Author's Reply

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In summary, this is a significant study that has our full attention. Because the expression location of a protein is closely related to its function, we would be glad to hear from the authors.

METHODS FOR IMMUNOFLUORESCENCE STAINING

The kidney tissues from male C57BL mice and a renal biopsy specimen of a patient with minor glomerular lesion were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 3–4 μm sections. The slides were then deparaffinized, rehydrated, and antigen was retrieved using a citrate buffer. After blocking with goat serum for 30 minutes, the sections were incubated with diluted primary antibodies of RRAGD (rabbit anti-RRAGD, NBP2-32106, 1:2000) overnight at 4°C. Fluorophore-labeled secondary antibody (goat anti-rabbit, ab150077, 1:1000; Abcam) and 4′,6-diamidino-2-phenylindole (1:1000) were used for nuclear staining. The images were captured using a microscope (Eclipse 80i; Nikon, Tokyo, Japan) with a digital camera (DS-U1; Nikon).

DISCLOSURES

L. Chen reports serving as a member of the American Society of Nephrology (ASN), European Dialysis and Transplant Association, International Society for Peritoneal Dialysis, and International Society of Nephrology; receiving research funding from Baxter; and serving as associate editor of *JASN*. T. Ma reports serving as a member of the ASN and European Renal Association Community. L. Zhang reports serving as a member of the ASN and European Renal Association Community.

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The funders had no role in study design, data collection, analysis, and manuscript preparation.

AUTHOR CONTRIBUTIONS

CRediT Taxonomy T. Ma was responsible for formal analysis and methodology, and wrote the original draft; T. Ma and L. Zhang were responsible for investigation; L. Zhang and L. Chen were responsible for resources; L. Chen was responsible for project administration, supervision, and validation; and all authors were responsible for conceptualization and reviewed and edited the manuscript.

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See related reply, "Authors' Reply: The Subcellular Localization of RRAGD," on pages 1048–1049, and original article, "mTOR-Activating Mutations in RRAGD are Causative for Kidney Tubulopathy and Cardiomyopathy," in Vol. 32, Iss. 11, on pages 2885–2899.

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Author's Reply: The Subcellular Localization of RRAGD

We thank Ma *et al.* for their interest in our recent report, which identified mutations in *RRAGD* as a cause for kidney tubulopathy and dilated cardiomyopathy.[1] In their letter, Ma *et al.* report RagD staining in the nucleus of tubular epithelial cells. As the authors correctly note, the RagD staining in our manuscript is predominantly located in the cytoplasm of epithelial cells in the thick ascending limb of the loop of Henle (TAL) and the distal convoluted tubule (DCT) segments of the kidney.[1] Our findings are in line with mRNA expression data from the mouse and the rat nephron, and human single-cell RNA sequencing data which demonstrate high expression in these segments.[2–4]

Our manuscript reports immunohistochemistry of mouse kidney tissue because we have good segmental markers against mouse proteins available and the tissue can be better fixated due to perfusions. However, we have also analyzed human kidney cortex samples, which confirm the mouse stainings. In these human kidney sections, RagD expression is observed predominantly in the cytoplasm (Figure 1).

Indeed, the Rag GTPase complex is extensively described as a cytoplasmic nutrient sensor, where it resides at the lysosomal membrane and detects leucine and arginine amino acids.[5] Upon amino acid binding, the Rag complex recruits mammalian target of rapamycin (mTOR) to the lysosomal membrane and activates the mTOR signaling pathway.[6]

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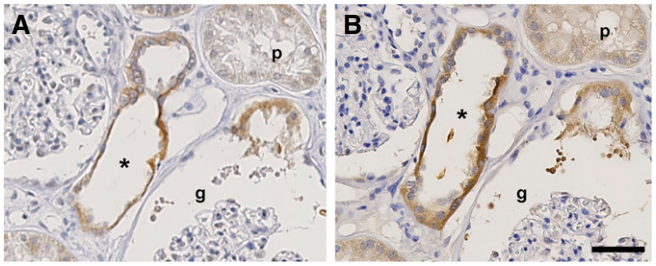

Figure 1. RagD is expressed in the cytoplasm of the distal convoluted tubule. Serial sections (as notified by the asterisk located in the same renal tubule) of human kidney were incubated with primary antibodies against (A) RagD or (B) uromodulin. The RagD protein was detected in uromodulin-positive the thick ascending limb of the loop of Henle (*). No significant signal was observed in the proximal tubule (p) or the glomerulus (g). Scale bar, 50 μm.

Although cytoplasmic localization is, therefore, expected, there are reports of nuclear translocation of Rag proteins *via* the nuclear pore complex.[7] In HEK293 cells, it has been demonstrated that RagC requires nuclear passage to fully activate mTOR signaling.[7] Moreover, the metformin analogue phenformin could prevent this nuclear translocation, suggesting intracellular signaling pathways may determine the nuclear versus cytoplasmic expression of Rag proteins. Consequently, differences in subcellular Rag expression may be explained by the metabolic status of the assessed kidney tissue. Therefore, the interesting observation of Ma *et al.* spikes an interest in further investigations of metabolic regulation of the Rag complex in the kidney.

DISCLOSURES

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AUTHOR CONTRIBUTIONS

J. de Baaij wrote the original draft; J. de Baaij, F. Jouret, N. Knoers, and K.-P. Schlingmann reviewed and edited the manuscript; and F. Jouret was responsible for investigation.

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