SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Phosphorylation Analysis of Heterologously-expressed Nedd4-2- HEK293 cells were transfected with FLAG-tagged Nedd4-2 or empty vector using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Twenty-four hours after transfection, cells were harvested in 500 μL lysis buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, and 1% Nonidet P-40] containing protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM, benzamidine, and 1x Complete Protease Inhibitor Cocktail (Roche)] and phosphatase inhibitors [25 mM sodium fluoride, 2 μM microcystin-LR, and 1x Phosphatase-Inhibitor Cocktail Sets I and II (Calbiochem)]. Lysates were immunoprecipitated with anti-FLAG affinity resin (Sigma-Aldrich), eluted with 3X FLAG-peptide (Sigma), and concentrated using a MicroCon YM-30 centrifugation filter column (Millipore). Samples were separated by SDS-PAGE and stained with Coomassie Blue. A band corresponding to FLAG-Nedd4-2 was cut from the gel and in-gel digested with trypsin, chymotrypsin, or N-asparaginase prior to analysis by mass spectrometry. This experiment was repeated with cells treated with vehicle or LY294002 (EMD/Calbiochem), a general inhibitor of PI-3 kinases, or co-transfected with constitutively active mouse SGK1 (S422D).

Mass Spectrometric Identification of Nedd4-2 Phosphorylation Sites-Enzymatic digests of Nedd4-2 were separated by reverse phase chromatography using a Nano-1D system (Eksigent) at a flow rate of 400 nl/min, employing a gradient from 0 to 33% Acetonitrile/0.1% formic acid over 36 minutes, before ramping up to 50% acetonitrile. Liquid chromatography was interfaced online with a QSTAR XL mass spectrometer (MDS Sciex/Applied Biosystems) or a Linear Ion Trap-Fourier Transform Ion Cyclotron Resonance Instrument (LTQ-FT) (Thermo). Data on the QSTAR were acquired in an information-dependent manner where a 0.8-s survey mass spectrometry (MS) scan was followed by a 1.6-s tandem MS analysis of the most intense multiply charged ion. Dynamic exclusion of ions previously selected for tandem MS analysis was employed. For data acquired on the LTQ-FT, MS data was acquired in the ion cyclotron. Then, the most intense peak was selected for a selected ion monitoring scan to get accurate mass, followed by MSMS fragmentation in the LTQ linear ion trap. Mass spectrometry data were converted to peaklists using the Mascot.dll version 1.6b20 for QSTAR data and the Mascot Distiller version 1.1 for LTQ-FT data, then searched using a developmental version of Protein Prospector (version 4.25.4), which has essentially the same functionality as the subsequently released version 5.0(45). Peaklists were searched assuming fully enzyme-specific cleavage with up to two missed cleavages. Precursor and fragment maximum mass deviations of 100 ppm and 0.1 Da respectively were employed for QSTAR data, and 10 ppm and 0.6 Da respectively for LTQ-FT data. Data were initially searched against *Xenopus* and Human entries in the UniprotKB database (www.uniprot.org) downloaded on 19th April 2007 (83320 entries), allowing for cysteine carbamidomethylation as a constant modification and methionine oxidation, protein N-terminal acetylation and peptide N-terminal pyroglutamic acid formation from glutamine residues as variable modifications. A subsequent search was then performed against only those proteins for which at least one peptide was identified with an Expectation value of less than 0.01. In this second level search serine and threonine phosphorylations were also considered, as well as lysine ubiquitination.

For quantitation of changes in phosphorylation stoichiometry, the ratio of the intensity of peaks in extracted ion chromatograms of phosphorylated and non-phosphorylated versions of the same peptides acquired on the QSTAR were compared. Recombinant GST-Nedd4-2, incubated with or without purified JNK1 *in vitro* was used for identifying additional potential JNK1-dependent phosphorylation sites using phosphopeptide enrichment (Custom Biologics).

Co-immunoprecipitation of Nedd4-2 and 14-3-3 in HEK293 Cells-
Cells were transfected with FLAG-tagged Nedd4-2, SGK1 S422D, HA-tagged mouse 14-3-3η, and/or empty vector using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were harvested in 500 μl lysis buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, and 1% Nonidet P-40]

containing protease inhibitors and phosphatase inhibitors as described above. Immunoprecipitation was performed with anti-FLAG antibody conjugated to resin (Sigma-Aldrich), and samples were separated by SDS-PAGE and immunoblotted with anti-FLAG or anti-HA-horseradish peroxidase 3F10 (Roche).

Co-immunoprecipitation of Nedd4-2 and ENaC in HEK293 Cells- Reciprocal co-immunoprecipitation studies of Nedd4-2 and each of the three ENaC subunits were performed as described previously (19), except that parental HEK293 cells were transfected with either WT or mutant FLAG-tagged Nedd4-2 along with epitope-tagged mouse α–, β–, and γ–ENaC subunits. Only one of the three ENaC subunits had a V5 epitope tag for each transfection; the other epitope tags were hemagglutinin (HA) for α-ENaC and β-ENaC and c-myc for γ-ENaC. The relative binding affinities for each reciprocal co-immunoprecipitation were calculated by dividing the co-immunoprecipitated protein signal by the signal for that protein in the cellular lysate, which was then normalized to the amount of immunoprecipitated protein for that condition (by dividing by that signal on the same membrane). For each experimental condition, the binding measured with mutant Nedd4-2 was quantitated and expressed relative to that with WT Nedd4-2.

SUPPLEMENTARY FIGURE LEGENDS

Suppl. Fig. 1. Mass spectrometry analysis of Nedd4-2 phosphorylation. Nedd4-2 protein was purified by 1D PAGE, and the corresponding bands were excised from the gel and in-gel digested with either trypsin or Asp-N. Peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on both a QSTAR and a LTQ-FT. (A)-(F) Representative spectra of peptides encompassing each of the identified phosphorylated residues in Nedd4-2. Arrows on peptide indicate C-terminal fragment ions (y#) and N-terminal fragment ions (b#). Each fragment ion corresponds to a peak with a particular relative abundance and mass-to-charge (m/z) ratio. Arrows superimposed on the spectra indicate a peak which identifies the modification by phosphorylation. In some cases the data were insufficient to identify the exact residue of modification: one of serines 179-181 and one of serines 471 and 475 were phosphorylated. See *Supplementary Experimental Procedures* for details. (G) The arrow indicates the band excised from a representative Coomassie-stained gel for mass spectrometry.

Suppl. Fig. 2. Decreased inhibition of ENaC by mutant Nedd4-2. To test the potential functional consequences of phosphorylation at Ser-293 on ENaC, we co-expressed α, β, and γ-ENaC and N-terminal FLAG-tagged Nedd4-2, either wild-type (WT, gray) or mutant (S293A, black) Nedd4-2 in *Xenopus* oocytes (2 ng of each cRNA). (A) Amiloride-sensitive currents were measured by the two-electrode voltage clamp technique, and current-voltage (I-V) plots were obtained under the various conditions for each experiment. Oocytes expressing WT or S293A Nedd4-2 had a conductance of 7.3μS or 16.3 μS respectively. (B) At -100 mV, ENaC currents with Nedd4-2 compared to ENaC alone were 90% decreased for WT Nedd4-2 but only 80% for S293A Nedd4-2 (*$p < 0.001$, vs. ENaC alone; #$p = 0.01$, vs. WT; N=4 batches, n=22-41 eggs). (C) For these studies, the expression levels of various FLAG-Nedd4-2 proteins in oocytes were verified by Western blot, and currents shown were normalized for protein expression as quantitated by densitometry of the bands relative to β-actin.

Suppl. Fig. 3. Functional effects of WT vs. S293A Nedd4-2. (A) Co-immunoprecipitation of 14-3-3η with Nedd4-2 in HEK293 cells. FLAG-Nedd4-2 constructs and HA-tagged 14-3-3η were co-expressed and immunoprecipitated with anti-FLAG beads followed by immunostaining with both anti-FLAG and anti-HA antibodies. A representative blot is shown. Densitometric analysis of three independent experiments showed no change between WT and S293A Nedd4-2 interaction with 14-3-3η. (B) S293A Nedd4-2 mutant does not modulate AMPK-dependent inhibition of ENaC. *Xenopus* oocyte co-expression assay with co-expression αβγ-ENaC and WT or S293A Nedd4-2. Injection of 32 nL/oocyte of vehicle (K-gluconate, white bars) vs. 40 mM K-ZMP, an AMPK activator (gray bars) 1-3 h prior to measurements

caused significant inhibition of amiloride-sensitive ENaC currents (*$p < 0.03$, N= 4 batches, n= 16-21 oocytes), but there was no significant difference between WT vs. S293A Nedd4-2. (C) Co-immunoprecipitation of Nedd4-2 with ENaC subunits. HEK293 cells were co-transfected with differentially tagged αβγ-ENaC and WT or S293A Nedd4-2. With immunoprecipitation of either FLAG-tagged Nedd4-2 (gray bars) or V5-tagged ENaC subunit (black bars), there was no significant difference in interaction of any of the ENaC subunits with WT vs. S293A Nedd4-2. Densitometric analysis is shown (N=4-6 replicate assays). (D) Ubiquitination assay in HEK293 cells. Co-transfection of vector (V) or Nedd4-2 construct (WT, S293A, or ligase-deficient C938S) with differentially-tagged αβγ-ENaC. Cell dissociation followed by immunoprecipitation of ENaC subunit (α-ENaC, light gray bars, β-ENaC, medium gray bars, or γ-ENaC, black bars) and immunoblotting with anti-ubiquitin antibody. Densitometry is shown, (*$p < 0.03$ for each subunit vs. V, N = 3 experiments).

Suppl. Fig. 4. SGK1 phosphorylates WT and triple mutant Nedd4-2 with comparable efficacy. FLAG-tagged WT or mutant Nedd4-2 was transfected and immunoprecipitated from HEK293 cells, followed by an *in vitro* kinase assay with or without SGK1. Densitometry of relative phosphorylation is shown (N = 5 experiments).

Suppl. Fig. 5. Co-immunoprecipitation of Nedd4-2 with ENaC subunits. Differentially tagged αβγ-ENaC and WT or triple mutant Nedd4-2 were co-transfected into HEK293 cells. (A) IP of either FLAG-tagged Nedd4-2 (gray bars) or V5-tagged ENaC subunit (black bars) followed by immunoblot analysis with anti-V5 or anti-FLAG antibodies, respectively, showed no significant difference in interaction of any of the ENaC subunits with WT vs. triple mutant Nedd4-2. (B) Densitometry comparing binding signal of ENaC subunits to triple mutant relative to WT Nedd4-2 as measured by reciprocal co-immunoprecipitations (N = 3 experiments).