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Androgen-responsive long non-coding RNA *CTBP1-AS* promotes prostate cancer

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.
This manuscript was transferred from another journal where it was originally reviewed. Since the original reviews are not subject to EMBO's transparent review process policy, those reports and author response cannot be published.)

Editor: Anke Sparmann

1st Editorial Decision 19 December 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83681-T) to our editorial office. I apologize for the delay in reaching a decision. Your study has been seen by two reviewers, and due to the conflicting nature of their recommendations, I wanted to clarify certain concerns through cross-referee commenting.

Referee #1 is in general supportive of publication in The EMBO Journal, pending revisions that address the specific concerns raised. Referee #2 is more skeptical and questions the overall relevance of your observations. However, given the positive evaluation of reviewer #1 and the extensive revisions you underwent in order to address previous reviewer's comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referees' concerns must be fully addressed where necessary.

Specifically, both reviewers agree that the role of CTBP1-AS in prostate cancer progression should be more rigorously established by conducting additional in vitro experiments mentioned by reviewer #2. Furthermore, the effects of PSF and CTBP1-AS on p53 and SMAD3 should be functionally connected to the cell biological consequences. On the other hand, we feel that an in-depth look into impact on response to hormone therapy or the development of castration resistance called for by reviewer #2 (points 1, 2 and 4), albeit very interesting, is beyond the scope of the current manuscript.

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I would also like to point out that the manuscript needs to be substantially reformatted. Although The EMBO Journal does not have strict limitations on supplemental material, we prefer not to publish manuscripts in which a substantial amount of data is hidden in the supplements. Please consider carefully which of the supplemental figures are essential to the central message of your paper. Referee #1 provides suggestions at the end of her/his report, and there is still the option to move some of the truly critical data to the main body of the paper.

I appreciate that this revision will entail time-consuming experimentation and re-writing, and given the history of this manuscript, I would understand if you decide to seek rapid publication elsewhere. However, in case you do embark on revisions for our journal, please take the specified demands into careful consideration. I would like to add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

Please do not hesitate to contact me should any particular argument require further clarification!

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1

In the manuscript titled, "Androgen-responsive antisense long non-coding RNA CTBP1-AS promotes prostate cancer growth by epigenetic regulation", Takayama and colleagues characterized the novel functions of a long non-coding RNA CTBP1-AS in prostate cancer. The authors showed that CTBP1-AS is induced by androgen and expressed predominantly in the nucleus. Using siRNA knockdown, the authors provided evidence that CTBP1-AS is not only a cis-acting transcriptional repressor of CTBP1, but also a trans-regulatory activator of AR target genes. They also examined the use of CTBP1-AS as a potential target for CRPC treatment, and observed that knockdown of the RNA inhibited castration-resistant tumor growth in a nude mice xenograft model. Takayama et al. further proposed that CTBP1-AS represses genes, including CTBP1, by recruiting the HDAC-Sin3A complex to modify the chromatin. Finally, the authors showed that CTBP1-AS is associated with PSF and that this RNA-protein complex cooperatively mediates the repression of cell cycle regulators to promote cell cycle progression.

Since the role of long non-coding RNAs as regulators of transcription at either cis- or trans- level is no longer novel, the significance of this paper lies in the identification of novel regulators of AR signaling in prostate cancer. Aside from several issues highlighted below, the authors have provided extensive data (including in vivo studies, multiple cell lines) to support their model.

Major issues:

1. While CTBP1 has been well-established as a transcriptional repressor, its role in prostate cancer is less clear. In a recent article by Wang et al. in Neoplasia (2012 Oct; 14(10): 905-14), it is shown by both real-time qPCR and immunohistochemical analysis that CTBP1 is overexpressed in metastatic prostate cancer and that knockdown of CTBP1 reduces prostate tumor growth in vivo - findings which contradict with the current authors' claim.

In Suppl Fig 10a (RNA levels in microdissected tissue), it appears CTBP1-AS is overexpressed in only a subset of cancer samples, while CTBP1 is consistently underexpressed. The authors must better address the negative correlation between levels of CTBP1-AS and CTBP1, to support their sense-antisense transcriptional regulation hypothesis.

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The authors could also have made use of publicly available RNA-Seq datasets to validate their results.

2. Evidence to support the cooperative nature of CTBP1-AS and PSF in global suppression of genes with functions in cancer progression and metastasis is limited.

It is apparent CTBP1-AS is required for the recruitment of PSF and HDACs at certain sites, but what about genome-wide (or at least the 139 genes whose repression was reversed by both CTBP1-AS and PSF depletion)? And have the authors checked if CTBP1-AS binds to any other protein? A ChIRP-Seq experiment would have provided insight to sites of CTBP1-AS RNA-chromatin interaction or additional binding protein partners of CTBP1-AS.

Also, knockdown of PSF led to a reduction in cell cycle progression, but it was not shown if knockdown of CTBP1-AS would result in a similar phenotype? Although both PSF and CTBP1-AS knockdown brought about an increase in SMAD3 and p53, it is not clear if this is sufficient to bring about the observed decrease in cell cycle progression?

Minor issues:

1. No scale bars in Fig 1h & 3k and several Suppl Figs.
2. Legend for Fig 3f & j should have been "CTBP1 protein expression in tumours transfected with siCTBP1-AS"
3. The Supplementary section will benefit from further proof-reading.

Suggestions:

1. Suppl Fig 5a should suffice in showing the androgen induction of CTBP1-AS at 1 nM (physiologic level of) R1881 in AR-positive cell lines. Suppl 3 and 4 are not essential.
2. Suppl Fig 11 is not pertinent to the main conclusions.
3. Suppl Fig 15 and 16 could be merged into a single Fig.

Referee #2

The role of long non-coding RNAs in human malignancy has been underexplored, and the present study focuses on CTBP1-AS, a long non-coding RNA located in the AS region of the C-terminal binding protein 1 (CTBP1) in prostate cancer. Using a variety of in vitro model systems, it is concluded that there are functional androgen receptor (AR) regulated anti-sense non-coding RNAs in the CTBP1 locus: CTBP1-AS modulation of CTBP1 is suggested to correlate with poor prognosis in human disease; CTBP1-AS is concluded to regulate AR activity; histone modification induced by PSF associated with CTBP1-AS regulates CTBP1 expression; PSF regulates androgen mediated repression of cell cycle regulators; and the trans-regulatory functions of CTBP1-AS and PSF modulates cell cycle inhibitors. Together it is concluded that the studies provide insight into the function of non-coding RNAs in malignancy of the prostate.

Critique: The concept that androgens and AR may regulate transcripts that include non-coding RNAs is not surprising, but the function of androgen mediated non-coding RNA in prostate cancer has not been well explored. This is a major gap in the field, given the reliance of tumors on AR for progression to castration-resistant, aggressive disease. However, there are a number of major concerns that temper enthusiasm for the findings, and raise questions about the overall relevance of the observations. Concerns are:

1. -Cell proliferation assays to assess the effect of CTBP1 overexpression are not compelling as shown, and are critical to establishing a cellular effect for the putative corepressor. There appears to be little significant effect on cell doubling based on the data shown. Reliance on MTS assays or other mitochondrial-based assays as an indirect measure of cell population doublings is not appropriate, especially given the propensity of androgens to alter mitochondrial size shape and function. Finally, the link between CTBP1 and AR dependent proliferation is generallt reliant on a single cell line; specificity should be addressed in AR negative lines, and by assessing the impact on

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cell proliferation in both hormone therapy sensitive and castration resistant models. On balance the major conclusion that CTBP1 modulates AR-dependent cell proliferation is not well considered and does not support the data shown.

2. Studying the impact of CTBP1 and CTBP1-AS in clinical disease is of importance given the preliminary findings. However, much of the data in figure 2 and figure S 11 are suggestive rather than conclusive. In figure 2, specificity for the immunohistochemistry is not demonstrated, and the IHC shown is not compelling. Clinicopathological parameters are not well considered; for example, it should be determined whether CTBP1 correlated with Ki67 levels, AR levels, or most importantly PSA levels, as would be predicted from the overall model. Moreover, the concept that CTBP1 could inform response to hormone therapy is not at all supported by the data shown-- the cohort utilized addresses potential effects on "response" to neoadjuvant hormone therapy prior to surgery, which is not considered to be an indicator of any particular factors' affect on response to hormone therapy. Rather, these studies would need to be performed using a cohort of patients that received definitive hormone therapy for metastatic disease. The same concerns also exist within this cohort with regard to a lack of consideration for clinicopathological parameters known to impact outcome, and which would inform status of AR activity.

3. In figure 3, studies modulating CTBP1-AS should be accompanied by rigorous analysis of AR target gene function, both in the presence of DHT or in combination with bicalutamide or MDV3100. Reliance on luciferase assays to readout for impact on AR in these models raises concern. The animal models are provocative that begin in figure 3H, but is quite surprising that injection of siRNA again CTBP1-AS could be effective enough to impact tumor growth. These studies should be performed more carefully, says so as to establish reproducibility and specificity of the effects. Moreover, the impact on AR signaling should be carefully determined in these tumor specimens.

4. In contrast to what is stated, studies in Figure 3 do not address the role of CTBP1 or CTBP1-AS in cancer progression, and should be interpreted appropriately. Progression in this disease is monitored by assessing impact on de novo development of castration-resistance in hormone-therapy sensitive models after manipulation or challenge.

5. There is a concern about the timing of ChIP analyses in Figure 4, which were performed 24 hrs post-DHT stimulation. Notably, AR can be detected at sites of action in 1-2 hrs post-DHT, and peaks at target genes shortly thereafter. There is an overarching concern that the latent timepoint is detecting secondary event and effects on chromatin, rather than proximal effects associated with AR regulation.

6. Examining the effects on cell cycle of PSF in S30, S34, S36, etc is an important part of the study but the experimental strategy is lacking in rigor. Traces should also be shown, and bivariate flow cytometry performed in order to more accurately assess active S-phase/impact on cell cycle regulation. Moreover, cells in S36 appear to be non-viable, and there is a concern about the interpretation of the data.

7. Links in Figure 5 to p53 and SMAD3 are interesting, but are not well connected to the purported biological findings (regulation of AR-dependent cell cycle control or tumor growth). This significantly reduces the impact of the findings.

Other:
1. ChIP data should be reported as percent input rather than fold enrichment, so as to objectively compare relative occupancy with previous studies of AR and CTBP1 function. Moreover, it is not clear why agonists, for example R1881 or DHT, recruit putative corepressers. This is typically observed only in the presence of anti-androgens such as bicalutamide.

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1st Revision - authors' response 14 March 2013

We appreciate the referees for the thoughtful and highly constructive comments. We have addressed all the referees' comments in the revised version.

Rev#1

In the manuscript titled, "Androgen-responsive antisense long non-coding RNA CTBP1-AS promotes prostate cancer growth by epigenetic regulation", Takayama and colleagues characterized the novel functions of a long non-coding RNA CTBP1-AS in prostate cancer. The authors showed that CTBP1-AS is induced by androgen and expressed predominantly in the nucleus. Using siRNA knockdown, the authors provided evidence that CTBP1-AS is not only a cis-acting transcriptional repressor of CTBP1, but also a trans-regulatory activator of AR target genes. They also examined the use of CTBP1-AS as a potential target for CRPC treatment, and observed that knockdown of the RNA inhibited castration-resistant tumor growth in a nude mice xenograft model. Takayama et al. further proposed that CTBP1-AS represses genes, including CTBP1, by recruiting the HDAC-Sin3A complex to modify the chromatin. Finally, the authors showed that CTBP1-AS is associated with PSF and that this RNA-protein complex cooperatively mediates the repression of cell cycle regulators to promote cell cycle progression.

Since the role of long non-coding RNAs as regulators of transcription at either cis- or trans- level is no longer novel, the significance of this paper lies in the identification of novel regulators of AR signaling in prostate cancer. Aside from several issues highlighted below, the authors have provided extensive data (including in vivo studies, multiple cell lines) to support their model.

Major

1. While CTBP1 has been well-established as a transcriptional repressor, its role in prostate cancer is less clear. In a recent article by Wang et al. in Neoplasia (2012 Oct; 14(10): 905-14), it is shown by both real-time qPCR and immunohistochemical analysis that CTBP1 is overexpressed in metastatic prostate cancer and that knockdown of CTBP1 reduces prostate tumor growth in vivo - findings which contradict with the current authors' claim.

In Suppl Fig 10a (RNA levels in microdissected tissue), it appears CTBP1-AS is overexpressed in only a subset of cancer samples, while CTBP1 is consistently underexpressed. The authors must better address the negative correlation between levels of CTBP1-AS and CTBP1, to support their sense-antisense transcriptional regulation hypothesis.

The authors could also have made use of publicly available RNA-Seq datasets to validate their results.

Ans 1: As mentioned by this reviewer, we agree that the observation by Wang *et al.* in *Neoplasia* was different from our findings. We assume that the discrepancy may be derived from the difference of experimental designs, cell lines and cancer samples.

1) In our study, we used RNAs for cancerous regions strictly segregated from benign regions by using laser capture microdissection (LCM), to minimize the contamination of non-cancer cells. As far as we read the description by Wang *et al.*, it is not clear whether they used RNAs derived from the samples collected by LCM.
2) In the study by Wang *et al.*, they evaluated the expression levels of CTBP1 by Western blot analysis and observed CTBP1 protein expression was increased in metastatic prostate cancer samples compared with benign. Instead, we analyzed the expression levels of CTBP1 protein by calculating labeling index in each case to evaluate epithelial cell specific expression of CTBP1 in immunohistochemical analysis, as we concern lysates from prostate tissues are basically the mixture of epithelial and stromal cells. The discrepancy between their study and ours may come from the difference of methodology or the cohorts, nevertheless, we consider that it may be important to assess the role of CTBP1 in prostate cancer in a cell type-specific manner.
3) In their study, migration assay was performed in AR-negative prostate cancer cells. We also found that CTBP1 overexpression repressed the proliferation of AR-positive LNCaP and VCaP cells whereas did not the growth of AR-negative DU145 cells, suggesting that the status of AR

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expression is related to CTBP1 functions. As CTBP1 protein expression was upregulated in a couple of their MET samples, AR expression might be reduced in those tissues. A previous report may be consistent with their result as AR signaling was rather repressed in distal metastatic tissues (Stanbrough M et al., 2006). There is also a difference in the timing of obtaining metastatic samples between their study and ours, the former at autopsy whereas the latter at radical prostatectomy.

As criticized by this reviewer, we added 9 more samples (N = 16 in total) in the RNA expression study in microdissected tissues and carefully compared the expression of *CTBP1-AS* with CTBP1 (new Figure 2J). We showed that *CTBP1-AS* expression level correlates with CTBP1 repression in cancer. This sense-antisense regulatory association was also confirmed by IHC and ISH studies.

As suggested by this reviewer, *in silico* analysis based on the public RNA-seq data will provide useful findings. In a pair-ended directional RNA-seq study using pairs of normal and carcinoma tissue RNAs from 10 prostate cancer patients, CTBP1 mRNA was not overexpressed in any of the 10 cancer samples (<1.2-fold) and rather downregulated (<0.8-fold) in 5 of the 10 cancer samples based on the calculation of all the sequence tags mapped within CTBP1 exons (Kannan et al., PNAS108: 9172-7, 2011, NCBI accession number: GSE 22260). In the study, it is notable that *CTBP1-AS* expression was upregulated by >2-fold in 5 of the 10 cancer samples (Kannan et al., 2011). Thus we consider that the data for CTBP1 and *CTBP1-AS* expression in the RNA-seq study are well consistent with our findings in terms of the cancer-associated role of *CTBP1-AS*. So far few RNA-seq datasets for clinical tumor samples are publicly available, thus the accumulation of RNA-seq data by future studies will reveal the precise expression profiles of CTBP1 and *CTBP1-AS* in clinical cancer samples.

2. Evidence to support the cooperative nature of CTBP1-AS and PSF in global suppression of genes with functions in cancer progression and metastasis is limited.

It is apparent CTBP1-AS is required for the recruitment of PSF and HDACs at certain sites, but what about genome-wide (or at least the 139 genes whose repression was reversed by both CTBP1-AS and PSF depletion)? And have the authors checked if CTBP1-AS binds to any other protein? A ChIRP-Seq experiment would have provided insight to sites of CTBP1-AS RNA-chromatin interaction or additional binding protein partners of CTBP1-AS.

Also, knockdown of PSF led to a reduction in cell cycle progression, but it was not shown if knockdown of CTBP1-AS would result in a similar phenotype? Although both PSF and CTBP1-AS knockdown brought about an increase in SMAD3 and p53, it is not clear if this is sufficient to bring about the observed decrease in cell cycle progression?

Ans 2: As suggested by this reviewer, we performed ChIP analysis to speculate the global effects of *CTBP1-AS* knockdown on PSF binding and further analyzed epigenetic modulation by si*CTBP1-AS* at certain promoter regions in terms of PSF recruitment and histone acetylation status (Figure 8C). As exemplified by 5 PSF binding regions (< 50 kb from TSS) of genes randomly selected from the ChIP-seq data, we showed that si*CTBP1-AS* significantly suppressed PSF recruitment and increased histone acetylation levels. The additional data will provide evidence to support the cooperative nature of *CTBP1-AS* and PSF in global suppression of genes in prostate cancer cells.

As questioned by this reviewer in regard to the interaction of any other protein with *CTBP1-AS*, we investigated whether another RNA-binding factor NONO cooperates with *CTBP1-AS* using RIP assay. Compared with the interaction of *CTBP1-AS* with PSF, NONO weakly but substantially associates with the ncRNA. As suggested by the reviewer, ChIRP-Seq will be a future plan to obtain more insights to sites of *CTBP1-AS* RNA-chromatin interaction or additional binding protein partners of *CTBP1-AS*.

In terms of the role of *CTBP1-AS* in cell cycle progression, additional FACS analysis revealed that *CTBP1-AS* knockdown also reduced cell cycle progression as observed in PSF knockdown (new Figure 6E and Supplementary figure 16C). Consistently, cell cycle progression was shown in *CTBP1-AS* overexpressing cells vice versa (new Supplementary figure 6E).

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In regard to the contribution of SMAD3 and p53 to the decrease in cell cycle progression by PSF and *CTBP1-AS* knockdown, we showed that knockdown of these tumor suppresors partially reversed the siPSF-dependent cell cycle retardation (new Figure 7J-L, new Supplementary figure 21C). The result suggests that other cell cycle regulators will be also included in the PSF/ *CTBP1-AS* system, nevertheless, we consider that SMAD3 and p53 will be important targets for the PSF/ *CTBP1-AS* pathway to be functionally suppressed during AR-dependent cell cycle progression.

Minor issues:

1. No scale bars in Fig 1h & 3k and several Suppl Figs.

RE: We add scale bars in the microscopic figures as suggested.

2. Legend for Fig 3f & j should have been "CTBP1 protein expression in tumours transfected with siCTBP1-AS".

RE: We corrected the legends for the previous Fig. 3f (new Figure 3L) and 3j (new Figure 4I) as pointed out by the reviewer.

3. The Supplementary section will benefit from further proof-reading.

Suggestions:

1. Suppl Fig 5a should suffice in showing the androgen induction of CTBP1-AS at 1 nM (physiologic level of) R1881 in AR-positive cell lines. Suppl 3 and 4 are not essential.
2. Suppl Fig 11 is not pertinent to the main conclusions.
3. Suppl Fig 15 and 16 could be merged into a single Fig.

RE: We moved some supplementary figures important for main conclusions to main figures (Supplementary Fig1 a, Supplementary Fig2a, b, c, Supplementary Fig 4a, c, d, Supplementary Fig 6 d, f, Supplementary Fig 7a-c, Supplementary Fig 10a, Supplementary Fig 12a, Supplementary Fig 13e-g, Supplementary Fig 17 a, b, d, Supplementary Fig 21, 22, Supplementary Fig 30c, d, Supplementary Fi 31a, Supplementary Fig 35d of former version). We deleted some figures which are not essential (Supplementary Fig1b, c, Supplementary Fig3, Supplementary Fig 4b, Supplementary Fig 6a, e, Supplementary Fig8a, b, c, Supplementary Fig 11, Supplementary Fig 17c, Supplementary Fig26a, Supplementary Fi 31b-d, Supplementary Fig 36, Supplementary Fig 38c of former version) or combined two figures (Supplementary Fig 15, 16, Supplementary Fig 18, 19 of former version) as suggested by the reviewer.

Rev#2
The role of long non-coding RNAs in human malignancy has been underexplored, and the present study focuses on CTBP1-AS, a long non-coding RNA located in the AS region of the C-terminal binding protein 1 (CTBP1) in prostate cancer. Using a variety of in vitro model systems, it is concluded that there are functional androgen receptor (AR) regulated anti-sense non-coding RNAs in the CTBP1 locus: CTBP1-AS modulation of CTBP1 is suggested to correlate with poor prognosis in human disease; CTBP1-AS is concluded to regulate AR activity; histone modification induced by PSF associated with CTBP1-AS regulates CTBP1 expression; PSF regulates androgen mediated repression of cell cycle regulators; and the trans-regulatory functions of CTBP1-AS and PSF modulates cell cycle inhibitors. Together it is concluded that the studies provide insight into the function of non-coding RNAs in malignancy of the prostate.

Critique: The concept that androgens and AR may regulate transcripts that include non-coding RNAs is not surprising, but the function of androgen mediated non-coding RNA in prostate cancer has not been well explored. This is a major gap in the field, given the reliance of tumors on AR for progression to castration-resistant, aggressive disease. However, there are a number of major concerns that temper enthusiasm for the findings, and raise questions about the overall relevance of the observations. Concerns are:

1. *-Cell proliferation assays to assess the effect of CTBP1 overexpression are not compelling as shown, and are critical to establishing a cellular effect for the putative corepressor. There*

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appears to be little significant effect on cell doubling based on the data shown. Reliance on MTS assays or other mitochondrial-based assays as an indirect measure of cell population doublings is not appropriate, especially given the propensity of androgens to alter mitochondrial size shape and function. Finally, the link between CTBP1 and AR dependent proliferation is generally reliant on a single cell line; specificity should be addressed in AR negative lines, and by assessing the impact on cell proliferation in both hormone therapy sensitive and castration resistant models. On balance the major conclusion that CTBP1 modulates AR-dependent cell proliferation is not well considered and does not support the data shown.

Ans 1: As pointed out by this reviewer in terms of methodology, we also performed the cell number counting to validate the effect of CTBP1 on cell proliferation analyzed by MTS assay and confirmed that the alteration of cell numbers was basically proportional to the results of MTS assay (new Supplementary figure 5). To address the concern in regard to the usage of a single cell line in this study, we further evaluate the effect of CTBP1 overexpression in other cell lines including hormone-sensitive VCaP cells and a castration-resistant model LTAD cells, as well as AR-negative DU145 cells. We showed that CTBP1 overexpression repressed the proliferation of VCaP and LTAD cells whereas did not the growth of DU145 cells based on the data of MTS assay and cell counting (new Supplementary figure 5F, G). In addition, we confirmed that siRNA-mediated knockdown of CTBP1 promoted the proliferation of VCaP cells (new Supplementary figure 5D). As far as we studied, that the growth-inhibitory effect of CTBP1 has been shown in AR-positive prostate cancer cells. Future studies will reveal the impact of CTBP1 on cell proliferation also in other AR-negative cells or castration-resistant models.

2. Studying the impact of CTBP1 and CTBP1-AS in clinical disease is of importance given the preliminary findings. However, much of the data in figure 2 and figure S 11 are suggestive rather than conclusive. In figure 2, specificity for the immunohistochemistry is not demonstrated, and the IHC shown is not compelling. Clinicopathological parameters are not well considered; for example, it should be determined whether CTBP1 correlated with Ki67 levels, AR levels, or most importantly PSA levels, as would be predicted from the overall model. Moreover, the concept that CTBP1 could inform response to hormone therapy is not at all supported by the data shown-- the cohort utilized addresses potential effects on "response" to neoadjuvant hormone therapy prior to surgery, which is not considered to be an indicator of any particular factors' affect on response to hormone therapy. Rather, these studies would need to be performed using a cohort of patients that received definitive hormone therapy for metastatic disease. The same concerns also exist within this cohort with regard to a lack of consideration for clinicopathological parameters known to impact outcome, and which would inform status of AR activity.

Ans 2: In terms of the specificity of the anti-CTBP1 used in this study, we confirmed that the antibody was specific to CTBP1 and did not detect CTBP2 by Western blot analysis in cells transfected with either gene (new Supplementary figure 2B). It is notable that this antibody could detect the expression of CTBP1 by IHC in a breast cancer specimen, in which no substantial background IHC staining was shown by mouse normal IgG instead of anti-CTBP1(new Supplementary figure 2A).

As suggested by this reviewer, we added the data of clinicopathological study showing the association between CTBP1/*CTBP1-AS* expression and various parameters (Supplementary table III-V of the revised manuscript). We showed that high expression levels of *CTBP1-AS* were associated with AR status ($P = 0.009$, when *CTBP1-AS* expression was categorized in 2 groups as strong versus negative and weak, new Supplementary table V) and Gleason scores ($P = 0.004$ new Supplementary table V**)** in clinical specimens, suggesting a role of *CTBP1-AS* in the cancer progression. In contrast, CTBP1 expression was not significantly correlated with Gleason scores, PSA levels, and AR status at the time of radical prostatectomy. Nevertheless, we showed that high expression of CTBP1 is significantly associated with better prognosis in PSA-free survival in our population (new Supplementary Figure 2D), suggesting a tumor suppressive role of CTBP1 in clinical prostate cancers.

In regard to the types of cohorts for clinicopathological study, we understand that it will be better to use the population who received hormone therapy after metastasis rather than the cohort with the

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neoadjuvant therapy as shown in the previous Supplementarymental figure 11. Thus we delete the data of the cohort with neoadjuvant therapy in the revised manuscript.

3. In figure 3, studies modulating CTBP1-AS should be accompanied by rigorous analysis of AR target gene function, both in the presence of DHT or in combination with bicalutamide or MDV3100. Reliance on luciferase assays to readout for impact on AR in these models raises concern. The animal models are provocative that begin in figure 3H, but is quite surprising that injection of siRNA again CTBP1-AS could be effective enough to impact tumor growth. These studies should be performed more carefully, says so as to establish reproducibility and specificity of the effects. Moreover, the impact on AR signaling should be carefully determined in these tumor specimens.

Ans 3: As criticized by this reviewer, we performed rigorous analyses including expression studies and cell proliferation assay in the presence of ligands to assess the *CTBP1-AS* effect on the AR signaling. In qRT-PCR study, *CTBP1-AS* knockdown by si*CTBP1-AS* #2 and #3 inhibited the DHT-induced elevation of AR-regulated gene expression in LNCaP cells, and the extent of repression by siRNAs was almost the same as that by bicalutamide treatment (new Supplementary figure 9A). Similar knowkdown effects were also shown for more numbers of genes in LNCaP and VCaP cells (new Figure 3N and Supplementary figure 9A). In terms of tumor suppressor genes including CTBP1, SMAD3, and p53, the R1881-dependent repression of gene expression was reversed by the transfection of si*CTBP1-AS* in LNCaP as well as VCaP cells (new Supplementary figure 8D). In microarray study, we revealed that 60% of R1881-upregulated genes (>2-fold) were substantially repressed by si*CTBP1-AS* treatment (<0.75-fold) (new Figure 3N). Similar results were confirmed in the experiments with si*CTBP1-AS* #2 in both LNCaP and VCaP cells (new Suplementary figure 8C). *CTBP1-AS* knockdown substantially reduced cell proliferation in the presence of DHT (new Figure 3H).

As also concerned by this reviewer, we carefully checked the reproducibility and specificity of the studies using si*CTBP1-AS* in mice inoculated with LNCaP and LTAD cells. We also performed the tumor formation experiments using LNCaP cells overexpressing *CTBP1-AS* (new Figure 4E). Based on the integrated results of loss- and gain-of-function studies for *CTBP1-AS*, we consider that the long ncRNA is a bona-fide modulator that facilites the AR signaling in prostate cancer.

4. In contrast to what is stated, studies in Figure 3 do not address the role of CTBP1 or CTBP1-AS in cancer progression, and should be interpreted appropriately. Progression in this disease is monitored by assessing impact on de novo development of castration-resistance in hormone-therapy sensitive models after manipulation or challenge.

Ans 4: In regard to the concern by this reviewer in terms of the experimental systems in the study of the role of *CTBP1-AS*, we consider that LTAD cells in the present study could be assumed as a model that mimics the development of castration-resistance from hormone-sensitive system, as our LTAD cells were generated by the same method as LNCaP-abl cells, which were LNCaP-derived cells cultured in the long-term androgen ablation (Culing et al., 1999, Wang et al., 2009). Nevertheless, we avoid some of the definitive expressions in the revised manuscript, as further studies will be required to conclude whether *CTBP1-AS* is critical for *de novo* development of CRPC.

5. There is a concern about the timing of ChIP analyses in Figure 4, which were performed 24 hrs post-DHT stimulation. Notably, AR can be detected at sites of action in 1-2 hrs post-DHT, and peaks at target genes shortly thereafter. There is an overarching concern that the latent timepoint is detecting secondary event and effects on chromatin, rather than proximal effects associated with AR regulation.

Ans 5: We performed ChIP assay at 24 h after R1881 treatment in Figure 5 of the revised text (previous Figure 4), because we would like to evaluate the AR signaling at the timing when *CTBP1-AS* expression was maximal while CTBP1 expression was minimal as shown in Figure 1. To assess the concern by this reviewer, we re-examined AR recruitment at the CTBP1 AR binding site and histone acetylation level at CTBP1 promoter region in LNCaP cells at both 1 h and 24 h after DHT treatment (new Supplementary Figure 10G). As suggested by this reviewer, AR binding was substantially observed even at 1 h after DHT treatment. Substantial repression of histone acetylation

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at CTBP1 promoter, however, was shown at 24 h after androgen stimulation. Thus we consider that the gradual amplification of AR signaling can be achieved by the upregulation of *CTBP1-AS* and subsequent recruitment of PSF complexes, which will take time more than 1-2 h after androgen stimulation. We assume that the precise time-dependent alteration of AR occupancy and histone modification in future will provide more interesting evidence for the contribution of *CTBP1-AS* to the AR signaling in prostate cancer cells.

6. Examining the effects on cell cycle of PSF in S30, S34, S36, etc is an important part of the study but the experimental strategy is lacking in rigor. Traces should also be shown, and bivariate flow cytometry performed in order to more accurately assess active S-phase/impact on cell cycle regulation. Moreover, cells in S36 appear to be non-viable, and there is a concern about the interpretation of the data.

Ans 6: As this reviewer commented that our cell cycle analysis is lacking in rigor, we performed bivariate FACS experiments in both LNCaP and VCaP cells, labeling active S-phase cells by BrdU. The cell cycle analysis showed that the populations of S-phase cells were reduced by siPSF treatment (new Figure 6E and Supplementary Figure 16C). Traces of histogram of cell cycle profiling are now shown in new Figure 6.

We agree that the shPSF stable cells in the previous Supplementary Figure 36 were likely non-viable as pointed out by the reviewer. Thus we deleted the data from the revised manuscript.

7. Links in Figure 5 to p53 and SMAD3 are interesting, but are not well connected to the purported biological findings (regulation of AR-dependent cell cycle control or tumor growth). This significantly reduces the impact of the findings.

Ans 7: Although this reviewer commented that the study of p53 and SMAD3 is not well connected to our biological findings, the negative functions of these genes in the AR-regulated transcriptional activity have been previously reported in prostate cancer cells (Shenk et al., 2001, Hayes et al., 2001, new Supplementary figure 22). In the present study, we show that androgen-dependent PSF function downregulates p53 and SMAD3, which can be reversed by the knockdown of PSF or *CTBP1-AS*. To obtain further evidence for the p53- or SMAD3-dependent alteration of the AR signaling, we performed additional MTS assay in LNCaP and VCaP cells and showed that the knockdown of p53 or SMAD3 substantially promoted cell proliferation (new Figure 7H-J, Supplementary figure 21C, D) and partially reversed the siPSF-dependent repression of androgen-induced cell proliferation (new Figure 7K). We further observed knockdown of SMAD3 and p53 reversed partially cell cycle inhibition in LNCaP cells by siPSF (new Figure 7L). Taken together, we consider that the inhibitory functions of these tumor suppressor genes are regulated by the PSF-mediated AR signaling and contribute to the progression of cell cycle and cell proliferation.

Other
1. ChIP data should be reported as percent input rather than fold enrichment, so as to objectively compare relative occupancy with previous studies of AR and CTBP1 function. Moreover, it is not clear why agonists, for example R1881 or DHT, recruit putative corepressers. This is typically observed only in the presence of anti-androgens such as bicalutamide.

Ans 8: As suggested by this reviewer, we now show ChIP data as percent input in new main figures and Supplementary Figure 9.

As suggested by the reviewer and also reported in the past reports (ref. #1, #2), nuclear receptor corepressor proteins NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic and thyroid hormone receptors) have been shown to interact with the antagonist-occupied AR complexes including HDACs. Interestingly, recruitment of some corepressors can be also observed in the regulation of the agonist-mediated gene induction (#3). In addition, ligand-dependent nuclear receptor corepressor (LCOR) is known to interact with nuclear receptor family in the presence of agonist by both HDAC-dependent and -independent mechanism, and this corepressor also interacts with CTBP1 ligand-dependently (#4). In the recent reports of global analysis of corepressor complexes, ERG, HDAC and EZH2 have been shown to be recruited to AR binding sites ligand-dependently and to exert the repressive functions to AR-induced transcription

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(#5). Collectively, it is assumed that agonist-dependent AR activation involves both coactivators and corepressors in the regulation of gene expression.

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