

# Cardiomyocyte OTUD1 drives diabetic cardiomyopathy via directly deubiquitinating AMPK $\alpha$ 2 and inducing mitochondrial dysfunction

Corresponding Author: Professor Guang Liang

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript describes the identification and characterization of the role of OTUD1 in diabetic cardiomyopathy. The study initiated with scRNA-seq of single heart cells from wild type mice and a T2DM mouse model. The study identified OTUD1 as the most upregulated DUB, which was then validated and studied in detail. The studies show that OTUD1 binds AMPK $\alpha$ 2 and removes K63 linked ubiquitin chains from two lysines. This is consistent with reported ubiquitin linkage preferences of OTUD1. Mutagenesis studies confirm the requirement of the catalytic cysteine of OTUD1. Functionally, reversing these effects of OTUD1 alleviated diabetic cardiomyopathy. Overall this is a strong study that includes both mechanistic and efficacy studies, and in general multiple experiments to corroborate most results.

There are some minor concerns/requests prior to publication.

Fig 1. Please comment on whether other OTU DUBs are similarly overexpressed and if so, confirm they are not modulated in KD studies.

Fig. 4. Given diabetic OTUD1 mice cardiac cells have higher ROS, and DUB catalytic cysteines can be inactivated by oxidation, was the possible oxidation, or resistance to oxidation, of OTUD1 considered as potentially relevant.

Fig. 6. It is hard to tell from the description and representation of co-IP results in panels a-b how significantly AMPK $\alpha$ 2 was enriched and whether other relevant proteins were also identified. A volcano plot would enable easier visualization of results.

Fig. 6. The domains of AMPK $\alpha$ 2 important for interaction with OTUD1 was investigated but the requisite domains of OTUD1 for the interaction were not and would add to understanding of the role of OTUD1 and how to think about perturbing the interaction between the two proteins. Along similar lines, is OTUD1 phosphorylation/dephosphorylation part of the mechanism.

Reviewer #2

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for addressing my questions and concerns. Recommend for publication.

Reviewer #2

(Remarks to the Author)

I am satisfied with the Authors responses and have no further question.

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## Responses to the Reviewers' comments

**Title:** Cardiomyocyte OTUD1 drives diabetic cardiomyopathy via directly deubiquitinating AMPK $\alpha$ 2 to inhibit AMPK activity and induce mitochondrial dysfunction

**Manuscript Number:** NCOMMS-24-76628

### Reviewer #1:

**Synopsis:** This manuscript describes the identification and characterization of the role of OTUD1 in diabetic cardiomyopathy. The study initiated with scRNA-seq of single heart cells from wild type mice and a T2DM mouse model. The study identified OTUD1 as the most upregulated DUB, which was then validated and studied in detail. The studies show that OTUD1 binds AMPK $\alpha$ 2 and removes K63 linked ubiquitin chains from two lysines. This is consistent with reported ubiquitin linkage preferences of OTUD1. Mutagenesis studies confirm the requirement of the catalytic cysteine of OTUD1. Functionally, reversing these effects of OTUD1 alleviated diabetic cardiomyopathy. **Overall, this is a strong study that includes both mechanistic and efficacy studies, and in general multiple experiments to corroborate most results.**

There are some minor concerns/requests prior to publication.

**Reviewer #1-1:** Fig 1. Please comment on whether other OTU DUBs are similarly overexpressed and if so, confirm they are not modulated in KD studies.

**Response:** Thanks. To address this concern, we analyzed the expression levels of OTU subfamily members in T2DM heart tissues using the scRNA-sequence data. As demonstrated in the new Supplemental Table 1, *Otud1* gene exhibited the highest upregulation (Log2FC [T2DM/Ctrl] = 1.14) and the most predominant expression in cardiomyocytes among all OTU subfamily members. Therefore, we focused on OTUD1 in this study. This information has been added to the “Results” section in the revised manuscript.

#### New Supplemental Table 1

| Gene name      | Log2FC<br>(T2DM/Ctrl) | Expression in CMs<br>(%) |
|----------------|-----------------------|--------------------------|
| <i>Otud1</i>   | 1.14                  | 39.42                    |
| <i>Tnfaip3</i> | 0.84                  | 0.40                     |
| <i>Yod1</i>    | 0.67                  | 16.29                    |
| <i>Otud4</i>   | 0.62                  | 16.43                    |
| <i>Zranb1</i>  | 0.53                  | 13.79                    |
| <i>Otulin</i>  | 0.49                  | 14.34                    |
| <i>Otud6b</i>  | 0.23                  | 10.40                    |
| <i>Zranb3</i>  | 0.13                  | 18.43                    |
| <i>Otud7b</i>  | 0.12                  | 17.22                    |

**Reviewer #1-2:** Fig. 4. Given diabetic OTUD1 mice cardiac cells have higher ROS, and DUB catalytic cysteines can be inactivated by oxidation, was the possible oxidation, or resistance to oxidation, of OTUD1 considered as potentially relevant.

**Response:** Thank you. Accordingly, we evaluated the deubiquitinating activity of OTUD1 using an assay kit containing a fluorescent universal ubiquitin substrate (Ubiquitin-Rhodamine 110) in NRPCs with or without HG+PA incubation for 24 h. As shown in the new Supplementary Fig. 1b, OTUD1's deubiquitinase activity was significantly increased (about 1.7-fold) in HG+PA-challenged NRPCs than that in the control NRPCs. On the other side, we also noted that HG+PA stimulation for 24 h induced a 1.7-fold increase in OTUD1 protein level in NRPCs (Fig. 1j). These results suggests that HG+PA-increased deubiquitinating activity of OTUD1 mainly attributes to the increased protein level of OTUD1, while the ROS level under HG+PA environment may have a minimal impact on the deubiquitinating activity of OTUD1 in cardiomyocytes.

New Supplementary Fig. 1b

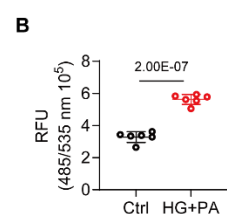
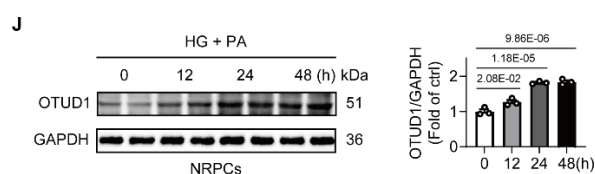


Fig. 1J



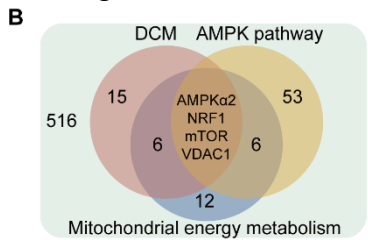
**Reviewer #1-3:** Fig. 6. It is hard to tell from the description and representation of co-IP results in panels a-b how significantly AMPKα2 was enriched and whether other relevant proteins were also identified. A volcano plot would enable easier visualization of results.

**Response:** Thank you. To identify the specifically targeted substrate of OTUD1 in cardiomyocytes, we performed immunoprecipitation and mass spectrometry analysis (Fig. 6a). We obtained 608 proteins potentially binding to OTUD1. Since OTUD1 regulates diabetic cardiomyocyte hypertrophy through modulation of AMPK pathway and mitochondrial energy metabolism, we checked the 608 potential OTUD1-binding proteins and found that, among these 608 ones, only 4 proteins have been reported to be related with DCM, AMPK pathway, and mitochondrial energy metabolism, including AMPKα2 (*Curr Med Chem*, 2019, 26: 5207-5229; *Redox Biol*, 2018, 15: 335-346), NRF1 (*Exp Mol Med*, 2023, 55: 767-778; *Free Radic Biol Med*, 2025, 227: 210-220), mTOR (*Tissue Cell*, 2024, 90:102478; *Mol Med Rep*, 2025, 31: 64), and VDAC1 (*Int J Cardiol*, 2023, 372: 101-109; *Phytomedicine*, 2024, 128: 155313; *J Neurotrauma*,

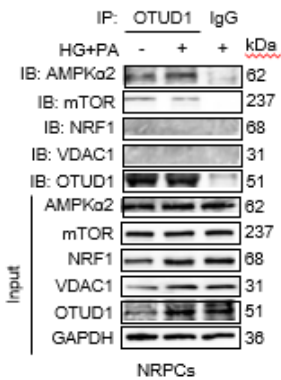
2022, 39: 560-576). Please see the data in the new Fig. 6b.

We then confirmed the interactions between these 4 potential substrates and OTUD1 in NRPCs, respectively. We found that only AMPK $\alpha$ 2 and mTOR bound to OTUD1, but NRF1 or VDAC1 showed no obvious interaction with OTUD1 in NRPCs (new Supplementary Fig. 7a). Notably, the interaction of AMPK $\alpha$ 2-OTUD1 but not mTOR-OTUD1 was enhanced by HG+PA stimulation in NRPCs (new Supplementary Fig. 7a), suggesting that AMPK $\alpha$ 2-OTUD1 interaction in cardiomyocytes may be more related to DCM progress. Therefore, we selected AMPK $\alpha$ 2 as the direct substrate of OTUD1 for further investigation. These data have been provided in the revised “Results” section.

New Fig.6b



New Supplementary Fig.7a

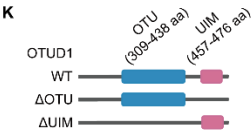


**Reviewer #1-4:** Fig. 6. The domains of AMPK $\alpha$ 2 important for interaction with OTUD1 was investigated but the requisite domains of OTUD1 for the interaction were not and would add to understanding of the role of OTUD1 and how to think about perturbing the interaction between the two proteins. Along similar lines, is OTUD1 phosphorylation/dephosphorylation part of the mechanism.

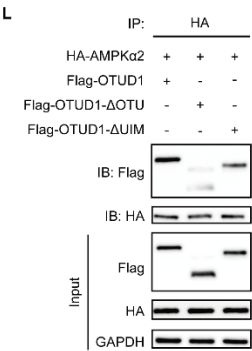
**Response:** Thank you. To identify the OTUD1 protein domains responsible for AMPK $\alpha$ 2-OTUD1 interaction, truncated mutants of OTUD1 were constructed. As shown in new Fig. 6k, OTUD1 protein contains an ovarian tumor (OTU) domain and a ubiquitin-interacting motif (UIM). The immunoprecipitation assay showed that AMPK $\alpha$ 2 interacted with the OUT domain of OTUD1 (new Fig. 6l). These new data have been provided in the revised “Results” section.

New Fig.6k-1

**K**



**L**



**Reviewer #2:**

**Synopsis:** The authors examined the role of a deubiquitinating enzyme (DUB), ovarian tumor domain containing protein (OTUD1) in diabetic cardiomyopathy. In hearts from mouse with diabetes, they found an increased OTUD1 and knockout of this enzyme prevented cardiac hypertrophy and dysfunction in both T1D and T2 mouse with diabetes by restoring cardiac AMPK activity and mitochondrial function. In vitro, OTUD1 caused mitochondrial injuries and hypertrophy in cardiomyocytes by negatively influencing AMPK activity by directly binding to the kinase domain of the AMPK $\alpha$ 2 subunit and deubiquitinating it. The study concludes that this is a new OTUD1-AMPK axis that drives diabetic cardiomyopathy (DCM) and modulating this interaction may be an effective strategy for diabetic cardiomyopathy.

**Reviewer #2-1:** In general, the paper was well written, with a significant amount of data generated.

**Response:** Thank you for your positive comment.

**Reviewer #2-2:** The introduction has a statement which says “Although conventional cardiovascular drugs are available for the management of DCM”. Is that really true. Treatment with GLP-1 receptor agonists and SGLT2 inhibitors have cardioprotective effects in T2D patients, but I am not sure if they are used for the management of DCM (or in T1D).

**Response:** Thank you. We apologize for the confusing expression. In fact, the clinical treatment of DCM primarily focuses on blood glucose control and improving heart function rather than directly treating DCM itself. As you mentioned, GLP-1 receptor agonists and SGLT2 inhibitors have shown cardioprotective effects in T2D patients. However, they are not currently considered standard treatments for DCM, although they are recommended in some diabetic treatment guidelines in cases of diabetes with concomitant heart diseases (*Drugs*, 2024, 84: 1347-1364; *Diabetes Care*, 2025, 48: S207-S238). We have changed the sentence “management of DCM” to “management of heart failure in diabetic patients” in the “Introduction” section.

**Reviewer #2-3:** One of the premises of this study is that diabetic mice have reduced AMPK activity in their heart with mitochondrial instability and cardiac dysfunction. Yet metformin, a classical drug that is used to treat T2D patients, and is a known AMPK activator, is really cardiac neutral. It was difficult for me to reconcile the impact of AMPK and DCM keeping this in mind.

**Response:** Thank you. The cardiac neutral effect of metformin is primarily attributed to its dose-dependent pharmacokinetics and multi-target mechanisms. After oral administration, metformin exhibits preferential distribution in the liver, kidneys, and gastrointestinal tract, with limited cardiac tissue penetration (*Clin Pharmacokinet*, 2011, 50: 81-98; *Trends Pharmacol Sci*, 2020, 41: 868-881). This limited cardiac distribution, coupled with its rapid systemic clearance (plasma half-life = 2.7 h; *Diabet Med*, 2016, 33: 105-10), results in subtherapeutic concentrations and insufficient exposure duration of metformin in cardiac tissue to exert direct cardioprotective effects.

Furthermore, metformin has multiple molecular targets in addition to AMPK, including mitochondrial complex I, protein phosphatase 2A (PP2A), and TNF- $\alpha$  signaling pathways. These diverse targets mediate multiple biological processes, ranging from regulating mitochondrial function and gluconeogenesis, anti-cancer, and anti-inflammation (*Cancer Cell*, 2019, 35: 798-815; *Redox Biol*, 2024, 75: 103266; *Sci Adv*, 2024, 10: 5466). The pleiotropic nature of metformin suggests that its effects on hearts are possibly mediated through AMPK-independent pathways, potentially involving systemic metabolic modulation and indirect cardioprotective mechanisms (*Nat Rev Endocrinol*, 2023, 19: 460-476; *Trends Pharmacol Sci*, 2020, 41: 868-881; *Diabetologia*, 2016, 59: 426-35).

In addition, the p-AMPK level is typically decreased in diabetic mouse hearts, and we summarized them in the following Table R1. It has reported that fibroblast growth factor 1 (FGF1) prevents DCM via activating AMPK, while these cardioprotective effects are blunted in the AMPK $\alpha$ 2 null mice (*Circulation*, 2022, 146: 1537-1557; *Signal Transduct Target Ther*, 2021, 133). Therefore, targeting AMPK activation in cardiomyocytes represents a promising strategy for the treatment of DCM. We have discussed this point in the revised manuscript.

Table R1. The AMPK levels in heart tissues of DCM model:

| Model                                  | Animals            | Molding cycle | p-AMPK profile            | Reference                                      |
|----------------------------------------|--------------------|---------------|---------------------------|------------------------------------------------|
| T2DM<br>(HFD + STZ, 35 mg/kg, 3 times) | C57BL/6 mouse      | 12 weeks      | p-AMPK protein ↓          | PMID: 36134579,<br><i>Circulaiton</i> .        |
| T2DM<br>(HFD + STZ, 75 mg/kg, once)    | C57BL/6 mouse      | 16 weeks      | p-AMPK protein ↓          | PMID: 35909160,<br><i>Sig Trans Tar Ther</i> . |
| T2DM                                   | <i>db/db</i> mouse | 26 weeks      | p-AMPK protein ↓          | PMID: 33762571,<br><i>Sig Trans Tar Ther</i> . |
| T2DM                                   | <i>db/db</i> mouse | 28 weeks      | p-AMPK protein ↓          | PMID: 38218357,<br><i>Pharmacol Res</i> .      |
| T2DM                                   | <i>db/db</i> mouse | 12 weeks      | p-AMPK protein ↓          | PMID: 38222788,<br><i>World J Diabetes</i> .   |
| T1DM<br>(STZ, 50 mg/kg, 5 times)       | C57BL/6 mouse      | 16 weeks      | p-AMPK $\alpha$ protein ↓ | PMID: 37625569,<br><i>J Adv Res</i> .          |
| T1DM<br>(STZ, 50 mg/kg, 5 times)       | C57BL/6 mouse      | 8 weeks       | p-AMPK protein ↓          | PMID: 31182921,<br><i>Int J Biol Sci</i> .     |
| T1DM<br>(STZ, 50 mg/kg, 5 times)       | C57BL/6 mouse      | 18 weeks      | p-AMPK protein ↓          | PMID: 35176461,<br><i>Bio Bi Acta Mol</i> .    |

**Reviewer #2-4:** High glucose and palmitic acid stimulate OTUD1 in rat neonatal primary cardiomyocytes. Much of the work is done in mouse hearts yet this experiment is done using rat tissue. Why?

**Response:** Thank you. We employed neonatal rat primary cardiomyocytes (NRPCs) for *in vitro* experiments based on their well-established advantages in cardiovascular research. This selection was supported by the following scientific and technical considerations:

(1) Experimental Suitability and Robustness

NRPCs are extensively utilized in cardiac research due to their superior survival rates

and high viability under culture conditions. Compared to mouse cardiomyocytes, NRPCs exhibit greater ease of isolation and maintenance, as mouse cardiomyocytes are often limited by technical challenges, resulting in lower yields of viable cells.

(2) Enhanced Reproducibility

The protocols for isolating and culturing NRPCs are more standardized and reproducible across laboratories. This ensures consistent experimental outcomes and facilitates better comparability with published studies.

(3) Practical Feasibility

Conducting *in vitro* studies using primary mouse cardiomyocytes presents significant technical challenges. For the same number of animals, the yield of viable cells from mouse hearts is substantially lower than that from rat hearts. Therefore, NRPCs were chosen to meet the experimental scale and reproducibility requirements essential for our study.

Therefore, it is widely accepted to do *in vitro* experiments using NRPCs in basic research in cardiology. To further substantiate our experimental approach, we have compiled a comprehensive list of previous publications supporting the combined use of NRPCs and mouse models in cardiac research (Table R2).

Table R2: The primary rat cardiomyocytes used in the cardiology study.

| Purpose                                                    | <i>In vitro</i> | <i>In vivo</i> | Related studies                                               |
|------------------------------------------------------------|-----------------|----------------|---------------------------------------------------------------|
| Diabetic cardiomyopathy                                    | NRPCs           | Mice           | <i>Circulation</i> , 2024, 149: 684-706<br>PMID: 37994595     |
| Cardiac ischemia/reperfusion injury study                  | NRPCs           | Mice           | <i>Nat Commun</i> , 2024, 15: 9667<br>PMID: 39516487          |
| The mechanisms of ferroptosis regulation in cardiomyocyte  | NRPCs           | Mice           | <i>Circulation</i> , 2023, 147: 1444-1460<br>PMID: 369879240  |
| Diabetic cardiomyopathy                                    | NRPCs           | Mice           | <i>Signal Trans Tar Ther</i> , 2022, 7: 259<br>PMID: 35909160 |
| Diabetic cardiomyopathy                                    | NRPCs           | Mice           | <i>Diabetes</i> , 2022, 71: 795-811<br>PMID: 35043173         |
| Doxorubicin-induced heart failure                          | NRPCs           | Mice           | <i>Eur Heart J</i> , 2022, 43: 4496-4511<br>PMID: 35758064    |
| Cardiomyocyte proliferation                                | NRPCs           | Mice           | <i>Cell Res</i> , 2021, 31: 450-462<br>PMID: 32973339         |
| Arrhythmias and explore the underlying molecular mechanism | NRPCs           | Mice           | <i>Circulation</i> , 2018, 138: 1330-1342<br>PMID: 296505430  |
| Antihypertrophic treatment in the heart                    | NRPCs           | Mice           | <i>Circulation</i> , 2011, 124: 2702-2715<br>PMID: 220826740  |

In addition, to further validate our findings in mouse cardiomyocytes, we also tried to isolate few neonatal mouse primary cardiomyocytes (NMPCs) and replicated some key experiments using NMPCs. Specifically, we conducted WB and RT-qPCR analyses to verify whether HG+PA induced OTUD1 overexpression and whether HG+PA-induced hypertrophic response in NMPCs involved OTUD1. As shown in the Fig. R1A-B, HG+PA stimulation upregulated OTUD1 expression in NMPCs in a time-dependent manner. The RT-qPCR analysis revealed that OTUD1 knockdown effectively attenuated HG+PA-induced hypertrophic responses in NMPCs (Fig. R1C).

Fig.R1

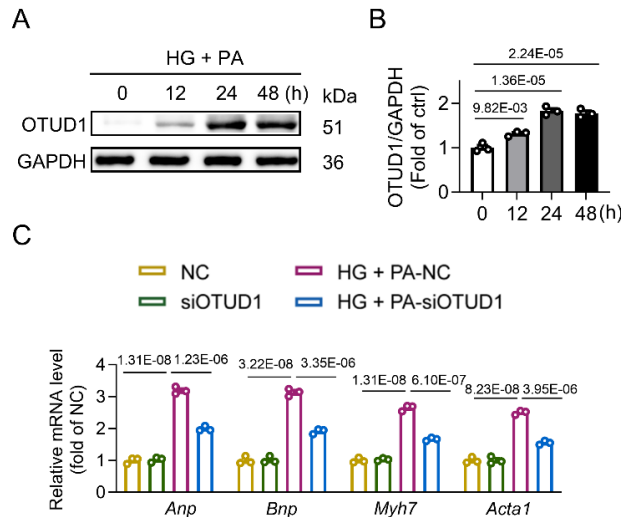


**Figure R1.** (A) The NMPCs were stimulated with HG (33 mM) and PA (100 μM) for different time points to detect OTUD1 expression. (B) Quantification of protein levels of OTUD1 in A. (C) The NMPCs transfected with siRNA targeting OTUD1 (siOTUD1) or scrambled negative control (NC) were stimulated with HG (33 mM) and PA (100 μM) for 24 h. RT-qPCR analysis of hypertrophic genes (*Anp*, *Bnp*, *Myh7*, *Acta1*) in NMPCs. Data are presented as mean ± SEM (n = 3); For B-C, *P* values were determined by one-way ANOVA followed by Tukey post hoc tests.

**Reviewer #2-5:** I was hard pressed to locate some experimental details. It would have been nice to see some of the phenotype of the *db/db* animals (blood glucose, insulin lipid profile, etc.). Plus, with the cardiomyocyte incubation studies, what were the glucose and FA concentrations? And why were you incubating with FA when I was not sure if the plasma levels of FA and triglycerides were actually increased in their diabetic models.

**Response:** Thank you. The phenotype of *db/db* mice, including serum insulin, total cholesterol, and triglyceride, has been examined as suggested. As shown in Supplementary Fig.8b-c, neither OTUD1 nor AMPKα2 knockdown affected the hyperglycemia and body-weight-gain profile in *db/db* mice. The serum levels of insulin, total cholesterol, and triglyceride indicate that AAV9-mediated OTUD1 knockdown in hearts significantly blunted diabetes-induced metabolic disorder in *db/db* mice (updated Supplementary Table 5). However, knockdown of OTUD1 failed to reduce these parameters in AAV9-shAMPKα2 treated diabetic mice (updated Supplementary Table 5). These data have been provided in the revised manuscript.

For cardiomyocyte stimulation, 33 mM D-glucose plus 100 μM palmitic acid (PA) served as the HG+PA group. The addition of exogenous glucose and PA as stimulator is commonly used to simulate the diabetic microenvironment, which is a widely recognized cellular model for diabetic complication studies (*Cardiovascular Diabetology*, 2023, 22: 25; *Journal of Agricultural and Food Chemistry*, 2024, 72: 10339-10354; *Cardiovascular Diabetology*, 2023, 22: 107).

We have added these experimental details in the revised manuscript.

Updated Supplemental Table 5

|                              | <i>db/db</i><br>AAV9-<br>Vector | <i>db/db</i><br>AAV9-<br>shOTUD1 | <i>db/db</i><br>AAV9-<br>shAMPK $\alpha$ 2 | <i>db/db</i><br>AAV9-<br>shAMPK $\alpha$ 2+shOTUD1 |
|------------------------------|---------------------------------|----------------------------------|--------------------------------------------|----------------------------------------------------|
| Serum insulin<br>(ng/mL)     | 13.5 $\pm$ 0.6                  | 7.5 $\pm$ 0.3*                   | 13.7 $\pm$ 0.5                             | 13.2 $\pm$ 0.7 <sup>#, ns</sup>                    |
| Total cholesterol<br>(mg/dL) | 162.1 $\pm$ 2.0                 | 100.8 $\pm$ 2.3*                 | 158.7 $\pm$ 2.5                            | 161.8 $\pm$ 2.1 <sup>#, ns</sup>                   |
| Triglyceride<br>(mg/dL)      | 59.8 $\pm$ 1.1                  | 36.8 $\pm$ 1.6*                  | 61.2 $\pm$ 1.6                             | 59.7 $\pm$ 1.7 <sup>#, ns</sup>                    |

Data presented as Mean  $\pm$  SEM. N = 6. \* $P$  < 0.05 compared to *db/db*-AAV9-Vector; <sup>#</sup> $P$  < 0.05 compared to *db/db*-AAV9- shOTUD1; <sup>ns</sup> $P$  > 0.05 compared to *db/db*-AAV9-shAMPK $\alpha$ 2; ns, no significance.

**Reviewer #2-6:** One of the models used a small-dose streptozotocin (STZ; 35 mg/kg) combined with high fat diet (HFD) feeding. What was the route of administration of STZ and what was the composition of the high fat (palmitic acid, oleic acids, etc.). Were these animals' insulin resistant?

**Response:** Thank you. The mice received STZ (35 mg/kg) by intraperitoneal injection for 3 consecutive days. The high-fat diet consists of 60.3 kcal% fat, which contains palmitic acid, oleic acid, stearic acid, linoleic acid, and  $\alpha$ -linolenic acid. In addition, the levels of serum insulin were detected in STZ combining HFD feeding mice. OTUD1<sup>fl/fl</sup> T2DM mice showed increased serum insulin levels compared to OTUD1<sup>fl/fl</sup> sham mice (updated Supplementary Table 2). This information has been added in the revised manuscript.

Updated Supplemental Table 2

|                          | WT-Sham         | OTUD1 CKO-Sham  | WT-T2DM          | OTUD1 CKO-T2DM               |
|--------------------------|-----------------|-----------------|------------------|------------------------------|
| Serum insulin<br>(ng/mL) | 0.58 $\pm$ 0.02 | 0.56 $\pm$ 0.02 | 0.81 $\pm$ 0.02* | 0.73 $\pm$ 0.01 <sup>#</sup> |

Data are presented as Mean  $\pm$  SEM. N = 6. \* $P$  < 0.05 compared to WT-Sham; <sup>#</sup> $P$  < 0.05 compared to WT-T2DM.

**Reviewer #2-7:** “The T1DM mouse model was established using high-dose STZ injection (120 mg/kg)”. I was surprised by this result. Generally, when using a single high dose of STZ to induce diabetes in mice, 200 mg/kg has been found to be directly toxic to pancreatic  $\beta$ -cells, rapidly causing diabetes, with blood glucose concentrations of >500 mg/dl within 48 hr (Like & Rossini, 1976). This is because mice are generally resistant to STZ-induced diabetes and thus the higher dose.

**Response:** Thank you. In the last decades, a lot of publications have reported that a single injection of STZ at 100-120 mg/kg is widely used to effectively induce T1DM in mice (*Nat Commun*, 2020, 11: 2148; *Theranostics*, 2019, 9: 4287-4307; *Journal of Hepatology*, 2014, 61: 1328-1336; *International Immunopharmacology*, 2023, 124: 110984; *Reproductive Biology and Endocrinology*, 2022, 20: 45).

**Reviewer #2-8:** Many of the figure legends lacked experimental details. Concentrations

used, duration of incubations, etc. It would be helpful if these details were included in the appropriate figure legends.

**Response:** Thank you for your suggestion. All figure legends have been carefully checked, and experimental details have been provided in the revised manuscript.

**Reviewer #2-9:** In rats made diabetic with 55 mg/kg STZ and kept for 6-weeks, AMPK and ACC phosphorylation were comparable in control and diabetic hearts. Even when these rats were injected with 100 mg/kg STZ, and kept for 4-days, there was no difference in cardiac AMPK and ACC phosphorylation in diabetic rats compared to control. In fact in this paper, the only time AMPK phosphorylation changed was when AMPK was measured in acutely diabetic animals made diabetic with 55 mg/kg and kept for 4 days. AMPK and ACC phosphorylation were significantly higher in these particular diabetic hearts (J Mol Cell Cardiol 2007 Feb;42(2):333-42). So again, some discussion of these conflicting data should be provided.

**Response:** Thank you. Similar question is raised in Reviewer #2-3. Please see our response to **Reviewer #2-3**. We checked a large number of publications reporting AMPK activity changes in diabetic mouse hearts and summarized them in the Table R1.

In addition, diabetes (sustained hyperglycemia) for more than 4 weeks starts to induce cardiac complication in mice and the cardiac dysfunction appears after 8-week sustained hyperglycemia. We acknowledge that the changes of p-AMPK levels in diabetic heart tissues depend on the duration of diabetes. Generally, the p-AMPK level is typically decreased in diabetic mouse hearts at a relatively long disease duration (from 8 weeks to 28 weeks), when the hearts has been damaged by sustained hyperglycemia.

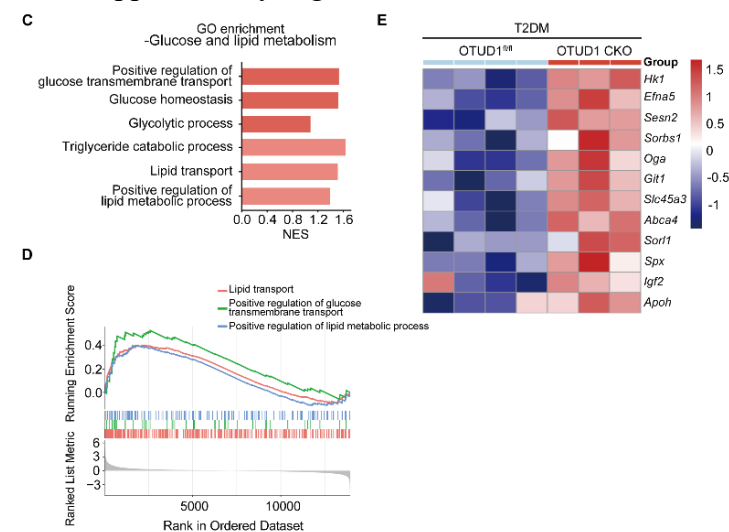
The cardiac AMPK activity is not affected during the early stage of diabetes when no cardiac complication occurs, while AMPK activity will be decreased during the later stage of diabetes when myocardial injury occurs. In other words, AMPK inactivation is a pathological feature in diabetic cardiomyopathy. We have discussed this point in the revised manuscript.

**Reviewer #2-10:** The more current concept is that pathological hypertrophy impairs cardiac energetics, shifting the substrate preference of the heart from FA to glucose, in addition to supplying glucose-derived aspartate for nucleotide and protein synthesis which drives hypertrophic growth of cardiomyocytes. Increasing FA oxidation rates reduces glucose utilization and its flux to anabolic precursors, provides for substantial ATP generation, and precludes pathological remodeling. What is the status of glucose utilization in your models of diabetes and following knockout of OTUD1?

**Response:** Thank you for raising this question. Our transcriptomic analysis may provide some evidences addressing this question. KEGG and GO enrichment analysis of our RNA-seq data showed significant enrichment of signaling pathways related glucose and lipid metabolism (new Supplementary Fig. 5c). GSEA further found upregulation of AMPK, and glucose/lipid metabolic and transport pathway in OTUD1-deficient diabetic hearts (NES > 1, *P* value < 0.05; Fig. 4a, and new Supplementary Fig. 5d). The expressed levels of genes regulating glucose and lipid metabolism in diabetic

hearts were changed by OTUD1 knockout (new Supplementary Fig. 5e). To substantiate these findings, we assessed the protein expression of key metabolic regulators in mouse heart tissues following 16 weeks of HFD intervention. Acetyl-CoA carboxylase (ACC), a downstream target of AMPK, is key regulator for fatty acid oxidation (*Cell Death Dis*, 2018, 9: 227). Hexokinase-1 (HK-1), known as the rate-limiting enzyme of glycolysis and subsequent glucose metabolism, plays a pivotal role in maintaining glucose homeostasis (*Trends Endocrinol Metab*, 2022, 33: 665-667). As expected, T2DM mice exhibited significant decreases in the phosphorylation levels of cardiac AMPK and ACC, and HK-1, while OTUD1 deficiency in cardiomyocytes reversed these changes in diabetic hearts, indicating OTUD1 CKO improves lipid/glucose metabolism and cardiac energetics (updated Fig. 4b). Moreover, we found that HG+PA-challenged cardiomyocytes had lower phosphorylated levels of AMPK<sup>T172</sup> and ACC, and HK-1 expression, while gene silencing of OTUD1 abrogated this alteration (updated Fig. 5a and Supplementary Fig. 6c). Not surprisingly, OTUD1 overexpression further decreased the levels of p-AMPK<sup>T172</sup>, p-ACC, and HK-1 in NRPCs challenged with HG+PA (updated Fig. 5a and Supplementary Fig. 6c). These data suggest that diabetic hearts exhibit dual metabolic insults, including disrupted fatty acid oxidation and impaired glucose utilization. OTUD1 deficiency both *in vivo* and *in vitro* enhanced both fatty acid oxidation capacity and glycolytic flux.

#### New Supplementary Fig. 5c-e



#### Updated Fig. 4b, 5a, and Supplementary Fig.6c

**Figure 4B**

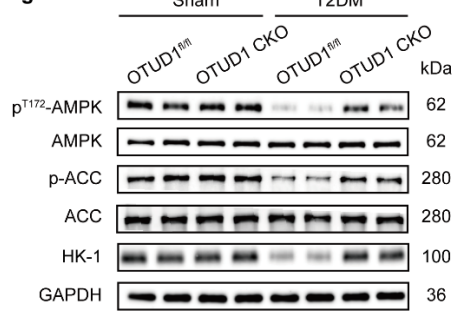
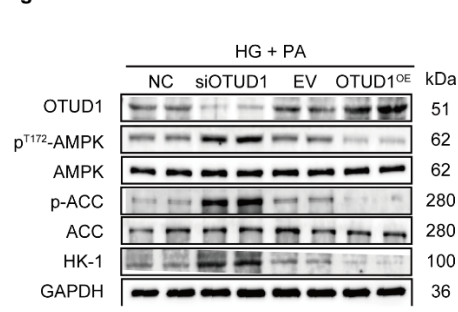
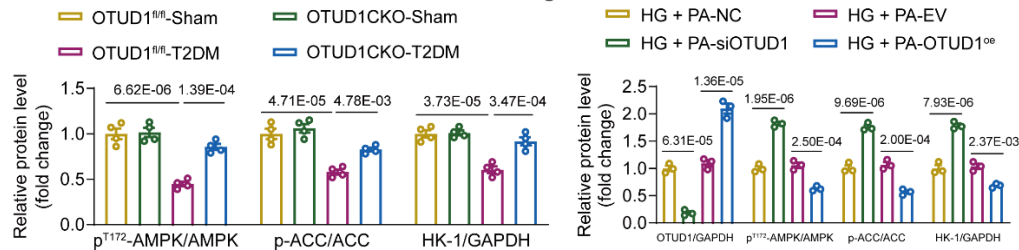


**Figure 5A**



**SFigure 6C**



**Reviewer #2-11:** I liked the experiments and the results obtained related to OTUD1 and binding to AMPKalpha2 and its contribution towards diabetic cardiomyopathy.

**Response:** Thank you very much.

**Reviewer #2-12:** The manuscript should be carefully proofread.

**Response:** Thank you. We have carefully proofread the manuscript, and all changes have been highlighted using red font.

## Summary

The authors examined the role of a deubiquitinating enzyme (DUB), ovarian tumor domain-containing protein (OTUD1) in diabetic cardiomyopathy. In hearts from mouse with diabetes, they found an increased OTUD1 and knockout of this enzyme prevented cardiac hypertrophy and dysfunction in both T1D and T2 mouse with diabetes by restoring cardiac AMPK activity and mitochondrial function. In vitro, OTUD1 caused mitochondrial injuries and hypertrophy in cardiomyocytes by negatively influencing AMPK activity by directly binding to the kinase domain of the AMPK $\alpha$ 2 subunit and deubiquitinating it. The study concludes that this is a new OTUD1-AMPK axis that drives diabetic cardiomyopathy (DCM) and modulating this interaction may be an effective strategy for diabetic cardiomyopathy.

## Evaluation

1. In general, the paper was well written, with a significant amount of data generated.
2. The introduction has a statement which says "Although conventional cardiovascular drugs are available for the management of DCM". Is that really true. Treatment with GLP-1 receptor agonists and SGLT2 inhibitors have cardioprotective effects in T2D patients, but I am not sure if they are used for the management of DCM (or in T1D).
3. One of the premises of this study is that diabetic mice have reduced AMPK activity in their heart with mitochondrial instability and cardiac dysfunction. Yet metformin, a classical drug that is used to treat T2D patients, and is a known AMPK activator, is really cardiac neutral. It was difficult for me to reconcile the impact of AMPK and DCM keeping this in mind.
4. High glucose and palmitic acid stimulate OTUD1 in rat neonatal primary cardiomyocytes. Much of the work is done in mouse hearts yet this experiment is done using rat tissue. Why?
5. I was hard pressed to locate some experimental details. It would have been nice to see some of the phenotype of the *db/db* animals (blood glucose, insulin lipid profile, etc.). Plus, with the cardiomyocyte incubation studies, what were the glucose and FA concentrations? And why were you incubating with FA when I was not sure if the plasma levels of FA and triglycerides were actually increased in their diabetic models.
6. One of the models used a small-dose streptozotocin (STZ; 35 mg/kg) combined with high fat diet (HFD) feeding. What was the route of administration of STZ and what was the composition of the high fat (palmitic acid, oleic acids, etc.). Were these animals' insulin resistant?
7. "*The T1DM mouse model was established using high-dose STZ injection (120 mg/kg)*". I was surprised by this result. Generally, when using a single high dose of STZ to induce diabetes in mice, 200 mg/kg has been found to be directly toxic to pancreatic  $\beta$ -cells, rapidly causing diabetes, with blood glucose concentrations of >500 mg/dl within 48 hr (Like & Rossini, 1976). This is because mice are generally resistant to STZ-induced diabetes and thus the higher dose.
8. Many of the figure legends lacked experimental details. Concentrations used, duration of incubations, etc. It would be helpful if these details were included in the appropriate figure legends.

9. In rats made diabetic with 55 mg/kg STZ and kept for 6-weeks, AMPK and ACC phosphorylation were comparable in control and diabetic hearts. Even when these rats were injected with 100 mg/kg STZ, and kept for 4-days, there was no difference in cardiac AMPK and ACC phosphorylation in diabetic rats compared to control. In fact in this paper, the only time AMPK phosphorylation changed was when AMPK was measured in acutely diabetic animals made diabetic with 55 mg/kg and kept for 4 days. AMPK and ACC phosphorylation were significantly higher in these particular diabetic hearts (J Mol Cell Cardiol 2007 Feb;42(2):333-42). So again, some discussion of these conflicting data should be provided.
10. The more current concept is that pathological hypertrophy impairs cardiac energetics, shifting the substrate preference of the heart from FA to glucose, in addition to supplying glucose-derived aspartate for nucleotide and protein synthesis which drives hypertrophic growth of cardiomyocytes. Increasing FA oxidation rates reduces glucose utilization and its flux to anabolic precursors, provides for substantial ATP generation, and precludes pathological remodeling. What is the status of glucose utilization in your models of diabetes and following knockout of OTUD1?
11. I liked the experiments and the results obtained related to OTUD1 and binding to AMPK $\alpha$ 2 and its contribution towards diabetic cardiomyopathy.
12. The manuscript should be carefully proofread.