Author's response to reviews

**Title:** Absence of Chloride Intracellular Channel 4 (CLIC4) Predisposes to Acute Kidney Injury But Has Minimal Impact on Recovery

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**Author's response to reviews:** see over
Thank you for considering our manuscript, *Absence of Chloride Intracellular Channel 4 (CLIC4) Predisposes to Acute Kidney Injury But Has Minimal Impact on Recovery*, for publication in BMC-Nephrology. We appreciate the thoughtful comments of the reviewers and have revised the manuscript substantially in response.

The major changes to the paper are:

1. Addition of negative control images to figures 1 and two. These images are of identically processed and stained material from Clic4 null mice, imaged using identical confocal microscope parameters.
2. Addition of quantification of western blot signals for CLICs 1 and 5 at baseline and following acute injury WT and Clic4 null kidney (figures 13 and 15).
3. Presentation of the pSMAD quantification normalized to total SMAD in the same mice (figure 14).
4. The images of immunostaining have been revised in an effort to make the observations more apparent.

Specific responses to reviewer comments:

**Dr. Yuspa:**

1. Negative controls for the immunolocaliation of CLIC4 are now included in figures 1 and 2.
2. We agree that further testing of the nature of glomerular filtration barrier in the CLIC4 knockouts would be of interest, but we feel that is beyond the reasonable scope of this report. We have added several qualifications to the discussion of the proteinuria results as suggested by Dr. Long (see below and page 28 of the manuscript).
3. We added characterization of expression levels of the other CLICs that are known to be expressed in the kidney (see figures 13 and 15). The results are interesting and do not suggest a simple model in which upregulation of the other CLICs compensates for absence of CLIC4, discussed in the revised manuscript (page 31).
4. Normalization of pSMAD to total SMAD is now presented in figure 14. This analysis confirms that SMAD2-3 phosphorylation following injury is not significantly different between the kidneys of WT and Clic4 null mice.

**Dr. Long:**

1. We agree an assessment of renal angiogenesis in WT and Clic4 null embryos during renal development would be very interesting, but we think these experiments are beyond the reasonable scope of this paper. Assessing levels of angiogenesis and/or expression of angiogenic mediators in the un-injured adult mice is less likely to be clearly informative since steady state levels of vessel growth and turnover are low. For example, levels of VEGF isoforms
and angiopoietins in the hind limb muscles of adult Clic4 null mice have been previously reported to be not different from those of WT even though they have measurable difference in their vascular density, but the absence of CLIC4 was shown to decrease the rise in VEGF and angiopoietins following ischemic injury (Chalothorn et al., Circ Res 2009, 105: 89-98).

2. We have added the appropriate caveats to the discussion regarding proteinuria (page 28 of the revised manuscript). While the substantial fraction of creatinine clearance due to tubular secretion mentioned by Dr. Long does limit the value of serum creatinine level as an indicator of GFR, it is still valid to normalize urine protein to urine creatinine as a quantitative indicator of proteinuria as long as urinary excretion remains the primary means of creatinine disposal. Whether it gets into the urine by filtration or by secretion does not matter as long as the mouse is in a steady state and all the creatinine produced by muscle is excreted in the urine. The important assumption is that rate of creatinine production by muscle is constant and is the same between WT and Clic4 nulls. While we have no reason to expect it to be different, we have not confirmed that to be the case, and this is noted in the manuscript. We cannot make statements about absolute amounts of protein per 24 hours since we do not know the rate of creatinine generation by the mice. However, we should be able to make valid quantitative comparisons between mice as long as the rate of creatinine generation is constant.

3. We did not assess proteinuria following injury. The steady state assumptions would not apply during acute kidney injury, rendering spot urine protein to creatinine ratios uninterpretable; the number of mice in the study precluded for us an attempt at timed urine collections for the entire group.

   There was no statistically significant difference in the response to injury between genders although there was a trend to more severe injury in the females of both genotypes. The male and female mice are now distinguished in figure 8 and these observations are now noted in the results (page 18 of the revised manuscript).

4. We have added a discussion of the potential role of angiogenesis in acute kidney injury with appropriate references as requested (pages 4, 5 and 27). As noted by Dr. Long, maintenance of the peritubular capillary network following injury is known to be important in recovery from AKI and active angiogenesis plays a role. One could assess angiogenic mediators following injury, but since functional and morphologic recovery was not different between WT and Clic4 nulls, and with the wide variability in degree of injury within each group, interpretation of any possible result would be of uncertain significance. Consequently, we chose not to invest the resources to make those measurements. We think a more well-behaved experimental model would be necessary to investigate effect of absence of CLIC4 on active angiogenesis following injury.

Minor: As requested, we have added some further explanation to the statistics section (page 11-12) and specified the age of the mice used for immunostaining.

Dr. Dockrell:

1. Citations have been added.
2. Negative controls have been added to figures 1 and 2.
3. We have added a low power image (figure 1) that shows a number of LTA-positive proximal tubule segments that lack the apical staining for CLIC4 and also shows LTA negative distal tubule segments.

4. We have edited the figure to show nuclear staining more clearly.

5. Table numbering has been corrected.

6. Medium changed to Median – thanks!

7. The LTA staining is more apparent in revised figure, which also now includes a LTA-negative tubule fragment for comparison.

8. We now use a cutoff of BUN>200 for identifying severe injury in both considering the susceptibility to initial injury in the text, and examining the recovery of severe kidney injury as presented in new figure 10B. In the previous version of the manuscript, we had used a lower threshold for the recovery figure because I was concerned that the low number of WT mice that had BUN values over 200 and survived to day 21 weakened the statistics. However, the point is actually made quite well with favorable statistics using the higher threshold as now presented. We agree the resulting consistency of definitions makes the paper clearer.

9. Severity of acute injury is defined by BUN on day 2; severity of chronic residual renal dysfunction is defined by BUN on day 21. The observation we are trying to make is that mice with higher initial injury (day 2 BUN) tended to have more chronic residual renal dysfunction (higher day 21 BUN). We have revised the text to make this more clear (page 19 of the revised manuscript).

10. The method to determine percentage fibrosis is now presented in the methods section (page 10 of the revised manuscript).

11. There are two parts to the statement, both of which I think are defensible and supported by the data: 1. It is clear we do not see a substantial re-distribution of CLIC4 to the nuclei of either proximal tubule cells or peritubular capillary endothelial cells. 2. While we did not directly measure kinetics of the P-SMADs, the prediction of a CLIC4-mediated prolonged half life is that the phosphorylated form will be more abundant at any time point following injury in the WT than the Clic4 null. While we do see a tendency to lower levels of the P-SMAD at all time points in the absence of CLIC4, this never reaches the 95% confidence level. Furthermore, we see no difference in the expected end result of TGF signaling through SMADs, i.e, scarring, fibrosis, and residual renal injury. Therefore, I think the statement is fair and supported by the data.

We hope with these revisions, the manuscript is now acceptable for publication in BMC-Nephrology.

Sincerely yours

John C. Edwards, for the authors.