Dear Editor,

Thank you very much for your fair evaluation of our manuscript entitled “Functional coupling of V-ATPase and CLC-5”. This manuscript has been improved according to the suggestions of reviewers. All changes below are now highlighted in red in the revised manuscript.

1. Author names and institutions have been updated. We have added a coauthor, Kyoji Moriya and his institution in page 1.

2. We have provided more details of the address for the University of Tokyo Hospital in pages 1-2.

3. We have added the journal title “*Pflugers Archiv : European journal of physiology* “ to Reference No.11 in page 16.

We feel that the revised manuscript is a suitable response to the comments. We believe that it is now ready for publication in World Journal of Nephrology.

Sincerely yours,

Nobuhiko Satoh

Response to Reviewer 1

Thank you very much for careful reading and giving insightful comments.

Major

1. *"In the HEK293 cells, the hypotonicity-induced V-type ATPase activity was negligible in the vector-transfected cells despite the expression of V-ATPase in the membrane. What is the explanation for this phenomenon? There are some discussion on this issue, but insufficient."*

Thank you very much for pointing out an important issue. While involvement with cell-type specific factors cannot be excluded, lack of V-ATPase activity in HEK293 cells may support the functional coupling of V-ATPase and CLC-5 at the plasma membrane. This point is now clarified in pages 12-13.

2. *"In the same context, for general readers of the Journal, it would be more helpful to explain the hypothetical schematic model for the present results in terms with the pathophysiological mechanisms of Dent's disease. Comparison with the previous conventional model would be better."*

In accordance with the reviewer's suggestion, we have added hypothetical schematic models for the functional coupling between V-ATPase and CLC-5 in both endosomes and plasma membrane as Fig. 9, which is now cited in the discussion section (page 14).

3. *The quality of immunoblot data shown in Fig. 3c is poor. It is requested to perform the experiment again.*

According to your suggestion, we performed additional immunoblotting experiments to renew Fig. 3c. To reflect the results of the experiment, we also modified the corresponding summary data in Fig. 3d.

4. *Fig. 6d, e: the acidification by Na^+ removal appeared spontaneous reversed before the hypotonic stress. It is requested to clarify the time durations for the calculation of the pH change slopes in Fig. 6e.*

In these experiments, we estimated V-ATPase activities by calculating the rates of cell

pH recovery during the initial 30 seconds. This point is now clarified in page 8.

5. *The summarized data in Fig. 7 showed statistical significance. However, considering the sizes of error bars in Fig. 7b, it may appear questionable. Please specify the P-value for this comparison (Fig. 7b). In addition, it has to be stated whether these analysis were paired or unpaired t-test.*

Thank you very much for pointing out this important point. We used unpaired t-test for analyses and the P-value was 0.046. These points are now clarified in page 27. During the re-investigation of original data, we also found that the sizes of error bars were incorrectly presented. We now corrected the values of standard error in Fig. 7b from 0.056 to 0.038 for day 0 and from 0.049 to 0.030 for day 1. We deeply apologize for this mistake.

Minor

1. *Reference format should be checked carefully.*

In accordance with your comment, we have checked and corrected the reference format.

Response to Reviewer 2

Thank you very much for careful reading and giving insightful comments.

Major

1. *The intriguing question will be whether endocytosis is actually affected by this mutant as a small V-ATPase activity was still observed in vitro. Furthermore, their acute observations may not be kept in in vivo condition where many compensatory functions will happen as NHE and other anion channels. Please discuss such limitations of the current study and propose future plans and directions.*

We agree that it is unclear whether our present *in vitro* data directly correspond to *in vivo* condition where many compensatory functions will happen. To clarify this issue, future studies on E211Q knock-in mice or proximal tubular cells from the patient carrying E211Q mutation will be required. This point is now clarified in page 13.

2. *Are there any phenotypic differences in the patient with E211Q from other patients*

with Dent's disease in general, which may be relevant to the results of this study? In other words, are there any expected phenotypes in this patient based on the results?

No, there are not. As shown in the section of patient description in page 4, the patient with E211Q mutation presented with the typical clinical phenotypes of Dent's disease.

3. *It will be expected that the function of endosomes is compromised by the inefficient acidification with E211Q mutation. Are there any additive effects by bafilomycin or even negative effects by hypotonicity in vitro?*

As shown in Figures 3 and 5, E211Q mutant still induced the small bafilomycin-sensitive component. As shown in Fig. 3b, hypotonicity did not induce the negative effect on cells transfected with E211Q.

Minor

1. *Were there any correlations between the endosomal V-ATPase and the plasma membrane V-ATPase in Fig. 4 and 5?*

As you pointed out, there seemed to be some correlations between the endosomal V-ATPase and the plasma membrane V-ATPase. In both situations, the V-ATPase activation by the CLC-5 mutants were less than that by WT. Because the methods used to estimate the V-ATPase activities in these two situations are not identical, it is rather difficult to directly compare the V-ATPase activities at endosome and plasma membrane.

2. *The baseline fluctuations of the cell pH in Fig. 3a seem to be larger than those of other figures. Any reasons?*

This is due to the different optical properties of the photometric systems for cell pH measurement. The fluorescence microscope equipped with MetaFluor 7.7 software used for PT experiments had superior signal-to-noise ratio to the OSP-10 system used for HEK293 experiments (Fig. 3a).

3. *The overnight incubated proximal tubules may have a selection bias. How high were the V-ATPase activities of fresh isolated proximal tubules?*

The overnight incubation indeed reduced the V-ATPase activities. To clarify this point, we now added data for the basal V-ATPase activities of PTs in isotonic solution in day 0 and day 1 as Fig. 7c. As already shown in Fig. 7b, however, the hypotonicity-induced V-ATPase activities were relatively preserved after the overnight incubation.

4. *The comments on the anesthesia of the animals will be needed with the permission by the authority.*

Thank you for pointing out an important matter. We have provided additional information about the animal treatment in the methods section.

List of the changes

We highlighted all the changes listed below in red throughout the revised manuscript.

1. To explain negligible hypotonicity-induced V-ATPase activity in vector-transfected HEK293 cells, we slightly modified the main text in page 12 and added the following text in page 13. New text: While the involvement of cell-type specific factors cannot be excluded, lack of basal V-ATPase activity in HEK293 cells may support the functional coupling of V-ATPase and CLC-5 at the plasma membrane as will be discussed below.

2. We added a new figure depicting hypothetical schematic models for the functional coupling between V-ATPase and CLC-5 in endosomes and plasma membrane as Fig. 9. This new figure was cited in page 14 and its legends were inserted in pages 27-28.

3. We performed additional western blot analysis to renew Fig. 3c. Accordingly, statistical parameters in Fig. 3d were slightly modified with additional densitometry data as shown in page 25.

4. In accordance with the reviewer's request, we clarified how to calculate the V-ATPase activities in page 8.

5. We corrected the values of standard error in Fig. 7b from 0.056 to 0.038 for day 0 and from 0.049 to 0.030 for day 1, adding p-value in page 27.

6. We described limitations of our present study and future directions in page 14 as follows. New text: On the other hand, it is unclear whether our present in vitro data directly corresponds to in vivo condition where many compensatory functions will happen. To clarify this issue, future studies on E211Q knock-in mice or PT cells from the patient carrying the E211Q mutation will be required.

7. We added data for the basal V-ATPase activities of PTs in isotonic solution in day 0 and day 1 as Fig. 7c. Additional figure legends was inserted in page 27.

8. In accordance with the reviewer's suggestion, we clarified that unpaired t-test was used for analyses in page 8.

9. In accordance with the reviewer’s suggestion, we added information about the animal treatment in page 4.

10. We checked and corrected the reference format throughout the manuscript.