Cardiovascular Research

Altered proteasome function in right ventricular hypertrophy

--Manuscript Draft--

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Abstract:	Background - In patients with pulmonary hypertension, right ventricular hypertrophy (RVH) is a detrimental condition that ultimately results in right heart failure and death. The ubiquitin proteasome system has been identified as a major protein degradation system to regulate cardiac remodelling in the left heart. Its role in right heart hypertrophy, however, is still ambiguous. Methods and results - RVH was induced in mice by pulmonary artery banding (PAB). Both, expression and activity of the proteasome was found to be upregulated in the hypertrophied right ventricle compared to healthy controls. Catalytic inhibition of the

	proteasome by the two proteasome inhibitors Bortezomib (BTZ) and ONX-0912 partially improved RVH both in preventive and therapeutic applications. Native gel analysis revealed that specifically the 26S proteasome complexes were activated in experimental RVH. Increased assembly of 26S proteasomes was accompanied by elevated expression of Rpn6, a rate-limiting subunit of 26S proteasome assembly, in hypertrophied cardiomyocytes of the right heart. Intriguingly, patients with RVH also showed increased expression of Rpn6 in hypertrophied cardiomyocytes of the right ventricle as identified by immunohistochemical staining. Conclusions - Our data demonstrate that alterations in expression and activity of proteasomal subunits play a critical role in the development of RVH. Moreover, this study provides an improved understanding on the selective activation of the 26S proteasome in RVH that might be driven by the rate-limiting subunit Rpn6. In RVH, Rpn6 therefore represents a more specific target to interfere with proteasome function than the commonly used catalytic proteasome inhibitors.
Suggested Reviewers:	
Opposed Reviewers:	

Altered proteasome function in right ventricular hypertrophy

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Abstract

Background - In patients with pulmonary hypertension, right ventricular hypertrophy (RVH) is a detrimental condition that ultimately results in right heart failure and death. The ubiquitin proteasome system has been identified as a major protein degradation system to regulate cardiac remodelling in the left heart. Its role in right heart hypertrophy, however, is still ambiguous.

Methods and results - RVH was induced in mice by pulmonary artery banding (PAB). Both, expression and activity of the proteasome was found to be upregulated in the hypertrophied right ventricle compared to healthy controls. Catalytic inhibition of the proteasome by the two proteasome inhibitors Bortezomib (BTZ) and ONX-0912 partially improved RVH both in preventive and therapeutic applications. Native gel analysis revealed that specifically the 26S proteasome complexes were activated in experimental RVH. Increased assembly of 26S proteasomes was accompanied by elevated expression of Rpn6, a rate-limiting subunit of 26S proteasome assembly, in hypertrophied cardiomyocytes of the right heart. Intriguingly, patients with RVH also showed increased expression of Rpn6 in hypertrophied cardiomyocytes of the right ventricle as identified by immunohistochemical staining.

Conclusions - Our data demonstrate that alterations in expression and activity of proteasomal subunits play a critical role in the development of RVH. Moreover, this study provides an improved understanding on the selective activation of the 26S proteasome in RVH that might be driven by the rate-limiting subunit Rpn6. In RVH, Rpn6 therefore represents a more specific target to interfere with proteasome function than the commonly used catalytic proteasome inhibitors.

Key words: Proteasome; proteasome inhibition; Rpn6; pulmonary artery banding; right ventricular hypertrophy.

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23. November 2018/Az.:

MS: CVR-2017-981

To the Editor,
Cardiovascular Research
Giessen, Germany, 23.11.2018

Dear Editors, Karin R. Sipido and Tomasz J. Guzik,

Please find attached our revised version of the manuscript entitled: *"Altered proteasome function in right ventricular hypertrophy"*.

As you asked, we refer to the current manuscript number: **CVR-2017-981**.

In addition to the revised version of the manuscript with highlighted changes, we have prepared a point-by-point reply to the reviewers and editors as a separate file.

The revised manuscript, or part of it, has neither been published (except in form of abstract or thesis) nor is currently under consideration for publication by any other journal; and all co-authors have read the manuscript and approved its submission to *Cardiovascular Research*.

We hope that the revised manuscript is eligible for publication in your prestigious journal.

Thank you very much in advance.

Kindest regards,

Prof. Dr. Ralph Schermuly (on behalf of all authors)

Reviewer #1: This is an interesting report on the role of the proteasome in the RV hypertrophy induced by pulmonary artery banding. They provide evidence of increased proteasome activity, and in particular upregulation of the regulatory unit Rpn6, which was also seen in human RV samples from patients with RVH. As well, they show that proteasome inhibitors reduced adverse remodeling and improved RV function in this model of compensated RV hypertrophy.

R #1: *We thank to the reviewer for overall positive evaluation of our manuscript.*

Specific comments:

1. The PAB model is a good model for compensated RVH, but increased afterload is usually well tolerated and unlike PH models, rarely progresses to right heart failure (Am J Respir Cell Mol Biol 2011;45(6):1239-1247). It would be important to assess the response to proteasome inhibition in a model that is prone to RHF.

R1: *We are grateful to the reviewer for raising this important question. In our manuscript we outlined in the beginning that the focus of this study is on early time points of right ventricular pathology during compensated RVH. We believe that it is important to learn more about the earlier phases of this disease to understand disease progression to the later stages. In addition, we agree that the PAB model is usually considered as a model for RVH rather than right heart failure (RHF). However, the literature indicates that this model can also serve as a model of RHF, depending on the size of constriction (Borgdorff et al, Heart Fail Rev (2015) 20: 475-491). Furthermore, in a classical model of pulmonary hypertension (PH), such as the monocrotaline (MCT) model, it has previously been shown that proteasome inhibition (bortezomib) leads to improvement of hemodynamics and reduction of RVH (Kim et al, Am J Respir Cell Mol Biol. 2012. 47:698-708; Zhu et al, Life Sci. 2017. 173:36-42 and Zhang et al, Am J Physiol Cell Physiol 2016. 311: C482-97). Also, it has been demonstrated that carfilzomib (ONX-0912 used in our study as its analogue) is beneficial in the SU5416/hypoxia model of PH (Wang et al, Cardiovasc Res. 2016. 110: 188-99). Based on this, we did not intend to merely repeat these studies but rather focused on a new RVH mouse model and early disease stages. However, we fully agree with the reviewer that this is an important issue and future studies should be designed to focus on this aspect.*

2. The effects of the proteasome inhibitors were fairly modest. Based on the cited literature, it has been reported that proteosomal activity is in fact reduced in heart failure, and increased in hypertrophy. Would there be long term concerns that long-term inhibition could worsen function? Again this underlines the need to study in a more relevant PH model.

R2: *We thank the reviewer for this important comment. Indeed, proteasome activity is differentially regulated in the diseased heart depending on the stage of heart disease. Any use of catalytic proteasome inhibitors for long-term treatment in left or right heart disease thus faces the problem of disease worsening as the proteasomal system is chronically inhibited and may thus no longer be able to fulfill its essential functions in protein quality control and regulated protein turnover. We have addressed this dilemma of cell- and time-specific effects of proteasome inhibition previously (Meiners S, et al., Med Res Rev. 2008 Mar;28(2):309-27). For that reason, we believe it is crucial to find more specific treatment options beyond an overall catalytic proteasome inhibition as previously proposed (Gaczynska and Osmulski Curr Top Med Chem 2015;**15**:2056–2067). In our present study, we have identified the 26S proteasome as a potential new therapeutic target. Targeting the assembly of the 19S complex via Rpn6 might provide a more specific approach to interfere with hypertrophy-associated*

proteasome activation. We have previously shown that partial silencing of Rpn6 in primary fibroblasts prevents TGFβ-induced activation of fibroblasts into the myofibroblast (Semren et al., Am J Respir Crit Care Med. 2015 Nov 1;192(9):1089-101). This indicates in a proof-of-concept approach that a more specific targeting of 26S proteasome activity might be a promising therapeutic strategy. We have stressed this issue in the discussion of the revised manuscript version (marked in red, page 19).

3. Figure 6A: The staining for Rpn6 is very patchy, both in the sham and PAB groups. Areas of high expression are immediately adjacent to regions of little or no expression. Can the authors provide an explanation for this? How many mice were studied?

R3: *We thank the reviewer for this comment. Immunohistochemistry (IHC) for Rpn6 and tropomyosin was performed in **10** sham-treated mice and **10** PAB mice, and the expression patterns were similar among all mice in the respective groups. Please note that the staining of both Rpn6 and tropomyosin are patchy in the sham group. However, in the PAB group, Rpn6- as well as tropomyosin immunostaining reveal more diffuse distribution in the cytoplasm of cardiomyocytes, which might be indicative of disease-associated phenotypic alterations of cardiomyocytes following PAB. These IHC-results presumably mirror the enhanced 26S proteasome activities in RVH. Interestingly, in humans, expression of Rpn6 is only faint in tropomyosin expressing cardiomyocytes of normal heart tissue, whereas it is eminently induced throughout the cytoplasm of tropomyosin-positive cardiomyocytes of hearts from patients with RVH. With regard to different pattern of Rpn6-IHC-staining in human tissue compared to mice, we have no explanation for this phenomenon and can only speculate that species differences might be the reason. We have addressed this issue in the revised manuscript version (marked in red, pages 13 and 14).*

4. Human samples: How many controls were studied and how were these obtained? All but one patient had predominately left heart disease which may not be analogous to the RVH in PH. They need to provide quantitation by WB analysis of protein levels in the tissue. Also, they need to provide relevant demographic information for the patients. What was the source of the "normal" RV tissue? Interestingly the patchy appearance of staining was not present in the human tissue.

R4: *We thank the reviewer for this important question and apologize for the missing data on the patients' samples. In total, we analyzed paraffin sections of 7 right and 6 left ventricle biopsy samples from patients with diagnosed pulmonary hypertension as well as right ventricle biopsies from three non-PAH and non-DCM controls who were analyzed for suspected myocarditis. Unfortunately, we were not able to obtain more than paraffin embedded material of endomyocardial biopsies and were thus unable to perform Western blot analysis of patients' heart samples. Except for the age and sex, we do not have further demographic data on these patients. According to the reviewer's comment, we have now included the available information into the revised manuscript (pages 7 and 8; and supplementary table 1).*

5. The authors need to elaborate more on the pharmacodynamics of these agents. Are they suggesting that twice weekly dosing will avoid the long-term adverse effects of these agents? If so why? In their study they are only delivering the drugs over a maximum of 3 weeks and they do not seem to be assessing any off target effects.

R5: *We thank the reviewer for pointing out the important issue of pharmacodynamics. Pharmacodynamics of bortezomib is quite well studied in vivo while ONX-0912 is a 2nd*

generation inhibitor – an orally available version of carfilzomib - and less data are available on the in vivo pharmacodynamics of this inhibitor. The main difference between these two inhibitors is their mode of action. While bortezomib acts as a reversible proteasome inhibitor, ONX-0912 binds the catalytic active site irreversibly and is highly specific only for the CT-L active site (Dick and Fleming Drug Discov Today. 2010 Mar;15(5-6):243-9). Accordingly, the dosing of these inhibitors has to take into account this differential activity as well as the half-life of the proteasome in the target cell which determines recovery from irreversible inhibition by assembly of new proteasomes. The doses we chose for our study here are quite low compared to other published studies. Several reports applied bortezomib in mice at higher doses and for longer time points without observing toxic-side effects (e.g. Manning et al., J Immunol. 2015 Feb 15; 194(4): 1695–1701; Wagner-Ballon et al., Blood. 2007 Jul 1;110(1):345-53). Zeniya et al. applied bortezomib at similar doses as in our study but for 8 weeks and did not observe any hematological alterations (Zeniya et al., Sci Rep. 2017 Oct 12;7(1):13086). Moreover, our own results revealed that 0.5 mg/kg application of bortezomib inhibits the degradation of a proteasome GFP reporter in the left heart of mice 24 hours after application (Wilck et al., Arterioscler Thromb Vasc Biol. 2012 Jun;32(6):1418-26). These data prompted us to choose the dose of 0.5 mg/kg as an effective but seemingly non-toxic inhibitor dose.
Regarding the dosing of ONX-0912, we also applied a very low dose which was based on a recent study from our lab showing that the beta5 specific inhibition of the 20S proteasome by ONX-0912 causes less toxicity compared to bortezomib in vitro (Semren et al., PLoS One. 2015 Sep 4;10(9):e0136188). In this study, we also confirmed prolonged inhibition of the proteasome after 96 hours arguing in favor of a twice weekly dosing of the inhibitor. Our finding confirmed data from a tumor study in which ONX-0912 proved to be less toxic compared to bortezomib (Chauhn et al., Blood. 2010 Dec 2;116(23):4906-15). In this study, a dosing of 50 mg/kg given orally at two consecutive days every week for 4 weeks, resulted in reduced tumor burden and increased survival of mice without any off-target effects. In conclusion, in our present study we carefully chose inhibitor doses that were non-toxic and would allow prolonged and repeated treatment without inducing any off-target effects. We have included this information about the dosing issues in the revised manuscript (marked in red, pages 10, 15 and 16).

6. In the discussion, the authors hypothesize that "proteasome function in RVH versus RHF is related to the extent of maladaptive cardiac remodeling". This begs the question of what would be the effect of further inhibition of UPS in RHF. To the extent that reduced proteasome activity contributes to HF, then this could be detrimental. This could a major problem for the translation of this therapy to the clinic since it is the patients with RHF that are most need of treatments. I would strongly suggest that they also explore a model of decompensated RV remodelling to assess this experimentally.

R6: *We thank the reviewer for pointing this out and would like to refer to our reply **R1** of reviewer 1. We fully agree with the reviewer that this is an important issue and future studies should be designed to reply to this question.*

7. Figure 4. The methodology for assessing and quantifying fibrosis needs to be included. Why is the sham collagen levels so variable? In Fig 4F, collagen staining was less than in other PAB sham controls, suggesting this may be spurious.

R7: *In agreement with the reviewer, we have added additional information for assessing and quantifying the fibrosis in the revised manuscript version (Supplement, page 4). This methodology has been developed in our group almost a decade ago and since then we have*

published it in several papers (Kosanovic et al, Respir Res. 2011; 23; 12:87; Lang et al, PLoS One. 2012; 7:e43433; Kojonazarov et al, Int J Cardiol. 2013. 167: 2630-7; Kosanovic et al, Eur Respir J. 2015; 46: 1084-94; Janssen et al, Biomed Res Int. 2015; 2015:438403; Luitel et al, Physiol Rep. 2017; 5(6)…). Therefore, it is a well-established method. However, as for any other computer software-based histo-morphometric technique, this approach is also semi-quantitative and semi-automatic, so the existence of such variations is normal and expected. In addition, we believe that variations in the range from approx. 0.7 to 1.5 (% of collagen content) among the sham groups are fully acceptable. Similarly, the collagen levels of 2.7 to 6 % among the PAB groups are also in an acceptable range of variations. Finally, in addition to the fact that the assessment of the collagen content is indeed semi-quantitative and semi-automatic approach, the further potential source of variations may also appear since it is not possible to investigate exactly the same part of the right ventricle (RV) in every animal. Basically, after the heart is taken out, approx. one half of the RV is used for histology and the other half is used for molecular biology. Although we tried our best to cut exactly at the same place in every animal, it is realistic to expect that a bit different pieces of the RV tissue may appear. Knowing that there are differences in the tissue pattern and structure in the base versus apex of the RV, such variations are reasonable.

Reviewer #2: The role of the ubiquitin proteasome system during the development of right ventricular hypertrophy (RVH) and progression to failure (RVF) is less studied than its left-sided counterpart. Here Heitmeier et al. describe a detrimental role of proteasome activation during RVH, which was partially alleviated by proteasome inhibition. These findings differ to studies published in 2013 by Rajagopalan et al. and Fessart et al., which the authors attribute to the stage of RVH and RVF studied. Here the authors report a novel finding that the expression of Rpn6 is increased in RVH, which facilitates increased proteasome assembly and activity. For the most part this is a well-written manuscript and well designed study, however there are some areas that should be addressed to increase its clarity.

R #2: *We are thankful to the reviewer for overall positive impression about our study.*

8. The authors mention they get right ventricle samples from human patients that was part of routine diagnostics but fail to elaborate on their patient population. How was the tissue collected? What stage of right ventricular hypertrophy/failure were the patients in? What treatment regimens were the patients on?

R8: *We thank the reviewer for this important question which we have addressed also as response **R4** to reviewer #1. In total, we analyzed paraffin sections of 7 right and 6 left ventricle biopsy samples from patients with diagnosed pulmonary hypertension as well as right ventricle biopsies from three non-PAH and non-DCM controls who were analyzed for suspected myocarditis. Except for the age and sex, we do not have further demographic data on these patients. Unfortunately, we do not have exact information on the treatment regimen of these patients as this information is not part of the routine for standard pathological analysis. According to the reviewers' suggestion we have now included all relevant and available patients' data in the revised manuscript (pages 7 and 8; and supplementary table 1).*

9. How do the authors reconcile the effect differences between the two proteasome inhibitors? The data is the data but some rationale is needed. The authors are reporting that bortezomib is more effective as a preventative treatment but ONX is a better therapeutic intervention.

Proteasome inhibition with bortezomib as a therapeutic intervention appeared to have a trend of increased collagen content (Figure 4F). These results that seem to oppose the author's hypothesis.

R9: *We agree with the reviewer that the effects of bortezomib and ONX are somewhat different. Of note, the figure 4F refers to the therapeutic intervention where the ONX has been found more effective. Therefore, the finding of very slight and not significant increase of collagen content fits with the fact that bortezomib achieved less efficacy in the therapeutic treatment regimen compared to ONX. The most reasonable explanation for this observation is that these two inhibitors have distinct inhibitory activities on the proteasome. As already explained in our response to reviewer #1* ***R5****, bortezomib reversibly inhibits the beta 5 and beta 1 active sites of the 20S proteasome while ONX irreversibly blocks only the beta 5 activity (Dick and Fleming Drug Discov Today. 2010 Mar;15(5-6):243-9). Accordingly, the cellular effects of these two inhibitors can be quite different depending on the effective dose and cell type as recently investigated by us (Semren et al., PLoS One. 2015 Sep 4;10(9):e0136188). It is, however, difficult to dissect these effects on the cellular level in vivo as we used total tissue lysates to perform molecular analysis. We have addressed this issue in the revised manuscript (marked in red, pages 10 and 16).*

10. Others have presumed that proteasome inhibition may be effective by inhibiting activation of the NF-kB pathway. What are the I-kB and NF-kB levels in RVH +/- proteasome inhibitors compared to sham?

R10: *We are very thankful to the reviewer for this important point. In agreement with the reviewer's suggestion, we have investigated I-kB alpha and beta levels in our right heart protein lysates. Unfortunately, we were unable to prepare nuclear extracts from our right heart tissue to investigate whether nuclear translocation of NF-kB had been blocked by proteasome inhibitor treatment of the mice. As shown in the supplemental figure 11B of our revised manuscript, we indeed observed accumulation of I-kB alpha but not beta in the right hearts of the bortezomib treated mice while ONX treatment had no effect (data not shown). These data thus support the differential effect of the two inhibitors and suggest that activation of NF-kB might be inhibited in bortezomib-treated PAB mice and have been included in the revised manuscript (page 16).*

11. Prior studies have reported an up regulation of autophagy as a compensatory mechanism with proteasome impairment/inhibition. Is autophagy increased in this model of proteasome inhibition to elicit cardioprotection?

R11: *We thank the reviewer for this suggestion and we have accordingly determined levels of LC3 I and II as an indicator of autophagosome formation. We only detected the LC3 I form in the right hearts of sham, PAB and PAB-inhibitor treated mice but no LC3 II forms indicating that autophagy is not grossly activated. This observation is in line with the only minor overall inhibition of proteasome activity, which most likely does not trigger any major compensatory proteostatic mechanism (Vilchez et al., Nat Commun. 2014 Dec 8;5:5659). We have included these data into the supplement of our revised manuscript and discussed the results (supplementary figure 11B and pages 15 and 16 in the main manuscript).*

12. Increased ubiquitinated proteins and protein aggregates are hallmarks of cardiomyopathy and heart failure; thought to accelerate disease pathogenesis. The authors fail to report total ubiquitinated proteins and protein aggregates with proteasome inhibition.

R12: *We agree with the reviewer that we did not include analysis of polyubiquitinated proteins as a readout for proteasome inhibition in our manuscript. As shown in the Figure 5 (revised figure 4), we observed accumulation of poly-ubi proteins in PAB indicative of increased 26S proteasome activity and proteasome turnover (Semren et al., Am J Respir Crit Care Med. 2015 Nov 1; 192(9):1089-101; Vilchez et al., Nature. 2012; 489(7415):304-8). We now also measured polyubiquitinated proteins in the inhibitor treated mice as suggested by the reviewer. However, we were unable to detect any significant differences in overall poly-ubiquitin levels when we compared PAB and PAB- inhibitor treated mice (see revised supplementary figure 11A). This finding is again in line with our mild inhibitor treatment that will not grossly disturb protein homeostasis and result in accumulation of polyubiquitinated soluble proteins (Meiners S, et al., Med Res Rev. 2008 Mar; 28(2):309-27).*

13. Grammatical errors
Results section- clipping of the pulmonary artery

R13: *Thank you very much. We have corrected the grammatical error in the revised manuscript version.*

Reviewer #3: The authors aim to identify altered regulation of the proteasome in RV hypertrophy induced by pulmonary artery banding in mice. They find upregulation of the proteasome in the hypertrophied heart of the mice with RVH as well as in hearts form patients with various forms of pulmonary hypertension. Furthermore, proteasome inhibition improved RV function in their mice model.

R #3: *We thank to reviewer for the nice summary of our work.*

Major comments:

14. Figure 2 shows a large variation between samples particularly in the PAB group. Is this related to any hemodynamic, echographic or histological parameters of severity of PAB?

R14: *In agreement with the reviewer´s suggestion, we have analyzed the correlations between the proteolytic activities of the proteasome (C-L and CT-L; figure 2 (revised figure 1)) and important histological, functional, hemodynamic and echocardiographic parameters. There was a significant positive correlation between the C-L and CT-L, and several histological and morphometric parameters: cardiomyocyte hypertrophy (Pearson test, p=0.02 (C-L and cardiomyocyte size) and p=0.03 (CT-L and cardiomyocyte size); collagen content (Pearson test, p=0.02 (C-L and collagen content) and p=0.005 (CT-L and collagen content) and inner diameter of the RV (Pearson test, p=0.0008 (C-L and RVID) and p=0.002 (CT-L and RVID). In addition, there was a significant negative correlation between the C-L and CT-L, and TAPSE (Pearson test, p=0.0009 (C-L and TAPSE) and p=0.001 (CT-L and TAPSE). Finally, there was no correlation between the C-L and CT-L, and systolic blood pressure (SBP). Therefore, increased activity of the proteasome is associated with severity of the RV hypertrophy.*

15. In figures 3 and 4, it appears that 4 different Control-PAB groups are used, each treated with the appropriate vehicle. Although echographic findings are very similar between Control

groups, particularly collagen content seems very variable (figure 4F vs other PAB groups, and supplemental Fig 4), which may influence the conclusion of the treatment. Please comment.

R15: *We thank reviewer for this observation. However, as usual for any other computer software-based histo-morphometric technique, this approach is semi-quantitative and semi-automatic, so the existence of such variations is normal and expected. In general, we believe that variation in the range from approx. 2.7 to 6 (% of collagen content) among the PAB groups is fully acceptable. Finally, in addition to the fact that the assessment of the collagen content is indeed semi-quantitative and semi-automatic approach, another potential source of variations may come from the fact that it is not possible to investigate exactly the same part of the right ventricle (RV) in every animal. Basically, after the heart is taken out, approx. one half of the RV is used for histology and the other half is used for molecular biology. Although we tried the best to cut exactly at the same place in every animal, it is realistic to expect that a bit different pieces of the RV tissue may appear. Knowing that there are differences in the tissue pattern and structure in the base versus apex of the RV, such variations are reasonable.*

16. Please define colors of immunohistochemical staining in figure 6. Also, staining seems to be very patchy across the sample. Is the typical for Rpn6? Please also show quantification for the different patients. Patients seem to represent a very diverse group of PH patients. Is there a correlation with severity/ origin of PH? How relevant are these patients for patients with PH?

R16: *In agreement with the reviewer, we have defined the colors of immunohistochemical staining in the figure 6 (revised figure 5) in the revised manuscript (pages 13, 14 and 29). With regard to the pattern of Rpn6 expression, we would like to mention that immunohistochemistry (IHC) for Rpn6 and tropomyosin was performed in **10** sham-treated mice and **10** PAB mice, and the expression patterns were similar among all mice in the respective groups. Please note that the staining of both Rpn6 and tropomyosin are patchy in the sham group. However, in the PAB group, Rpn6- as well as tropomyosin immunostaining reveal more diffuse distribution in the cytoplasm of cardiomyocytes, which might be indicative of disease-associated phenotypic alterations of cardiomyocytes following PAB. These IHC-results presumably mirror the enhanced 26S proteasome activities in RVH. Interestingly, in humans, expression of Rpn6 is only faint in tropomyosin expressing cardiomyocytes of normal heart tissue, whereas it is eminently induced throughout the cytoplasm of tropomyosin-positive cardiomyocytes of hearts from patients with RVH. With regard to different pattern of Rpn6-IHC-staining in human tissue compared to mice, we have no explanation for this phenomenon and can only speculate that species differences might be the reason. We have addressed this issue in the revised manuscript version (marked in red, pages 13 and 14).*
We agree with the reviewer that the group of patients is very diverse, but would like to point out the general difficulty of obtaining human RV biopsies. With regard to the correlation of disease severity and Rpn6 staining in PAH patients, we are not able to provide solid data as the IHC staining is only semi-quantitative and the numbers of samples is too low and too diverse (7 RV, 6 LV PAH samples and 3RV controls). Moreover, we did not have standardized information on the severity of PAH and treatment regimens.

Minor comments:

17. Please provide details regarding severity of PAB in methods section, and hemodynamic measurements of PAB severity (i.e RVSP) in results section.

R17: *We have provided the RVSP values in the revised manuscript version, as requested by the reviewer (supplementary figure 10).*

18. Is collagen content of the RV measured as interstitial collagen, perivascular collagen or both?

R18: *The collagen content of the RV has been measured with focus on interstitial collagen fraction. We have included this information in the revised manuscript version (supplement, page 4).*

19. Data from supplemental Figs 5 and 6 should be added to figs 3 and 4.

R19: *We thank the reviewer for suggestion, but we would like to keep them separately. Otherwise, the figures 3 and 4 would be overcrowded.*

Editors

The subject is of potential interest but there are a few major concerns that will require further work. Conceptually, as pointed out, the model may offer a somewhat limited take on the human pathology and this needs to be further explored to ensure that there is no bias with unjustified conclusions for human disease. The experimental design and control groups need further clarification. On the one hand it appears as if the in vivo control/PAB data presented in Figure 1 are identical to the control/PAB data in Fig.3 and 4. On the other hand, there are discrepancies between the baseline data for microscopy, i.e. CM size and in particular collagen.

E1: *We thank to the editors for the summary and we have tried to address all these issues, as written in the reply to the reviewers and in the revised manuscript version.*

The pharmacology needs more documentation. How specific - off-target effects?

E2: *We have address this point in the reply to the reviewers and in the revised manuscript in* ***R5*** *and* ***R9****.*

Lastly, the human data are poorly developed and need better documentation, of the subjects and (semi)quantification of the measurements. RV endomyocardial biopsies in some of the cases were for diagnosis of LV disease and taken from the septum? How does this compare to the mouse data - septum or RV free wall?

E3: *Also, we have address the issues with regard to the human data in the revised manuscript version and in the reply to the reviewers* ***R4, R8 and R16****.*

- The timeline in Suppl Fig.1 should include the groups of mice and how they were assigned/used.

E4: *The revised supplementary figure 1 was prepared in agreement with the editors´ suggestion (page 3).*

If the treatment experiments were done in parallel and the same controls were used for the

two drugs/treatments, an ANOVA is needed. Then there no need to show the baseline data separately but a detailed Table overview could be included instead.

E5: *We fully agree with the editors. Therefore, we have presented the baseline data in the form of the table (supplementary table 2, page 20).*

Please adhere to the Instructions to Authors and show all data as scatter/dot plots.

E6: *In agreement with the editors, we have presented our data as scatter/dot plots in the revised manuscript version.*

The WBs in Figure 5 appear overexposed – please provide better images.

E7: *In general, due to the low protein amounts the WBs were difficult and we have already provided the best images available using the high-sensitivity ECL detection system and film development. We agree with the editor that WB signals are slightly overexposed in the PAB group, but would like to point out that signals are not overexposed in the sham group. As such the densitometric analysis would rather underestimate the differences between the two groups.*

Altered proteasome function in right ventricular hypertrophy

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Abstract

Background - In patients with pulmonary hypertension, right ventricular hypertrophy (RVH) is a detrimental condition that ultimately results in right heart failure and death. The ubiquitin proteasome system has been identified as a major protein degradation system to regulate cardiac remodelling in the left heart. Its role in right heart hypertrophy, however, is still ambiguous.

Methods and results - RVH was induced in mice by pulmonary artery banding (PAB). Both, expression and activity of the proteasome was found to be upregulated in the hypertrophied right ventricle compared to healthy controls. Catalytic inhibition of the proteasome by the two proteasome inhibitors Bortezomib (BTZ) and ONX-0912 partially improved RVH both in preventive and therapeutic applications. Native gel analysis revealed that specifically the 26S proteasome complexes were activated in experimental RVH. Increased assembly of 26S proteasomes was accompanied by elevated expression of Rpn6, a rate-limiting subunit of 26S proteasome assembly, in hypertrophied cardiomyocytes of the right heart. Intriguingly, patients with RVH also showed increased expression of Rpn6 in hypertrophied cardiomyocytes of the right ventricle as identified by immunohistochemical staining.

Conclusions - Our data demonstrate that alterations in expression and activity of proteasomal subunits play a critical role in the development of RVH. Moreover, this study provides an improved understanding on the selective activation of the 26S proteasome in RVH that might be driven by the rate-limiting subunit Rpn6. In RVH, Rpn6 therefore represents a more specific target to interfere with proteasome function than the commonly used catalytic proteasome inhibitors.

Key words: Proteasome; proteasome inhibition; Rpn6; pulmonary artery banding; right ventricular hypertrophy.

Introduction

Right ventricular hypertrophy (RVH) is of important prognostic value for the outcome of patients with pulmonary hypertension (PH)[1] and several cardiomyopathies[2]. Although there are different causes of PH, they all share the pathological feature of alterations in the pulmonary vasculature (remodelling)[3] which causes elevated pulmonary arterial pressure. The right ventricle (RV) adapts to this increased afterload with hypertrophy (Cor pulmonale)[4]. With progression of the disease, the adaptive hypertrophy of the myocardium shifts to a maladaptive state of cardiac remodelling. A pathological hallmark is the appearance of cardiac fibrosis[4]. The change in tissue structure, namely increased size of cardiomyocytes and overproduction of extracellular matrix, subsequently alters conduction of electric signals and the mechanical function of the heart. Eventually this results in right heart failure (RHF)[5] which represents one of the most common causes of death in patients with PH[6]. Standard therapies in left heart diseases (LHD) have so far failed to improve RV maladaptive remodeling[7]. In conclusion, there is an urgent need for new therapeutic strategies to target RV dysfunction by preventing or reversing cardiac remodelling.

One hallmark of cardiomyocyte hypertrophy is altered protein homeostasis that involves an overall increase in protein synthesis but also in protein degradation[8]. The ubiquitin-proteasome system is the major systems for intracellular protein degradation[9]. For that, proteins are first tagged with polyubiquitin chains and later degraded by the 26S proteasome. The protein degrading proteasome consists of a 20S catalytic core and one (26S proteasome) or two (30S proteasome) 19S regulatory caps. The 20S catalytic core forms a barrel that comprises three catalytic active sites. In a caspase-, trypsin- or chymotrypsin-like manner (C-L, T-L or CT-L), the catalytic subunits β1, β2 and β5 cleave the target proteins after acidic, basic or hydrophobic ami-

no acids, respectively[10]. Proteasome activators bind to the 20S catalytic core and facilitate opening of its - usually closed – pores, and allow substrate entry[11]. Among the known five proteasome activators, the 19S regulator (also called PA700) mediates ATP-dependent degradation of ubiquitin-tagged proteins[12]. The 19S proteasome is involved in the recognition and binding of the protein substrate to funnel it into the, now opened, 20S core[13].

The ubiquitin proteasome system is critically involved in numerous cellular processes such as cell cycle control, transcriptional regulation, stress and immune responses, and disposal of misfolded proteins[14]. It has been found dysregulated in numerous diseases including cancer, inflammation, fibrosis, PH and cardiomyopathies[15]. The 26S proteasome has been comprehensively analysed by proteomic analyses in samples of whole hearts[16]. Proteasome expression and activity was differentially regulated in hypertrophic versus failing hearts; while proteasome function was found to be upregulated in experimental left ventricular hypertrophy (LVH) and dilative cardiomyopathy in patients, proteasome activity was impaired in failing left hearts[17]. Specific catalytic proteasome inhibitors have been applied in different experimental models of LVH where it successfully counteracted development and also reversed established LVH[18]. Similarly, they have recently been tested in experimental models of RVH and right heart failure (RHF) with controversial results; while the proteasome inhibitor Bortezomib alleviated RVH[19], proteasome inhibition partially augmented RHF[20].

In this study, we identify the activation of the proteasome as a contributing factor to development and maintenance of RVH. Specifically, we observed activation of the 26S proteasome and overexpression of the 19S subunit Rpn6 in experimental RVH

and in right hearts of patients with PH. These data identify Rpn6 as a novel potential target to specifically interfere with 26S proteasome activation in RVH.

Methods

For details on the immunohistochemical, protein, activity and RNA analyses of mouse and human tissue, the reader is referred to the online supplement.

In vivo studies

All animal experiments were approved by the local ethics committee of the Regierungspraesidium Giessen and were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The PAB surgery to provoke RVH, echocardiographic assessments and hemodynamic measurements were performed as reported previously[21]. The proteasome inhibitors Bortezomib (BTZ) and ONX-0912 were used for preventive (*BTZprev* and *ONXprev*) and therapeutic treatment (*BTZther* and *ONXther*). Three weeks after PAB, the mice were sacrificed and histology and protein/mRNA analysis were performed for various targets in RV tissue. For profound details of the model, drugs and molecular analyses the reader is referred to the online supplement.

Anaesthesia, analgesia and euthanasia

The below described general anaesthesia via inhalation was used for echocardiography, PAB-surgery and hemodynamic measurements. First, the mouse was placed for about 5 minutes into an induction chamber where it was exposed to a mix of 5% isoflurane and 100% oxygen (Vetequip inhalation anaesthesia system SN:3470). Once the righting and the pinch-toe reflexes were negative, the mouse was placed supine on the corresponding examining table. Next dexpanthenol was placed onto the eyes to avoid their dehydration. A rectal probe recorded the body temperature which was maintained at 37°C by a warming plate. Together with chest movement

and pinch-toe reflex these parameters served as anaesthetic monitoring. Anaesthesia was maintained with 2.0% isoflurane-oxygen mix, delivered either by nose cone (echocardiography, hemodynamic measurements) or via tracheal tube (PAB surgery). Ventilation was carried out at 120-150/min, a breath volume of 200-250µl and 2 L flow. After PAB-surgery, isoflurane was turned off while keeping the ventilation with oxygen. The mouse was disconnected time and again to check if it was already breathing spontaneously. In this case the tube was removed and the mouse put into a cage under an infrared lamp. The whole procedure from induction to emergence of the anaesthesia took 25 to 35 minutes.

Concerning pain management, 30-45 minutes before PAB-surgery the mouse received 0.1 mg/kg Buprenorphine (Temgesic, Essex Pharma GmbH, Munich) subcutaneously (SC). For the next 48 hours after surgery the mouse received 0.1 mg/kg buprenorphine SC BID (bis in die) and 2.0 mg/kg carprofen over the drinking water. All mice were checked daily according to a score sheet with a special interest in the development of clinical signs for right heart failure (inactivity, ruffled fur, dyspnea, ascites).

Euthanasia took place after hemodynamic measurements; the heart catheter was gently removed from the still anaesthetised mouse before it was sacrificed by incision of the heart to bleed out.

Human material

Human tissue samples of right ventricles were obtained for routine histological diagnostics. Paraffin sections of 7 right and 6 left ventricle biopsy samples from patients with diagnosed PH were analyzed, as well as right ventricle biopsies from three non-

PAH and non-DCM controls that were investigated for suspected myocarditis. Tissue that was not needed any more for diagnostic approaches was used for staining in this study. The patients gave informed consent for histological and immunohistological examinations, and the human tissue samples usage was approved by the ethics committee of the University of Tuebingen that conforms the declaration of Helsinki. For further information on patients´ characteristics, please refer to the supplement (supplementary table 1).

Statistical analysis

The presented data comprise the means and standard error means (SEM). Significance was determined by one-way ANOVA and Newman-Keuls-test. The comparison of two groups was analysed by the unpaired t-test. Significance was defined as $p \leq 0.05$. Statistical details and sample size are indicated in the figure legends.

Results

Impaired function of RV is accompanied by structural changes

Mice that underwent PAB surgery successfully developed RVH within three weeks as determined by RV mass in relation to tibia length (RV/TL, supplementary table 2). Echocardiography revealed an increase in both inner diameter (RVID) and wall thickness (RVWT) of RV in *PAB* compared to *sham* (supplementary table 2). The relatively thin RV, compared to the left ventricle (LV) that is used to work in a high-pressure system[22], reacts quickly with a dilatation to the banding of the pulmonary artery. The increase in RVWT additionally demonstrates the development of RVH in our mouse model. The mice did not show any clinical signs for RHF during daily checks. To assess heart function, tricuspid annular plane systolic excursion (TAPSE) and cardiac index (CI) were measured by echocardiography (supplementary table 2). Both parameters were significantly decreased in *PAB* compared to *sham* animals indicating a deterioration of heart function in *PAB* mice. Histology revealed an increase in cardiomyocyte size (supplementary table 2) as well as in collagen content in the RV (supplementary table 2) of *PAB* mice compared to healthy controls. Taken together, these functional and structural data reveal development of RVH in the absence of heart failure in our mouse model of PAB.

Proteasome activity is increased in RVH

The proteolytic activity of the proteasome in RV was determined using luminescent substrates for the β1 and β5 active sites. As depicted in figure 1A, the caspase like (C-L), as well as the chymotrypsin like (CT-L) activity were significantly increased by more than three fold in *PAB* compared to *sham* mice. We confirmed this pronounced

increase in proteasomal activity using a second method. The use of activity based probes (ABP) allows discrimination of several active sites of the proteasome upon binding of a fluorescently labelled probe [23]. This ABP acts like a specific proteasome inhibitor as it covalently binds to the active-site threonine of the catalytic subunits of the proteasome[24]. As such, the extent of binding of the labelled ABP is a direct measure for the number of active sites present and can be quantified for the different catalytic subunits after separation of proteasome complexes by SDS-PAGE (figure 1B). ABP analysis unambiguously confirmed concerted activation of the activity of all three catalytic subunits β1, β2 and β5 in the RV of the *PAB* mice compared to *sham*.

Proteasome inhibition attenuates development of RVH

In order to investigate whether this increase in proteasome activity contributes to the development of RVH in PAB, we applied two distinct proteasome inhibitors. The reversible proteasome inhibitor Bortezomib (BTZ) has been FDA approved in 2003 as a first generation inhibitor for the treatment of multiple myeloma patients and was used in several preclinical models of human diseases[25]. In contrast, the carfilzomib analogue ONX-0912 represents a second-generation proteasome inhibitor. It is applied orally[26] and binds irreversibly only to the β5 active site of the proteasome[25]. Preventive treatment of *PAB* mice twice a week with either BTZ (0.5 mg/kg BW intraperitoneally) or ONX-0912 (50 mg/kg applied by oral gavage) did not significantly alter the ratio of RV mass to tibia length (RV/TL, supplementary figures 2A,C). In contrast, echocardiography demonstrated significant improvement of RV structure (RVID, figures 2A,C) and function (TAPSE, figures 2B,D) while RV free wall thickness was not altered (RVWT, supplementary figures 2B,D). The cardiac index (CI, supplementary figures 2E,G) increased slightly but significantly at constant heart rate

(HR, supplementary figures 2F,H) only in the preventively ONX-treated group. Histology showed that preventive treatment with BTZ led to a reduction of cardiomyocyte size (figure 2E) while results did not reach significance in the *ONXprev* group compared to the placebo-treated *PAB* group (figure 2G). Collagen content showed a tendency to decrease in the BTZ and ONX-0912 treated groups compared to the *PAB* placebo control (figures 2F,H). Constant systolic and diastolic blood pressure (SBP, DBP, supplementary figure 3) indicated that both proteasome inhibitors had no considerable systemic effects on hemodynamic parameters. In summary, these findings suggest that catalytic proteasome inhibition had beneficial effects on RV morphology and function in experimental RVH.

Therapeutic proteasome inhibition reduces RVH

In a next step, we tested both inhibitors for their therapeutic effects on established RVH (*BTZther* and *ONXther*). To confirm development of RVH in our mouse model, we performed echocardiography after one week of PAB surgery and compared right heart parameters to echocardiographic data at baseline (supplementary figure 5); *PAB* mice had already developed pronounced changes in RV structure and function (supplementary figure 5). The animals now received either BTZ or ONX-0912 as described above to investigate the possible therapeutic effect of proteasome inhibition on established RVH. Final echocardiography was performed two weeks later. CI and HR changed neither in *BTZther* nor in *ONXther* compared to *PAB* (supplementary figures 6E-H). As in the preventive approach, therapeutic treatment with BTZ (*BTZther*) significantly reduced RVID compared to *PAB* (figure 3A) while RVWT did not change (supplementary figure 6B). In the therapeutic ONX group, proteasome inhibition had beneficial effects on RVH as evidenced by significant reduction of

RVID (figure 3C) and RVWT (supplementary figure 6D) compared to *PAB*. TAPSE improved significantly in both *ONXther* and *BTZther* (figures 3B,D), but only *ONXther* reduced RV/TL (supplementary figure 6C). Treatment with BTZ influenced neither cardiomyocyte size nor collagen content (figures 3E,F). In contrast, both histopathological parameters were significantly reduced upon the treatment with ONX (figures 3G,H). In summary, these findings suggest that therapeutic application of proteasome inhibitors improved RV structure and function in experimental RVH, with ONX being more effective than BTZ.

26S proteasome activity augmented in RVH

These data support a potential therapeutic value of catalytic proteasome inhibitors in the therapy of RVH. A major drawback of the therapeutic application of proteasome inhibitors, however, are their systemic side effects such as neuropathy[27] and cardiac proteotoxicity[28]. Targeting distinct proteasomal complexes by interfering with the assembly of the 20S core complex and its proteasome activators was recently proposed as a more specific approach to reduce side effects[29]. In order to better understand proteasome activation in RVH, we analysed the formation of proteasome complexes in the right heart of *PAB* mice compared to healthy controls. Western blot analysis and subsequent densitometry revealed increased levels of the catalytic subunit β1 as well as of subunit α3 (figure 4A) in *PAB* mice. Moreover, expression of the 19S regulatory subunits Rpt5 and Rpn6 was also clearly elevated in the hypertrophied RV of *PAB* mice (figure 4A). These findings were accompanied by augmented activity of singly and doubly capped 26S proteasome complexes as revealed by labelling of active proteasome complexes in native gels using the aforementioned ABPs (figure 4B). Enhanced 26S proteasome activity resulted in increased turnover

of ubiquitinated substrates as indicated by elevated levels of polyubiquitinated proteins (figure 4A). These data indicate that an elevation in proteasomal activity is due to an increased activity of the 26S proteasomes which mediate ubiquitin-dependent protein degradation in the hypertrophied RV[12].

Rpn6 increased in hypertrophied RV tissue

We corroborated our data by focusing on the cell-specific expression of the 19S subunit Rpn6 in hypertrophied RV. Recent publications have identified Rpn6 as a major regulator for the assembly and activation of the 26S proteasome complexes[30]. Cell-specific expression of Rpn6 in diseased tissue may be used as a surrogate marker for elevated 26S proteasome activity[31]. Immunohistochemical staining for Rpn6 in the RV of *sham* and *PAB* mice revealed co-localization of Rpn6 (red colour) with tropomyosin (brown colour), indicating expression of Rpn6 in the cytoplasm of cardiomyocytes (figure 5A). The staining of both Rpn6 and tropomyosin was patchy in the *sham* group. However, in the *PAB* group, Rpn6 as well as tropomyosin immunostaining revealed more diffuse distribution in the cytoplasm of cardiomyocytes, which might be indicative of disease-associated phenotypic alterations of cardiomyocytes following *PAB*. We observed an increase in Rpn6 staining in RVH of *PAB* mice compared to healthy tissue of *sham* mice, which goes in line with our quantitative Western blot data for Rpn6. These data suggest that elevated expression of Rpn6 contributes to increased 26S proteasome activity in hypertrophied cardiomyocytes in mice. In order to apply this finding to human RVH, we stained biopsies of RV tissue obtained from patients with RV cardiomyopathies for Rpn6 (figure 5B). Of note, Rpn6 (pale red colour) expression was again co-localized to cardiomyocytes and appeared to be elevated in hypertrophied heart tissue of patients with RVH. Interestingly, in humans,

expression of Rpn6 was only faint in tropomyosin (dark red colour) expressing cardiomyocytes of normal heart tissue, whereas it was eminently induced throughout the cytoplasm of tropomyosin-positive cardiomyocytes of hearts from patients with RVH.

Discussion

Proteasome inhibition partially reduces RVH

Despite the success of proteasome inhibition in the field of hematopoietic tumours, severe side effects were reported since the first approval of BTZ in 2003 as third treatment of multiple myeloma[32]. Application over a longer period resulted in accumulation of misfolded and nonsense proteins[32]. From research on BTZ, neuropathies were well-known[27], but case reports described also signs of cardiac toxicity after the treatment with both reversible[28,33] and irreversible[34] proteasome inhibitors which reversed upon termination of therapy[35]. This was taken into consideration when designing this study by choosing a sequential design to adjust the right dose and administering both proteasome inhibitors only twice weekly. We carefully chose inhibitor doses that were non-toxic and would allow prolonged and repeated treatment without inducing any off-target effect. Several reports applied BTZ in mice at higher doses and for longer time points without observing toxic-side effects[36,37,38]. Regarding the dosing of ONX, we applied a low dose and repetitive dosing based on data from our lab and others[39,40].

The groups of Hedhli[41] and Stansfield[42] had reported that proteasome inhibition in experimental LVH not only decreased, but also prevented cardiac remodelling. Our data for RVH provide evidence that treatment with BTZ or ONX only partially improves RV function and structure, suggesting that partial proteasome inhibition in the beginning of RVH may at least delay the development of RHF. On the molecular level, the applied inhibitor doses did not induce pronounced accumulation of polyubiquitinated proteins in the RV (supplementary figure 11A) and also did not result in a compensatory induction of autophagy (supplementary figure 11B)[43], suggesting that

indeed the proteasome is only partially inhibited. The beneficial effects of partial proteasome inhibition have previously been discussed[14]. Of note, BTZ treatment induced accumulation of the NF-κB inhibitor IκBα – but not IκBβ – in the RVs of BTZ treated mice indicating suppression of inflammatory NF-κB signalling, a well-established effect of proteasome inhibition (supplementary figure 11B)[44]. Interestingly, we observed that ONX was more efficient in recovering RVH function and structure than BTZ. This effect might be related to the distinct pharmacologic features of the boronate inhibitor BTZ and the α, β-epoxyketone inhibitor ONX-0912[45]. While BTZ reversibly inhibits both the chymotrypsin- and caspase-like activity, ONX-0912 is selective for the chymotrypsin-like activity which it inhibits irreversibly[27]. Moreover, off-target effects have been described for BTZ but are not evident for the ONX-0912 analogue carfilzomib which has also recently been approved for treatment of multiple myeloma[27]. These distinct pharmacological features of the two applied proteasome inhibitors BTZ and ONX-0912 may thus account for the observed effects as supported by our previous comparative study on lung fibrosis[39]. A comparative analysis on the therapeutic effects of reversible versus irreversible proteasome inhibitors on heart function, like in our study, has not been performed before and adds to the understanding of the differential effects of distinct proteasome inhibitors[14].

Proteasome activity increased in RVH

We provide evidence that the catalytic activity of the proteasome increases in experimental RVH. This is in contrast to two other studies reporting a decrease in proteasome function; Rajagopalan et al. observed that proteasome activity was decreased in mice with PAB induced RVH. This was associated with upregulation of key regulators for the UPS such as several E3 ligases and the 19S subunit Rpt5[46].

Fessart et al. also observed an impairment of the proteasome system in rats with hypoxia- or monocrotaline-induced RVH[20]. Importantly, both studies analysed animals that had developed clinical signs of RHF in contrast to our model of RVH. We thus hypothesize that proteasome function in RVH versus RHF is related to the extent of maladaptive cardiac remodelling. This has also been suggested for the left heart: In TAC-induced left heart hypertrophy, Ranek et al.[47] observed an increase in the activity of the proteasome within two weeks. Heart function and proteasome activity, however, deteriorated within the following weeks until heart failure eventuated. Tsukamoto et al. corroborated this finding in experimental LVH and in LVH samples of patients with heart failure[48]. These data indicate that the activity of the proteasome is related to the stage of myocardial remodelling with enhanced activity upon adaptive hypertrophic remodelling and loss of proteasome function accompanying progressive heart failure. We propose that this concept also holds true for right heart remodelling; adaptive RVH is accompanied by an increase in proteasomal activity whereas at later stage of maladaptive cardiac remodelling not only heart but also proteasome function deteriorates. Accordingly, proteasome inhibitors may have opposite effects, depending on the stage of cardiac remodelling. During adaptive cardiac remodelling they act protective and even reverse right and left heart remodelling while at maladaptive remodelling catalytic proteasome inhibitors will have detrimental effects. Any further inhibition of the proteasome will then result in cardiac proteotoxicity and provoke a hypertrophied right heart to fail[47]. According to this concept, determination of proteasome activity in RV or LV biopsies emerge as a novel discriminating marker for defining progressive stages of heart dysfunction with the potential to define a personalized therapeutic regimen for patients with PAH or cardiomyopathies.

Increased Assembly of the 26S Proteasome in RVH

Our analysis of proteasome expression and function revealed that the increased proteasome activity in hypertrophied RV can be ascribed to augmented formation and activity of 26S proteasome complexes. Activation of 26S proteasomes was accompanied by an increased turnover of polyubiquitinated substrates as shown previously[8]. This finding is also in line with the recently described positive feedback of the mTOR pathway to the proteasome that links increased protein synthesis to activation of proteasomal protein degradation[49]. In this study, Zhang et al. demonstrated mTOR-dependent transcriptional activation of the proteasome via the transcription factor Nrf1[49]. In contrast, we were unable to observe concerted induction of proteasomal gene expression on the mRNA level. The only proteasomal gene that we found transcriptionally activated in experimental RVH was the 19S regulatory subunit Rpn6 (supplementary figure 9). Of note, this subunit has been proposed to act as a structural clamp that stabilizes the interaction of the 19S regulatory complex with the 20S catalytic core[50]. In line with these structural data, Rpn6 has been identified as a rate-limiting subunit for regulating assembly of 26S proteasomes in differentiating embryonic stem cells and myofibroblasts[30,31]. The particular role of Rpn6 for the activation of 26S proteasome function is also supported by the recent observation that phosphorylation of Rpn6 by protein kinase A enhances assembly of 26S proteasome complexes[51]. Taken together, these data strongly support the notion that Rpn6 is a potential marker for enhanced 26S proteasome activity. In line with this concept, increased 26S proteasome activity was accompanied by Rpn6 overexpression in hypertrophied RVs of *PAB* mice with predominant localization to hypertrophied cardiomyocytes as determined by quantitative Western blot and semi-quantitative immunohistochemistry analysis. We also observed Rpn6 to be highly expressed in hypertro-

phied cardiomyocytes in RV of human patients. This finding opens the possibility that immunohistochemical detection of Rpn6 might be used as a surrogate biomarker for elevated proteasome activity in order to discriminate progressive stages of heart dysfunction as proposed above.

Moreover, identification of Rpn6 as an important regulator for 26S proteasome activity suggests that this subunit might serve as a novel distinct target to specifically interfere with 26S proteasome activation. Indeed, genetically induced overexpression of Rpn6 conferred resistance of C. elegans worms to proteotoxic stress[52]. Conversely, silencing of Rpn6 altered differentiation of human embryonic stem cells[30] and counteracted TGFβ-induced myodifferentiation of human lung fibroblasts[31]. These studies suggest that targeting the assembly of the 19S complex via Rpn6 might provide a more specific approach to interfere with hypertrophy-associated 26S proteasome activation compared to the indiscriminate inhibition of all proteasome complexes using catalytic proteasome inhibitors.

Conclusion

Our study revealed that

- proteasome expression and activities increased in experimental RVH,
- proteasome inhibition had beneficial therapeutic effects on RVH with ONX-0912 being more potent than BTZ,
- 26S proteasome formation increased via the regulatory unit Rpn6, making Rpn6 a more specific target for proteasome interference than catalytic inhibition.

To the best of our knowledge, we provide for the first time evidence that there is an upregulation of Rpn6 in experimental RVH. Moreover, Rpn6 was found when investigating RV tissue of human hypertrophied hearts, indicating the relevance of this animal model to the human situation. Future investigations can focus on Rpn6 as new, more specific therapeutic target for proteasome interference.

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1 LITERATURE CITED

1. Thenappan T, Gomberg-Maitland M. Epidemiology of pulmonary hypertension and right ventricular failure in left heart failure. *Curr Heart Fail Rep* 2014;**11**:428–435.
2. Ryan JJ, Tedford RJ. Diagnosing and treating the failing right heart. *Curr Opin Cardiol* 2015;**30**:292–300.
3. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 2009;**297**:L1013-L1032.
4. Bogaard HJ, Abe K, Vonk Noordegraaf A, Voelkel NF. The Right Ventricle Under Pressure. *Chest* 2009;**135**:794–804.
5. Schermuly RT, Ghofrani HA, Wilkins MR, Grimminger F. Mechanisms of disease: pulmonary arterial hypertension. *Nat Rev Cardiol* 2011;**8**:443–455.
6. Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, Naeije R, Newman J, Oudiz RJ, Provencher S, Torbicki A, Voelkel NF, Hassoun PM. Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. *J Am Coll Cardiol* 2013;**62**:D22-D33.
7. Walker LA, Buttrick PM. The right ventricle: biologic insights and response to disease: updated. *Curr Cardiol Rev* 2013:73–81.
8. Mearini G, Schlossarek S, Willis MS, Carrier L. The ubiquitin-proteasome system in cardiac dysfunction. *Biochim Biophys Acta* 2008;**1782**:749–763.
9. Hershko A, Ciechanover A. The Ubiquitin System. *Annu Rev Biochem.* 1998;**67**:425–479.
10. Groll M, Ditzel L, Löwe J, Stock D, Bochtler M, Bartunik HD, Huber R. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 1997;**386**:463–471.
11. Stadtmueller BM, Hill CP. Proteasome activators. *Mol Cell* 2011;**41**:8–19.
12. Meiners S, Keller IE, Semren N, Caniard A. Regulation of the proteasome: Evaluating the lung proteasome as a new therapeutic target. *Antioxid Redox Signal* 2014;**21**:2364–2382.
13. Lander GC, Martin A, Nogales E. The proteasome under the microscope: the regulatory particle in focus. *Curr Opin Struct Biol* 2013;**23**:243–251.
14. Meiners S, Ludwig A, Stangl V, Stangl K. Proteasome inhibitors: Poisons and remedies. *Med Res Rev.* 2008;**28**:309–327.
15. Schmidt M, Finley D. Regulation of proteasome activity in health and disease. *Biochim Biophys Acta* 2014;**1843**:13–25.
16. Gomes AV, Zong C, Edmondson RD, Li X, Stefani E, Zhang J, Jones RC, Thyparambil S, Wang GW, Qiao X, Bardag-Gorce F, Ping P. Mapping the murine cardiac 26S proteasome complexes. *Circ Res* 2006;**99**:362–371.

17. Predmore JM, Wang P, Davis F, Bartolone S, Westfall MV, Dyke DB, Pagani F, Powell SR, Day SM. Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies. *Circulation* 2010;**121**:997–1004.
18. Drews O. The left and right ventricle in the grip of protein degradation: similarities and unique patterns in regulation. *J Mol Cell Cardiol* 2014;**72**:52–55.
19. Wang X, Ibrahim YF, Das D, Zungu-Edmondson M, Shults NV, Suzuki YJ. Carfilzomib reverses pulmonary arterial hypertension. *Cardiovasc Res* 2016;**110**:188–199.
20. Fessart D, Martin-Negrier ML, Claverol S, Thiolat ML, Crevel H, Toussaint C, Bonneu M, Muller B, Savineau JP, Delom F. Proteomic remodeling of proteasome in right heart failure. *J Mol Cell Cardiol* 2014;**66**:41–52.
21. Shi L, Kojonazarov B, Elgheznawy A, Popp R, Dahal BK, Böhm M, Pullamsetti SS, Ghofrani HA, Gödecke A, Jungmann A, Katus HA, Müller OJ, Schermuly RT, Fisslthaler B, Seeger W, Fleming I. miR-223–IGF-IR signaling in hypoxia- and load-induced right ventricular failure: a novel therapeutic approach. *Cardiovasc Res* 2016;**111:**184-193.
22. Apostolakis S, Konstantinides S. The right ventricle in health and disease: insights into physiology, pathophysiology and diagnostic management. *Cardiology* 2012;**121**:263–273.
23. Verdoes M, Florea BI, Menendez-Benito V, Maynard CJ, Witte MD, van der Linden WA, van den Nieuwendijk AM, Hofmann T, Berkers CR, van Leeuwen FW, Groothuis TA, Leeuwenburgh MA, Ovaa H, Neefjes JJ, Filippov DV, van der Marel GA, Dantuma NP, Overkleeft HS. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem Biol* 2006;**13**:1217–1226.
24. Cravatt BF, Wright AT, Kozarich JW. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu Rev Biochem* 2008;**77**:383–414.
25. Dick LR, Fleming PE. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discov Today* 2010;**15**:243–249.
26. Obrist F, Manic G, Kroemer G, Vitale I, Galluzzi L. Trial Watch: Proteasomal inhibitors for anticancer therapy. *Mol Cell Oncol* 2015;**2**:e974463.
27. Huber EM, Heinemeyer W, Groll M. Bortezomib-resistant mutant proteasomes: structural and biochemical evaluation with carfilzomib and ONX 0914. *Structure* 2015;**23**:407–417.
28. Bockorny M, Chakravarty S, Schulman P, Bockorny B, Bona R. Severe heart failure after bortezomib treatment in a patient with multiple myeloma: a case report and review of the literature. *Acta Haematol* 2012;**128**:244–247.
29. Gaczynska M, Osmulski P. Targeting Protein-Protein Interactions in the Proteasome Super-Assemblies. *Curr Top Med Chem* 2015;**15**:2056–2067.

30. Vilchez D, Boyer L, Morantte I, Lutz M, Merkwirth C, Joyce D, Spencer B, Page L, Masliah E, Berggren WT, Gage FH, Dillin A. Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. *Nature* 2012;**489**:304–308.
31. Semren N, Welk V, Korfei M, Keller IE, Fernandez IE, Adler H, Günther A, Eickelberg O, Meiners S. Regulation of 26S Proteasome Activity in Pulmonary Fibrosis. *Am J Respir Crit Care Med* 2015;**192**:1089–1101.
32. Adams J. The development of proteasome inhibitors as anticancer drugs. Review. *Cancer Cell* 2004;**5**:417–421.
33. Voortman J, Giaccone G. Severe reversible cardiac failure after bortezomib treatment combined with chemotherapy in a non-small cell lung cancer patient: a case report. *BMC Cancer* 2006;**6**:129.
34. Hasinoff BB, Patel D, Wu X. Molecular Mechanisms of the Cardiotoxicity of the Proteasomal-Targeted Drugs Bortezomib and Carfilzomib. *Cardiovasc Toxicol* 2017;**17**:237-250.
35. Grandin EW, Ky B, Cornell RF, Carver J, Lenihan DJ. Patterns of cardiac toxicity associated with irreversible proteasome inhibition in the treatment of multiple myeloma. *J Card Fail* 2015;**21**:138–144.
36. Manning ML, Mason-Osann E, Onda M, Pastan I. Bortezomib reduces pre-existing antibodies to recombinant immunotoxins in mice. *J Immunol* 2015;**194**:1695-1701.
37. Wagner-Ballon O, Pisani DF, Gastinne T, Tulliez M, Chaligne R, Lacout C, Aurade F, Villeval JL, Gonin P, Vainchenker W, Giraudier S. Proteasome inhibitor bortezomib impairs both myelofibrosis and osteosclerosis induced by high thrombopoietin levels in mice. *Blood* 2007;**110**:345-353.
38. Zeniya M, Mori T, Yui N, Nomura N, Mandai S, Isobe K, Chiga M, Sohara E, Rai T, Uchida S. The proteasome inhibitor bortezomib attenuates renal fibrosis in mice via the suppression of TGF-β1. *Sci Rep* 2017;**7**:13086.
39. Semren N, Habel-Ungewitter NC, Fernandez IE, Königshoff M, Eickelberg O, Stöger T, Meiners S. Validation of the 2nd generation proteasome inhibitor oprozomib for local therapy therapy of pulmonary fibrosis. *PLoS One* 2015;**10**:e0136188.
40. Chauhan D, Singh AV, Aujay M, Kirk CJ, Bandi M, Ciccarelli B, Raje N, Richardson P, Anderson KC. A novel orally active proteasome inhibitor ONX 0912 triggers in vitro and in vivo cytotoxicity in multiple myeloma. *Blood* 2010;**116**:4906-4915.
41. Hedhli N, Lizano P, Hong C, Fritzky LF, Dhar SK, Liu H, Tian Y, Gao S, Madura K, Vatner SF, Depre C. Proteasome inhibition decreases cardiac remodeling after initiation of pressure overload. *Am J Physiol Heart Circ Physiol* 2008;**295**:H1385-H1393.

42. Stansfield WE, Tang RH, Moss NC, Baldwin AS, Willis MS, Selzman CH. Proteasome inhibition promotes regression of left ventricular hypertrophy. *Am J Physiol Heart Circ Physiol* 2008;**294**:H645-H650.
43. Korolchuk VI, Menzies FM, Rubinsztein DC. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. *FEBS Lett* 2010;**584**:1393-1398.
44. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001;**107**:135-142.
45. Dou QP, Zonder JA. Overview of Proteasome Inhibitor-Based Anti-cancer Therapies: Perspective on Bortezomib and Second Generation Proteasome Inhibitors versus Future Generation Inhibitors of Ubiquitin-Proteasome System. *Curr Cancer Drug Targets* 2014;**14**:517–536.
46. Rajagopalan V, Zhao M, Reddy S, Fajardo G, Wang X, Dewey S, Gomes AV, Bernstein D. Altered ubiquitin-proteasome signaling in right ventricular hypertrophy and failure. *Am J Physiol Heart Circ Physiol* 2013;**305**:H551-H562.
47. Ranek MJ, Zheng H, Huang W, Kumarapeli AR, Li J, Liu J, Wang X. Genetically induced moderate inhibition of 20S proteasomes in cardiomyocytes facilitates heart failure in mice during systolic overload. *J Mol Cell Cardiol* 2015;**85**:273–281.
48. Tsukamoto O, Minamino T, Okada K, Shintani Y, Takashima S, Kato H, Liao Y, Okazaki H, Asai M, Hirata A, Fujita M, Asano Y, Yamazaki S, Asanuma H, Hori M, Kitakaze M. Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice. *Biochem Biophys Res Commun* 2006;**340**:1125–1133.
49. Zhang Y, Nicholatos J, Dreier JR, Ricoult SJ, Widenmaier SB, Hotamisligil GS, Kwiatkowski DJ, Manning BD. Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature* 2014;**513**:440–443.
50. Pathare GR, Nagy I, Bohn S, Unverdorben P, Hubert A, Körner R, Nickell S, Lasker K, Sali A, Tamura T, Nishioka T, Förster F, Baumeister W, Bracher A. The proteasomal subunit Rpn6 is a molecular clamp holding the core and regulatory subcomplexes together. *Proc Natl Acad Sci U S A* 2012;**109**:149–154.
51. Lokireddy S, Kukushkin NV, Goldberg AL. cAMP-induced phosphorylation of 26S proteasomes on Rpn6/PSMD11 enhances their activity and the degradation of misfolded proteins. *Proc Natl Acad Sci U S A* 2015;**112**:85.
52. Vilchez D, Morantte I, Liu Z, Douglas PM, Merkwirth C, Rodrigues AP, Manning G, Dillin A. RPN-6 determines C. elegans longevity under proteotoxic stress conditions. *Nature* 2012;**489**:263–268.

Figure legends

Figure 1: Increased proteasome activity upon RVH

A: Three weeks after pulmonary artery banding (*PAB*,n=9-11), proteasome activity assay revealed an increase in caspase-like (**C-L**) and chymotrypsin-like activity (**CT-L**) in hypertrophied right ventricles (**RV**) compared to the healthy control group (*sham*,n=9-11). **B**: Representative gel showing RV extracts labelled under native conditions with the activity based probe (**ABP**) MV151 and separation of catalytic active subunits of the proteasome by SDS gel electrophoresis with the standard proteolytic subunits **β1, β2** and **β5** and the immunoproteasome subunits **β1i, β2i, β5i**. **C**: Densitometry quantified the overall increase in catalytic activity of the proteasome in PAB compared to *sham* as determined by ABP labelling and gel electrophoresis. *Significance compared to control group (*sham*); Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

Figure 2: Preventive proteasome inhibition partly improved function and structure of RV

A, C: Three weeks after pulmonary artery banding (**PAB**), preventive proteasome inhibition by Bortezomib (*BTZprev*,n=9-11) and ONX (*ONXprev*,n=9-11) significantly reduced the internal diameter of the right ventricle (**RVID**) compared to the placebo-treated group (*PAB*,n=9-11). **B, D**: Both proteasome inhibitors significantly increased right ventricular function as determined by systolic excursion of the tricuspid annular plane (**TAPSE**). **E, G**: Preventive proteasome inhibition with Bortezomib (*BTZprev*) but not ONX (*ONXprev*) resulted in a decrease in cardiomyocyte cross-sectional area (**CM CSA**) compared to placebo-treated PAB controls. **F, H**: Analysis of the **collagen content** revealed a slight decrease in *PAB* mice treated with proteasome inhibitors. Staining with WGA-FITC for cardiomyocyte size measurement and Sirius Red for collagen detection see supplement 4. §Significance compared to placebo-treated *PAB* group (n=9-11), *significance compared to healthy control group (*sham*,n=9-11); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

Figure 3: Therapeutic proteasome inhibition reduced experimental RVH

A, C: Three weeks after pulmonary artery banding (**PAB**), therapeutic proteasome inhibition by Bortezomib (*BTZther*,n=8-10) and ONX (*ONXther*,n=8-10) significantly reduced the internal diameter of the right ventricle (**RVID**) compared to the placebo-treated control group (*PAB*,n=8-10). **B, D**: both proteasome inhibitors significantly increased right ventricular function as determined by systolic excursion of the tricuspid annular plane (**TAPSE**). **E, G**: Therapeutic proteasome inhibition with ONX (*ONXther*,n=8-10) but not BTZ (*BTZther*,n=8-10) led to a significant decrease in car-

diomyocyte cross-sectional area (**CM CSA**) compared to placebo-treated PAB controls (*PAB*, n=8-10). **F, H**: Collagen content significantly decreased in RV that had been treated therapeutically with ONX (*ONXther*,n=8-10, H), but not in BTZ treated RV (*BTZther*,n=8-10,F). Staining with WGA-FITC for cardiomyocyte size measurement and Sirius Red for collagen detection see supplement 8. §Significance compared to placebo-treated control group (*PAB*,n=8-10), *significance compared to healthy control group (*sham*,n=8-10); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

Figure 4: Augmented 26S proteasome activity in RVH

A: Three weeks after pulmonary artery banding (**PAB**), Western blotting of murine RV tissue (*PAB*,n=6) revealed an increase in the proteasomal subunits α3, β1, Rpt5 and Rpn6 as well as in K48-linked polyubiquitinated proteins (K48-Ubi) compared to the control group (*sham*,n=6). For quantification see densitometric analysis of subunit expression, normalised to the respective β-actin loading control. **B**: Labelling of RV extracts with the activity based probe (**ABP**) MV151 and subsequent native gel analysis revealed an increase in the singly and doubly capped 26 and 30S proteasome complexes compared to *sham*. For densitometric analysis, signals for 26S, 30S and 20S complexes were determined and compared to that of *sham* controls. **20S** catalytic core proteasome; **30S proteasomes** contain two regulatory 19S caps at both sides of the 20S catalytic core while **26S proteasomes** contain only one 19S cap on one 20S proteasome. *Significance of *PAB* compared to healthy control group (*sham*); Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

Figure 5: Expression of Rpn6 in normal and diseased hearts of (A) mice and (B) humans

A: Pulmonary artery banding (**PAB**) was used to induce Right Ventricular Hypertrophy (**RVH**) in mice. Mice with RVH revealed cytoplasmic overexpression of Rpn6 in cardiomyocytes (red stain, upper row) which were identified in a parallel section with the cell-specific marker protein tropomyosin (brown stain, lower row). **B**: Tropomyosin-expressing cardiomyocytes (dark red stain, lower row) from patients with RVH showed diffuse cytoplasmic overexpression of Rpn6 (pale red stain, upper row) as compared to normal hearts.

Figure 1

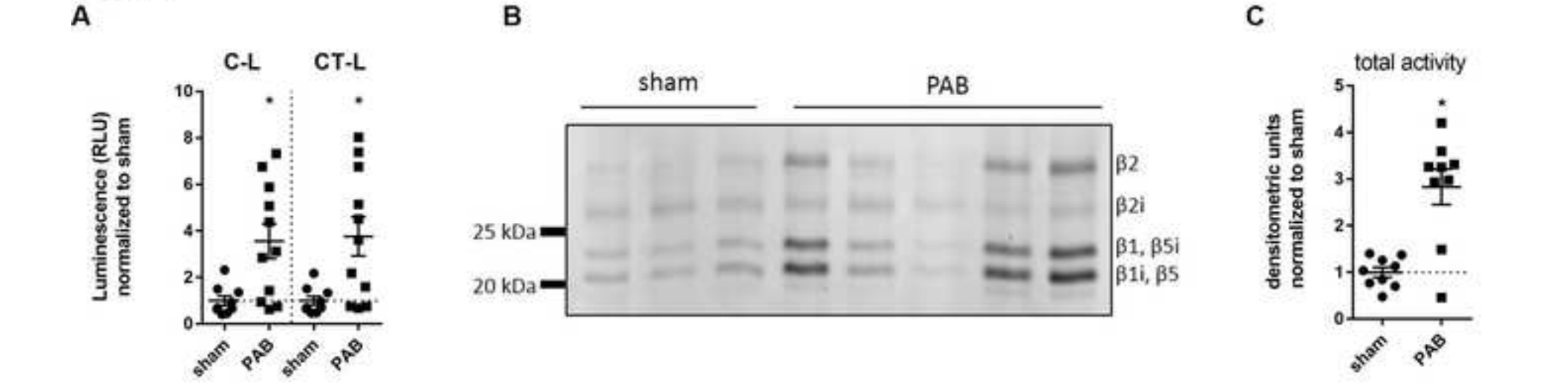


Figure 2

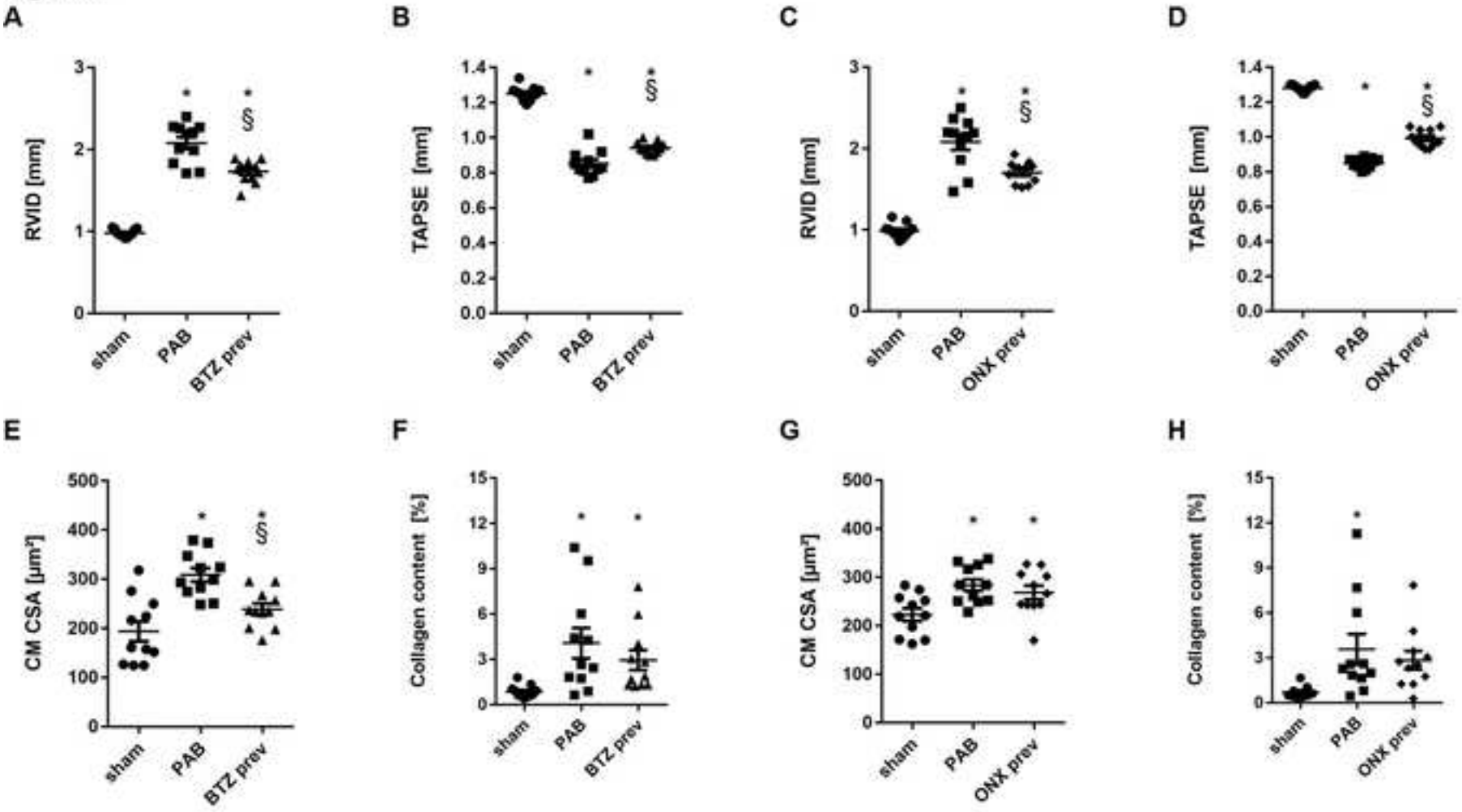


Figure 3

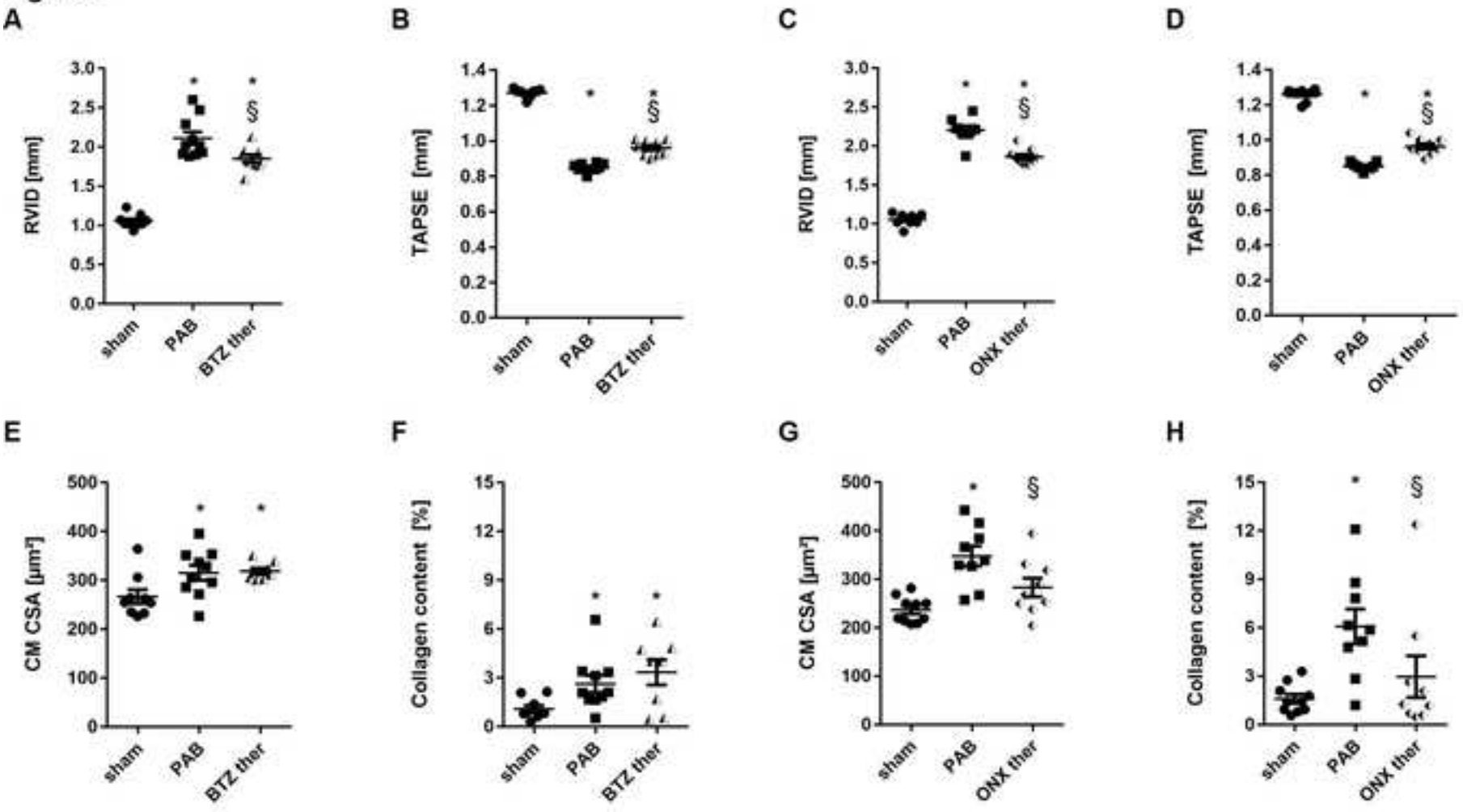
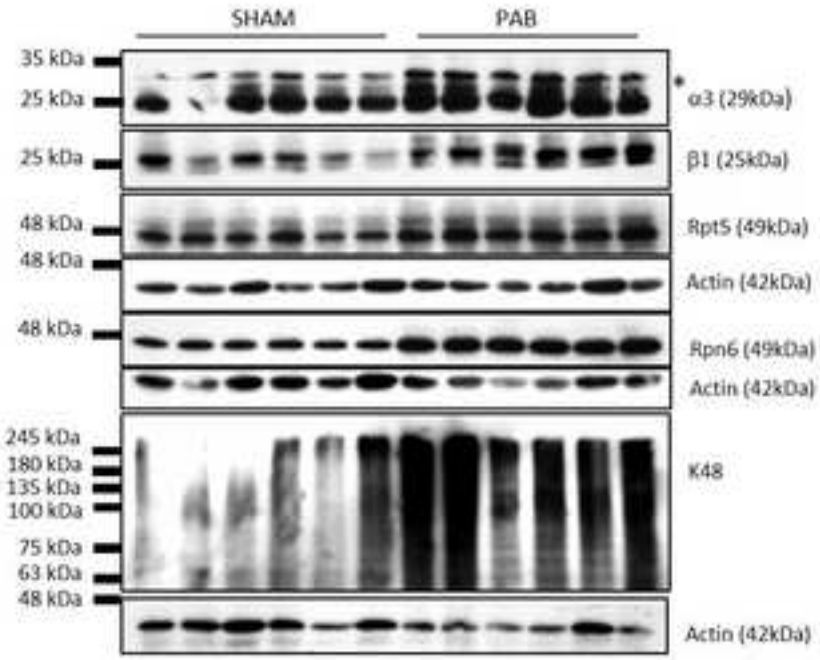
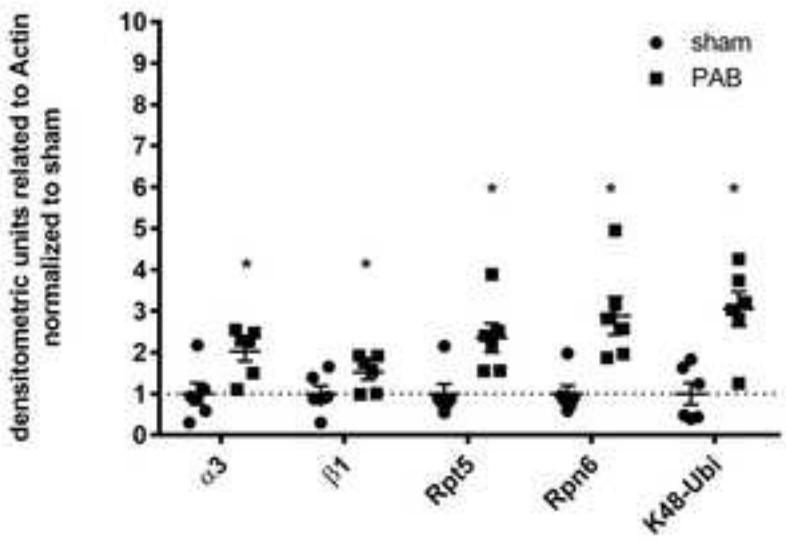


Figure 4

A





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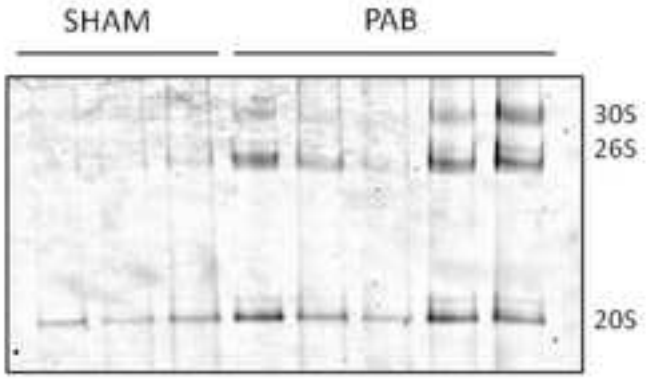


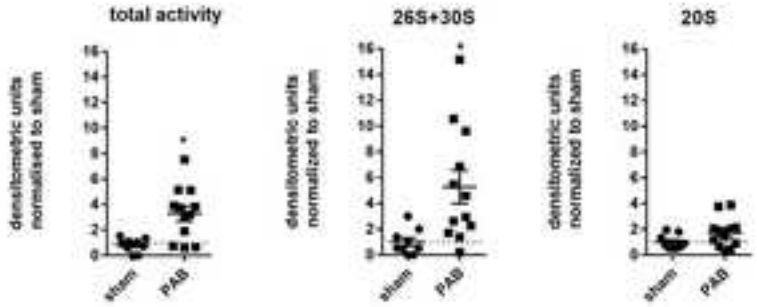
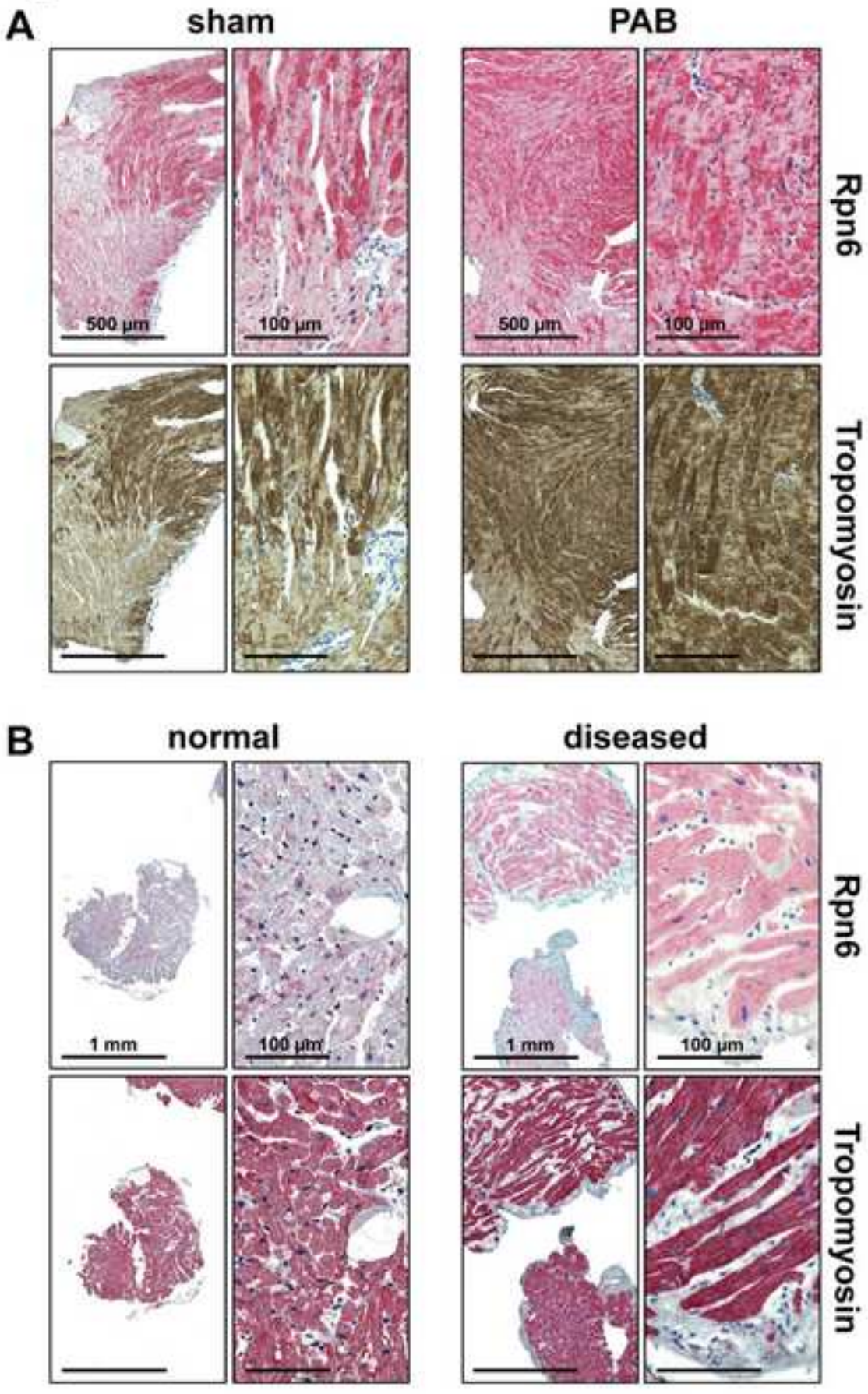


Figure 5



Altered proteasome function in right ventricular hypertrophy

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ONLINE SUPPLEMENT MATERIAL

Material and methods

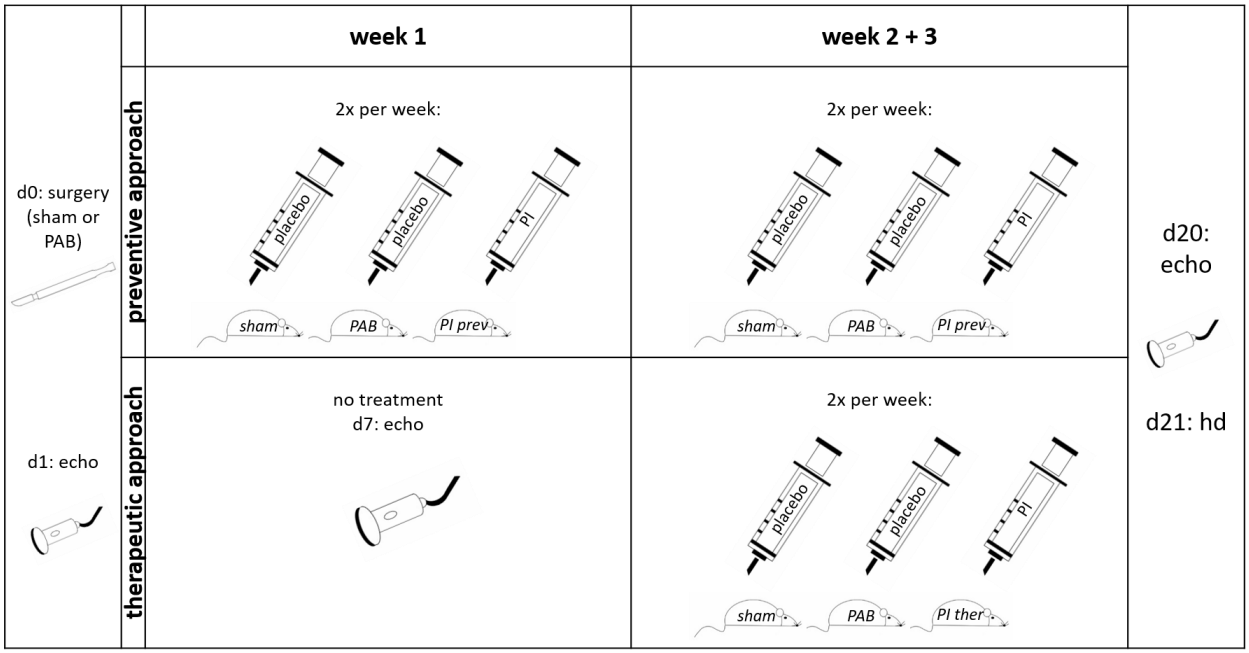
In vivo studies

Pathogen-free male C57/Bl6N mice, 20-25g, 12 weeks, by Charles River, were housed in the local animal facility five days before the start of experiments. Pulmonary artery banding (PAB) was performed to provoke right ventricular hypertrophy (RVH) by reducing the cross-sectional area (CSA) of the artery to 0.3 mm (about 66% of original CSA). During thoracotomy a titanium clip was placed around the pulmonary trunk as described previously[1–5]. The mice received either six (preventive approach) or four (therapeutic approach) drug treatments during the next three weeks (Supplementary figure 1). Placebo-treated animals served as sick control group (*PAB*). Sham surgery was performed the same way as described for PAB surgery without placing a titanium clip. These mice served as healthy control group (sham). All mice were checked daily according to a score sheet with special focus on the development of clinical signs for right heart failure (inactivity, ruffled fur, dyspnoea, ascites)[6].

One day before and three weeks after surgery echocardiography was performed as described previously[2,3,7–10]. During the therapeutic approach, drug administration only started when hypertrophy had already developed as determined by an additional echocardiography one week after PAB surgery. The images were acquired with a VEVO2100 high resolution imaging system (Visual Sonics, Toronto, Canada) using a 40 MHz MicroScan linear array transducer MS550D. Calculations were performed offline with the according software Vevo LAB. The following parameters were derived: internal diameter of the right ventricle (RVID, [mm]), wall thickness of its free wall (RVWT, [mm]), tricuspid annular plane systolic excursion (TAPSE, [mm]) and

cardiac index (CI, $[l/(min*g)]$). Hemodynamic measurements via Millar-catheter were performed three weeks after PAB surgery in a previously reported manner[4,11,12]. Subsequently the mice were sacrificed; tibia, lung, heart, liver, blood and pulmonary artery were harvested and RV mass to tibia length (RV/TL, $[g/mm*10^{-4}]$) was determined.

A



Bortezomib ip 0.5mg/kg ONX po 50mg/kg

Supplementary figure 1: Scheme for the preventive and therapeutic approaches

A: Each preventive and therapeutic approach took three weeks during which pulmonary artery banding (**PAB**) or sham surgery, two (preventive approach) or three (therapeutic approach) echocardiographies (**echo**) and hemodynamic measurements (**hd**) were performed. During the preventive approach (first row), the mice received six treatments with the proteasome inhibitors (**PI**) Bortezomib (BTZ) or ONX-0912. In the therapeutic approach, the treatment started after right ventricular hypertrophy (RVH) had already developed (second row).

d day, **echo** echocardiography, **hd** hemodynamic measurements, **PI** proteasome inhibitor, **BTZ** proteasome inhibitor Bortezomib, **ONX** proteasome inhibitor ONX, **ip** intraperitoneal, **po** oral application, **prev** preventive treatment **ther** therapeutic treatment.

Proteasome inhibitors and solvent controls

Bortezomib (BTZ, Velcade by Millennium, Cambridge, USA), a class-III proteasome inhibitor[13], was diluted in isotonic saline solution in a concentration of 0.5 mg/kg bodyweight (BW). Mannitol was mixed in a 0.9 fold amount of original BTZ sample weight in saline and served as placebo. Both compounds were applied intraperitoneally (IP). ONX-0912 (ONX, Onyx Pharmaceuticals, South San Francisco, USA) is an epoxyketone and a successor to carfilzomib[14]. ONX was suspended in 1% carboxyl methylcellulose (CMC) at a concentration of 50 mg/kg BW. Furthermore, CMC served as a vehicle for placebo treatment and was administered at 1% orally. The different administration routes (IP or orally) and solvents (mannitol solution or CMC) made it necessary to obtain a healthy (sham) and sick (PAB) control group for each BTZ and ONX treatment.

Histology and morphometry

After fixating the tissue[7], different staining were performed on paraffin-embedded RV slices of 3 µm. Staining with Picrosirius Red determined collagen (red, [%]), a marker for fibrosis, as previously described[4,7,9,11,12]. In this staining, collagen is stained red whereas the other tissue appears yellow. The area of stained interstitial collagen was measured with the help of Leica QWin software, a well-established morphometric method in our research group[4,7,9,11,12]. At least twenty fields of view per slide were analysed meander-like at 400× magnification. Analysis was performed blinded. Collagen content per RV was calculated as the mean of all measured images.

To assess the cross-sectional area of cardiomyocytes (CM CSA, [µm]), a parameter for hypertrophy, RV sections were stained with wheat germ agglutinin (WGA),

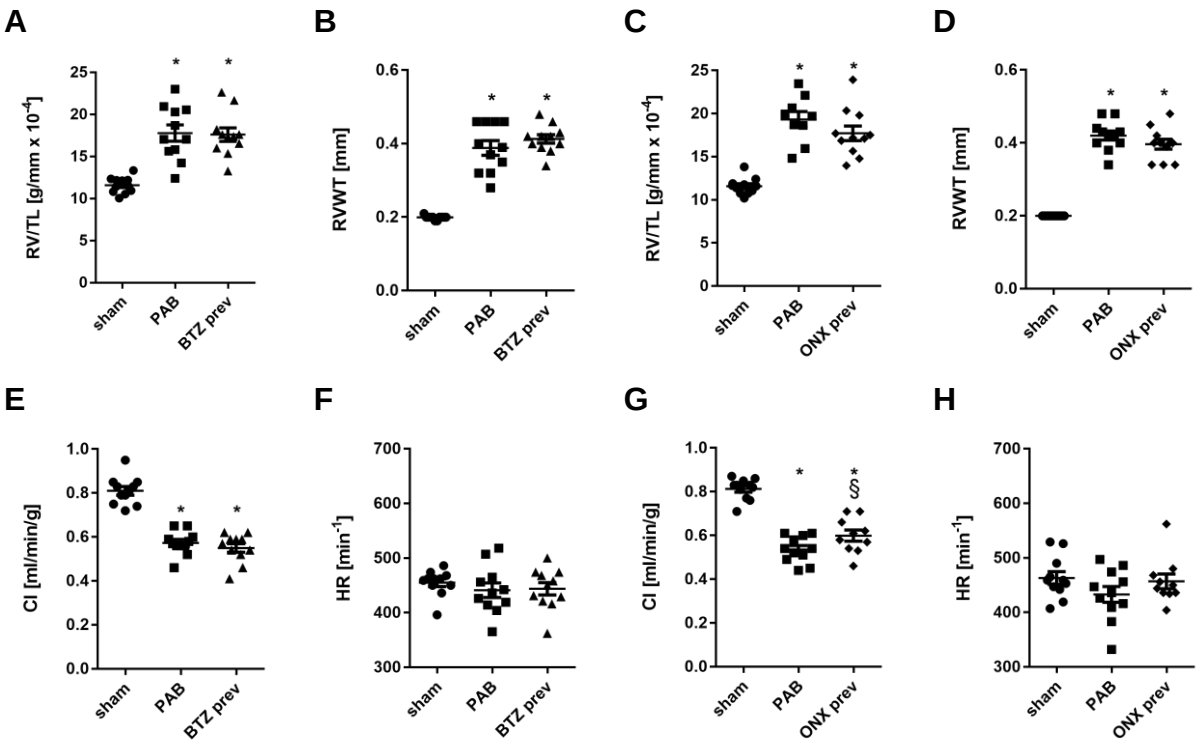
fluorescein isothiocyanate (FITC) and 4′,6-diamidino-2-phenylindole (DAPI). This staining is well established at our institute as reported before[2,3,7]. CSA of at least 100 cardiomyocytes was measured with the help of Leica QWin software before calculating the mean CSA per RV. IHC-staining for Rpn6 and tropomyosin were performed according to a previously described protocol[15]. Each analysis was performed in a blinded manner.

Protein and mRNA analysis

Protein biochemistry and mRNA analysis was performed as described elsewhere[15]. Tissue was lysed in TSDG buffer containing 0.2% detergent (NP-40). Tissue was homogenized and protein concentration was determined using the Pierce bicinchoninic acid assay. To determine the activity of the catalytic subunits of the proteasome, the Proteasome-Glo Assay kit (Promega, Fitchburg, USA) and the activity based probe MV151 were used as described before[16]. In-gel proteasome activity and protein bands were quantified using the volume tool of Image Lab software and normalised to sham mice.

For quantitative analyses of the mRNA level via real time quantitative polymerase chain reaction (qRT-PCR), the SYBR Green LC480 System was used. The expression of the target genes were normalised to the 60S ribosomal protein L19 (RPL19) as a housekeeping gene.

Results



Supplementary figure 2: Right ventricular structure and function upon preventive proteasome inhibition

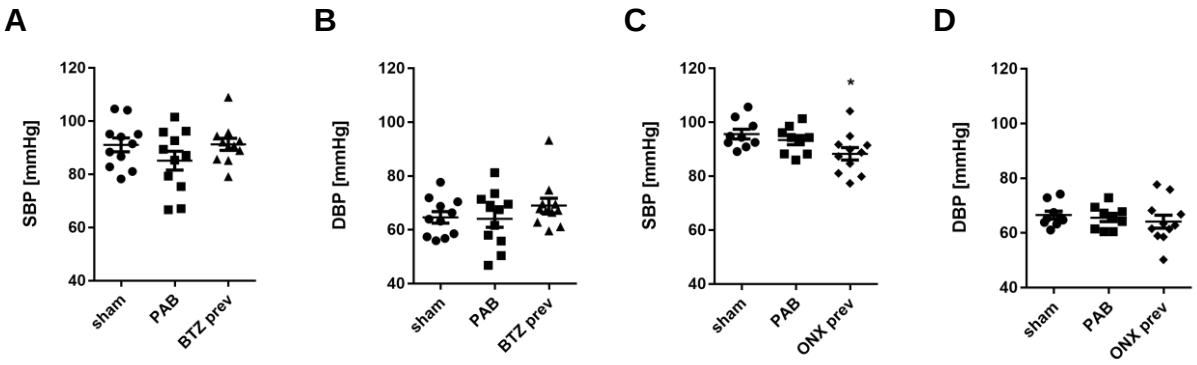
A, C: Three weeks after pulmonary artery banding (PAB), the mass of the right ventricle (**RV/TL**) increased in PAB groups (**PAB**, n=11), indicating the development of right ventricular hypertrophy. Preventive proteasome inhibition by Bortezomib (**BTZprev**, n=11) or ONX (**ONXprev**, n=11) did not influence the mass. **TL** tibia length

B, D: RV wall thickness (**RVWT**) was not influenced by proteasome inhibition.

E-F: Preventive proteasome inhibition by Bortezomib (BTZprev) altered neither cardiac index (**CI**) nor heart rate (**HR**) when compared to the placebo-treated control group PAB.

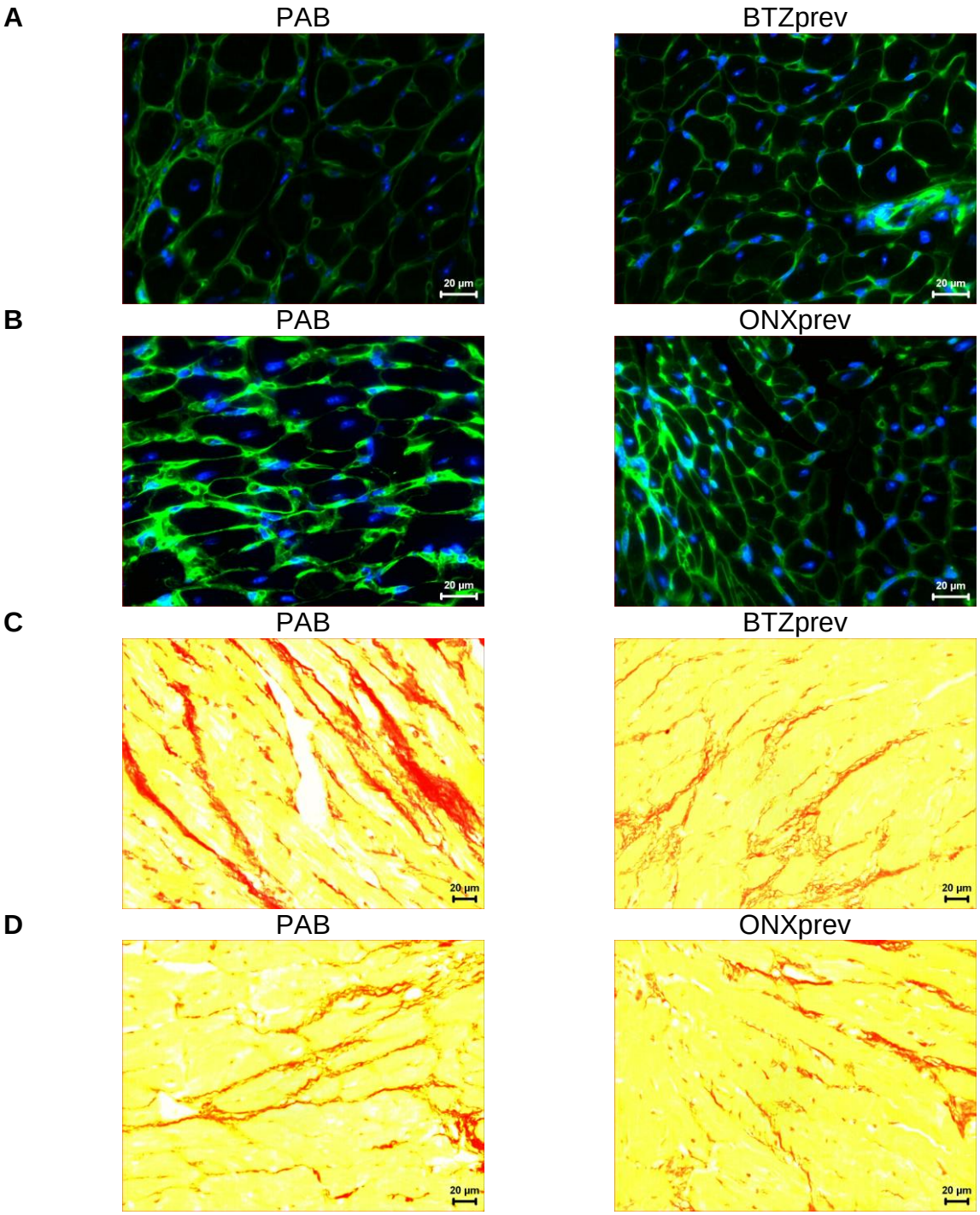
G-H: Preventive proteasome inhibition by ONX (ONXprev) improved CI significantly while HR remained constant in comparison to PAB. §Significance compared to

placebo-treated PAB group, *significance compared to healthy control group (**sham**, n=11); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 3: Hemodynamic parameters upon preventive proteasome inhibition

Three weeks after pulmonary artery banding (PAB), hemodynamic measurements were derived by right heart catheterization in mice. Preventive treatment with proteasome inhibitors (**A, B:** BTZprev, **C, D:** ONXprev n=9-11) did not change the systemic blood pressure (**SBP, DBP**) in comparison to placebo-treated PAB controls (**PAB**, n=9-11). *Significance compared to healthy control group (**sham**, n=11); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

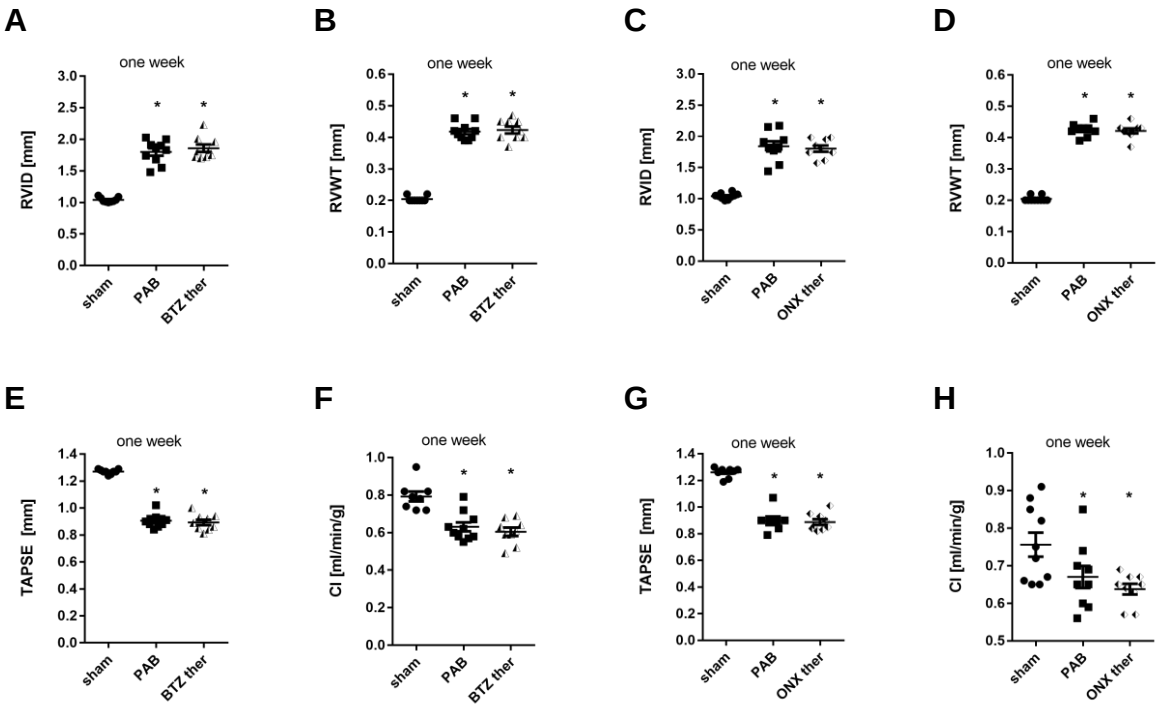


Supplementary figure 4: Effects of preventive treatment on cardiomyocyte size and collagen content

A, B: Three weeks after pulmonary artery banding (PAB), murine right ventricles (RV) were stained with WGA (wheat germ agglutinin), FITC (fluorescein isothiocyanate) and DAPI (4′,6-diamidino-2-phenylindole) to assess the size of cardiomyocytes (green).

Preventive proteasome inhibition with Bortezomib (**BTZprev**, n=9-11), but not with ONX (**ONXprev**, n=9-11), led to a decrease in cardiomyocyte cross-sectional area (**CM CSA**) in comparison to placebo-treated PAB controls (**PAB**, n=9-11).

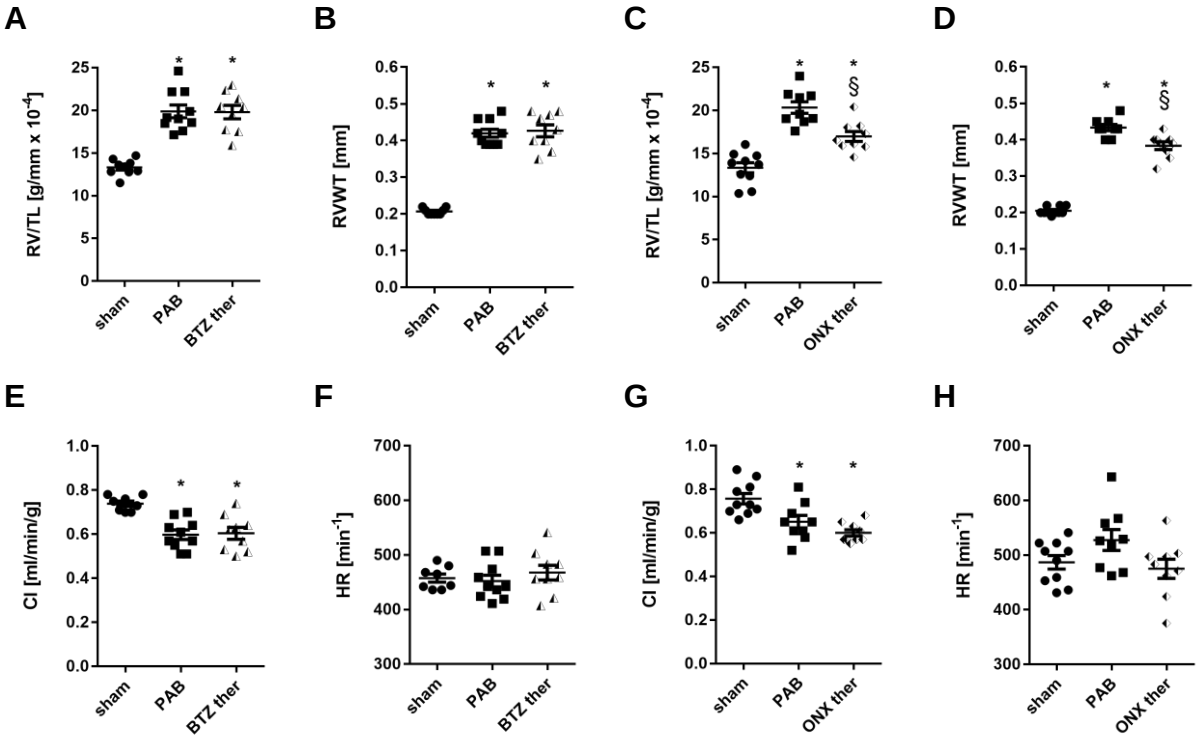
C, D: Staining with Sirius red for collagen detection (red) revealed a slight decrease of the collagen content in RV of mice that had been treated with proteasome inhibitors. Scale bars = 20 µm.



Supplementary figure 5: Development of RVH one week after PAB

A-D: During the therapeutic approach, echocardiography was performed one week after pulmonary artery banding (PAB), but before treatment started. Operated mice (**PAB**, **BTZther**, **ONXther**, n=7-10) had developed right ventricular hypertrophy (**RVID**, **RVWT**) compared to the healthy control group (**sham**, n=7-10).

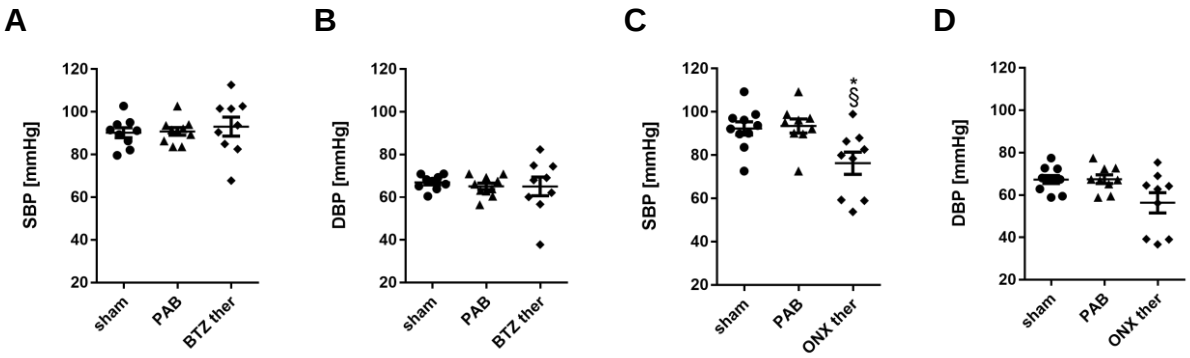
E-H: Furthermore, there was a decrease in (right) heart function (**TAPSE**, **CI**) in mice with PAB (**PAB**, **BTZther**, **ONXther**, n=7-10). The heart rate remained constant (data not shown). Afterwards, treatment with the proteasome inhibitors Bortezomib (BTZ) or ONX started. **RVID** right ventricular internal diameter, **RVWT** right ventricular wall thickness, **TAPSE** tricuspid annular plane systolic excursion, **CI** cardiac index. *Significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 6: Right ventricle structure and function upon therapeutic approach

A-D: Three weeks after pulmonary artery banding (**PAB**), the mass of the right ventricle in relation to tibia length (**RV/TL**) and its free wall (**RVWT**) increased in PAB groups (**PAB**, n=9-11), indicating the development of right ventricular hypertrophy. Therapeutic proteasome inhibition by ONX (**ONXther**, n=9-11) significantly reduced RV/TL and RVWT.

E-H: Therapeutic proteasome inhibition by Bortezomib (**BTZther**) and ONX (**ONXther**) altered neither cardiac index (**CI**) nor heart rate (**HR**) when compared to the placebo-treated control group PAB. **ther** therapeutic administration of drugs. §Significance compared to placebo-treated control group (**PAB,** n=8-10), *significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

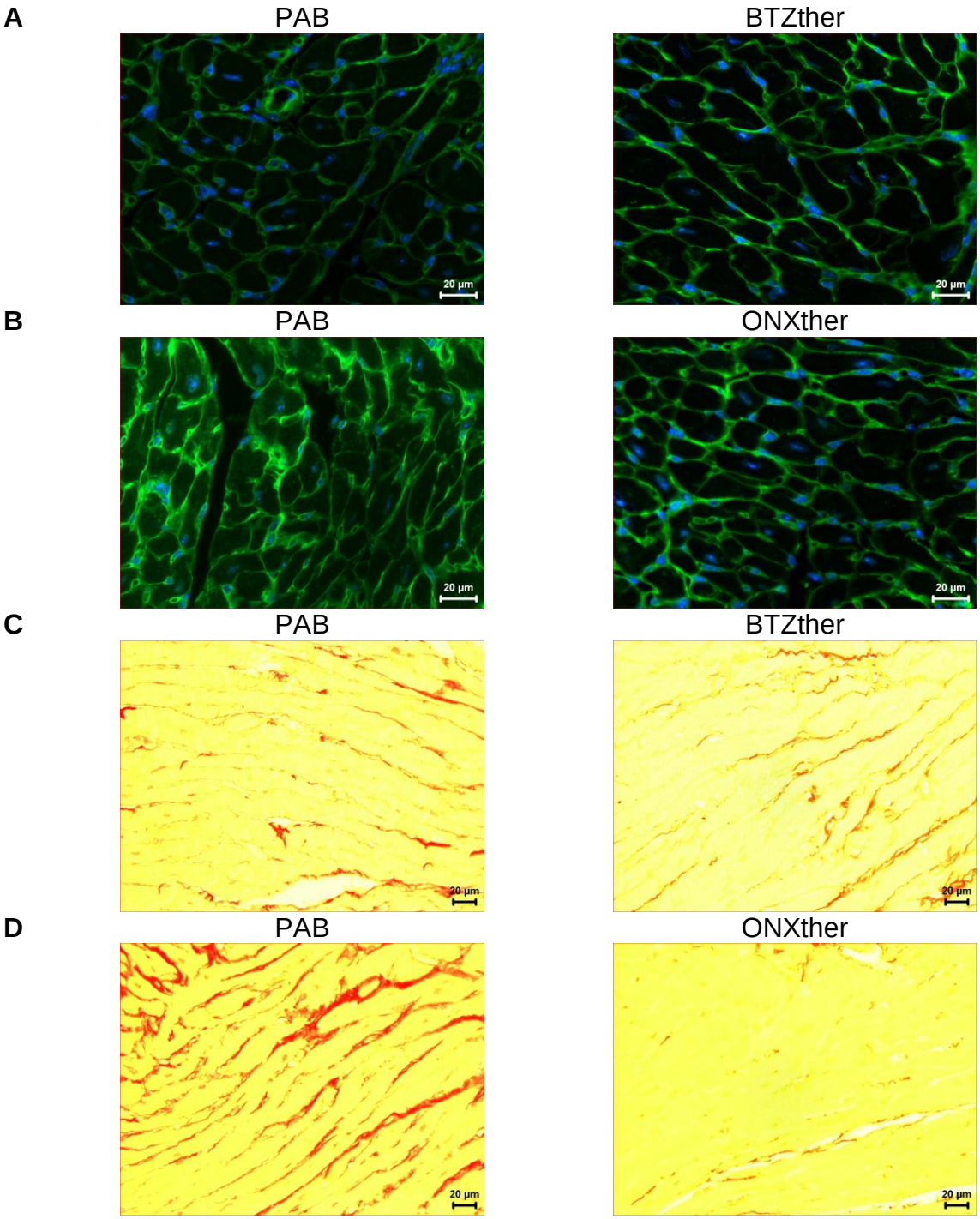


Supplementary figure 7: Hemodynamic parameters upon therapeutic proteasome inhibition

Three weeks after pulmonary artery banding (PAB), hemodynamic measurements were derived by right heart catheterization.

A, B: Therapeutic treatment of mice with the proteasome inhibitor Bortezomib (**BTZther**, n=8-10) changed neither systolic nor diastolic blood pressure (**SBP, DBP**) compared to placebo-treated PAB controls (**PAB**, n=8-10).

C, D: Mice that had received the proteasome inhibitor ONX (**ONXther**, n=8-10) showed a decrease in systolic blood pressure (SBP). §Significance compared to placebo-treated control group (**PAB,** n=8-10), *significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

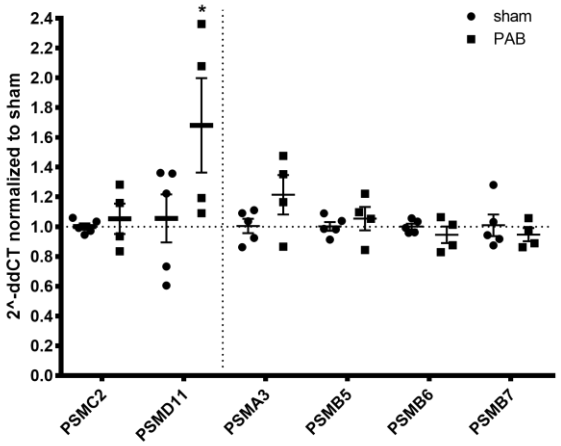


Supplementary figure 8: Effects on cardiomyocyte size and collagen content by therapeutic proteasome inhibition

A, B: Three weeks after pulmonary artery banding (PAB), murine right ventricles (RV) were stained with WGA (wheat germ agglutinin), FITC (fluorescein isothiocyanate) and DAPI (4′,6-diamidino-2-phenylindole) to assess the size of cardiomyocytes (green). Therapeutic proteasome inhibition with ONX (**ONXther**, n=8-10) led to a significant decrease in cardiomyocyte cross-sectional area (**CM CSA**) compared to

placebo-treated PAB controls (**PAB**, n=8-10). This did not apply for the treatment with proteasome inhibitor BTZ (**BTZther**, n=8-10).

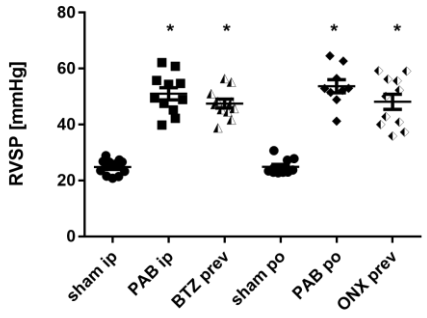
C, D: Staining with Sirius red for collagen detection (red) revealed a significant decrease of collagen content in RV that had been treated therapeutically with ONX (**ONXther**). Scale bars = 20 µm.



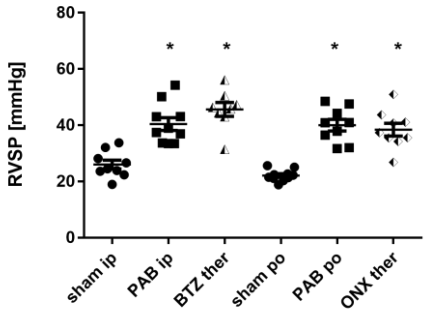
Supplementary figure 9: mRNA expression of the Rpn6 encoding gene PSMD11 increased in RVH

qRT-PCR analysis showed no alterations in mRNA expression of proteasomal subunits in RVH except for PSMD11; PSMD11 encodes for Rpn6, a subunit of the 19S proteasome which is involved in the assembly of the 26S proteasome. **PSMC2** gene name for Rpt1; **PSMA3** gene that encodes subunit α3; **PSMB5**, **PSMB6**, **PSMB7** encode subunit β5, β6 and β7, respectively. *****Significance compared to control group (sham); Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/- SEM.

A

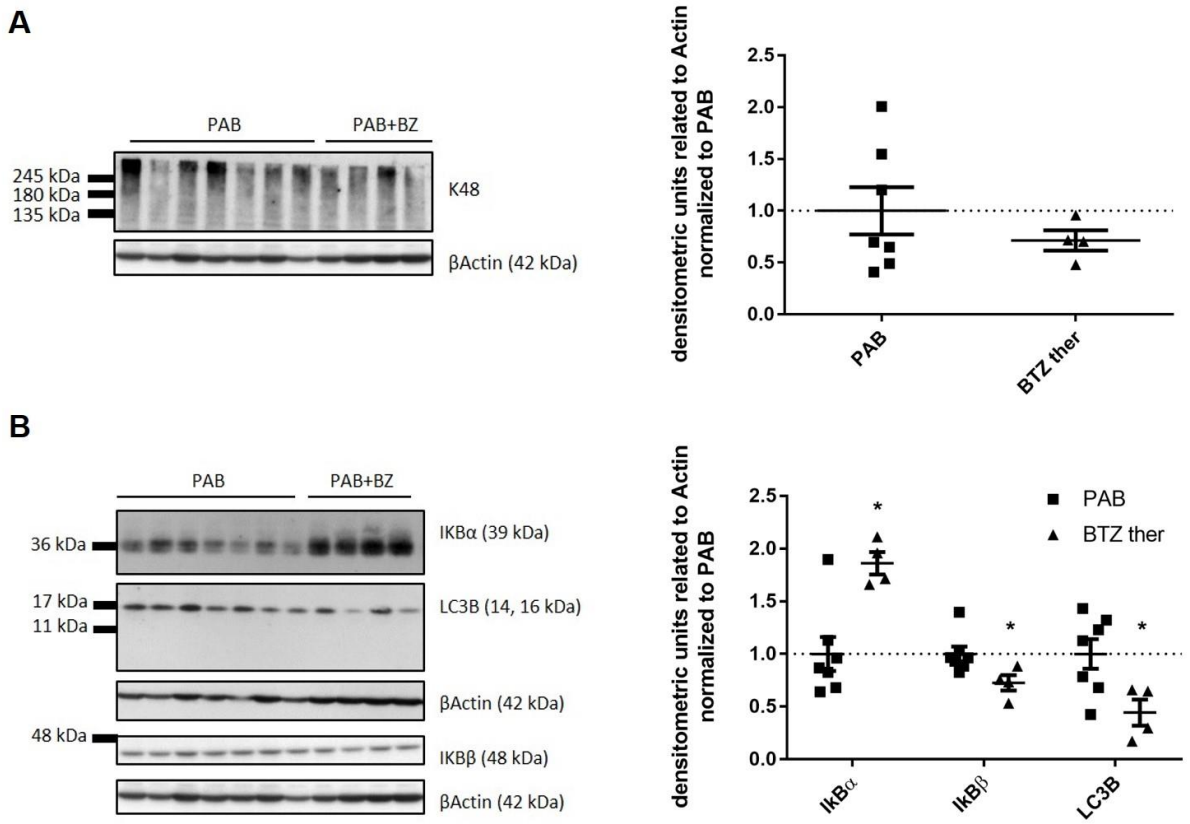


B



Supplementary figure 10: Right ventricular systolic blood pressure (RVSP)

Hemodynamic measurements revealed a significant difference of the PAB groups (PAB ip, PAB po) and the treated groups (BTZprev / ther, ONXprev / ther) in comparison to the healthy control (sham). **Ip** intraperitoneal injection, **po** per os; n=8-11, One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 11: Proteostasis and inflammatory signaling upon bortezomib (BTZ) treatment in PAB mice

A: Western blotting of murine right ventricle tissues of BTZ-treated PAB mice (**BTZther**, n=4) for K48-linked polyubiquitinated proteins (K48-Ubi) compared to the control group (**PAB**, n=7). **B:** Western botting of the same tissue for IκBα, IκBβ and the autophagy marker LC3B. For quantification see densitometric analysis of subunit expression, normalised to the respective β-Actin loading control. Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

Supplementary table 1: Patients' data for immunohistochemical analysis of right ventricle biopsies

Year of Birth	Sex	Diagnosis
controls		
1934	m	RV endomyocardial biopsies were taken to exclude myocarditis, normal LV function. Histological findings: mild lipomatosis cordis in the right ventricular myocardium. No myocarditis or cardiomyopathy. No cardiac virus infection.
1954	f	RV endomyocardial biopsies were taken to exclude myocarditis. No pathological findings, no myocarditis, no cardiomyopathy.
na	na	RV endomyocardial biopsies were taken to exclude myocarditis. No pathological findings, no myocarditis, no cardiomyopathy.
PAH		
1951	m	RV endomyocardial biopsies. Case history: LVEF 38%; pulmonary hypertension, AV block I°, obesity. Histological and immunohistochemical investigations revealed reactive hypertrophy of myocytes due to pulmonary hypertension, interstitial fibrosis and a mild inflammation. No arrhythmogenic RV cardiomyopathy. No cardiac virus infection.
1940	m	RV endomyocardial biopsies. Case history: LVEF 29%, moderate pulmonary hypertension, DCM of uncertain aetiology, no coronary heart disease.

		Histological and immunohistochemical investigations revealed hypertrophic myocytes, a few apoptotic myocytes and interstitial fibrosis. No myocarditis. No cardiac virus infection.
2010	f	RV endomyocardial biopsies. Case history: LVEF normal, pulmonary hypertension, status post occlusion of ASD II, suspected non-compaction cardiomyopathy of RV, secondary hypertrophy. Histological and immunohistochemical investigations revealed a moderate reactive hypertrophy due to pulmonary hypertension. No non-compaction cardiomyopathy, no myocarditis. No cardiac virus infection.
1943	m	RV endomyocardial biopsies to exclude potential storage disorders or a restrictive cardiomyopathy. Case history: LVEF 60%, pulmonary hypertension, NYHA II-III, atrial fibrillation. Histological and immunohistochemical investigations revealed findings consistent with a hypertensive heart disease, with hypertrophic myocytes and small arterioles with hypertrophied walls. No cardiac virus infection.
1959	m	RV endomyocardial biopsies to exclude myocarditis. Case history: LVEF 20%, pulmonary hypertension, NYHA II-III. Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with DCM.

		No cardiac virus infection. No acute or chronic myocarditis, no amyloidosis.
1963	m	RV endomyocardial biopsies to exclude myocarditis. Case history: LVEF 15%, pulmonary hypertension, manifested DCM Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with DCM. No cardiac virus infection. No acute or chronic myocarditis, no amyloidosis.
1964	f	RV endomyocardial biopsies to exclude amyloidosis. Case history: LVEF < 20%, restrictive cardiomyopathy with left ventricular NYHA III-IV. Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with restrictive cardiomyopathy with reactive hypertrophy and diffuse interstitial fibrosis. Mild inflammation consistent with suspected chronic lymphocytic myocarditis.

Supplementary table 2: Deterioration of RV structure and function after pulmonary artery banding (PAB)

Groups	RV/TL	RVID	RVWT	TAPSE	CI	CM CSA	CC
	*g/mm*10^{-4}*	*mm*	*mm*	*mm*	*ml/min/g*	*µm²*	*%*
sham	11.59 ± 0.29	0.98 ± 0.01	0.19 ± 0.00	1.25 ± 0.01	0.81 ± 0.02	193.9 ± 20.2	0.86 ± 0.13
PAB	17.79* ± 0.97	2.08* ± 0.07	0.38* ± 0.02	0.85* ± 0.02	0.57* ± 0.02	308.9* ± 13.6	4.08* ± 1.00

RV/TL right ventricular mass to tibia length **RVID** right ventricular internal diameter **RVWT** right ventricular wall thickness **TAPSE** tricuspid annular plane systolic excursion **CI** cardiac index **CM CSA** cardiomyocyte cross-sectional area **CC** collagen content. *Significance compared to control group (sham,n=11); Unpaired t-test (p≤0.05). Results are presented as mean+/-SEM.

Literature Cited

1. Kreymborg Kg, Uchida S, Gellert P, Schneider A, Boettger T, Voswinckel R *et al.* Identification of right heart-enriched genes in a murine model of chronic outflow tract obstruction. *J Mol Cell Cardiol* 2010;**49**:598–605.
2. Kojonazarov B, Luitel H, Sydykov A, Dahal BK, Paul-Clark MJ, Bonvini S *et al.* The peroxisome proliferator-activated receptor beta/delta agonist GW0742 has direct protective effects on right heart hypertrophy. *Pulmonary Circulation* 2013;**3**:926–935.
3. Kojonazarov B, Sydykov A, Pullamsetti SS, Luitel H, Dahal BK, Kosanovic D *et al.* Effects of multikinase inhibitors on pressure overload-induced right ventricular remodeling. *Int J Cardiol* 2013;**167**:2630–2637.
4. Janssen W, Schymura Y, Novoyatleva T, Kojonazarov B, Boehm M, Wietelmann A *et al.* 5-HT2B receptor antagonists inhibit fibrosis and protect from RV heart failure. *Biomed Res Int* 2015;**2015**:438403.
5. Shi L, Kojonazarov B, Elgheznawy A, Popp R, Dahal BK, Böhm M *et al.* miR-223–IGF-IR signaling in hypoxia- and load-induced right ventricular failure: a novel therapeutic approach. *Cardiovascular Research* 2016;**111**.
6. Borgdorff MAJ, Koop AMC, Bloks VW, Dickinson MG, Steendijk P, Sillje HHW *et al.* Clinical symptoms of right ventricular failure in experimental chronic pressure load are associated with progressive diastolic dysfunction. *J Mol Cell Cardiol* 2015;**79**:244–253.
7. Kosanovic D, Kojonazarov B, Luitel H, Dahal BK, Sydykov A, Cornitescu T *et al.* Therapeutic efficacy of TBC3711 in monocrotaline-induced pulmonary hypertension. *Respir Res* 2011;**12**:87.
8. Sklepkiewicz P, Shiomi T, Kaur R, Sun J, Kwon S, Mercer B *et al.* Loss of secreted frizzled-related protein-1 leads to deterioration of cardiac function in mice and plays a role in human cardiomyopathy. *Circ Heart Fail* 2015;**8**:362–372.
9. Pullamsetti SS, Doebele C, Fischer A, Savai R, Kojonazarov B, Dahal BK *et al.* Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension. *Am J Respir Crit Care Med* 2012;**185**:409–419.
10. Szardien S, Nef HM, Voss S, Troidl C, Liebetrau C, Hoffmann J *et al.* Regression of cardiac hypertrophy by granulocyte colony-stimulating factor-stimulated interleukin-1beta synthesis. *European Heart Journal* 2012;**33**:595–605.

11. Werner F, Kojonazarov B, Gassner B, Abesser M, Schuh K, Volker K *et al.* Endothelial actions of atrial natriuretic peptide prevent pulmonary hypertension in mice. *Basic Res Cardiol* 2016;**111**:22.
12. Pradhan K, Sydykov A, Tian X, Mamazhakypov A, Neupane B, Luitel H *et al.* Soluble guanylate cyclase stimulator riociguat and phosphodiesterase 5 inhibitor sildenafil ameliorate pulmonary hypertension due to left heart disease in mice. *Int J Cardiol* 2016;**216**:85–91.
13. Dick LR, Fleming PE. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discov Today* 2010;**15**:243–249.
14. Zhou H-J, Aujay MA, Bennett MK, Dajee M, Demo SD, Fang Y *et al.* Design and synthesis of an orally bioavailable and selective peptide epoxyketone proteasome inhibitor (PR-047). *J Med Chem* 2009;**52**:3028–3038.
15. Semren N, Welk V, Korfei M, Keller IE, Fernandez IE, Adler H *et al.* Regulation of 26S Proteasome Activity in Pulmonary Fibrosis. *Am J Respir Crit Care Med* 2015;**192**:1089–1101.
16. Keller IE, Vosyka O, Takenaka S, Kloss A, Dahlmann B, Willems LI *et al.* Regulation of immunoproteasome function in the lung. *Sci Rep* 2015;**5**:10230.