressed transcription unit by ≥10kb. Signals above and below the x-axis are sense and antisense reads, respectively. The y-axis scale is RPKM. TSS=transcription start site; TES=transcription end site (this marks the PAS position). This is an average of two biological replicates.

D. Heatmap representation of the data in C, which displays signal as a log2 fold change (log2FC) in RBBP6 depleted versus un-depleted conditions. This is an average of two biological replicates.

Figure 1B shows POINT-seq coverage over *NEDD1* including the upstream antisense transcript. RBBP6 loss causes a termination defect beyond the PAS shown by the extended POINT-seq signal beyond the *NEDD1* gene. However, RBBP6 does not affect the termination of upstream antisense RNA which is not extended when RBBP6 is depleted. Because RBBP6 loss induces strong termination defects beyond the PAS, we generalised its effects using genes separated from their neighbours by ≥10kb. Metaplot and heatmap analyses across these 1316 genes confirm that RBBP6 loss causes a general termination defect downstream of protein-coding genes but has little impact on antisense transcription (**Figures 1C and D**). Although many antisense transcripts contain multiple AAUAAA sequences, most still terminate RBBP6-independently (**Supplementary Figures 1B and C**). Similarly, we recently showed that the PAS-dependent 5'◊3' exonucleolytic torpedo terminator, XRN2, does not affect antisense transcriptional termination[9]. Therefore, although some antisense transcripts are polyadenylated, most antisense transcription can terminate using PAS-independent mechanisms. Consequently, PAS-dependent termination cannot fully explain the promoter-proximal attenuation of antisense transcription.

Integrator depletion increases and extends antisense transcription

Our data argue that PAS-independent termination mechanisms control a large fraction of antisense transcription. A major PAS-independent termination pathway is driven by the Integrator complex, which was first identified as the 3' end processing complex for snRNAs[23].

Several reports show that Integrator terminates transcription from most promoters, including those that initiate antisense transcription, so it might affect promoter directionality[11,16,17]. To analyse this, we tagged its endonucleolytic subunit (INTS11) with a dTAG degron[24], which enables rapid depletion of its catalytic activity (**Figure 2A**). We then performed POINT-seq on *INTS11-dTAG* cells depleted or not of INTS11 to assay the global impact of INTS11 on RNAPII transcription. Integrator is established to control the transcription of snRNAs, and this was detected in our POINT-seq, which demonstrates a clear extension of read density at *RNU5A-1* and *RNU5B-1* loci (**Figure 2B**).

As exemplified by *PGK1* (**Figure 2C**), INTS11 loss does not affect the termination of protein-coding transcription but causes a strong upregulation of antisense transcription. Meta-analysis of the same gene set used for RBBP6 POINT-seq shows the generality of antisense transcriptional attenuation via INTS11 (**Figure 2D**). A heatmap analysis of transcription 3kb upstream and downstream of the same transcription start sites (TSSs) demonstrates the dominant impact of INTS11 on antisense vs. sense transcription at most of these promoters (**Figure 2E**). Although the transcription over protein-coding genes is less affected by INTS11 loss, an increase in promoter-proximal transcription is apparent in some cases (**Supplemental Figure 2A**). The most strongly affected genes are lowly expressed, which is consistent with recent findings (**Supplemental Figure 2B**)[12–17]. Overall, INTS11 frequently attenuates antisense transcription whereas a smaller fraction of sense transcription is affected. RNAPII.

The hypersensitivity of antisense transcripts to INTS11 might be due to the makeup of antisense RNAPII or some other promoter feature. To interrogate this further, we analysed two other promoter classes: those where protein-coding transcripts are initiated in both directions and those that initiate the bidirectional transcription of unstable enhancer (e)RNAs. In the former case, both transcripts are extended and stable like most sense transcripts at directional promoters. In the latter case, both are short and unstable like most antisense transcripts at directional promoters. When both directions are protein-coding, INTS11 depletion causes modest reductions in transcription (**Supplemental Figure 2C**). Conversely, eRNA transcription is bidirectionally upregulated upon INTS11 elimination like the antisense transcripts in (**Supplemental Figure 2D**). We conclude that short ncRNAs are more strongly affected by INTS11 than protein-coding transcripts. At directional promoters this results in the attenuation of antisense transcription.

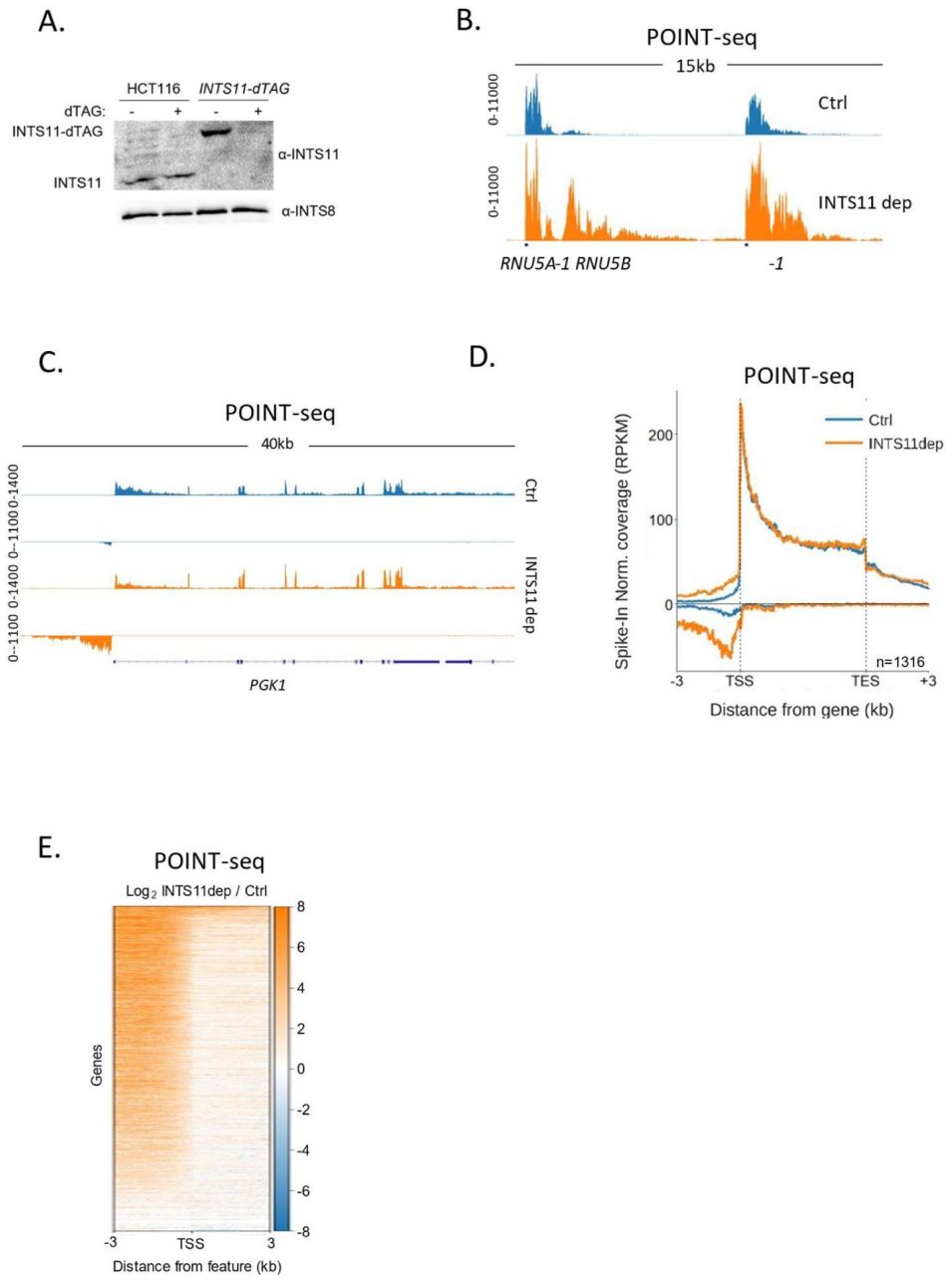

FIGURE 2

INTS11 loss disrupts the termination of antisense transcription.

A. Western blot demonstrating homozygous tagging of *INTS11* with dTAG and the depletion of INTS11-dTAG after 1.5hr treatment with dTAGv-1. INTS8 serves as a loading control.

B. Genome browser track showing POINT-seq signal over *RNU5A-1* and *RNU5B-1* in *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1. Note, that INTS11 depletion induces a termination defect in each case. Y-axis shows RPKM following spike-in normalisation.

C. Genome browser track showing POINT-seq signal over *PGK1* and its upstream antisense region in *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1. Y-axis shows RPKM following spike-in normalisation.

D. Metaplot of POINT-seq data from *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1. This shows the same 1316 genes used in **Figure 1C**. Signals above and below the x-axis are sense and antisense reads, respectively. Y-axis shows RPKM following spike-in normalisation. This is an average of three biological replicates.

E. Heatmap representation of the data in D, which displays signal as a log2 fold change (log2FC) in INTS11 depleted versus undepleted conditions over a region 3kb upstream and downstream of annotated TSSs. This is an average of three biological replicates.

Transcription initiates more efficiently in the sense direction

The increased antisense POINT-seq signal following INTS11 loss is consistent with defects in transcriptional termination. However, it could result from increased transcriptional initiation. We were also interested in whether preferential sense initiation could contribute to mammalian promoter directionality as was proposed in budding yeast[4]. To precisely resolve directional aspects of initiation and assay any impact of INTS11, we devised a variant of POINT-seq called short (s)POINT. Briefly, sPOINT follows the POINT-seq protocol, but library preparation employs the selective amplification of 5' capped RNAs <150nts (**Figure 3A**, **Supplemental Figure 3A**, Experimental Procedures). The sPOINT signal over *PGK1* exemplifies this and demonstrates a very restricted signal close to the TSS (**Figure 3B**). **Figure 3C** compares the meta profile of POINT- and sPOINT-seq on 684 well-expressed and well-spaced (≥10kb from neighbours) genes to highlight the full read coverage obtained by POINT-seq and the tightly restricted, TSS-proximal, sPOINT signal. Because most capped RNAPII-associated RNA <150nts are promoter-proximal, sPOINT effectively assays the promoter-proximal RNAPII pause.

The sPOINT-seq metaplot in **Figure 3B** shows a higher signal in the sense direction, suggesting that more efficient transcriptional initiation establishes promoter directionality. To assay this further and test the impact of INTS11, we performed sPOINT-seq in *INTS11-dTAG* cells treated or not with dTAGv-1 and plotted the coverage over the promoters of well-expressed protein-coding genes (3060 promoters, **Figure 3D**). As sPOINT-seq maps the 5' and 3' ends of these reads, precise TSSs are mapped at single-nucleotide resolution and plotted in the lower meta profile in **Figure 3D**. This analysis once again shows a higher sPOINT signal in the sense direction. In addition, the lower TSS mapped plot also shows that sense transcription initiated in a more focused manner compared to in the antisense direction. **Figure 3E** (*PGK1*) and **supplemental figure 3B** (*ACTB*) exemplify these features on individual genes, and the metaplot in **Figure 3F** highlights the dispersed nature of antisense TSSs. Quantitation of the TSS-derived sense vs. antisense signal confirms a higher read count in the sense direction in untreated cells (**Supplemental Figure 3C**). The most noticeable (although still modest) effect of INTS11 loss on sPOINT-seq profiles is a mild signal reduction. This shows that INTS11 depletion does not generally enhance transcriptional initiation in either direction. This slight reduction in sPOINT-seq signal could result from less transcriptional initiation or less efficient pausing if, for example, INTS11 loss allows more RNAPII to escape the promoter. Although a lower resolution technique, RNAPII ChIP-seq confirmed these promoter characteristics and the mild impact of INTS11 (**Supplemental Figures 3D and E**). Overall, these data show that directionality might be established by more efficient initiation in the sense direction.

CDK9 inhibition sensitises sense transcription to INTS11

After transcription initiates, further control of directionality is evident because sense transcription goes further than antisense transcription. If INTS11 regulates this, an opposing force is required to promote elongation. INTS11 affects transcription very early, occupies promoters, and becomes less active as RNAPII moves into elongation[16–18,25,26]. Therefore, if INTS11 is counteracted to allow sense transcription, any responsible mechanism needs to act early. One of the first transcriptional checkpoints involves the phosphorylation of RNAPII and other factors by CDK9, which releases promoter-proximally paused RNAPII into elongation[27]. During this process, the Integrator-associated phosphatase, PP2A, antagonizes CDK9 and presumably regulates the sensitivity of RNAPII to INTS11[18]. Because Integrator phosphatase has little effect on antisense transcription[16], we hypothesised that INTS11 sensitivity and CDK9 activity are inversely correlated to maintain directionality after initiation.

Our hypothesis predicts that sense transcription will be attenuated via INTS11 when CDK9 is inactive. To test this genome-wide, we depleted INTS11 from *INTS11-dTAG* cells in the presence or absence of a specific CDK9 inhibitor (NVP-2[28]) and performed POINT-seq. As exemplified by

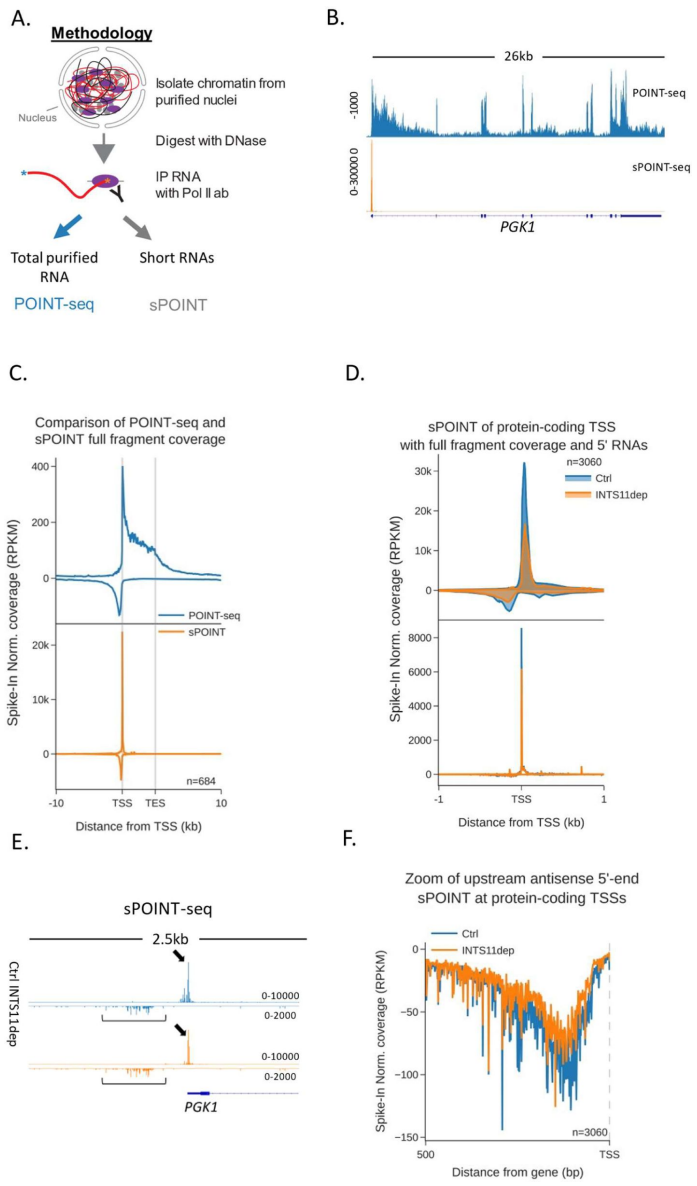

FIGURE 3

Sense direction transcription initiation is more efficient and focused.

A. Schematic of sPOINT-seq protocol. The POINT-seq protocol is followed, in which chromatin is isolated and engaged RNAPII is immunoprecipitated. Short transcripts are preferentially amplified during library preparation (see Experimental Procedures for full details).

B. Comparison of POINT-(top trace) and sPOINT-seq (lower trace) on *PGK1*. Y-axis units are RPKM.

C. Metaplot comparison of POINT-(top plot) and sPOINT-seq (lower plot) profiles across the 684 highest expressed protein-coding that are separated from expressed transcription units by ≥10kb (top ~50%). Signals above and below the x-axis are sense and antisense reads, respectively. Y-axis shows RPKM following spike-in normalisation.

D. Top metaplot shows full read coverage for sPOINT-seq performed in *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1 at the promoters of the top expressed 20% of protein-coding genes. The lower metaplot is the same data but only the 5' end of each read is plotted. The y-axis signals are RPKM following spike-in normalisation. Two biological replicates of sPOINT were performed.

E. Genome browser track of *PGK1* promoter region in sPOINT-seq. This showcases the focused sense TSS (black arrows) and the dispersed antisense reads (brackets). Note the higher y-axis scale (RPKM) for sense vs. antisense.

F. Metaplot zoom of the antisense TSS signals deriving from the lower plot in D. This makes clear the dispersed sites of initiation. The Y-axis scale is RPKM following spike-in normalisation. Two biological replicates of sPOINT were performed.

TARDBP, the depletion of INTS11 alone caused an antisense transcriptional termination defect with a milder impact on the protein-coding sense direction (**Figure 4A**). As expected, NVP-2 treatment reduced transcription over the protein-coding gene body. Antisense transcription also displays some CDK9 sensitivity. Importantly, in NVP-2-treated cells, INTS11 loss increased transcription in both directions. This contrasts with the dominant antisense effect deriving from just depleting INTS11 (refer to **Figure 2**). As such, CDK9 activity prevents sense transcription from being attenuated by INTS11. This is a genome-wide trend as shown in the metaplots in **Figures 4B and C**. A heatmap of the effect of INTS11 loss after CDK9 inhibition shows the bidirectional upregulation of transcription at the same promoters assayed in **Figure 2** (**Figure 4D**). Although this experiment employed longer INTS11 depletion than that in **Figure 2** (2.5hr vs. 1.5hr to allow concurrent CDK9 inhibition), its antisense effects remain general, and the affected protein-coding genes strongly overlapped (**Supplemental Figure 4A-C**). Lastly, when CDK9 and INTS11 are both compromised, the POINT-seq signal remains higher in the sense vs. antisense direction (black line, **Figure 4C**). This is consistent with our sPOINT-based finding that initiation of sense transcription is generally more efficient.

Following CDK9 inhibition and INTS11 loss, the largest recovery of protein-coding transcription is often over the 5' end of genes. This presumably reflects poor elongation without CDK9 activity even when INTS11 is absent. RNAPII elongation is associated with phosphorylation of the C-terminal domain (CTD) of its largest subunit, RPB1, and most frequently occurs on Serine 5 or 2 (Ser5/2p) of its heptad repeat[29,30]. We assayed the effects of CDK9 inhibition and INTS11 depletion on these two modifications by western blotting (**Figure 4E**). As previously shown[28], CDK9 inhibition by NVP-2 suppresses Ser2p. However, both Ser5p and Ser2p are enhanced by INTS11 loss whether CDK9 is active or not. Thus, unattenuated transcription after INTS11 depletion is associated with some CTD phosphorylation. Where NVP-2 is employed, kinases redundant with CDK9 presumably phosphorylate RNAPII.

We confirmed the CDK9-mediated suppression of INTS11 on four selected protein-coding genes using an alternative inhibitor, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (**Supplemental Figure 4D**). Interestingly, inhibition of CDK7 with THZ2 sensitised the same selected protein-coding transcripts to INTS11 attenuation (**Supplemental Figure 4E**). This may be because CDK7 is required to activate CDK9[31], or because Integrator additionally targets RNAPII when CDK7 is inactive. This result reinforces the idea that the successful execution of early transcriptional checkpoints may overcome Integrator-mediated attenuation.

If CDK9 counteracts INTS11, its recruitment should prevent transcriptional attenuation by Integrator. To assay this, we employed a plasmid where transcription is driven by the human immunodeficiency virus (HIV) promoter. Transcription from the HIV promoter results in synthesis of the trans-activating response (TAR) element and promoter-proximal RNAPII pausing. Pause release requires the trans-activator of transcription (TAT), which promotes RNAPII elongation by recruiting CDK9[32]. INTS11 suppresses transcription from the HIV promoter when TAT is absent[33]. To assay whether CDK9 affects this process, *INTS11-dTAG* cells were transfected with the HIV reporter with or without TAT before treatment or not with dTAGv-1. Transcription from the reporter was then analysed by qRT-PCR (**Figure 4F**). INTS11 loss induces HIV transcription in the absence of TAT. However, TAT strongly stimulates transcription (~200 fold) and desensitises it to INTS11 loss (slightly less reporter RNA was recovered). Therefore, TAT-mediated CDK9 recruitment alleviates INTS11-dependent attenuation of transcription. In line with endogenous protein-coding genes, CDK9 inhibition sensitises TAT-activated transcription to INTS11 (**Supplemental Figure 4F**). Overall, our data strongly suggest that CDK9 activity counteracts transcriptional attenuation by INTS11.

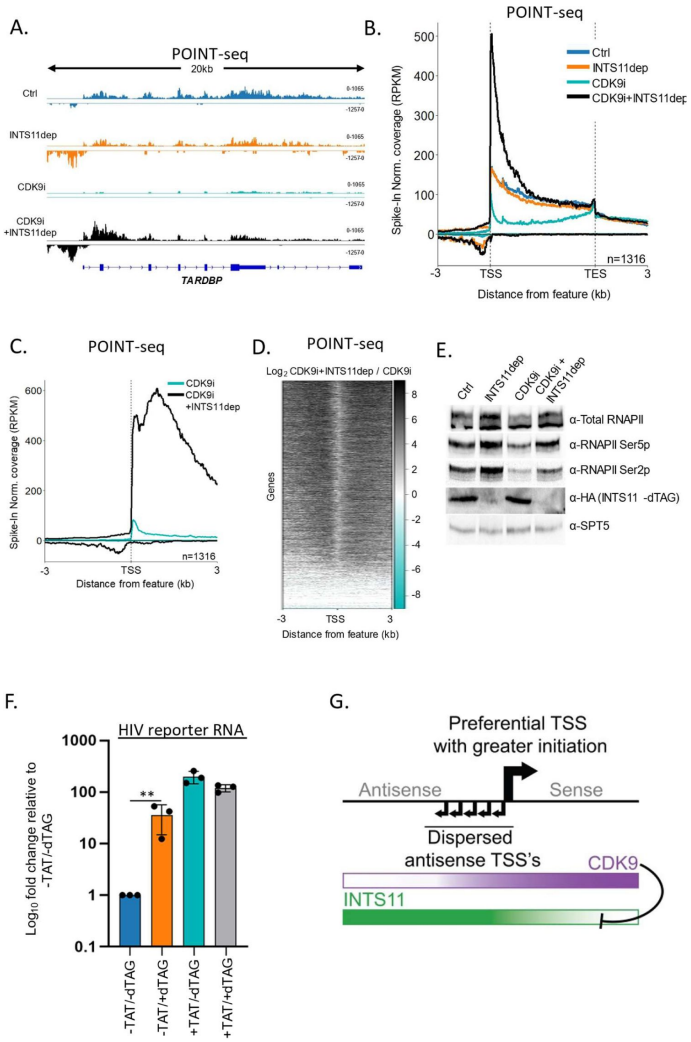

FIGURE 4

Some promoter directionality is lost when CDK9 is inhibited.

A. Genome browser track of *TARDBP* in POINT-seq data derived from *INTS11-dTAG* cells either untreated, dTAG treated, NVP-2 treated or dTAG and NVP-2 treated (2.5 hr). Signals above and below the Y-axis are sense and antisense reads, respectively. The Y-axis scale shows RPKM following spike-in normalisation.

B. Metaplot of POINT-seq in *INTS11-dTAG* cells depleted or not of INTS11 and treated or not with NVP-2 (2.5 hr). This uses the same gene set as **Figure 1C**. The regions 3kb upstream and downstream of genes are included. Y-axis units are RPKM following spike-in normalisation. This is an average of two biological replicates.

C. Metaplot of the same CDK9i + and -dTAG data shown in B but zoomed into the region 3kb upstream and downstream of the TSS. This is an average of three biological replicates.

D. Heatmap representation of the data in C, which displays signal as a log2 fold change (log2FC) in INTS11 depleted versus un-depleted conditions covering a region 3kb upstream and downstream of the TSS. This is an average of three biological replicates.

E. Western blot for total RNAPII and RNAPII phosphorylated on Ser2/5 (Ser2/5p) in *INTS11-dTAG* cells treated or not with dTAGv-1 and/or NVP-2 (as indicated). dTAG-mediated INTS11 depletion is shown in the anti-HA blot and SPT5 us used as a loading control.

F. qRT-PCR analysis of *INTS11-dTAG* cells transfected with the HIV reporter construct with or without TAT then depleted or not of INTS11. Quantitation shows signals relative to those obtained in the presence of INTS11 and the absence of TAT after normalising to MALAT1 RNA. n=3. Error bars show standard deviation. ** denoted p=0.01. Note that INTS11 depletion was performed concurrently with transection (14hr in total).

G. Model for promoter directionality depicting higher levels of focused transcriptional initiation in the sense direction together with opposing gradients of CDK9 and INTS11 activity that peak in sense and antisense directions, respectively.

Discussion

Most studies on mammalian promoter directionality have focused on understanding how antisense transcription terminates and how RNAPII elongation is enabled in the sense direction. However, we show that preferential sense transcriptional initiation significantly contributes toward the directionality of many mammalian promoters. More analyses are required to elucidate the mechanism, but we hypothesise that a unidirectional arrangement of at least some core promoter elements favours transcription in the protein-coding direction. Consistently, the evolution of promoter elements explains how a bidirectional promoter "ground state" acquired directionality in yeast[4]. Consistent with our finding, focused transcription initiation is associated with highly expressed mammalian genes and promoters with clearly defined elements (e.g. TATA)[34]. Thus, the lower level and dispersed nature of antisense initiation may reflect the suboptimal orientation of promoter elements or the opportunistic transcription of open chromatin in promoter regions.

We hypothesise that INTS11 controls transcriptional attenuation via its endonuclease activity. An inactive mutant of INTS11 (E203Q) is widely used to interrogate its catalytic function[23]; however, we discovered that it poorly associates with other Integrator components compared to wild-type INTS11 (**Supplemental Figure 4G**). Therefore, when employed in cells, it may not isolate the effects of INTS11 activity from those requiring an intact Integrator complex. Similarly, catalytic mutations in the highly related PAS endonuclease, CPSF73, disrupt its association with other cleavage and polyadenylation components[35]. In light of this, it is formally possible that non-catalytic consequences of INTS11 loss explain the upregulation of antisense transcription.

How CDK9 activity opposes INTS11 is unresolved, but INTS11 and SPT5 are adjacent in the RNAPII: Integrator structure[36]. As SPT5 is a prominent substrate of CDK9[37–39], its phosphorylation might evict INTS11 or prevent its association with the complex. Consistently, Integrator is enriched on promoters whereas phosphorylated SPT5 (SPT5p) is most prevalent during elongation over protein-coding gene bodies[25,38]. Furthermore, although antisense RNAPII occupancy is increased by INTS11 loss, published data suggest that this is not paralleled by increases in its Ser5p and Ser2p forms[16]. As these modifications require CDK7 and CDK9, this provides further support that these activities are less prevalent for antisense vs. sense transcription.

While INTS11 and CDK9 activities are the clearest on antisense and sense transcription, respectively, this is not binary. Some antisense transcription is NVP-2 sensitive, which indicates CDK9 activity on some RNAPII (**Figure 4** and[40]). CDK9-sensitive antisense transcription could undergo INTS11-independent attenuation, which might account for the polyadenylated antisense RNA observed previously[10,41]. Consistently, some antisense transcription is targeted by the poly(A) exosome targeting connection, which operates on cleaved and polyadenylated transcripts[42,43]. Reciprocally, promoter-proximal transcription from lowly expressed protein-coding transcription is INTS11-sensitive, which could reflect low CDK9 activity at those loci. We and others recently described the Restrictor complex as restraining antisense transcription[9,44,45]. Unlike Integrator, which associates with NELF-bound RNAPII[36], Restrictor is proximal to the distinct PAF-bound RNAPII[9]. Thus, various complexes may target different forms of RNAPII before and after CDK9 activity. Even so, our data suggests that Integrator influences a greater volume of antisense RNAPII than the cleavage and polyadenylation pathway.

In addition to CDK9, multiple studies demonstrate a role for U1 snRNA in promoting elongation through protein-coding genes[5,6,8,46]. U1 promotes RNAPII elongation and shields transcription from early PAS-dependent termination and the Restrictor complex[5,9]. The U1-mediated suppression of premature PAS-dependent termination inspired the original model of promoter directionality. As U1 sites are rarer in antisense RNAs, their termination was

hypothesised to be driven by early PASs that would consequently be active. Our RBBP6 data (and other published data on PAS termination factors[9]) argues that a large fraction of antisense transcriptional termination is PAS-independent. Nevertheless, the lack of U1 sites may limit RNAPII elongation and render it sensitive to Integrator (or Restrictor). Accordingly, it will be interesting to test whether U1 regulates the sensitivity of transcription to Integrator.

In conclusion, we provide a new model for mammalian promoter directionality and the subsequent control of bidirectional transcription (**Figure 4G**). sPOINT demonstrates more efficient sense transcription initiation, which establishes directionality. Early events dictate the decision to elongate or attenuate transcription. In the sense direction, CDK9 activity prevents attenuation by Integrator to favour productive elongation, whereas antisense transcription is hypersensitive to INTS11 and terminates early. As INTS11 occupies most promoters and CDK9 activity is near-universally required to achieve protein-coding transcription, this model can generally explain how directionality is initiated and maintained. Our new sPOINT-seq approach will be valuable in elucidating additional aspects of promoter-proximal transcriptional regulation.

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Author contributions

Conceptualization J.D.E, S.W; data curation J.D.E, J.B, L.D, C.E; formal analysis J.D.E., J.B., L.D; C.E and S.W; methodology, J.D.E. and S.W.; investigation, J.D.E., J.B., L.D; C.E and S.W.; supervision and funding acquisition, S.W.; writing and editing, J.D.E., J.B., L.D; C.E and S.W.

Experimental procedures

Sequencing data

Deposited at Gene Expression Omnibus under accession: GSE243266.

Cloning

HIV reporter constructs were made by removing the CMV promoter, the entire β-globin sequence, and its PAS from a pcDNA5 FRT/TO plasmid containing the WT β-globin (βWT) gene[47] and inserting an HIV promoter and downstream TAR element derived from βΔ5-7[48]. *INTS11* targeting constructs were modified from those we previously described to generate *INTS11-SMASh* cells[49]. The SMASh tag was removed and replaced with 2xHA dTAG derived from Addgene plasmid 91792[24]. Guide RNA expressing Cas9 plasmids to modify *INTS11* or *RBBP6* were made by inserting annealed oligonucleotides, containing the targeting sequence, into px330[50] digested with BbsI.

Cell culture and cell lines

HCT116 cells were maintained in DMEM supplemented with penicillin/streptomycin at 37°C, 5% CO_2. *dTAG-RBBP6* cells were generated using the "CHoP in" protocol[51]. Briefly, a 24-well dish was transfected with 250ng of px330[50] containing the *RBBP6*-targeting guide and 250ng of PCR

product containing the dTAG degron preceded by a blasticidin or puromycin selection marker (derived from Addgene plasmid 91792 and 91793[24]). Jetprime (Polyplus) was used for transfection. Three days later, cells were passaged into media containing 10µg/ml Blasticidin/1µg/ml Puromycin and colonies were PCR screened ~10 days later. *INTS11-dTAG* cells were generated by homology-directed repair. A 6-well dish of cells was transfected with 1µg px330 containing the *INTS11*-targeting guide (described in[49]) and 1µg each of the repair templates. Three days later, cells were passaged into media containing 30µg/ml Hygromycin and 800µg/ml G418. ~10 days later, colonies were picked and screened by PCR. 1µM dTAGv-1 (Tocris) was used for 1-14hrs (see figure legends for timings used in each experiment); NVP-2 was used at 250nM for 2.5hr; DRB was used at 100µM for 2.5hr; THZ2 was used at 5µM for 2.5hr.

POINT-seq and sPOINT-seq

For POINT-seq, we followed the protocol provided in[22]. The only modification was that we started with a confluent 10cm dish of cells and performed the immunoprecipitation with 6µg of anti-RNAPII. ~2% cell volume of Drosophila S2 cells was included as a spike in control. Libraries were prepared using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs). sPOINT was performed in the same manner with the following difference: a confluent 15cm dish of cells was used and 10µg of anti-RNAPII. In sPOINT, the immunoprecipitated and DNAse treated RNA was treated with Terminator™ 5-Phosphate-Dependent Exonuclease (lucigen) to remove any uncapped transcripts, and libraries were prepared with the SMARTer® smRNA-Seq Kit for Illumina (Takara Bio) to selectively capture transcripts <150nts.

ChIP-seq

For each experiment, 1×10cm dish of cells was used. Protein: DNA crosslinks were formed by adding Formaldehyde (1% v/v) to culture media for 10 mins then quenching with 125mM glycine. Cells were rinsed 2x with PBS, scraped off the dish, and pelleted in 10ml PBS at 500xg for 5 mins. We then employed the simple ChIP enzymatic kit (Cell Signalling Technologies) to fragment chromatin and purify RNAPII-bound DNA. We followed the kit protocol except for conjugating 5µg of anti-total RNAPII to sheep anti-mouse dynabeads (Life Technologies). Sequencing libraries were generated using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina.

Total/chromatin-associated RNA isolation and qRT-PCR

For total RNA, a 24-well dish of cells was transfected with 100ng HIV reporter plasmid and, where co-transfected, 50ng TAT plasmid[52] using Jetprime (Polyplus) following the manufacturer's protocol. Media was refreshed 5 hours post-transfection and dTAGv-1 was added where appropriate. The next day, total RNA was isolated using Trizol (Thermo Fisher) following the manufacturer's protocol. For chromatin-associated RNA (for **Supplemental Figure 4F**), a 6 well dish of cells was transfected with 300ng HIV reporter plasmid and 150ng TAT. Pelleted cells were resuspended in 800µl hypotonic lysis buffer (HLB: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl and 2.5 mM $MgCl_2$, 0.5% NP40) and underlayered with 200µl HLB +10% sucrose. Nuclei were isolated by centrifugation for 5 mins at 500xg. These were resuspended in 100µl NUN1 (20 mM Tris-HCl (pH 7.9), 75 mM NaCl, 0.5 mM EDTA, and 50% Glycerol). After addition of 1ml of NUN2 (20 mM HEPES-KOH (pH 7.6), 300 mM NaCl, 0.2 mM EDTA, 7.5 mM $MgCl_2$, 1% NP-40, 1 M Urea) chromatin was isolated by 10 min incubation on ice followed by 10 min centrifugation at 13000rpm. RNA was isolated from the chromatin pellet using Trizol following the manufacturers' instructions. For qRT-PCR of total and chromatin-associated RNA, RNA was DNase treated then 1µg was reverse transcribed using Protoscript II reverse transcriptase (New England Biolabs). qPCR was performed using LUNA SYBR green reagent (New England Biolabs) on a Qiagen Rotorgene instrument. Quantitative analysis used the ΔCT method.

Co-immunoprecipitation

For each transfection, a semi-confluent 10cm dish of cells was transfected with 5µg of plasmid expressing flag tagged wild type or E203Q INTS11. Immunoprecipitation was based on the ELCAP protocol[53]. The following day, cells were washed 2x in PBS, scrapped into 10ml PBS and spun down for 5 mins at 500xg. Pelleted cells were resuspended in 800µl hypotonic lysis buffer low NP40 (HLB: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl and 2.5 mM $MgCl_2$, 0.1% NP40) and underlayered with 200µl HLB low NP40 +10% sucrose. Nuclei were resuspended in 1ml Chromatin digestion buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 10% (v/v) glycerol, 150 mM NaCl, 0.1% (v/v) NP-40 and 250 U/mL Benzonase) and incubated for 1hr at 4°C. Debris was pelleted at 13000rpm for 10 mins and supernatant was incubated with 20µl of anti-flag magnetic beads (Sigma) for 2hrs at 4°C. Beads were washed 6x with ice-cold chromatin digestion buffer and samples were eluted by boiling in protein loading buffer (100 mM Tris-Cl (pH 6.8) 4% (w/v) sodium dodecyl sulfate 0.2% (w/v) bromophenol blue 20% (v/v) glycerol) before polyacrylamide separation and western blotting.

qRT-PCR primers and gRNA target sites

See supplemental table 1.

Bioinformatics

POINT-Seq alignment and visualisation

A metagene list of genes with no overlapping regions within 10kb of any other expressed transcription unit (1316). Adapters were removed from raw reads using Trim Galore! and mapped to GRCh38 using HISAT2 using default parameters. Biological replicates were normalised and merged using SAMtools merge. Split strand metagene plots were produced using Drosophila spike-in normalised sense and antisense (scaled to -1) bigwig coverage files separately with further graphical processing performed in R. For heat maps, computematrix (DeepTools) was used to generate score files from the normalised bigwig files using the 10kb non-overlapping gene list. A log2 ratio (depletion/control) was applied to identify changes in reads. Plots were redrawn in R; parameters used for each heat map are detailed in figure legends.

sPOINT

For sPOINT TSS metaplots showing full-length and 5' derived coverage, gene lists were determined by selecting principal protein-coding transcript isoforms from gencode v42 human annotation – specifically, those containing both "appris_principal_1" and "Ensembl_canonical" labels (15301 in total). The top 20% expressed (based on -dTAG) were used to generate meta profiles (3060 TSSs in total). The 684 genes used to exemplify the difference between POINT and sPOINT coverage are the top 50% of expressed genes (based on POINT-seq in *INTS11-dTAG)* cells, which are separated from neighbouring transcription usints by at least 10kb.

Antisense PAS analysis

Bed files from **Figure 1C** were edited to map the antisense transcript from each gene by fitting each gene's start and end point to 3kb upstream of their TSS. Sequences underlying these regions were obtained via BEDTools getFASTA (hg38) and consensus PAS (AATAAA) enumerated in R. PAS frequency per transcript was plotted using ggplot2. Heatmaps for transcripts containing > 1 or no PAS motifs were plotted with DeepTools.

ChIP-seq alignment and plotting

Adapters were removed from raw reads using Trim Galore! and mapped to GRCh38 using HISAT2 with default parameters. Reads were also mapped to Dm6 to identify spike-in signal. Reads with MAPQ score of ≤30 were removed with SAMtools[54]. Peaks were called using MACS2 in paired-end mode[55]. Metaplots were created from S2 scaled, merged replicate -log10 q-value bigwig coverage using DeepTools with further processing performed within R. For **Supplementary Figure 3E**, plots used the same gene list described above for sPOINT promoter analyses (see sPOINT section above).

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
cell line (*D. melanogaster*)	S2	This paper	FLYB:FBtc0000181; RRID:CVCL_Z992	Cell line maintained in N. Perrimon lab; FlyBase symbol:

				S2-DRSC.
Cell line (H. Sapiens)	HCT116	In house	This paper	
Cell line (H. Sapiens)	*INTS11-dTAG*	In house	This paper	
Cell line (H. Sapiens)	*dTAG-RBBP6*	In house	This paper	
Antibody	INTS11	Abbexa	abx234340	WB: 1:500
Antibody	INTS10	Proteintech	Cat# 15271-1-AP RRID:AB_2127260	WB: 1:3000
Antibody	INTS8	Proteintech	Cat# 18802-1-AP RRID:AB_10597250	WB: 1:1000
Antibody	INTS9	Proteintech	Cat# 11657-1-AP RRID:AB_2127514	WB: 1:1000
Antibody	INTS1	Bethyl	Cat# A300-361A-T RRID:A	WB: 1:500

			B_2632121 (Now discontinued)	
Antibody	HA	Cell Signalling	Cat# 3724 RRID:AB_1549585	WB: 1:2000
Antibody	RNAPII Ser2p	Active Motif	Cat# 61084 RRID:AB_2687450	WB: 1:2000
Antibody	RNAPII Ser5p	Millipore	Cat# 04-1572-I RRID:AB_2801296	WB: 1:2000
Antibody	Total RNAPII	Hiroshi Kimura Lab	N/A	WB: 1:10,000 IP: 5-10ug (Ab:Beads)
Antibody	SPT5	Santa Cruz Biotech	Cat# sc-136075 RRID:AB_2286723	WB: 1:1000
Antibody	Flag	Proteintech	Cat# 20543-1-AP RRID:AB_11232216	WB: 1:5000

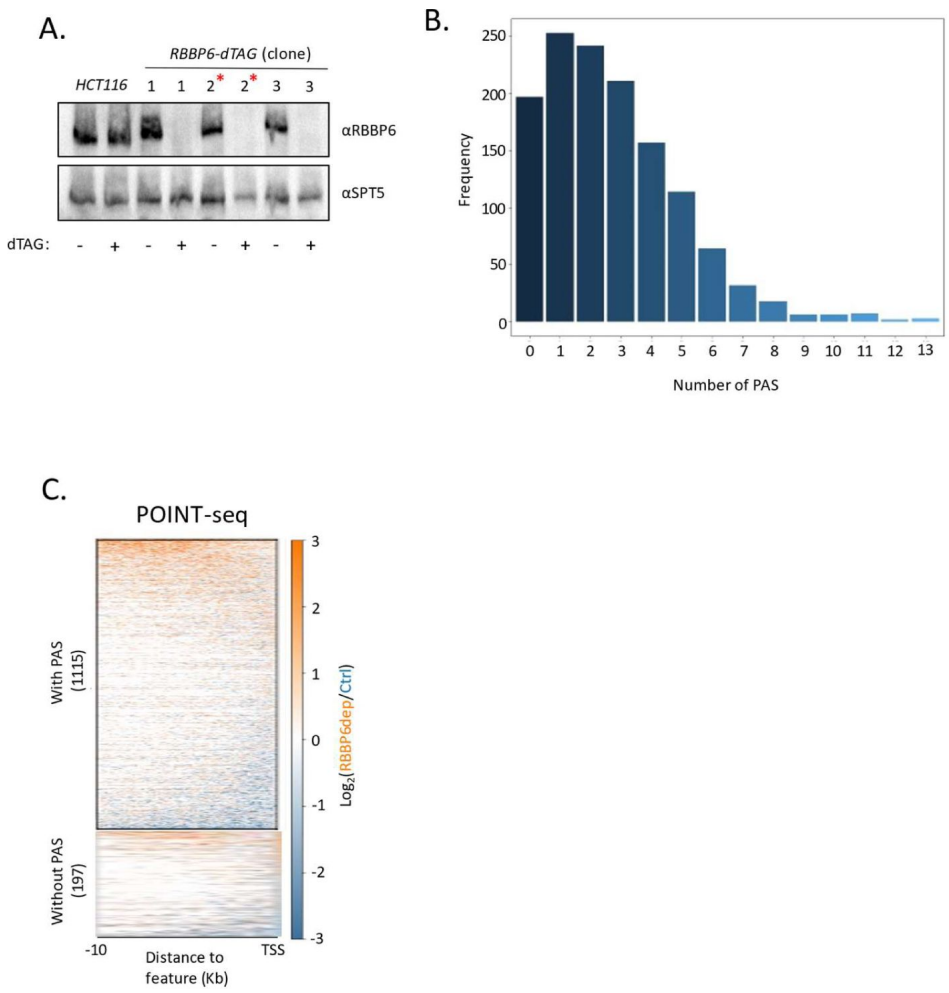
Antibody	anti-Rabbit	Proteintech	Cat# SA00001-2 RRID:AB_2722564	WB: 1:2000
Antibody	anti-Mouse	Proteintech	Cat# SA00001-1-A RRID:AB_2890995	WB: 1:2000
Antibody	anti-Rat	Thermofisher	Cat #A18865 RRID:AB_2535642	WB: 1:2000
Recombinant DNA reagent	px300	Addgene	RRID #: Addgene_42230	
Recombinant DNA reagent	pCRIS-PITChv2-BSD-dTAG (BRD4)	Addgene	RRID:Addgene_91792	
Recombinant DNA reagent	pCRIS-PITChv2-PURO-dTAG (BRD4)	Addgene	RRID:Addgene_91793	
Transfected construct	TAT	[52]		
Transfected construct	INTS11 WT and E203Q	Twist	This paper	Supplemental Table 1
Transfected construct	HIV reporter	In house	This paper	

Commercial assay, kit	Terminator 5-phosphate dependant exonuclease	Lucigen	Cat #TER51020	
Commercial assay, kit	SMARTer smRNA-seq kit for illumina	Takara	Cat #635031	
Commercial assay, kit	Dynabeads M280 sheep anti-mouse	Thermofisher	Cat #11202D	
Commercial assay, kit	anti-FLAG/DYKDDDDK magnetic beads	MilliporeSigma	Cat #M8823	
Commercial assay, kit	SimpleChIP® Plus Enzymatic Chromatin kit	Cell Signalling	Cat #9005	
Commercial assay, kit	NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®	NEB	Cat #E7645S	
Commercial assay, kit	JetPRIME	PolyPlus	Cat #114-01	
Commercial assay, kit	NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina	NEB	Cat #E7765	
Commercial assay, kit	Protoscript II reverse transcriptase	NEB	Cat #M0368	
Commercial assay, kit	LUNA qPCR reagent	NEB	Cat #M3003	

Commercial assay, kit	Ampure XP beads	Beckman Coulter	Cat #A63880	
Other	Benzonase	Sigma	Cat #E1014-5KU	
Chemical compound, drug	dTAGv-1	Tocris	Cat #2624313-15-9	
Chemical compound, drug	NVP2	Merck	Cat #SML3069-5MG	
Chemical compound, drug	THZ2	Medchem Express	Cat #HY-12280	
Chemical compound, drug	DRB	Merck	Cat #D1916	
Chemical compound, drug	Trizol Reagent	Thermofisher	Cat #15596026	
Commercial assay, kit	Turbo DNAse kit	Thermofisher	Cat #AM2238	
software, algorithm	Bamtools	[56]	NA	NA
software, algorithm	BEDtools	[57]	NA	NA
software, algorithm	Cutadapt	Other	NA	https://doi.org/10.14806/ej.17.1.200

software, algorithm	Deeptools	[58]	NA	NA
software, algorithm	FastQC	Other	NA	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
software, algorithm	Hisat2	[59]	NA	NA
software, algorithm	IGV	[60]	NA	NA
software, algorithm	MACS2	[55]	NA	NA
software, algorithm	MultiQC	[61]	NA	NA
software, algorithm	R	Other	NA	https://cran.r-project.org/
software, algorithm	Rstudio	Other	NA	https://www.rstudio.com/
software, algorithm	SAMTools	[54]	NA	NA
software, algorithm	Trim_galore!	Other	NA	https://github.com/FelixKrueger/TrimGalore/

Key Resources Table

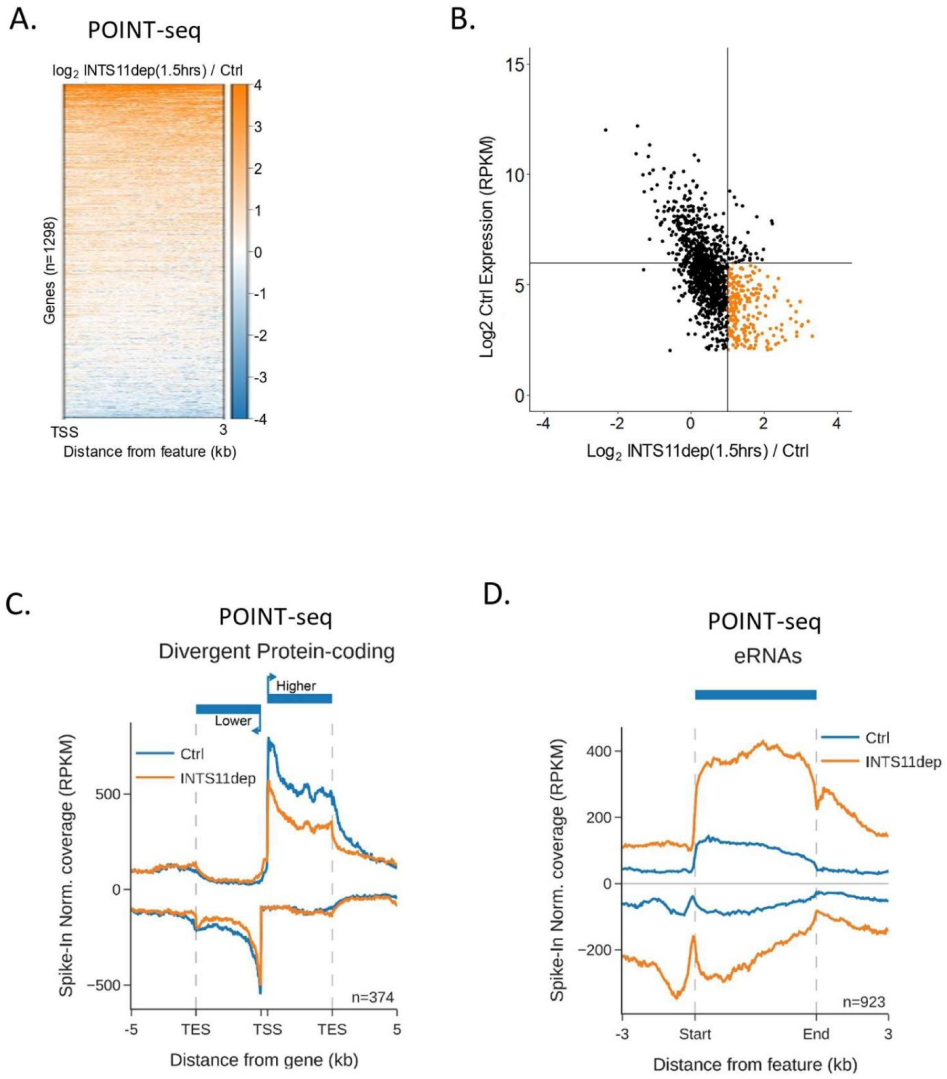


SUPPLEMENTAL FIGURE 1, related to figure 1.

A. Western blot showing three separate *dTAG-RBBP6* cell clones. In each case, homozygous tagging is demonstrated by the size shift versus endogenous RBBP6 (HCT116 lanes). Tagged RBBP6 is completely depleted by 2hr treatment with dTAGv-1 whereas endogenous RBBP6 is unaffected. SPT5 serves as a loading control. Clone number 2 (red asterisk) was selected for the experiments in **Figure 1**.

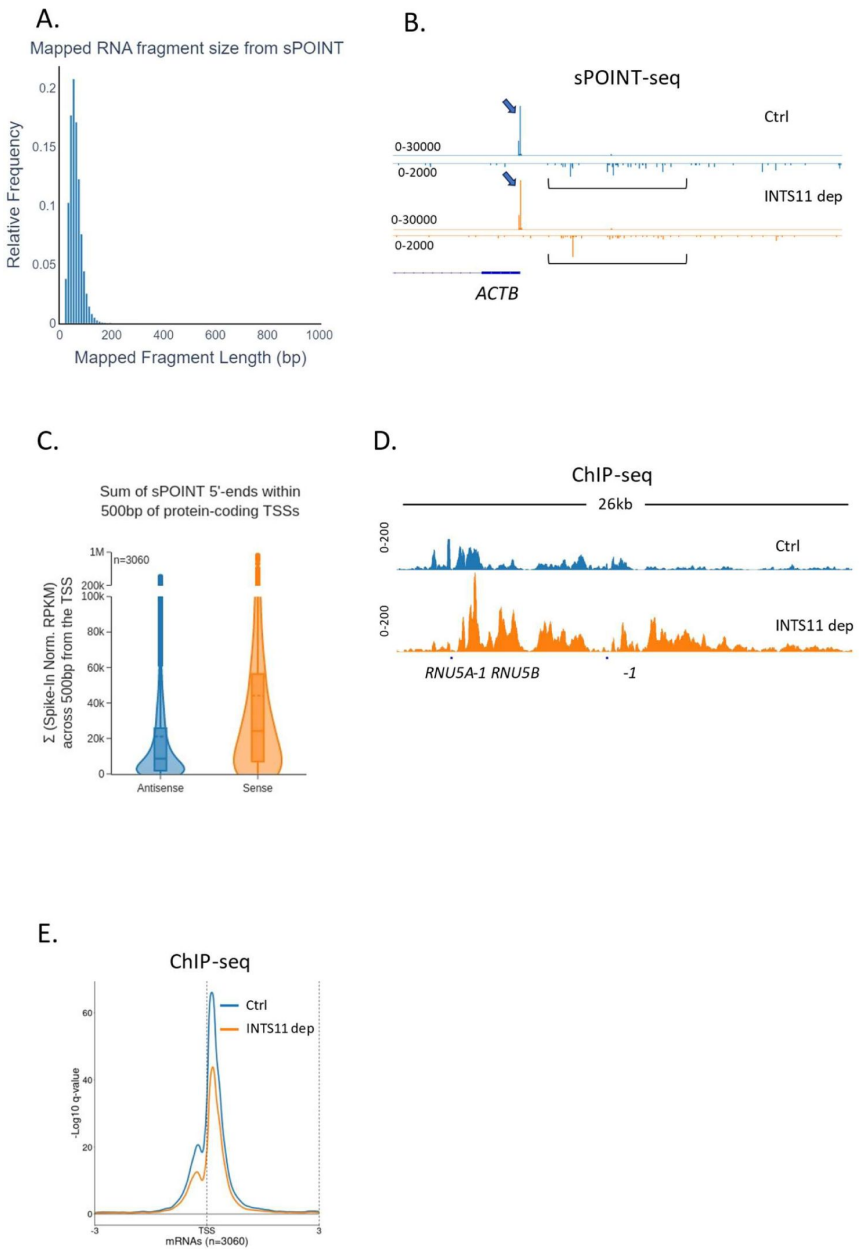
B. Graph plotting the number of AAUAAA sequences in antisense transcripts derived from a 3kb window upstream of TSSs. Y-axis is the number of transcripts, and the x-axis shows the AAUAAA count per transcript.

C. Heatmap of control or RBBP6-depleted POINT-seq showing the RBBP6 effect on antisense transcripts without an AAUAAA or those that contain at least one AAUAAA. Most are unaffected by RBBP6 loss. The y-axis is RPKM.



SUPPLEMENTAL FIGURE 2, related to figure 2.

A. Heatmap analysis of the 1316 gene set used in Figure 1C but focused on promoter-proximal transcription of the sense direction. Note the change in scale (Log2FC - INTS11/+INTS11) to reflect the smaller effect sizes vs. those for antisense transcription from the same gene set.
B. Comparison of INTS11-dependent changes in promoter-proximal protein-coding transcription with the expression level of each gene using the 1316 gene set as **Figure 1C**. X-axis shows Log2FC in levels (-INTS11/+INTS11) and y axis shows expression level. Note that genes with the largest increase in promoter-proximal signal following INTS11 loss are low expressed (coloured orange). Data derives from POINT-seq following treatment or not with dTAGv-1 (1.5hr).
C. Metaplot of POINT-seq data from *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1. These are protein-coding genes arranged head-to-head. Signals above and below the x-axis are sense and antisense reads, respectively. Y-axis shows RPKM following spike-in normalisation.
D. Metaplot of POINT-seq data from *INTS11-dTAG* cells treated or not (1.5hr) with dTAGv-1. This shows RNAs derived from enhancer clusters, which generally initiate transcription bidirectionally. Signals above and below the x-axis are sense and antisense reads, respectively. Y-axis shows RPKM following spike-in normalisation.



SUPPLEMENTAL FIGURE 3, related to figure 3.

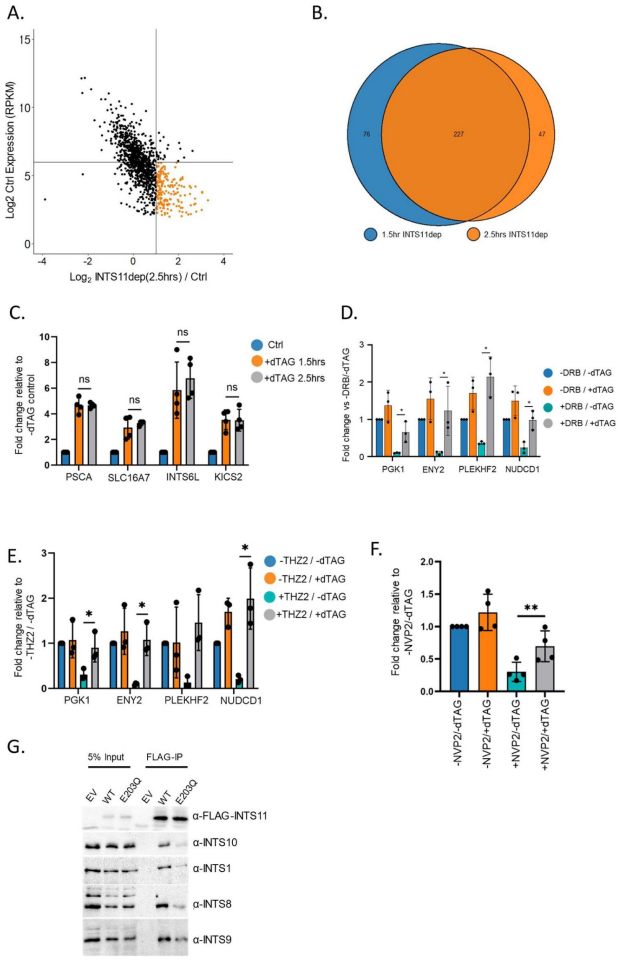
A. Plot showing the size distribution of fragments mapped by sPOINT-seq. This demonstrates its selectivity toward transcripts <150nts.

B. Genome browser track of *ACTB* promoter region in sPOINT-seq from *INTS11-dTAG* cells treated or not with dTAGv-1 (1.5 hr). This showcases the focused sense TSS (black arrows) and the dispersed antisense reads (brackets). Note the different y-axis scales (RPKM) for sense vs. antisense.

C. Violin plot of the sum sPOINT 5'end spike-in normalised RPKM signal across a 500bp window from the protein-coding TSSs shown in Figure 3D (n=3060). These samples are untreated with dTAG to quantitate the normal levels of sense vs. antisense initiation/pausing in a window -/+ 500bp from the TSS in respective directions for antisense and sense. This shows more initiation/pausing in sense vs. antisense directions.

D. Genome track of *RNU5A-1* and *RNU5B-1* in RNAPII ChIP-seq performed on *INTS11-dTAG* cells treated or not with dTAGv-1 (2hr). INTS11 depletion causes RNAPII build-up beyond both genes indicating a transcription termination defect. The Y-axis scale is RPKM.

E. Metaplot of RNAPII occupancy of protein-coding TSS regions (the same gene set employed for the sPOINT analysis in Figure 3) derived from RNAPII ChIP-seq performed on *INTS11-dTAG* cells treated or not with dTAGv-1 (2hr). The Y-axis scale is log10 qvalue of peak pileup values normalised to spike in control.



SUPPLEMENTAL FIGURE 4, related to figure 4.

A. Comparison of INTS11-dependent changes in promoter-proximal protein-coding transcription with the expression level of each gene. The gene set is equivalent to the analogous panel in **Supplemental Figure 2B**. The x-axis shows Log2FC in levels (-INTS11/+INTS11) and the y-axis shows expression level. Note that genes with the largest increase in promoter-proximal signal following INTS11 loss are low expressed (coloured orange). Data derives from POINT-seq following treatment or not with dTAGv-1 (2.5hr).

B. Venn diagram showing the number of protein-coding genes where promoter-proximal POINT-seq signal increases by ≥Log2FC of 1 after INTS11 loss after 1.5h or 2.5h dTAGv-1 treatment. A strong overlap is seen indicating reproducible effects at the two time points.

C. qRT-PCR analysis of PSCA, SLC16A7, INTS6L, and KICS2 pre-mRNAs in *INTS11-dTAG* cells treated or not with dTAGv-1 for 1.5hr or 2.5hr. To enrich nascent transcripts, primers detect intronic RNA. Quantitation shows fold change versus spliced actin relative to untreated samples. Error bars show standard deviation. n=4. n.s denotes not significant.

D. qRT-PCR analysis of PGK1, ENY2, PLEKHF2, and NUDCD1 pre-mRNAs in *INTS11-dTAG* cells treated or not with dTAGv-1 and at the same time exposed or not to DRB (all 2.5hr). To enrich nascent transcripts, primers detect intronic RNA. Quantitation shows fold change versus spliced actin relative to samples untreated with dTAGv-1 or DRB. DRB treatment substantially reduces signal, which is restored when INTS11 is co-depleted. Error bars show standard deviation. n=3, * denotes p≤0.05.

E. As for C but following treatment with the CDK7 inhibitor, THZ1, and/or depletion of INTS11 with dTAGv-1 (all treatments 2.5hr). Error bars show standard deviation. n=3, * denotes p≤0.05.

F. qRT-PCR analysis of chromatin-associated (nascent) RNA isolated from *INTS11-dTAG* cells transfected with the HIV reporter construct together with TAT then treated or not with INTS11 and/or NVP-2. Quantitation shows signals relative to those obtained in untreated cells after normalising to MALAT1 RNA. n=4. Error bars show standard deviation. **denotes p≤0.01.

G. Flag immunoprecipitation performed in *INTS11-dTAG* cells transfected with flag-tagged wild-type or E203Q INTS11. The gel shows input and co-precipitated samples probed for components of different Integrator modules (tail module – INTS10; backbone module – INTS1; phosphatase module – INTS8 and cleavage module – INTS9). These components are poorly associated with E203Q INTS11 vs. wild type.

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Reviewer #1 (Public Review):

Summary:

In this manuscript, Eaton et al. examine the regulation of transcription directionality using a powerful genomic approach (more about the methodology below).
Their data challenge the notion that the polyadenylation signal-reading Cleavage and Polyadenylation (CPA) complex is responsible for controlling promoter directionality by terminating antisense transcription. Namely, depletion of the required CPA factor RBBP6 has little effect on antisense transcription measured by POINT. They find instead that initiation is intrinsically preferential in the sense direction and additionally maintained by the activities of an alternative processing complex called Integrator, together with the kinase CDK9. In the presence of CDK9 activity, depletion of Integrator endoribonuclease INTS11 leads to globally increased transcription in the antisense direction, and minor effects in the sense direction.

However, CDK9 inhibition reveals that sense transcription is also sensitive to INS11 depletion. The authors suggest that CDK9 activity is stronger in the sense direction, preventing INTS11-mediated premature termination of sense transcripts.

Strengths:

The combination of acute depletion of the studied factors using degron approaches (important to limit possible secondary effects), together with novel and very sensitive nascent transcriptomics methods POINT and sPOINT is very powerful. The applied spike-in normalization means the analysis is more rigorous than most. Using this methodology allowed the authors to revisit the interesting question of how promoter/transcription directionality is determined.

The data quality appears very good and the fact that both global analysis as well as numerous gene-specific examples are shown makes it convincing.

The manuscript is well written and hence a pleasure to read.

Weaknesses:

The bias in transcriptional initiation directionality remains to be elucidated.

Conclusion/assessment:

This important work substantially advances our understanding of the mechanisms governing the directionality of human promoters. The evidence supporting the claims of the authors is compelling, with a.o. the use of advanced nascent transcriptomics including spike-in normalization controls and acute protein depletion using degron approaches.

In my opinion the authors' conclusions are well supported.

Not only the manuscript but also the data generated will be useful to the wide community of researchers studying transcriptional regulation. Also, the POINT-derived novel sPOINT method described here is very valuable and can positively impact work in the field.

https://doi.org/10.7554/eLife.92764.2.sa2

Reviewer #2 (Public Review):

Summary:

Eaton and colleagues use targeted protein degradation coupled with nascent transcription mapping to highlight a role for the integrator component INST11 in terminating antisense transcription. They find that upon inhibition of CDK9, INST11 can terminate both antisense and sense transcription - leading to a model whereby INST11 can terminate antisense transcription and the activity of CDK9 protects sense transcription from INST11-mediated termination. They further develop a new method called sPOINT which selectively amplifies nascent 5' capped RNAs and find that transcription initiation is more efficient in the sense direction than in the antisense direction. This is an excellent paper which uses elegant experimental design and innovative technologies to uncover a novel regulatory step in the control of transcriptional directionality.

Strengths:

One of the major strengths of this work is that the authors endogenously tag two of their proteins of interest - RBBP6 and INST11. This tag allows them to rapidly degrade these proteins - increasing the likelihood that any effects they see are primary effects of protein

depletion rather than secondary effects. Another strength of this work is that the authors immunoprecipitate RNAPII and sequence extracted full length RNA (POINT-seq) allowing them to map nascent transcription. A technical advance from this work is the development of sPOINT which allows the selective amplification of 5' capped RNAs < 150 nucleotides, allowing the direction of transcription initiation to be resolved.

Weaknesses:

While the authors provide strong evidence that INST11 and CDK9 play important roles in determining promoter directionality, their data suggests that when INST11 is degraded and CDK9 is inhibited there remains a bias in favour of sense transcription (Figure 4B and C). This suggests that there are other unknown factors that promote sense transcription over antisense transcription and future work could look to identify these.

https://doi.org/10.7554/eLife.92764.2.sa1

Reviewer #3 (Public Review):

Summary:

Using protein degradation approach, Eaton et al show that INST11 can terminate the sense and anti-sense transcription but higher activity of CDK9 in sense direction protects it from INS11-dependent termination. They developed sPOINT-seq that detects nascent 5'-capped RNA. The technique allowed them to reveal robust transcription initiation of sense-RNA as compared to anti-sense.

Strengths:

The strength of paper is acute degradation of proteins, eliminating the off-target effects. Further, the paper uses elegant approaches such as POINT and sPOINT-seq to measure nascent RNA and 5'-capped short RNA. Together, the combination of these three allowed the authors to make clean interpretations of data.

Weaknesses:

While manuscript is well written, the details on panel is not sufficient. The methods can be more elaborate for better understanding. Additional discussion on how authors findings contradict the existing model of anti-sense transcription termination should be added.

in the revised manuscript, authors have added details on panels and elaborated method and other sections for better understanding.

https://doi.org/10.7554/eLife.92764.2.sa0

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, Eaton et al. examine the regulation of transcription directionality using a powerful genomic approach (more about the methodology below). Their data*

> *challenge the notion that the polyadenylation signal-reading Cleavage and Polyadenylation (CPA) complex is responsible for controlling promoter directionality by terminating antisense transcription. Namely, depletion of the required CPA factor RBBP6 has little effect on antisense transcription measured by POINT. They find instead that initiation is intrinsically preferential in the sense direction and additionally maintained by the activities of an alternative processing complex called Integrator, together with the kinase CDK9. In the presence of CDK9 activity, depletion of Integrator endoribonuclease INTS11 leads to globally increased transcription in the antisense direction, and minor effects in the sense direction. However, CDK9 inhibition reveals that sense transcription is also sensitive to INS11 depletion. The authors suggest that CDK9 activity is stronger in the sense direction, preventing INTS11-mediated premature termination of sense transcrpts.*
>
> *Strengths:*
>
> *The combination of acute depletion of the studied factors using degron approaches (important to limit possible secondary effects), together with novel and very sensitive nascent transcriptomics methods POINT and sPOINT is very powerful. The applied spike-in normalization means the analysis is more rigorous than most. Using this methodology allowed the authors to revisit the interesting question of how promoter/transcription directionality is determined.*
>
> *The data quality appears very good and the fact that both global analysis as well as numerous gene-specific examples are shown makes it convincing.*
>
> *The manuscript is well written and hence a pleasure to read.*

We appreciate this positive assessment.

> *Weaknesses:*
>
> *I am slightly worried about the reproducibility of the data - it is unclear to me from the manuscript if and which experiments were performed in replicate (lack of table with genomic experiments and GEO access, mentioned in more detail in below recommendations to authors), and the methods could be more detailed.*

All sequencing data was deposited with GEO. Multiple biological replicates were performed for each sequencing experiment. Bigwig files are presented as a table in the GEO submissions. This data has now been made public.

> *A separate discussion section would be useful, particularly since the data provided challenge some concepts in the field. How do the authors interpret U1 data from the Dreyfuss lab in light of their results? How about the known PAS-density directionality bias (more PAS present in antisense direction than in sense) - could the differential PAS density be still relevant to transcription directionality?*

As suggested, we have expanded our discussion to relate our findings to existing data. We think the results from the Dreyfuss lab are very important and highlight the role of U1 snRNA in enforcing transcriptional elongation. It does this in part by shielding PAS sequences. Recent work from our lab also shows that U1 snRNA opposes the Restrictor complex and PNUTS, which otherwise suppress transcription (Estell et al., Mol Cell 2023). Most recently, the Adelman lab has demonstrated that U1 snRNA generally enhances transcription elongation (Mimoso and Adelman., Mol Cell 2023). Our work does not challenge and is not inconsistent with these studies.

The role of U1 in opposing PAS-dependent termination inspired the idea that antisense transcriptional termination may utilise PASs. This was because such regions are rich in AAUAAA and comparatively poor in U1 binding sites. However, our RBBP6 depletion and POINT-seq data suggest that PAS-dependent termination is uncommon in the antisense direction. As such, other mechanisms suppress antisense transcription and influence promoter directionality. In our paper, we propose a major role for the Integrator complex.

We do not completely rule out antisense PAS activity and discuss the prior work that identified polyadenylated antisense transcripts. Nevertheless, this was detected by oligo-dT primed RT-PCR/Northern blotting, which cannot determine the fraction of non-polyadenylated RNA that could result from PAS-independent termination (e.g. by Integrator). To do that requires an analysis of total nascent transcription as achieved by our POINT-seq. Based on these experiments, Integrator depletion has a greater impact on antisense transcription than RBBP6 depletion.

> *I find that the provided evidence for promoter directionality to be for the most part due to preferential initiation in the sense direction should be stressed more. This is in my eyes the strongest effect and is somehow brushed under the rug.*

We agree that this is an important finding and incorporated it into the title and abstract. As the reviewer recommends, we now highlight it further in the new discussion.

> *References 12-17 report an effect of Integrator on 5' of protein-coding genes, while data in Figure 2 appears contradictory. Then, experiments in Figure 4 show a global effect of INST11 depletion on promoter-proximal sense transcription. In my opinion, data from the 2.5h time-point of depletion should be shown alongside 1.5h in Figure 2 so that it is clear that the authors found an effect similar to the above references. I find the current presentation somehow misleading.*

We are grateful for this suggestion and present new analyses demonstrating that our experiment in Figure 2 concurs with previous findings (Supplemental Figures 2A and B). Our original heatmap (Figure 2E) shows a very strong and general antisense effect of INTS11 loss. On the same scale, the effects in the sense direction are not as apparent, which is also the case using metaplots. New supplemental figure 2A now shows sense transcription from this experiment in isolation and on a lower scale, demonstrating that a subset of genes shows promoter-proximal increases in transcription following INTS11 depletion. This is smaller and less general than the antisense effect but consistent with previous findings. Indeed, our new analysis in supplemental figure 2B shows that affected protein-coding genes are lowly expressed, in line with Hu et al., Mol Cell 2023. This explains why a sense effect is not as apparent by metaplot, for which highly expressed genes contribute the most signal.

As a result of our analyses, we are confident that the apparently larger effect at the 2.5hr timepoint (Figure 4) that we initially reported is due to experimental variability and not greater effects of extended INTS11 depletion. Overlaying the 1.5h and 2.5h datasets (Supplemental Figure 4B) revealed a similar number of affected protein-coding genes with a strong (83%) overlap between the affected genes. To support this, we performed qPCR on four affected protein-coding transcripts which revealed no significant difference in the level of INTS11 effect after 2.5h vs 1.5h (Supplemental Figure 4C).

We now present data for merged replicates in Figures 2 and 4 which reveal very similar average profiles for -INTS11 vs +INTS11 at both timepoints. Overall, we believe that we have resolved this discrepancy by showing that it amounts to experimental variability and because the most acutely affected protein-coding genes are lowly expressed. As detailed above, we

show this in multiple ways (and validate by qPCR) We have revised the text accordingly and removed our original speculation that differences reflected the timeframe of INTS11 loss.

> *Conclusion/assessment:*
>
> *This important work substantially advances our understanding of the mechanisms governing the directionality of human promoters. The evidence supporting the claims of the authors is compelling, with among others the use of advanced nascent transcriptomics including spike-in normalization controls and acute protein depletion using degron approaches.*
>
> *In my opinion, the authors' conclusions are in general well supported.*
>
> *Not only the manuscript but also the data generated will be useful to the wide community of researchers studying transcriptional regulation. Also, the POINT-derived novel sPOINT method described here is very valuable and can positively impact work in the field.*

We are grateful for the reviewers' positive assessment of our study.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Eaton and colleagues use targeted protein degradation coupled with nascent transcription mapping to highlight a role for the integrator component INST11 in terminating antisense transcription. They find that upon inhibition of CDK9, INST11 can terminate both antisense and sense transcription - leading to a model whereby INST11 can terminate antisense transcription and the activity of CDK9 protects sense transcription from INST11-mediated termination. They further develop a new method called sPOINT which selectively amplifies nascent 5' capped RNAs and find that transcription initiation is more efficient in the sense direction than in the antisense direction. This is an excellent paper that uses elegant experimental design and innovative technologies to uncover a novel regulatory step in the control of transcriptional directionality.*
>
> *Strengths:*
>
> *One of the major strengths of this work is that the authors endogenously tag two of their proteins of interest - RBBP6 and INST11. This tag allows them to rapidly degrade these proteins - increasing the likelihood that any effects they see are primary effects of protein depletion rather than secondary effects. Another strength of this work is that the authors immunoprecipitate RNAPII and sequence extracted full-length RNA (POINT-seq) allowing them to map nascent transcription. A technical advance from this work is the development of sPOINT which allows the selective amplification of 5' capped RNAs < 150 nucleotides, allowing the direction of transcription initiation to be resolved.*

We appreciate this positive assessment.

> *Weaknesses:*
>
> *While the authors provide strong evidence that INST11 and CDK9 play important roles in determining promoter directionality, their data suggests that when INST11 is degraded and CDK9 is inhibited there remains a bias in favour of sense transcription (Figures 4B and C). This suggests that there are other unknown factors that promote sense transcription over antisense transcription and future work could look to identify these.*

We agree that other (so far, unknown) factors promote sense transcription over antisense, which was demonstrated by our short POINT. We have provided an expanded discussion on this in the revision. In our opinion, demonstrating that sense transcription is driven by preferential initiation in that direction is a key finding and we agree that the identification of the underlying mechanism constitutes an interesting avenue for future study.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Using a protein degradation approach, Eaton et al show that INST11 can terminate the sense and anti-sense transcription but higher activity of CDK9 in the sense direction protects it from INS11-dependent termination. They developed sPOINT-seq that detects nascent 5'-capped RNA. The technique allowed them to reveal robust transcription initiation of sense-RNA as compared to anti-sense.*
>
> *Strengths:*
>
> *The strength of the paper is the acute degradation of proteins, eliminating the off-target effects. Further, the paper uses elegant approaches such as POINT and sPOINT-seq to measure nascent RNA and 5'-capped short RNA. Together, the combination of these three allowed the authors to make clean interpretations of data.*

We appreciate this positive assessment.

> *Weaknesses:*
>
> *While the manuscript is well written, the details on the panel are not sufficient. The methods could be elaborated to aid understanding. Additional discussion on how the authors' findings contradict the existing model of anti-sense transcription termination should be added.*

We have added more detail to the figure panels, which we hope will help readers to navigate the paper more easily. Specifically, the assay employed for each experiment is indicated in each figure panel. As requested, we provide a new and separate discussion section in the revision.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Congratulations on this important piece of work!*
>
> *Some specific suggestions.*
>
> *MAJOR*
>
> *-The data are not available (Accession "GSE243266" is currently private and is scheduled to be released on Sep 01, 2026.) This should be corrected and as a minimum, the raw sequencing files as well as the spike-in scaled bigwig files should be provided in GEO.*

We have made the data public. Raw and bigwig files are provided as part of the GEO upload.

> *MINOR*
>
> *- It would be useful for readers if you could include catalog numbers of the reagents used in the study.*

We have included this information in our revision.

> *- A table in experimental procedures summarizing the genomic experiments performed in this study as well as published ones reanalyzed here would be helpful.*

This is now provided as part of the resources table.

> *- It would be easier for reviewers to evaluate the manuscript if the figure legends were included together with the figures on one page. This is now allowed by most journals.*

We have used this formatting in the revision.

> *- Providing some captions for the results sections would be helpful.*

We have included subheadings as suggested.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Generally, I would suggest writing the experiment-type above panels where it is not immediately obvious what they are so a reader can appreciate the figures without referencing the legend. E.g. write POINT-seq on Figure 1B just to make it obvious to someone looking at the figures what methodology they are looking at. Likewise, you could write RNAPII ChIP-seq for Supplementary Figures 3D and 3E.*

We have carried out this recommendation.

> *Can a y-axis be indicated on POINT-seq genome browser tracks? This could make them easier to interpret.*

Y-axis scales are provided as RPKM as stated in the figure legends.

> *The authors could address/speculate in the text why there is less POINT-seq signal for the antisense transcript in the treatment condition in Figure 1B? Or could consider including a different example locus where this is not the case for clarity.*

Acute depletion of poly(A) factors (like RBBP6) results in a strong read-through beyond the poly(A) signal of protein-coding genes as Figure 1 shows. However, it also causes a reduction in transcription levels, which can be seen in the figure and is correctly noted by the reviewer in this comment. We see this with other poly(A) factor depletions (e.g. CPSF73 and CPSF30 – Eaton et al., 2020 and Estell et al., 2021) and other labs have observed this too (e.g for CPSF73-dTAG depletion (Cugusi et al., Mol Cell 2022)). Plausible reasons include a limited pool of free RNAPII due to impaired transcriptional termination or limited nucleotide availability due to their incorporation within long read-through transcripts. For these reasons, we have retained the example in Figure 1B as a typical representation of the effect. Moreover, the heatmap in Figure 1D fairly represents the spectrum of effects following RBBP6 loss – highlighting the strong read-through beyond poly(A) signals and the marginal antisense effects.

> *"The established effect of INTS11 at snRNAs was detected in our POINT-seq data and demonstrates the efficacy of this approach (Figure 2B)." The authors could explain this point more clearly in the text and describe the data - e.g. As expected, depletion of INTS11 leads to increased POINT-seq signal at the 3' end of snRNAs, consistent with defects in transcriptional termination. This is highlighted by the RNU5A-1 and RNU5B-1 loci (Figure 2B).*

We agree and have added more context to clarify this.

> *I would suggest adjusting the scale of the heatmap in Figure 2E - I think it would be easier to interpret if the value of 0 was white - with >0 a gradient of orange and <0 a gradient of blue (as is done in Figure 1C). I think making this change would make the point as written in the text clearer i.e. "heatmap analysis demonstrates the dominant impact of INTS11 on antisense versus sense transcription at most promoters (Figure 2E)." I'm assuming most of the sense transcription would be white (more clearly unchanging) when the scale is adjusted.*

We agree and have done this. The reviewer is correct that most sense transcription is unchanged by INTS11 loss. However, as we alluded to in the original submission, a subset of transcripts shows a promoter-proximal increase after INTS11 depletion. We have expanded the analyses of this effect (see responses to other comments) but stress that it is neither as general nor as large as the antisense effect.

> *The authors make the point that there is mildly increased transcription over the 5' end of some genes upon INST11 depletion and show a track (Supplementary Fig 2A). It is not immediately obvious from the presentation of the meta-analysis in Figure 2D how generalisable this statement is. Perhaps the size of the panel or thickness of the lines in Figure 2D could be adjusted so that the peak of the control (in blue) could be seen. Perhaps an arrow indicating the peak could be added? I'm assuming the peak at the TSS is slightly lower in the control compared to INST11 depletion based on the authors' statement.*

We have provided multiple new analyses of this data to highlight where there are promoter-proximal effects of INTS11 loss in the sense direction. Please see our response to the public review of reviewer 1 and new supplemental figures 2A, 2B, 4A and 4B which highlight the sense transcription increased in the absence of INTS11.

> *The authors label Figure 4 "Promoters lose their directionality when CDK9 is inhibited" - but in INST11 depleted cells treated with CDK9i they find that there still is a bias towards sense transcription. Suggested edit "Some promoter directionality is lost when CDK9 is inhibited" or similar.*

We agree and have made this change.

> *The authors conclude that INTS11-mediated effects are the result of perturbation of the catalytic activities of Integrator, the authors should perform rescue experiments with the catalytically dead E203Q-INTS11 mutant.*

This is a very good suggestion and something we had intended to pursue. However, as we will describe below (and shown in Supplemental Figure 4G), there were confounding issues with this experiment.

The E203Q mutant of INTS11 is widely used in the literature to test for catalytic functions of INTS11. However, we have found that this mutation impairs the ability of INTS11 to bind other Integrator modules in cells. Based on co-immunoprecipitation of flag-tagged WT and E203Q derivatives, INTS1 (backbone module), 10 (tail module), and 8 (phosphatase module) all show reduced binding to E203Q vs. WT. Because E203Q INTS11 is defective in forming Integrator complexes, rescue experiments might not fully distinguish the effects of INTS11 activity from those caused by defects in complex assembly. While this may at first seem unexpected, in the analogous 3’ end processing complex, catalytic mutants of CPSF73 (which

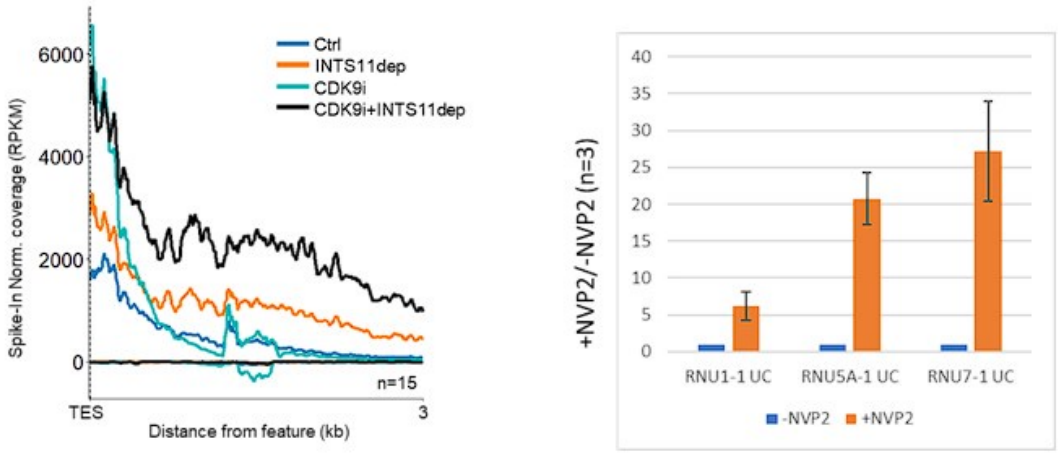
is highly related to INTS11) negatively affect its interaction with other complex members (Kolev and Steitz, EMBO Reports 2005).

We hypothesise that INTS11 activity is most likely involved in attenuating promoter-proximal transcription, but we cannot formally rule out other explanations and discuss this in our revision. Regardless of how INTS11 attenuates transcription, our main conclusion is on its requirement to terminate antisense transcription whether this involves its cleavage activity or not.

> *The authors suggest that CDK9 modulates INTS11 activity/assembly and suggest this may be related to SPT5. Is there an effect of CDK9 inhibition on the snRNA's highlighted in Figure 2B?*

We believe that snRNAs are different from protein-coding genes concerning CDK9 function. Shona Murphy's lab previously showed that, unlike protein-coding genes, snRNA transcription is insensitive to CDK9 inhibition, and that snRNA processing is impaired by CDK9 inhibition (Medlin et al., EMBO 2003 and EMBO 2005). We reproduce these findings by metaanalysis of 15 highly expressed and well-separated snRNAs and by qRT-PCR of unprocessed RNU1-1, RNU5A-1 and RNU7-1 snRNA following CDK9 inhibition. We observe snRNA read-through by POINT-seq following INTS11 loss whether CDK9 is inhibited or not (left panel, below). Note the higher TES proximal signal in CDK9i conditions, which likely reflects the accumulation of unprocessed snRNA as validated by qPCR for three example snRNAs (right panel, below).

Author response image 1.



> *For Figure 4, would similar results be observed using inhibitors targeting other transcriptional CDKs such as CDK7,12/13?*

In response to this suggestion, we analysed four selected protein-coding transcripts (the same 4 that we used to validate the CDK9i results) by qRT-PCR in a background of CDK7 inhibition using the THZ2 compound (new Supplemental Figure 4E). THZ2 suppresses transcription from these genes as expected. Interestingly, expression is restored by co-depleting Integrator, recapitulating our findings with CDK9 inhibition. As CDK7 is the CDK-activating kinase for CDK9, its inhibition will also inhibit CDK9 so THZ2 may simply hit this pathway upstream of where CDK9 inhibitors. Second, CDK7 may independently shield transcription from INTS11. We allude to both interesting possibilities.

> *What happens to the phosphorylation state of anti-sense engaged RNAPII when INTS11 is acutely depleted and/or CDK9 is inhibited? This could be measured by including Ser5 and Ser2 antibodies in the sPOINT-seq assay and complemented with Western Blot analysis.*

We have performed the western blot for Ser5 and Ser2 phosphorylation as suggested. Both signals are mildly enhanced by INTS11 loss, which is consistent with generally increased transcription. Ser2p is strongly reduced by CDK9 inhibition, which is consistent with the loss of nascent transcription in this condition. Interestingly, both modifications are partly recovered when INTS11 is depleted in conjunction with CDK9 inhibition. This is consistent with the effects that we see on POINT-seq and shows that the recovered transcription is associated with some phosphorylation of RNAPII CTD. This presumably reflects the action(s) of kinases that can act redundantly with CDK9.

We have not performed POINT-seq with Ser5p and Ser2p antibodies under these various conditions. Our rationale is that our existing data uses an antibody that captures all RNAPII (regardless of its phosphorylation status), which we feel most comprehensively assays transcription in either direction. Moreover, the lab of Fei Chen (Hu et al., Mol Cell 2023) recently published Ser5p and Ser2p ChIP-seq following INTS11 loss. By ChIP-seq, they observe a bigger increase in antisense RNAPII occupancy vs. sense providing independent and orthogonal support for our POINT-seq data. Interestingly, this antisense increase is not paralleled by proportional increases in Ser5p or Ser2p signals. This suggests that the unattenuated antisense transcription resulting from INTS11 loss does not have high Ser5p or Ser2p. Since CDK7 and 9 are major Ser5 and 2 kinases, this supports our model that their activity is less prevalent for antisense transcription. We now discuss these data in our revision.

> *The HIV reporter RNA experiments should be performed with the CDK9 inhibitor added to the experimental conditions. Presumably CDK9 inhibition would result in no upregulation of the reporter upon addition of TAT and/or dTAG. Perhaps the amount of TAT should be reduced to still have a dynamic window in which changes can be detected. It is possible that reporter activation is simply at a maximum. Can anti-sense transcription be measured from the reporter?*

We have performed the requested CDK9 inhibitor experiment to confirm that TAT-activated transcription from the HIV promoter is CDK9-dependent (new supplemental figure 4F). Consistent with previous literature on HIV transcription, CDK9 inhibition attenuates TAT-activated transcription. Importantly, and in line with our other experiments, depletion of INTS11 results in significant restoration of transcription from the HIV promoter when CDK9 is inhibited. Thus, TAT-activated transcription is CDK9-dependent and, as for endogenous genes, CDK9 prevents attenuation by INTS11.

While TAT-activated transcription is high, we do not think that the plasmid is saturated. When considering this question, we revisited previous experiments using this system to study RNA processing (Dye et al., Mol Cell 1999, Cell 2001, Mol Cell 2006). In these cases, mutations in splice sites or polyadenylation sites have a strong effect on RNA processing and transcription around HIV reporter plasmids. Effects on transcription and RNA processing are; therefore, apparent in the appropriate context. In contrast, we find that the complete elimination of INTS11 has no impact on RNA output from the HIV reporter. Our original experiment assessing the impact of INTS11 loss in +TAT conditions used total RNA. One possibility is that this allows non-nascent RNA to accumulate which might confound our interpretation of INTS11 effects on ongoing transcription. However, the new experiment described in the paragraph above was performed on chromatin-associated (nascent) RNA to rule this out. This again shows no impact of INTS11 loss on HIV promoter-derived transcription in the presence of TAT.

To our knowledge, antisense transcription is not routinely assayed from plasmids. They generally employ very strong promoters (e.g. CMV, HIV) to drive sense transcription. Crucially, their circular nature means that RNAPII going around the plasmid could interfere with antisense transcription coming the other way which does not happen in a linear genomic context. This is why we restricted our use of plasmids to looking at the effects of stimulated CDK9 recruitment (via TAT) on transcription rather than promoter directionality.

> *The authors should clearly state how many replicates were performed for the genomics experiments. Ideally, a signal should be quantified and compared statistically rather than relying on average profiles only.*

We have stated the replicate numbers for sequencing experiments in the relevant figure legends. All sequencing experiments were performed in at least two biological replicates, but often three. In addition, we validated their key conclusions by qPCR or with orthogonal sequencing approaches.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The authors provide strong evidence in support of their claims.*
>
> *ChIP-seq of pol2S5 and S2 upon INST11 and CDK9 inhibition will strengthen the observation that transcription in the sense direction is more efficient.*

We view the analysis of total RNAPII as the most unbiased way of establishing how much RNAPII is going one way or the other. Importantly, ChIP-seq was very recently performed for Ser2p and Ser5p RNAPII derivatives in the lab of Fei Chen (Hu et al., Mol Cell 2023). Their data shows that loss of INTS11 increases the occupancy of total RNAPII in the antisense direction more than in the sense direction, which is consistent with our finding. Interestingly, the increased antisense RNAPII was not paralleled with an increase in Ser2p or Ser5p. This suggests that, following INTS11 loss, the unattenuated antisense transcription is not associated with full/normal Ser2p or Ser5p. These modifications are normally established by CDK7 and 9; therefore, this published ChIP-seq suggests that they are not fully active on antisense transcription when INTS11 is lost. This supports our overall model that CDK9 (and potentially CDK7 as suggested for a small number of genes in new Supplemental Figure 4E) is more active in the sense direction to prevent INTS11-dependent attenuation. We now discuss these data in our revision.

> *In Supplementary Figure 2, the eRNA expression increases upon INST11 degradation, I wonder if the effects of this will be appreciated on cognate promoters? Can the authors test some enhancer:promoter pairs?*

We noticed that some genes (e.g. MYC) that are regulated by enhancers show reduced transcription in the absence of INTS11. Whilst this could suggest a correlation, the transcription of other genes (e.g. ACTB and GAPDH) is also reduced by INTS11 loss although they are not regulated by enhancers. A detailed and extensive analysis would be required to establish any link between INTS11-regulated enhancer transcription and the transcription of genes from their cognate promoters. We agree that this would be interesting, but it seems beyond the scope of our short report on promoter directionality.

> *Line 111, meta plot was done of 1316 genes. Details on this number should be provided. Overall, the details of methods and analysis need improvement. The layout of panels and labelling on graphs can be improved.*

We have now explained the 1316 gene set. In essence, these are the genes separated from an expressed neighbour by at least 10kb. This distance was selected because depletion of RBBP6 induces extensive read-through transcription beyond the polyadenylation site of protein-coding genes. To avoid including genes affected by transcriptional read-through from nearby transcription units we selected those with a 10kb gap between them. This was the only selection criteria so is unlikely to induce any unintended biases. Finally, we have added more information to the figure panels and their legends, which we hope will make our manuscript more accessible.

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