



## Renal Peroxiredoxin 6 interacts with Anion Exchanger 1 and plays a novel role in pH homeostasis

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3 **Renal Peroxiredoxin 6 interacts with Anion Exchanger 1 and plays a novel role in pH**  
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5 **homeostasis**  
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## Abstract

Peroxiredoxin 6 (PRDX6) is one of six members of the PRDX family, which have peroxidase and antioxidant activity. PRDX6 is unique, containing only one conserved cysteine residue (C47) rather than the two found in the others. A yeast two-hybrid screen elicited PRDX6 as a potential binding partner of the C-terminal tail of anion exchanger 1 (AE1), a Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger basolaterally expressed in renal  $\alpha$ -intercalated cells. PRDX6 immunostaining in human kidney was both cytoplasmic and peripheral and co-localized with kAE1. Analysis of native protein showed the protein was largely monomeric, whereas expressed tagged protein was more dimeric. Two methionine oxidation sites were identified. *In vitro and ex vivo* pulldowns and immunoprecipitation assays confirmed interaction with AE1; mutation of the conserved C47 resulted in loss of interaction. We found that *Prdx6*<sup>-/-</sup> mice have a baseline acidosis with a major respiratory component and greater Ae1 expression than *Prdx6*<sup>+/+</sup> animals. After an oral acid challenge, *Prdx6* expression increased in +/+ mice, with preservation of Ae1, but Ae1 expression significantly decreased in -/- animals. Kidneys from acidified mice showed widespread proximal tubular vacuolation in +/+ but not -/- animals. siRNA induced knockdown of PRDX6 in mammalian cells resulted in reduction of both total and cell membrane AE1 levels. These data suggest that the PRDX6-AE1 interaction contributes to maintenance of AE1 during cellular stress such as metabolic acidosis.

(1498 characters)

## Introduction

Anion exchanger 1 (AE1), also known as Band 3, is a sodium-independent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger member of the SLC4 gene family expressed in erythrocytes (eAE1) and at the basolateral surface of intercalated cells in the kidney (kAE1). AE1 plays a crucial role in intracellular and systemic pH regulation, and maintenance of intracellular  $\text{Cl}^-$  levels and cell volume.<sup>1</sup> Compared with eAE1, human kAE1 lacks the N-terminal 65 amino acids.<sup>2</sup> This truncation is known to preclude kAE1 protein binding to some known eAE1 partners (such as aldolase and ankyrin), but does not interfere with anion exchange.<sup>3, 4, 5, 6, 7</sup> The C-terminus of AE1 (AE1(C)), containing residues 876-911, has proved interesting, with the elucidation firstly of AE1(C) mutations that result in distal renal tubular acidosis, including a truncating mutation (AE1(C) $\Delta$ 11) and a point mutation (M909T) causing aberrant kAE1 membrane targeting, and secondly, C-terminal protein binding partners including GAPDH, the sodium pump's  $\beta$ 1 subunit, kinesin 3B, carbonic anhydrase and AP1.<sup>8, 9, 10, 11, 12, 13, 14, 15, 16, 17</sup>

To investigate the protein binding of AE1(C), a yeast two-hybrid screen was designed using both intact AE1(C)WT and AE1(C) $\Delta$ 11 against a human kidney cDNA library.<sup>13</sup> A number of candidate binding partners were identified, including peroxiredoxin 6 (PRDX6), which we investigated in this work.

The peroxiredoxins, a family of at least 6 members in mammals, have peroxidase activity, catalyzing  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and acting as antioxidant proteins. While maintaining these functions, PRDX6 is also uniquely capable of phospholipase  $\text{A}_2$  activity, hydrolyzing the *sn*-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids.<sup>18</sup> PRDX6 has only one conserved cysteine (C47) that is buried within the protein's tertiary structure,<sup>19</sup> whereas the other PRDX family members all contain two conserved cysteine residues and

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3 typically form homodimers.<sup>20</sup> The crystal structure of PRDX6 reveals the exposed catalytic  
4 triad for phospholipase A<sub>2</sub> activity (His26, Ser32, Asp140) and the buried conserved  
5 cysteine (C47), which is the catalytic site for peroxidase activity;<sup>18</sup> other structural work  
6 indicates that heterodimerization with glutathione accompanies PRDX6's activity.<sup>21</sup>  
7  
8 Northern and Western blotting have shown significant tissue expression of PRDX6 in brain,  
9 heart, kidney, lung and testis,<sup>22</sup> and PRDX6 expression has recently been identified in all  
10 sections of the nephron.<sup>23</sup> Here we report the interaction between AE1 and PRDX6 in the  
11 kidney, and demonstrate a role for renal PRDX6 in pH homeostasis.  
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## 25 Results

### 26 *PRDX6 identified as a potential binding partner for AE1(C)*

27  
28 Initial evidence of an interaction between AE1 and PRDX6 from yeast two-hybrid screening  
29 was validated by obtaining colonies following specific mating of both the AE1(C)WT and  
30 AE1(C) $\Delta$ 11 bait strains, but not p53 vector strain alone, with the PRDX6 strain (Figure 1A).  
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32 This indicates that the interaction takes place upstream of the R901 truncation site within  
33 AE1's 36-residue C-terminal cytoplasmic domain.  
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### 42 *PRDX6 is present in human and mouse kidney*

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44 Localization of the two proteins in human kidney was examined using dual immunostaining.  
45 As expected, kAE1 was constrained to the basolateral surface of intercalated cells (Figure  
46 1B-F, red), while PRDX6 (green) was observed in all cells and its distribution was more  
47 widespread in both cytoplasm and at the cell periphery. Clear co-localization of PRDX6 and  
48 kAE1 was observed (Figure 1F). Absence of staining when primary antibodies were omitted  
49 confirmed specificity (C). Further confirmation of the PRDX6 antibodies' specificity was  
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3 demonstrated on *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> kidney sections and lysates (Supplementary Figure  
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6 1).

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8 Analysis of native PRDX6 expression in human and mouse kidney under non-reducing  
9  
10 conditions demonstrated largely monomeric expression as a doublet (~26 kDa) with a  
11  
12 small amount of dimer (~52 kDa) (Figure 2A). Reducing conditions yielded an expected  
13  
14 single monomeric band. Given the embedded location of the cysteine, any *in vivo*  
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16 dimerization is most likely via non-covalent bonding.<sup>19</sup>  
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#### 18 19 20 *PRDX6 co-immunoprecipitates with kAE1*

21  
22 *In vivo* confirmation of the kAE1-PRDX6 association was provided by co-  
23  
24 immunoprecipitation (co-IP) of the two from human kidney membrane preparations. The  
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26 completed IP was probed with Bric 170 (anti-AE1 antibody) and a band was observed in  
27  
28 the bead-bound anti-PRDX6 lane (Figure 2B) but not in either of the two control lanes  
29  
30 (beads alone and a bead-bound extraneous precipitating antibody (PI3K)). However,  
31  
32 although these results supported an *in vivo* interaction between kAE1 and PRDX6, they  
33  
34 could not differentiate between direct and indirect linkage between the two proteins.  
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#### 39 40 *Protein expression and HisPRDX6 mutagenesis*

41  
42 Expressed and purified N-terminal histidine-tagged PRDX6 (HisPRDX6) run on a coomassie  
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44 gel under non-reducing conditions yielded approximately equal ratios of monomeric (~26  
45  
46 kDa) and dimeric (~52 kDa) states (Figure 2C), in contrast to the behaviour of the untagged  
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48 protein (where monomer prevailed; Figure 2A). Mass spectrometry of all four bands  
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50 revealed the purified tagged protein in four different oxidation states via the two  
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52 methionine residues per molecule (Supplementary Figure 2); this likely also accounts for  
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54 the doublets observed in Figure 2A.  
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3 The single conserved cysteine (C47) was next mutated to alanine (HisPRDX6-C47A) and  
4 here monomer prevailed over dimer (Figure 2C). *In vitro* dimerization is potentially  
5 influenced by both non-covalent bonding between two HisPRDX6 proteins and by the  
6 interaction between N-terminal His tags.<sup>24</sup>  
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#### 10 11 12 *Binding of PRDX6 to AE1 confirmed by pull-down*

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14 To further support the interaction between AE1 and PRDX6, a pull-down using purified  
15 bead-bound HisPRDX6 to precipitate kAE1 from a human kidney membrane protein  
16 fraction was also performed, showing that HisPRDX6 precipitates kAE1. When mutagenized  
17 HisPRDX6-C47A was used, a loss of binding to kAE1 was demonstrated (Figure 2D). Beads  
18 alone provided a negative control.  
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#### 27 28 *Direct protein-protein interaction*

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30 The direct interaction between purified HisPRDX6 and expressed and purified N-terminal  
31 maltose binding protein-tagged AE1(C) (MBP-AE1(C)) was assessed by pull-down using the  
32 two recombinant proteins under non-reducing conditions. Bead bound MBP-AE1(C) or MBP  
33 alone were incubated with HisPRDX6, and precipitated proteins were analyzed by Western  
34 blotting using anti-PRDX6. The same blot was re-probed for MBP as a loading control. As  
35 with the previous *in vitro* assay, HisPRDX6 was seen largely in its dimeric form (Figure 2E).  
36 Again mutagenized HisPRDX6-C47A showed a loss of binding (Figure 2F). Supplementary  
37 Figure 3 confirms the successful expression of AE1-MBP, although partial degradation or  
38 aggregation of MBP cannot be excluded in these preparations.  
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52 Thus both indirect and direct pull-down techniques demonstrated loss of kAE1 binding to  
53 the mutant HisPRDX6. Given the known inaccessible location of C47 within the protein,<sup>19</sup> it  
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3 is likely that non-covalent binding between kAe1 and the tertiary PRDX6 structure is  
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5 disrupted by the C47A mutation.  
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#### 8 *Prdx6 and kAe1 expression variation in Prdx6<sup>+/+</sup> and Prdx6<sup>-/-</sup> mice*

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10 *Prdx6<sup>-/-</sup>* mice are reported as having no readily evident phenotype under ambient  
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12 conditions<sup>25</sup> but males have reduced fertility.<sup>26</sup> Here we found that systemic pH in knockout  
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14 mice was lower than that of wild type animals (+/+, 7.31 ± 0.03; -/- 7.07 ± 0.03, p<0.01;  
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16 Figure 3A, t=0), with elevated pCO<sub>2</sub> (Supplementary Table 1) indicating a respiratory  
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18 component. This was accompanied by significantly more kAe1 protein at baseline in *Prdx6<sup>-/-</sup>*  
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20 animals than wild-type animals (Figure 3B, t=0 hr), implying some, but not sufficient,  
21  
22 attempt at renal compensation. Since kAe1 is critically involved in pH homeostasis, we next  
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24 investigated the ability of these animals to handle oral acid loading with NH<sub>4</sub>Cl,<sup>27</sup> examining  
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26 kAe1 and/or Prdx6 expression. Both *Prdx6<sup>+/+</sup>* and *Prdx6<sup>-/-</sup>* animals responded to acid  
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28 loading with a drop in pH (Figure 3A); the nadir was reached more rapidly (by 1 hr) in  
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30 *Prdx6<sup>-/-</sup>* than in *Prdx6<sup>+/+</sup>* animals (2 hr post-gavage). At 3 hr post gavage, Prdx6 expression  
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32 was significantly increased in *Prdx6<sup>+/+</sup>* animals (Figure 3C), while a transient but non-  
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34 significant rise in kAe1 expression had resolved (Figure 3D). Interestingly, kAe1 levels  
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36 dropped significantly at 3 hr post gavage in the *Prdx6<sup>-/-</sup>* mice (Figure 3B).  
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38 Additionally, at 48 hours post-gavage, *Prdx6<sup>+/+</sup>* animals had returned to their baseline pH;  
39  
40 however, *Prdx6<sup>-/-</sup>* mice significantly overshoot and failed to return to their baseline pH  
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42 (Figure 3A). Taken together, these results suggest that Prdx6 plays a role in protecting kAe1  
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44 during metabolic acidification and works within the molecular pathways that maintain and  
45  
46 restore pH.  
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#### 55 *Cellular changes in acidified Prdx6<sup>+/+</sup> and Prdx6<sup>-/-</sup> mice*



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3 In support of this, when wild-type mice were acidified, widespread cellular vacuolation  
4 developed in their kidneys (Figure 4A, white arrows), which resolved when pH normalized,  
5  
6 but in the knockout animals (n=3) at the same time points, vacuolation was minimal.  
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10 Staining with megalin showed vacuolation to be in the proximal tubule and not in other  
11 nephron segments (Figure 4B) with ~30% of proximal tubules affected. Vacuolation did not  
12 conform to known organelles including lysosomes and autophagosomes (Supplementary  
13 Figure 4A-E). Apoptosis was not detected by TUNEL staining (data not shown) and there  
14 was no detectable activated Caspase-3 in either genotype by 3 hrs post gavage  
15 (Supplementary Figure 4F,G). Staining for Ae1 in wild type and knockout mouse kidney  
16 revealed that it is maintained in a basolateral location in the absence of Prdx6 (Figure 4C  
17 and D respectively).  
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#### 20 *siRNA knockdown of PRDX6 leads to loss of kAE1 in mammalian cells*

21 HEK- $\Delta$ pMEP-eGFP-kAE1 cells stably express GFP-tagged kAE1 evenly on the plasma  
22 membrane.<sup>14</sup> Using a specific human siRNA for PRDX6, ~80% knockdown of expression  
23 was achieved whereas a control oligo had no effect (Figure 5A); the knockdown did not  
24 cause a difference in viability between the treatment groups. Immunolocalization following  
25 knockdown showed preservation of kAE1 appearances (Figure 5B) but total levels of kAE1  
26 were lower in knockdown cells than control cells (Figure 5C). Surface biotinylation  
27 confirmed that surface kAE1 was depleted in approximately the same ratio as total kAE1  
28 (Figure 5D). These data indicate that PRDX6 is required to maintain the amount of kAE1  
29 present in the cell but does not affect its trafficking to the cell membrane.  
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## 32 **Discussion**

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3 This study includes the first data concerning PRDX6 in native human kidney. To date, most  
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5 work regarding PRDX6 function has been in tissues other than the kidney and/or in  
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7 rodents; rodent renal PRDX6 expression was recognized in 2001<sup>22, 28</sup> and there is evidence  
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9 for PRDX6 up-regulation in renal cancer.<sup>29</sup> While expression of all members of the Prdx  
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11 superfamily has been identified within the distal nephron of rats,<sup>23, 28</sup> our yeast two-hybrid  
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13 screen only elicited PRDX6 as a potential binding partner for AE1. As well as *ex vivo* and *in*  
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15 *vitro* experimental verification of an interaction between PRDX6 and the C-terminus of AE1,  
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17 our work demonstrates a role for PRDX6 in maintenance of acid-base homeostasis, in which  
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19 AE1 also plays an important part.  
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24 Unlike the other peroxiredoxins, PRDX6 does not form a homodimer through a disulfide  
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26 bond of the single conserved cysteine; any *in vitro* dimers are rather either through  
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28 hydrogen bonds or intermolecular interactions between residues in  $\beta$ -strands of the two  
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30 monomers.<sup>19, 21</sup> Our results from *ex vivo* kidney bear this out, with a minimal amount of  
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32 dimerization observed. We wished to better understand the role of the C47 residue, a  
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34 known catalytic site; thus it was mutagenized to alanine in a His-tagged PRDX6 construct.  
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36 This did dimerize *in vitro*, whether or not the cysteine was present, further confirming  
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38 previous assertions that dimerization is not via C47. The likeliest explanation for this  
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40 difference is *in vitro* interaction between tags,<sup>24</sup> however we cannot exclude the possibility  
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42 that removing a cysteine residue may cause sufficient conformational change to affect  
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44 intermolecular interactions. Importantly, complete loss of PRDX6-AE1 interaction was  
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46 observed when the cysteine was absent, in both *in vitro* and *ex vivo* experiments. This  
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48 implies that C47 is required for normal folding of a separate binding moiety in the PRDX6  
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3 structure. Very recently, PRDX6 structural differences based upon PLA<sub>2</sub> versus peroxidase  
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5 catalytic activity have also been shown, indicating flexibility of PRDX6 structure.<sup>30</sup>  
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8 For *in vivo* understanding, we pursued murine studies. *Prdx6*<sup>-/-</sup> mice have a resting acidosis  
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10 that is largely respiratory, with correspondingly a greater expression of kAe1 compared to  
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12 *Prdx6*<sup>+/+</sup> animals. These results are in line firstly with *Prdx6*'s known expression in the lung,  
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14 and secondly with the role of kAe1 to reabsorb bicarbonate into the blood to increase pH.  
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17 Exogenously induced metabolic acidosis in knockout animals led to a drop in kAe1  
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19 expression, but from a higher baseline than in WT animals. This suggests firstly that Ae1 is  
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21 also interacting with other stabilising protein(s) enabling it to contribute to compensation  
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23 for chronic acidosis, and secondly that in the acute situation, *Prdx6* stabilizes kAe1, so  
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25 without *Prdx6*, kAe1 expression cannot be maintained under increasingly acidotic  
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27 conditions. Systemic pH in fact overshoot in *Prdx6*<sup>-/-</sup> compared to <sup>+/+</sup> animals following acute  
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29 acidification, again suggesting that normal compensatory mechanisms are not in place.  
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33 We were not surprised that *Prdx6*<sup>-/-</sup> animals showed a baseline increase in Ae1 abundance,  
34  
35 since many other interacting and chaperone proteins are involved in the response to  
36  
37 acidosis. It is known that on the basis of an initially normal acid-base status, AE1 increases  
38  
39 at the cell surface in response to an induced acidosis via cellular trafficking and its total  
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41 amount trends (not significantly) to an increase.<sup>31, 32</sup> However, quantitative responses to  
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43 the situation in our mice, where there was already an underlying respiratory acidotic  
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45 component, do not appear in the literature for comparison. We speculate that the difference  
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47 in kinetics between PRDX6 WT and KO animals is due to an abnormal cellular stress  
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49 response. It is also likely that PRDX6 may interact with other proteins in the response  
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51 pathway.  
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3 In support of PRDX6 playing a stabilizing role, siRNA knockdowns of PRDX6 in HEK cells  
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5 stably expressing kAE1 demonstrated a total reduction of kAE1 expression but a similarly  
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7 proportionate amount localizing to the cell surface as in control cells. Unfortunately, further  
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9 correlations between *in vitro* and physiologic data could not be made because the buffered  
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11 pH of cell culture media (7.5-8) is significantly different from the chronically acidic systemic  
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13 pH in the mouse model and we could not mimic this *in vitro*.  
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16  
17 Whether it is PRDX6's phospholipase or peroxidase activity that is important for kAE1  
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19 maintenance in intercalated cells during an acid challenge is unfortunately unanswerable in  
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21 the absence of an intercalated cell line in which to explore it, although either mechanism is  
22  
23 plausible. In addition, purified native or recombinant enzyme *in vitro* generally lacks GSH  
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25 peroxidase activity because of oxidation of its single conserved cysteine.<sup>21</sup> Maximal PRDX6  
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27 enzymatic activity has been shown to be pH dependent with PLA2 activity optimal at pH 4  
28  
29 versus peroxidase activity at pH 7 – 7.4.<sup>33</sup> Despite this difference, it has been suggested that  
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31 it is PLA2 activity that is important for function.<sup>18</sup> However, others indicate that PRDX6  
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33 peroxidase activity is crucial for gene expression via ROS stabilization.<sup>26, 34</sup> kAE1  
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35 stabilization could either be due to PRDX6 stabilizing the cell membrane through PLA2  
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37 activity, which would explain kAE1 expression decreasing in acidified mice, or decreased  
38  
39 kAE1 expression could be due to decreased gene expression resulting from increased ROS  
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41 with decreased pH.  
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49 Histological changes were noted in the kidneys of acutely acidified *Prdx6*<sup>+/+</sup> animals that  
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51 were not found in *Prdx6*<sup>-/-</sup> mice. Neither strain showed cellular vacuolation at baseline;  
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53 however, after NH<sub>4</sub>Cl treatment, *Prdx6*<sup>+/+</sup> mice showed considerable amounts of proximal  
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55 tubular cellular vacuolation, which did not develop in knockout animals. The vacuoles did  
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3 not conform to any known organelles or lipid storage vacuoles. Cellular vacuolization is a  
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5 known response to various forms of cellular stress<sup>35, 36</sup> and addition of NH<sub>4</sub>Cl to chick  
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7 fibroblast cells causes reversible vacuolation with no effect on functionality.<sup>36</sup> These  
8  
9 authors suggested a mechanism involving sequestration of base left from NH<sub>4</sub>Cl  
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11 dissociation, to minimise alkalinization of the cytosol. Additionally, NH<sub>4</sub>Cl inhibits the  
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13 maturation of lysosomes, which would normally sequester hydrogen ions.<sup>37</sup> Thus, another  
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15 possibility is that vacuoles directly sequester hydrogen ions. However, in this scenario, we  
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17 would have expected vacuolation to be present at baseline in -/- animals but possibly not to  
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19 worsen. A further hypothesis would be that vacuoles sequester oxidized glutathione in an  
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21 effort to maintain the cell's reducing capacity, a phenomenon which is seen in yeast,<sup>38</sup> but  
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23 which the lack of PRDX6 would render inefficient.  
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30 In summary these data, together with earlier studies of ours and others, suggest that while  
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32 Prdx6 cannot be the sole Ae1 stabilising protein (such that Ae1 can contribute to  
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34 compensation for chronic acidosis in Prdx6<sup>-/-</sup> animals), in the acute situation, Prdx6 plays a  
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36 direct or indirect role in stabilising kAe1. Thus without Prdx6, kAe1 expression cannot be  
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38 maintained under increasingly acidotic conditions and the recovery from acidosis is also  
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40 compromised.  
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## 46 **Materials and Methods**

### 47 *Yeast Two-Hybrid Assay*

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50 AE1(C)WT (residues 876-911) or AE1(C) $\Delta$ 11 (residues 876-900) were used as bait protein  
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52 to screen the Pre-Transformed Human Kidney Matchmaker cDNA Library (Clontech) as  
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54 previously described.<sup>13</sup> Confirmation of potential interaction with PRDX6 was assessed by  
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3 mating back to the original bait of either AE1(C)WT, AE1(C) $\Delta$ 11 or a control vector (p53).

4  
5 Confirmed clones were sequenced and identified through BLAST searches

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7  
8 (blast.ncbi.nlm.nih.gov).

### 9 10 *Histological and Immunofluorescence Staining*

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12 With ethical approval from the Cambridge Biomedical Research Centre Tissue Bank, 10%  
13 formalin fixed, paraffin wax-embedded normal human kidney discarded following tumour  
14 removal was obtained. After de-paraffinization and rehydration in an ethanol series,  
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proteinase K antigen retrieval was undertaken (50  $\mu$ g/ml) for 6 minutes at room  
temperature. Following washing and blocking, primary antibodies were applied overnight  
at 4°C (anti-PRDX6 (Abcam 59543) at 1:500; Bric 170 (IBGRL 9450) at 1:100).

Fluorochrome conjugated secondary antibodies (1:200) were applied for 1hr at room  
temperature. After mounting with Vector Shield Mounting Medium (Vector Laboratories)  
slides were examined using a Zeiss LSM880 confocal microscope.

Mouse kidneys were fixed in 10% neutral buffer formalin, paraffin-embedded and  
sectioned before hematoxylin/eosin staining. Primary antibodies for immunolocalisation  
were against megalin (sc-16478) at 1:200, LC3 (NB100-2220 Novus) at 1:500, LAMP2  
(ab13524) at 1:50, calreticulin (ab92516) at 1:250, perilipin (cs9349) at 1:250 and EEA1  
(ab2900) at 1:500, PRDX6 (ab16947 or ab59543) at 1:500, AE1 (gift of C. Wagner) at  
1:1000. TUNEL staining was carried out using a colorimetric TUNEL analysis kit (DeadEnd,  
Promega). The amount of vacuolation was assessed by counting the percentage of megalin  
positive tubules that displayed vacuolation in a 1000  $\mu$ m<sup>2</sup> area of cortex.

### *Co-Immunoprecipitation*

1  
2  
3 Protein A beads were incubated with either PRDX6 antibody (Abcam 16947), PI3K antibody  
4 (Abcam 86714) as the control antibody or no antibody in solubilization buffer (20 mM Tris,  
5  
6 pH 7.4, 150 mM NaCl, 1% NP40, 2 mM PMSF, EDTA-free Protease Inhibitor Cocktail (Roche),  
7  
8 10% glycerol) overnight at 4°C. Human kidney total protein fraction was solubilized with  
9  
10 the solubilization buffer (30 mins, 4°C) before centrifugation. Recovered supernatant, after  
11  
12 addition of 20% PEG, was incubated with the bead samples at 4°C for 4 h. The beads were  
13  
14 washed with Buffer A (20 mM Tris, pH 7.4, 5 mM NaN<sub>3</sub>, 0.1% n-Nonyl-β-D-glucopyranoside  
15  
16 (n-NDG)) and Buffer B (Buffer A + 500 mM NaCl). Bound proteins were eluted from beads  
17  
18 by incubating in SDS sample buffer [0.175 M Tris/HCl (pH 6.8), 5.14% SDS, 18% glycerol,  
19  
20 0.3 M DTT, 0.006% bromophenol blue] for 5 min at 95°C. Samples were subjected to  
21  
22 SDS/PAGE and Western blotting before probing with Bric 170.  
23  
24  
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### 30 *Expression of Wild Type and mutagenized HisPRDX6*

31  
32 *PRDX6* coding sequence inserted into a modified pRSET-A vector (N-terminal His tag, gift  
33  
34 from J. Clarke) was used to express full-length N-terminal His-tagged PRDX6 (HisPRDX6) in  
35  
36 BL21 cells according to standard methods. Expressed proteins were visualized following  
37  
38 purification on Ni-NTA resin and Western Blotting of SDS-PAGE gels. The procedure was  
39  
40 repeated with a construct created using the QuikChange Site-Directed Mutagenesis kit  
41  
42 (Stratagene) to alter the 47<sup>th</sup> residue in the PRDX6 coding sequence from cysteine to  
43  
44 alanine (HisPRDX6-C47A).  
45  
46  
47  
48

### 49 *Mass Spectrometry*

50  
51 HisPRDX6 was separated on a 12% SDS-PAGE gel under non-reducing conditions and  
52  
53 stained with InstantBlue (Expedeon). The bands were excised and individually trypsin  
54  
55  
56  
57  
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1  
2  
3 digested. The peptides were analyzed with a MALDI-TOF-TOF mass spectrometer (ABI  
4  
5 4800 model, Applied Biosystems) and identified using the Mascot database.  
6  
7

#### 8 *Expression of MBP and MBP-AE1(C)*

9

10 pMAL-c4X vector alone was used to express MBP; and *AE1(C)* (residues 876-911) coding  
11  
12 sequence inserted into pMAL-c4X polylinker vector (N-terminal MBP tag) was used to  
13  
14 express full-length N-terminal MBP-tagged *AE1(C)* (MBP-*AE1(C)*) in BL21 cells according to  
15  
16 standard methods. Expressed proteins were visualized following purification on maltose  
17  
18 resin and Western Blotting of SDS-PAGE gels (Supplementary Figure 3).  
19  
20  
21

#### 22 *HisPRDX6 Pulldown*

23

24  
25 Equal moles of HisPRDX6 wild type (WT) or HisPRDX6-C47A were immobilized on Ni-NTA  
26  
27 beads. Human kidney total fractions were solubilized with solubilization buffer (10 mM Tris,  
28  
29 pH 7.4, 1 mM EDTA, 1 mM DTT, 1.5% n-NDG, 10% glycerol) for 30 mins at 4°C prior to  
30  
31 centrifugation (Beckman Ultracentrifuge, 100,000 x g, 1 h, 4°C). The solubilized kidney  
32  
33 fractions were added to the bead bound fusion proteins and incubated at 4°C overnight. The  
34  
35 beads were washed with Buffer A (1X PBS, 0.1% Triton X-100) and Buffer B (Buffer A + 500  
36  
37 mM NaCl). Bound proteins were eluted from beads by incubating in SDS sample buffer for 5  
38  
39 min at 95°C and analyzed by SDS-PAGE and Western blot using Bric 170.  
40  
41  
42  
43

#### 44 *Direct MBP-AE1(C) and HisPRDX6 Pulldowns*

45

46  
47 Equal moles of MBP-*AE1(C)* or MBP alone were incubated with amylose resin for 3+ hours  
48  
49 at 4°C. Equivalent amounts of HisPRDX6 ligand were added to beads with bound MBP-  
50  
51 *AE1(C)* or MBP. NaCl (final concentration of 250 mM) was added to each tube. The  
52  
53 combined constructs were incubated overnight at 4°C. The beads were washed (20 mM  
54  
55 Tris-HCl, pH 7.4 buffer) containing 250-500 mM NaCl. Bound proteins were eluted from  
56  
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1  
2  
3 beads by incubating in SDS sample buffer for 5 min at 95°C and analyzed by Western blot  
4  
5 using anti-PRDX6 antibody (Abcam 16947). Blots were reprobbed with anti-MBP (NEB  
6  
7 8032S) to verify loading.  
8  
9

#### 10 *Acute acid challenge Prdx6<sup>+/+</sup> and Prdx6<sup>-/-</sup> animals*

11  
12 Groups of adult *Prdx6<sup>+/+</sup>* and *Prdx6<sup>-/-</sup>* mice were maintained on standard diets with ad lib  
13  
14 access to food and water. Animals were gavage-fed either water or 0.28M NH<sub>4</sub>Cl (0.8 g  
15  
16 NH<sub>4</sub>Cl/kg of body weight in a maximum volume of 10 mL/kg). After gavage, groups of mice  
17  
18 were terminally anesthetized and arterial blood collected at various time points and blood  
19  
20 analyzed on an i-STAT portable clinical analyzer (Abbott).  
21  
22  
23

#### 24 *Protein Fractionation and Western Blotting*

25  
26 Mouse kidneys were homogenized in 500 µl of cold homogenization buffer (10 mM Tris pH  
27  
28 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM PMSF, and protease inhibitors) followed  
29  
30 by centrifugation and collection of the total protein fraction (supernatant). Lysate  
31  
32 concentration was estimated using a BioRad protein binding assay (BioRad) and subjected  
33  
34 to SDS/PAGE and Western blot. Primary antibodies used were PRDX6 (Abcam 16947), AE1  
35  
36 (gift of C. Wagner) or Caspase-3 (9665 Cell Signalling) with actin (Abcam 6276) as a loading  
37  
38 control. Prdx6 analysis was carried out by combining the density of both the monomer and  
39  
40 dimer forms relative to actin loading control.  
41  
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#### 46 *Protein expression in mammalian cells, knockdown, biotinylation and staining*

47  
48 Stable HEK-ΔpMEP-GFP-kAE1 cells were cultured on tissue culture plates (for knockdown)  
49  
50 or glass cover slips (for staining) in DMEM (Sigma) supplemented with 10% FBS, penicillin  
51  
52 (100 U/ml)/streptomycin (100 µg/ml), and L-glutamine (2mM) at 37°C with a 5% CO<sub>2</sub>  
53  
54 atmosphere in a humidified incubator. GFP-kAE1 expression was induced with the addition  
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3 of 100 mM ZnCl<sub>2</sub> and 2 mM CdCl<sub>2</sub>. Endogenous PRDX6 expression was depleted with a  
4  
5 siRNA oligonucleotide (117286; Ambion). The oligonucleotide, or a nonspecific siRNA  
6  
7 (Silencer Negative Control No. 1 siRNA; 4611 Ambion) were transfected into the cells at a  
8  
9 final concentration of 50 nM using Lipofectamine RNAiMAX reagents (Invitrogen). 48 hours  
10  
11 after transfection the cells were evaluated by Western blot of total cell lysates or  
12  
13 biotinylation of the plasma membrane using a Cell Surface Protein Isolation Kit (Pierce)  
14  
15 followed by Western blot. Antibodies used were Bric170, PRDX6 (Abcam 16947) and α-  
16  
17 tubulin (Sigma T9026) as a loading control. For immunofluorescence the cells were fixed in  
18  
19 4% paraformaldehyde and then permeabilized with PBS containing 0.1% Triton X-100.  
20  
21 After blocking in 1% BSA the cells were incubated with PRDX6 (Abcam16947) for 1hr and  
22  
23 after washing a fluorochrome conjugated secondary antibody was applied. The slides were  
24  
25 mounted with Vectashield medium (Vector Laboratories).  
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### 35 **Disclosure**

36  
37 No author has any disclosures.  
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40 Supplementary information is available at *Kidney International's* website.  
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For Peer Review Only

## Titles and Legends

### Figure 1. Confirmation of PRDX6 as binding candidate of kAE1 and co-staining of

**intercalated cell.** (A) Yeast mating test of both AE1(C)WT and AE1(C) $\Delta$ 11 with PRDX6 in yeast cells shows protein potential interaction indicated by the blue colonies on selection plates with no cell growth of the negative control (p53 empty vector). (B-F)

Immunofluorescence in human renal collecting duct. PRDX6 (green) and AE1 (red) co-localize in a number of collecting duct cells (B) with absence of staining when primary antisera were omitted (C). An enlarged image of an intercalated cell shows widespread PRDX6 expression (D) and basolateral AE1 (Bric 170) expression (E). Merged panel (F) indicates co-localization of PRDX6 and AE1 at the basolateral surface. (Scale bars = 20  $\mu$ m)

### Figure 2. *In vitro* and *ex vivo* experimental evidence of PRDX6/AE1 interaction and

**loss of interaction with mutagenized PRDX6.** (A) PRDX6 from solubilized human kidney

fraction (HKT) immunoblotted under reducing conditions (+) and non-reducing conditions (-), showing PRDX6 in monomer only and monomer and dimer forms respectively; Prdx6

from mouse kidney fraction (MKT) immunoblotted under non-reducing conditions shows monomer and dimer forms. (B) Co-immunoprecipitation by bead bound PRDX6 antibody of

kAE1 from solubilized membrane protein fractions of human kidney. Negative controls (no antibody and PI3K antibody) did not immunoprecipitate kAE1, with the input (human

kidney membrane) providing a control for the Western blot. (C) Coomassie stained gel of expressed HisPRDX6 (1.5  $\mu$ g) and HisPRDX6-C47A (4.4  $\mu$ g) showing monomer and dimer

forms of the protein. (D) Immobilized HisPRDX6-WT and HisPRDX6-C47A proteins

incubated with human kidney membrane showed HisPRDX6-WT could precipitate kAE1;

HisPRDX6-C47A or beads alone could not. (E-F) Immobilized MBP-AE1(C) was able to



1  
2  
3 directly pull down HisPRDX6-WT (E) indicating binding but this interaction was not  
4  
5 replicated with HisPRDX6-C47A (F). MBP alone does not interact with either form of PRDX6.  
6  
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8  
9 **Figure 3. Prdx6 and kAe1 expression studies in acidified *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> mice.**

10  
11 (A) pH of terminally collected whole-blood from *Prdx6*<sup>+/+</sup> (grey circles) and *Prdx6*<sup>-/-</sup> (black  
12 squares) animals at time points after acid gavage. (B) Baseline kAe1 expression  
13  
14 (normalized to actin and relative to WT as 100% at t=0hr) is significantly greater in *Prdx6*<sup>-/-</sup>  
15 animals compared to *Prdx6*<sup>+/+</sup> animals and at 3hrs post gavage, *Prdx6*<sup>-/-</sup> animals have  
16 significantly decreased expression. (C) Prdx6 expression (combined monomer and dimer  
17 normalized to actin relative to t=0hr) is maximally increased in *Prdx6*<sup>+/+</sup> animals 3 hr post  
18 gavage. (D) kAe1 expression in *Prdx6*<sup>+/+</sup> animals is not significantly changed. (all time points  
19 refer to hours post gavage, error bars represent  $\pm$  standard error, \* = p<0.05, \*\* = p<0.01)  
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32 **Figure 4. Cellular changes in acidified adult *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> mouse kidney. (A)**

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34 H+E stained mouse renal cortex of *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> at baseline, 3 hours and 48 hours  
35 after acid gavage showing tubular vacuolation in *Prdx6*<sup>+/+</sup> animals (white arrowheads)  
36 which later resolved. This vacuolation was not evident in the cortex of *Prdx6*<sup>-/-</sup> animals after  
37 the same treatment. (B) Megalin staining (red) confirmed the proximal tubular location of  
38 the vacuoles (white arrowheads). Ae1's basolateral location (green) was similarly  
39 maintained in *Prdx6*<sup>+/+</sup> (C) and *Prdx6*<sup>-/-</sup> (D) animals. (Scale bar = 20  $\mu$ m)  
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50 **Figure 5. PRDX6 knockdown in mammalian cells. (A)** Stably expressing HEK- $\Delta$ pMEP-

51 eGFP-kAE1 cells transfected with siRNA against PRDX6 (knockdown) or negative control  
52 siRNA (control) over 48 hours show ~80% knockdown of PRDX6 protein. (B) Surface  
53  
54 membrane localization of GFP-tagged kAE1 (green) was preserved in both control and  
55  
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1  
2  
3 knockdown cells and colocalization (yellow) with PRDX6 (red) was observed; scale bars =  
4  
5 20  $\mu$ m. (C) Total GFP-kAE1 and (D) cell surface GFP-kAE1 are reduced by similar amounts  
6  
7 when PRDX6 is knocked down. Tubulin was used for normalization, with control oligo  
8  
9 results set at 100%, and blots are representative of 3 experiments. (Error bars indicate  
10  
11 SEM)  
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19  
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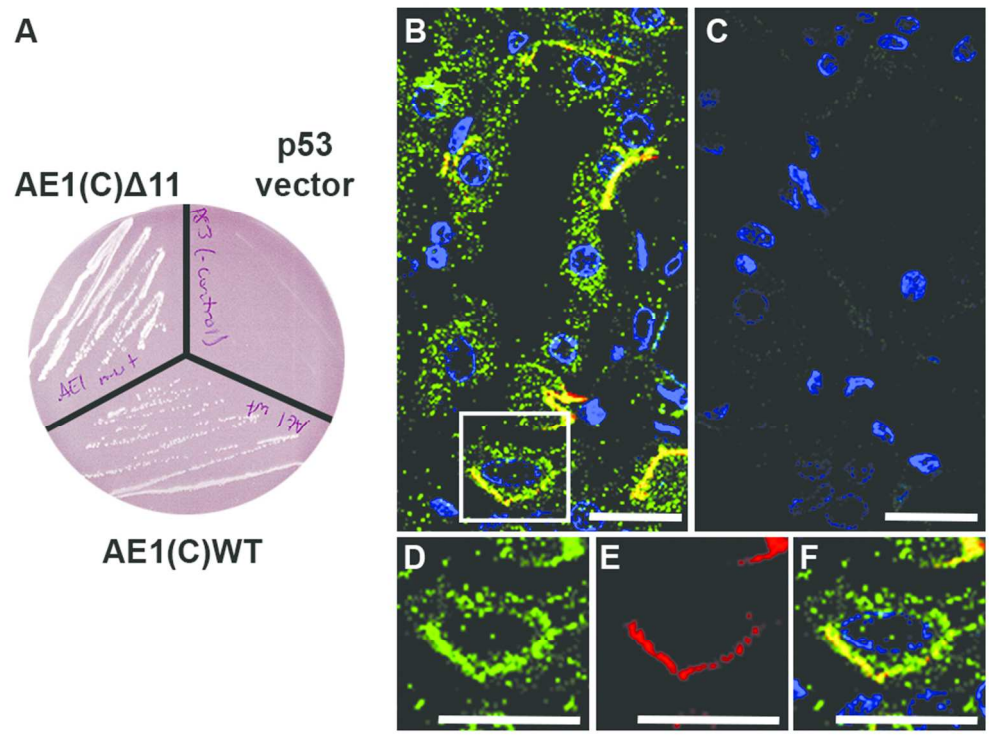


Figure 1  
86x64mm (300 x 300 DPI)

View Only

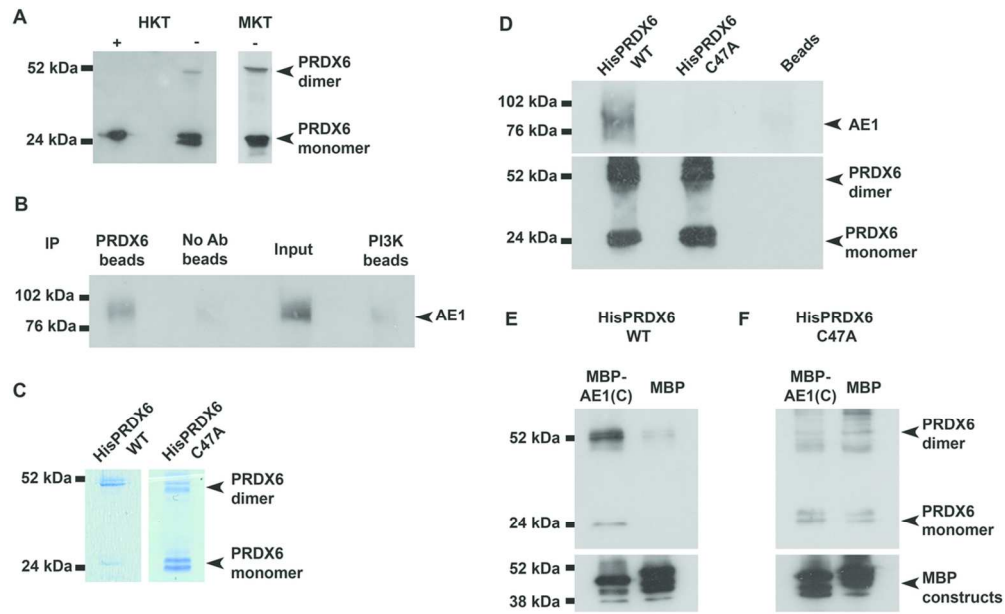


Figure 2  
109x67mm (300 x 300 DPI)

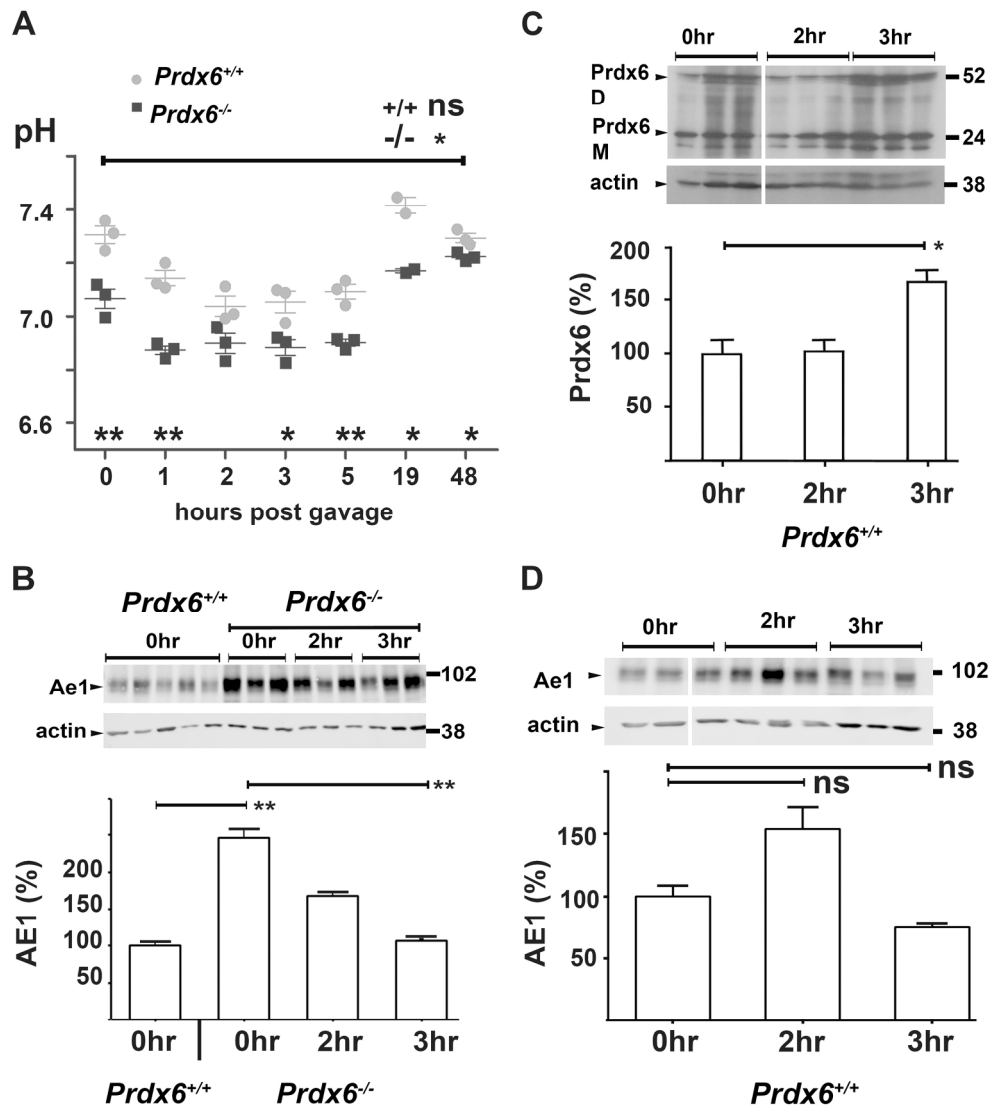


Figure 3  
96x107mm (600 x 600 DPI)



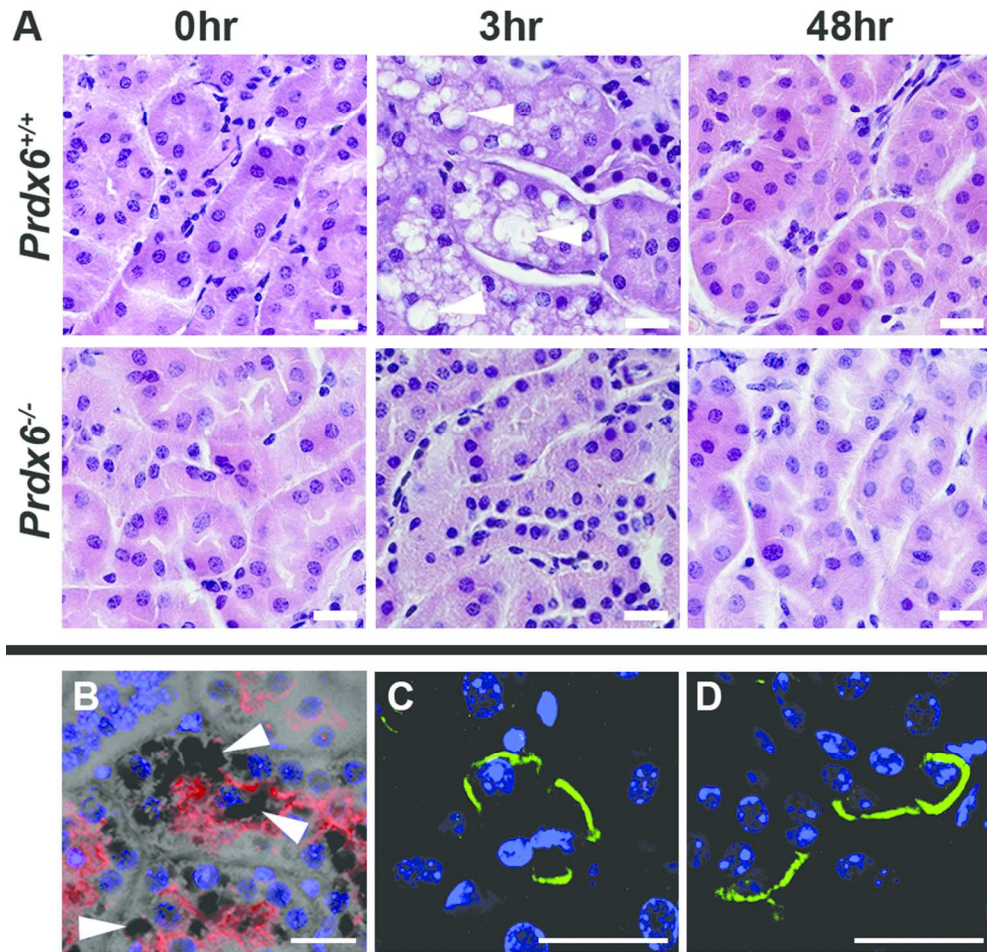


Figure 4  
85x82mm (300 x 300 DPI)

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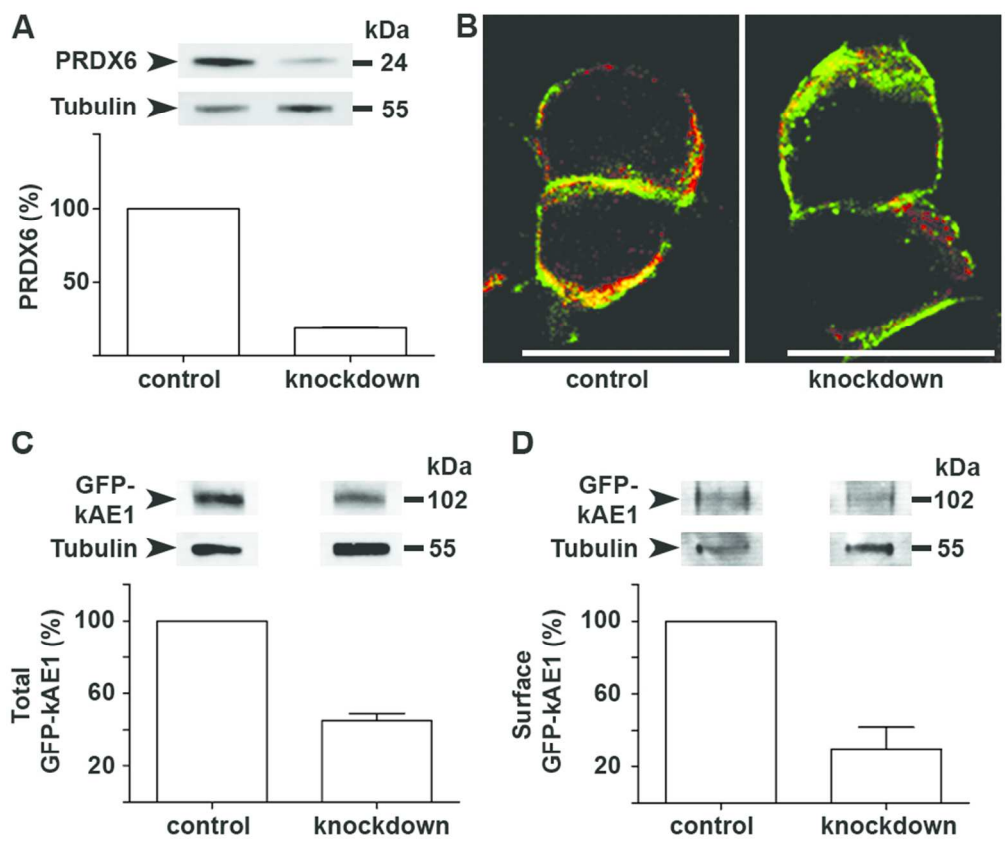


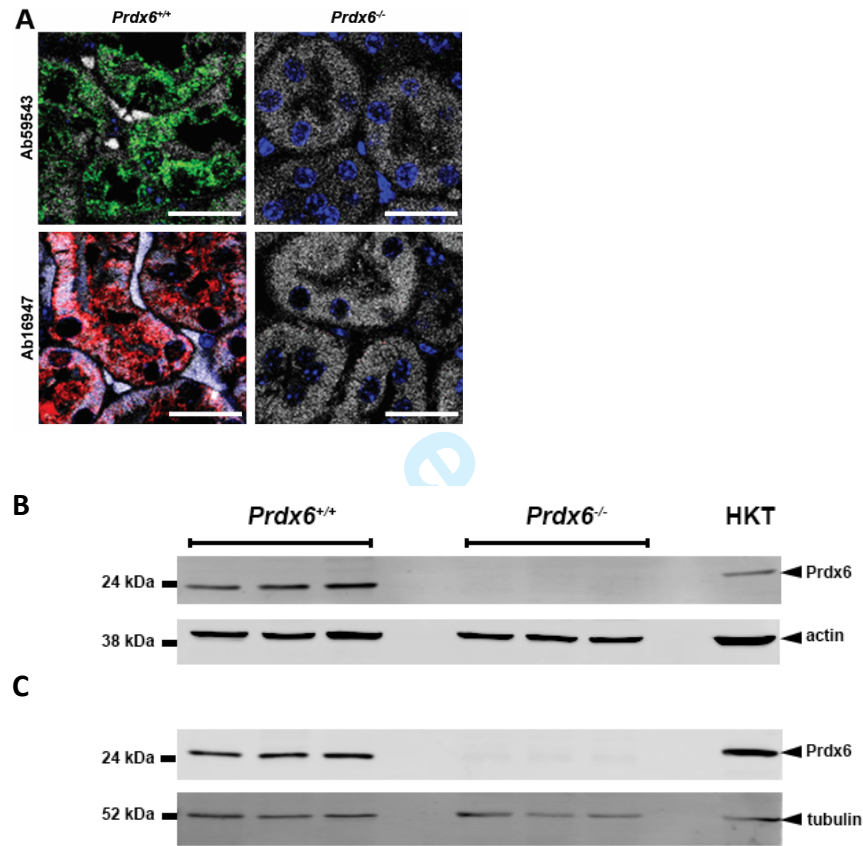
Figure 5  
86x73mm (300 x 300 DPI)

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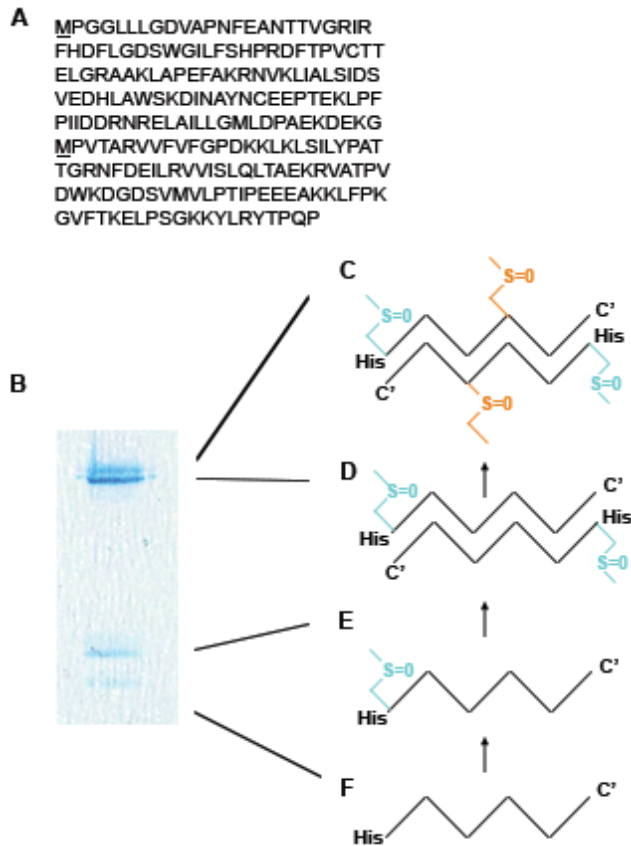


Supplementary Figure 1. Confirmation of antibody specificity by immunofluorescence and Western blotting



Kidney sections from *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> mice were used to test the specificity of the anti PRDX6 antibodies Ab59543 (green) and Ab16947 (red) (A) Scale bars: 20  $\mu$ m. Western blots with three samples each of *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> total kidney lysate with a human kidney total (HKT) lysate as a control were probed with Ab59543 (B) and Ab16947 (C). Actin and tubulin were used as loading controls respectively. The sample preparations were carried out under reducing conditions.

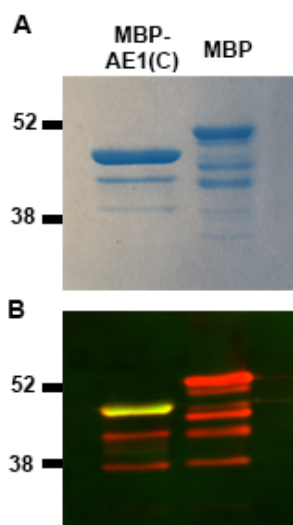
Supplementary Figure 2. Summarized MALDI-TOF-TOF results of the expressed construct PRDX6 monomer and dimer doublet bands and their oxidation states.



(A) Amino acid sequence of PRDX6 (A) with oxidised methionine residues (M1 and M127) identified by Mascot software underlined. (B) Coomassie-stained gel of expressed purified HisPRDX6 showing the protein in a range of oxidation states and (C-F) schematics showing the different oxidation states of HisPRDX6 as indicated by Mascot data. The native HisPRDX6 oxidation state corresponds to the lowest band separated on a gel (F) with (E) thru (C) representing each ascending excised band of HisPRDX6 and the corresponding oxidation of methionines and the dimerization of the protein.

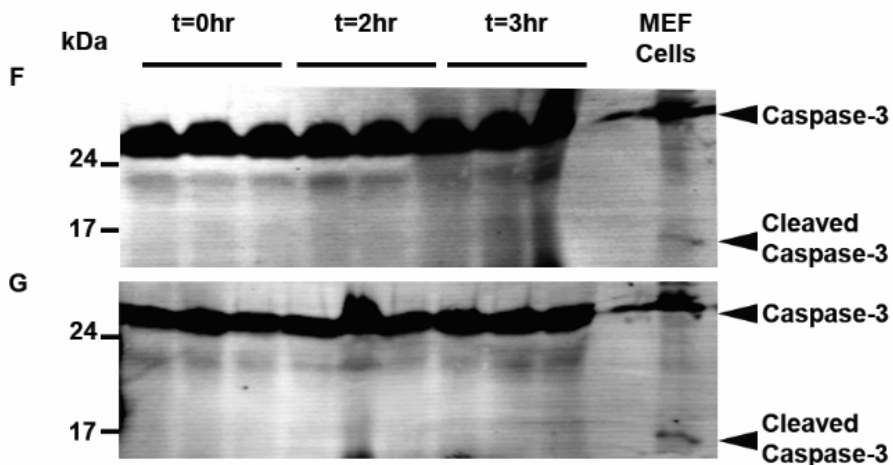
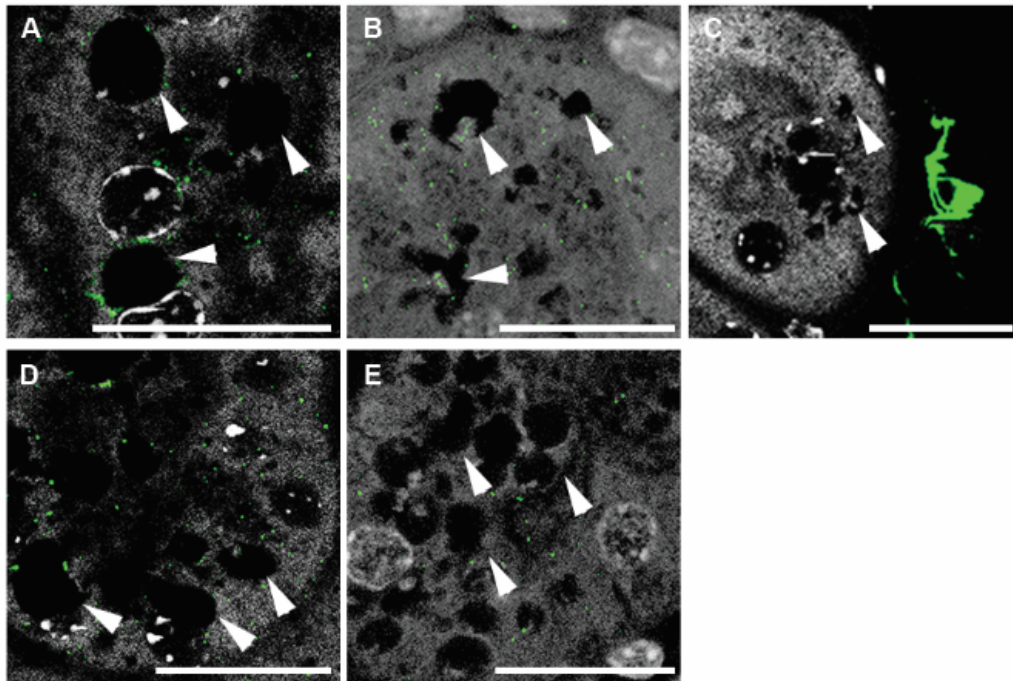


## Supplementary Figure 3. MBP-AE1(C) and MBP protein expression.



(A) Coomassie-stained gel of expressed and purified MBP-AE1(C) and MBP. (B) Merged Western blot image of the samples probed simultaneously for AE1 (green) and MBP (red) using a fluorescent conjugated secondary dye. Yellow staining represents overlapping signals.

## Supplementary Figure 4. Vacuole characterization and detection of apoptosis



Kidney sections from acid gavaged *Prdx6*<sup>+/+</sup> mice showing vacuolation (white arrowheads) were stained for (A) calreticulin, a soluble endoplasmic reticulum protein; (B) LC3, an autophagosomal marker; (C) perilipin, a lipid storage protein; (D) EEA1, an early endosomal marker; and (E) LAMP2, a lysosomal marker. All staining in green, scale bars = 20  $\mu$ m.

A Western blot for activated Caspase-3 (17/19 kDa cleaved fragment) was performed on *Prdx6*<sup>+/+</sup> (F) and *Prdx6*<sup>-/-</sup> (G) mouse kidney lysates after acid gavage showed no detectable activation (n=3 for each group). Staurosporine-treated mouse embryonic fibroblasts (MEF) cells provided a control for the blot.

**Supplementary Table 1. Blood biochemistry of adult *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> animals**

	<i>Prdx6</i> <sup>+/+</sup>	<i>Prdx6</i> <sup>-/-</sup>	P
pH	7.31 ± 0.03	7.07 ± 0.04	0.0080
HCO <sub>3</sub> <sup>-</sup> (mM)	20.3 ± 0.29	18.8 ± 1.48	ns
pCO <sub>2</sub> (kPa)	5.5 ± 0.38	8.7 ± 0.11	0.0012

Physiological blood parameters of adult *Prdx6*<sup>+/+</sup> and *Prdx6*<sup>-/-</sup> animals on a standard diet.

All values are mean ± SEM. n=3 animals.