nature portfolio

Peer Review File

Stepwise release of Activin-A from its inhibitory prodomain is modulated by cysteines and requires furin coexpression to promote melanoma growth

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Pinjusic et al. investigates Activin A processing and the consequences for its molecular interactions and biological activity.
The manuscript is not an easy read (due to the multiple constructs and controls) but it is an excellent example how painstaking, rigorous work can help to gain a detailed understanding of the molecular processing of a protein that plays crucial roles in many areas of physiology and pathophysiology including - but not limited to - cancer.
I have no major concerns and only a minor suggestion:
In light of the multiple papers reporting activin A and/or follistatin expression changes in cancer (and their link to prognosis) and the focus on melanoma growth in the title, it would be interesting to include a brief discussion of what is known about alterations of furin or related PCs in cancer.

Reviewer #2 (Remarks to the Author):

In the present manuscript, the authors investigate the cleavage and maturation of activin-A. To do this, they use the B16F1 mouse melanoma cell line as a model, because this cell line does not express INHBA (which encodes activin-A), allowing ectopic expression of WT or a mutated form of this. In this manuscript, the authors investigate the cleavage and maturation of activin-A. They use the B16F1 mouse melanoma cell line as a model because this cell line does not express INHBA (which encodes activin-A), allowing ectopic expression of WT or a mutant form of this gene. To investigate the processing of Activin-A, the authors derived B16 clones knocked out for Furin, the major protease involved in Activin-A cleavage, together or not with the knockout of PCSK7, the only Furin-related protease expressed in this cell line. The demonstration of how Activin-A is matured and how disruption of this maturation affects its signalling is compelling. However, the conclusions about melanoma biology are not well supported. For example, the introduction cites reports claiming that circulating levels of activin-A correlate with cachexia, but they use a cancer cell line that does not express the gene. To investigate the processing of Activin-A, the authors derived B16 clones knocked out for Furin, the major protease involved in Activin-A cleavage, together or not with the knockout of PCSK7, the only Furin-related protease expressed in this cell line. The demonstration of how Activin-A is matured and how disruption of this maturation affects IS signalling is compelling. However, the conclusions about melanoma biology are not well supported. For example, the introduction cites reports claiming that circulating levels of activin-A correlate with cachexia, but they use a cancer cell line that does naturally express it.

Specific points:
1. The main cell line used in this study is B16F1 melanoma, but the claims made here apply to melanoma in general. B16F1 are an unconventional type of melanoma because they are mutated on b-catenin, which makes them very aggressive even without Activin-A. Looking at the TCGA data (https://www.cbioportal.org/comparison/survival?comparisonId=667d6b1083e9543d61910894&u

nselectedGroups=%5B%5D), the expression of INHBA does not seem to affect patient survival. Therefore, the author should strengthen her argument for a possible role of Activin-A in melanoma. Here B16F1 is (well) used as a test tube, but the data do not really provide insight into melanoma biology.

2. Fig. 1D, are there any differences in proliferation on plastic between B16F1 WT and B16F1-bA? The author should provide a proliferation assay. Also, the data in Figure 1D should be accompanied by a graph showing the time taken to reach the initial tumour volume.
3. Figure 1 is puzzling. Figure 1D shows that the B16F1-bA-transplanted tumour grows faster than the B16F1-WT tumour. On the other hand, no differences are seen between B16FurKo-bA and B16FurKo WT. However, the amount of circulating A30 form is similar between B16F1-bA and B16FurKo-bA. The author should provide more data to prove that melanoma produced Activin-A has a systemic effect on cachexia.
The graph showing the weight of the mice should be given in grams and not as a percentage.

Mino points out:
- Figure 1 A and B:
Even if the cell lines have been previously characterised, the authors should show Western blots of both Furin and PC-7 expression in all cell lines.
- The author uses RT-PCR to profile Pcsk gene expression in B16F1 (Fig. 1D). This method is rather outdated and not quantitative. Publicly available RNAseq databases such as GSE154115 could be used to strengthen their claim.
-In line 380, the authors claim that B
- B16F1/B16-F1should be harmonised.

COMMSBIO-24-2189-T, response to referees

Reviewer #1 (Remarks to the Author):

The manuscript by Pinjusic et al. investigates Activin A processing and the consequences for its molecular interactions and biological activity.

The manuscript is not an easy read (due to the multiple constructs and controls) but it is an excellent example how painstaking, rigorous work can help to gain a detailed understanding of the molecular processing of a protein that plays crucial roles in many areas of physiology and pathophysiology including - but not limited to - cancer.

We are very grateful that the reviewer took the time and appreciated that our attention even to painstaking details was worth the effort. Since the subject was complex, and in an effort to facilitate the reading, we now tried to further simplify or shorten sentences wherever possible throughout the text of the revised manuscript. In all instances where such changes were not merely about grammar, we highlighted them in color and, where necessary, compare them below in the point-by-point answer to the original version to indicate how we tried to de-convolute any unnecessarily complex statements without changing any conclusions.

I have no major concerns and only a minor suggestion:

In light of the multiple papers reporting activin A and/or follistatin expression changes in cancer (and their link to prognosis) and the focus on melanoma growth in the title, it would be interesting to include a brief discussion of what is known about alterations of furin or related PCs in cancer.

Indeed, this important aspect was glaringly missing: Thanks for pointing this out! Since the large number of articles implicating PCs in the processing of multiple cancer-relevant substrates in various tumor types, a few additional introductory lines in the revised Discussion on p16 (lines 10-13) now refer readers to two recent authoritative reviews on this topic (new references 63 & 64).

Reviewer #2 (Remarks to the Author):

In the present manuscript, the authors investigate the cleavage and maturation of activin-A. To do this, they use the B16F1 mouse melanoma cell line as a model, because this cell line does not express INHBA (which encodes activin-A), allowing ectopic expression of WT or a mutated form of this. To investigate the processing of Activin-A, the authors derived B16 clones knocked out for Furin, the major protease involved in Activin-A cleavage, together or not with the knockout of PCSK7, the only Furin-related protease expressed in this cell line. The demonstration of how Activin-A is matured and how disruption of this maturation affects its signalling is compelling.

We are grateful that the reviewer found the take home message of this study to be convincing.

However, the conclusions about melanoma biology are not well supported. For example, the introduction cites reports claiming that circulating levels of activin-A correlate with cachexia, but they use a cancer cell line that does not express the gene.

The reviewer is correct that the present study is about Activin-A processing and its role in signaling. A function of activin signaling in cachexia is supported by numerous earlier studies in various mouse models (starting with the seminal discoveries by the cited references 8 and

9), and by a strong correlation between circulating Activin-A levels and poor prognosis in human patients across multiple cancer types (reviewed in the cited reference 7).

To address the point raised by this reviewer, the revised Introduction now states on line 14 of p3 that both a correlation of Activin-A levels with poor prognosis and the ability of Activin-A to induce cachexia have been previously demonstrated *across multiple tumor types*. Please note that we cautiously stated that Activin-A *can* induce cachexia since direct evidence comes primarily from animal studies. In patients, current data are still primarily of a correlative nature because available therapeutic strategies rely on ligand traps which can also block related anorexic factors such as GDF11 and Myostatin (reviewed in ref. 7). We also would like to point out that the Introduction at this point has deliberately not yet mentioned melanoma since a link of Activin-A to cachexia was initially inferred from other tumor types. We subsequently reported a similar effect of Activin-A both in our B16F1-βA gain of function model *and at endogenous expression levels in human C8161 melanoma xenografts*. To better highlight that cachexia can be induced even by endogenous Activin-A (and also in melanoma models), the revised Introduction now explicitly mentions this original observation by stating that also the *"…blockade of endogenous Activin-A in a human melanoma xenograft model protected mice against muscle wasting [15]."* (p3, line 29).

A role for systemic activin signaling in mediating muscle wasting is generally accepted (pharma companies interested in anti-cancer and anti-aging therapies heavily invest in this pathway as a therapeutic target). Therefore, and since the cited references 7-8 and 15 are still valid and up to date, we have not cited any of the many additional individual studies that have further corroborated them in the meantime. Rather than making any new claim that cachexia is linked to or correlating with circulating Activin-A levels, the only experiment related on this subject in our present study focused on using a previously characterized, established syngeneic grafting model to conclusively test whether or not a *known* tumor-promoting function of Activin-A and the *known* release of Activin-A into the circulation depend on cell-autonomous cleavage by furin within the melanoma cells themselves.

Our novel (and perhaps surprising) finding is not that Activin-A promotes cachexia, but rather that its mature form in the circulation and its known systemic effect (marked here by sudden loss of body weight near the endpoint that ethically limits how long we are allowed to grow these tumors) were sustained even by *Furin* knockout tumors. In all of the DNA sequencing-validated CRISPR clones examined, cell autonomous Activin-A processing was severely inhibited, and the associated tumor growth was specifically and completely abolished, thus ruling out a CRISPR artifact.

Specific points:

1. The main cell line used in this study is B16F1 melanoma, but the claims made here apply to melanoma in general. B16F1 are an unconventional type of melanoma because they are mutated on b-catenin, which makes them very aggressive even without Activin-A. Looking at the TCGA data (https://www.cbioportal.org/comparison/survival?comparisonId=667d6b1083e9543d61910894&unselectedGroups=%5B%5D), the expression of INHBA does not seem to affect patient survival. Therefore, the author should strengthen her argument for a possible role of Activin-A in melanoma.

To address this point, we revised the Introduction on p3 to more clearly explain the known role of Activin-A in human melanoma (p3, lines 26-30). As described there, we previously reported that *INHBA* is among the top upregulated genes in immune checkpoint therapy-resistant melanoma patients, and that Activin-A promotes immune evasion and immunotherapy resistance across several syngeneic mouse melanoma grafting models, also at endogenous

expression levels (ref. 22). Therefore, and since *INHBA* mRNA expression is a negative predictor in several tumor types (see https://www.proteinatlas.org/ENSG00000122641-INHBA/pathology), innovative strategies to block Activin-A signaling are urgently needed.

The reviewer is correct that human melanoma is not among those tumors where survival anticorrelates with INHBA *transcription*. Instead, as we have shown previously and mention in the revised Introduction, it correlates with increased expression of the Activin-A antagonist FST (ref 15), consistent with our working model that melanoma hijack Activin-A signaling by post-transcriptional mechanisms. We also added there the information that other authors in the meantime independently confirmed that poor prognosis in human melanoma correlates with upregulation of Activin-A at the protein level both in cancer cells and, to a lesser extent, in macrophages, and with an associated immunosuppressive phenotype (ref. 24). Taken together, these studies have already validated the clinical relevance of Activin-A in melanoma. Therefore, this is not the theme anymore of the present study. Instead, we here focused on investigating how Activin-A is activated post-transcriptionally in this and possibly other tumor types. The present work advances the field by showing that interfering with precursor processing holds considerable potential, but that our findings on furin-independent processing should be taken into account.

Here B16F1 is (well) used as a test tube, but the data do not really provide insight into melanoma biology.

The reviewer is correct that here we (intentionally) use primarily the B16-F1 model to study the regulation of precursor processing since a role for Activin-A in human melanoma has already been established previously: As mentioned above, poor survival in human melanoma of the TCGA dataset correlates with lower FST levels (ref 15). Secondly, elevated *INHBA* mRNA levels in patients correlate with resistance to anti-PD1 therapy (ref. 22). Thirdly, IF staining in two cohorts of melanoma patients showed that poor survival also strongly correlates with Activin-A upregulation at the protein level both in tumor cells and, independently (and apparently to a lesser extent), in macrophages (ref. 24).

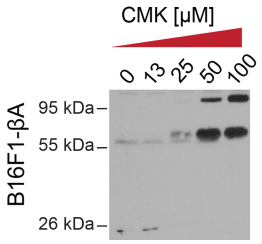
Here, we tested the role of furin in a specific aspect of melanoma biology by analyzing for the first time a tumor model in which endogenous furin was genetically inactivated by CRISPR/Cas9 editing. Specifically, our goal was to test the influence on Activin-A function. To this end, we had to use a mouse model because in human xenografts, we previously found that the role of Activin-A in promoting primary and metastatic tumor growth is suppressed by the absence of adaptive immunity (ref. 15).

In addition, we here validated that knockdown of furin also similarly stabilizes uncleaved proActivin-A in human melanoma cells, together with half-processed intermediate (**Fig. 1C**). However, to further characterize the role of this furin-independent hemicleavage and its impact on receptor binding and signaling, it was crucial to switch to our previously validated mouse model where furin was completely and permanently inactivated alone or together with PC7. PC7 is the only other PC family member present in B16-F1 cells, besides furin, and the same was true after furin and/or PC7 knockout (**Fig. S1D**). the knockouts inactivated all alleles of both of these genes (see below), and this inactivation had been independently confirmed both by gene sequencing and by independent analysis of our FRET reporter substrate (ref. 32). Furin and PC7 are also the only PCs present in normal human melanocytes (PMID: 25545474). By contrast, in human cancer cells, even traces of other PCs could potentially interfere with the analysis, and the ploidy of furin and any other PCs could be variable and even less stable than in the widely used B16 models.

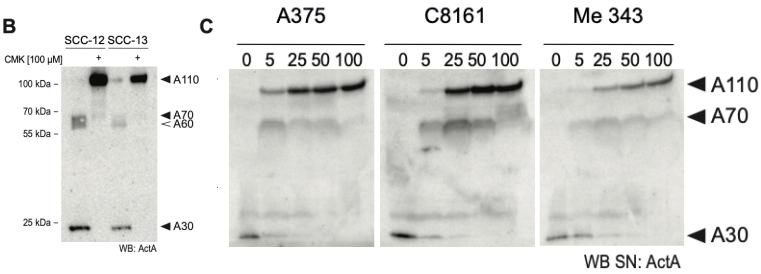
To further address the role of furin in human cancer cells, we now also compared several available human melanoma and squamous cell carcinoma cell lines to our mouse model in terms of their response the general PC inhibitor dec-RVKR-cmk (CMK). This new data in

figure 1 of the co-submitted revised manuscript confirms that the differential sensitivity of the two βA subunits of Activin-A to inhibition by increasing doses of CMK in our engineered murine cancer cells (Fig. 2A) faithfully recapitulates the behavior of endogenous Activin-A in all of the human cell lines examined:

Left: B16F1-βA cells (Fig 2A of the present manuscript):



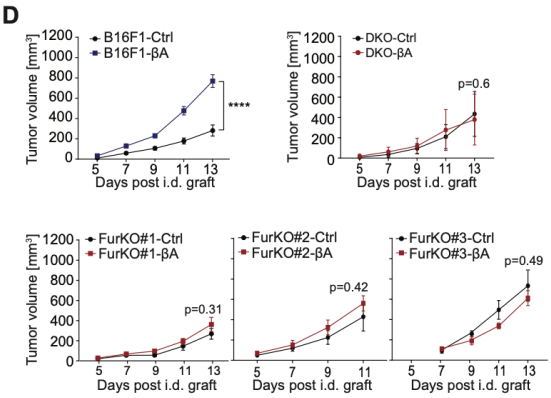
Right: Human squamous cell carcinoma (SCC) and melanoma cell lines (A375, C8161, Me343):



2. Fig. 1D, are there any differences in proliferation on plastic between B16F1 WT and B16F1-bA? The author should provide a proliferation assay.
The data provided in Suppl. Fig. S1E showed that the *in vitro* proliferation of these and of FurKO and DKO cell lines with βA vs Ctrl lentivirus was neither changed by βA nor by the furin or PC7 knockout.

Also, the data in Figure 1D should be accompanied by a graph showing the time taken to reach the initial tumour volume.

We did not understand what the reviewer meant by "initial" tumor volumes. The tumor growth curves in Fig. 1D (image to the right) *are* graphs of the time it took for tumors of any of the indicated genotypes to reach the endpoint (day 13 or, in the fastest growing CRISPR clone FurKO#2, day 11), see image below. The earliest time point examined is when tumors reached the minimal size to become measurable.



Since clonal sublines can be expected to differ somewhat from one another in terms of tumor growth rates over the course this time window, it was indeed important to compare multiple independent CRISPR clones, and we chose this approach to display the data because it clearly shows that regardless of such clonal differences, the deletion of furin abolished the βA-induced tumor growth advantage in all of them.

3. Figure 1 is puzzling. Figure 1D shows that the B16F1-bA-transplanted tumour grows faster than the B16F1-WT tumour. On the other hand, no differences are seen between B16FurKo-bA and B16FurKo WT. However, the amount of circulating A30 form is similar between B16F1-bA and B16FurKo-bA. The author should provide more data to prove that melanoma produced Activin-A has a systemic effect on cachexia. The graph showing the weight of the mice should be given in grams and not as a percentage.

In case the reviewer questions whether Activin-A induces muscle wasting (?), we would like to refer to our explanation in the answer to his/her summary statement above. The cited references 7-9 and a large body of follow-up literature leave no doubt that circulating Activin-

A has such systemic activity, see e.g. Roh et al., Sci. Transl. Med. 2019 (PMID: 30842316) and Chen et al., PNAS 2017 (PMID: 28607086).

Our lab does not study cachexia, but we are obliged to measure body weights as one of the parameters of animal well-being due to this well known devastating systemic effect of Activin-A. Our data in Fig. 1F show the relative changes of the body weight in each mouse at the endpoint relative to its body weight at the time of grafting. Since the actual body weight of each mouse measured in grams is unique, plots of actual BW would be impossible to read. Instead, it is common to plot the relative changes as we do it here. But to address this point of the reviewer, the revised figure legend now states more clearly: *(E, F) Comparison of (E) tumor masses and (F) changes in body weight of each tumor recipient analyzed in (D) at the endpoint (day 13 or, in the case of the fastest growing CRISPR clone FurKO#2, day 11) relative to its body weight at the time of grafting.*

On p6 (lines 4-9) of the main text, we also revised the description of our analysis of the effect of Activin-A secretion on the body weight in hosts of furin-deficient tumor grafts to state more precisely that the systemic effect of βA expression was clearly furin-independent in two of the FurKO clones analyzed, whereas the loss of stastitical significance of a similar effect in the two other clones correlates with their slower tumor formation. Accordingly, the revised text states: *"Importantly, a comparison of body weight of the hosts before tumor grafting and at the endpoint revealed that the systemic effect of βA expression was maintained nonetheless, as clearly seen in FurKO#2 and FurKO#3 tumors (**Fig. 1F**). In hosts of βA-expressing FurKO#1 and DKO tumors, a similar trend did not reach statistical significance. These two CRISPR clones form tumors more slowly than parental βA-expressing cells (**Fig. 1D**), despite comparable cell proliferation in vitro (**Fig. S1E**), suggesting they did not quickly enough form tumors of sufficient size for cachexia to manifest before reaching the endpoint."*

Since the βA-induced onset of cachexia and an associated decrease in BW does not overtly manifest before day 11, it is indeed highly plausible that tumor size (i.e. the size of the *source* of anorectic/cachetic factors and not just their steady state concentrations at the endpoint) must reach a critical threshold to trigger this deadly disease. However, we should point out that our ethics committee is very strict concerning our animal experiments that induce cachexia. Since it is difficult to justify the suffering imposed by this constraint, they require that animals are euthanized before they lose more than 15% of their BW. This is a key reason why we are not allowed to grow such βA-expressing tumors longer than up to 13 days, and why our lab does not study cachexia.

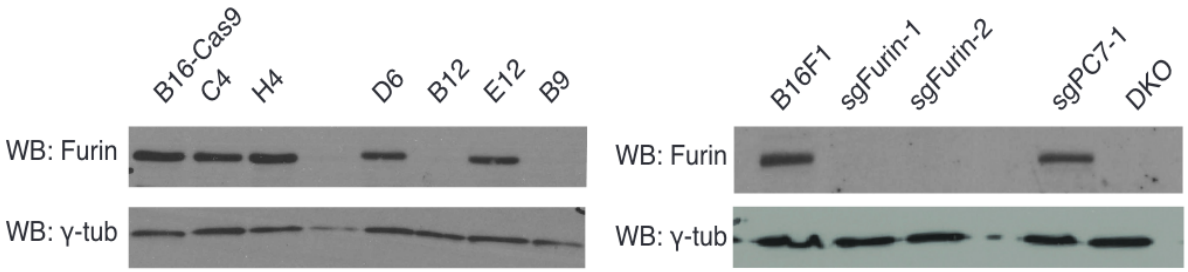
Minor points out:

- Figure 1 A and B:

Even if the cell lines have been previously characterised, the authors should show Western blots of both Furin and PC-7 expression in all cell lines.

As we are not aware of any suitable commercial PC7 antibodies, and since Western blots are not as informative as direct gene sequencing, our CRISPR clones were validated by sequencing the targeted region. As described in the cited reference 32, all clones contained frame-shifting indel mutations in all alleles of *Furin* and/or *Pcsk7* (in DKO cells). In cytosolic proteins, indel mutations could be problematic if in-frame alternative start codons can give rise to truncated functional proteins. However, in case of furin and PC7, this would not be an issue since any such truncated versions would remain stuck in the ER due to the lack of a signal sequence and truncation of their prodomains.

As an independent functional validation, we analyzed the effects on processing of a variety of substrates, including a pan-PC specific FRET biosensor. The results confirmed beyond any doubt that our FurKO and DKO cells, respectively, lack furin alone or together with PC7. For furin, Western blotting also confirmed the absence of detectable furin protein both in the 3 FurKO clones (see figure below, left panel) and in DKO cells (last lane of the panel on the right).



Since these data are published (ref. 32), we did not repeat these blots again. Instead, our present finding that absence of furin correlated with inhibition of Activin-A maturation in all of our clones further corroborates the conclusion of our previously published data that our CRISPR editing worked as expected.

- The author uses RT-PCR to profile Pcsk gene expression in B16F1 (Fig. 1D). This method is rather outdated and not quantitative. Publicly available RNAseq databases such as GSE154115 could be used to strengthen their claim.

The claim of the data in figure S1D is to show that even a saturating RT-PCR at the endpoint failed to detect transcripts of any PCs other than furin and PC7. Quantification of the absence of detectable signal in our cells would make no sense to us. We did not attempt to access public data or to analyze it because we would not want to rely on data from someone else's batch of cells, or on RNA-seq where it tends to be primarily a matter of sequencing depth whether or not a transcript is reported as present or absent. More importantly, the key question here was whether furin and/or PC7 deletion leads to compensatory detectable expression of another PC family member, which was not the case. This result was key for us to decide that a screen for proteases mediating furin-independent Activin-A hemicleavage was warranted. In our co-submitted manuscript, such a screen identified kallikrein-8.

-In line 380, the authors claim that B

On line 380, we described that binding of BMPRII-Fc to A70 was not detected. However, it seems that the reviewer decided to delete the question related to this finding.

- B16F1/B16-F1should be harmonised.

To respect the meaning of the "F1", and in line with the literature, we consistently refer to B16-F1 when we talk about the unmodified parental cell line. By contrast, to clearly indicate where we modified them by lentiviral βA or empty Ctrl vectors, or by CRISPR editing, we aimed to consistently distinguish the resulting sublines from parental cells by deliberately omitting the dash between B16 and F1. We believe this is important for readers to be able to recognize when we use unmodified parental cells. Figure S1D is a good example, and its legend explains that we refer to the parental cells when we leave the dash in place. This definition can also be inferred from the Methods section (p17, lines 11-12).

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I thank the authors for addressing my suggestions. I have no further concerns and recommend publication.