SUPPLEMENTAL MATERIALS AND METHODS

Antibodies

Commercially available primary antibodies to the following proteins were used as directed: annexin II (Santa Cruz, sc-1924); BiP (Stressgen, SPA-826 and Abcam ab21685); GDP dissociation inhibitor 2 (Abcam, ab59163); heat shock cognate 70 (Stressmarq, SPC-102C/D); heat shock protein 70 (Stressmarq, SMC-100A/B); protein phosphatase 1 catalytic subunit, beta isoform (Abcam, ab53315); protein phosphatase 2A catalytic subunit (Cell Signaling, 2259); RAN (Cell Signaling, 4462).

As members of the same hsp70 family, antibodies to hsc70 and hsp70 were chosen for minimal cross-reactivity. The amino acid sequences of the peptide immunogens used to generate these antibodies were unique upon BLAST analysis. The following purified, recombinant proteins were purchased from Stressmarq: BiP (SPR-107), heat shock protein 70 (SPR-103), and heat shock cognate 70 (SPR-106) for analysis of antibody specificity to Hsp70 and Hsc70 (see Supplemental Figure 4).

Rabbit IgG directed against the AQP2 C-terminus was used as previously described [1]. Affinity purified rabbit IgG directed against NaPi-2 was used as a negative control for co-immunoprecipitation experiments because this protein is not expressed in the rat inner medulla [2,3].

Fluorescent secondary antibodies against rabbit, mouse, goat, and chicken were purchased from Licor (Lincoln, NE) and Rockland. HRP-conjugated TrueBlot anti-rabbit antibodies were purchased from eBioscience (San Diego, CA) for development of selected co-immuoprecipitation immunoblots.

Peptides and Proteins

Three biotinylated AQP2 COOH-terminal peptides were synthesized by AnaSpec (San Jose, CA). All had the following amino acid sequence: Biotin-LC-CEPDTDWEEREVRRRQSVELHSPQSLPRGSKA. Verification of the correct sequence was provided by mass spectrometric analysis, and the use of the nonphospho, pS256, and pS261 peptides has been described previously [4]. Peptides were weighed using a microbalance. Equality of the peptides concentrations was confirmed by dot blot analysis using fluorescently-conjugated streptavidin (IRDye 800, Rockland, Gilbertsville, PA) to detect the biotinylated C-termini (see Supplemental Figure 3).

Circular Dichroism of Phospho Peptides

Circular dichroism was performed using a Jasco J-715 spectropolarimeter. Three sets of biotinylated peptide samples (nonphos, pS256, and pS261) were prepared in 1X PBS. Samples were scanned from 200 to 260 nm with a band width of 0.5 nm, response time of 0.5 sec at a scanning speed of 50 nm/minute in a 0.2 cm cell with 10 accumulations per sample. CD angle and voltage were measured.

Buffered Solutions

Cytosolic buffer was designed to mimic intracellular osmolality, pH, and divalent cation concentrations. This buffer consisted of 180 mM Tris HCl (pH 7.4), 2×10^{-4} mM Ca^{++}, 0.8 mM Mg^{++}, 1X Halt Phosphatase Inhibitor Cocktail (Pierce), Complete Mini Protease Inhibitor tablets (Roche, 1 tablet/10 ml), and dithiothreitol (0.5 mg/ml). The complete solution was titrated to pH 7.2 using a pH meter. Final osmolality was approximately 330 mosm/kg.

RIPA buffer was prepared by adding enough 5X RIPA to cytosolic buffer in order to achieve 1X RIPA, which consisted of cytosolic buffer plus the following: 1% Triton X, 0.1% SDS,

and 0.5% sodium deoxycholate. The solution was titrated to pH 7.2 using 10N HCl and then1N HCl for fine adjustments.

1X Laemmli elution buffer was prepared to contain 1.5% SDS, 50 mM Tris (pH 6.8), and 6% glycerol.

Inner Medullary Collecting Duct Tubule Isolation

IMCD tubule isolation has been described previously [5,6]. Pathogen-free male Sprague-Dawley rats (Taconic Farm, Germantown, NY) weighing 200-250 g were maintained on an autoclaved pelleted rodent chow (413110–75-56, Zeigler Bros., Gardners, PA) and ad libitum drinking water. All experiments were conducted in accord with an animal protocol approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (ACUC protocol number H-0110).

For AQP2 COOH-terminal peptide pull-downs, rats were provided sucrose water (250 mosm/kg) for two days in order to reduce endogenous AQP2 content. Urine was collected. The average osmolality was found to be 387 (± 485 SD) mosm/kg (N=26). For all other experiments, rats were provided with *ad libitum* water.

After incubation, isolated IMCD cells were pelleted at 60 xg for 20 seconds and washed twice with sucrose solution. For AQP2 COOH-terminal peptide pull-downs, a third wash was performed with cytosolic buffer not containing inhibitors or DTT and a fourth with cytosolic buffer containing inhibitors and DTT. For co-immunoprecipitations of annexin II and protein phosphatase 1C, a third wash was performed in sucrose. Cells were then pelleted at 16,000 xg for 30 seconds. Pellets were resuspended in cytosolic buffer, centrifuged again, and pellets resuspended in 0.1X RIPA cytosolic buffer. For co-immunoprecipitation of BiP, a third wash was performed in bicarbonate solution (pH 7.4) that had been centrifuged at 16,000 xg. Cells were incubated with dDAVP as described below and then pelleted. Bicarbonate supernatant was

aspirated, cells were resuspended in cytosolic buffer, spun, and finally lysed in 0.1X RIPA cytosolic buffer and homogenized as described below using a Potter-Elvehjem homogenizer.

Estimate of AQP2 Abundance

As a background study, we estimated the abundance of AQP2 in IMCD lysates. This was important to ensure that the amount of AQP2 COOH-terminal peptide for pull-down experiments was within a reasonable physiological range. To achieve this aim, a known molar amount of nonphosphorylated biotinylated AQP2 COOH-terminal peptide was conjugated to ovalbumin via a maleimide linkage and quenched with L-cysteine. Peptide samples were first conjugated to ovalbumin in order to achieve maximal membrane retention on transfer (experimentation with unconjugated peptide resulted in appreciable transfer of material through a second and even third nitrocellulose membrane). Varying dilutions of the AQP2 COOH-terminal peptide-ovalbumin conjugate were analyzed by immunoblotting using an antibody directed against the AQP2 C-terminus to generate a standard curve of AQP2 COOH-terminal peptide molar amount vs. signal intensity, taking into account AnaSpec's product data claim that lyophilized peptides are approximately 80% pure. Given known protein concentrations in IMCD lysates evaluated by BCA assay, we estimated an abundance of approximately 0.0015 nmol AQP2 per microgram total protein of IMCD prep. (see Supplemental Figure 1).

We further estimated a recovery of 250 μg total protein per rat inner medulla (IM). Thus, a single IM may contain 0.375 nmol AQP2. This corresponds, given an AQP2 COOH-terminal peptide molecular weight of approximately 4200 g/mol, to approximately 1.6 μg peptide per IM, or 3.2 μg peptide for 2 IM's from a single rat. Since, Anaspec estimates 80% lyophilized peptide purity, approximately 4 μg AQP2 COOH-terminal peptide peptide should correspond to the total abundance of AQP2 in a single rat. Biotinylated AQP2 COOH-terminal peptide pull-down

experiments utilized no more than 50 μg peptide per rat. Appropriate corresponding volumes of Dynabeads were used to capture biotinylated peptides.

Pull-Downs Using Biotinylated AQP2 COOH-terminal Peptides

IMCD tubules were isolated as described above. Lysis was performed using a Potter-Elvehjem homogenizer (10 cycles, 15 second pulses) in cytosolic buffer. Lysates were then spun at 200,000 xg for 1 h at 4C to separate cytosolic from membrane fractions. Cytosolic fractions were aspirated and saved. A small amount of cytosol was used as a positive control in confirmatory immunoblot analysis of mass spectrometric data. Assuming an initial IMCD isolate volume of about 20 μl/rat, dilution of cytosol ranged from approximately five to eight-fold. To this diluted cytosol, enough 5X RIPA (see above) was added to achieve 1X.

Remaining cytosol in cytosolic buffer was divided evenly among four arms in 1.7 ml tubes. To each of these four arms, an equal molar amount of biotin (no peptide control), biotinylated nonphosporylated AQP2 C-teriminal peptide, pS256 peptide, or pS261 peptide was added. Across experiments, amounts ranged from 20 to 50 ug peptide (see estimate of AQP2 abundance above). Early experiments used a 100-fold molar excess of biotin to streptavidin, but results did not differ from subsequent experiments that used approximately equal molar amounts. Samples were vortexed and microcentrifuged gently. AQP2 COOH-terminal peptides and cytosol were incubated overnight at room temperature in a test tube rack with gentle rocking.

The following day, pre-washed (cytosolic buffer with 1X RIPA) MyOne Streptavidin C1 Dynabeads (Invitrogen) were added to each arm in a ratio of 1 μg peptide/10 μl Dynabeads, or an approximately 1:1 ratio of biotin to streptavidin. Samples were incubated at room temperature with gentle rocking and periodic vortexing for two hours. Samples were washed four times with 500 μl cytosolic buffer with 1X RIPA as follows: twice by incubating 30 minutes

with gentle rocking and periodic vortexing, followed by twice for 15 minutes with gentle rocking and periodic vortexing.

Elution was performed by incubating samples with 150 μl 1X Laemmli Buffer (without DTT) for 30 minutes at 37C, followed by a final rinse with 25 μl 1X Laemmli Buffer. Prior to SDS Page analysis, samples were dried to approximately 30 μl so that the entire sample could be loaded in a single well.

Wash, Binding, and Elution Conditions for Pull-Down Experiments

Flow-throughs, washes, and eluates were saved, run on reducing SDS-PAGE gels, and silver stained. Peptide immobilization was attempted with both sulfhydryl linkage to sepharose beads and streptavidin-biotin binding on a magnetic bead platform. Variations in lysate/peptide binding conditions included incubating with or without varying concentrations of detergent-containing RIPA solution. Incubations were also performed in the lowest workable volume in order to mimic physiologic binding conditions. Based upon a volume extimate of 9.5 μl per homogenized IMCD, we diluted IMCD lysates approximately 5 to 8-fold.

Varying wash conditions included PBS washes, gentle detergent (0.2% CHAPS), high salt, low salt, gentle heating (to 37°C), and cytosolic buffer (See Supplemental Figure 3). Washes were performed with or without vortexing until we chose the former, more stringent condition. Elution was performed under harsh conditions in order to achieve maximal efficacy (1X Laemmli Buffer with heating to 37°C).

Separation by 1-D SDS-PAGE and Trypsinization

Eluted samples from AQP2 COOH-terminal peptide pull-downs were run on 10-20% SDS-PAGE gels (Biorad, Hercules, CA). Gels were then stained with Coomassie blue. Entire lanes corresponding to eluates from each AQP2 COOH-terminal peptide were cut in parallel

fashion and prepared for LC-MS/MS as described previously [7]. Slices were destained with 25 mM NH_4HCO_3/50% ACN for 10-min intervals until entirely destained. Gel samples were dried and then reduced with 10 mM DTT in 25 mM NH_4HCO_3 for 1 h at 56°C. The supernatant was removed, and an aqueous solution containing 55 mM iodoacetamide in 25 mM NH_4HCO_3 was added for 45 min in darkness at RT to alkylate the reduced cysteine residues. The supernatant was then removed, and gels were washed with 25 mM NH_4HCO_3 for 10 min. Gel pieces were dehydrated with 25 mM NH_4HCO_3/50%ACN and dried. Proteins were trypsinized using 12.5 ng/μl sequencing grade modified trypsin (Promega, Madison, WI) diluted in 25 mM NH_4HCO_3 and incubated at 37°C for 16 h. Peptides were extracted from the gel in a 50% ACN/ 0.5% formic acid solution, then dried, and reconstituted with 0.1% formic acid. Samples were then cleaned by ZipTip.

LC-MS/MS Protein Identification and Analysis

LC-MS/MS was performed as described previously [8]. ZipTipped trypic peptides were injected into a reversed-phase liquid chromatographic (LC) column (PicoFrit™, Biobasic C18, New Objective, Woodburn, MA) to stratify sample proteins before delivery to an LTQ tandem mass spectrometer (MS/MS, Thermo Electron Corp., San Jose, CA) via a nanoelectrospray ion source. The spectra with a total ion current greater than 10,000 were used to search for matches to peptides in a concatenated RefSeq database using Bioworks software (Version 3.1, Thermo Electron Corp.) based on the Sequest algorithm. The concatenated RefSeq database was composed of forward protein sequences and reversed protein sequences derived from the NCBI using in-house software. The search parameters included: 1) precursor ion mass tolerance less than 2 amu, 2) fragment ion mass tolerance less than 1 amu, 3) up to 3 missed tryptic cleavages allowed, and 4) amino acid modifications: cysteine carboxyamidomethylation (plus 57.05 amu), methionine oxidation (plus 15.99 amu), and cysteine modification with

acrylamide (plus 14 amu). For protein identifications, in-house software was used to filter the matched peptide sequences using target decoy analysis to achieve < 2% false discovery rate.

Label-Free Quantification Using QUOIL Software

Spectral counting was applied to screen for differential abundances of proteins of interest in AQP2 COOH-terminal peptide pull-down eluates. Proteins of interest were further analyzed for relative quantification by label-free quantification using QUOIL software, which calculated the ratios of the areas of the reconstructed peptide LC elution profiles from multiple samples [9]. The peptide mass tolerance was set to 0.5 Da. The minimal signal-to-noise threshold was set at 1.5 fold. Noise was subtracted from the calculation of relative peptide abundance.

Immunoblotting

Samples in Laemmli buffer with DTT were heated to 65°C for 10 minutes; sample in Laemmli plus any percentage RIPA buffer were heated at 37°C for 10 minutes. All samples were run on Criterion Tris-HCl polyacrylamide gels (Biorad) in 1X Tris/Glycine/SDS buffer. Transfers were performed at constant amperage (0.25 mAmp) for 1 hour in Fairbanks Tris running buffer. All membranes were blocked for at least one hour at room temperature using Odyssey block with 1.5 mM sodium azide with rocking. Primary antibody incubation was performed in Odyssey block with 0.1% Tween and 1.5 mM sodium azide overnight with rocking. Secondary incubation was performed in the same buffer but incubated for one hour at room temperature. Immunoblots were scanned and band density quantified using the Licor Odyssey fluorescence system. Co-IPs for annexin II, PP1C, and BiP were developed on Kodak XAR film.

Immunoprecipitation of AQP2 from native IMCD

Rat IMCD cell isolation was performed as above. For co-immunoprecipitation of BiP, cells were first incubated in 10^{-9} M dDAVP for 20 minutes in a bicarbonate solution (pH 7.4)

under 5% CO_2. Isolated cells were lysed by Potter-Elvehjem homogenization in the presence of 0.1% Triton X-100, 0.01% SDS, and 0.05% sodium deoxycholate in cytosolic buffer. Some lysates were pre-clarified by centrifugation at 10,000 xg for 15 minutes at 4°C. No difference was observed between co-IPs with or without pre-clarification. Total protein was measured in lysates using the BCA assay, and 100 μg total protein was used per co-immunoprecipitation sample Enough DTT was then added to achieve a concentration of 0.5 μg/ml. AQP2 was immunoprecipitated using a rabbit IgG antibody directed against a region of the COOH-terminus of AQP2, upstream from the polyphosphorylated region [1]. An unrelated rabbit IgG directed against the proximal tubule sodium-phosphate co-transporter 2 (NaPi-2)—affinity purified according to the same protocol—was used as a negative control. Antibodies were tested for specificity: anti-AQP2 but not anti-NaPi-2 pulled down AQP2; NaPi-2 recognized no IMCD proteins on Western blotting (data not shown).

For 100 μg total IMCD lysate,15 μg IgG (control or anti-AQP2) was used in a total volume of 200 μl per co-immunoprecipitation sample incubation. For these incubations, 100 μg total IMCD protein was incubated in 200 μl cytosolic buffer containing protease and phosphatase inhibitors and DTT as above in the presence of 200 μg Triton X-100, 20 μg SDS, and 100 μg sodium deoxycholate. The detergent amount was chosen based on the rule-of-thumb stating a requirement of at least 2 mg nonionic detergent per 1 mg membrane protein for full solubilization [10]. Lysate and antibody were incubated overnight at 4°C with end-over-end rocking. Lysate-antibody complex was added to 100 μl of pre-washed protein G-coated Dynabeads. Capture was performed by end-over-end rocking for 1 hour at 4°C. Dynabeads were then washed 4 times with 500 μl of the same solution.

Elution was performed twice in 2X Laemmli Buffer with 77 mg/ml DTT at 37°C for 30 minutes, and eluates were pooled. Eluates were then run on SDS-PAGE reducing gels and subjected to immunoblotting.

Immunoprecipitation of AQP2 from stably-transfected MDCK cells.

Generation of constructs, stable transfection and cell culture was performed as previously described [1]. For immunoprecipitation, cells were grown to confluency in a T-25 flask, and treated with 25μM forskolin for 30min where indicated. Cells were washed 3x with ice-cold PBS then subsequently scraped and homogenized in RIPA buffer (final concentration; 0.2% SDS, 300mM NaCl, 1mM EDTA, 100mM Tris (pH7.5), 1% Na deoxycholate, 2% Nonidet P-40) containing protease and phosphatase inhibitors. After estimation of protein concentration, 200μg cell lysate was subject to immunoprecipitation overnight at 4°C with an NH2-terminal AQP2 antibody (N20, Santa Cruz) using the *Catch and Release System* (Upstate). Experiments were performed in duplicate and repeated at least 3 times on different days. Immunoblotting was performed as described above. To confirm equal amounts of immunoprecipitated AQP2 in the different cell lines, immunoblotting using an antibody recognizing total AQP2 (K5007) was performed. Densitometry values for the co-immunoprecipitated proteins were normalized to total AQP2 in each individual IP sample, thus facilitating comparisons both within and between experiments.

Preparation of tissue for immunogold electron microscopy

Fixed tissue blocks from the kidney inner medulla were infiltrated with 2.3 M sucrose for 30 min, and frozen in liquid nitrogen. Frozen tissue blocks were subjected to cryosubstitution and Lowicryl HM20 embedding. Lowicryl sections of 80 nm were cut on a Reichert Ultracut S and were preincubated with 0.05 M Tris, pH 7.4, 0.1% Triton-X-100 (TBST) containing 0.1% sodium borohydride and 0.05M glycine followed by incubation with TBST containing 0.2% skimmed milk. Preincubation was followed by incubation with BiP (1:250, Abcam ab21685); protein phosphatase 1 catalytic subunit, beta isoform (1:100, Abcam, ab53315);annexin II (1:500, Santa Cruz), and where necessary chicken anti-AQP2 (1:2000

dilution). Labeling was visualized with goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-chicken IgG conjugated to colloidal gold particles. Grids were stained with uranyl acetate for 10 min and with lead citrate for 5 s.

Immunolabeling of kidney sections and confocal laser scanning microscopy

This technique has been described in detail previously (Fenton 2007 AJP Renal V2R paper). Primary antibodies used were BiP (1:1000, Abcam ab21685); protein phosphatase 1 catalytic subunit, beta isoform (1:1000, Abcam, ab53315); annexin II (1:2000, Santa Cruz) and chicken anti-AQP2 (1:5000 dilution). A Leica TCS SL (SP2) laser confocal microscope and Leica Confocal Software was used for imaging of the kidney tissue sections. Images were taken using a HCX PL APO 63x oil objective lens.

Statistical Analysis

Statistical analysis was conducted using InStat3. For immunoblot confirmation of mass spectrometric results, ANOVA analysis was performed with Bonferroni comparisons of multiple data sets.

Reference List

1. Hoffert, J. D.; Fenton, R. A.; Moeller, H. B.; Simons, B.; Tchapyjnikov, D.; McDill, B. W.; Yu, M. J.; Pistikun, T.; Chen, F.; Knepper, M. A. Vasopressin-stimulated increase in phosphorylation at ser-269 potentiates plasma membrane retention of aquaporin-2. *J. Biol. Chem.* **2008,** *283*, 24617-24627.

2. Kim, G. H.; Martin, S. W.; Fernandez-Llama, P.; Masilamani, S.; Packer, R. K.; Knepper, M. A. Long-term regulation of renal Na-dependent cotransporters and ENaC: response to altered acid-base intake. *Am. J. Physiol Renal Physiol* **2000,** *279* (3), F459-F467.

3. Turban, S.; Beutler, K. T.; Morris, R. G.; Masilamani, S.; Fenton, R. A.; Knepper, M. A.; Packer, R. K. Long-term regulation of proximal tubule acid-base transporter abundance by angiotensin II. *Kidney Int.* **2006,** *70* (4), 660-668.

4. Hoffert, J. D.; Nielsen, J.; Yu, M. J.; Pisitkun, T.; Schleicher, S. M.; Nielsen, S.; Knepper, M. A. Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. *Am. J. Physiol Renal Physiol* **2007,** *292* (2), F691-F700.

5. Stokes, J. B.; Grupp, C.; Kinne, R. K. Purification of rat papillary collecting duct cells: functional and metabolic assessment. *Am J Physiol* **1987,** *253* (2 Pt 2), F251-F262.

6. Pisitkun, T.; Bieniek, J.; Tchapyjnikov, D.; Wang, G.; Wu, W. W.; Shen, R. F.; Knepper, M. A. High-throughput identification of IMCD proteins using LC-MS/MS. *Physiol Genomics* **2006,** *25* (2), 263-276.

7. Pisitkun, T.; Shen, R. F.; Knepper, M. A. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. U. S. A* **2004,** *101* (36), 13368-13373.

8. Yu, M. J.; Pisitkun, T.; Wang, G.; Aranda, J. F.; Gonzales, P. A.; Tchapyjnikov, D.; Shen, R. F.; Alonso, M. A.; Knepper, M. A. Large-scale quantitative LC-MS/MS analysis of detergent-resistant membrane proteins from rat renal collecting duct. *Am. J. Physiol Cell Physiol* **2008**.

9. Wang, G.; Wu, W. W.; Zeng, W.; Chou, C. L.; Shen, R. F. Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and application with complex proteomes. *J. Proteome. Res.* **2006,** *5* (5), 1214-1223.

10. Scopes, R. K. Protein Purification: Principles and Practice. 41. 1994. New York, NY, Springer Science + Business Media, Inc.
Ref Type: Generic

SUPPLEMENTAL LEGENDS

Supplemental Table 1. Protein identifications and unique peptides that are consistent with these identifications. Some peptides were identified multiple times, so only uniquely identified proteins are included. Some peptides may be consistent with more than one protein but all were identified by QUOIL's BLAST functionality as matches for proteins listed.

Supplemental Figure 1. Estimate of AQP2 abundance. Varying concentrations of known molar amounts of nonphosphorylated AQP2 COOH-terminal peptide were conjugated to ovalbumin to generate a standard curve. Two different amounts of IMCD lysate were run on the same gel. Transferred membranes were immunoblotted with an anti-AQP2 antibody. Based on the standard curve, the approximate molar amount of AQP2 per microgram IMCD was determined.

Supplemental Figure 2. Peptide dot blot. Biotinylated AQP2 COOH-terminal peptides were weighed multiple times for multiple pull-down experiments subjected to mass spectrometry or analysis by immnoblotting. One set of weighed peptides (nonphospho, pS256, and pS261) were analyzed by dot-blotting to confirm approximately equal concentrations and rule out significant weighing error or differences in concentration of peptides in their lyophilized forms. Blots were probed with fluorophore-coupled streptavidin.

Supplemental Figure 3. AQP2 COOH-terminal peptide pull-downs: wash, binding, and elution conditions. In order to optimize the biotinylated peptide pull-down experiments, we experimented with varying incubation and wash conditions. Top panel shows silver staining of eluates obtained by pull-down conditions prior to optimization. Incubation of AQP2 COOH-terminal peptides with IMCD cell lysates was performed in cytosolic buffer. Samples were washed twice with cytosolic buffer, followed by two washings with 1X RIPA in cytosolic buffer. All washings employed periodic vortexing. This set of samples was submitted to mass spectrometric analysis as experiment 1. Bottom panel shows eluates obtained under optimized conditions, in which AQP2 COOH-terminal peptides and IMCD cell lysates were incubated in 1X RIPA with cytosolic buffer and washed only in the same. Samples obtained in this way were submitted to mass spectrometric analysis as experiments 2-4. All washings employed periodic vortexing.

Supplemental Figure 4. Evaluation of Anti-hsc70 and hsp70 specificity. Equal amounts of purified recombinant hsc70, hsp70, and BiP proteins were run alongside IMCD lysate for immunoblotting using either anti-hsc70 or anti-hsp70. Selectivity of anti-hsp70 for hsp70 over hsc70 was quantified using the Li-cor Odyssey fluorescent scanning system.

Supplemental Figure 5. Identified proteins discarded based on poor ion chromatogram quality. Hundreds of proteins were identified by mass spectrometric analysis of AQP2 COOH-terminal pull down experimental eluates. MS1 ion chromatogram quality, including high signal-to-noise ratio and clean curves suggesting the absence of contaminant proteins were key inclusive criteria. Shown are ion chromatograms for proteins that were identified but discarded based on quality.

Supplemental Figure 6. Absence of PP2Ac in eluates. As in quantification by immunoblotting, eluates were probed for protein phosphatase 2A catalytic subunit. While present in the positive control, this protein was not identifiable in the eluates corresponding to any of the AQP2 COOH-terminal phospho forms. N=2.

Supplemental Figure 7. Co-immunoprecipitation of AQP2 with BiP, PP1c, and annexin-2 in MDCK cells. Whole cell lysates from MDCK cells stably expressing either WT-AQP2 or S256A-AQP2, under control conditions (con) or after forskolin (for) stimulation were subjected to co-immunoprecipitation. A)

Representative immunoblots. BiP pulldown increases after forskolin stimulation, but decreases after an S256A mutation. Forskolin has no effect on PP1c pulldown, but an S256A mutation decreases interaction. Annexin-2 pulldown increases after forskolin stimulation, but decreases after an S256A mutation. B) Relative quantitation of co-immunoprecipitated proteins in different MDCK cells (n=6). To facilitate comparisons both within and between experiments, individual densitometry values (arbitary units) were normalized to WT-AQP2 control.

Supplemental Figure 8. Co-immunoprecipitation of AQP2 with annexin II, PP1c, and BiP from native IMCD. This figure includes the additional trial of the co-IP of AQP2 and BiP. Unrelated IgG = NaPi-2.

Supplemental Table 1

Unique Peptide Amino Acid Sequences Identified

Heat Shock Cognate 70	Experiment Number
DAGTIAGLNVLR	1
FELTGIPPAPR	1
GPAVGIDLGTTYSCVGVFQHGK	1
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	1
NQTAEKEEFEHQQK	1
NSLESYAFNMK	1
SFYPEEVSSMVLTK	1
TVTNAVVTVPAYFNDSQR	1
MVNHFIAEFK	1
DAGTIAGLNVLR	2
EIAEAYLGK	2
FELTGIPPAPR	2
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	2
MKEIAEAYLGK	2
NQTAEKEEFEHQQK	2
NQVAMNPTNTVFDAK	2
NQVAMNPTNTVFDAK	2
NSLESYAFNMK	2
SFYPEEVSSMVLTK	2
TVTNAVVTVPAYFNDSQR	2
VCNPIITK	2
VQVEYKGETK	2
FDDAVVQSDMK	2
LSKEDIER	2
RFDDAVVQSDMK	2
CNEIISWLDK	3
DAGTIAGLNVLR	3
EIAEAYLGK	3
FELTGIPPAPR	3
GPAVGIDLGTTYSCVGVFQHGK	3
HWPFMVVNDAGRPK	3
HWPFMVVNDAGRPK	3
LDKSQIHDIVLVGGSTR	3
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	3
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	3
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	3
MKEIAEAYLGK	3
MKEIAEAYLGK	3
NQTAEKEEFEHQQK	3
NQVAMNPTNTVFDAK	3
NQVAMNPTNTVFDAK	3
NSLESYAFNMK	3
NSLESYAFNMK	3
SENVQDLLLLDVTPLSLGIETAGGVMTVLIK	3

SFYPEEVSSMVLTK	3
SFYPEEVSSMVLTK	3
SINPDEAVAYGAAVQAAILSGDK	3
SQIHDIVLVGGSTR	3
TVTNAVVTVPAYFNDSQR	3
VCNPIITK	3
VQVEYKGETK	3
FDDAVVQSDMK	3
FDDAVVQSDMK	3
LSKEDIER	3
MVNHFIAEFK	3
MVNHFIAEFKR	3
MVNHFIAEFK	3
MVQEAEKYK	3
RFDDAVVQSDMK	3
RFDDAVVQSDMK	3
TLSSSTQASIEIDSLYEGIDFYTSITR	3
CNEIISWLDK	4
DAGTIAGLNVLR	4
FELTGIPPAPR	4
HWPFMVVNDAGRPK	4
HWPFMVVNDAGRPK	4
LDKSQIHDIVLVGGSTR	4
LYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD	4
MKEIAEAYLGK	4
NQTAEKEEFEHQQK	4
NQVAMNPTNTVFDAK	4
NQVAMNPTNTVFDAK	4
NSLESYAFNMK	4
NSLESYAFNMK	4
SFYPEEVSSMVLTK	4
SFYPEEVSSMVLTK	4
SINPDEAVAYGAAVQAAILSGDK	4
SQIHDIVLVGGSTR	4
TVTNAVVTVPAYFNDSQR	4
VCNPIITK	4
VQVEYKGETK	4
LSKEDIER	4
MVNHFIAEFK	4
MVQEAEKYK	4
MVNHFIAEFK	4
RFDDAVVQSDMK	4

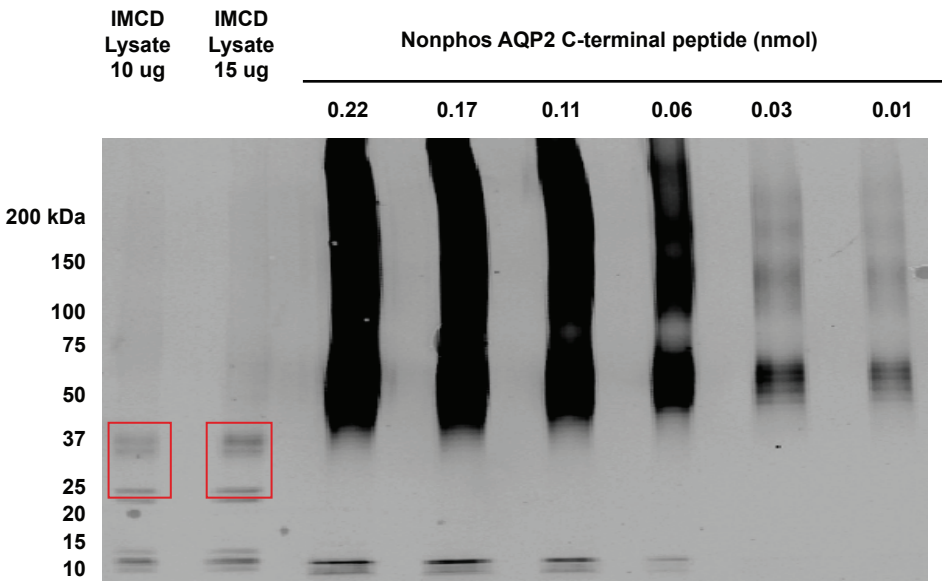
Heat Shock Protein 70 Isoform 1	**Experiment Number**
NQVALNPQNTVFDAK	1
NQVALNPQNTVFDAK	2
GGSGSGPTIEEVD	3
LLQDFFNGR	3
NQVALNPQNTVFDAK	3
QTQTFTTYSDNQPGVLIQVYEGER	3
VQVNYKGENR	3
ARFEELCSDLFR	3
IINEPTAAAIAYGLDR	3

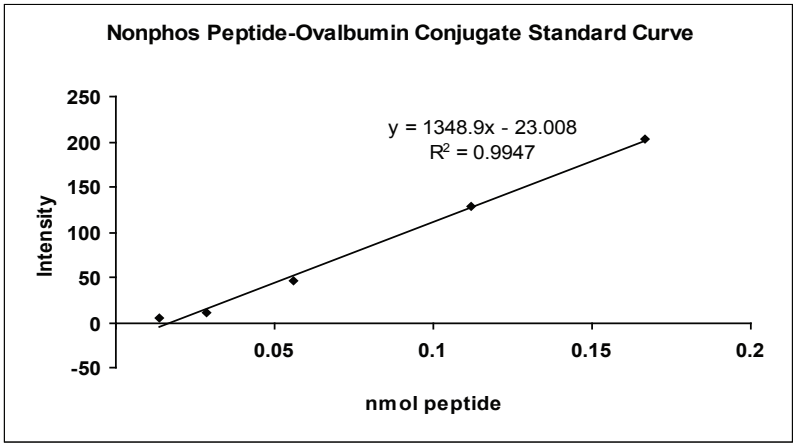
SFYPEEISSMVLTK	3
SFYPEEISSMVLTK	3
VCNPIISGLYQGAGAPGAGGFGAQAPK	3
YKAEDEVQR	3
LLQDFFNGR	4
NQVALNPQNTVFDAK	4
QTQTFTTYSDNQPGVLIQVYEGER	4
SAVEDEGLK	4
ARFEELCSDLFR	4
IINEPTAAAIAYGLDR	4
SFYPEEISSMVLTK	4
SFYPEEISSMVLTK	4
YKAEDEVQR	4
Heat Shock Protein 70 Isoform 2	**Experiment Number**
DAGTITGLNVLR	1
LLQDFFNGK	1
FEELNADLFR	1
KFEDATVQSDMK	1
DAGTITGLNVLR	2
FEDATVQSDMK	2
LLQDFFNGK	2
LYQGGPGGGGSSGGPTIEEVD	2
NAVESYTYNIK	2
NQVAMNPTNTIFDAK	2
STAGDTHLGGEDFDNR	2
FEELNADLFR	2
KFEDATVQSDMK	2
DAGTITGLNVLR	3
LDKGQIQEIVLVGGSTR	3
LLQDFFNGK	3
LYQGGPGGGGSSGGPTIEEVD	3
NAVESYTYNIK	3
NQVAMNPTNTIFDAK	3
STAGDTHLGGEDFDNR	3
TFFPEEISSMVLTK	3
TFFPEEISSMVLTK	3
VQSAVITVPAYFNDSQR	3
ARFEELNADLFR	3
FEELNADLFR	3
IINEPTAAAIAYGLDKK	3
MVSHLAEEFKR	3
CQEVINWLDR	4
DAGTITGLNVLR	4
GQIQEIVLVGGSTR	4
LDKGQIQEIVLVGGSTR	4
LLQDFFNGK	4
NQVAMNPTNTIFDAK	4
STAGDTHLGGEDFDNR	4
TFFPEEISSMVLTK	4
TFFPEEISSMVLTK	4
VQSAVITVPAYFNDSQR	4
VQVEYK	4

ARFEELNADLFR	4
FEELNADLFR	4
IINEPTAAAIAYGLDKK	4
LSKDDIDR	4
MVSHLAEEFKR	4
BiP	**Experiment Number**
ELEEIVQPIISK	1
NELESYAYSLK	1
DAGTIAGLNVMR	2
ITITNDQNR	2
LYGSGGPPPTGEEDTSEKDEL	2
NQLTSNPENTVFDAK	2
SQIFSTASDNQPTVTIK	2
VLEDSDLK	2
VYEGERPLTK	2
ITPSYVAFTPEGER	2
NELESYAYSLK	2
TWNDPSVQQDIK	2
ITITNDQNR	3
MKETAEAYLGKK	3
TFAPEEISAMVLTK	3
TKPYIQVDIGGGQTK	3
VTHAVVTVPAYFNDAQR	3
VYEGERPLTK	3
GVPQIEVTFEIDVNGILR	3
ITPSYVAFTPEGER	3
DAGTIAGLNVMR	4
ELEEIVQPIISK	4
IEWLESHQDADIEDFKAK	4
ITITNDQNR	4
SQIFSTASDNQPTVTIK	4
TFAPEEISAMVLTK	4
VTHAVVTVPAYFNDAQR	4
VYEGERPLTK	4
ITPSYVAFTPEGER	4
NELESYAYSLK	4
Annexin II	**Experiment Number**
SYSPYDMLESIR	1
AEDGSVIDYELIDQDAR	1
TNQELQEINR	1
ALLYLCGGDD	2
DIISDTSGEFR	2
GVDEVTIVNILTNR	2
LLVALAK	2
RAEDGSVIDYELIDQDAR	2
SLYYFIQQDTK	2
SYSPYDMLESIR	2
TPAQYDASELK	2
WISIMTER	2
AEDGSVIDYELIDQDAR	2
KLLVALAK	2
QDIAFAYQR	2

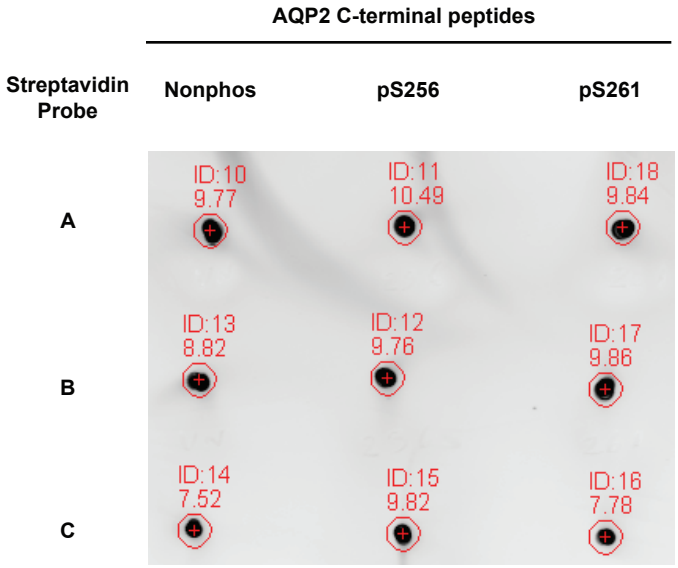
TNQELQEINR	2
ALLYLCGGDD	3
DIISDTSGEFR	3
DIISDTSGEFRK	3
GLGTDEDSLIEIICSR	3
GVDEVTIVNILTNR	3
LLVALAK	3
LSLEGDHSTPPSAYGSVKPYTNFDAER	3
RAEDGSVIDYELIDQDAR	3
SALSGHLETVMLGLLK	3
SLYYFIQQDTK	3
SYSPYDMLESIR	3
SYSPYDMLESIR	3
TDLEKDIISDTSGEFR	3
TDLEKDIISDTSGEFRK	3
TKGVDEVTIVNILTNR	3
TPAQYDASELK	3
WISIMTER	3
WISIMTER	3
AEDGSVIDYELIDQDAR	3
DALNIETAIK	3
ELYDAGVKR	3
QDIAFAYQR	3
TNQELQEINR	3
GVDEVTIVNILTNR	4
Protein Phosphatase 1 Catalytic Subunit	**Experiment Number**
IVQMTEAEVR	2
IKYPENFFLLR	3
IVQMTEAEVR	3
TFTDCFNCLPIAAIVDEK	3
TFTDCFNCLPIAAIVDEK	4
GDP Dissociation Inhibitor 2	**Experiment Number**
DLGTDSQIFISR	2
MLLFTEVTR	2
VIEGSFVYK	2
DLGTDSQIFISR	3
TFEGVDPK	3
FVSISDLFVPK	4
MLLFTEVTR	4
Ras-Related Nuclear Protein (RAN)	**Experiment Number**
FNVWDTAGQEK	3
SNYNFEKPFLWLAR	3
VCENIPIVLCGNK	3
FNVWDTAGQEK	4
NLQYYDISAK	4
VCENIPIVLCGNK	4

Supplemental Figure 1. Estimate of AQP2 Abundance in IMCD Lysates

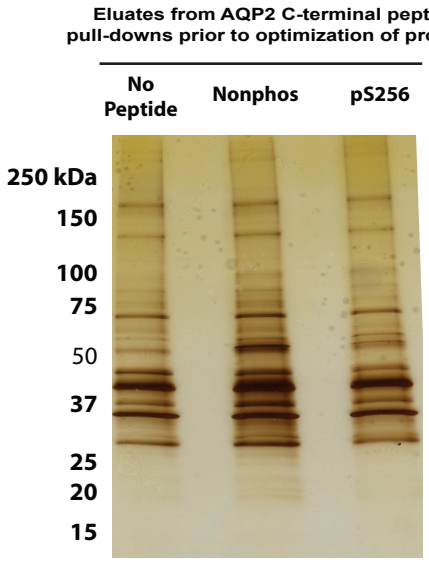


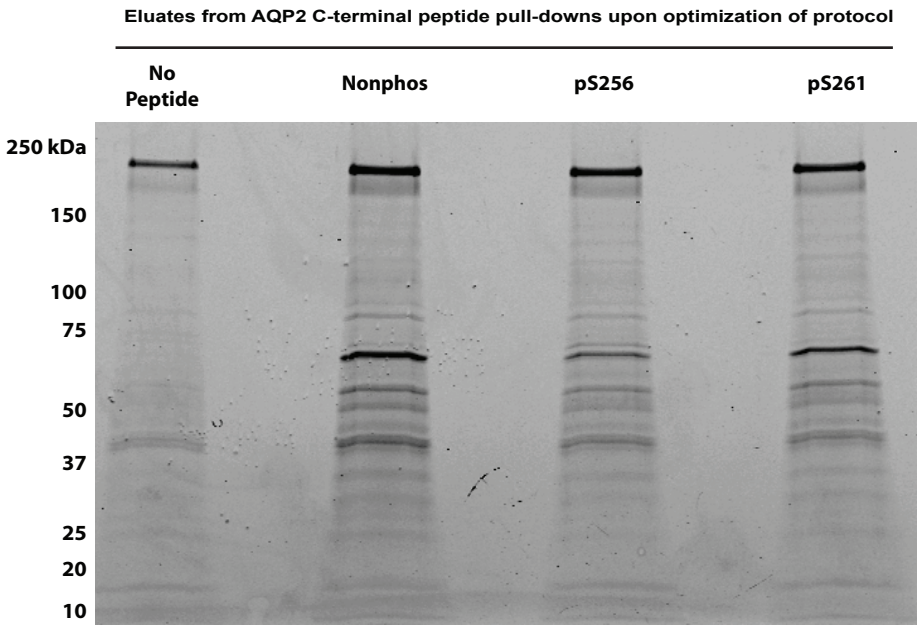


Supplemental Figure 2. Peptide Dot Blots

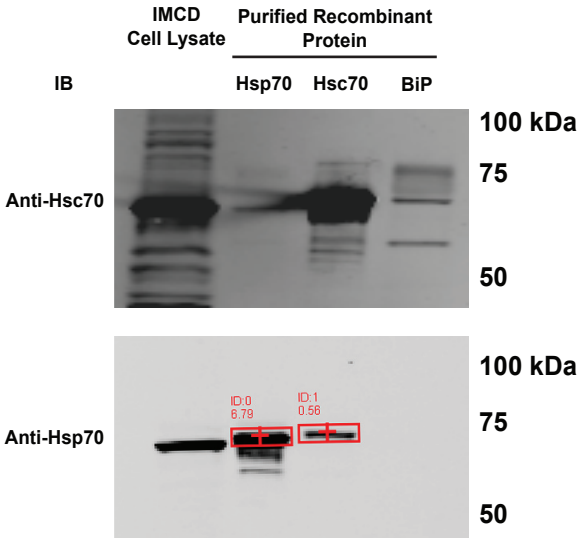


Supplemental Fig 3. AQP2 COOH-terminal peptide pull-downs: wash, binding, and elution conditions

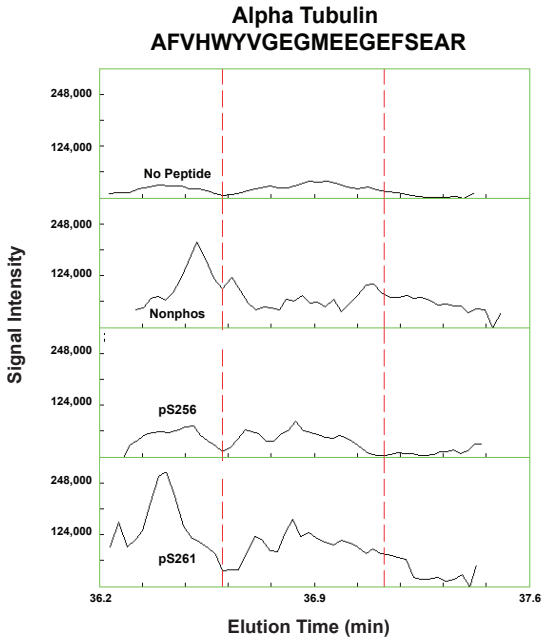


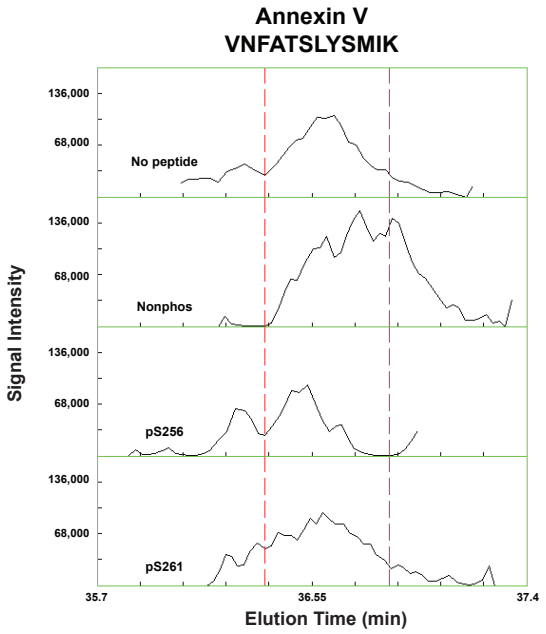


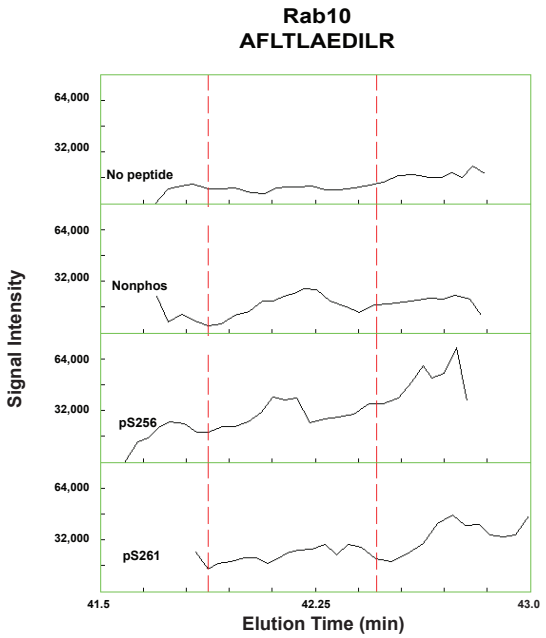
Supplemental Figure 4. Evaluation of Anti-Hsc70 and Anti-Hsp70 Specificity



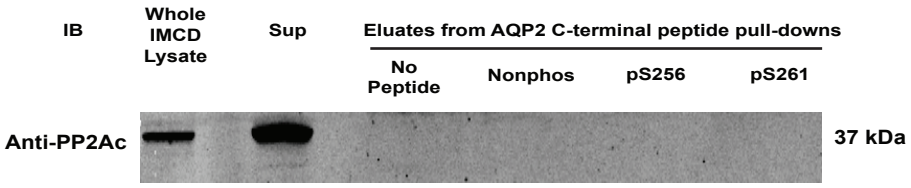
Supplemental Figure 5. Identified Proteins Discarded Based on Spectra



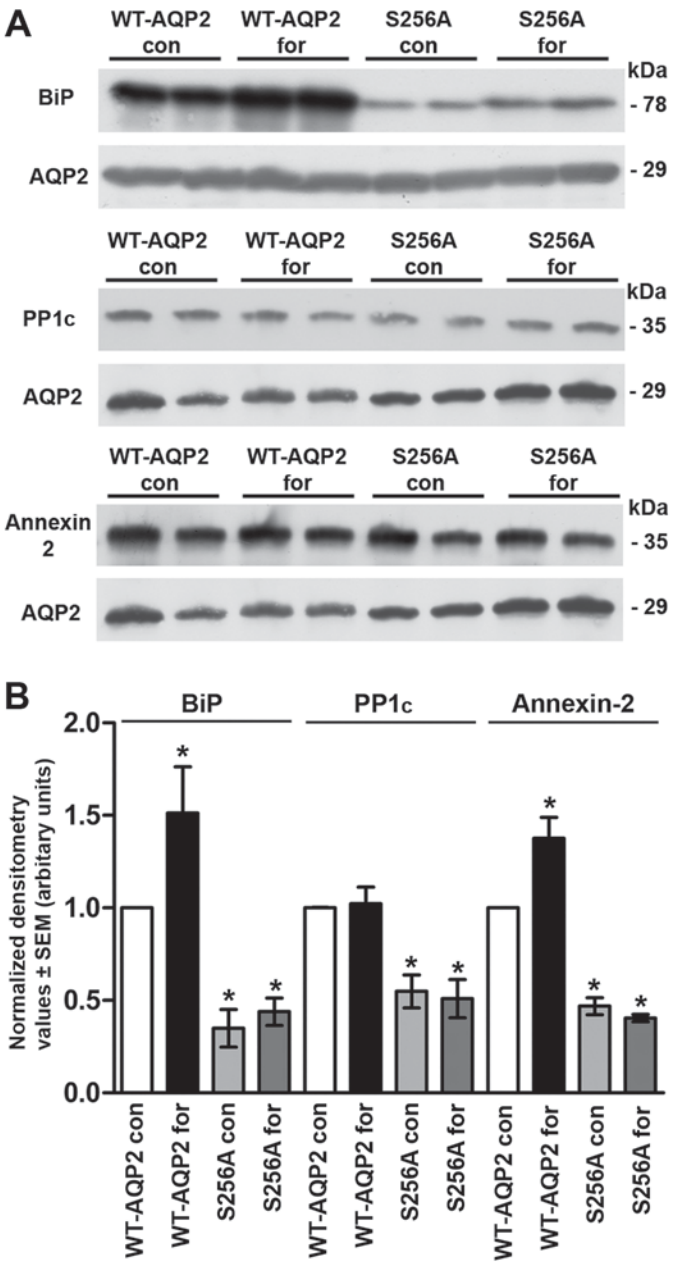




Supplemental Fig 6. Absence of PP2Ac in AQP2 COOH-Terminal Peptide Pull-Down Eluates



Supplemental Figure 7



Supplemental Figure 8