nature portfolio

Peer Review File

ATP dynamics as a predictor of future podocyte structure and function after acute ischemic kidney injury in female mice

Corresponding Author: Professor Motoko Yanagita

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)
This study is dedicated to investigate the in vivo ATP dynamics using FRET-based ATP biosensor in ischemia reperfusion mouse model of acute kidney injury (AKI). The authors demonstrated that decreased ATP levels were recovered in podocytes after reperfusion in short-term ischemia, but long-term ischemia caused insufficient ATP recovery in podocytes, which correlated with increased levels of mitochondrial fission and foot processes effacement. Additionally, this study suggests using ATP dynamics as a marker of podocyte injury in AKI.
This is one of the top 5% studies, which clearly shows results and answers all the possible emerging questions while reading within the next figure. Together with the investigation of ATP dynamics in podocytes in AKI, the authors also demonstrated ATP changes in other compartments of the nephron and compared ATP changes in podocytes and endothelial cells of glomeruli. In general, this is an interesting study that significantly contributes to our understanding of podocyte injury in AKI. While I have no big concerns about this study, some minor concerns should be addressed.

1. Figure 8B shows MitoTracker red staining and claims that mitochondrial membrane potential was reduced in podocytes cultured with oligomycin A and 2-deoxy-D-glucose. I would suggest to measure quantitative mitochondrial membrane potential using TMRM probe instead.
2. The manuscript has a paucity of information on the inhibitor reagent used in the study. A revision should include the rationale for the agent chosen, the dosing regimen employed based on known mechanism, pharmacokinetics, protein binding, and IC50s. The known specificity of the inhibitor should also be discussed regarding off target effects, particularly those that may impact on the interpretation of your findings. In the methods, please add information on the longevity of Midivi-1 treatment.
3. The authors showed that ATP depletion and mitochondrial injury are the main contributors to podocyte foot processes effacement and that no significant podocyte loss has been observed in the chronic phase of ischemic reperfusion. However, it would be important to at least discuss the phenomenon of podocyte death absence in the given experimental conditions as mitochondrial dysfunction is known to have a significant impact on cell survival.

Reviewer #2

(Remarks to the Author)
The MS by Yanagita and co-workers reports an in vitro and in vivo investigation of intracellular ATP changes during ischemia in kidney podocytes. The Authors took advantage of a genetically-modified mouse systemically expressing a genetically-encoded FRET-based sensor of intracellular ATP. Thanks to this experimental model, they were able to in vivo measure by multyphoton microscopy intracellular ATP during ischemia and in the post-ischemic recovery phase, and to correlate ATP levels to podocyte morphology and to mitochondrial structure in vitro. This study exploits a range of state-of-the-art techniques and sophisticated animal models to address an important pathophysiological issue, and the results might have important practical implications.
My main concerns are detailed below.

1. Podocyte ATP levels are shown as ratios, in most cases between 0.4 and 2.5. I understand that an in vivo calibration to translate ratios into the actual ATP (mM)concentration is very difficult, if not impossible, yet I believe that an in vitro

calibration would at least provide a gross indication of the range of ATP changes under these different experimental conditions.

2. On the same line of reasoning, it would be helpful showing the effects on the ATP ratio of known mitochondrial uncouplers, such as for example FCCP. This is important because this would provide a demonstration of how the experimental model reacts to agents causing a well predictable effect. For example, FCCP could be administered via the renal artery after exteriorization of the kidney, or intraperitoneally via the same route as Mdivi-1. After this procedure the mouse should be of course euthanized, but this would provide an indication of the response of the GO-Ateam2 biosensor to known ATP perturbing agents.

3. It would be helpful if changes in fluorescence shown in Fig. 1D were quantitated with an usual image analysis program.

4. Fig. 3, A and B, reportedly shows ATP changes in the podocytes. Image magnification, especially in A, is very low, thus I wonder how podocytes were discriminated from the other cellular components of the glomeruli. I must say that even the Nphs 1-Ateam pictures shown in Fig. 5 are not terribly informative. Just as a control, why the Authors don't show images of kidney tubules from the Nhps 1-Ateam and the Tie2-Ateam mice? This should provide a clear discrimination with the kidney tubules being fluorescent only in the Tie2-Ateam mouse.

5. It is difficult to appreciate the "ATP hyper-recovery" (pg 7, line 1) from the images shown in Fig. 4. I fear that the Authors overinterpreted their data.

6. At pg 7, lines 12 and following, the Authors state that they were able to identify cells with lower intracellular ATP levels, and lower GO-Ateam2 expression, that they assign to a different lineage than the podocytes. This is not clear to me. If GO-Ateam2 is systemically expressed, I would tink that all glomerular cells should express it to more or less the same level.

7. Again, at pg 7, bottom lines, the Authors conclude that the cells that undergo slow ATP decline after ischemia are neither endothelial cells nor podocytes, thus they are presumably mesangial cells. I think that the Authors should discuss how this would affect overall intracellular ATP changes in the glomerulus given the equivocal identification of the cellular source of the GO-Ateam2 signal.

8. I think that TEM pictures showing mitochondrial fragmentation in Fig. 6 should be implemented with a wide size and higher resolution image showing a detailed mitochondrial structure (e.g. christae).

9. Images showing mitochondrial fragmentation showed in Fig. 8, B and C, are not very informative. Using MitoTracker Red to investigate mitochondrial morphology is not the best technical approach because, being MitoTracker Red potential sensitive, fully depolarized mitochondria will be weakly (or not at all) fluorescent and thus escape fragmentation analysis. I wonder why they did not use the potential-insensitive MitoTracker Green.

10. I am not convinced from the data supporting the reversal of mitochondrial fragmentation by Mdivi-1. Despite the commendable quantitation of mitochondrial roundness and foot process width shown in Fig. 8G, I am not satisfied with the images shown in Fig. 8C. I cannot appreciate any clear differences between panels a, c and b. Better and more informative images should be provided.

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors demonstrate that ATP depletion leading to mitochondrial fragmentation in podocytes leads to podocyte effacement after ischemic AKI. They provide supporting data using FRET-based ATP biosensor as well as cell culture studies using immortalized mouse podocytes. While there is some interesting data provided in this manuscript, there are several major concerns that significantly dampen the overall enthusiasm for this proposal.

Major Comments:
1) A major concern is the lack of novelty as other laboratories have demonstrated that mitochondrial fragmentation exacerbated podocyte injury – ex. – Wang et al. Cell metabolism 2012.
2) While there is likely mitochondrial fragmentation that might occur in podocytes, the biological significance in contributing to significant proteinuria is a major concern in this IRI model. Furthermore, the proteinuria shown in Figure 1 is not biologically significant as it relates to podocyte injury—could be potentially as a result of injured proximal tubule's ability to reuptake albumin.
3) Are there any endothelial changes in Figure 1?
4) Since there is a difference in level of fluorescence for imaging glomeruli, where all the glomeruli imaged at the same level in Figure 3?
5) Specificity of ATP fluorescence in DT and CD is not provided with costaining, would demonstrate specificity.
6) Most IRI models are extended to 60 min – raises concern for validity of duration of IRI as representative of previously reported IR models.
7) In Figure 5, there is no evaluation of mesangial cells – which also contribute to the changes in ATP observed. Also, the duration of IRI is unclear in Figure 5.
8) Figures 6 and 7 are not consistent in their level of mitochondrial roundness observed for 30 minutes. Also, is there any biochemical evidence of mitochondrial fragmentation specificity to podocytes (i.e., isolation of podocytes and measuring p-DRP1 levels)?

9) Immortalized podocytes are not ideal to assess for changes in mitochondrial fission, primary cultured podocytes would be more representative. In addition, energy utilization has been previously reported to be different in immortalized podocytes as compared to primary podocytes.
10) The use of Mdivi-1 is not specific to podocytes and improvements might not be a direct result of preventing mitochondrial fragmentation specifically in podocytes.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised version of the manuscript, the authors have adequately addressed the original concerns and I don't have any more comments.

Reviewer #2

(Remarks to the Author)
No further comments.

Reviewer #3

(Remarks to the Author)
In this revision, the authors demonstrate that ATP depletion leading to mitochondrial fragmentation in podocytes leads to podocyte effacement after ischemic AKI. They provide key supporting data using FRET-based ATP biosensor as well as cell culture studies using immortalized mouse podocytes. While the authors have demonstrated novelty in the use of FRET-based ATP biosensor to study mitochondrial dynamics, there are still some major concerns:

1. While the authors respond that this is the first study to demonstrate that mitochondrial fragmentation exacerbates podocyte injury post IRI (AKI model), the biological significance remains still a concern. They now reframe the discussion to include “moderate” albuminuria which is still an overreach with an increase from 40 to 70 mg/g. Furthermore, the expression levels of the canonical podocyte markers are minimally reduced (figure 1). Additionally, in Figure 1, the authors only show albuminuria and podocyte marker data for one time point post-IRI (45 min). It would be more convincing to show albuminuria levels at shorter and longer times post-ischemia and reperfusion phase (figure 4) to demonstrate the biological significance.

2. In this revision, the authors provide evidence of the protective effects of Drp1 ko in cultured podocytes, which has been previously reported (Wang et al. Cell metabolism 2012), thereby reducing the novelty. Ultimately, demonstrating that conditional knockdown of Drp1 or mutant pDrp1 in podocytes will be protective post-IRI in mice will be necessary to demonstrate the impact of podocyte mitochondrial fragmentation in AKI. Additionally, recent study from cell reports demonstrates that podocyte-specific Drp1 ko mouse has no major phenotype (Brinkkoetter et al. 2019 Cell Reports).

Point by point responses to the reviewers

Reviewer #1 (Remarks to the Author):

This study is dedicated to investigate the in vivo ATP dynamics using FRET-based ATP biosensor in ischemia reperfusion mouse model of acute kidney injury (AKI). The authors demonstrated that decreased ATP levels were recovered in podocytes after reperfusion in short-term ischemia, but long-term ischemia caused insufficient ATP recovery in podocytes, which correlated with increased levels of mitochondrial fission and foot processes effacement. Additionally, this study suggests using ATP dynamics as a marker of podocyte injury in AKI.
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Reply:
Thank you for your strongly positive and supporting comments and valuable suggestions that have helped us to improve our manuscript.

1. Figure 8B shows MitoTracker red staining and claims that mitochondrial membrane potential was reduced in podocytes cultured with oligomycin A and 2-deoxy-D-glucose. I would suggest to measure quantitative mitochondrial membrane potential using TMRM probe instead.

Reply:
Thank you for the important comment. According to your suggestion, we used TMRM probe to show the reduction in mitochondrial membrane potential in cultured podocytes after ATP depletion stress. We observed decrease in TMRM intensity in podocytes after ATP depletion treatment (Figure 8, B and E). We also used MitoTracker Green according to the suggestion of Reviewer 2 to show morphological change of mitochondria more clearly. Podocyte mitochondria, which were labelled by MitoTracker Green, showed significant fragmentation during and after ATP depletion, which was ameliorated by Mdivi-1 treatment (Figure 8, B, E, H, and K).

2. The manuscript has a paucity of information on the inhibitor reagent used in the study. A revision should include the rationale for the agent chosen, the dosing regimen employed based on known

mechanism, pharmacokinetics, protein binding, and IC50s. The known specificity of the inhibitor should also be discussed regarding off target effects, particularly those that may impact on the interpretation of your findings. In the methods, please add information on the longevity of Midivi-1 treatment.

Reply:

Thank you for the important and insightful comment. We added an explanation regarding the reasons for choosing oligomycin A and 2-deoxy-D-glucose as reagents for ATP depletion in the Methods (see below for details). Dosing of these reagents was determined referring to our experiments with slice culture of murine kidney, in which more than 10 uM of oligomycin A and more than 20 mM of 2-deoxy-D-glucose were required for sufficient inhibition of ATP production (Figure 1 for the reviewers). We did not include any information on how the dosage of the inhibitors were determined in the current manuscript because these slice culture experiments are part of another manuscript that is under consideration elsewhere. However, if the reviewer suggests, we can include this information/statement as data not shown in the current manuscript.

IC50 for oligomycin A was reported to be 0.21-3.8 uM (J Antibiot. 2017; 70: 871). As for 2-deoxy-D-glucose, IC50 ranges 1-10 mM at 24 hours depending on cell lines (Oncotarget. 2017; 8(19): 30978). A previous study showed sufficient reduction in ATP after supplementation of 10-100 mM 2-deoxy-D-glucose in cultured podocytes (Sci Rep. 2015; 5: 18575). Additionally, the combination of 10 uM oligomycin A and 20 mM 2-deoxy-D-glucose was also employed in another publication (Cell Death Differ. 2005; 12: 1390). To the best of our knowledge, off-target effects of oligomycin A and 2-deoxy-D-glucose have not been shown. We could not find data about protein biding of oligomycin A and 2-deoxy-D-glucose.

Hence, the following sentences were added to the Methods section.

"We used Oligomycin A and 2-deoxy-D-glucose to inhibit ATP production. Oligomycin A directly inhibits ATP synthase, and 2-deoxy-D-glucose inhibits the glycolytic pathway because its metabolite is not a substrate for glucose-6-phsophate dehydrogenase or phosphohexose isomerase. Dosing of oligomycin A and 2-deoxy-D-glucose were determined based on reported IC50 (oligomycin A: 0.21-3.8[62]; 2-deoxy-D-glucose: 1-10 mM[63]) and other previous studies[20, 36]." (page 18, line 10)

As for Mdivi-1, we used this reagent because Mdivi-1 specifically inhibits the mitochondrial fission protein DRP1. Dosing of Mdivi1 from previous reports varies from 1 uM to 100 uM (Dev Neurobiol. 2017; 77(11): 1260). First, we decided to use 100 uM after a pilot study, in which we found 100 uM was slightly more protective than 10 uM for cultured podocytes after ATP depletion stress. The data sheet of the reagent states that IC50 is 1-10 uM. For *in vivo* experiments, we used 50 mg/kg as the

dose per injection, which is in line with previous reports that used murine models of kidney injury (Am J Physiol Renal Physiol. 2018; 314: F798. Am J Soc Nephrol. 2016; 27: 2733), whereby Mdivi-1 was administrated daily for 7 days or every other day for 8 weeks, both intraperitoneally. Based on these protocols, we decided to perform daily intraperitoneal injection for 14 days to maximize the therapeutic effect. A previous study reports that the half-life of Mdivi-1 in animal models is 12 hours (Molecular Neurobiology. 2016; 53: 240). We could not find data about protein binding of Mdivi-1.
Off-target effect of Mdivi-1 has been discussed by Bordt and colleagues recently (Dev Cell. 2017; 40: 583). They reported that Mdivi-1 has an inhibitory effect of mitochondrial complex I with higher concentration (>50 uM). Compounds that inhibit complex I, including metformin, are implicated with improvement of glycolytic activity and improved energy metabolism. Thus, it is possible that the high concentration of Mdivi-1 we used in the previous version of Figure 8 could have additional benefit through the off-target effect. To minimize off-target effects, we repeated the experiments using 50 uM Mdivi-1, and observed a similar efficacy as was observed with the concentration of 100 uM (Figure 8, H-M).

We added the sentences below to the Methods, Results and Discussion sections, respectively:
"To investigate the role of mitochondrial fragmentation in podocytes, we treated podocytes with Mdivi-1, a GTPase inhibitor that specifically inhibits the mitochondrial fission protein DRP1." (page 9, line 4)
"Regimen of Mdivi-1 treatment was determined based on previous studies and the estimated half-life (12 hours)[25, 26, 59]." (page 17, line 19)
"Dosing of Mdivi-1 supplementation for *in vitro* and *ex vivo* experiments was determined based on IC50 indicated on its datasheet (1-10 uM, Selleck Chemicals) and a pilot study." (page 18, line 19)
"We cannot exclude the involvement of possible beneficial off-target effect of Mdivi-1, as was recently reported by Bordt and colleagues, whereby Mdivi-1 reversibly inhibits mitochondrial complex I and reduces reactive oxygen species production only at higher concentrations[44]." (page 14, line 22)

3. The authors showed that ATP depletion and mitochondrial injury are the main contributors to podocyte foot processes effacement and that no significant podocyte loss has been observed in the chronic phase of ischemic reperfusion. However, it would be important to at least discuss the phenomenon of podocyte death absence in the given experimental conditions as mitochondrial dysfunction is known to have a significant impact on cell survival.

Reply:
Thank you for the very insightful comment. As you mention, mitochondrial dysfunction is well-known trigger of cell death. Indeed, previous studies that used Mdivi-1or podocyte-specific deletion of *Drp1*

have shown that suppression of mitochondrial fragmentation reduces podocyte apoptosis in the diabetic nephropathy and in the aldosterone induced-nephropathy models (Am J Physiol Renal Physiol. 2018; 314: F798. Am J Soc Nephrol. 2016; 27: 2733). In contrast, in our study, we did not observe significant podocyte loss despite severe mitochondrial fragmentation. This discrepancy may be due to difference in the experimental models or the duration of observation. Szeto and colleagues demonstrated that glomerulosclerosis and interstitial fibrosis significantly progressed 8 months after bilateral 45 minutes ischemia, compared to 1 month after the injury (J Am Soc Nephrol. 2017; 28: 1437). Given that podocyte loss is a major cause of glomerulosclerosis, podocyte loss after ischemia injury, which was not visible at the time of our analysis (day 14), could progress slowly but continuously thereafter.

We have now added the sentences below:

"We did not observe significant podocyte loss despite the signs of severe mitochondrial fragmentation, a well-known trigger of podocyte death[49]. However, podocyte injury that we observed on day 14 of IRI could progress over time. A previous study showed that glomerulosclerosis, a consequence of significant podocyte loss, increases 8 months after IRI in rats, compared to 1 month after IRI, and is significantly reduced by SS-31 treatment, which enhances mitochondrial ATP production in podocytes[50]. Therefore, it remains unclear how mitochondrial dysfunction after ischemic injury influences the long-term viability of podocytes." (page 14, line 22)

Reviewer #2 (Remarks to the Author):

The MS by Yanagita and co-workers reports an in vitro and in vivo investigation of intracellular ATP changes during ischemia in kidney podocytes. The Authors took advantage of a genetically-modified mouse systemically expressing a genetically-encoded FRET-based sensor of intracellular ATP. Thanks to this experimental model, they were able to in vivo measure by multyphoton microscopy intracellular ATP during ischemia and in the post-ischemic recovery phase, and to correlate ATP levels to podocyte morphology and to mitochondrial structure in vitro. This study exploits a range of state-of-the-art techniques and sophisticated animal models to address an important pathophysiological issue, and the results might have important practical implications.
My main concerns are detailed below.

Reply:

Thank you for your positive and valuable comments that have helped us to improve our manuscript.

1. Podocyte ATP levels are shown as ratios, in most cases between 0.4 and 2.5. I understand that an in vivo calibration to translate ratios into the actual ATP (mM)concentration is very difficult, if not

impossible, yet I believe that an in vitro calibration would at least provide a gross indication of the range of ATP changes under these different experimental conditions.

Reply:

Thank you for the insightful comment. Based on your suggestion, we performed *in vitro* calibration experiments using primary cultured murine embryonic fibroblasts derived from GO-ATeam2 mice (Supplemental Figure 1B, C), and made an equation to estimate actual intracellular ATP concentration *in vivo*, which is detailed in the Method section as follows:

"Estimation of intracellular ATP concentrations

Murine embryonic fibroblasts (MEFs) were freshly isolated from GO-ATeam2 mouse embryos at E13.5. For estimation of intracellular ATP concentration, MEFs that were treated by Seahorse XF Plasma membrane permeabilizer (1 nM; Agilent Technologies, Santa Clara, CA, USA) for 10 minutes were observed in calculation buffer containing arbitrary concentrations of ATP (Thermo Fisher Scientific). The plot was fitted with a regression equation; [ATP] mM = (ATP^{ratio} − 0.553)/0.449" (page 19, line 7).

Estimated intracellular ATP concentration is described in the Results section as follows:
"Intracellular ATP concentrations of kidney cells ranged from 3 mM to 4.2 mM (Supplemental Figure 1D)." (page 6, line 13)

2. On the same line of reasoning, it would be helpful showing the effects on the ATP ratio of known mitochondrial uncouplers, such as for example FCCP. This is important because this would provide a demonstration of how the experimental model reacts to agents causing a well predictable effect. For example, FCCP could be administered via the renal artery after exteriorization of the kidney, or intraperitoneally via the same route as Mdivi-1. After this procedure the mouse should be of course euthanized, but this would provide an indication of the response of the GO-Ateam2 biosensor to known ATP perturbing agents.

Reply:

Thank you for the important and insightful suggestion. In accordance to your suggestion, we tried FCCP administration during ATP imaging. Based on a previous study (Int J Mol Sci. 2022; 23(3): 1320), we set two doses (standard dose: 1mg/kg, and high dose: 10mg/kg). Standard dose injection slightly reduced kidney blood flow, but did not alter the kidney ATP levels (Figure 2 for reviewer, A), while high dose injection led to the death of the mice during anesthesia. Indeed, we previously experienced significant decrease in the cardiac output and blood perfusion in the kidney when using a

combination of anesthesia and the systemic administration of reagents that suppress mitochondrial ATP production such as oligomycin A or antimycin.

Therefore, we established a new local approach for drug administration to the kidney under ATP imaging conditions (e.g., under anesthetization) by filling the space between the kidney and the imaging glass with the drug solution. Surface application of Oligomycin A (10 uM) or Antimycin (36 uM) induced significant ATP decrease in proximal tubules without hemodynamic alterations (Figure 2B for the reviewers). This indicates that this experimental model responded to the drugs and caused predictable effects as you kindly suggested. Furthermore, as you suggested, we added an experiment using FCCP, however, surface application of FCCP (20 uM) induced only a slight decrease in proximal tubular ATP levels. Although the reason for the insufficient reaction is unclear, the electron gradient produced by electron transport chain in proximal tubules could be too robust for surface application of FCCP to reduce under the functional level. Additionally, the effects on reducing ATP by surface application of reagents was attenuated as we went deeper into the tissue, likely due to insufficient diffusion of the drugs, making it difficult to investigate the effect of oligomycin A inhibiting ATP production on podocytes *in vivo*.

3. It would be helpful if changes in fluorescence shown in Fig. 1D were quantitated with an usual image analysis program.

Reply:

Thank you for your comment. We quantified the fluorescence data in Figure 1D using MetaMorph, which, as far as we know, is a widely used software for image analysis and showed the data in Figure 1F. To clarify this point, we added the description of analysis software in the Method section as follows:

"Mean fluorescence intensity of podocyte markers was assessed with MetaMorph software." (page 21, line 15)

4. Fig. 3, A and B, reportedly shows ATP changes in the podocytes. Image magnification, especially in A, is very low, thus I wonder how podocytes were discriminated from the other cellular components of the glomeruli.

Reply:

We apologize for the confusion. In Figure 3A, we intended to show that there is no significant variation in ATP changes between glomeruli. As you mentioned, analyzing podocyte ATP in these images is difficult. We assessed podocyte ATP in magnified views, as shown in Figure 3B. To clarify this point, we changed the wording in the relevant text and added a sentence as follows:

“To investigate how ATP dynamics in glomeruli are affected during ischemia, we performed time-lapse imaging. First, we focused on the variation of overall ATP changes between glomeruli before dissecting cell-type specific ATP dynamics.” (Page 6, line 15)

I must say that even the Nphs 1-Ateam pictures shown in Fig. 5 are not terribly informative. Just as a control, why the Authors don't show images of kidney tubules from the Nhps 1-Ateam and the Tie2-Ateam mice? This should provide a clear discrimination with the kidney tubules being fluorescent only in the Tie2-Ateam mouse.

Reply:
Thank you for the important comment. The two strains used in Figure 5, *Nphs1*-ATeam and *Tie2*-ATeam mice, express the ATP biosensor in a cell type-specific manner in podocytes and endothelial cells, respectively, but not in tubules. Co-staining of the cell-type markers and ATP biosensor shown in Supplemental Figure 3 indicates the cell-type specific expression of the biosensor and its absence in proximal tubules. Indeed, the two-photon microscopy image of *Tie2*-ATeam mice in Figure 5D shows low level fluorescence in the tubules, but this is autofluorescence in the proximal tubules. Since *Tie2*-ATeam mice have lower biosensor expression than *Nphs1*-ATeam mice, stronger laser power is needed to visualize the biosensor, which likely causes more prominent autofluorescence in the adjacent proximal tubules. The images from kidney surface of wild-type, *Nphs1*-ATeam and *Tie2*-ATeam mice showed similar levels of fluorescence in proximal tubules, which also supported the absence of ATP biosensor in proximal tubules (Figure 2C for reviewers).

5. It is difficult to appreciate the "ATP hyper-recovery" (pg 7, line 1) from the images shown in Fig. 4. I fear that the Authors overinterpreted their data.

Reply:
Thank you for your advice. We have removed the relevant sentence about "ATP hyper-recovery".

6. At pg 7, lines 12 and following, the Authors state that they were able to identify cells with lower intracellular ATP levels, and lower GO-Ateam2 expression, that they assign to a different lineage than the podocytes. This is not clear to me. If GO-Ateam2 is systemically expressed, I would think that all glomerular cells should express it to more or less the same level.

Reply:

Thank you for your important comment. The CAG promoter and biosensor gene (GO-ATeam2) are knocked in the ROSA26 locus in GO-ATeam2 mice, thus, the biosensor expression is influenced by the CAG promoter. The expression of the gene downstream of CAG promoter is reported to be heterogenous among several organs, including the kidney, as well as among cell types (FEBS Letter. 2000; 470(3): 263). For example, this paper shows that EGFP expression under the CAG promoter is strong in cardiac and skeletal muscle, while it is low in the brain.
Based on your comment, we added the sentences below in the manuscript (Page 6, line 3).
"In Go-ATeam2 mice, the expression of the biosensor is under control of the CAG promoter inserted in the ROSA26 locus[18]. The expression of CAG promoter has been previously reported to be heterogenous in several organs including the kidney, as well as among different cell types[19]."

7. Again, at pg 7, bottom lines, the Authors conclude that the cells that undergo slow ATP decline after ischemia are neither endothelial cells nor podocytes, thus they are presumably mesangial cells. I think that the Authors should discuss how this would affect overall intracellular ATP changes in the glomerulus given the equivocal identification of the cellular source of the GO-Ateam2 signal.

Reply:
Thank you for your helpful suggestion. As you suggested, ATP decline rate after ischemia varies among different cell types in glomeruli. We picked up peripherally located cells showing strong fluorescence (based on high expression of the biosensor in podocytes shown in Figure 2D) with cellular processes in magnified view to analyze podocyte ATP (Figure 2C, Figure 3B and C, Figure 4). We also confirmed that ATP dynamics of such cells during ischemia reperfusion was similar to the ones we observed in *Nphs1*-ATeam (Figure 3C and 4B vs. 5E and G). Since we analyzed intracellular ATP levels at a single cell level in all experiments and analyzed ATP levels of each cell type separately in Figure 5, we consider that ATP levels of particular cell types does not directly influence those of neighboring cells.

8. I think that TEM pictures showing mitochondrial fragmentation in Fig. 6 should be implemented with a wide size and higher resolution image showing a detailed mitochondrial structure (e.g. christae).

Reply:
Thank you for the important suggestion. We added wide-view images to Supplemental Figure 5A and Supplemental Figure 6A, according to your suggestion. We also added magnified views of mitochondria focusing on detailed mitochondrial structure to Figure 6. Podocyte mitochondria are smaller and their crista is less distinct compared to those in proximal tubules.

9. Images showing mitochondrial fragmentation showed in Fig. 8, B and C, are not very informative. Using MitoTracker Red to investigate mitochondrial morphology is not the best technical approach because, being MitoTracker Red potential sensitive, fully depolarized mitochondria will be weakly (or not at all) fluorescent and thus escape fragmentation analysis. I wonder why they did not use the potential-insensitive MitoTracker Green.

Reply:

Thank you for the valuable suggestion. Based on your advice, we have redone all the experiments in Figure 8 using MitoTracker Green. Podocyte mitochondria, which were labelled by MitoTracker Green showed significant fragmentation during and after ATP depletion, which was ameliorated by Mdivi-1 treatment (Figure 8, B, E, H, and K). We have also changed the description of Methods to MitoTracker Green as follows:

"To assess the mitochondrial membrane potential and morphology, TMRM (100 nM; Thermo Fisher Scientific, Waltham, MA, USA) and MitoTracker Green (200 nM; Thermo Fisher Scientific) were added to the medium 30 minutes before observation." (Page 18, line 21)

10. I am not convinced from the data supporting the reversal of mitochondrial fragmentation by Mdivi-1. Despite the commendable quantitation of mitochondrial roundness and foot process width shown in Fig. 8G, I am not satisfied with the images shown in Fig. 8C. I cannot appreciate any clear differences between panels a, c and b. Better and more informative images should be provided.

Reply:

Thank you for the important comment. Based on your advice, we performed experiments in previous Figure 8C using MitoTracker Green and revised the figure (new Figure 8H). We also included the data of primary podocytes according to the suggestion of Reviewer 3, which showed the similar results (new Figure 8K). We hope that these figures are satisfactory for the reviewer. As for the quantification, in the revised manuscript, we utilized "aspect ratios" instead of "mitochondria roundness" for *in vitro* analysis. In the previous manuscript, we analyzed "roundness" by manually tracing mitochondrial outlines and calculating mitochondrial roundness. In the revision, we performed many new *in vitro* experiments using podocyte culture where mitochondria were denser and more abundant than TEM images, making it difficult to manually trace these data. Thus, we employed the morphology analysis function of ImageJ software, which can calculate aspect ratios of objects. Decreased aspect ratios in mitochondria suggests increased mitochondrial fission. Aspect ratios of podocyte mitochondria were significantly decreased by ATP depletion stress, and Mdivi-1 treatment ameliorated that decrement,

suggesting that Mdivi-1 alleviates mitochondrial fragmentation after ATP depletion (new Figure 8, H, I, K, and L).

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors demonstrate that ATP depletion leading to mitochondrial fragmentation in podocytes leads to podocyte effacement after ischemic AKI. They provide supporting data using FRET-based ATP biosensor as well as cell culture studies using immortalized mouse podocytes. While there is some interesting data provided in this manuscript, there are several major concerns that significantly dampen the overall enthusiasm for this proposal.

Reply:
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Major Comments:
1) A major concern is the lack of novelty as other laboratories have demonstrated that mitochondrial fragmentation exacerbated podocyte injury – ex. – Wang et al. Cell metabolism 2012.

Reply:
Thank you for the important comment. As you pointed out, pathological importance of mitochondrial fragmentation in podocytes has been documented by previous works (Cell Metabolism. 2012; 15(2):186-200, Am J Physiol Renal Physiol. 2018; 314: F798, Am J Soc Nephrol. 2016; 27: 2733). However, investigation focusing on mitochondrial dysfunction of podocytes after acute kidney injury (AKI), especially ischemia reperfusion injury (IRI) model, does not exist to the best of our knowledge. More importantly, we believe that our research is unique as the first study that revealed podocyte ATP dynamics during ischemia reperfusion at a single cell resolution and demonstrated the link between podocyte ATP dynamics in acute phase and podocyte structure and function in chronic phase. Therefore, our study adds a new understanding of ATP dynamics and mitochondrial dysfunction of podocyte in ischemic AKI.
The novelty of ATP Imaging in glomeruli is discussed in Discussion (Page12, line 12).
We also added a sentence below (Page12, line 4).
"Mitochondrial dysfunction is also an important pathological feature in podocyte injury[24,25,26], however, investigations focusing on mitochondrial dysfunction of podocytes after AKI, especially in the IRI model, have not existed todate. Importantly, our study reveals for the first time podocyte ATP

dynamics during ischemia reperfusion at a single cell resolution, demonstrating the link between podocyte ATP dynamics in the acute phase and podocyte structure and function in the chronic phase."

2) While there is likely mitochondrial fragmentation that might occur in podocytes, the biological significance in contributing to significant proteinuria is a major concern in this IRI model. Furthermore, the proteinuria shown in Figure 1 is not biologically significant as it relates to podocyte injury—could be potentially as a result of injured proximal tubule's ability to reuptake albumin.

Reply:

Thank you for raising this important point. As you have pointed out, it is difficult to rule out the contribution of tubular injury to the development of proteinuria in our experimental model. However, we believe that in this paper, we have been able to show evidence of podocyte injury, besides proteinuria, by electron microscopy and immunohistochemistry (Figure 1 A-F) and by using our unique ATP visualization techniques (Figure 2-7). Other groups also previously reported that IRI induces podocyte foot process effacement in murine kidney (J Biol Chem. 2008; 283(51): 35579. Biochem Biophys Res Commun. 2015; 461: 413). Epidemiological data also showed proteinuria and glomerulosclerosis after AKI and kidney transplantation (Transplantation. 2003; 76: 421. Kidney Int. 2018; 93: 460. J Am Soc Nephrol. 2023; 34: 346.). Taken together, we believe that our findings have a certain significance.

Taking into account your suggestion, we have now added the following sentence into the Discussion: "Because the degree of proteinuria in our model was moderate, we cannot exclude the contribution of decreased protein absorption in injured tubules. However, given that our data also directly demonstrated podocyte injury, and given that clinical evidence shows proteinuria and glomerulosclerosis after AKI and kidney transplantation[5, 7, 45, 46, 47, 48, 49], it is highly likely that changes in podocyte ATP levels during ischemia reperfusion is strongly linked to podocyte injury in the chronic phase." (page15, line 9)

3) Are there any endothelial changes in Figure 1?

Reply:

Thank you for the important comment. The majority of endothelial cells lacked clear signs of injury even after 60 minutes ischemia reperfusion injury, while a small number of glomeruli exhibited mild signs of endothelial injury, including cellular swelling and loss of fenestration, (Supplemental Figure 6) (Page 9, line 6). Signs of endothelial injury after shorter ischemia (≤ 45 minutes) were not evident.

4) Since there is a difference in level of fluorescence for imaging glomeruli, where all the glomeruli imaged at the same level in Figure 3?

Reply:

Thank you for your comment. The glomeruli in Figure 3 were imaged at the same laser power and depth (approximately 45 μm deep from the kidney surface) in order to image multiple glomeruli in a single field of view. Since glomeruli are located at various depths, some of the glomeruli measured in this field of view may be located slightly deeper than others, and those glomeruli may appear slightly darker. When measuring ATP ratios in glomeruli, we use a higher magnification and focused on each glomerulus one at a time for imaging.

5) Specificity of ATP fluorescence in DT and CD is not provided with costaining, would demonstrate specificity.

Reply:

Thank you for your comment and we are sorry for not being clear enough.

We identified distal tubules and collecting ducts according to the method that we established in our previous publication utilizing GO-ATeam2 mice (Yamamoto S et al. J Am Soc Nephrol. 2020 Dec;31(12):2855-2869.). In this paper, we identified these segments by co-staining of the GO-ATeam2 biosensor and cell type markers as well as by live imaging after injection of fluorescence-labelled dextran. We extracted the figures from the publication for your reference (Figure 3 for the reviewers) (Yamamoto S, Yanagita M, et al. J Am Soc Nephrol. 2020; 31: 2855).

We also added the sentences below in the Methods section:

"Distal tubules and collecting ducts were identified according to the criteria used in our previous work[17]. Briefly, we identified these segments by co-staining of the GO-ATeam2 biosensor and cell type markers as well as by live imaging after injection of fluorescence-labelled dextran[17]." (page 20, line 11)

6) Most IRI models are extended to 60 min – raises concern for validity of duration of IRI as representative of previously reported IR models.

Reply:

Thank you for the important comment. As you pointed out, ischemia duration longer than 45 minutes is unusual for the IRI model, but this may be because most of the previous studies were primarily focused on tubular injury. Since information of glomerular pathology in IRI was scarce, we attempted

ischemia durations ranging from 15 to 60 minutes and observed changes in cellular structures and ATP levels in podocytes. We found that acute changes in podocyte structure occur with the ischemia longer than 30 minutes (Figure 6) and chronic changes in podocyte structure occur with the ischemia longer than 45 minutes (Figure 7). Thus, we used 45 minutes ischemia model as a model for ischemic podocyte injury in Figure 3, 5, and 10. For clarity, a description of the ischemia duration was added in the relevant parts of the manuscript and the figures.

7) In Figure 5, there is no evaluation of mesangial cells – which also contribute to the changes in ATP observed. Also, the duration of IRI is unclear in Figure 5.

Reply:

Thank you for your important comments.

In the previous version of our manuscript, we did not perform experiments on mesangial cells due to the lack of a mesangial cell-specific Cre. But since you brought this to our attention, we have searched for a mouse line that can be used as a mesangial cell-specific Cre.

$FOXD1^{+}$ progenitor cells are known to differentiate into glomerular mesangial cells in addition to pericytes and vascular smooth muscle cells (Kidney Int Supple 2014; 4: 26-33). We therefore obtained *Foxd1*-Cre mice and crossed them with GO-ATeam2 flox/+ mice. Unfortunately, the ATeam probe was expressed not only in mesangial cells but also in podocytes in these mice, which was not sufficiently suitable for the analysis of mesangial ATP dynamics (Figure 4 for the reviewers).

Experiments with *Nphs1*-ATeam and *Tie2*-ATeam demonstrated that both podocytes and endothelial cells have relatively fast ATP decline during ischemia (Figure 5, D and E). Throughout these experiments, we did not observe cells with slow ATP decline such as ones shown in Figure 5A (asterisks). Because labelling efficiencies in *Nphs1*-ATeam mice and *Tie2*-Ateam mice were high enough (Supplemental Figure 3), we believe it is unlikely that some podocytes or endothelial cells that escaped biosensor labelling showed slow ATP decline like Figure5A asterisks.

In addition, ischemia time in Figure 5 is 45 minutes. We clarified this in Figure legend as well.

We think contamination of ATP signals from mesangial cells on ATP measurement of podocytes would be minimal, because we measured podocyte ATP by creating ROI around peripherally located cells that had characteristic strong fluorescence and cellular processes, which are quite distinct from mesangial cells. For clarity, we added images of ROIs created for Figure 2 C and 5D in Supplemental figure 1A and 3, C and D, respectively. Furthermore, we employed *Nphs1*-ATeam mice, which express ATeam biosensor only in podocytes and confirmed similar ATP dynamics to that observed in podocytes in GO-ATeam2 mice.

8) Figures 6 and 7 are not consistent in their level of mitochondrial roundness observed for 30 minutes. Also, is there any biochemical evidence of mitochondrial fragmentation specificity to podocytes (i.e., isolation of podocytes and measuring p-DRP1 levels)?

Reply:

Thank you for your comment and constructive suggestions. Since mitochondrial roundness in Figure 6 and Figure 7 were measured on day 0 (30 minutes after reperfusion) and on day14, respectively, we think that mitochondria might partially recover between the two time points, resulting in a difference between the values of Figure 6C and Figure 7C.

Regarding the biochemical evidence of mitochondrial fragmentation in podocytes, measuring p-DRP1 in isolated podocytes is technically challenging due to the low cell volume and possible dephosphorylation during isolation process. Instead, we performed immunostaining of DRP1 and podocyte marker, and found that DRP1 staining in podocytes increased as early as 1 hour after ischemia reperfusion (Supplemental Figure 5B).

To further demonstrate the participation of podocyte DRP1 in the pathological condition, we performed the experiments using DRP1 knockdown in cultured podocytes. As shown in newly added Figure 9, DRP1 knockdown ameliorated mitochondrial fragmentation and stress fiber loss in cultured podocytes after ATP depletion injury.

Because mitochondrial fragmentation was also reported in proximal tubules in a previous study (J Am Soc Nephrol. 2018; 29: 194), it is difficult to exclude its influence on podocyte structure and function. However, upregulation of DRP1 in podocytes *in vivo* and DRP1 knockdown experiments using cultured podocytes support our hypothesis that DRP1-mediated mitochondrial fragmentation in podocytes plays, at least in part, an important role in podocyte injury after IRI.

The following sentences were added to the Methods, Results and Discussion.

“To further demonstrate the participation of podocyte DRP1 in the pathological conditions, we performed the experiments using *Drp1* knockdown in cultured podocytes. Knockdown mediated by siRNA targeting *Drp1* reduced protein levels in cultured podocytes (Figure 9, A and B). Importantly, *Drp1* knockdown ameliorated mitochondrial fragmentation and stress fiber loss in cultured podocytes after ATP depletion injury (Figure 9, C, D and E).” (page 10, line 11)

“the protective effect induced by *Drp1* knockdown in cultured podocytes suggests that DRP1 and mitochondrial fragmentation in podocytes play pivotal roles in podocyte injury after ischemia.” (page 14, line 25)

"*Knockdown by siRNA*

Nontargeting control oligonucleotide (D-001810-10-05) and siRNA against mouse *Drp1* (L-054815-01-005) were purchased from Horizon Discovery (Cambridge, UK). Transfection of siRNA oligonucleotide was performed using DharmaFECT-2 siRNA transfection reagent (Horizon Discovery) per manufacturer's instructions." (page 19, line 1)

9) Immortalized podocytes are not ideal to assess for changes in mitochondrial fission, primary cultured podocytes would be more representative. In addition, energy utilization has been previously reported to be different in immortalized podocytes as compared to primary podocytes.

Reply:

Thank you for the important suggestion. To address your comment, we have now performed experiments using primary cultured podocytes and observed similar results (Figure 8 E-G, K-M).

10) The use of Mdivi-1 is not specific to podocytes and improvements might not be a direct result of preventing mitochondrial fragmentation specifically in podocytes.

Reply:

Thank you for the important and valuable comment. We cannot exclude that Mdivi-1 could ameliorate mitochondrial injury not only in podocytes but also in other cell types such as proximal tubular cells. Increased hydrostatic pressure and cytokine secretion have been suggested as the cause of secondary glomerular injury after tubular injury (Am J Physiol Renal Physiol. 2018; 314: F154. Front Immunol. 11: 606488). Thus, to dissect the primary effect of Mdivi1 on podocytes from secondary beneficial effects on other cell types, we employed a kidney slice culture system, which is a classic method that can exclude confounding effects such as hydrostatic changes and neurohormonal mediators, while preserving 3-D tissue organization (Methods Cell Biol. 2019; 153: 185). We have established an *ex vivo* IRI model using this kidney slice culture system, and have found that it can indeed mimic ATP dynamics during *in vivo* IRI (paper submitted to another journal).

As shown in Figure 10 D, E and F, Mdivi-1 treatment was efficient in preserving foot processes and mitochondrial structure in this *ex vivo* IRI model as well as in *in vivo* IRI, strongly supporting the likelihood that Mdivi-1 exerts a direct protective effect on podocytes.

Obviously, as an inevitable limitation of pharmacological experiments, we cannot fully exclude the possibility that protective effect of Mdivi-1 in other cell types affect the phenotypes of podocytes. Nonetheless, these pharmacological experiments are of clinical relevance and the results of our *in vitro* and *ex vivo* analyses suggest that Mdivi-1 directly protects podocytes at least in part.

We added the following description of the experiments with slice culture into the Methods, Results and Discussion:

"We also employed a newly established *ex vivo* IRI model to minimize the potential influence of Mdivi1 on adjacent injured tubules and hemodynamic changes after IRI (Figure 10D, manuscript submitted). After 45-minutes deprivation of oxygen and energy resource and 4-h of recovery phase, podocytes in cultured kidney slices showed significant mitochondrial fission and foot process effacement (Figure 10, E and F). In contrast, glomeruli treated by Mdivi-1 showed mitigation of mitochondrial fission and foot process effacement in podocytes, strongly supporting the likelihood that Mdivi-1 exerts a direct protective effect on podocytes (Figure 10, E and F). These results suggest that alleviating mitochondrial fragmentation is protective for the podocyte cytoskeleton after ATP-depletion stress *in vitro*, in *ex vivo* model, and *in vivo.*" (page 11, line 3)

"*Ex vivo* slice culture is a classic method that can exclude confounding effects such as changes of hydrostatic pressure and neurohormonal mediators, while preserving 3-D tissue organization[45]. Although increased hydrostatic pressure and cytokine secretion have been suggested as the causes of secondary glomerular injury after tubular injury[46, 47], our *ex vivo* model has the advantage of being able to exclude them." (page 15, line 3)

"*Ex vivo IRI model*

We established an *ex vivo* IRI model (manuscript submitted). After adequate anesthesia using isoflurane, kidneys of 10-week-old male wild type mice were harvested and decapsulated. They were immediately sliced at 300 μm with a vibratome (VT1000S; Leica Microsystems, Wetzlar, Germany) in ice-cold buffer that was with 95% O_2 and 5% CO_2 and contained 97.5mM NaCl, 5mM KCl, 0.24mM NaH_2PO_4, 0.96mM Na_2HPO_4, 10mM CH_3COONa, 25mM $NaHCO_3$, 10mM glucose, 5mM Na pyruvate, 0.6mM $MgSO_4$, and 1mM $CaCl_2$, as previously reported with a few modifications[71]. To induce ischemia-like conditions, the normal buffer was replaced by the ischemia buffer, which contained no energy sources and was saturated with nitrogen, as previously reported in the heart and cell culture[72, 73]. To induce reperfusion-like conditions, ischemia buffer was replaced by normal buffer, and oxygenation was resumed." (page22, line 22)

First, we would like to thank the handling editor and reviewers for providing supportive and insightful comments and suggestions, which has helped us significantly improve our manuscript.

Reviewer #1 (Remarks to the Author):
In the revised version of the manuscript, the authors have adequately addressed the original concerns and I don't have any more comments.

Reviewer #2 (Remarks to the Author):
No further comments.

Response to comments from Reviewer #1 and #2:

We sincerely appreciate both Reviewers 1 and 2 for their comments and being satisfied with our revisions, which have significantly improved our paper.

Reviewer #3 (Remarks to the Author):
In this revision, the authors demonstrate that ATP depletion leading to mitochondrial fragmentation in podocytes leads to podocyte effacement after ischemic AKI. They provide key supporting data using FRET-based ATP biosensor as well as cell culture studies using immortalized mouse podocytes. While the authors have demonstrated novelty in the use of FRET-based ATP biosensor to study mitochondrial dynamics, there are still some major concerns:

1. While the authors respond that this is the first study to demonstrate that mitochondrial fragmentation exacerbates podocyte injury post IRI (AKI model), the biological significance remains still a concern. They now reframe the discussion to include "moderate" albuminuria which is still an overreach with an increase from 40 to 70 mg/g. Furthermore, the expression levels of the canonical podocyte markers are minimally reduced (figure 1). Additionally, in Figure 1, the authors only show albuminuria and podocyte marker data for one time point post-IRI (45 min). It would be more convincing to show albuminuria levels at shorter and longer times post-ischemia and reperfusion phase (figure 4) to demonstrate the biological significance.

Response to the comments:

We thank the Reviewer 3 for valuable suggestions. To address these concerns, we have now

assessed albuminuria in both acute and chronic phases. We demonstrate that the increase in albuminuria after IRI was significant as early as day 7 and persisted for at least four weeks (Figure 1H).

We also performed electron microscopy of podocytes four weeks after IRI and found significant foot process effacement in the IRI group (Figure 1, I and J). We also assessed PAS staining and found that some glomeruli of post-IRI kidneys showed shrinkage of capillary tufts and sclerotic changes (Supplemental figure 1), which resembled ischemic glomeruli after kidney transplantation (J Am Soc Nephrol. 2023. 34; 346.).

Taking into consideration that the criteria for chronic kidney disease (CKD) are proteinuria or decreased eGFR lasting more than three months in human and the difference in life span between mice and humans, the persistence of proteinuria and podocyte injury even one month after IRI in mice is clearly biologically significant. Hence, our current study sheds light on glomerular injury, which has been underestimated in the pathophysiology of AKI to CKD transition.

One point noteworthy to mention is that in order to minimize post-surgery effects such as inflammation and dehydration, we set a 24-hour recovery phase between unilateral ureteral obstruction surgery and the start of 24-hour urine collection in our original submission. We noticed that the description of the recovery phase was missing in the original Methods section and the graphical description (Figure 1G) and sincerely apologize for the omission. We have now revised the Methods section (p. 20, l.16-19) and graphical description accordingly (Figure 1G).

2. In this revision, the authors provide evidence of the protective effects of Drp1 ko in cultured podocytes, which has been previously reported (Wang et al. Cell metabolism 2012), thereby reducing the novelty. Ultimately, demonstrating that conditional knockdown of Drp1 or mutant pDrp1 in podocytes will be protective post-IRI in mice will be necessary to demonstrate the impact of podocyte mitochondrial fragmentation in AKI. Additionally, recent study from cell reports demonstrates that podocyte-specific Drp1 ko mouse has no major phenotype (Brinkkoetter et al. 2019 Cell Reports).

Response to the comments

We thank the Reviewer for raising this point. We are certainly aware of the data present in the two papers (Wang et al. 2012 Cell Metabolism and Brinkkoetter et al. 2019 Cell Reports) described by the Reviewer. However, we would like to respectfully point out to the Reviewer that from our understanding of the literature, neither paper has not shown/investigated the role of Drp1 in

ischemic injury or ischemic injury-mimicking conditions.

Wang et al. (Cell Metabolism 2012) have shown protective effects of *Drp1* knockdown in the context of diabetic stress. As for Brinkkoetter et al. (Cell Reports 2019), they demonstrate that conditional *Drp1* knockout mice showed no significant phenotypes in podocytes within 12 months— but they did not induce any insult to the kidneys. Therefore, we consider that their data does not exclude the possible contribution of Drp1 in an ischemic reperfusion model. Hence, based on our findings, we assume that conditional *Drp1* knockout may still ameliorate podocyte injury after ischemic reperfusion. To clarify these ambiguities, we have now included an additional section in the Discussion describing these differences with our study as follows (p. 15, l. 2-7):

"Similar protective effects have also been observed in podocytes deficient for *Drp1* under diabetic stress[24, 25], though the exact underlying mechanisms remain unclear. Considering that the conditional knockout of *Drp1* alone seems to show no significant phenotype during a 12-month observation window[35], this suggests that the pathophysiological role of DRP1 in podocytes may only become apparent upon stress-induced conditions. Our results indicate that DRP1 may be a potential therapeutic target to prevent podocyte injury after IRI, though further direct validation will be necessary (see below for details)."

As mentioned by the Reviewer, we appreciate the importance of understanding further the molecular mechanisms behind our observations. We agree that further analysis using podocyte-specific conditional knockout of *Drp1* could shed more insights into the mechanistic basis of these protective effects. However, we would also like to respectfully point out that in our revised manuscript, we performed *Drp1* knockdown experiments using cultured podocytes— thereby validating our original data using the DRP1 inhibitor. Hence, we believe this should be sufficient supportive evidence of the protective effects of inhibiting DRP1 on IRI. We mentioned the limitations of our experimental setup in the Discussion section as follows (p. 17, l.3-5):

"Finally, to conclusively determine the role of DRP1 in podocyte injury after IRI, our findings need to be further validated using *Drp1* conditional knockout mice, which warrants future investigation."

First, we would like to thank the handling editor and reviewers for providing supportive and insightful comments and suggestions, which has helped us significantly improve our manuscript.

Reviewer #1 (Remarks to the Author):
In the revised version of the manuscript, the authors have adequately addressed the original concerns and I don't have any more comments.

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1. While the authors respond that this is the first study to demonstrate that mitochondrial fragmentation exacerbates podocyte injury post IRI (AKI model), the biological significance remains still a concern. They now reframe the discussion to include "moderate" albuminuria which is still an overreach with an increase from 40 to 70 mg/g. Furthermore, the expression levels of the canonical podocyte markers are minimally reduced (figure 1). Additionally, in Figure 1, the authors only show albuminuria and podocyte marker data for one time point post-IRI (45 min). It would be more convincing to show albuminuria levels at shorter and longer times post-ischemia and reperfusion phase (figure 4) to demonstrate the biological significance.

Response to the comments:
We thank the Reviewer 3 for valuable suggestions. We added several conditions for urinary albumin measurement (ischemia severity and timing of measurement) to address the concern. First, we analyzed urinary albumin after various severities of IRI, and found that albuminuria

levels increased as ischemia time prolonged (Figure 7D), which is the same trend to the ones seen in foot process width measurement and mitochondrial fragmentation analysis (Figure 7, B and C). We also demonstrated that the increase in albuminuria after IRI was significant as early as on day 7 and persisted for at least four weeks (Figure 1H). We also added histological assessments of the kidneys four weeks after IRI, and observed persisting foot process effacement in electron microscopic analysis (Figure 1I) and sclerotic changes of glomeruli in PAS staining (Supplemental Figure 1). Taking into consideration that the definition for chronic kidney disease (CKD) in humans are kidney damage (including proteinuria and histological signs) or decreased eGFR lasting more than three months in human and the difference in life span between mice and humans, we consider that the persistence of proteinuria and podocyte injury even one month after IRI in mice is biologically significant.

Taken together, we believe that these results support the biological significance of our findings. Hence, our current study sheds light on glomerular injury, which has been underestimated in the pathophysiology of AKI to CKD transition.

One point noteworthy to mention is that in order to minimize post-surgery effects such as inflammation and dehydration, we set a 24-hour recovery phase between unilateral ureteral obstruction surgery and the start of 24-hour urine collection in our original submission. We noticed that the description of the recovery phase was missing in the original Methods section and the graphical description (Figure 1G) and sincerely apologize for the omission. We have now revised the Methods section (p. 20, l.16-18) and graphical description accordingly (Figure 1G).

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Response to the comments

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ischemic injury-mimicking conditions.

Wang et al. (Cell Metabolism 2012) have shown protective effects of *Drp1* knockdown in the context of high glucose and overexpression of constitutive active ROCK1. As for Brinkkoetter et al. (Cell Reports 2019), they demonstrate that conditional *Drp1* knockout mice showed no significant phenotypes in podocytes within 12 months— but they did not induce any insult to the kidneys. Therefore, we consider that their data does not exclude the possible contribution of Drp1 in an ischemic reperfusion model. Hence, based on our findings, we assume that conditional *Drp1* knockout may still ameliorate podocyte injury after ischemic reperfusion. To clarify these ambiguities, we have now included an additional section in the Discussion describing these differences with our study as follows (p. 15, l. 2-7):

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As mentioned by the Reviewer, we appreciate the importance of understanding further the molecular mechanisms behind our observations. We agree that further analysis using podocyte-specific conditional knockout of *Drp1* could shed more insights into the mechanistic basis of these protective effects. However, we would also like to respectfully point out that in our revised manuscript, we performed *Drp1* knockdown experiments using cultured podocytes— thereby validating our original data using the DRP1 inhibitor. Hence, we believe this should be sufficient supportive evidence of the protective effects of inhibiting DRP1 on IRI. We mentioned the limitations of our experimental setup in the Discussion section as follows (p. 17, l.5-7):

"Finally, to conclusively determine the role of DRP1 in podocyte injury after IRI, our findings need to be further validated using *Drp1* conditional knockout mice, which warrants future investigation."