nature portfolio

Peer Review File

Single-molecule imaging reveals RNA polymerase II dynamics and TAF1-dependent promoter-proximal pause release

Corresponding Author: Professor Robert Coleman

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Haque et al Review
Haque et al. use an endogenously tagged RNAPII system to investigate single-molecule RNAPII dwell time in live cells and provide a nice confirmation of the rapid turnover of pol II at promoters and the inefficient transition to productive elongation reported previously using complementary approaches (eg refs 7, 8). In addition to authors report substantial heterogeneity between cells in the fraction of initiation events that transition to a stable state that they equate with transcription elongation. Also like a number of previous studies they report local concentrations of active pol II transcription that they call "hotspots"
The major new finding reported here is a significant clarification of the role of TAF1/TFIID in elongation control. The authors present a convincing argument that contrary to a previous report, TAF1 facilitates the transition from the paused to the productively elongating state.
Specific comments.
1. The authors distinguish shorter and longer-lived promoter-bound states. It would be useful to discuss the possible nature of these states in a little more detail.
2. It would be useful in Fig. 2g to define explicitly Initiation and elongation efficiency and pausing index.
3. The current definition of elongation efficiency measures only the fraction of cells entering the long-lived engaged state and does not account for global RNAPII dwell time in promoter-bound or initiated states. A potential concern is that reducing the initiated state could indirectly decrease the engaged state, leading to an apparent reduction in elongation efficiency without directly affecting elongation. Could the authors discuss why elongation efficiency is not calculated as a ratio of the engaged to initiated state, rather than relying solely on the engaged state?
4. The connection of TAF1 and phospho-SPT5 is interesting, but it is unclear if TAF1 facilitates SPT5 phosphorylation (as stated in Fig. 7) or just that TAF1 is required indirectly for SPT5 phosphorylation. The Fig.7 legend should be modified to reflect this uncertaintly.
5. The authors should compare and contrast their observation of "pol II hotspots" with previously reported "transcription factories" (Ghamori et al Genes Dev 2013, 26:767, Iborra et al 1996, J. Cell Sci, 109:1427) and pol II "clusters" (Cho et al eLife2016; 5:e13617). They should carefully justify any introduction of a new term into an already crowded and somewhat confusing body of terminology. I do not find the term "hotspot" to be a particularly clarifying or enlightening one.
6. These statements in the text (lines 281-284) are confusing and should be revised:
"In contrast, elongation efficiency was reduced (Fig. 5f), indicating that Pol II molecules that escape the promoter are defective in entering a long-lived, gene-body state."
If a pol II molecule "escapes the promoter" by definition it enters into a state of productive elongation. The alternative to escape is premature termination.
" This inefficiency in transitioning to a stably engaged state could reflect a disruption in promoter-proximal pausing".
If the transition to a stably engaged state is inefficient the implication is not that promoter proximal pausing is disrupted but rather that it is enhanced.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In the manuscript entitled “TAF1 facilitates promoter proximal pause release of RNA Polymerase II”, Haque et al. employ single-molecule imaging to characterize RNA Pol II transcription in U2OS cells. Using a SNAP-tag knock-in to track polymerase residence time, they use GRID analysis to convincingly delineate populations of polymerases at the stages of initiation, pausing and elongation with the help of selective inhibitors (Triptolide, NVP-2). They find that among chromatin binding events lasting for around 10 seconds, only a small fraction proceeds to initiate, pause and eventually engage in productive elongation. Surprisingly, they find that cells are heterogenous in their overall elongation efficiency, and this heterogeneity cannot be explained by differences in the cell cycle stage. Furthermore, sites of frequent Pol II binding, referred to as hotspots, are sites of high transcription efficiency. Finally, using a PROTAC approach to acutely degrade TAF1, the authors find that TAF1 is necessary for cells to achieve high elongation efficiency. Since most of the TAF subunits remain unaffected and phospho-SPT5 levels are decreased, they conclude that TAF1 depletion leads to impaired promoter-proximal pause release.

Overall, the imaging data and analysis are compelling and offer insights into the efficiency by which Pol II undergoes initiation, pausing and productive elongation. They are consistent with previous studies, e.g. measuring apparent half-lives of Pol II (e.g. Teves et al. 2018), and the observation that cells vary in their overall rates is surprising and interesting. The data on TAF1 also make sense but are less comprehensive, especially given that TFIID is well studied as initiation factor.

Specific points:

While the overall story makes sense, it is not clear why the title specifically focuses on the broad conclusion on TAF1. Since the paper’s strength resides in the imaging approach and the comprehensive analysis of polymerase residence time, it would be helpful to at least mention the single-molecule imaging technique in the title.

The finding that acute TAF1 degradation does not impact global initiation efficiency is intriguing and should be discussed in the light of prior studies who have examined the loss of TAF1 with more traditional approaches. Is there evidence that TFIID without TAF1 is indeed functional or could the maintained initiation rate after TAF1 depletion be partially explained by TATA promoters that can initiate without TFIID? TATA-containing promoters have been shown to be less dependent on TFIID (e.g. Petrenko et al. 2019) and may even show larger and prolonged transcriptional bursts after TAF1 depletion (Pennington et al. 2013).

The TAF1 and phospho-SPT5 link would also benefit from additional discussion. The link is based on the finding that most TFIID subunits are unaffected by TAF1 degradation, but was this also true for TAF7 in the mass spectrometry analysis? Was TAF7 detectable in the DMSO samples? In vitro studies have shown that TAF7 enhances P-TEFb-mediated phosphorylation of SPT5 (e.g. Gegonne et al, 2008). Mentioning TAF7 would help contextualize the finding with regard to the current literature and would strengthen the connection between TFIID and pause release.

The model presented in figure 7 appears inaccurate. Given that TFIID’s TAF1/TAF2/TAF7 typically bind 10-25 bp downstream of the TSS, the pause site should be located closer to it. The pause site should be located 30-80 bp downstream of the TSS, but based on the helical turns in the figure, this looks quite a bit further away. It is recommended to revise the model figure and perhaps to consider adding some imaging results as summary.

Minor points:

- The term “promoter (transient)” in the GRID analysis figures is misleading. These short-lived signals may be any transient, non-productive interactions with chromatin, which could occur at promoters but also at enhancers. A more neutral term such as “transient binding” would be more accurate.
- Supplementary figure 6 is currently not referenced in the main text. It should be integrated in the result paragraph on Pol II hotspots analysis.
- The difference in TAF1 degradation levels between the two concentrations of HaloPROTAC3 appears minimal. This point could be moved to the Supplement.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
Haque et. al introduce a live cell imaging method to analyze RNA Polymerase II (Pol II) binding events and dynamics. Using their imaging technique, they identify different dwell times of PolI II on the genome and attribute these to Pol II in different states of the transcription cycle (i.e. initiated, paused, elongating) that agree with previous orthogonal methods. Consistent with prior studies, the authors find that many Pol II initiation events do not lead to the long-lived Pol II binding associated with productive elongation. The authors address the heterogeneity of transcription efficiency between different cells and show that this is not dependent on cell cycle differences. The origin of such heterogeneity thus remains unclear (see below). Applying their method further they work to define the role of TAF1 in initiation and promoter proximal pause release. Overall, the experimental system has strengths and the questions addressed are important. However, there are shortfalls in the experimental approaches (e.g., drug treatments). Also, the data presentation and terminology in this manuscript that need to be remedied before this work would be suitable for publication.
Concerns:
1) Although the abstract and results section focus on the step of pausing and pause release, the process of pausing is not adequately described in the introduction. This will make the manuscript difficult to follow for many readers. I suggest a considerable overhaul of the introduction to more accurately describe the steps following initiation, including a clear presentation of pausing - including the role of SPT5 in pausing (SPT5 is mentioned extensively in the results, but is poorly introduced). The introduction should also describe the steps that allow for pause release. P-TEFb / CDK9 is also discussed in the results, but not well introduced. Further, the authors consistently suggest that paused Pol II 'dissociates' from DNA, and never accurately describe that the paused complex is subjected to active termination by the integrator complex. The inadequacies of the introduction should be fixed to better put the findings in context.
2) Several terms used in this manuscript raise confusion:
Within the results section, it is very confusing that the authors refer to the intermediate state as 'initiated' but then interpret changes to this state as reflecting alterations in pausing. If the authors think that this state can be measured to infer pausing duration, they should refer to it as the paused state, rather than 'initiated'.
The authors use the term 'engaged' in several places to refer to the productive elongation state, which is confusing. In the transcription field, engaged means that Pol II is associated with the DNA template and nascent RNA. The paused Pol II is engaged. Thus, the term used confounds the paused and actively elongating states.
3) This work includes several nice technological advances over previous work attempting to measure the kinetics of Pol II occupancy on the genome. However, the authors make a problematic over-simplification. They assume that all Pol II binding events that last > a few seconds occur at 'promoters'. This is surely not the case, since Pol II initiates at ~15K mRNA promoters and 80-100K regulatory elements (enhancers, noncoding RNAs) across the genome. Thus, the majority of binding and initiation events measured here occur at noncoding or regulatory RNA loci, not mRNA promoters. It has been repeatedly shown that Pol II binding is much more stable at promoters than enhancers, and here the authors are confounding the different types of sites. To avoid misleading the field, the authors must alter their wording - including in the abstract. To my mind, being able to measure all types of Pol II binding events isn't a weakness of the work - but the authors do need to make it extremely clear that they are not just looking at mRNA promoters, but are measuring Pol II dynamics at all possible initiation sites, that occur anywhere in the genome.
4) Along these lines, I suspect that the hotspots observed that show more efficient initiation and elongation are in fact active mRNA promoters, and most of the really short lived Pol II binding reflects the complexes formed at enhancers and noncoding RNAs where Pol II is known to be unstable. This would be useful to note and discuss.
5) The authors show no decrease in cell viability based on their tagging of Pol II and TAF1, which is promising. However, the cells used in this study have nonetheless been through multiple rounds of CRISPR and selection, raising concerns about their overall genome stability. Based on the methods section, it appears that the authors are using a pool of sequentially CRIPSR-targeted cells, which may harbor an array of off-target events. Although using a pool of CRISPR-targeted cells is often beneficial, I worry in this case that some of the variability seen among cells could arise from the genome engineering- perhaps some cells have lower (or higher) expression of TAF1 or Pol II that leads to differences in initiation efficiency? This is important to evaluate. I recommend that the authors select a single cell clone and perform imaging of Pol II in this clone to see if - for a cell population with identical genotype- they observe variability in transcription efficiencies.
6) Regarding the use of transcription inhibitors, the authors claim that "If these populations represent transcription independent Pol II, no changes will occur after cells are treated with transcription-specific inhibitors." This assumption does not hold for the Trp conditions used, which cause significant degradation of Pol II. Any dynamics of Pol II will be affected when half the population is degraded, eliciting a big reduction in overall signal. Two hours of Trp treatment is just too long for reliable measurements of transcription kinetics. The amount of cellular feedback that occurs after transcription has been shut off for 2 hours and so much Pol II is degraded are huge! I don't think any detailed mechanistic conclusions can be drawn from experiments involving such substantive perturbation of transcription. The authors should evaluate cells after 15-30 min of Trp treatment, prior to such large scale alterations in polymerase levels and cell biology.
7) It is similarly concerning that the authors observe less initiated / paused Pol II in cells treated with NVP2. As the authors must know, previous work clearly shows that NVP2 treatment increases Pol II engaged in pausing, because it impairs pause release. The fact that the authors don't see this makes me worry that their 2 hour treatment with NVP2 is also having unwanted effects on the cells. 15-30 min treatment with NVP2 is more than enough to block CKD9 activity, and 2h begins to cause feedback and affect cell health. The authors should include a much shorter treatment with NVP2, which hopefully will allow them to see the increase in paused Pol II expected from this treatment. Seeing this anticipated result would really help the authors case that they are correctly attributing the intermediate state as the initiated and paused state.
8) The authors conclusion that loss of TAF1 impairs pause release hinges on a reported increase in pausing index. However, the data shown indicate that this arises uniquely from a decrease in long-lived polymerase binding events, presumed to represent elongation. However, if pause release is affected, one would anticipate an increase in the

intermediate state that represents paused Pol II. As the authors note, the previous PRO-seq from the Taatjes lab showed an increase in paused Pol II upon TAF1 depletion, consistent with increased pausing. Why is this not seen in the current data?
9) In Fig. 6b the authors use a decrease in SPT5 phosphorylation to support the model that TAF1 facilitates SPT5 phosphorylation enabling pause release. I think this would be further supported by blotting for SPT5 phosphorylation in the washout experiment in Fig. 6e. Demonstrating that the loss of TAF1 is directly linked to this decrease and that it increases as TAF1 does after washout.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My criticisms have been well addressed

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In the revised manuscript, “Single-molecule imaging reveals RNA Polymerase II dynamics and TAF1-dependent promoter-proximal pause release,” Haque et al. have addressed the concerns raised by all reviewers overall well. This has improved the quality of the manuscript, but unfortunately makes the manuscript now much harder to read.

Among the improvements are that the different events identified in their GRID analysis have now been renamed and are more understandable. They show the spectrum GRID analysis following TAF1 degradation and show that TAF7 is not degraded during the TAF1-HaloPROTAC. These findings show that the reduction in productive Pol II elongation within gene bodies is not caused by a global reduction in transcription initiation, but rather by a defect in promoter-proximal pause release. This strengthens the argument that TAF1 is involved in pause release.

While it is appreciated that the authors were very responsive to the reviewers’ comments, adding substantially more information to the main body of the manuscript does not necessarily make it better. I recommend shortening and streamlining the main text so that the manuscript is more accessible.

Specific points:

In the Introduction, substantial additional information has been included to describe the process of promoter-proximal pausing and pause release. This reads more like a review and lacks a clear connection to the study itself. The manuscript would read more easily and focus the reader’s attention if the authors would explain their motivation for developing the technique and analyzing Pol II dynamics earlier and introduce only the background information necessary to understand the questions, results and conclusions. There is no need to elaborate on all the molecular details of each process.

The last two paragraphs of the section “Variability in gene body entry is not explained by cell-cycle state” seem somewhat out of place and not directly related to the section title. Reorganizing the section could improve its overall coherence.

In the section “Single-cell analysis reveals heterogeneity in gene body entry of Pol II”, the additional information regarding Triptolide and NVP-2 treatments somewhat blurs the main message that gene body entry is a highly heterogeneous step across cells. This is an example where the requested additions do not improve the clarity of the section and instead make this section more difficult to read.

In Figure 7, the proposed model suggests that TAF1 is part of promoter-bound TFIID when mediating SPT5 phosphorylation. Since the TFIID complex and promoter-proximally paused Pol II might clash when simultaneously occupying the same DNA molecule (at least in Drosophila: https://www.nature.com/articles/ng.3867), is it possible that TAF1 may function independently of the full promoter-bound TFIID complex during pause release?

In the section “Loss of TAF1 significantly impairs Pol II entry into the gene body state,” adding a sentence linking the proposed role of TAF1 in pause release with the absence of TAF7 degradation would help clarify that the loss of productive elongation is specifically caused by TAF1 depletion rather than by an off-target effect resulting in TAF7 degradation.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The authors have done a very nice job of revising the manuscript, clarifying the terminology, adding new experiments with shorter drug treatments, and testing alternative hypotheses for the heterogeneity observed. I find their results compelling, and think their clarification of the role of TAF1/ TFIID in pausing will be valuable to the field.
At this point, I am generally supportive of publication after several changes have been made:
1) Data from the 2 hour Triptolide experiments really should be removed from the manuscript (Figures 2e in particular). The data shown in 2e erroneously suggest that Triptolide treatment causes a loss of RNAPII binding events. As acknowledged in the text, this result reflects the degradation of RNAPII caused by this overly-long Trp treatment, and not a biological insight into RNAPII behavior. I am very concerned that keeping the data shown in Figure 2e in the manuscript, along with the inaccurate conclusion that would arise from this extended Trp treatment, is misleading for the field.
The authors now have much cleaner data to include, but curiously only show the more accurate / interpretable data in a supplemental figure. This is confusing.
I strongly recommend only including data from timepoints when the RNAPII has not been degraded.
2) There are some remaining areas where the text is unclear or inaccurate: in particular, the authors should consider the following:
Line 70-71: with most Pol II molecules dissociating during the early stages of promoter engagement
This would more accurately be stated as: “during the early stages of chromatin engagement” since most Pol II binding isn’t occurring at gene promoters but instead other sites of open chromatin.

Line 113-114: We show that Pol II entry into the gene body is globally inefficient and heterogeneous between cells.
This would best be changed to “Pol II entry into productive elongation”, since most of the sites being measured are not actually genes, but instead enhancers, short non-coding RNAs, etc.

As a note, I am harping on this point because I think there is an important distinction between the Pol II transitions that occur at mRNA promoters and enhancers/ncRNAs that needs to be acknowledged. I saw how detrimental to the field it was when papers were published that smeared the behaviors of Pol II at promoters and enhancers together as if they were the same, and am hoping we can avoid that in future by using clearer language.

3) A number of studies have been performed to estimate the average (or median) pausing duration, with most estimates in the range of 2-5 minutes. See work from Matt Simon (Zimmer, Mol Cell 2021) Dirk Schubeler (Krebs, Mol Cell 2017), John Lis (Jonkers, eLife, 2014). These assays used methods like PRO-seq or short-capped RNA sequencing that are very good at detecting paused Pol II (better than TT-seq or mNET-seq), and these estimates are broadly considered to be accurate. Thus, I suggest that the authors cite the consensus estimate of ~several minutes for the stability of paused Pol II, rather than one specific paper (Gressel 2019), that gives estimates that are somewhat outliers in the field. Please note that in the work by Gressel and colleagues the authors did not consider premature termination as an option in their modeling, which influenced the accuracy of their half life measurements. I think it’s fine for the authors to keep using ~90 seconds as a threshold for ‘productive elongation’, but they should be aware that a good deal of paused polymerase has a lifetime in this range.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I am happy with the changes that were made to the text. Great job!

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The authors have responded well to my comments and the manuscript is now suitable for publication.

(Remarks on code availability)

We sincerely thank the reviewers for their thoughtful and constructive comments, which have substantially improved the clarity of the manuscript and strengthened our conclusions. In response, we have made several major revisions throughout the paper. Most notably, we have redefined the Pol II populations identified by GRID as Transient binding (formerly "Promoter unstable"), Unstable early engagement (formerly "Promoter stable"), Pause instability (formerly "Initiated"), and Gene body (formerly "Engaged"). These revised terms more accurately reflect the dissociation-rate populations, including the longest-lived population, which may also be influenced by photobleaching at the experimental limit. To avoid over-interpretation, we now state explicitly throughout the manuscript that our measurements reflect global Pol II chromatin engagements and include binding events at multiple classes of genomic loci, including noncoding regions such as enhancers. In addition, we have removed the original 'Initiation efficiency' metric and renamed 'Elongation efficiency' as 'Percent Pol II entering the gene body state'. We conservatively removed the initiation efficiency metric because we could not definitively assign a precise dwell-time threshold from promoter escape based upon our acute triptolide treatment.

We address specific comments point by point in red below, with numbered line references in **bold green**. We have ensured that "All Markup" is enabled in Track Changes so that the line references cited in our responses are displayed accurately.

Reviewer #1 (Remarks to the Author):

Haque et al. use an endogenously tagged RNAPII system to investigate single-molecule RNAPII dwell time in live cells and provide a nice confirmation of the rapid turnover of pol II at promoters and the inefficient transition to productive elongation reported previously using complementary approaches (eg refs 7, 8). In addition to authors report substantial heterogeneity between cells in the fraction of initiation events that transition to a stable state that they equate with transcription elongation. Also like a number of previous studies they report local concentrations of active pol II transcription that they call "hotspots"
The major new finding reported here is a significant clarification of the role of TAF1/TFIID in elongation control. The authors present a convincing argument that contrary to a previous report, TAF1 facilitates the transition from the paused to the productively elongating state.
Specific comments.

1. The authors distinguish shorter and longer-lived promoter-bound states. It would be useful to discuss the possible nature of these states in a little more detail.

We thank the reviewer for this helpful suggestion. We have renamed these short-lived populations as "Transient binding" and "Unstable early engagement". We interpret the "Transient binding" population as short-lived Pol II binding events that briefly probe chromatin while searching for genomic targets. This interpretation is based on the very short residence time of this population (~1.3 s) relative to our imaging interval, consistent with highly transient interactions (Lines **167 – 169**). We further interpret the "Unstable

early engagement" population (residence time ~10.1 s) as including Pol II complexes that may have initiated synthesis of very short (<10 nt) RNAs. Previous work has shown that such complexes are particularly unstable relative to both the preinitiation complex, in which Pol II has not yet synthesized nascent RNA, and the early elongation complex, in which Pol II has synthesized at least 10 nucleotides. We have added this in the results section of the revised manuscript (Lines **202 – 207**).

2. It would be useful in Fig. 2g to define explicitly Initiation and elongation efficiency and pausing index.

We thank the reviewer for this suggestion. In the revised manuscript, Fig. 2g is now Supplementary Fig. 5c, and we explicitly define the % Pol II entering the gene body state (formerly elongation efficiency) and pausing index next to this panel. In addition, to make these terms easier to locate and interpret, we consolidated the definitions of % Pol II entering the gene body and pausing index into one location in the results section, under "Single-cell analysis reveals heterogeneity in gene body entry of Pol II" (Lines **256 – 265 and 272 – 274**).

3. The current definition of elongation efficiency measures only the fraction of cells entering the long-lived engaged state and does not account for global RNAPII dwell time in promoter-bound or initiated states. A potential concern is that reducing the initiated state could indirectly decrease the engaged state, leading to an apparent reduction in elongation efficiency without directly affecting elongation. Could the authors discuss why elongation efficiency is not calculated as a ratio of the engaged to initiated state, rather than relying solely on the engaged state?

We agree that the original "elongation efficiency" metric could be influenced by changes in upstream dissociation events, such that a reduction in this value would not necessarily indicate a direct defect in elongation itself. We therefore concluded that this terminology was too interpretive. Because our assay does not directly measure how efficiently Pol II transcribes once it has initiated, we have revised the terminology to more accurately describe the underlying measurement. Specifically, we now define events lasting >90 s as Pol II binding events that can enter the gene body state. This quantity reflects the percentage of all detected Pol II binding events that reach the long-lived gene body state and will therefore decrease if more Pol II molecules dissociate at earlier steps of transcription.

4. The connection of TAF1 and phospho-SPT5 is interesting, but it is unclear if TAF1 facilitates SPT5 phosphorylation (as stated in Fig. 7) or just that TAF1 is required indirectly for SPT5 phosphorylation. The Fig.7 legend should be modified to reflect this uncertaintly.

We thank the reviewer for this helpful suggestion. We see that our original wording may have implied a mechanistic conclusion that is not directly supported by the data. In the revised manuscript, we modified the Fig. 7 legend to clarify that, although TAF1 is required

for phospho-SPT5, the precise mechanism by which TAF1 enables SPT5 phosphorylation remains unknown. We also revised Fig. 7 for clarity by simplifying the text and using visual guides to highlight the main findings. In addition, we now discuss a possible indirect mechanism in the discussion section. Specifically, we cite prior work showing that TAF1 physically interacts with BRD4, which is involved in recruitment of P-TEFb to paused Pol II complexes (Lines **1062 – 1071**).

5. The authors should compare and contrast their observation of "pol II hotspots" with previously reported "transcription factories" (Ghamori et al Genes Dev 2013, 26:767, Iborra et al 1996, J. Cell Sci, 109:1427) and pol II "clusters" (Cho et al eLife2016; 5:e13617). They should carefully justify any introduction of a new term into an already crowded and somewhat confusing body of terminology. I do not find the term "hotspot" to be a particularly clarifying or enlightening one.

We thank the reviewer for this important suggestion. We carefully reviewed the studies cited by the reviewer, along with additional related literature, and concluded that our observations are best described using the existing term **"**Pol II clusters**"** rather than **"**hotspots**".** In the revised manuscript, we have used this terminology throughout the text and figures. We also added a section comparing our observations with previously described transcription factories and Pol II clusters, to better clarify the relationship between our findings and prior work (Lines **469 – 480**).

6. These statements in the text (lines 281-284) are confusing and should be revised:
"In contrast, elongation efficiency was reduced (Fig. 5f), indicating that Pol II molecules that escape the promoter are defective in entering a long-lived, gene-body state."
If a pol II molecule "escapes the promoter" by definition it enters into a state of productive elongation. The alternative to escape is premature termination. " This inefficiency in transitioning to a stably engaged state could reflect a disruption in promoter-proximal pausing". If the transition to a stably engaged state is inefficient the implication is not that promoter proximal pausing is disrupted but rather that it is enhanced.

We agree that the original wording is confusing. We therefore revised these sentences to read: "We observed a decrease in the percentage of Pol II entering the gene body state (Fig. 5d), which could reflect impaired pause release or a reduced rate of initiation upon TAF1 loss" (Lines **386 – 388**).

(Remarks on code availability)

Reviewer #2 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.(Remarks on code availability)

Reviewer #3 (Remarks to the Author)

In the manuscript entitled “TAF1 facilitates promoter proximal pause release of RNA Polymerase II”, Haque et al. employ single-molecule imaging to characterize RNA Pol II transcription in U2OS cells. Using a SNAP-tag knock-in to track polymerase residence time, they use GRID analysis to convincingly delineate populations of polymerases at the stages of initiation, pausing and elongation with the help of selective inhibitors (Triptolide, NVP-2). They find that among chromatin binding events lasting for around 10 seconds, only a small fraction proceeds to initiate, pause and eventually engage in productive elongation. Surprisingly, they find that cells are heterogenous in their overall elongation efficiency, and this heterogeneity cannot be explained by differences in the cell cycle stage. Furthermore, sites of frequent Pol II binding, referred to as hotspots, are sites of high transcription efficiency. Finally, using a PROTAC approach to acutely degrade TAF1, the authors find that TAF1 is necessary for cells to achieve high elongation efficiency. Since most of the TAF subunits remain unaffected and phospho-SPT5 levels are decreased, they conclude that TAF1 depletion leads to impaired promoter-proximal pause release.

Overall, the imaging data and analysis are compelling and offer insights into the efficiency by which Pol II undergoes initiation, pausing and productive elongation. They are consistent with previous studies, e.g. measuring apparent half-lives of Pol II (e.g. Teves et al. 2018), and the observation that cells vary in their overall rates is surprising and interesting. The data on TAF1 also make sense but are less comprehensive, especially given that TFIID is well studied as initiation factor.

Specific points:

While the overall story makes sense, it is not clear why the title specifically focuses on the broad conclusion on TAF1. Since the paper’s strength resides in the imaging approach and the comprehensive analysis of polymerase residence time, it would be helpful to at least mention the single-molecule imaging technique in the title.

We thank the reviewer for this helpful suggestion and agree that the title should better reflect the single-molecule imaging approach, which is central to the study. At the same time, we felt it was important for the title to retain the major biological conclusion regarding the role of TAF1 in promoter-proximal pause release. We therefore revised the title to: “Single-molecule imaging reveals RNA Polymerase II dynamics and TAF1-dependent promoter-proximal pause release.”

The finding that acute TAF1 degradation does not impact global initiation efficiency is intriguing and should be discussed in the light of prior studies who have examined the loss of TAF1 with more traditional approaches. Is there evidence that TFIID without TAF1 is indeed functional or could the maintained initiation rate after TAF1 depletion be partially explained by TATA promoters that can initiate without TFIID? TATA-containing promoters have been shown to be less dependent on TFIID (e.g. Petrenko et al. 2019) and may even

show larger and prolonged transcriptional bursts after TAF1 depletion (Pennington et al. 2013).

We thank the reviewer for this important point. The interpretation of the original initiation efficiency metric required caution, particularly in light of prior work demonstrating promoter-specific dependence on TAF1 and TFIID. In the revised manuscript, we have removed the initiation efficiency metric, as we concluded that the ~40 s Pol II dissociation event identified by GRID may not be an appropriate threshold for defining initiation efficiency. Instead, we interpret this time point more conservatively as reflecting promoter-proximal pause instability, a state in which Pol II is subject to active termination by the Integrator complex. Accordingly, we have revised the manuscript to focus on the effects of TAF1 loss on pausing and entry into the gene body state.

With that said, we cannot at this time distinguish if Pol II binding to the genome, after depletion of TAF1, is driven by 1.) TBP alone or a TBP related factor, 2.) a partial TFIID complex lacking TAF1 and TBP (Pennington et. al., 2019), or 3.) The SAGA complex. We have included this information in the discussion when we talk about residual Pol II binding in clusters after TAF1 loss (Lines **505 – 511**).

We are hesitant to draw too many conclusions about the role of TAF1 in transcriptional bursting from the Pennington et. al., 2013 study since the assays were completed after a 3-day depletion of TAF1, which can't rule out indirect and compensatory effects.

Recent studies by the Hahn (Donczew et. al., eLife 2020) and Pugh (Mittal et. al., Genes & Dev. 2022) labs that rapidly deplete TAF1 have shown that TFIID is likely involved in PIC assembly at nearly all promoters in yeast. Furthermore, a significant proportion of TAF1 protein remains stably bound to inducible (likely TATA containing) promoters even after the bulk of TAF1 protein is removed from the nucleus using the anchor away system (Mittal et. al., 2022). This residual TAF1 protein, along with the presence of alternative core promoter binding complexes, likely complicates the interpretation of TAF1's role in Pol II recruitment and initiation in each of our systems.

The TAF1 and phospho-SPT5 link would also benefit from additional discussion. The link is based on the finding that most TFIID subunits are unaffected by TAF1 degradation, but was this also true for TAF7 in the mass spectrometry analysis? Was TAF7 detectable in the DMSO samples? In vitro studies have shown that TAF7 enhances P-TEFb-mediated phosphorylation of SPT5 (e.g. Gegonne et al, 2008). Mentioning TAF7 would help contextualize the finding with regard to the current literature and would strengthen the connection between TFIID and pause release.

This is an excellent suggestion. TAF7 is important to consider in light of prior work showing that TAF7 can enhance P-TEFb-mediated phosphorylation of SPT5, and may therefore help contextualize the connection between TFIID and pause release. In our mass spectrometry analysis, TAF7 was not detected. Because loss of TAF7 could provide an alternative

explanation for the pausing phenotype, we reprobed the time-course TAF1 degradation immunoblot (Formerly Fig. 5b, now Fig. 5a) using a TAF7-specific antibody to test whether HaloPROTAC3 treatment also reduced TAF7 levels. We found that TAF7 levels were not detectably affected under these conditions. This new result has been incorporated into the revised manuscript as Fig. 5a, with the full uncropped blot provided in the source data file. Finally, we cited the Gegonne et al. study in the introduction to better place our findings in the context of current literature (Lines **88 – 91**).

The model presented in figure 7 appears inaccurate. Given that TFIID's TAF1/TAF2/TAF7 typically bind 10-25 bp downstream of the TSS, the pause site should be located closer to it. The pause site should be located 30-80 bp downstream of the TSS, but based on the helical turns in the figure, this looks quite a bit further away. It is recommended to revise the model figure and perhaps to consider adding some imaging results as summary.

We appreciate the reviewer's attention to this detail. We have revised Fig. 7 to more accurately reflect the established positioning of the TFIID lobe C module (TAF1/TAF2/TAF7), located ~10–25 bp downstream of the TSS, and the promoter-proximal pause site, located ~30–80 bp downstream of the TSS. In addition, we revised Fig. 7 for clarity by substantially reducing the text and using visual guides to highlight the main findings. We also incorporated key imaging results into the model to better align with our new title, including the increased probability of Pol II entering the gene body state within Pol II clusters and the reduction in Pol II cluster number following TAF1 loss.

Minor points:

- The term "promoter (transient)" in the GRID analysis figures is misleading. These short-lived signals may be any transient, non-productive interactions with chromatin, which could occur at promoters but also at enhancers. A more neutral term such as "transient binding" would be more accurate.

We thank the reviewer for this suggestion and have renamed that population to 'Transient binding' accordingly in the text and across all associated figures. We highlight that these interactions reflect Pol II probing chromatin for its genomic targets (Lines **167 – 169**).

- Supplementary figure 6 is currently not referenced in the main text. It should be integrated in the result paragraph on Pol II hotspots analysis.

We thank the reviewer for catching this omission. We originally described and referenced this figure in the methods section, as it is primarily a quality-control figure rather than a result central to the interpretation of the Pol II cluster analysis. We now reference this panel in the results section of the revised manuscript (Line **341**).

- The difference in TAF1 degradation levels between the two concentrations of HaloPROTAC3 appears minimal. This point could be moved to the Supplement.

We thank the reviewer for this suggestion and have moved this panel from the main figure to Supplemental Fig. 9.

(Remarks on code availability)

Reviewer #4 (Remarks to the Author):

Haque et. al introduce a live cell imaging method to analyze RNA Polymerase II (Pol II) binding events and dynamics. Using their imaging technique, they identify different dwell times of Poll II on the genome and attribute these to Pol II in different states of the transcription cycle (i.e. initiated, paused, elongating) that agree with previous orthogonal methods. Consistent with prior studies, the authors find that many Pol II initiation events do not lead to the long-lived Pol II binding associated with productive elongation. The authors address the heterogeneity of transcription efficiency between different cells and show that this is not dependent on cell cycle differences. The origin of such heterogeneity thus remains unclear (see below). Applying their method further they work to define the role of TAF1 in initiation and promoter proximal pause release.
Overall, the experimental system has strengths and the questions addressed are important. However, there are shortfalls in the experimental approaches (e.g., drug treatments). Also, the data presentation and terminology in this manuscript that need to be remedied before this work would be suitable for publication. Concerns:

1) Although the abstract and results section focus on the step of pausing and pause release, the process of pausing is not adequately described in the introduction. This will make the manuscript difficult to follow for many readers. I suggest a considerable overhaul of the introduction to more accurately describe the steps following initiation, including a clear presentation of pausing - including the role of SPT5 in pausing (SPT5 is mentioned extensively in the results, but is poorly introduced). The introduction should also describe the steps that allow for pause release. P-TEFb / CDK9 is also discussed in the results, but not well introduced. Further, the authors consistently suggest that paused Pol II 'dissociates' from DNA, and never accurately describe that the paused complex is subjected to active termination by the integrator complex. The inadequacies of the introduction should be fixed to better put the findings in context.

We thank the reviewer for this important and constructive suggestion. We agree that the original introduction did not provide sufficient background on promoter-proximal pausing

and pause release, particularly for readers less familiar with these processes. In the revised manuscript, we made significant changes to the introduction to more clearly describe the steps following initiation, including promoter-proximal pausing, the role of SPT5/DSIF complex in establishing and maintaining the paused state, and the mechanisms that promote pause release (Lines **69 – 93**).

We also agree that our original wording of paused Pol II "dissociating" from DNA was inaccurate. In response, we revised the manuscript to more accurately describe this state in the context of active termination by the Integrator complex. Therefore, we renamed the "Initiated" population identified by GRID to "Pause instability" and cite the Integrator in the results (Lines **213 – 215**, **233 – 235**). Finally, we incorporated additional background in the introduction on Integrator-mediated control of paused Pol II, including the antagonistic role it plays to CDK9-dependent pause release (Lines **94 – 100**).

2) Several terms used in this manuscript raise confusion: Within the results section, it is very confusing that the authors refer to the intermediate state as 'initiated' but then interpret changes to this state as reflecting alterations in pausing. If the authors think that this state can be measured to infer pausing duration, they should refer to it as the paused state, rather than 'initiated'. The authors use the term 'engaged' in several places to refer to the productive elongation state, which is confusing. In the transcription field, engaged means that Pol II is associated with the DNA template and nascent RNA. The paused Pol II is engaged. Thus, the term used confounds the paused and actively elongating states.

We thank the reviewer for this important point and agree that the terms "Initiated" and "Engaged" were overly broad and could be misleading in the context of transcriptional state terminology. In particular, "Initiated" does not accurately describe the ~40 s population detected by GRID, because this population reflects a dissociation state rather than initiation per se. We have therefore renamed these populations "Pause instability" and "Gene body", respectively, to better reflect their kinetic properties and biological interpretation based on the GRID analysis and the photobleaching limit (Lines **213 – 223**).

3) This work includes several nice technological advances over previous work attempting to measure the kinetics of Pol II occupancy on the genome. However, the authors make a problematic over-simplification. They assume that all Pol II binding events that last > a few seconds occur at 'promoters'. This is surely not the case, since Pol II initiates at ~15K mRNA promoters and 80-100K regulatory elements (enhancers, noncoding RNAs) across the genome. Thus, the majority of binding and initiation events measured here occur at

noncoding or regulatory RNA loci, not mRNA promoters. It has been repeatedly shown that Pol II binding is much more stable at promoters than enhancers, and here the authors are confounding the different types of sites. To avoid misleading the field, the authors must alter their wording - including in the abstract. To my mind, being able to measure all types of Pol II binding events isn't a weakness of the work - but the authors do need to make it extremely clear that they are not just looking at mRNA promoters, but are measuring Pol II dynamics at all possible initiation sites, that occur anywhere in the genome.

We thank the reviewer for this critical point. We agree that our original wording was promoter-focused and could imply that all measured Pol II binding events occurred at mRNA promoters, which is certainly not the case. We concede that this was an oversight on our part as our original intention was to use terminology consistent with prior imaging studies of Pol II dynamics (Darzacq et. al. 2007 and Steuer et. al., 2018).

We agree that in this assay, our single-molecule measurements capture Pol II dynamics across multiple classes of genomic initiation sites, including mRNA promoters as well as noncoding and regulatory regions. We have therefore substantially revised the wording throughout the manuscript, including the abstract (Lines **46 – 48**), results (Lines **167 – 169**, **204 – 207**, **213 – 223**), and discussion (Lines **435 – 445**) sections to make this explicit and to avoid over-interpreting these populations as promoter-associated dissociation events.

4) Along these lines, I suspect that the hotspots observed that show more efficient initiation and elongation are in fact active mRNA promoters, and most of the really short lived Pol II binding reflects the complexes formed at enhancers and noncoding RNAs where Pol II is known to be unstable. This would be useful to note and discuss.

We thank the reviewer for this insightful comment. We agree that Pol II binding events occurring within Pol II clusters (formerly hotspots) may be enriched for active mRNA promoters, whereas shorter-lived binding events outside of clusters may include a greater contribution from enhancers and other noncoding regulatory loci, where Pol II is generally less stable. Although our current single-molecule measurements do not directly assign individual binding events to specific genomic element classes, we now discuss this interpretation in the revised manuscript (Lines **330 – 337**).

In addition, we performed a new analysis comparing cluster characteristics (cluster density, mean cluster size, and average number of binding events per cluster) with the percentage of Pol II entering the gene body state (formerly elongation efficiency) in Pol II clusters. Among these features, the average number of Pol II binding events per cluster showed a modest linear correlation, consistent with the idea that repeated local Pol II

cycling may support more efficient transcriptional progression. We relate this observation a prior study suggesting that frequent Pol II binding can help maintain TSS accessibility by keeping nucleosomes at bay, thus promoting transcription within these regions (Lines **479 – 480**).

5) The authors show no decrease in cell viability based on their tagging of Pol II and TAF1, which is promising. However, the cells used in this study have nonetheless been through multiple rounds of CRISPR and selection, raising concerns about their overall genome stability.

We appreciate the reviewers concern about the genome stability from our sequentially edited CRISPR-cell lines. To minimize the potential genetic drift associated with high passage number, we have kept the passage number as low as possible and used fresh U2-OS cells acquired directly from ATCC for cell lines used/engineered in this study.

Based on the methods section, it appears that the authors are using a pool of sequentially CRIPSR-targeted cells, which may harbor an array of off-target events. Although using a pool of CRISPR-targeted cells is often beneficial, I worry in this case that some of the variability seen among cells could arise from the genome engineering- perhaps some cells have lower (or higher) expression of TAF1 or Pol II that leads to differences in initiation efficiency? This is important to evaluate.

We thank the reviewer for raising this concern. To assess whether cell-to-cell variability in the percent of Pol II entering the gene body state could be driven by differences in Pol II or TAF1 abundance in the engineered population, we tested for correlations between per-cell gene body entry and an imaging-derived proxy for Pol II/TAF1 abundance (e.g., number of detected binding events per cell). Across cells, we did not observe a strong association between the number of Pol II/TAF1 binding events and the percentage of Pol II reaching the gene body state (Linear regression $r^2 \leq 0.06$, $p > 0.08$). This suggests that the observed heterogeneity is not primarily explained by variation in Pol II or TAF1 abundance in the engineered population. We now include this analysis as Supplementary Fig. 7a, b, and report these results in the revised manuscript (Lines **311 – 317**).

I recommend that the authors select a single cell clone and perform imaging of Pol II in this clone to see if - for a cell population with identical genotype- they observe variability in transcription efficiencies.

This is an excellent suggestion. To determine whether cell-to-cell variability in Pol II gene body entry persists within a genetically uniform population, we performed a limiting dilution assay to isolate cells derived from a single founder cell while minimizing passage number and culture time. Specifically, we seeded 0.3 cells per well directly into a 96-well imaging plate and cultured the cells in conditioned medium to support growth and reduce cellular stress. After approximately 2 weeks, we identified a well containing a colony derived from a single founder cell (Supplementary Fig. 7c). We then directly imaged cells within this clonal colony and measured the percentage of Pol II entering the gene body state. In parallel, we also imaged cells exogenously expressing an α-amanitin-resistant SNAP-tagged Pol II generated by plasmid expression and therefore independent of CRISPR-based genome editing. In both cases, we observed a similar spread in the percentage of Pol II entering the gene body state, indicating that repeated rounds of CRISPR targeting are unlikely to be the primary source of the observed variability (Supplementary Fig. 7d). The results of these experiments are reported in the revised manuscript (Lines **318 – 327**).

6) Regarding the use of transcription inhibitors, the authors claim that "If these populations represent transcription independent Pol II, no changes will occur after cells are treated with transcription-specific inhibitors." This assumption does not hold for the Trp conditions used, which cause significant degradation of Pol II. Any dynamics of Pol II will be affected when half the population is degraded, eliciting a big reduction in overall signal. Two hours of Trp treatment is just too long for reliable measurements of transcription kinetics. The amount of cellular feedback that occurs after transcription has been shut off for 2 hours and so much Pol II is degraded are huge! I don't think any detailed mechanistic conclusions can be drawn from experiments involving such substantive perturbation of transcription. The authors should evaluate cells after 15-30 min of Trp treatment, prior to such large-scale alterations in polymerase levels and cell biology.

We thank the reviewer for this important point and agree that our original Triptolide condition (1 μM for 2 hours) was too strong for mechanistic interpretation, given the substantial reduction in Pol II levels under those conditions. In response, we repeated these experiments using shorter, lower-dose Triptolide treatments designed to perturb transcription initiation while minimizing Pol II degradation. Under acute exposure (~30 min), we did not observe a detectable reduction in nuclear Pol II levels from both imaging and a western blot (Supplementary Fig. 3a, b). The event spectrum GRID analysis revealed little

to no change in the resolved Pol II dwell-time populations with Triptolide relative to control (Supplementary Fig. 3c).

However, under acute Triptolide treatment the state spectrum GRID analysis (see point 7 below) showed an increase in the 10 s population (Supplementary Fig. 3c) and the single-cell analysis revealed a reduction in the percentage of Pol II entering the gene body state, without a corresponding change in pausing index (Supplementary Fig. 5d, e). We also observed a time-dependent decrease in Pol II cluster number upon acute Triptolide treatment (Supplementary Fig. 8c). We now present these data more conservatively in the revised manuscript. In an abundance of caution, we no longer use the 2-hour Triptolide treatment as primary evidence to unambiguously assign the ~10 s population and have removed that interpretation from the revised manuscript.

7) It is similarly concerning that the authors observe less initiated / paused Pol II in cells treated with NVP2. As the authors must know, previous work clearly shows that NVP2 treatment increases Pol II engaged in pausing, because it impairs pause release. The fact that the authors don't see this makes me worry that their 2 hour treatment with NVP2 is also having unwanted effects on the cells.

We thank the reviewer for raising this important point and acknowledge that our prolonged NVP-2 treatment may be having off target effects. However, our original live-cell single-molecule imaging analysis does not measure pausing in a positional sense. Instead, the event spectrum GRID analysis reports the effective chromatin dissociation rates, which can reflect a mixture of transient binding interactions, premature termination, and/or other dissociation pathways.

In the original version of the manuscript, we report the event spectrum from the GRID analysis. The event spectrum is the frequency with which a dissociation event of a certain rate occurs during our imaging period (~17 minutes). During this imaging period, the vast majority of binding events were single frame events, with <6% lasting more than 40 seconds. In other words, there will always be fewer and fewer long lived binding events compared to shorter events. This is fundamentally different from genomics-based approaches, which are 'snapshot' assays that tell you the position of Pol II at an instantaneous point in time.

There is a way, however, to get a snapshot readout of dissociation rates using GRID. This is what's called the 'state spectrum' (Reisser, M., et al. *Scientific Reports*. 2020). The state spectrum provides information on the number of distinct Pol II binding states in an instantaneous snapshot in time. It does so by weighting the frequency of dissociation rates by the residence times. In other words, long lived binding events, which exist in multiple

frames, have a greater contribution towards the state spectrum than short-lived binding events. Thus, a state spectrum is more analogous to a genomics-based readout than an event spectrum.

Under 50 nM NVP-2 for 2-hours using a state spectrum analysis, we observe a decrease in the 'Gene body' population and a corresponding increase and redistribution toward shorter-lived components, most notably the 'Pause instability' state (new Fig. 2f, g). We include this result and biological interpretation in the revised manuscript (Lines **226 – 239**).

15-30 min treatment with NVP2 is more than enough to block CKD9 activity, and 2h begins to cause feedback and affect cell health. The authors should include a much shorter treatment with NVP2, which hopefully will allow them to see the increase in paused Pol II expected from this treatment. Seeing this anticipated result would really help the authors case that they are correctly attributing the intermediate state as the initiated and paused state.

We acknowledge the reviewer's concern that a 2-hour treatment with NVP2 may have off-target effects. To address this, we repeated the imaging using 500 nM of NVP-2 for 30-minutes. We observed a similar shift in the event and state spectrum after 30-minutes of NVP-2 treatment (new Supplementary Fig. 4a, b) as was observed after 2-hours of treatment. In addition, we found that the effects at the single-cell level on the transcription metrics (% Pol II entering gene body and pausing index) were similar to the 2-hour treatment (new Supplementary Fig. 5d, e).

Based on previously published reports, we believe that our current treatment of 50 nM NVP-2 for 2 hours is sufficient to demonstrate increased pausing and/or SPT5 dephosphorylation:

- (Olson, C. M., et al. *Nature Chemical Biology*. 2018) – Figure 5a, using 250 nM NVP-2 for 6 hours, they observed an increase in Pol II occupancy around the TSS in MOLT4 cells.
- (Aoi, Y., et al. *Molecular Cell*. 2021) – Supplemental Fig. S4C. Using 250 nM NVP-2 for 3 hours, they observe an increase in Pol II occupancy at the pause site in DLD-1 cells. Notably, 1 hour of 250 nM NVP-2 treatment followed by 2-hours of Auxin-induced degradation of NELF showed a redistribution of paused Pol II toward the gene body.
- (Parua, P. K., et al. *Nature Communications*. 2020) – Fig. 4d, ChIP-qPCR reveals a reduction in pSPT5 along the *MYC* gene after treating HCT116 cells with 250 nM NVP-2 for 1 hour.

- (Sun, R., et al. *Molecular Cell*. 2025) – Fig. 4F, Metagene plot from ChIP-seq of RPB1 in HCT116 cells. After treating cells with 250 nM NVP-2 for 1 hour, they observed an increase in Pol II occupancy around the TSS.

Thus, we have kept both drug treatment conditions in the manuscript and emphasize the state-spectrum analysis to demonstrate an increase in the paused state.

8) The authors conclusion that loss of TAF1 impairs pause release hinges on a reported increase in pausing index. However, the data shown indicate that this arises uniquely from a decrease in long-lived polymerase binding events, presumed to represent elongation. However, if pause release is affected, one would anticipate an increase in the intermediate state that represents paused Pol II. As the authors note, the previous PRO-seq from the Taatjes lab showed an increase in paused Pol II upon TAF1 depletion, consistent with increased pausing. Why is this not seen in the current data?

We thank the reviewer for raising this important point. We agree that if TAF1 loss impairs pause release, an increase in the paused population would be expected. In the revised manuscript, we now include the GRID state spectrum for HaloPROTAC3 treatment (new Fig. 6b), which reveals not only a decrease in the gene body fraction, but also an increase in the pause instability fraction, similar to NVP-2 treatment. This brings our live-cell imaging results into better agreement with the prior PRO-seq findings, which showed increased promoter-proximal Pol II upon TAF1 depletion.

9) In Fig. 6b the authors use a decrease in SPT5 phosphorylation to support the model that TAF1 facilitates SPT5 phosphorylation enabling pause release. I think this would be further supported by blotting for SPT5 phosphorylation in the washout experiment in Fig. 6e. Demonstrating that the loss of TAF1 is directly linked to this decrease and that it increases as TAF1 does after washout.

We thank the reviewer for this suggestion. In response, we examined phospho-SPT5 levels in the HaloPROTAC3 washout experiment (Supplementary Fig. 11a). Although recovery is modest by visual inspection at the early washout time point, densitometric quantification normalized to total SPT5 and Lamin A/C shows a progressive restoration of phospho-SPT5 following TAF1 recovery, increasing from ~45% under HaloPROTAC3 treatment to ~57% after 10 h washout and ~93% after 24 h washout (Supplementary Fig. 11b). At this time, it is unclear what threshold levels of pSPT5 are required to facilitate a measurable amount of pause release. We have now included this data in the revised manuscript.

We thank the reviewers for their comments and suggestions.

We address specific comments point by point in red below, with numbered line references in **bold green**. We have ensured that "All Markup" is enabled in Track Changes so that the line references cited in our responses are displayed accurately.

Reviewer #1 (Remarks to the Author):

My criticisms have been well addressed

Thank you.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, "Single-molecule imaging reveals RNA Polymerase II dynamics and TAF1-dependent promoter-proximal pause release," Haque et al. have addressed the concerns raised by all reviewers overall well. This has improved the quality of the manuscript, but unfortunately makes the manuscript now much harder to read.

Among the improvements are that the different events identified in their GRID analysis have now been renamed and are more understandable. They show the spectrum GRID analysis following TAF1 degradation and show that TAF7 is not degraded during the TAF1-HaloPROTAC. These findings show that the reduction in productive Pol II elongation within gene bodies is not caused by a global reduction in transcription initiation, but rather by a defect in promoter-proximal pause release. This strengthens the argument that TAF1 is involved in pause release.

While it is appreciated that the authors were very responsive to the reviewers' comments, adding substantially more information to the main body of the manuscript does not necessarily make it better. I recommend shortening and streamlining the main text so that the manuscript is more accessible.

Specific points:

In the Introduction, substantial additional information has been included to describe the process of promoter-proximal pausing and pause release. This reads more like a review and lacks a clear connection to the study itself. The manuscript would read more easily and focus the reader's attention if the authors would explain their motivation for developing the technique and analyzing Pol II dynamics earlier and introduce only the background information necessary to understand the questions, results and conclusions. There is no need to elaborate on all the molecular details of each process.

We thank the reviewer for this helpful suggestion and have revised the introduction accordingly. Specifically, we streamlined the text by removing mechanistic details about proteins and complexes that were not directly relevant to the questions addressed or conclusions drawn in this study. We retained a concise discussion of Integrator because Integrator-mediated premature termination provides important context for interpreting the pause instability population identified by GRID in the results, as suggested by reviewer 4. We also revised the introduction to more clearly connect the background on promoter-proximal pausing and pause release to the motivation for our single-molecule imaging approach. Overall, these changes have resulted in a decrease in the total number of lines in the introduction from 60 to 46.

The last two paragraphs of the section "Variability in gene body entry is not explained by cell-cycle state" seem somewhat out of place and not directly related to the section title. Reorganizing the section could improve its overall coherence.

Agreed. To improve the flow and coherence of this section, we have split it into two. The last two paragraphs are now part of a new section titled "Heterogeneity in productive elongation is independent of protein levels and genetic diversity". (Line **280–281**)

In the section "Single-cell analysis reveals heterogeneity in gene body entry of Pol II", the additional information regarding Triptolide and NVP-2 treatments somewhat blurs the main message that gene body entry is a highly heterogeneous step across cells. This is an example where the requested additions do not improve the clarity of the section and instead make this section more difficult to read.

We thank the reviewer for bringing this to our attention. We reported multiple Triptolide conditions in the revision to identify a concentration that perturbed transcription while maintaining Pol II levels. However, we can see how this is distracting since it reads more like a troubleshooting effort rather than a key result central to our story.

To improve the clarity of this section and the manuscript overall, we have removed select Triptolide datasets that produced nonsignificant changes or resulted in global loss of Pol II, as suggested by reviewer 4. We now only report the 30-min 500 nM Triptolide dataset in the manuscript, since it is the only condition where transcription was affected without leading

to nonspecific Pol II degradation. In addition, we added text to this section to emphasize the importance of the drug treatments in relation to the observed heterogeneity. (Line **252 – 254**)

In Figure 7, the proposed model suggests that TAF1 is part of promoter-bound TFIID when mediating SPT5 phosphorylation. Since the TFIID complex and promoter-proximally paused Pol II might clash when simultaneously occupying the same DNA molecule (at least in Drosophila: https://www.nature.com/articles/ng.3867), is it possible that TAF1 may function independently of the full promoter-bound TFIID complex during pause release?

We thank the reviewer for raising this interesting possibility. In human cells, it remains unclear whether TFIID remains fully or partially promoter-associated as Pol II transcribes through the DPE. It is also unclear whether TAF1 can function outside of the TFIID complex. Previous biochemical fractionation studies suggest that TAF1 is predominantly associated with TFIID, as TAF1 co-fractionated with other TFIID subunits in native cell extracts and did not elute by itself or as part of smaller complexes (Gegonne, PNAS 2008, Fig. 2C). In contrast, both TBP and TAF7 were detected in additional fractions. Based on these results and to our knowledge, there is no evidence to suggest that TAF1 can function independently of TFIID in vivo.

In the section “Loss of TAF1 significantly impairs Pol II entry into the gene body state,” adding a sentence linking the proposed role of TAF1 in pause release with the absence of TAF7 degradation would help clarify that the loss of productive elongation is specifically caused by TAF1 depletion rather than by an off-target effect resulting in TAF7 degradation.

We have added a sentence noting that the decrease in productive elongation is unlikely to arise due to off-target effects resulting from TAF7 loss. (Line **358 – 361**)

Reviewer #4 (Remarks to the Author):

The authors have done a very nice job of revising the manuscript, clarifying the terminology, adding new experiments with shorter drug treatments, and testing alternative hypotheses for the heterogeneity observed. I find their results compelling, and think their clarification of the role of TAF1/ TFIID in pausing will be valuable to the field.
At this point, I am generally supportive of publication after several changes have been made:

1) Data from the 2 hour Triptolide experiments really should be removed from the manuscript (Figures 2e in particular). The data shown in 2e erroneously suggest that Triptolide treatment causes a loss of RNAPII binding events. As acknowledged in the text, this result reflects the degradation of RNAPII caused by this overly-long Trp treatment, and not a biological insight into RNAPII behavior. I am very concerned that keeping the data shown in Figure 2e in the manuscript, along with the inaccurate conclusion that would

arise from this extended Trp treatment, is misleading for the field.
The authors now have much cleaner data to include, but curiously only show the more accurate / interpretable data in a supplemental figure. This is confusing.
I strongly recommend only including data from timepoints when the RNAPII has not been degraded.

We thank the reviewer for this suggestion. In response, we have removed the 2-hour Triptolide dataset, not just in Fig. 2e, but in all figures where it appears and have adjusted the main text/figure legends accordingly. In addition, we have removed some of the new Triptolide conditions which either failed to show changes in Pol II kinetics (low dose) or led to degradation of Pol II (high dose/extended incubation times) to further improve clarity. We now only report the 30-min 500 nM Triptolide condition in the main figures.

2) There are some remaining areas where the text is unclear or inaccurate: in particular, the authors should consider the following:
Line 70-71: with most Pol II molecules dissociating during the early stages of promoter engagement
This would more accurately be stated as: “during the early stages of chromatin engagement” since most Pol II binding isn’t occurring at gene promoters but instead other sites of open chromatin.

We agree that ‘chromatin engagement’ is more appropriate and have made that change in the revised manuscript (Line **88**).

Line 113-114: We show that Pol II entry into the gene body is globally inefficient and heterogeneous between cells.
This would best be changed to “Pol II entry into productive elongation”, since most of the sites being measured are not actually genes, but instead enhancers, short non-coding RNAs, etc.

We have changed that line to now read ‘We show that Pol II entry into productive elongation…’. (Line **100**)

As a note, I am harping on this point because I think there is an important distinction between the Pol II transitions that occur at mRNA promoters and enhancers/ncRNAs that needs to be acknowledged. I saw how detrimental to the field it was when papers were published that smeared the behaviors of Pol II at promoters and enhancers together as if they were the same, and am hoping we can avoid that in future by using clearer language.

We acknowledge that the current terminology can be misleading, since “Gene body” implies transcription in protein-coding genes, which we cannot specifically capture with our assay. We sincerely thank the reviewer for sharing their experience to emphasize this point. We have therefore made substantial terminology changes throughout the manuscript. Most notably, we now define the most stable fraction identified by GRID as

"productive elongation" rather than "gene body," and have updated the associated figures, figure legends, and text accordingly. We have also changed "% Pol II entering gene body" to "% Pol II entering productive elongation" in the single-cell analysis. Finally, we changed the label in our model (Fig. 7) from "Gene body" to "Productive elongation" to better align with our imaging results.

3) A number of studies have been performed to estimate the average (or median) pausing duration, with most estimates in the range of 2-5 minutes. See work from Matt Simon (Zimmer, Mol Cell 2021) Dirk Schubeler (Krebs, Mol Cell 2017), John Lis (Jonkers, eLife, 2014). These assays used methods like PRO-seq or short-capped RNA sequencing that are very good at detecting paused Pol II (better than TT-seq or mNET-seq), and these estimates are broadly considered to be accurate. Thus, I suggest that the authors cite the consensus estimate of ~several minutes for the stability of paused Pol II, rather than one specific paper (Gressel 2019), that gives estimates that are somewhat outliers in the field. Please note that in the work by Gressel and colleagues the authors did not consider premature termination as an option in their modeling, which influenced the accuracy of their half life measurements. I think it's fine for the authors to keep using ~90 seconds as a threshold for 'productive elongation', but they should be aware that a good deal of paused polymerase has a lifetime in this range.

We thank the reviewer for bringing this to our attention and have modified the text and cited those sources accordingly. (Line **231 - 232**)