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Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer
Corresponding author name(s): Professor Claudio Sette

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:

26th Oct 2021

Dear Professor Sette,

Thank you again for submitting your manuscript "The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer". I apologize for the delay while we awaited the reports (copied below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that all 3 reviewers acknowledge the potential interest of the work, and each offers suggestions to improve data presentation or to strengthen the central findings. Reviewer #1 queries aspects of the functional and physical interactions of Sam68 and XRN2 that need to be addressed in the text, while #2 requests additional control experiments to strengthen the supporting data. Reviewer #3 is more critical, and finds that the current data don't fully support the conclusions drawn, which limits the scientific advance provided by the study. The reviewer offers alternative explanations for the assays supporting the model that Sam68 and XRN2 function as a complex and that XRN2 recruits Sam68 as proposed. Reviewer #3 also finds that analysis of clinical datasets is needed to conclude that the factors contribute to prostate cancer. Editorially, we agree that these suggestions would strengthen the findings, and ask that they be incorporated in a revised manuscript.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We appreciate the requested revisions are extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the last revision, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets

can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

With kind regards,

Beth

Beth Moorefield, Ph.D.
Senior Editor
Nature Structural & Molecular Biology

Referee expertise:

Referee #1: Sam68 function

Referee #2: Sam68;ApA in diff'n/cancer

Referee #3: ApA in diff'n/cancer

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

Pieraccioli et al., identify the 5'-3' exonuclease XRN2 as an interactor of the Sam68 RNA binding protein. They report XRN2 is overexpressed in patient prostate cancers and correlates with elevated Sam68. The promoter of the XRN2 genes like the promoter of Sam68 contains E-boxes and is regulated transcriptionally by MYC. 3'READS analysis in PC cells uncovered a widespread impact of Sam68 and XRN2 on polyadenylation regulation, with an extensive functional overlap between these proteins. XRN2 promoted the recruitment of Sam68 to the polyadenylation region of regulated transcripts.

The Sam68/XRN2 complex preferentially represses recognition of strong pAs at the distal end of the 3'UTR, thus favoring usage of sub-optimal proximal pAs. Pieraccioli et al. show that this regulation has an impact on the expression of cell cycle related genes, by promoting expression of APA isoforms with higher translational efficiency and increasing the expression of proteins involved in G1/S progression, like MCM10 and ORC2. They define a molecular mechanism involved in APA regulation, which is directly linked to shortening of the 3'UTR of transcripts for cell cycle proteins and supports cell proliferation.

Overall, the experiments are convincing and well-conceived and provide a new role for Sam68 on pA selection dependent on XRN2. As Sam68 is an RNA binding protein, why does it need XRN2 to access certain APAs? Is the scaffolding function of XRN2 required, ie can exonuclease-dead XRN2 also regulate Sam68 binding as in Fig 5? Therefore, is XRN2 activity required? What portion of Sam68 interacts with the C-terminus of XRN2? Finally, is RNA needed for interaction?

Reviewer #2:
Remarks to the Author:
A. Summary of key results. The overall message of this paper is that high levels of Sam68 and XRN2 proteins in prostate cancers are driven by Myc and cause changes in polyA site selection. This occurs particularly by repressing the use of downstream polyA sites via a competitive mechanism with the polyA machinery, and thereby increasing use of upstream sites.
B. Originality and significance. This manuscript provides a mechanism that results in shorter UTR lengths that are not subject to translational regulation. The significance of this data is that it provides a new mechanistic basis for the switch to shorter UTRs that have been observed in cancers, and ties in upregulation of Sam68 in several cancers with molecular events important in cancer cells. The focus is on prostate cancer. These are important topics, and this current study provides a mechanism that will help interpretation of a lot of the literature. I think it will also catalyse future work in this area. There is good consideration of previous work, especially since the format of this journal is consistent with being short and precise.
C. Data and methodology. The authors use a very nice approach – identification of a novel interaction partner of Sam68 protein followed by molecular dissection in a clinically relevant scenario, and testing for expression of key target proteins by polysome analysis. The data quality is high – both in the main text and supplementary. The experimental approach and analysis is mainly rigorous. There is room for some improvement to make the case even more strongly. The co-IPs to show protein interactions seem clear, but should be done in the presence of a nuclease to confirm that the observed protein interaction partners are not tethered by RNA. The same applies for the pull downs. Maybe a nuclease was already included, but this was not clearly mentioned in the methods. The clinical expression data seem very clear. Recurrence-free survival plots are shown for MCM10 and ORC2. Similar plots should be shown for Sam68 and XRN2 to investigate if these are also correlated with survival (presumably

they are but not definitely). The 3' read experiments seem strong, but validation experiments should include 3' RACE to show that specific sites are being differentially used, not just qPCR (e.g. as shown in Figure S6).
D. Statistics. I am not well placed to assess the statistics. From my knowledge it seems their approach is satisfactory.
E. Conclusions. These seem robust (but see above for extra controls).
F. Suggested improvements: I am not entirely sure why Sam68 association with the polyA machinery fits into the mechanism? I can see why the XRN2 interaction does, but could interaction with the other components of the polyA machinery suggest a more complex mechanism involving other proteins in addition to XRN2?
G. References. One of the papers cited (Nimura et al 2016 eLife) shows that Xrn2 knockdown increases the expression of Atp2a2 and Tnnt2 mRNAs with long UTRs. Could the authors check in their data for these genes (if they are expressed in PCa) since as I understand it this would be the reverse of their prediction, and if so discuss why there might be differences between prostate cancer and heart development.
H. Clarity and context. The text is extremely well written and would be very easy for basic scientists and clinicians at all levels to read and understand.

Reviewer #3:
Remarks to the Author:
This manuscript reported a series of experiments that provided evidence that Xrn2 and Sam68 regulates mRNA alternative polyadenylation (ApA) and in turn cell cycle progress in prostate cancer cells. Prior to this work, the authors had reported a role for Sam68 in alternative polyadenylation regulation in development. The current study brought Xrn2 into the mix and the authors concluded the following: 1. The Sam68/Xrn2 complex regulates ApA globally; 2) Xrn2 promotes the recruitment of Sam68, which competes with CPSF for binding to the polyA signal; 3) Sam68 and Xrn2 are regulated by Myc and they module cell cycle progression through ApA regulation of cell cycle factors such as Mcm10 and Orc2. Although the data presented are of interest to the field, it fell short of supporting their model and does not represent a significant advance that merit publication in NSMB. Below are my main points:

1. I was convinced by the evidence presented that Xrn2 and Sam68 are associated with each other either directly or indirectly, and correlation has been detected between their expression patterns. However, I was not convinced that they regulate ApA together as a complex. Based on the co-IP patterns shown in Fig. 4B, Sam68 and Xrn2 displayed overlapping but also distinct binding patterns with polyA factors, furthering raising the question as to whether they function as a complex or they merely have overlapping functions in ApA regulation.
2. The authors concluded that Xrn2 helps to recruit Samp68 to its target transcripts. The main evidence is that Sam68 CLIP signals decrease in Xrn2-depleted cells on a set of tested transcripts. However, Xrn2 is required generally for transcription termination and its depletion has been shown to broadly impact transcription. So the decrease in Sam68 CLIP signals in Xrn2-depleted cells could very well be an indirect effect of mis-regulated transcription activities in these cells and is, thus, insufficient to suggest that Xrn2 helps to recruit Sam68. Additionally as Xrn2 is generally involved in transcription termination, it is unclear how it could help recruit an RNA-binding protein to find it specific targets.
3. The proposal that Sam68 regulates ApA by competing with CPSF for binding to the polyA signal was primarily based on the partially overlapping CLIP signals of Sam68 and CPSF30. However, similar

overlap was observed in up-, down- and un-regulated polyA sites (Supp Fig. 7 C-H). In all cases, Samp68 signals were higher than that of CPSF30. Thus this overlap does not seem to explain the effect of Sam68 on these ApA events. Experiments using orthogonal systems are needed to test the competition model.
4. The authors suggest that the Sam68/Xrn2 functions in ApA regulation is relevant to cancer based on several lines of indirect evidence. Previous studies have shown that many RBPs are regulated by Myc. Given the essential nature of Xrn2, it is not surprising that its depletion negatively impact cell growth. Thus these evidence is not sufficient to support a role of either Xrn2 or Sam68 in prostate cancer. Does the activation, repression, or mutations in Sam68 or Xrn2 directly modulate prostate cancer development and/or metastasis? In vitro and/or in vivo evidence and analysis of clinical dataset are needed to reach a conclusion as to whether these factors contribute to prostate cancer.

Author Rebuttal to Initial comments

Reviewer #1:

Overall, the experiments are convincing and well-conceived and provide a new role for Sam68 on pA selection dependent on XRN2."

We thank the Reviewer for the positive comments. In this revised version we have addresses her/his concerns.

Q1. "*As Sam68 is an RNA binding protein, why does it need XRN2 to access certain APAs?"*

A1. This result was indeed unexpected, as the APA targets of Sam68 are characterized by an enrichment of Sam68 binding motifs (U/A-rich sequences). We found, however, that a substantial fraction of nuclear Sam68 (~40%) is associated with the chromatin and that this localization is significantly reduced in LNCaP cells depleted of XRN2 (Figure 5B). Noteworthy, much of the RNA processing events, including cleavage/polyadenylation, occurs while the nascent transcripts are still bound to the chromatin (Herzel L, et al. Nat Rev Mol Cell Biol. 2017). Thus, our result suggests that, although Sam68 is likely capable to independently bind its targets RNAs as an RNA binding protein (RBP), in vivo binding of Sam68 to transcripts is facilitated by its XRN2-dependent recruitment on the chromatin. In this regard, a recent report has shown that the majority of the previously called "chromatin-bound" nascent transcripts are actually associated with the nuclear matrix. In particular, this work showed that a fraction of nascent transcripts is regulated by APA when associated to the nuclear matrix and that this interaction allows their nuclear retention and may favor splicing of weak introns and selection of sub-optimal pA sites (Tang P, et al., Nat Struct Mol Biol. 2022). Interestingly, Sam68 was identified among the RBPs associated with the nuclear

matrix (see Supplementary Table 1 in Tang et al., Nat. Struc. Mol. Biol. 2022), suggesting that it may contribute to nuclear retention of nascent transcripts. In light of this observation, we have now tested whether Sam68 is indeed associated with the nuclear matrix in PC cells. By applying the same subcellular fractionation protocol, we first confirmed the interaction of Sam68 with the nuclear matrix in LNCaP cells and then showed that XRN2 is also associated with this compartment. Importantly, the association of Sam68 with the nuclear matrix is significantly reduced in cells depleted of XRN2 (new **Supplementary Figure 7A**). Thus, we propose that XRN2 plays a scaffold function in APA regulation by bridging Sam68 to the nuclear matrix and promoting its stable interaction with target pre-mRNAs in the nucleus.

Q2. "*Is the scaffolding function of XRN2 required, ie can exonuclease-dead XRN2 also regulate Sam68 binding as in Fig 5? Therefore, is XRN2 activity required?"*

A2. As the Reviewer mentions, XRN2 was previously proposed to also act as scaffolding protein that promotes the recruitment of DNA repair proteins to sites of double strand breaks (Morales et al., Plos Genet 2016). To address the question of the Reviewer, we have now generated a catalytically inactive XRN2 isoform (XRN2-D235A) (previously described in Fong N., et al., Mol Cell 2015). LNCaP cells were stably silenced for XRN2 expression by lentivirus-delivered expression of a sh-RNA targeting the 3'UTR of the endogenous XRN2 transcript and then transfected with constructs encoding either wild type (XRN2-WT) or mutated (XRN2-D235A) XRN2 isoforms. In the new **Figure 5E and Supplementary Figure 7B**, we show that XRN2-D235A behaves like wild-type XRN2 and is fully competent to rescue the APA defects observed in LNCaP cells depleted of the endogenous XRN2. Moreover, CLIP assays showed that XRN2-D235A was also capable to rescue the binding of Sam68 to target transcripts in LNCaP cells (new **Figure 5G** and **new Supplementary Figure 7E**). Thus, we conclude that the exonuclease activity of XRN2 is not required for its function in APA. Together with the observations reported above, our data support a "structural" role of XRN2 in the complex, which acts by bridging Sam68 to the nuclear matrix and favors its role in pA selection. This hypothesis is now also reported at the beginning of the Discussion section of the revised manuscript (page 14).

Q3. "What portion of Sam68 interacts with the C-terminus of XRN2? Finally, is RNA needed for interaction?"

A3. To define the region of Sam68 required for XRN2 binding, we have performed GST-pulldown experiments. We found that XRN2 interacts with the C-terminal portion of Sam68. Indeed, deletion of the last 11 residues (GST-Sam68-351-434) dramatically reduces binding of XRN2. Further deletion of additional 34 residues in the C-terminus (GST-Sam68-351-400) caused only a mild additional effect (new **Supplemental Figure 2B**). Regarding the requirement of RNA, co-immunoprecipitation experiments

indicated that the Sam68/XRN2 interaction was not affected by the presence of RNase A, suggesting a direct protein-protein interaction (new **Figure 1F**).

Reviewer #2:

The experimental approach and analysis is mainly rigorous. There is room for some improvement to make the case even more strongly.

We thank the Reviewer for the interest shown in our work and the constructive comments. We have now addressed the issues raised to our original manuscript as detailed below.

Q1. "The co-IPs to show protein interactions seem clear, but should be done in the presence of a nuclease to confirm that the observed protein interaction partners are not tethered by RNA. The same applies for the pull downs. Maybe a nuclease was already included, but this was not clearly mentioned in the methods".

A1. The original data were obtained in the absence of RNase. We have now repeated the co-immunoprecipitation experiment in the presence or absence of RNase A and found that the interaction of XRN2 and Sam68 is not affected by nuclease treatment (new **Figure 1F**). Since we now found that the region of Sam68 that interacts with XRN2 in the in vitro pull-down assays does not bind RNA (new **Supplemental Figure 2B**), as it lacks the KH domain, RNase A was not added to these assays to confirm the interaction.

Q2. "*The clinical expression data seem very clear. Recurrence-free survival plots are shown for MCM10 and ORC2. Similar plots should be shown for Sam68 and XRN2 to investigate if these are also correlated with survival (presumably they are but not definitely).*"

A2. To address this question, we first analyzed the correlation between Sam68 and XRN2 and the Gleason score of patients, which is directly associated with disease progression and malignancy in PC (Sathianathen NJ, et al. Nat Rev Urol. 2018). Interestingly, concomitant high expression of Sam68 and XRN2 in PC patients was significantly associated with high Gleason score and advanced disease, whereas up-regulation of only one of these proteins was not (new **Figure 2D**).

We next tested the association of Sam68 and XRN2 with progression free survival (PFS) in PC patients for whom clinical data are present in the TCGA database. Although neither Sam68 nor XRN2 expression was significantly associated with PFS, we found that patients displaying concomitant high expression of Sam68 and XRN2 had a trend for shorter PFS (see **Figure 1 for Reviewer 2**). Nevertheless, the difference was not statistically significant, likely due to the small sample size.

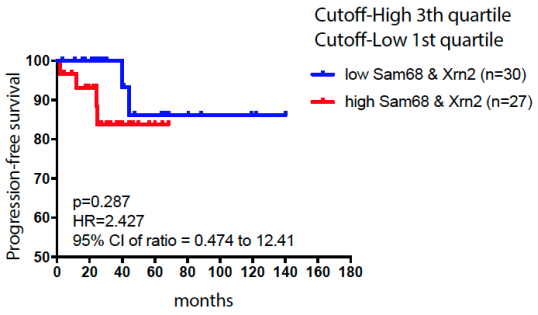

Figure 1. Progression-free survival of PC patients retrieved from TCGA database (The Molecular Taxonomy of Primary Prostate Cancer. Cell 2015, Cancer Genome Atlas Research Network). Patients were clustered according to

Q3. "Validation experiments should include 3' RACE"

A3. As suggested by the Reviewer, we have now added the validation of some APA events by 3'RACE assays. We selected four 3'UTR events that could be amplified with a single reaction. Indeed, most 3'UTR alternative pAs are too distant to be amplified with a single pair of primers. These qualitative analyses confirmed the 3'READS and quantitative PCR analyses (see new **Supplemental Figure 5E**).

Q4: "I am not entirely sure why Sam68 association with the polyA machinery fits into the mechanism? I can see why the XRN2 interaction does, but could interaction with the other components of the polyA machinery suggest a more complex mechanism involving other proteins in addition to XRN2"?

A4. The co-immunoprecipitation of Sam68 with proteins of the cleavage and polyadenylation (C/P) complex was tested at the beginning of this study to start investigating the possible involvement of Sam68 in APA regulation. We were intrigued by the observation that genome-wide CLIP signals for Sam68 showed a sharp peak at the 3'-end of the transcription units, suggesting a widespread role in 3'-end processing of nascent transcripts. We agree with the Reviewer that it is possible that Sam68 also plays additional roles in the C/P complex that involve its interaction with other protein components. However, our data suggest that a substantial part of its impact on APA in PC cells requires the interaction with XRN2. This notion is supported by the very extensive (n=1117) and highly significant (P=0) overlap between the APA events regulated by Sam68 and XRN2 (**Figure 4D**). Moreover, all these events are modulated in the same direction by depletion of either Sam68 or XRN2, with no additional effect observed when both proteins are concomitantly depleted (**Figure 4E**). It is also interesting to mention that a substantial fraction of nuclear Sam68 (~40%) is associated with the chromatin in an XRN2-dependent manner (**Figure 5B**).

Since much of the nuclear RNA processing events, including cleavage/polyadenylation, occurs while the nascent transcripts are still bound to the chromatin (Herzel L, et al. Nat Rev Mol Cell Biol. 2017), our results suggest that XRN2 bridges Sam68 to the chromatin and facilitates its interaction with target pre-mRNAs. Indeed, depletion of XRN2 also reduces binding of Sam68 near the regulated pAs, as measured by CLIP assays (**Figure 5F** and **Supplemental Figure 7C**). Collectively, these observations strongly suggest that Sam68 and XRN2 forms a functional complex involved in APA regulation of hundreds of genes in PC cells. For this reason, our present work has focused on mechanistic and functional aspects of this interaction. Nevertheless, both Sam68 and XRN2 also affect non-overlapping APA events in PC cells (**Figure 4D**). Thus, it is likely that Sam68 (as well as XRN2) also forms other complexes that are targeted to a different subset of pre-mRNAs. Future studies will be addressed to elucidate more thoroughly the role of Sam68 in the functions of the C/P complex.

Q5: "One of the papers cited (Nimura et al 2016 eLife) shows that Xrn2 knockdown increases the expression of Atp2a2 and Tnnt2 mRNAs with long UTRs. Could the authors check in their data for these genes (if they are expressed in PCa) since as I understand it this would be the reverse of their prediction, and if so discuss why there might be differences between prostate cancer and heart development".

A5. We have checked these two genes in our dataset. The *TNNT2* gene is not expressed in LNCaP cells. Regarding *ATP2A2*, Nimura and co-authors showed that silencing of XRN2 increases the expression of the Atp2a2 transcript with long 3'UTR without altering expression of coding gene (red box in their Figure 1-figure supplement 8 reporting the reads obtained by conventional RNA-seq analysis; panel A in the **Figure 2 for Reviewer 2** below). This result is in line with the preferential lengthening of transcripts that we also observe upon depletion of XRN2 (and Sam68). Indeed, our 3'READS-Seq indicates increased usage of a more distal pA upon XRN2 silencing. In the human gene, this is annotated as a middle pA because there is an additional distal-most pA located just few base pairs downstream of it (panel B in the **Figure for Reviewer 2** below, middle PAS is indicated with the black box). Moreover, *ATP2A2* is not regulated at the gene expression level in XRN2 depleted cells, like in mouse cardiac cells. Collectively, our data are consistent with the effect of XRN2 to promote an ATP2A2 APA variant with a longer 3'UTR, as also proposed by Nimura and co-authors.

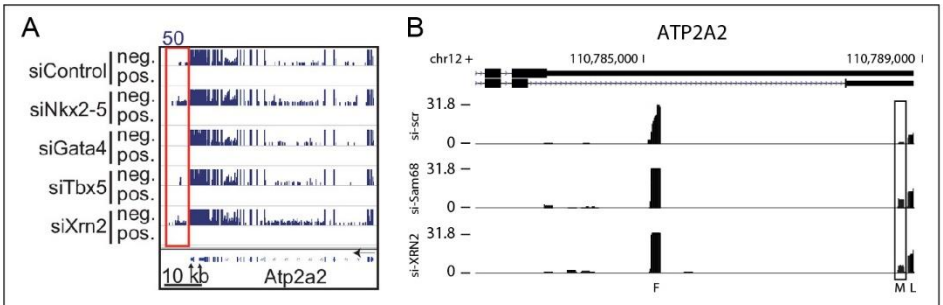

Figure 2. A) Genome browser representation of strand-specific RNA-seq of Atp2a2 genes from Nimura et al 2016 eLife with the indicated siRNAs. **B)** Genome browser representation of 3'READS showing 3'UTR-APA regulation of Atp2a2 event with the indicated siRNAs. Letters F (First), M (Middle) and L (Last) indicate polyadenylation sites.

Reviewer #3:

Although the data presented are of interest to the field, it fell short of supporting their model and does not represent a significant advance that merit publication in NSMB. Below are my main points.

Q1. "*I was convinced by the evidence presented that Xrn2 and Sam68 are associated with each other either directly or indirectly, and correlation has been detected between their expression patterns. However, I was not convinced that they regulate ApA together as a complex. Based on the co-IP patterns shown in Fig. 4B, Sam68 and Xrn2 displayed overlapping but also distinct binding patterns with polyA factors, furthering raising the question as to whether they function as a complex or they merely have overlapping functions in ApA regulation".*

A1. We agree with the Reviewer that it is possible that Sam68 and XRN2 could also play additional roles in the cleavage and polyadenylation (C/P) complex that involve their interaction with other protein components. However, our data suggest that a substantial part of their impact on APA in PC cells requires their functional interaction. Our conclusion is based on several observations. First, we found an extensive and highly significant (n=1117, p=0) overlap in the APA events regulated by the two proteins (**Figure 4D**). Second, and more importantly, all these common APA events were regulated in the same direction by depletion of either Sam68 or XRN2 (**Figure 4E**), which suggest that the two proteins cooperate to the same mechanism of regulation. Third, concomitant depletion of Sam68 and XRN2 does not increase the effect observed by depletion of just one of the two proteins (**Figure 4F,G** and **Supplemental Figure 6D**), suggesting that they need to act together to exert an effect. Fourth, depletion of XRN2 reduces binding of Sam68 to its target transcripts (new **Figure 5F** and new **Supplemental Figure 7C**). Moreover, as requested by Reviewer 1, we have now also tested whether XRN2 acts as scaffold to facilitate the role of Sam68 in APA regulation. Our new results indicate that ectopic expression of both wild type or catalytically inactive XRN2 (D235A) is sufficient to rescue the binding of Sam68 to its target transcripts and the APA pattern of control cells (new **Figure 5E,G** and **Supplementary Figure 7B,E**). It is also interesting to mention that a substantial fraction of nuclear Sam68 (~40%) is associated with the chromatin in an XRN2-dependent manner (**Figure 5B**). Since much of the nuclear RNA processing events, including cleavage/polyadenylation, occurs while the nascent transcripts are still bound to the chromatin (Herzel L, et al. Nat Rev Mol Cell Biol. 2017), our results suggest that XRN2 bridges Sam68 to the chromatin and facilitates its interaction with target pre-mRNAs. Collectively, these findings support our conclusion that Sam68 and XRN2 act as a complex in the APA regulation of selected targets.

Regarding the partially distinct binding patterns with polyA factors, it is important to note that, although they co-regulate a large fraction of APA events, Sam68 and XRN2 also show some specificity (i.e. targets

regulated exclusively by one of the two proteins, see Venn diagram in **Figure 4D**). Thus, it is possible that they also take part to other C/P subcomplexes independently of their interaction. Nevertheless, our study was not focused on the identification of the specific interactors of the two proteins. The co-immunoprecipitation experiments were performed in a very early stage of the project, when we wanted to evaluate whether Sam68 and XRN2 may play a role in APA before performing the 3'READS seq. The observation that they associate with other factors involved in APA regulation prompted us to perform the subsequent experiments described in the manuscript.

Q2. "The authors concluded that Xrn2 helps to recruit Sam68 to its target transcripts. The main evidence is that Sam68 CLIP signals decrease in Xrn2-depleted cells on a set of tested transcripts. However, Xrn2 is required generally for transcription termination and its depletion has been shown to broadly impact transcription. So the decrease in Sam68 CLIP signals in Xrn2-depleted cells could very well be an indirect effect of mis-regulated transcription activities in these cells and is, thus, insufficient to suggest that Xrn2 helps to recruit Sam68".

A2. The results of our CLIP assays are reported as percentage of RNA input for the same region of the transcript. Thus, the data takes into account possible differences in overall expression of the transcript. Moreover, CLIP assays for CPSF30 indicated that its binding to these target transcripts was increased by XRN2 depletion (see new **Figure 6K** and **Supplemental Figure 8L**), suggesting that expression of these transcripts is not overall repressed. Nevertheless, we agree with the Reviewer that if a transcript is strongly downregulated in a specific treatment, the ability of an RNA binding protein (RBP) to associate with it could be reduced. To test this possibility, we evaluated the transcription levels of some of the targets that we have investigated by CLIP assays. To make sure that transcription of these targets was ongoing in cells silenced for XRN2, we pulse-labeled the cells with 4sU for one hour before harvesting them. As shown in the new **Supplementary Figure 7D**, direct evaluation of the nascent transcripts for twelve of the targets tested by CLIP assays did not reveal significant changes in their transcription. This experiment indicates that, under our conditions, XRN2 depletion does not broadly impact on transcription of the genes regulated by APA and is in line with the 3'READS seq analysis, which indicated that the majority of the APA targets of Sam68/XRN2 are not regulated at the gene expression level (**Supplementary Figure 5D**), and with our quantitative real time PCR analysis of the expression levels of MCM10 and ORC2 shown in **Figure 7E**.

Q3. "Additionally as Xrn2 is generally involved in transcription termination, it is unclear how it could help recruit an RNA-binding protein to find it specific targets".

A3. This result was indeed unexpected, as the APA targets of Sam68 are characterized by an enrichment of Sam68 binding motifs (U/A-rich sequences). However, as mentioned above, Sam68 is associated with the

chromatin in LNCaP cells and this localization is significantly reduced in absence of XRN2 (**Figure 5B,C**). Noteworthy, much of the RNA processing events, including cleavage/polyadenylation, occurs while the nascent transcripts are still bound to the chromatin (Herzel L, et al. Nat Rev Mol Cell Biol. 2017). Since XRN2 is implicated in transcription termination and it is associated with the RNAPII complex on the chromatin (Proudfoot NJ, Science 2016), we asked whether XRN2 is required for the interaction of Sam68 with RNAPII during transcription elongation. However, co-immunoprecipitation experiments indicated that depletion of XRN2 did not significantly affect the interaction of RNAPII with Sam68 **(**see **Figure 1 for Reviewer 3** below**)**.

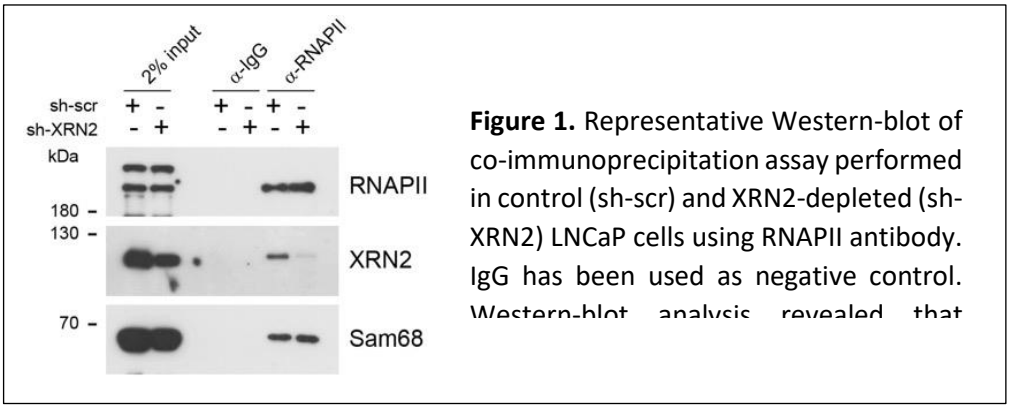

Figure 1. Representative Western-blot of co-immunoprecipitation assay performed in control (sh-scr) and XRN2-depleted (sh-XRN2) LNCaP cells using RNAPII antibody. IgG has been used as negative control. Western-blot analysis revealed that

Next, since a recent report has shown that the majority of the "chromatin-bound" nascent transcripts are actually associated with the nuclear matrix (Tang P, et al., Nat Struct Mol Biol. 2022), we tested whether XRN2 was important to tether Sam68 to the nuclear matrix. In particular, this work showed that a fraction of nascent transcripts is regulated by APA when associated to the nuclear matrix. Tethering to the nuclear matrix allows nuclear retention of these transcripts and may favor splicing of weak introns and selection of sub-optimal pA sites. Interestingly, Sam68 was reported among the RBPs associated with these nascent transcripts in the nuclear matrix (see the mass spectrometry data in Supplementary Table 1 of Tang et al., Nat. Struc. Mol. Biol. 2022), suggesting that it may contribute to their nuclear retention. By applying the same subcellular fractionation protocol, we first confirmed by Western blot analysis the interaction of Sam68 with the nuclear matrix in LNCaP cells and then showed that XRN2 is also associated with this compartment. Importantly, the association of Sam68 with the nuclear matrix is reduced in cells depleted of XRN2, whereas depletion of Sam68 does not affect the association of XRN2 with the nuclear matrix (new **Supplementary Figure 7A**). This result suggests that, although Sam68 is likely capable to independently bind its targets as an RBP, in vivo binding of Sam68 to transcripts is facilitated by its XRN2-dependent recruitment on the nuclear matrix. Thus, we propose that XRN2 plays a scaffold function in APA regulation by bridging Sam68 to the nuclear matrix and promoting its stable interaction with target pre-mRNAs in the nucleus. This notion is also supported by the evidence that a catalytically inactive XRN2 mutant rescues binding of Sam68 to its target transcripts, as well as the APA defect observed in XRN2-depleted cells. Collectively, our data indicate that the effects of XRN2 on APA regulation requires its scaffolding function (Morales et al., Plos Genet 2016) rather than its exonuclease activity.

Q4. "The proposal that Sam68 regulates ApA by competing with CPSF for binding to the polyA signal was primarily based on the partially overlapping CLIP signals of Sam68 and CPSF30. However, similar overlap was observed in up-, down- and un-regulated polyA sites (Supp Fig. 7 C-H). In all cases, Sam68 signals were higher than that of CPSF30. Thus, this overlap does not seem to explain the effect of Sam68 on these ApA events."

A4. Regarding the CLIP signals in the metagene analyses, we showed that the Sam68 peak is higher in up-regulated pAs with respect to its peak in down- and un-regulated pAs. In particular, the binding profile of Sam68 in down-regulated pAs is very spread in the whole region upstream of the CS, without showing a specific peak. This observation is also in line with the specific enrichment of Sam68 binding sites near up-regulated pAs and not the down-regulated pAs (**Figure 6F**). Accordingly, our CLIP assays showed that the binding of Sam68 to up-regulated pAs is significantly stronger that its binding to either down- or un-regulated pAs (new **Figure 5F** and new **Supplementary Figure 7C**). This result has now been confirmed in four additional targets (*RCC2*, *NFAT*, *SETD6* and *LAMC1*) that we have evaluated by CLIP (see new **Supplementary Figure 7C**; in total n=10). Thus, Sam68 preferentially binds to pAs that are up-regulated upon its depletion.

Regarding the different height of the Sam68 and CPSF30 peaks, it is worth mentioning that the amplitude of CLIP signal peaks achieved using different antibodies cannot be compared, as they may depend on the relative affinity of the antibodies for their specific targets. The analyses in the old **Supplemental Figure 7C-H** were aimed at evaluating whether the binding of Sam68 overlapped with that of other C/P components. Our analyses indicated that Sam68 binding overlaps with that of CPSF30 (peak around -25 nucleotides from cleavage site) but not with that of CSTF64 (peak around +15 nucleotides from cleavage site). Moreover, it also indicated that both Sam68 and CPSF30 bind preferentially to up-regulated pAs with respect to down- and un-regulated pAs (see new **Supplemental Figure 8D,E**). However, we understand that the previous representation was mis-leading. Thus, to avoid conveying erroneous information we have now omitted the overlap analyses (old **Supplementary Figure 7F-H**). The similar position of the CPSF30 and Sam68 peaks in up-regulated pAs is now only mentioned in the text (page 11). Moreover, we now provide evidence for the competition between these proteins by using an orthogonal system (see next point).

Q5. "Experiments using orthogonal systems are needed to test the competition model".

A5. We agree with the Reviewer that the competition model was not sufficiently supported by experimental evidence. Indeed, although we observed increased CLIP signals for CPSF30 near the up-regulated pAs upon depletion of Sam68 or XRN2, this effect may be the consequence and not the cause of the selection of that specific pAs. Moreover, albeit at lower level, this effect was also observed in some of the down-regulated pAs.

To confirm the hypothesis that Sam68 exerts its effects by binding to its cognate RNA motifs nearby the up-regulated pAs, we have now generated a minigene model of its target gene *FLNB*. The minigene contains the second to last exon, the last intron, the last exon carrying the alternative p-pA and d-pA, and 200 nucleotides of the genomic region downstream of the d-pA, which were cloned into the pCI vector lacking the SV40 pA site (see new **Figure 6H**). The minigene recapitulated the regulation observed in endogenous gene, as overexpression of Sam68 promoted the usage of the p-pA. On the other hand, overexpression of CPSF30 exerted the opposite effect and enhanced usage of the d-pA while repressing the p-pA (new **Figure 6I**).

Importantly, binding of Sam68 in proximity of the FLNB d-pA was required for its repression, as mutation of the consensus motifs flanking the d-pA abolished its effect on APA (new **Supplemental Figure 8F,G**). Moreover, in vitro pull-down assays using an RNA probe encompassing the FLNB d-pA indicated that Sam68 efficiently bound to this sequence, whereas CPSF30 binding was barely detectable. However, mutation of the Sam68 consensus motifs in this probe completely suppressed Sam68 binding and substantially increased binding of CPSF30 (new **Supplemental Figure 8H**). This observation further suggested the competition between these two factors for the d-pA. In support of this hypothesis, we now also show that transfection of increasing doses of CPSF30 was sufficient to relieve the repression exerted by Sam68 on the d-pA in the FLNB minigene, and completely suppressed usage of the weaker p-pA (new **Figure 6J**). Together with the increased in vivo binding of CPSF30 to the d-pA region in cell depleted of Sam68 (new **Figure 6K** and **Supplemental Figure 8L**), these results support the competition model between Sam68 and CPSF.

Q6. "The authors suggest that the Sam68/Xrn2 functions in ApA regulation is relevant to cancer based on several lines of indirect evidence. Previous studies have shown that many RBPs are regulated by Myc. Given the essential nature of Xrn2, it is not surprising that its depletion negatively impact cell growth. Thus, this evidence is not sufficient to support a role of either Xrn2 or Sam68 in prostate cancer. Does the activation, repression, or mutations in Sam68 or Xrn2 directly modulate prostate cancer development and/or metastasis? In vitro and/or in vivo evidence and analysis of clinical dataset are needed to reach a conclusion as to whether these factors contribute to prostate cancer".

A6: We apologize for having generated confusion regarding this issue. The main message of our study is not related to the contribution of Sam68 and XRN2 to prostate cancer (PC) progression, but rather their contribution to APA regulation in PC cells and the impact of this regulation on proliferation. Indeed, tumor progression was not mentioned in the title of the original manuscript. Nevertheless, we understand that the message conveyed in the original manuscript could be mis-interpreted and we have changed the text (Abstract and Discussion) to avoid confusion and wrong statements.

Regarding the impact of XRN2 on PC proliferation, our results indicate that depletion of XRN2 reduces but does not abolish the proliferation of PC cells, which do not undergo massive apoptosis (see new **Supplemental Figure 9B**). Moreover, we observed a similar effect on PC cell proliferation by reducing the cellular level of selected targets of Sam68 and XRN2, such as MCM10. Notably, the level of knockdown of MCM10 in our experiments is similar to that observed in cells silenced for either Sam68 or XRN2. These observations suggest that the effects reported are specific and not due to indirect consequences of general cell death. We have now clarified this issue in the revised text (page 12).

Regarding the physiological impact of Sam68 and XRN2 on PC, we now report the association of Sam68 and XRN2 expression with Gleason score, which measures disease progression and malignancy in PC patients (Sathianathen NJ, et al. Nat Rev Urol. 2018). Interestingly, only the concomitant up-regulation of Sam68 and XRN2 is significantly associated ($p<0.0001$) with advanced PC stages (high Gleason score), whereas single up-regulation of either Sam68 or XRN2 is not (new **Figure 2D**). These results further suggest that Sam68 and XRN2 cooperate in exerting their function in PC cells.

Nevertheless, since the main message of our study is not related to PC progression, but rather on the role of Sam68 and XRN2 in APA regulation and the impact of APA regulation on cell cycle progression, we have now toned down the statements relative to the impact of the Sam68/XRN2 complex on tumorigenesis (see the last sentence of the Abstract and of the Results and the Discussion section at page 15).

Decision Letter, first revision:

Our ref: NSMB-A45281A

11th Jul 2022

Dear Dr. Sette,

Thank you for submitting your revised manuscript entitled "The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer" (NSMB-A45281A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements within two weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Structural & Molecular Biology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Tiago

Tiago Faial, PhD
Consulting Editor
Nature Structural & Molecular Biology

Reviewer #1 (Remarks to the Author):

The authors have addressed all my comments.

Reviewer #2 (Remarks to the Author):

I am happy that the authors have addressed the issues I raised in my initial review with new experimentation, and find these responses satisfactorily addressed the points I raised.

I find the data convincing and the paper nice to read, and it should appeal to a wide range of readers.

Just a minor issue, principal component analysis is referred to incorrectly as principle component analysis.

Decision Letter, final checks:

Our ref: NSMB-A45281A

28th Jul 2022

Dear Dr. Sette,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer" (NSMB-A45281A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Structural & Molecular Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Sophia Frank
Editorial Assistant
Nature Structural & Molecular Biology
nsmb@us.nature.com

On behalf of

Tiago Faial
Editor
Nature Structural & Molecular Biology

Reviewer #1:
Remarks to the Author:
The authors have addressed all my comments.

Reviewer #2:
Remarks to the Author:
I am happy that the authors have addressed the issues I raised in my initial review with new experimentation, and find these responses satisfactorily addressed the points I raised.

I find the data convincing and the paper nice to read, and it should appeal to a wide range of readers.

Just a minor issue, principal component analysis is referred to incorrectly as principle component analysis.

Reviewer #3:
None

Final Decision Letter:

Dear Dr. Sette,

We are now happy to accept your revised paper "The transcriptional terminator XRN2 and the RNA binding protein Sam68 link alternative polyadenylation to cell cycle progression in prostate cancer" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Carolina

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