**Supplemental information**

## Concise Methods

### Generation of Ncc T58M knock-in mice

The experimental protocols used in the present study were approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan. To generate Ncc T58M knock-in mice, the targeting vector was prepared by the gap-repair technique.[1] Mouse *Slc12a3* genomic DNA encoding Ncc was isolated from a 129/Sv genomic DNA BAC library. The nonsense mutation Ncc T58M (TCT to TAG) was introduced by PCR when preparing the targeting vector. The entire sequence was verified by sequencing. The targeting vector was then transfected into R1 ES cells (129X1/SvJ x 129S1) by electroporation. After selection with 240μg/ml G418 and 2μM gancyclovir, correctly targeted ES clones were selected by Southern blotting. The neo cassette was excised by transfecting the Cre-expression plasmid into the targeted $Ncc^{flox/+}$ ES cells. The $Ncc^{T58M/+}$ ES cells were selected by PCR (Primers, F: 5'-TGC CCA ACA TGT TCT GTC AT TA-3'; R: 5'-CGG TGC CTG GAC AAT ATG TA-3'), and injected into C57BL/6 blastocysts. Chimeric males were bred with C57BL/6 females to produce heterozygous $Ncc^{T58M/+}$ mice (F1). Homozygous $Ncc^{T58M/T58M}$ mice (F2) were produced by mating $Ncc^{T58M/+}$ mice (F1) with each other. WT, $Ncc^{T58M/+}$ and $Ncc^{T58M/T58M}$ littermates (F2) were bred

and tail genomic DNA applied for genotyping by PCR.

## Results

### Generation of Ncc T58M knock-in mice

To generate Ncc T58M knock-in mice, we used homologous recombination in ES cells to create a mutant allele in which exon 1 of the *Slc12a3* gene encoding Ncc was replaced by a cassette expressing the selectable marker neomycin transferase (neo) followed by the mutant exon 1 (T58M) (Supplemental Figure S1). Twenty recombinant ES cells were found to be $Ncc^{flox/+}$ confirmed by Southern blot (Supplemental Figure S1). The neo cassette was excised by transfecting the Cre-expression plasmid into the selected $Ncc^{flox/+}$ ES cells and then confirmed by Southern blot. We obtained 4 chimeric mice from two different selected $Ncc^{T58M/+}$ clones (D6 and E7) and then crossed them with C57BL/6 mice to produce mutant $Ncc^{T58M/+}$ progeny. The $Ncc^{T58M/T58M}$ mice were then generated by crossing $Ncc^{T58M/+}$ littermates. The genotype of the offspring was verified by PCR amplification (Supplemental Figure 1C) and direct sequencing of exon 1 of the *Slc12a3* genomic DNA (Supplemental Figure S1). Because there were no differences in gross appearance and phenotype between D6 and E7 Ncc T58M knock-in mice, the D6 strain was selected for this study. $Ncc^{T58M/+}$ and $Ncc^{T58M/T58M}$ mice grew normally and were indistinguishable from wild-type (WT) littermates in appearance, behavior and

fertility.

**Reference**

1. Liu, P, Jenkins, NA, Copeland, NG: A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res,* 13: 476-484, 2003.

**Figure legends**

**Supplemental Figure 1. Generation of Ncc T58M knock-in mice**

**(A)** Targeting strategy for generating Ncc T58M knock-in mice. The diagram shows the wild-type (WT) *Slc12a3* locus encoding Ncc, the targeting construct, and the targeted locus before (flox) and after Cre recombination (T58M). **(B)** Homologous recombination verified by Southern blotting of TthIII I-digested genomic DNA derived from the selected ES cells (D6 and E7) before deleting neomycin transferase (neo) cassette. The 17.8-kb and 9.6-kb bands are derived from the WT and mutated (flox) alleles, respectively. C: control genomic DNA from WT ES cells. **(C)** Genomic DNA derived from Ncc T58M knock-in mice were used as template and a primer set flanking the remaining loxP site (arrow) was used for genotyping. The 229bp and 134bp bands represent the mutant (T58M) and WT alleles, respectively. **(D)** Direct sequencing of the PCR product covering the mutation site in exon 1 from WT, heterozygous (He), and homozygous (Ho) Ncc T58M knock-in mice.

**Supplemental Figure 2. p-Ncc expression in kidneys of $Ncc^{T58M/T58M}$ mice.**

(A) Semiquantitative immunoblot of p-Ncc (T53, T58, and S71) in crude membrane fractions from kidneys of wild-type (WT) and homozygous (Ho) Ncc T58M knock-in mice, and its semi-quantification by densitometry analysis ($n$ = 4/group). * denotes $p<0.05$ vs. WT comparison. (B) Immunofluorescence (IF) staining for p-Ncc (T53, T58, and S71). Representative IF micrographs of Ncc (low power field 200x and high

power field 1000x) in the cortices of WT and Ho mice.

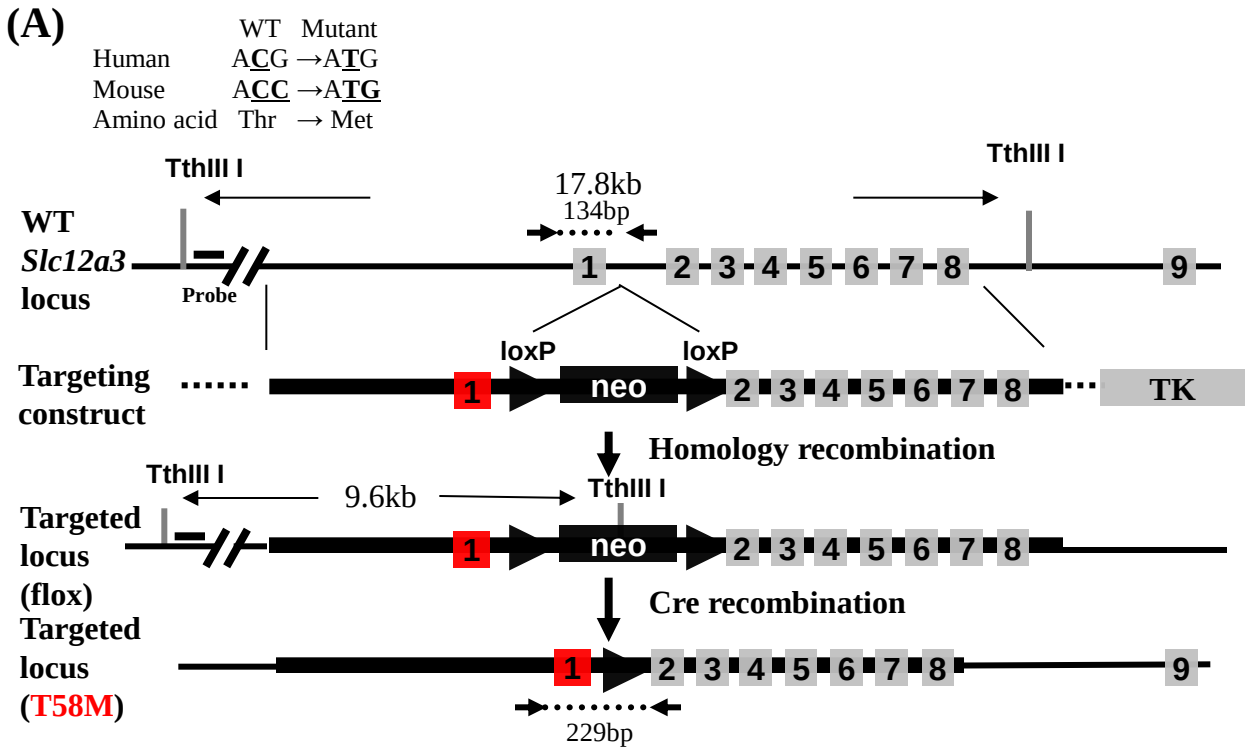
**Supplemental Figure 3. Total Nkcc2, p-Nkcc2 and ENaC( $\beta$ ) expression in kidneys of $Ncc^{T58M/T58M}$ mice.**

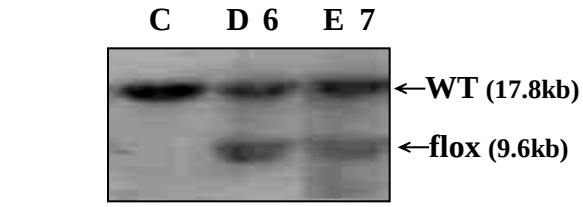
**(A)** Semiquantitative immunoblot of total Nkcc2, p-Nkcc2 (T96) and ENaC ( $\beta$ ) in crude membrane fractions from kidneys of wild-type (WT) and homozygous (Ho) Ncc T58M knock-in mice, and its semi-quantification by densitometry analysis ($n$ = 4/group). * denotes $p<0.05$ vs. WT comparison. **(B)** Immunofluorescence (IF) staining for total Nkcc2, p-Nkcc2 (T96) and ENaC(β). Representative IF micrographs of total Nkcc2, p-Nkcc2 and ENaC(β) (low power field 200x and high power field 1000x) in the outer medulla of WT and Ho mice.

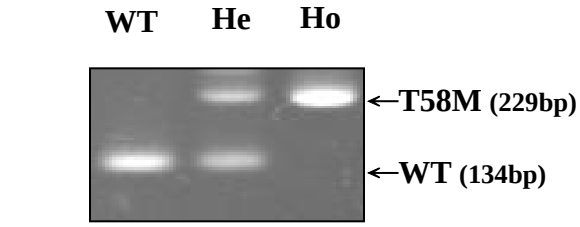
**Supplemental Figure 4. NCC mutation analysis**

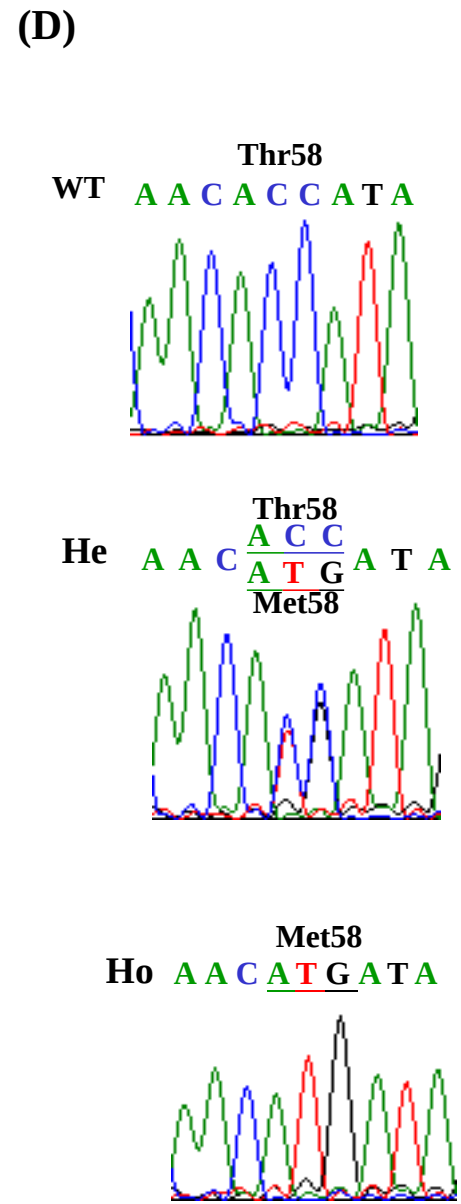
Schematic showing a representative family with NCC T60M mutation diagnosed by exon 1 sequencing directly and restriction fragment length polymorphism (RFLP) by PuvI. The patient number is the same showing in figure 5.

**Supplemental Fig. 1**

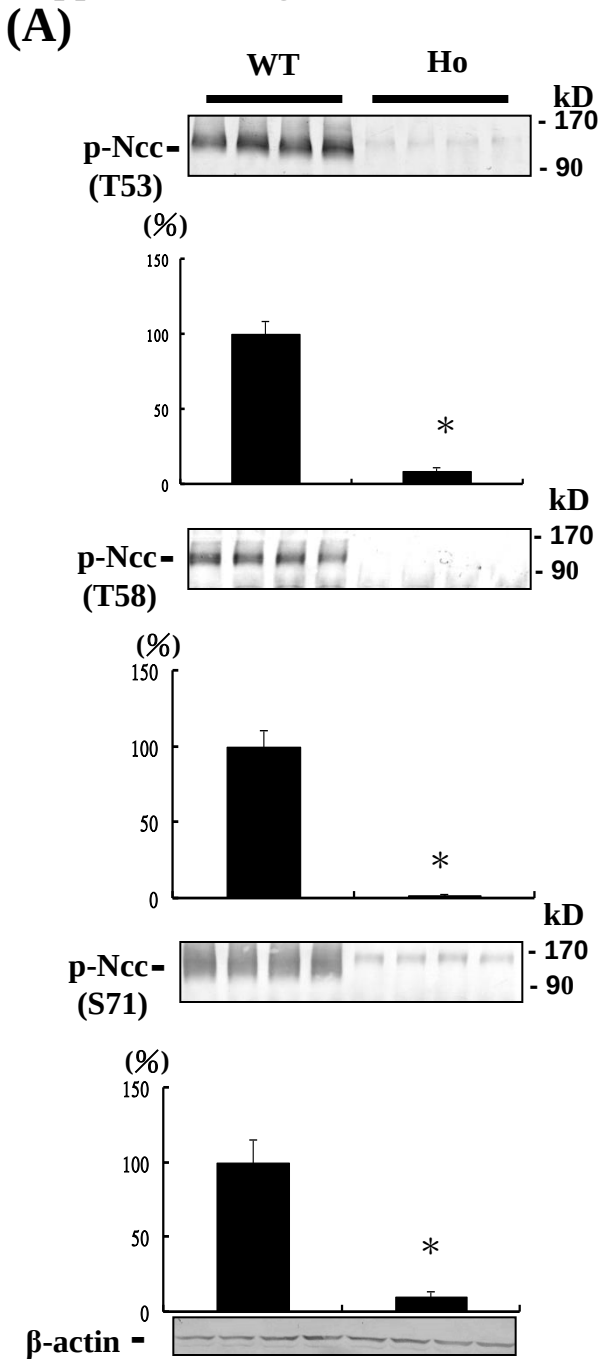


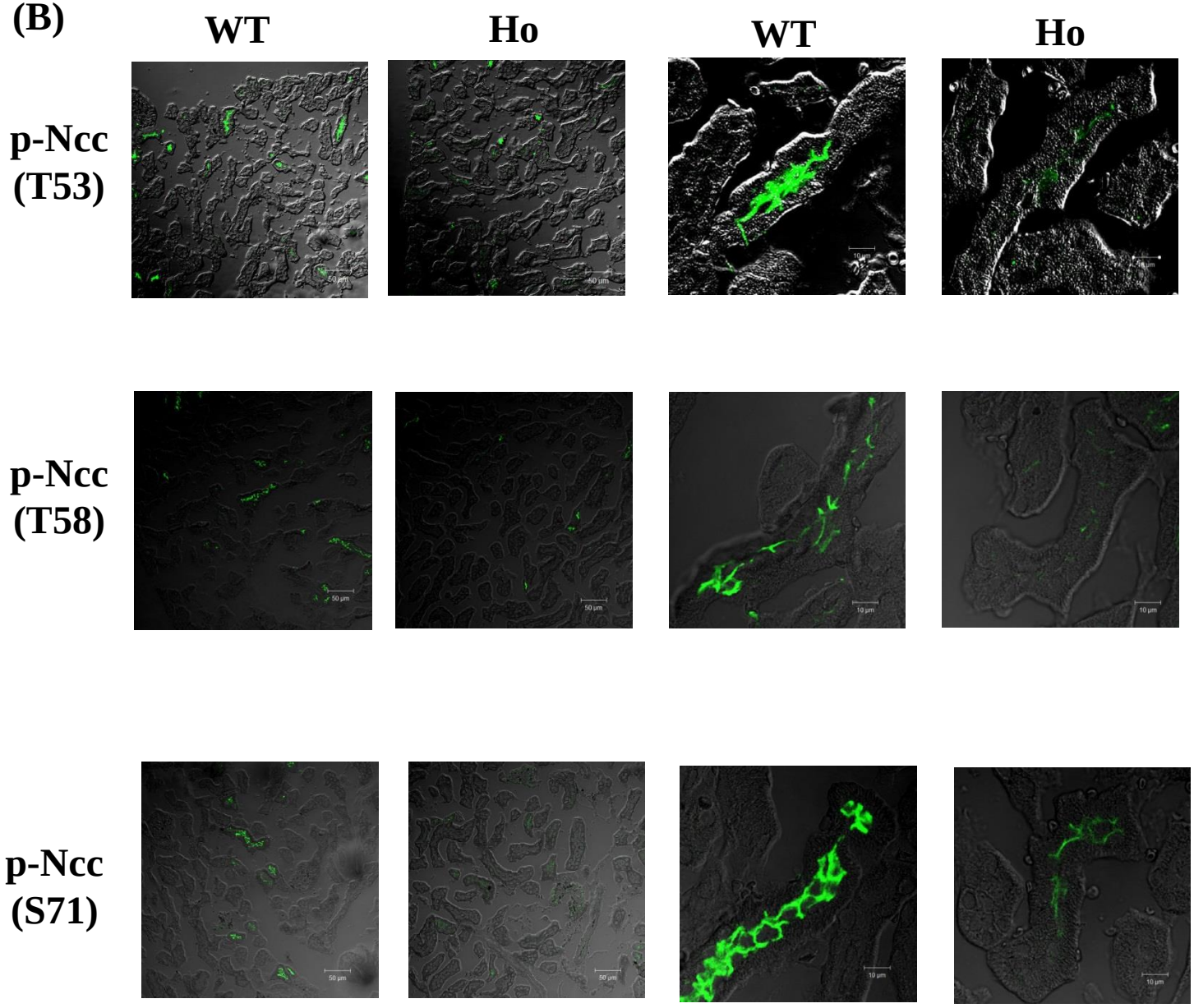



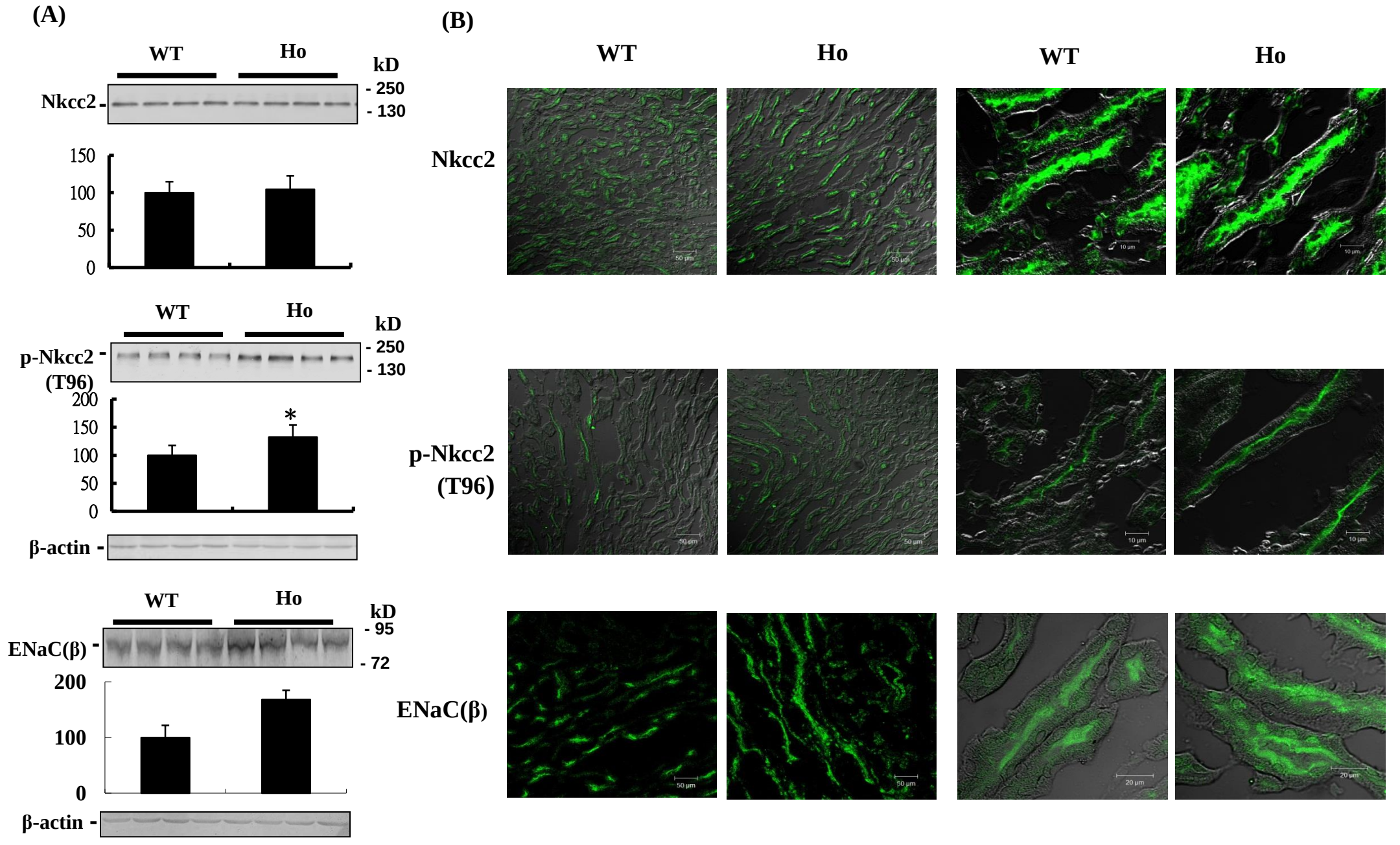



**Supplemental Fig. 2**



**Supplemental Fig. 3**



**Supplemental Fig. 4**