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Peer Review File

Vitamin D binding protein induces skeletal muscle atrophy and contributes to cancer-associated muscle wasting independently of vitamin D status

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Raiteri and colleagues hypothesize that vitamin D-binding protein, otherwise known as GC, exerts atrophic effects on muscle and hence contributes to muscle wasting during cachexia. Using cultured immortalized myotubes, GC KO mice, and cachexia models, the authors conclude that GC influences intracellular G-/F-actin ratios to induce mitophagy, mitochondrial dysfunction, muscle atrophy and reduced grip strength. While the idea that GC contributes to muscle wasting is potentially interesting, there are a number of flaws with the study and study design that dampen enthusiasm. In particular, the authors do not adequately show that GC directly influences muscle mass/function/respiration, as opposed to acting through changes in vitamin D status and whole-body metabolism.

- The introduction suggests that GC is upregulated in patients and animal models of pathologies susceptible to muscle-wasting. However, a lot of these findings likely reflect an increase in GC following liver injury, or in response to inflammation/trauma, rather than an effect of GC per se on muscle wasting.
- The studies in C2C12 myotubes were performed in serum free medium. However, C2C12 myotubes were differentiated in 2% foetal horse serum, have a large capacity to actively store vitamin D, and require vitamin D for many facets of mitochondrial function. An alternative explanation for the effects of GC on myotube diameter and mitochondrial energetics might be that GC binds and reduces vitamin D availability, exacerbated by lack of vitamin D in culture.
- The bar graphs in panel A and C look to have identical values even though the experimental conditions are different. This might be an inadvertent mistake, or conversely might reflect a highly reproducible finding. However, without showing individual datapoints, this needs some explanation.
- siRNA experiments are lacking data to show that silencing was effective in this particular preparation and experiment. Along similar lines, no experiments are provided to show that GC protein is in fact taken up into myotubes, beyond blocking megalin, which is non-selective for GC.
- The images and analysis in Figure 1I are unconvincing, since mitochondrial morphology cannot be properly observed. Better quality images are required, before re-analysis. Ditto for images of phalloidin and G-actin in Figure 4B, which do not show individual G-actin puncta and F-actin filaments, questioning whether the labelling worked.
- Figure 6C shows that GC KO LLC mice only possess increased grip strength on two out of the five days examined, implying that these mice might struggle to recover from physical exercise.
- Gene variants in GC are (variably) associated with differences in free/total vitamin D, as well as type 2 diabetes risk. Similarly, GC KO animals as well as GC treatment leads to changes in metabolism that would be expected to influence body weight, food intake and muscle mass. Supplementary Figure 3 shows a tendency toward decreased body weight but increased food intake following GC injection. These data are reflected by a loss in muscle mass following GC injection (Figure 5B and C). Notwithstanding the fact that the studies are underpowered, further experiments are needed to definitively show that effects of GC on muscle mass are not simply due to changes in metabolic status of the animals.
- GC KO animals have large reductions in 25(OH)D and 1,25(OH)2D, although are vitamin D sufficient on normal diet. Throughout, confounding effects of differences in free and total vitamin D need to be accounted for.
- Recent Mendelian randomization/PheWAS studies in healthy individuals show associations between GC, 25(OH)D and autoimmune disease. Is there human genetic evidence to support a link between GC and muscle mass and/or other relevant

GWAS traits?

Reviewer #2

(Remarks to the Author)
In this manuscript, Raiteri et al. examine the role of VDBP (vitamin D binding protein), also known as Gc, in muscle atrophy. The premise for these studies is the higher VDBP levels that are found in many disease conditions associated with wasting. VDBP binds vitamin D and therefore it may modulate muscle atrophy by perturbing vitamin D levels and homeostasis. By performing experiments in vitamin D-free conditions (in vitro) and in settings with no previously-reported changes in vitamin D levels (in vivo), the authors find a role for VDBP in muscle atrophy that appears to be independent from vitamin D levels. By using several assays, they report that VDBP perturbs intracellular actin dynamics, the function of the mitochondrial respiratory chain, mitochondrial fission and fusion, and mitophagy. Lastly, the authors inject VDBP intramuscularly and find that it promotes muscle wasting, fiber atrophy, and a decline in muscle force production in VDBP (GC) KO mice. Conversely, mice with VDBP (GC) KO are somewhat protected from cancer-induced muscle wasting, and this correlates with relevant gene expression changes. Overall, this interesting manuscript provides insight into the potential role of VDBP in muscle atrophy. There are however some major issues that limit the interpretation and/or insight provided by the data, as explained below. I encourage the authors to consider the points below to improve the manuscript.

Major points:
1) Mechanistically, it is unclear how VDBP induces muscle atrophy. The authors report that VDBP induces mitophagy (Fig. 1) and defects in mitochondrial respiratory functon(Fig. 3) and that it increases oxidative stress (Fig. 3) and mitochondrial fission (Fig. 5). Mitophagy is not necessarily negative as it is a quality control pathway, therefore it is unclear how it contributes to atrophy, also considering that the authors report that there are no changes in mitochondrial number (mtDNA; Fig. 3E), which would be expected if mitophagy leads to an overall loss of mitochondria. Therefore, it is currently unclear how perturbation of different mitochondrial functions contributes to muscle atrophy in response to VDBP.

2) In Fig. 5, the authors injected VDBP intramuscularly and compared it to contralateral (uninjected) controls and to other mice injected with saline. I would like to see all the raw data (rather than % changes) for all these groups. Currently the authors report only % changes for the saline and VDBP-injected groups but not for the contralateral controls.

3) In Figs. 5-6, the authors utilize grip strength as measurement of muscle force production but this method is notoriously highly variable and unreliable. The authors should utilize more robust methods to assess muscle force production such as in situ force measurements and, for cachexia experiments (Fig. 6), treadmill assays.

4) In Fig. 5C, the authors report that there are changes in the size of type IIa fibers but do not report whether there are changes in the more abundant type IIx and type IIb fibers of the TA muscle. A more comprehensive analysis of fiber size and type should be performed for the in vivo experiments in Fig. 5-6.

5) As explained above, it is unclear what is the significance of mitophagy (Fig. 5D-E) for VDBP-induced muscle atrophy, considering that the authors do not observe any decline in mitochondrial number (mtDNA levels in Fig. 3E).

6) In Figs. 6-7, the authors examine the role of VDBP in cancer cachexia and report that VDBP (gc) KO mice are somewhat protected from cancer-induced muscle wasting. However, Fig. 6K indicates that there is a ~25% lower tumor weight in the VDBP KO mice versus control mice. Although the authors report that this ~25% decline in tumor weight is not statistically significant, it is substantial enough to be biologically meaningful, i.e. it could be the reason for which VDBP (gc) KO mice appear to be somewhat protected from cancer-induced muscle wasting. Together with the other methodological caveats identified above for Fig. 5 and that also apply to Fig. 6 (i.e. lack of detailed fiber size/type analyses, suboptimal measurement of muscle force with grip strength assays), this reviewer is at present not convinced that VDBP (gc) KO protects from cancer-induced muscle wasting.

7) For a thorough evaluation of the data, it would be important that the authors display the raw data for muscle mass and body weight for all groups of mice, rather than reporting them as % changes.

8) The number of animals utilized for each assay in Fig. 6 seems variable within the same group for unclear reasons. For example, the LLC WT mice are n=3 in Fig. 6A, n=8 in Fig. 6E, n=5 in Fig. 6G, and n=4 in Fig. 6H. In general, it would be good to see the same, maximal number of animals for each group (such as n=8 for the LLC WT group) in all assays in Fig. 6.

9) While the authors have analyzed actin dynamics and a number of mitochondrial parameters in cell culture, their analysis in vivo in muscle is limited to mitochondrial fission in VDBP-injected mice and this is incomplete (Fig. 5E also needs quantitation) or absent (for Fig. 6). Therefore, it is unclear whether mitochondrial dysfunction(or changes in actin dynamics) occurs in vivo and whether it is a driver of VDBP-induced muscle wasting.

10) The authors report that VDBP (GC) expression increases by ~30% in the liver of mice injected with LLC cells. However, they should also explore whether VDBP is produced by LLC tumors.

11) While the authors report that they observe effects of VDBP that are independent from vitamin D levels in vivo (Fig. 6), they did not formally measure vitamin D levels but rather cite a previous publication. The authors should measure circulating as well as intramuscular vitamin D levels (which are likely more directly indicative of vitamin D bioavailability within skeletal

muscles), and whether these are modified by VDBP KO and cachexia.

Minor points:
1) The authors should indicate the total number of proteins that were detected by mass spec.
2) Many graphs do not display the individual values and/or error bars for all columns (for example, see control columns in Fig. 1).
3) Previous studies on the role of vitamin D on muscle atrophy and mitochondrial dysfunctions should be better discussed (for example see PMID: 30830277).
4) The authors should explain why the unusual Gusb and/or Ppif genes are used as reference for normalization in RT-qPCR experiments.
5) All the images should be quantified, including Fig. 5E.
6) Fig. 6 reports the body weight but the authors should specify whether this is the tumor-free body weight or not.

Reviewer #3

(Remarks to the Author)
Raiteri et al investigate to possible role of vitamin D binding protein (VDBP) in muscle atrophy and cancer cachexia. They combine in vitro and in vivo approaches to test to role of VDBP and its downstream pathway in muscle wasting. Intriguingly, their data suggest that VDBP perform a vitamin D-independent muscle atrophy activity. The study is very intriguing and could provide an original entry point to understand and treat cancer cachexia. Nevertheless, to better support their conclusions, the authors must address the following issues.

1. The entire study has been conducted using mouse samples. To support the relevance of findings to cancer-associated muscle wasting, they must demonstrate that VDBP is able to induce atrophy also of human muscle cells.

2. The authors must provide the specifics about the preparations of VDBP used for the study. For example, they indicate that a recombinant VDBP was used, but they provide a code for a human plasma purified protein. Moreover, they must demonstrate that the preparations of VDBP used for the study are highly pure and lack contaminants that can induce muscle atrophy. Related to this, the authors must perform a VDBP dose-response curve to demonstrate that the atrophic response is VDBP dose-dependent.

3. The Lrp2 knockdown is an important control and the authors must show that key defects displayed in Figures 2 and 3 are also rescued by reduction of megalin levels.

4. Based on Figure 2, there are relatively few proteins affected by VDBP treatment, and the actin/metabolism proteins are the minority. Moreover, the overlap between the proteins altered at the two timepoints appears small.

5. From Figure 3I-J it seems that mitoTEMPO has an effect also on cells not treated with VDBP. Hence, it is unclear if mitoTEMPO acts in the same pathway as VDBP or if we are seeing the results of two independent pathways.

6. The result of the grip test of Figure 5A is strange. Indeed, the control mice increase their strength over time, while VDBP maintain their strength. Therefore, the VDBP treated mice do not lose strength over time.

7. Based on Figure 5A, a significant difference between VDBP-treated and control mice is already present 2 days after VDBP delivery. Hence, why did the authors wait 8 days before sacrificing the mice to perform the other tests? There is concern that with a prolonged treatment a number of secondary effects could develop.

8. Is adipose tissue cachexia affected in the Gc KO mice of Figure 6?

9. Based on Figure 6K, there is an evident tendency for tumors to grow less in Gc KO mice. This must be, minimally, discussed by the authors since it could contribute to their results.

10. Is any muscle phenotype present in untreated Gc KO mice? For example, are the mice less susceptible to sarcopenia?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Many thanks for providing a revised manuscript. Whilst the authors have addressed a number of comments with further experiments and/or analysis, there are a few areas that still remain unclear.

- The MitoTracker images are better than before. However, analysis of mitochondrial area is confounded by the apparent decrease in myotube diameter following VDBP treatment. An alternative interpretation is that for their size, the VDBP-myotubes have the same number of mitochondria as control myotubes.

- PLA is used to show interactions between VDBP and DRP-1. Accurate PLA is contingent on the antibodies being specific. While negative control shows no PLA spots, there is presence of red (?) signal in Jas-treated samples (without VDBP). Normally, antibodies would be previously validated using siRNA of KO.

- An effect of VDBP treatment on 25(OH)D status was highlighted as a major potential confounder across the reviewers. The authors now provide measurement 25(OH)D using an ELISA kit. Suggesting that the kit might not be sufficiently sensitive and/or specific, there is only a 2-fold change in 25(OH)D in VDBP KO versus VDBP WT mice. Multiple previous studies have shown an almost 10-fold change (e.g. 10.1172/JCI5244).

- Following on from the above, 25(OH)D-saturated VDBP would be expected to release some 25(OH)D when added to 25(OH)D-free cells/media, which does not seem to be the case in the assays used in Figure 4F.

- Given the importance of 25(OH)D status for study interpretation, the authors should ideally use LC-MS which has the requisite sensitivity and specificity to report 25(OH)D and 1,25(OH)2D.

Reviewer #2

(Remarks to the Author)
The authors have improved this interesting manuscript and have addressed satisfactorily my previous concerns.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed my concerns. Congratulation on a well done study!

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors provide new data showing that the effects of VDBP on myotube atrophy are independent of 25(OH)D status. Thank you for this. The authors are often too certain about their conclusions, and it would be good to see various caveats inserted into the discussion e.g. intramuscular injection = high local VDBP levels, which might not reflect pathophysiological changes in circulating VDBP observed during cachexia; differences between extracellular versus intracellular VDBP levels and action; lack of human genetic support for a muscle phenotype etc. The cancer cachexia findings might also be related to the fact that VDBP increases energy expenditure, so loss of VDBP preseves muscle mass through indirect mechanisms. In light of this, and to better reflect the findings, the title should be changed to: "Local injection of Vitamin D binding protein induces skeletal muscle atrophy whereas its systemic deletion is muscle sparing". Or similar.

Reviewer #2

(Remarks to the Author)
I have no further comments, I support publication of this study

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thanks for making the suggested changes.

In response to the reviewers' feedback, we have revised the manuscript, adding new experimental data and refining the text throughout to enhance clarity and scientific rigor. Any data relevant to the reviewers' comments that do not appear in the main text are included here as additional editor/reviewer-only information.

Reviewers' comments: black
Our response: blue

Reviewer #1 (Remarks to the Author):

Raiteri and colleagues hypothesize that vitamin D-binding protein, otherwise known as GC, exerts atrophic effects on muscle and hence contributes to muscle wasting during cachexia. Using cultured immortalized myotubes, GC KO mice, and cachexia models, the authors conclude that GC influences intracellular G-/F-actin ratios to induce mitophagy, mitochondrial dysfunction, muscle atrophy and reduced grip strength. While the idea that GC contributes to muscle wasting is potentially interesting, there are a number of flaws with the study and study design that dampen enthusiasm. In particular, the authors do not adequately show that GC directly influences muscle mass/function/respiration, as opposed to acting through changes in vitamin D status and whole-body metabolism.

We are grateful to the Reviewer for recognizing the potential significance of our hypothesis regarding the role of VDBP/GC in muscle wasting and for the constructive remarks that spurred us to better understand the molecular mechanisms involved in its biological activity. We carefully considered all comments/indications and repeated or performed supplemental experiments to refine our work and dissect the involvement of vitamin D status and whole-body metabolism on the effects of VDBP on skeletal muscle.

1- The introduction suggests that GC is upregulated in patients and animal models of pathologies susceptible to muscle-wasting. However, a lot of these findings likely reflect an increase in GC following liver injury, or in response to inflammation/trauma, rather than an effect of GC per se on muscle wasting.

We agree with the Reviewer that the increase of GC/VDBP reported in the literature in pathologies associated with muscle wasting can derive from liver damage or inflammation. Nevertheless, independently of the cause of GC increase, our work aims to demonstrate the direct effect of GC on skeletal muscle and to understand the molecular mechanisms underlying its pro-atrophic activity.

2- The studies in C2C12 myotubes were performed in serum free medium. However, C2C12 myotubes were differentiated in 2% foetal horse serum, have a large capacity to actively store vitamin D, and require vitamin D for many facets of mitochondrial function. An alternative explanation for the effects of GC on myotube diameter and mitochondrial energetics might be that GC binds and reduces vitamin D availability, exacerbated by lack of vitamin D in culture.

We are grateful to the Reviewer for this comment, which has given us the possibility of addressing the important issue of the potential role of vitamin D and the interaction of VDBP/GC with vitamin D in the observed effects. To investigate the putative effect of VDBP/GC on the intracellular stores of

vitamin D, we treated the myotubes with VDBP pre-incubated with 25-hydroxy-vitamin D (25VD-bound VDBP) to avoid any accidental reduction of intracellular vitamin D that could affect cell trophism. We found that 25VD-bound VDBP had the same atrophic effect as that of VDBP alone (**Fig. 4D and E** in the revised manuscript). Furthermore, using a specific ELISA, we assessed potential changes in the amount of intracellular free vitamin D upon VDBP treatment. As shown in the **new Fig. 4F**, treatment with VDBP or 25VD-bound VDBP did not alter the intracellular levels of free 25VD. Besides the standards and internal controls of the kit, we also used a treatment with 25VD as a positive control to induce a change in the availability of intracellular free 25VD. These results, together with the fact that the F-actin stabilizer jasplakinolide, on the contrary, abrogates VDBP's atrophic effect, confirm that VDBP action is independent of vitamin D and highlight the involvement of the actin-binding activity of VDBP as the key mechanism of its biological activity in skeletal muscle cells.

3- The bar graphs in panel A and C look to have identical values even though the experimental conditions are different. This might be an inadvertent mistake, or conversely might reflect a highly reproducible finding. However, without showing individual datapoints, this needs some explanation.

We apologize for the lack of clarity in how some data were presented. To account for variability between independent experiments, we normalized the data relative to their corresponding control conditions. Specifically, myotube diameters were normalized to 100%, while densitometric values from Western blots were normalized to 1. While normalization may cause the control bars to have identical values across different panels, the underlying raw data are provided with the revised manuscript for full transparency (**Supplemental Data File S1**).

4- siRNA experiments are lacking data to show that silencing was effective in this particular preparation and experiment. Along similar lines, no experiments are provided to show that GC protein is in fact taken up into myotubes, beyond blocking megalin, which is non-selective for GC.

Following the Reviewer's remark, we have added new experiments to show the effective silencing of megalin by real-time PCR and IF (**Suppl. Fig. 2**). The specific uptake of VDBP/GC by myotubes was already reported by Abboud and colleagues who used a fluorescence-tagged VDBP [Abboud M et al. Endocrinology. 2013 154(9):3022-30. doi: 10.1210/en.2012-2245]. In the revised version of the work, we showed the increase of VDBP in lysates of VDBP-treated cells by Western blot, as well as the effect of Lrp2 silencing on it (new **Fig. 4A**). Also, we reported the effect of Lrp2 silencing on intracellular ROS increase (new **Fig. 4B**), in addition to the effect on myotube diameters (**Fig. 4C**).

5- The images and analysis in Figure 1I are unconvincing, since mitochondrial morphology cannot be properly observed. Better quality images are required, before re-analysis. Ditto for images of phalloidin and G-actin in Figure 4B, which do not show individual G-actin puncta and F-actin filaments, questioning whether the labelling worked.

We apologize for the suboptimal quality of some images in the original submission and have addressed this concern by repeating the relevant experiments and acquiring new images using confocal microscopy to improve resolution and data robustness. Specifically, the old Fig. 1I has been replaced by the **new Fig. 1H**, in which mitophagy is now assessed and quantified on the basis of the

colocalization of mitochondrial and lysosome markers. Regarding the F-actin and G-actin staining (original Fig. 4B), we have replaced the images with higher-resolution confocal images shown in the **new Fig. 4H**. We would like to note that, to our knowledge, the detection of G-actin with DNase I or similar labeling typically yields a diffuse staining pattern, rather than discrete puncta, as supported by published studies (e.g., PMID: 11810694; PMID: 23746641). Therefore, the absence of G-actin puncta is not indicative of a technical failure of the labeling.
We hope that the new images are more convincing in supporting our findings.

6- Figure 6C shows that GC KO LLC mice only possess increased grip strength on two out of the five days examined, implying that these mice might struggle to recover from physical exercise.
We thank the Reviewer for this observation and acknowledge that the grip strength data of Figure 6C were affected by experimental variability, likely due to insufficient pre-training of the animals. To address these issues, as we had to repeat the experiment, we improved the training protocol before measurements. As a result, our new data (**Fig. 7D**) indicate that Gc KO mice exhibit consistently better performance starting from week two post-LLC injection. Furthermore, we included an additional assessment of muscle performance using a treadmill endurance test (**Fig. 7E**), which confirmed superior physical performance in tumor-bearing Gc KO mice compared to tumor-bearing WT controls.
We believe that these refinements provide a more reliable and comprehensive assessment of muscle recovery and overall performance in Gc KO mice.

7- Gene variants in GC are (variably) associated with differences in free/total vitamin D, as well as type 2 diabetes risk. Similarly, GC KO animals as well as GC treatment leads to changes in metabolism that would be expected to influence body weight, food intake and muscle mass. Supplementary Figure 3 shows a tendency toward decreased body weight but increased food intake following GC injection. These data are reflected by a loss in muscle mass following GC injection (Figure 5B and C). Notwithstanding the fact that the studies are underpowered, further experiments are needed to definitively show that effects of GC on muscle mass are not simply due to changes in metabolic status of the animals.

We are thankful to the reviewer for these insightful comments that allowed us to further characterize Gc KO mice. We are aware of gene variants in GC that have been associated with higher fasting glucose and insulin levels, and impaired responses in glucose tolerance tests, suggesting a potential link between GC and type 2 diabetes. However, it is worth noting that ethnicity and environmental factors have not been excluded as a possible explanation (Wang G et al. BMJ Open. 2014;4(11):e005617. doi: 10.1136/bmjopen-2014-005617.).

Nevertheless, to directly address the concern that Gc KO animals may have altered metabolic status compared to WT mice, we performed additional metabolic assessments. Specifically, we conducted glucose and insulin tolerance tests and measured energy expenditure using indirect calorimetry in metabolic cages. These new data (**Suppl. Figure 4C and D**) revealed no significant differences between Gc KO and WT mice, suggesting that their systemic metabolic profiles are comparable.

Furthermore, in our revised experiments involving intramuscular VDBP injection (now presented in **Figure 6**), we modified the experimental design to control for potential systemic effects. To minimize inter-animal variability and exclude confounding influences from body weight or food intake, we directly compared the VDBP-injected tibialis anterior (TA) muscle with the contralateral saline-injected TA within the same animal. Importantly, the effect of GC was limited to the injected TA and did not affect adjacent muscles (**Suppl. Fig. 3**) nor local concentration of vitamin D (**Fig. 6T**), suggesting the lack of leakage from the injected site and further supporting the absence of any systemic effect of VDBP intramuscular injection. Taken together, these results support the conclusion that the effects of VDBP on muscle mass are not due to broader changes in animals' metabolic status.

Finally, while we acknowledge that in some experiments the sample size is limited, the differences observed between treated and control limbs were statistically significant and consistent with our previous findings.

8- GC KO animals have large reductions in 25(OH)D and 1,25(OH)2D, although are vitamin D sufficient on normal diet. Throughout, confounding effects of differences in free and total vitamin D need to be accounted for.

We thank the Reviewer for raising this important point and agree on the potential confounding effects of vitamin D levels in a complex system such as cancer cachexia and, more importantly, in the context of GC absence, which is known to alter vitamin D transport and distribution. To address this, we have measured free 25(OH)D levels in both blood and muscle samples from tumor-bearing mice and controls. Our new data (**Fig. 7C**) confirm previously published findings that the LLC cachexia model does not alter vitamin D levels. Moreover, our results show that LLC tumors do not further reduce the already lower vitamin D levels in GC KO mice. Notably, despite having lower vitamin D levels than WT mice, GC KO mice remain protected from cancer-induced muscle wasting. This finding, together with the in vitro results shown in **Fig. 4D-F**, further supports our conclusion that the effects of VDBP on skeletal muscle are independent of vitamin D availability.
Of note, we specifically measured only the free 25-hydroxy vitamin D (25VD) as our focus was on evaluating the immediate bioavailable pool potentially affected by VDBP, which, among the various VD metabolites, shows the highest binding affinity for the 25VD form (Bouillon R et al. 2020 doi: 10.3389/fendo.2019.00910).

9- Recent Mendelian randomization/PheWAS studies in healthy individuals show associations between GC, 25(OH)D and autoimmune disease. Is there human genetic evidence to support a link between GC and muscle mass and/or other relevant GWAS traits?
To our knowledge, to date, there are no GWAS studies directly linking GC gene variants and muscle mass. The recent systematic review on the impact of genetic variants related to vitamin D and autoimmunity (Trefilio et al. Heliyon. 2024 doi: 10.1016/j.heliyon.2024.e27700. PMID: 38689997) suggests that GC gene variants can influence vitamin D metabolism and transport, potentially affecting the risk and progression of various autoimmune diseases. Among the mentioned ones, inflammatory bowel disease (IBD) and Graves' Disease can lead to muscle wasting as a secondary effect. However, the paper does not explicitly discuss the connection between GC, these diseases,

and muscle wasting.

Reviewer #2 (Remarks to the Author):

In this manuscript, Raiteri et al. examine the role of VDBP (vitamin D binding protein), also known as Gc, in muscle atrophy. The premise for these studies is the higher VDBP levels that are found in many disease conditions associated with wasting. VDBP binds vitamin D and therefore it may modulate muscle atrophy by perturbing vitamin D levels and homeostasis. By performing experiments in vitamin D-free conditions (in vitro) and in settings with no previously-reported changes in vitamin D levels (in vivo), the authors find a role for VDBP in muscle atrophy that appears to be independent from vitamin D levels. By using several assays, they report that VDBP perturbs intracellular actin dynamics, the function of the mitochondrial respiratory chain, mitochondrial fission and fusion, and mitophagy. Lastly, the authors inject VDBP intramuscularly and find that it promotes muscle wasting, fiber atrophy, and a decline in muscle force production in VDBP (GC) KO mice. Conversely, mice with VDBP (GC) KO are somewhat protected from cancer-induced muscle wasting, and this correlates with relevant gene expression changes. Overall, this interesting manuscript provides insight into the potential role of VDBP in muscle atrophy. There are however some major issues that limit the interpretation and/or insight provided by the data, as explained below. I encourage the authors to consider the points below to improve the manuscript.

We sincerely appreciate the Reviewer's acknowledgment of the potential significance of our findings and for the overall interest in our work. We are thankful for the thoughtful evaluation of our manuscript and the constructive comments that have helped us to improve the clarity and robustness of our conclusions.
We acknowledge the Reviewer's concerns regarding some aspects of the data interpretation and presentation. In response, we have carefully addressed all the points raised, including performing additional experiments to elucidate the mechanisms through which VDBP induces muscle atrophy and strengthen our conclusions.

Major points:
1) Mechanistically, it is unclear how VDBP induces muscle atrophy. The authors report that VDBP induces mitophagy (Fig. 1) and defects in mitochondrial respiratory functon(Fig. 3) and that it increases oxidative stress (Fig. 3) and mitochondrial fission (Fig. 5). Mitophagy is not necessarily negative as it is a quality control pathway, therefore it is unclear how it contributes to atrophy, also considering that the authors report that there are no changes in mitochondrial number (mtDNA; Fig. 3E), which would be expected if mitophagy leads to an overall loss of mitochondria. Therefore, it is currently unclear how perturbation of different mitochondrial functions contributes to muscle atrophy in response to VDBP.

We are grateful to the Reviewer for this remark, which prompted us to assess further the role of mitophagy in our system. As supposed by the reviewer, we found that increased mitophagy was not a direct cause of atrophy but rather a compensatory mechanism to remove VDBP-damaged mitochondria. This interpretation is supported by the unchanged mitochondrial amount, as

measured by mtDNA content in vitro (**Fig. 3E**) and TOM20 immunofluorescence in vivo (**Fig. 6H and Q**), and by the enhanced expression of PGC1-α, which suggests increased mitochondrial biogenesis (**new Fig. 3F** in vitro and **Fig. 6P** in vivo).
Furthermore, our data now demonstrate that VDBP-induced muscle atrophy is primarily mediated by alterations in intracellular actin dynamics, which, in turn, appear to exacerbate mitochondrial fission and increase ROS production. Accordingly, the experiments with the mitochondrial-targeted antioxidant mitoTEMPO (**Fig. 3J-L**), the inhibitor of mitochondrial fission Mdivi-1 (**Fig. 5 E, F** in murine cells and **Fig. 5 H, I** in human cells), and the stabilizer of F-actin jasplakinolide (**Fig. 4I-K** and **Fig. 5D** in murine cells, **Fig. 5 H,I** in human cells) showed that each of these interventions was able to rescue the atrophic phenotype induced by VDBP.
We believe these findings clarify the mechanistic link between VDBP exposure, mitochondrial dysfunction, and muscle atrophy, thereby strengthening our overall conclusions.

2) In Fig. 5, the authors injected VDBP intramuscularly and compared it to contralateral (uninjected) controls and to other mice injected with saline. I would like to see all the **raw data** (rather than % changes) for all these groups. Currently the authors report only % changes for the saline and VDBP-injected groups but not for the contralateral controls.

We thank the reviewer for this remark, which, together with another Reviewer's comment, convinced us to modify the experimental design, thus improving the interpretability of the results. Specifically, ìn the revised version of the manuscript, we now compare the VDBP-injected TA muscle with the saline-injected contralateral TA within the same animal, thus allowing for a more robust intra-animal comparison and controls for potential systemic effects of VDBP injection. As requested, we now provide the raw data instead of percentage changes in the **new Fig. 6**. Furthermore, the complete raw datasets for all experiments are now available as part of the supplementary material (**Supplemental Data File S1**).

3) In Figs. 5-6, the authors utilize grip strength as measurement of muscle force production but this method is notoriously highly variable and unreliable. The authors should utilize more robust methods to assess muscle force production such as in situ force measurements and, for cachexia experiments (Fig. 6), treadmill assays.

We acknowledge the inherent variability of in vivo experiments, particularly in grip strength measurements. To overcome these limitations, we have taken several steps to enhance the reliability of our measurements. For a start, we repeated the experiments, ensuring adequate training before the actual testing for both the VDBP injection experiments (**new Fig. 6**) and the cachexia model (**new Fig. 7**). While we recognize that in situ force measurements would be a more robust and direct method to measure muscle force, we were unable to perform them due to the unavailability of the required equipment in our facility and the logistical challenges of transferring both animals and personnel to a different institution. Given these constraints, we used grip strength testing as the most feasible method available, as it allows force measurements from a single limb.

As for the cachexia experiments, we have addressed the reviewer's suggestion by complementing the grip test results (**Fig. 7D**) with a treadmill endurance assay (**Fig. 7E**), thus providing an additional

and independent functional readout to further support our conclusion that tumor-bearing KO mice preserve their muscle functionality compared to the tumor-bearing WT.

4) In Fig. 5C, the authors report that there are changes in the size of type IIa fibers but do not report whether there are changes in the more abundant type IIx and type IIb fibers of the TA muscle. A more comprehensive analysis of fiber size and type should be performed for the in vivo experiments in Fig. 5-6.

In response to the Reviewer's comment, we have expanded the analysis on fiber types. In the VDBP injection experiment, the changes in fiber IIa, IIx, and IIb after 48 h or 8 days post-injection are now reported in **Fig. 6C and M**, respectively. Unfortunately, in this experiment, the repeated intramuscular injections in the same site compromised muscle integrity and disrupted local architecture. As a result, the quantification of fiber areas according to their type in some sections yielded inconsistent or unreliable measurements, limiting the scope of the analysis in those specific samples. For the same reason, the overall CSA fiber distribution shown in **Fig. 6L** has been evaluated on selected, minimally damaged sections of the muscle slices and appears "bumpy" in aspect.

Regarding the cachexia experiment, we did not observe any significant alterations in muscle fiber type composition when comparing tumor-bearing WT and KO mice. This result is now presented in **Suppl. Fig. 4G**.

5) As explained above, it is unclear what is the **significance of mitophagy** (Fig. 5D-E) for VDBP-induced muscle atrophy, considering that the authors do not observe any decline in mitochondrial number (mtDNA levels in Fig. 3E).

We thank the Reviewer again for the comment, which stimulated us to complement the data presented in the previous version of the manuscript with new ones that allow now to interpret the activation of mitophagy as a compensatory response aimed at maintaining mitochondrial quality. Similarly to the in vitro results, in the in vivo VDBP injection experiments, we observed an upregulation of markers of both mitophagy (Bnip3) and mitochondrial biogenesis (Pgc1a). This upregulation was modest at 48 hours (a non-significant trend in **Fig. 6F-G**) and became significant at 8 days post-injection (**Fig. 6O-P**), suggesting the activation of mitochondrial quality control pathways over time.

In addition, Tom20 mean fluorescence intensity, a readout of mitochondrial content, remained unchanged in VDBP-injected limbs compared to saline-injected contralateral controls (**Fig. 6H and 6Q**), further supporting the notion that mitochondrial number is preserved, and that mitophagy is likely functioning to eliminate dysfunctional mitochondria without inducing a net loss of the mitochondrial pool.

6) In Figs. 6-7, the authors examine the role of VDBP in cancer cachexia and report that VDBP (gc) KO mice are somewhat protected from cancer-induced muscle wasting. However, Fig. 6K indicates that

there is a ~25% lower tumor weight in the VDBP KO mice versus control mice. Although the authors report that this ~25% decline in tumor weight is not statistically significant, it is substantial enough to be biologically meaningful, i.e. it could be the reason for which VDBP (gc) KO mice appear to be somewhat protected from cancer-induced muscle wasting. Together with the other methodological caveats identified above for Fig. 5 and that also apply to Fig. 6 (i.e. lack of detailed fiber size/type analyses, suboptimal measurement of muscle force with grip strength assays), this reviewer is at present not convinced that VDBP (gc) KO protects from cancer-induced muscle wasting.

We thank the Reviewer for these comments. We believe that the non-significant trend toward reduced tumor weight in VDBP KO mice in the original dataset (previous Fig. 6K) was likely a reflection of the variability of the in vivo experiments. Indeed, in the new experiment, performed to address the Reviewer's methodological concerns, the average tumor masses in the WT and KO mice are identical (**Suppl. Fig. 4F**), ruling out the possibility that the protection from muscle loss in KO mice could be a consequence of smaller tumors.

We also performed the analysis of muscle fiber type composition (reported in **Suppl. Fig. 4G**), which revealed no significant differences between KO and WT tumor-bearing animals.

We hope that the treadmill endurance test, added to complement and strengthen the grip test results (**Fig. 7E**), provides a more robust and functionally relevant assessment of muscle performance, thus reinforcing the validity of our conclusions.

7) For a thorough evaluation of the data, it would be important that the authors display the **raw data** for muscle mass and body weight for all groups of mice, rather than reporting them as % changes.
The muscle mass and body weights are now reported as raw data, and the complete raw datasets for all experiments are now available as part of the supplementary material (**Supplemental Data File S1**).

8) The number of animals utilized for each assay in Fig. 6 seems variable within the same group for unclear reasons. For example, the LLC WT mice are n=3 in Fig. 6A, n=8 in Fig. 6E, n=5 in Fig. 6G, and n=4 in Fig. 6H. In general, it would be good to see the same, maximal number of animals for each group (such as n=8 for the LLC WT group) in all assays in Fig. 6.

We thank the Reviewer for this comment, which allows us to explain the variation in animal numbers across different panels of Fig. 6 (**now Fig.7**). In particular, the mice shown in Fig. 6A (now **Fig. 7A,** complemented with data from the new experiment) were part of an initial exploratory experiment designed to determine whether tumor-bearing mice exhibited increased Gc/VDBP levels. Based on those findings, we subsequently designed experiments to specifically compare muscle wasting between WT and Gc KO mice. In many of these experiments, the primary comparison is indeed between tumor-bearing animals (WT vs. Gc KO). For this reason, the control groups were kept to a minimum, serving as reference points rather than as full experimental comparators. Furthermore, many analyses were performed on the same biological samples. In certain cases, samples were not

available for all analyses due to insufficient tissue quantity, sample degradation, or failed staining, resulting in variability in the number of animals across different assays.

9) While the authors have analyzed actin dynamics and a number of mitochondrial parameters in cell culture, their analysis in vivo in muscle is limited to mitochondrial fission in VDBP-injected mice and this is incomplete (Fig. 5E also needs quantitation) or absent (for Fig. 6). Therefore, it is unclear whether mitochondrial dysfuncton (or changes in actin dynamics) occurs in vivo and whether it is a driver of VDBP-induced muscle wasting.

We agree with the reviewer on the lack of a mechanistic link between our in vitro and in vivo findings and that the original version of the manuscript lacked sufficient evidence to establish that the mechanisms of VDBP-induced muscle atrophy observed in vitro also occur in vivo.

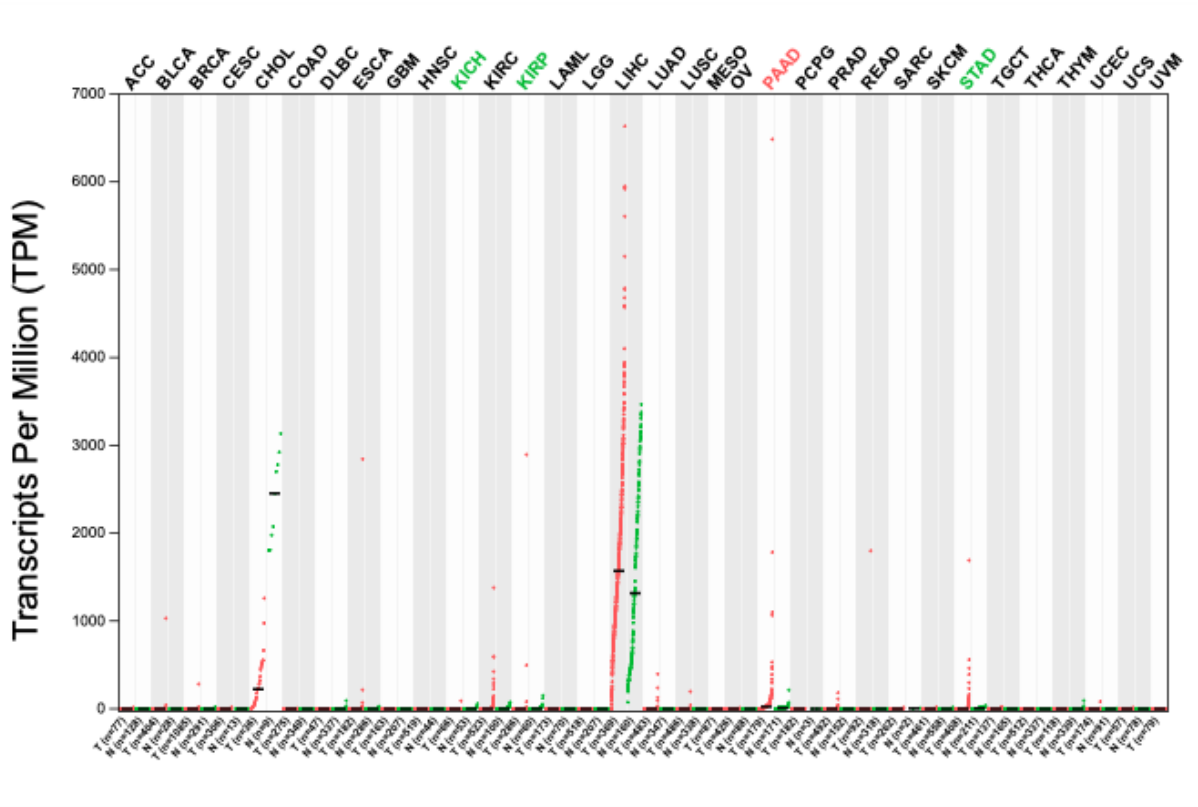
With the additional experiments or analyses performed, we tried to address this gap and confirm that the same key mechanism at the basis of VDBP-induced atrophy in C2C12 myotubes also occurred in vivo.

Similarly to the in vitro results, our additional experiments of VDBP intramuscular injection indicate that VDBP induces the alteration of G-/F-actin ratio in TA muscles, seen as G-actin accumulation using a dedicated biochemical assay kit (**Fig. 6D**). In addition, VDBP intramuscular injection induced the increase of the fission marker Mff and of mitochondrial fragmentation (**Fig. 6E and I** for the 48h and **Fig. 6N and R** for the 8-day endpoint), and impaired mitochondrial respiration (**Fig. 6J and S**).

An assessment of mitochondrial fragmentation and respiration was added to the cachexia experiment as well (**Fig.7L and M**). These data reinforce the conclusion that VDBP induces mitochondrial dysfunction in vivo, mirroring the cellular effects observed in cultured myotubes. However, we would like to clarify that the cachexia experiment was conceived only as a proof of concept that VDBP could contribute to the loss of muscle mass and function in a pathological condition associated with an increase in VDBP. We never proposed VDBP as the cause of cancer-associated muscle wasting. We apologize if we gave this impression and stressed this concept in the text.

10) The authors report that VDBP (GC) expression increases by ~30% in the liver of mice injected with LLC cells. However, they should also explore whether VDBP is produced by LLC tumors.

We are thankful for this stimulating cue. We checked the expression of Gc in LLC tumors by real-time PCR and found it almost undetectable compared to the liver. We added this in **Fig. 7A**.
More broadly, according to the human genome cancer atlas (TGCA), GC expression is, in general, barely detectable, with few exceptions, and, among those, only in pancreatic adenocarcinoma (PAAD, in red), there is a statistically significant increase compared to the corresponding healthy tissue.
GC expression in human cancers:



11) While the authors report that they observe effects of VDBP that are independent from vitamin D levels in vivo (Fig. 6), they did not formally measure vitamin D levels but rather cite a previous publication. The authors should measure circulating as well as intramuscular vitamin D levels (which are likely more directly indicative of vitamin D bioavailability within skeletal muscles), and whether these are modified by VDBP KO and cachexia.

We are grateful to the Reviewer for this comment, which has allowed us to address an important issue. Following the indication of this and another Reviewer, we measured the level of vitamin D (VD) in our systems and upon VDBP treatment, specifically of 25-dihydroxy-VD (25VD), as the potentially most affected by VDBP. We first investigated if VDBP could affect the intracellular amount of free 25VD in C2C12 myotubes, as VDBP-mediated sequestering of the anti-atrophic 25VD could account for increased susceptibility of cells to atrophy, and found no differences in VDBP-treated vs untreated cells and even in cells treated with a VDBP previously bound with 25VD (new **Fig. 4D-F**). In vivo, we confirmed the absence of changes in VD upon intramuscular injection of VDBP in KO mice (**Fig. 6T**). Likewise, cachexia induced by LLC cells did not alter free 25VD in WT nor KO mice (new **Fig. 7C**), although, as expected, Gc KO mice basally have less vitamin D than WT. This fact reinforces the tenet that it is not the reduction of vitamin D that induces atrophy, as Gc KO mice are more resistant to cancer-associated muscle loss.

Minor points:

1) The authors should indicate the total number of proteins that were detected by mass spec.

We added this information to the text.

2) Many graphs do not display the individual values and/or error bars for all columns (for example, see control columns in Fig. 1).
We apologize for any lack of clarity in the presentation of some data. Depending on the experiments, to account for variability between independent experiments, we normalized the results to their respective control conditions. Specifically, myotube diameters were expressed as a percentage relative to control (set at 100%), and densitometric values from Western blots were normalized to 1.

3) Previous studies on the role of vitamin D on muscle atrophy and mitochondrial dysfunctions should be better discussed (for example see PMID: 30830277).

We acknowledge the relevance of prior studies investigating the role of vitamin D in muscle atrophy and mitochondrial dysfunction, including the one referenced. However, we would like to emphasize that the primary aim of our study is not to explore the effects of vitamin D, but rather to investigate the vitamin D binding protein (VDBP) as a biologically active molecule with direct, vitamin D-independent effects on skeletal muscle.

We hope that the newly added analyses further support this distinction, demonstrating that the observed atrophic and mitochondrial effects of VDBP are independent of vitamin D.

4) The authors should explain why the unusual Gusb and/or Ppif genes are used as reference for normalization in RT-qPCR experiments.
The choice of Gusb and Ppif for in vitro and in vivo experiments, respectively, derived from a preliminary screening assessing several potential housekeeping genes (including the most commonly used Gapdh, 18S, Actb) for stability among samples in untreated and treated conditions and their level of expression, preferring genes with Ct values in the 25-27 range (similar to those of the target genes).

5) All the images should be quantified, including Fig. 5E.
All the images have now been quantified except for IP experiments (**Fig. 4G and 5B**), because they aimed to demonstrate the presence or absence of the VDBP-actin interaction rather than to measure precise changes in their levels, and the IF of megalin in **Supplementary Fig. 2** that was performed only to confirm the efficacy of gene silencing, which resulted in a complete loss of signal. Given the binary nature of this result (signal present vs. absent), we deemed that quantification was not necessary.

6) Fig. 6 reports the body weight but the authors should specify whether this is the tumor-free body weight or not.
We apologize for the lack of precision in the description. We have now specified that **Fig. 7F** of the revised version displays the tumor-free body weight of mice.

Reviewer #3 (Remarks to the Author):

Raiteri et al investigate to possible role of vitamin D binding protein (VDBP) in muscle atrophy and cancer cachexia. They combine in vitro and in vivo approaches to test to role of VDBP and its downstream pathway in muscle wasting. Intriguingly, their data suggest that VDBP perform a vitamin D-independent muscle atrophy activity. The study is very intriguing and could provide an original entry point to understand and treat cancer cachexia. Nevertheless, to better support their conclusions, the authors must address the following issues.

We are pleased that the Reviewer found the study intriguing and grateful for the constructive evaluation of our work. We have carefully addressed all the points raised, as detailed below, and, as a result, we believe the revised version of the manuscript presents more robust and well-supported conclusions.

1. The entire study has been conducted using mouse samples. To support the relevance of findings to cancer-associated muscle wasting, they must demonstrate that VDBP is able to induce atrophy also of human muscle cells.

We fully agree with the Reviewer on the importance of giving a proof of concept of the translationability on human cells of the results obtained in mouse-derived samples. To this aim, we performed a set of key experiments on human myotubes derived from primary human myoblasts. In detail, we tested the atrophic effect of VDBP and the protective effects of jasplakinolide and Mdivi-1 in these human muscle cells. The results confirmed that the mechanisms observed in mouse cells are also conserved in human myotubes. These data are shown in **Fig. 5G-I** of the revised manuscript and support the translational potential of our findings.

2. The authors must provide the specifics about the preparations of VDBP used for the study. For example, they indicate that a recombinant VDBP was used, but they provide a code for a human plasma purified protein. Moreover, they must demonstrate that the preparations of VDBP used for the study are highly pure and lack contaminants that can induce muscle atrophy. Related to this, the authors must perform a VDBP dose-response curve to demonstrate that the atrophic response is VDBP dose-dependent.

We apologize for the incorrect specifics provided regarding VDBP and for not having reported the dose-response curve used to determine the experimental dose. We amended both by correcting the Materials and Methods section and adding the dose-response curve (**Fig. 1A** of the revised manuscript). The purity level of VDBP provided by the producer was =>95% as determined by SDS-PAGE, making it highly unlikely that trace contaminants could account for the observed biological effects. In addition to the dose-dependent response observed in our experiments, the same results were obtained with another VDBP from a different company (data not shown), further supporting the notion that the effects are attributable to the compound itself rather than to impurities.

3. The Lrp2 knockdown is an important control and the authors must show that key defects displayed in Figures 2 and 3 are also rescued by reduction of megalin levels.

Following the reviewer's suggestion, we have repeated some key experiments after Lrp2 silencing. The new experiments include, in addition to the rescue of VDBP-induced atrophy in C2C12 myotubes (**Fig. 4C**), the impairment of VDBP-induced intracellular increase of VDBP itself (**Fig. 4A**), as a readout of VDBP entry in cells, and intracellular ROS increase (**Fig. 4B**).

4. Based on Figure 2, there are relatively few proteins affected by VDBP treatment, and the

actin/metabolism proteins are the minority. Moreover, the overlap between the proteins altered at the two timepoints appears small.
We agree with the reviewer regarding both aspects of the experiment shown in Figure 2. However, the proteomic analysis on C2C12 myotubes at 18h and 24h post-treatment was performed separately and, more importantly, with two different equipment with different sensitivities. We believe this to be the main reason for the poor overlap of detected proteins at the two timepoints. Nevertheless, this experiment was used uniquely to suggest the pathways potentially affected by VDBP, without further analysis on specific proteins.

5. From Figure 3I-J it seems that mitoTEMPO has an effect also on cells not treated with VDBP. Hence, it is unclear if mitoTEMPO acts in the same pathway as VDBP or if we are seeing the results of two independent pathways.
We believe that the very small and not statistically significant differences produced by mitoTEMPO alone in Fig. 3I-J (now **Fig. 3J-L**) simply reflect the normal experimental variability. However, we acknowledge that ROS act as signaling molecules; therefore, an excessive scavenging of ROS, just like excessive ROS production, could affect muscle homeostasis. Nevertheless, the protective effect of mitoTEMPO against VDBP-induced atrophy remains clear, and we interpret this as evidence that mitoTEMPO acts downstream of VDBP in the same pathway, mitigating its deleterious effects via ROS scavenging.

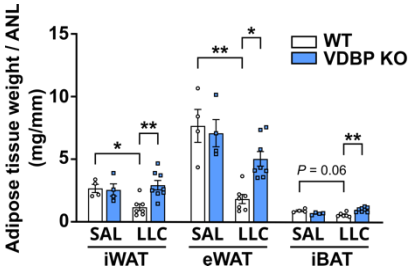
6. The result of the grip test of Figure 5A is strange. Indeed, the control mice increase their strength over time, while VDBP maintain their strength. Therefore, the VDBP treated mice do not lose strength over time.
We thank the Reviewer for this observation and acknowledge that the odd trend in grip strength data of Figure 5A, likely due to insufficient pre-training of the animals. To address these issues, when repeating the experiment, we improved the animal training before measurements. The new in vivo experiment shown in **Fig. 6A** gave a stable line for the saline-treated control limbs and a significantly decreased force in the VDBP-injected limbs.

7. Based on Figure 5A, a significant difference between VDBP-treated and control mice is already present 2 days after VDBP delivery. Hence, why did the authors wait 8 days before sacrificing the mice to perform the other tests? There is concern that with a prolonged treatment a number of secondary effects could develop.
We are grateful to the reviewer for this remark and acknowledge the potential development of secondary effects with a prolonged treatment. In our revised experiments involving intramuscular VDBP injection (now presented in **Figure 6**), we modified the experimental design to control for potential systemic effects and to minimize inter-animal variability and exclude confounding influences from body weight or food intake. Specifically, we now compared the VDBP-injected TA muscle with the saline-injected contralateral TA within the same animal. In addition, we provided support for the fact that the effect of GC was limited to the injected TA muscle and did not affect adjacent muscles (**Suppl. Fig. 3**) nor muscular concentration of vitamin D (**Fig. 6T**), suggesting the lack of leakage from the injected site and further supporting the absence of any systemic effect of VDBP intramuscular injection.

With the new experiment, we also analyzed a subset of animals at 48h post-injection, when a difference in strength is already visible. We now report some significant effects of VDBP injection (fiber type shift, **FIG. 6C**; Mff expression, **Fig. 6E**; mitochondrial fragmentation, **Fig. 6I**; LEAK phase of mitochondrial respiration, **Fig. 6J**), and a tendency in other parameters that became significant at 8 days post-injection, including the decrease in muscle mass (**Fig. 6K and L**), Bnip3 and PGC1a induction (**Fig. 6O and P**), and impairment of complex I in mitochondrial respiration (**Fig. 6S**).

8. Is adipose tissue cachexia affected in the Gc KO mice of Figure 6?

We thank the Reviewer for this insightful question. Although our data specifically focus on skeletal muscle, we have indeed observed adipose tissue preservation in tumor-bearing Gc KO mice compared to tumor-bearing WT controls, especially the inguinal and epididymal white adipose tissue (image below).



These findings raise important questions about other systemic effects of VDBP, including its role in regulating adipose tissue homeostasis, either directly or indirectly.

However, as VDBP effects on adipose tissue fall outside the scope of the present study, we have opted not to include these data in the current manuscript. We plan to address this aspect in a separate, dedicated study for a more thorough mechanistic investigation, also bearing in mind that VDBP binds fatty acids, and unsaturated fatty acids impair the binding of 1,25(OH)2D and 25OHD to VDBP, increasing the scenario complexity.

We hope the Reviewer understands this decision and agrees that maintaining a clear focus on skeletal muscle.

9. Based on Figure 6K, there is an evident tendency for tumors to grow less in Gc KO mice. This must be, minimally, discussed by the authors since it could contribute to their results.

We thank the Reviewer for pointing out this important consideration. The non-significant trend observed in Fig. 6K in the original manuscript was likely a reflection of the variability of the in vivo data. The data shown in **Suppl. Fig. 4F** of the revised version, obtained from a new experiment, the average tumor masses in the WT and KO mice were identical, ruling out the possibility that the protection from muscle loss in KO mice could be a consequence of smaller tumors.

10. Is any muscle phenotype present in untreated Gc KO mice? For example, are the mice less susceptible to sarcopenia?

We thank the Reviewer for this question. Both previous (Kew RR et al. J Trauma Acute Care Surg. 2018; 84(6):847-854. doi: 10.1097/TA.0000000000001875. PMID: 29554047) and our observations showed no significant differences in muscle phenotype in unchallenged KO mice. However, we have not yet studied the muscles of aged KO mice to assess if they are less prone to sarcopenia. We

foresee that this could be the case, since, in rats, there is an increase of Gc expression in the liver with aging, as shown in the following figure, that could contribute to the decline in muscle mass (data extracted from the paper by Shavlakadze T et al. Cell Rep. 2019;28(12):3263-3273.e3. doi: 10.1016/j.celrep.2019.08.043. PMID: 31533046.).

[Figure Redacted]

However, we would prefer to keep this very preliminary observation as a starting point for a future study.

In response to the feedback of Reviewer #1, we revised the manuscript by adding new experimental data and refining the text to improve clarity. Any data relevant to the Reviewer's comments that are not included in the main text are provided here as additional editor/reviewer-only information.

Reviewer's comments: black
Our response: blue

Reviewer #1 (Remarks to the Author):

Many thanks for providing a revised manuscript. Whilst the authors have addressed a number of comments with further experiments and/or analysis, there are a few areas that still remain unclear.

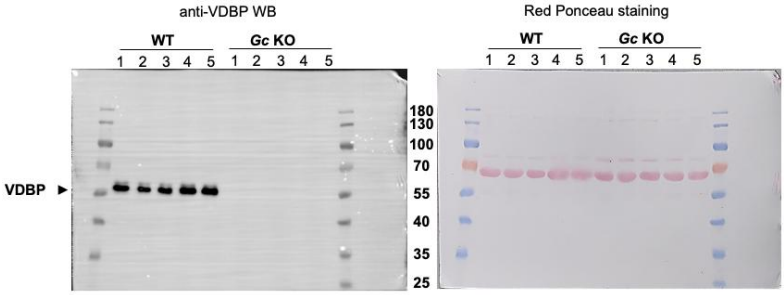
We appreciate the Reviewer's comments, which demonstrate a strong interest in key details of our work and have helped us further clarify certain points.

1- The MitoTracker images are better than before. However, analysis of mitochondrial area is confounded by the apparent decrease in myotube diameter following VDBP treatment. An alternative interpretation is that for their size, the VDBP-myotubes have the same number of mitochondria as control myotubes.

MitoTracker staining in Figs. 3H and 5G was used to evaluate mitochondrial fragmentation, and the decrease shown in the graph reflects the **average mitochondrial (fragment) size** rather than the total mitochondrial content per myotube. Consistent with the Reviewer's suggestion of unchanged mitochondrial abundance, we also reported no difference in mitochondrial content between VDBP-treated and control myotubes, as indicated by the lack of difference in the relative expression of the mitochondrial DNA gene Cox2, normalized to the genomic DNA gene Rps18 (Fig. 3E).

2- PLA is used to show interactions between VDBP and DRP-1. Accurate PLA is contingent on the antibodies being specific. While negative control shows no PLA spots, there is presence of red (?) signal in Jas-treated samples (without VDBP). Normally, antibodies would be previously validated using siRNA of KO.

We appreciate the reviewer's point that reliable PLA results depend on antibody specificity and appropriate controls. The specificity of the anti-VDBP and anti-DRP1 antibodies used for PLA was supported by Western blotting experiments, which showed clear bands at the expected molecular weights, consistent with specific target recognition. As an example, please see the image below showing the VDBP antibody used to assess the presence/absence of VDBP in blood from WT and *Gc* KO mice:



Regarding the PLA signals, although not visible in the representative image, a low level of puncta was observed even in the absence of exogenous VDBP. This is not unexpected, as our Western blots of whole-cell lysates and mitochondrial fractions (*e.g.*, Figs. 4A and 5A) show that cells contain detectable endogenous VDBP. Similarly, DRP1 is constitutively expressed and dynamically recruited during physiological mitochondrial fission.

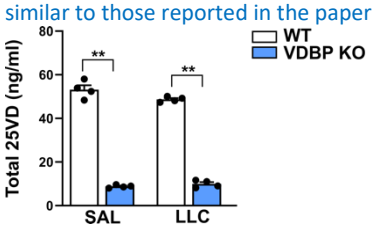
Concerning the Reviewer's observation of signal in JAS-treated samples, jasplakinolide stabilizes F-actin, which favors DRP1 recruitment to the mitochondrial outer membrane, facilitating the early steps of mitochondrial fission (doi: 10.1091/mbc.E16-03-0193). Therefore, the slight, non-significant increase in PLA puncta with JAS treatment is consistent with enhanced DRP1 recruitment driven by actin polymerization rather than nonspecific antibody binding.

3- An effect of VDBP treatment on 25(OH)D status was highlighted as a major potential confounder across the reviewers. The authors now provide measurement 25(OH)D using an ELISA kit. Suggesting that the kit might not be sufficiently sensitive

and/or specific, there is only a 2-fold change in 25(OH)D in VDBP KO versus VDBP WT mice. Multiple previous studies have shown an almost 10-fold change (e.g. 10.1172/JCI5244).

We thank the Reviewer for this comment and agree that several studies, such as the one cited, report higher differences in 25(OH)D between WT and *Gc* KO mice. However, those data were obtained by measuring **total 25(OH)D**, whereas the ELISA kit used in our experiments measured only the **free fraction**. We explicitly considered the free fraction because it is the form that could be sequestered by VDBP. Since the free 25(OH)D typically represents ~0.02–0.04% of the total form, total concentrations in the 20–50 ng/mL range correspond to roughly 4–20 pg/mL of free 25(OH)D, in line with the values shown in Fig. 7C of the manuscript.

Using an ELISA kit targeting **total 25VD**, we indeed obtain values and proportions of 25VD between WT and KO (shown below) similar to those reported in the paper cited by the Reviewer.



Nevertheless, in the revised version of the manuscript, we reported the **total 25VD values obtained by LC-MS analysis** (added panel in **Fig. 7C**).

4- Following on from the above, 25(OH)D-saturated VDBP would be expected to release some 25(OH)D when added to 25(OH)D-free cells/media, which does not seem to be the case in the assays used in Figure 4F.

We agree with the Reviewer that 25(OH)D-saturated VDBP would be expected to release some 25(OH)D when incorporated into 25(OH)D-free cells. However, the ELISA assay used in Fig. 4F detects only the **free form** of 25(OH)D; therefore, it does not recognize VDBP-bound 25(OH)D, which, furthermore, has been shown to remain associated with the VDBP inside the cell (doi: 10.1210/en.2012-2245).

To clarify this point and provide a comprehensive overview of VDBP's impact on intracellular 25(OH)D, we included in **Fig. 4F** of the revised manuscript the levels of **total** intracellular 25(OH)D measured using a total 25(OH)D ELISA assay. These data show detectable levels of total 25(OH)D after treatment with either 100 nM 25VD or 50 nM 25VD-VDBP, with proportions consistent with the lower 25VD content of the 25VD-VDBP complex.

Please note that the ELISAs routinely used in our laboratory, including the *Labor Diagnostika Nord 25-OH Vitamin D_3 Total ELISA kit* MS E-5800 and the *DiaSource 25OH Vitamin D Total ELISA KAP1971*), certify performance comparable to LC-MS (https://diasource-diagnostics.com/products/766-25oh-vitamin-d-total-elisa-96-tests).

We would like to emphasize, however, that even in conditions where the total 25(OH)D increases, the rise in ROS levels (Fig. 4D) and myotube atrophy (Fig. 4E) persist, demonstrating that the presence of 25(OH)D, whether free or bound, does not counteract the atrophic effect of VDBP, which is our main point in stating that the observed VDBP effects are independent of vitamin D status.

5- Given the importance of 25(OH)D status for study interpretation, the authors should ideally use LC-MS which has the requisite sensitivity and specificity to report 25(OH)D and 1,25(OH)2D.

We agree with the Reviewer that LC-MS is superior to other methods in terms of sensitivity and specificity for quantifying vitamin D metabolites. We analyzed the remaining blood and muscle samples from WT and *Gc* KO mice in the cachexia experiment using LC-MS and presented the results in the supplemental panel of **Fig. 7C**. We prioritized LC–MS quantification of total 25VD over 1,25VD, because total 25VD is the established indicator of vitamin D status in vivo, and because the remaining sample material was limited.

We verified that blood levels of total 25VD in KO vs. WT are similar to those previously reported in the literature, and that the presence of the LLC tumor does not alter the levels within the specific genotype, as previously reported (10.18632/oncotarget.15583).

We are confident that we have provided sufficient evidence that the molecular mechanisms through which VDBP acts on skeletal muscle are independent of vitamin D. Consistently, in vivo, VDBP KO mice, which have lower vitamin D levels, should be more prone to muscle wasting; however, they are actually protected, again demonstrating that vitamin D levels do not play a central role in this particular context.

Re: Point-by-Point Rebuttal for Manuscript NCOMMS-24-23151B "Vitamin D binding protein induces skeletal muscle atrophy and contributes to cancer-associated muscle wasting" by T. Raiteri et al.

In black: Reviewer's comments

In blue: our response

The authors provide new data showing that the effects of VDBP on myotube atrophy are independent of 25(OH)D status. Thank you for this. The authors are often too certain about their conclusions, and it would be good to see various caveats inserted into the discussion e.g. intramuscular injection = high local VDBP levels, which might not reflect pathophysiological changes in circulating VDBP observed during cachexia; differences between extracellular versus intracellular VDBP levels and action; lack of human genetic support for a muscle phenotype etc. The cancer cachexia findings might also be related to the fact that VDBP increases energy expenditure, so loss of VDBP preseves muscle mass through indirect mechanisms. In light of this, and to better reflect the findings, the title should be changed to: "Local injection of Vitamin D binding protein induces skeletal muscle atrophy whereas its systemic deletion is muscle sparing". Or similar.

We thank the Reviewer for the comments. Some of the highlighted caveats were already addressed in the manuscript's discussion (e.g., "The substantial loss of muscle mass in WT compared to Gc KO mice can be a consequence of the pro-inflammatory activity of VDBP, which can induce selective recruitment of neutrophils[5]."). However, in the revised manuscript's Discussion, we softened the wording of some conclusions and added a final paragraph outlining the most important limitations of the study, including some proposed by the Reviewer. All the points raised by the Reviewer are discussed below.

1. intramuscular injection = high local VDBP levels, which might not reflect pathophysiological changes in circulating VDBP observed during cachexia

The intramuscular VDBP injections were not performed to mimic pathological changes in circulating VDBP under disease conditions such as cachexia. Instead, our goal was to demonstrate that VDBP is both necessary and sufficient to cause muscle wasting in vivo. While the "necessary" condition was achieved by inducing cachexia in *Gc* KO mice (an experiment that indeed shows that the presence of VDBP is necessary to lose muscle mass), the injections served as a tool to establish the "sufficient" condition: adding VDBP where it was absent induced atrophy. Therefore, the experiment was explicitly designed to minimize any potential systemic effects of VDBP.

2. differences between extracellular versus intracellular VDBP levels and action

We agree that extracellular and intracellular VDBP pools may vary in abundance and function. In vitro, the loss of the atrophic response upon megalin silencing supports an uptake-dependent mechanism, indicating an intracellular site of action. However, we did not directly measure extracellular versus intracellular VDBP pools

in vivo and therefore cannot definitively rule out additional extracellular roles under pathophysiological conditions. We have updated the Discussion to clarify this distinction.

3. lack of human genetic support for a muscle phenotype

We agree that our study does not offer direct human genetic evidence linking VDBP variation to a skeletal muscle phenotype. However, key mechanistic findings were confirmed in human myotubes, providing experimental support for the relevance of circulating VDBP alterations in human conditions. However, establishing whether Gc variation or circulating VDBP predicts muscle loss and functional decline in patients will require targeted human genetic and clinical studies. We have revised the Discussion accordingly.

4. The cancer cachexia findings might also be related to the fact that VDBP increases energy expenditure, so loss of VDBP preserves muscle mass through indirect mechanisms

We agree that the lack of VDBP preserves skeletal muscle in cancer cachexia through both direct and indirect mechanisms, and we clearly stated this in the manuscript. Nevertheless, in the LLC-induced cachexia model we used, indirect calorimetry did not reveal genotype-dependent differences in energy expenditure at baseline or during tumor burden (**Supplementary Fig. 4: EE increased equally in WT and Gc KO tumor-bearing mice compared to controls**). Therefore, altered energy expenditure is unlikely to represent the primary mechanism for muscle mass preservation in our experimental conditions. To our knowledge, it has recently been reported that the absence of VDBP is associated with increased energy expenditure, but this evidence pertains to high-fat diet-fed mice (10.1172/jci.insight.195341), an unrelated non-cachectic setting not directly comparable to our experimental model.

5. To better reflect the findings, the title should be changed to: "Local injection of Vitamin D binding protein induces skeletal muscle atrophy whereas its systemic deletion is muscle sparing". Or similar.

We thank the Reviewer for suggesting a title change. Although we disagree with the need to change the title to emphasize only two specific experiments, as this would understate the broader significance and overall message of the study, we appreciate the suggestion and will revise the title to highlight that the observed effects of VDBP are independent of vitamin D status.