

## Original Paper

# AQP2 Abundance is Regulated by the E3-Ligase CHIP Via HSP70

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**Key Words**

AQP2 • CHIP E3ligase • MDM2 • HSP70 • 17AGG

**Abstract**

**Background/Aims:** AQP2 expression is mainly controlled by vasopressin-dependent changes in protein abundance which is in turn regulated by AQP2 ubiquitylation and degradation, however the proteins involved in these processes are largely unknown. Here, we investigated the potential role of the CHIP E3 ligase in AQP2 regulation. **Methods:** MCD4 cells and kidney slices were used to study the involvement of the E3 ligase CHIP on AQP2 protein abundance by cell homogenization and immunoprecipitation followed by immunoblotting. **Results:** We found that AQP2 complexes with CHIP in renal tissue. Expression of CHIP increased proteasomal degradation of AQP2 and HSP70 abundance, a molecular signature of HSP90 inhibition. Increased HSP70 level, secondary to CHIP expression, promoted ERK signaling resulting in increased AQP2 phosphorylation at S261. Phosphorylation of AQP2 at S256 and T269 were instead downregulated. Next, we investigated HSP70 interaction with AQP2, which is important for endocytosis. Compared with AQP2-wt, HSP70 binding decreased in AQP2-S256D and AQP2-S256D-S261D, while increased in AQP2-S256D-S261A. Surprisingly, expression of CHIP-delUbox, displaying a loss of E3 ligase activity, still induced AQP2 degradation, indicating that CHIP does not ubiquitylate and degrade AQP2 itself. Conversely, the AQP2 half-life was increased upon the expression of CHIP-delTPR a domain which binds Hsc70/HSP70 and HSP90. HSP70 has been reported to bind other E3 ligases such as MDM2. Notably, we found that co-expression of CHIP and MDM2 increased AQP2 degradation, whereas co-expression of CHIP with MDM2-delRING, an inactive form of MDM2, impaired AQP2 degradation. **Conclusion:** Our findings indicate CHIP as a master regulator of AQP2 degradation via HSP70 that has dual functions: (1) as chaperone for AQP2 and (2) as an anchoring protein for MDM2 E3 ligase, which is likely to be involved in AQP2 degradation

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## Introduction

Body water homeostasis is directly regulated by the hormone vasopressin (AVP) which controls the expression and the trafficking of the water channel AQP2. During dehydration and hypernatremia, AVP is secreted by the pituitary gland and binds type-2 receptor (V2R) at the basolateral membrane of renal collecting duct principal cells [1-4]. Activation of the cAMP/PKA signal transduction pathway stimulates the translocation of AQP2-bearing vesicles to the apical plasma membrane increasing luminal permeability [5-7]. This translocation is paralleled by AVP-dependent phosphorylation of AQP2 at serine-256 (pS256). Nevertheless, several studies have revealed that phosphorylation at S256 is not necessary for exocytosis as well as dephosphorylation is not required for endocytosis. Instead, it has been hypothesized that pS256 may play a role in controlling the kinetics of endocytosis [8]. Blocking clathrin-dependent endocytosis, by expressing GTPase-deficient dynamin mutation (K44A) or with methyl- $\beta$ -cyclodextrin, enhances the abundance at the plasma membrane of both wild-type AQP2 and AQP2 that cannot be phosphorylated at serine 256 (S256A) [9], indicating that the constitutive trafficking is not regulated by phosphorylation at S256. In the last few years, the understanding of regulation of AQP2 trafficking was improved by extensive investigations of Knepper's group demonstrating that AQP2 can also be phosphorylated at serine-261 (S261), serine-264 (S264) and serine/threonine-269 (S/T269) [10]. Time course of vasopressin-dependent AQP2 phosphorylation indicated that S256 phosphorylation is necessary as a priming event for phosphorylation at S264 and S/T269 or dephosphorylation at S261 [11]. Phosphorylation at S/T269 compartmentalized AQP2 exclusively in the apical plasma membrane therefore it can be considered a membrane retaining signature [11].

The expression level of integral membrane proteins in the plasma membrane results from a balance between exocytosis and endocytosis. Several studies revealed that accumulation of AQP2 at the apical plasma membrane can also occur in the absence of stimulation with vasopressin by inhibiting constitutive endocytosis [9] or by inducing actin depolymerization [12, 13]. However, the molecular machinery controlling docking and fusion of AQP2-bearing vesicles, as well as the endocytosis and degradation of AQP2 remains to be further clarified. The trafficking of AQP2 bearing vesicles involves actin remodeling, SNARE, Rab proteins and myosin as few examples of the complex group of proteins involved in regulating the trafficking of AQP2 [12-15]. It remains to be clarified whether these proteins play a role in controlling the constitutive trafficking or the vasopressin dependent AQP2 redistribution. Up to now, few studies have demonstrated the direct interaction between AQP2 and the components of trafficking machinery. Among them, the functional involvement of annexin-2 in the fusion process of AQP2 vesicles has been demonstrated [15]. Specifically, annexin-2 binds directly AQP2 [16] and associates to the lipid raft under forskolin-induced cAMP production [15]. Statins, cholesterol synthesis inhibitors, lowered raft cholesterol content and accumulated AQP2 at the plasma membrane by inhibiting the endocytosis via Rho proteins, independently of cAMP/PKA signaling [17, 18]. The endocytotic pathway is quite complex and involves selective protein-protein interactions. The heat shock 70 (HSP70) family members include multiple homologs such as the cytosolic stress-induced HSP70 (HSP72), Hsc70 and the ER-localized BiP [19, 20].

Importantly, HSP70 associates directly to AQP2 and controls the internalization process [21]. Functional knockdown of HSP70/hsc70 increased the abundance of AQP2 at the plasma membrane due to inhibition of endocytosis [21], which targets AQP2 in a recycling vesicles pool or to the degradation route. Short-chain ubiquitylation at lysine 270 (K270), enhances AQP2 endocytosis and lysosomal degradation [22]. Instead, AQP2 polyubiquitylation targets AQP2 for proteasomal degradation [23]. Bioinformatical studies proposed NEDD4 and NEDD4L, AMFR, STUB1, ITCH, ZFPL1 as possible E3 ligases responsible for AQP2 ubiquitylation [24]. Furthermore, transcriptome analysis and biochemical studies revealed that vasopressin stimulation changed the protein abundance of three E3 ligases named BRE1B, NEDD4, and CUL5 [25]. The present study aims to clarify

intracellular signals controlling AQP2 stability. Specifically, we found that the expression of the E3 ligase CHIP increases AQP2 degradation via HSP70, which interacts with AQP2 and anchors the E3 ligase MDM2, apparently involved in modulating AQP2 degradation. A novel pathway controlling AQP2 stability was therefore unraveled and we propose the E3-ligase CHIP and MDM2 novel players in modulating the abundance of AQP2.

## Materials and Methods

### *Chemicals and reagents*

All chemicals were purchased from Sigma-Aldrich (Milan, Italy). MG132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was obtained from AdipoGen AG (Liestal, Switzerland). 17AAG (17-(Allylamino)-17-demethoxygeldanamycin) was purchased from Santa Cruz Biotechnology (DBA, Milan Italy). PEI (Polyethylenimine, Linear, MW 25, 000) was purchased from Polysciences Inc. (Germany). Lipofectamine was purchased from Life Technologies (Monza, Italy).

### *Antibodies*

To detect the total amount of AQP2, antibodies against the 20 amino acid residue segment just N-terminal from the poly-phosphorylated region of rat AQP2 (CLKGLEPDTDWEEREVRRRQ) were used [26]. AQP2-pS256 and AQP2-pT269 antibodies were described by Trimpert et al [27]. AQP2-pS261 antibodies were purchased from Novus Biological (DBA, Milan Italy). Monoclonal antibody against CHIP, monoclonal antibody against c-Myc (9E10) and anti-MDM2 (C-18) were purchased from Santa Cruz Biotechnology (DBA, Milan Italy). Polyclonal antibody Anti-phospho-MAPKinase 1/2 (Erk 1/2) (Thr185/Tyr187, clone AW39) and monoclonal antibody Anti-Erk1/2 (p44/42, clone MK12) were bought from Millipore (Milan, Italy). Monoclonal antibody against HSP70 (C92F3A-5) was from Enzo Life Sciences (3V Chimica S.r.l., Roma, Italy). This antibody is specific for HSP70 family members (i.e. HSP72) but do not cross-react with Hsc70. Secondary goat anti-rabbit and goat anti-mouse antibodies were purchased from Sigma-Aldrich (Milan, Italy).

### *DNA constructs*

Myc-CHIP, Myc- $\Delta$  tetratricopeptide repeat (TPR) CHIP and Myc- $\Delta$ UboxCHIP were already described [28].

pcDNA3-MDM2 and pcDNA3-MDM2-del1-441 were obtained by Dr. Tullo [29]. The empty vector pCDNA 3.1, pCB6-hAQP2, pCB6-hAQP2-S256A, pCB6-hAQP2-S256D, pCB6-hAQP2-S261A, pCB6-hAQP2-S261D and pCB6-hAQP2-S256DS261D, pCB6-hAQP2-S256DS261A have been described [30, 31].

### *Cell culture and transfection*

Mouse cortical collecting duct M-1 and MCD4 cells [32], were grown in Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin, 100  $\mu$ g/ml streptomycin and 5  $\mu$ M dexamethasone at 37°C, 5% CO<sub>2</sub>.

Cells were transiently transfected with plasmids (0.4  $\mu$ g of DNA/cm<sup>2</sup>) encoding for CHIP-wt and its variants, using lipofectamine (1 mg/ml) according to the protocol provided by the manufacturer (Life Technologies, Monza, Italy). Experiments were performed 48 h post-transfection. Alternatively, cells were seeded a day prior to transfection at 80% confluence in 100 mm diameter Petri dishes. PEI 1  $\mu$ g/ $\mu$ l was dissolved with 150 mM NaCl (pH 7) and incubated for 5 min at room temperature. In parallel, 6  $\mu$ g of DNA were diluted in NaCl 150 mM. After 5 min, solutions were mixed, vortexed and incubated for 20 min at room temperature. Transfecting solution was then added to cells and incubated for 8 h in complete medium at 37°C, 5% CO<sub>2</sub>. Immunoprecipitation experiments were performed 48 h after transfection.

### *Treatment and cells preparation*

MCD4 cells were seeded in 12-well plates and assayed 48 h post transfection. Cells were left in the basal condition or stimulated with Chloroquine (0.1 mM) over night, with MG132 (10  $\mu$ M) for 4 hours or with 2  $\mu$ M 17-AAG for 2, 3 or 4 h, followed by the addition of 100  $\mu$ g/ml Cycloheximide for 1, 2 or 4 hours. Alternatively, cells were treated with FR180204 5  $\mu$ M for 30 min. Then, cells were scraped and sonicated.

The obtained lysates were resuspended in Laemmli's buffer 6X (350 mM Tris-HCl pH 6.8, 10% SDS, 50% Glycerol and 0.03% Bromophenol blue) in the presence of proteases (1 mM PMSF, 2 mg/ml leupeptin and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. The obtained homogenates were sonicated at 80 Hz amplitude for 10 sec and used for immunoblotting studies. Alternatively, cells were cultured in 100 mm diameter Petri dishes and grown to confluence for 3 days. Then, cells were washed twice in ice-cold PBS and scraped in ice-cold Isolation Medium (220 mM mannitol, 70 mM sucrose, 0, 5 M EGTA pH 8, 0, 5 M EDTA pH 8, 1 M Tris-HCl, pH 7.4) in the presence of proteases (1 mM PMSF, 2 mg/ml leupeptin and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. Cell suspension was homogenized manually with a mini-potter and spun at 8,000xg for 20 min. The supernatant was centrifuged at 17,000xg for 1 hour to obtain a pellet enriched in plasma membranes and the resulting supernatant was subjected to a last centrifugation step for 1 hour at 200,000xg in a Beckman Rotor TLA 120.1. The final pellet, enriched in intracellular vesicles, was gently resuspended in Isolation medium using a 30-gauge needle. Equal amount of proteins (30 µg/lane) were subjected to electrophoresis and immunoblotting.

### *Immunoprecipitation*

Immunoprecipitation experiments were performed as described [33]. Briefly, mice kidney medulla, fresh kidney slices, MCD4 or M1 cells were lysed with 1% Triton X-100, 150 mM NaCl, 25 mM HEPES (pH 7.4) in the presence of protease (1 mM PMSF, 2 mg/ml leupeptin and 2 mg/ml pepstatin A) and phosphatase (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. Lysates were clarified by centrifugation at 13,000 g for 15 minutes at 4°C. The obtained supernatants were precleared with protein A-sepharose suspension for 30 min under rotation at 4°C. Pre-cleared lysates were incubated overnight with anti-AQP2 or anti-HSP70 antibodies coupled to protein A-Sepharose. Immunocomplexes were washed three times, resuspended in Laemmli's buffer in not denaturing conditions and subjected to immunoblotting using anti-AQP2, anti-HSP70, anti-c-Myc and anti-MDM2 antibodies.

### *Kidney slices*

Studies in kidney slices were performed as reported [6]. Briefly, kidney were quickly removed, and thin transversal slices (500 µm), including the cortex and the medulla, were cut using a Mcllwain Tissue Chopper (Ted Pella Inc.; Redding, CA, United States). The slices were equilibrated for 10 min in Kidney Slices buffer containing 118 mM NaCl, 16 mM HEPES, 17 mM Na-HEPES, 14 mM Glucose, 3.2 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.8 mM MgSO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>. Subsequently, kidney slices were left in the same buffer at 37°C or incubated with 17AAG (10 µM) for 30 min. The treated sections were then homogenized with a mini-potter in ice-cold kidney slices buffer, in the presence of proteases (1 mM PMSF, 2 mg/ml leupeptin and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. Suspensions were then centrifuged at 12,000xg for 10 min at 4°C and the supernatants used for western blotting analysis.

### *Gel Electrophoresis and Western blotting*

Proteins were separated on 13% bis-tris acrylamide gels under reducing conditions. Protein bands were electrophoretically transferred onto Immobilon-P membranes (Millipore Corporate Headquarters, Billerica, USA) for Western blot analysis, blocked in TBS-Tween-20 containing 3% BSA and incubated with primary antibodies overnight. Immunoreactive bands were detected with secondary antibodies conjugated to horseradish peroxidase (HRP) obtained from Santa Cruz Biotechnologies (Tebu Bio, Milan, Italy). Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) with Chemidoc System (Bio-Rad Laboratories, Milan, Italy). Representative figures are shown. Densitometry analysis was performed with Scion Image. Data were summarized in histograms with GraphPad Prism (Graphpad Software Inc. La Jolla, CA, USA)

### *Statistical Analysis*

Data are reported as means ± SEMs. Statistical analysis was performed by one-way ANOVA and two-way ANOVA, followed by Tukey's multiple comparison test. When applicable, Student t-test student was also applied. A difference of P<0.05 was considered statistically significant.

## Results

### *AQP2 associates with CHIP in renal tissue*

Protein sequencing analysis revealed that AQP2 contains within its C-terminal domain the SXS motif which can bind the E3 ligase CHIP directly [34]. Co-immunoprecipitation experiments were performed to evaluate the putative interaction between AQP2 and the E3-ligase CHIP. To this end, MCD4 cells and mouse kidney lysates were incubated with anti-AQP2 antibodies, next, the immunocomplexes were precipitated with protein-A Sepharose and analyzed by immunoblotting with antibodies specific for CHIP. AQP2 antibodies were used as control of the immunoprecipitation experiments. The obtained results indicate that AQP2 can complex with CHIP in MCD4 cells (Fig. 1A) and in renal tissue (Fig. 1B).

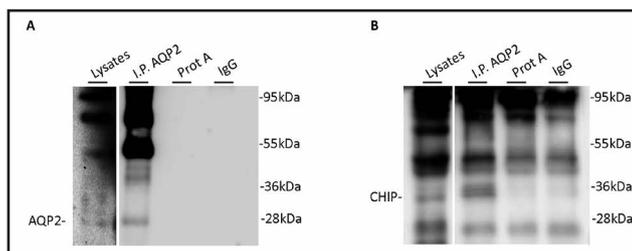
### *Expression of CHIP reduces AQP2 abundance*

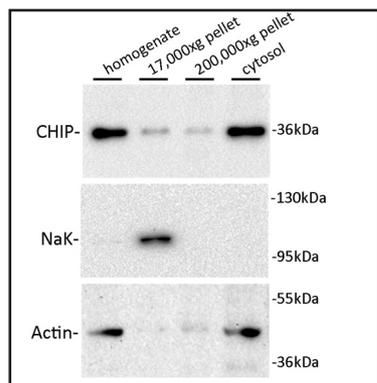
To investigate the possible involvement of CHIP in AQP2 expression levels, the mouse collecting duct cells M1, stably transfected with human AQP2, were used as experimental model (MCD4 cell line) [32]. Cellular fractionation experiments revealed that CHIP co-fractionated mainly in the cytosol with a weak expression in the 17, 000xg and in the 200, 000xg pellets (Fig. 2). To assess the functional role of CHIP on the cellular abundance of AQP2, MCD4 cells were transfected with Myc-CHIP. All experiments were performed 48 hours after transfection. The protein synthesis inhibitor cycloheximide (CHX, 100 µg/ml) was added to MCD4 to abolish de novo protein synthesis. The abundance of AQP2 at 1, 2 and 4 hours after CHX treatment was analyzed by immunoblotting (Fig. 3). Compared with MCD4 cells not expressing CHIP (mock, Fig. 3A), AQP2 abundance significantly decreased within one hour of CHX incubation in cells expressing CHIP-wt (Fig. 3B). Several studies showed that AQP2 can be targeted for degradation either to the lysosome pathway or to the proteasomal route [22, 23]. To evaluate whether the expression of CHIP marks AQP2 for degradation in the lysosomes, cells expressing CHIP were incubated overnight with chloroquine (0.1 mM), a known lysosome inhibitor (Fig. 4A). Chloroquine treatment did not impair the CHIP-induced decrease of AQP2 abundance. In contrast, a 4 hours incubation with the proteasome inhibitor MG132 (10 µM), abolished the CHIP-dependent decrease of AQP2 expression within one hour of CHX treatment (Fig. 4B).

### *Expression of CHIP increases HSP70 abundance*

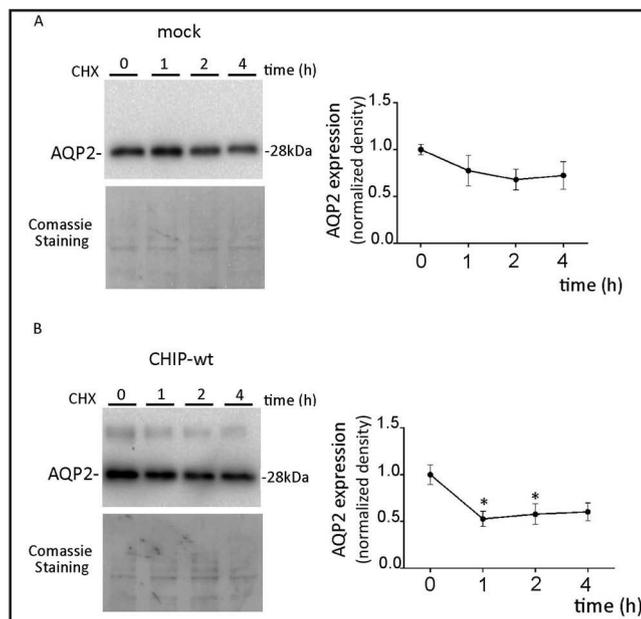
Protein homeostasis depends on controlled folding and protein degradation. Several co-chaperones play fundamental roles in regulating the transit of the client protein. Nevertheless, co-chaperones are also involved in ubiquitin-mediated proteasomal degradation. The C-terminal HSP70 binding protein (CHIP) associates with HSP70 and HSP90 through its tetratricopeptide domain (TRP) and acts as E3 ligase via the U-box domain which is a modified RING finger motif [35]. Moreover, CHIP increases the expression of HSP70 by stimulating HSF1 which is required for protection against stress-induced apoptosis [36]. Compared to mock cells, expression of CHIP significantly increases the abundance of HSP70 (Fig. 5). To exclude that the increased expression of HSP70 was

**Fig. 1.** AQP2 and CHIP co-immunoprecipitation in renal tissue. AQP2 was immunoprecipitated (I.P. AQP2) using pre-C-tail antibodies from MCD4 cells (A) and from fresh mouse kidney slices (B). Whole cell lysates were also subjected to precipitation with only protein A-coupled Sepharose (ProtA) or with nonspecific IgG. Immunocomplexes were analyzed by immunoblotting with antibodies specific for CHIP; anti-AQP2 antibodies were used as control of the immunoprecipitation experiments. The obtained results indicate that AQP2 can complex with CHIP in MCD4 cells and in renal tissue.





**Fig. 2.** Subcellular distribution of CHIP protein in MCD4 cells. MCD4 cells total homogenates, plasma membrane, intracellular vesicles and cytosol were isolated and analyzed by Western Blotting. Cellular fractionation experiments revealed that CHIP is mainly expressed in the homogenate and in the cytosol, although weak immunodetectable bands also appeared in the 17,000xg and in the 200,000xg pellets. Antibodies against Actin and NaK were used to confirm the purity of the isolated fractions.



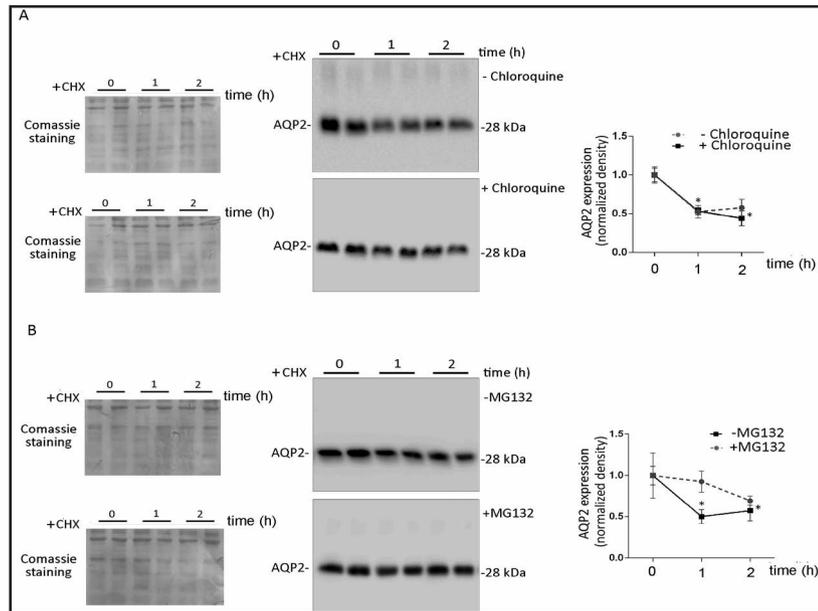
**Fig. 3.** AQP2 abundance in MCD4 cells decreased after protein synthesis inhibitor cycloheximide (CHX) treatment. Cells stably expressing AQP2 were transiently transfected with CHIP-wt. Cells were treated with 100  $\mu$ g/ml cycloheximide (CHX) for indicated time, and cell lysates were subjected to immunoblotting with anti-AQP2 antibodies. Equal amounts of protein (30  $\mu$ g/lane) were separated by gel electrophoresis and immunoblotted with anti-AQP2 antibody. The loading control by Coomassie staining is also shown. Densitometric analysis revealed that cycloheximide (CHX) caused a significant decrease in AQP2 expression ( $52.82 \pm 3.2\%$ ) within an hour of CHX incubation in cells expressing CHIP-wt (B) with respect to mock cells (A) ( $n=3$ ).

due to the transfection itself, MCD4 cells were further transfected with an empty vector (pcDNA3.1) containing CMV promoter but without CHIP cDNA. As shown in figure 5, the transfection with the empty plasmid did not alter the cellular abundance of HSP70, thereby indicating that CHIP itself leads to an increased abundance of HSP70.

#### *HSP90 inhibition reduces the cellular abundance of AQP2 in vitro and in ex vivo*

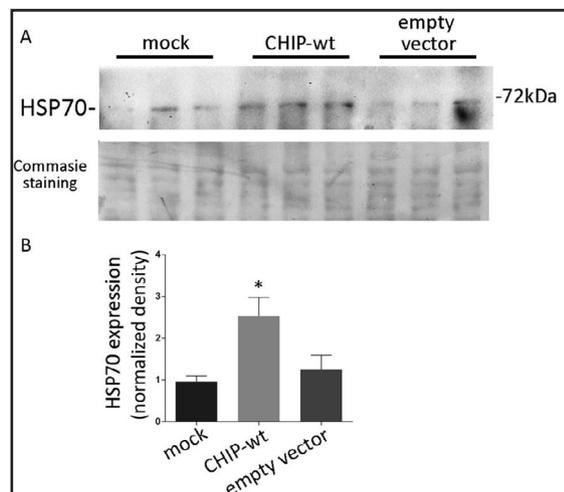
Based on previous studies, the increased expression of HSP70 can be considered a “molecular signature” of HSP90 inhibition [37]. Consistent with that, 17AAG, a known inhibitor of HSP90, significantly increased the cellular abundance of HSP70 [38]. To better define the involvement of these heat shock proteins on AQP2 stability through signaling activated by CHIP, MCD4 cells (mock) (Fig. 6A) and cells expressing CHIP (CHIP-wt) (Fig. 6B) were left untreated or incubated with 17AAG (2  $\mu$ M) for 2, 3 and 4 hours in the presence of CHX (100  $\mu$ g/ml). Treatment with 17AAG significantly reduced the abundance of AQP2 in cells not expressing CHIP (mock). In CHIP expressing cells, the expression of AQP2 was reduced regardless of 17AAG treatment. Moreover, in CHIP expressing cells, no additive effect on AQP2 degradation was observed under treatment with 17AAG, suggesting that 17AAG and expression of CHIP may converge on a similar intracellular pathway that involves the inhibition of HSP90. To assess the effect of 17AAG in renal tissue, *ex vivo* experiments were performed using fresh kidney slices (Fig. 7A). Renal slices were prepared and treated as described under experimental procedures. Immunoblotting analysis revealed that incubation with 17AAG significantly decreased the abundance of AQP2 compared with

**Fig. 4.** CHIP dependent AQP2 degradation was mediated by proteasomes. (A) Effect of lysosome inhibitor Chloroquine on AQP2 degradation. MCD4 cells transiently transfected with CHIP-wt were left under basal condition or incubated with Chloroquine 0.1 mM overnight. Cells were then treated with 100 µg/ml cycloheximide (CHX). Total lysates (30 µg/lane) were subjected to immunoblotting for total AQP2. The loading control by Coomassie staining is shown on the left of each panel. Densitometric analysis revealed that Chloroquine treatment did not impair the CHIP-induced decrease of AQP2 abundance (n=3). (B) Effect of the proteasome inhibitor MG132 on AQP2 degradation. Cells transiently transfected with CHIP-wt were left under basal condition or stimulated with 10 µM MG132 for 4 h, simultaneously 100 µg/ml cycloheximide (CHX) was added for 1 or 2 h. Total lysates were subjected to immunoblotting for total AQP2. Densitometric analysis revealed that incubation with MG132 abolished the CHIP-dependent decrease of AQP2 expression within an hour of CHX treatment (n=3).



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**Fig. 5.** Expression of CHIP increased HSP70 abundance. (A) MCD4 cells were transiently transfected with CHIP-wt or its empty vector pC-DNA 3.1. After transfection, cells were lysed and equal amounts of protein (30 µg/lane) were separated by gel electrophoresis and immunoblotted with anti-HSP70 antibody. The loading control by Coomassie staining is shown. (B) Densitometric analysis (means ± SEMs) revealed a significant increase of HSP70 in CHIP-wt expressing cells (n= 3, \*P<0.001) compared to mock.

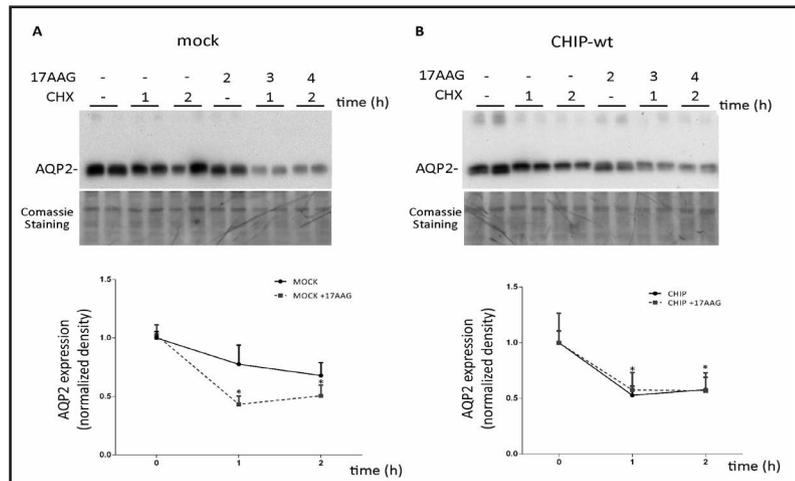


untreated slices (Fig. 7B). Together these data suggest that HSP90 inhibition is crucial to balance the total content of AQP2 in cells and in renal tissue.

*Expression of CHIP stimulates phosphorylation of ERK and changes the dynamic balance of the phosphorylation pattern of AQP2*

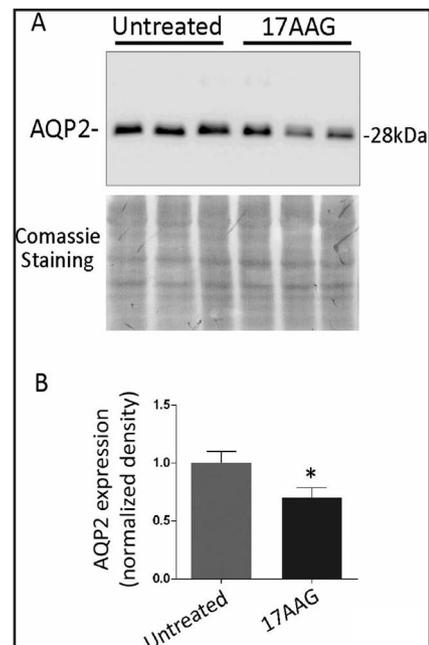
Several studies showed that treatment with 17AAG upregulates HSP70 [38, 39] that participates in intracellular signaling pathways by binding different co-chaperones such as CHIP [40]. HSP70 functions as ATP-dependent molecular chaperone through activation of the ERK signaling [41]. To test the hypothesis that HSP70 promotes ERK phosphorylation, secondary to the expression of CHIP, immunoblotting experiments were performed (Fig. 8A). To this end, MCD4 cells were left untransfected (mock) or transfected with CHIP-wt, as described in the experimental procedures. Immunoblotting studies revealed that exogenous expression of CHIP increases the phosphorylation of ERK compared to that observed in mock cells. Furthermore, incubation of CHIP expressing cells with FR180204, a selective ERK

**Fig. 6.** 17AAG-induced HSP90 inhibition promoted AQP2 degradation. MCD4 cells (mock (A) or expressing CHIP-wt (B)) were left untreated or incubated with 17AAG 2  $\mu$ M for 2, 3 or 4 hours in the presence of CHX 100  $\mu$ g/ml. Cells were lysed and equal amounts of protein (30  $\mu$ g/lane) were separated by gel electrophoresis and immunoblotted with anti-AQP2 antibody. The loading control by Coomassie staining



is shown. Statistical analysis of data (means  $\pm$  SEMs) revealed that treatment with 17AAG significantly reduced AQP2 abundance in mock cells similarly to that observed in CHIP-wt expressing cells. In CHIP expressing cells, AQP2 expression was reduced regardless of 17AAG treatment.

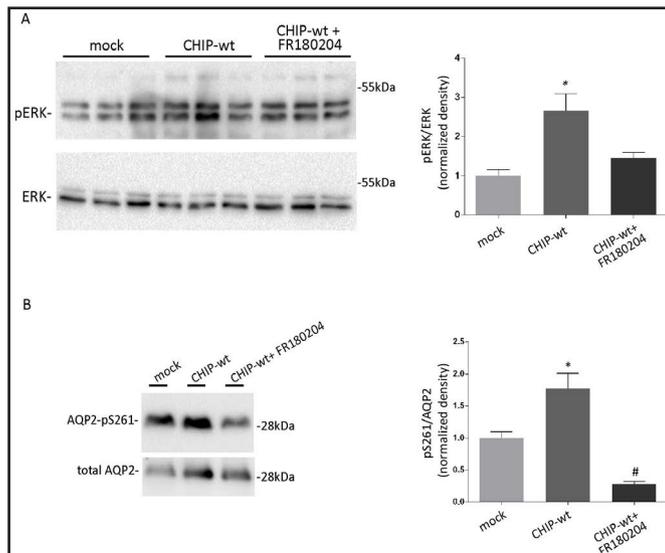
**Fig. 7.** Effect of 17AAG on AQP2 expression in mouse kidney slices. (A) Renal slices were treated with or without 17AAG (10  $\mu$ M) for 30 min. Kidney slices were lysed and equal amounts of protein (30  $\mu$ g/lane) were separated by gel electrophoresis and immunoblotted with anti-AQP2 antibody. The loading control by Coomassie staining is shown. (B) Signals were semiquantified by densitometry. Statistical analysis of data (means  $\pm$  SEMs) revealed that incubation with 17AAG significantly decreased AQP2 abundance with respect to untreated slices (Untreated=1 $\pm$ 0.1, 17AAG=0.7 $\pm$ 0.09, n= 3, \*P<0.01).



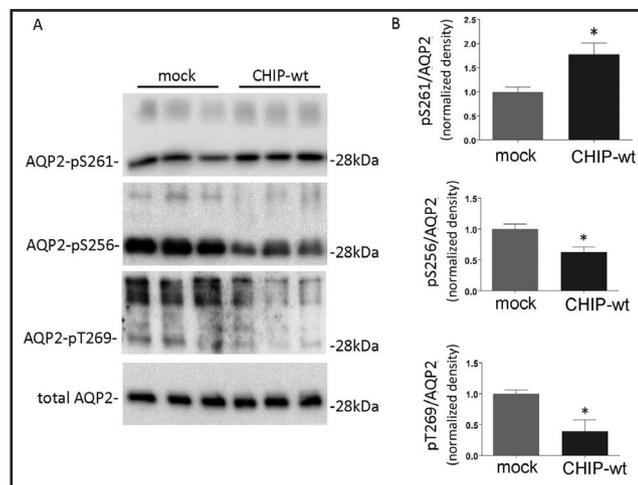
inhibitor, abolished the CHIP-dependent upregulation of ERK phosphorylation (Fig. 8A). Importantly, JNK and ERK can phosphorylate *in vitro* AQP2 at S261, a residue displaying a significant downregulation in its phosphorylation state under vasopressin stimulation *in vivo* [42]. Immunoblotting experiments (Fig. 8B) showed that the phosphorylation of AQP2 at S261 increased in CHIP expressing cells (CHIP-wt) compared to that observed in mock cells. CHIP-dependent phosphorylation of AQP2 at S261 was abolished in the presence of the ERK inhibitor FR180204 suggesting that ERK is the kinase committed to phosphorylate AQP2 at S261, secondary to CHIP expression.

Phosphorylation events are fundamental to control the cellular fate of AQP2 and thus urinary concentration. Inhibition of p38-MAPK reduced the phosphorylation of AQP2 at S261 preventing proteasomal degradation thereby enhancing AQP2 stability [23]. As stated above, phosphorylation at S256 is important but not essential for the insertion of AQP2 at the apical plasma membrane [43] and AQP2 is retained in the apical membrane when phosphorylated at S/T269. To evaluate whether the expression of CHIP (CHIP-wt) alters the phosphorylation balance of AQP2, immunoblotting studies were performed (Fig. 9A). Immunoblotting analysis revealed that the expression of CHIP significantly decreased the phosphorylation at S256 and T269, while increased the pS261 compared to the signals detected in cells not transfected with CHIP-wt (mock) (Fig. 9B).

**Fig. 8.** Expression of CHIP stimulated ERK phosphorylation and increased AQP2 phosphorylation at S261. (A) MCD4 cells were transfected with CHIP-wt and left under basal condition or stimulated with FR180204, a selective ERK inhibitor. Statistical analysis of data (means  $\pm$  SEMs) revealed that CHIP significantly increased ERK phosphorylation compared to that observed in mock cells (n=5, \*P<0.0001). Incubation of CHIP expressing cells with FR180204 abolished the CHIP-dependent upregulation of ERK phosphorylation. (B) MCD4 cells expressing CHIP-wt were left under basal condition or stimulated with FR180204. Endogenous expression of CHIP-wt was associated with a significant increase in AQP2 phosphorylation at S261 (AQP2-pS261) in cells expressing CHIP-wt respect to untransfected cells (n=5, \*P<0.01). Treatment of CHIP expressing cells with FR180204 abolished the CHIP-dependent increase of AQP2-pS261 (n=5, #P<0.0001).



**Fig. 9.** AQP2 phosphorylation balance was altered by CHIP expression. (A) MCD4 cells were left untransfected (mock) or transfected with CHIP-wt. After transfection, cells were lysates and subjected to immunoblot using antibodies against AQP2 phosphorylated at S256 (AQP2-pS256), S261 (AQP2-pS261) or T269 (AQP2-pT269). (B) Densitometric analysis (means  $\pm$  SEMs) revealed a significant increase of AQP2-pS261 in CHIP expressing cells (n= 3, \*P<0.005) and a significant decrease of AQP2-pS256 (n=3, \*P<0.01) and AQP2-T269 (n=3, \*P<0,05) compared to mock cells.

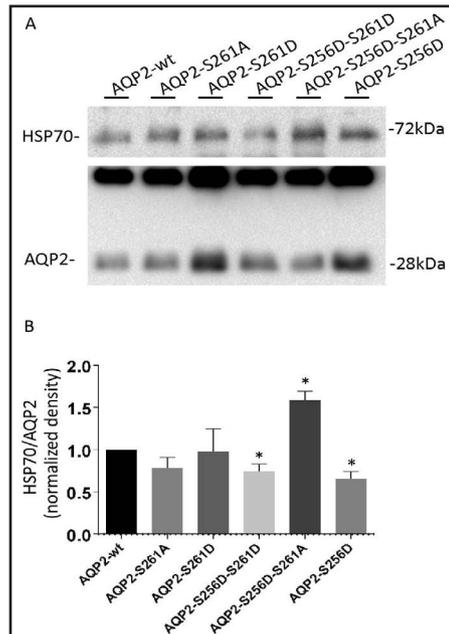


CHIP can switch HSP70/HSP90 complexes from protein folding to protein degradation via proteasomes [35]. Interestingly, HSP70 binds AQP2 directly and an intact S256 residue is crucial for this interaction [21]. Here, to investigate whether changes in AQP2 phosphorylation at S256 and S261 modulates the interaction with HSP70, several AQP2 mutants [30, 31] were transfected in M1 cells. Co-immunoprecipitation experiments showed that point mutations at S261 either with D, mimicking the constitutive phosphorylated form, or with A, mimicking the constitutive not phosphorylated protein, do not alter the interaction ability of AQP2 with HSP70 (Fig. 10A, lane 2-3). Conversely, similarly to what found by Lu et al [21], single mutation at S256 with D reduced the binding ability of AQP2 and HSP70. Moreover, double mutations at S256 and S261 with D (AQP2-S256D-S261D) also reduced this protein association similarly to what was observed in cells expressing the AQP2-S256D mutant. In contrast, double mutations at S256 with D and S261 with A (AQP2-S256D-S261A) increased the HSP70/AQP2 interaction. Densitometric analysis (Fig. 10B) reveals a significant upregulation of the HSP70/AQP2-S256D-S261A binding compared to HSP70/AQP2-wt ratio, fixed as 1 (Fig. 10 lane 5, AQP2-S256D-S261A: 1, 59  $\pm$  0.10 vs AQP2 wt: 1; p<0, 05).

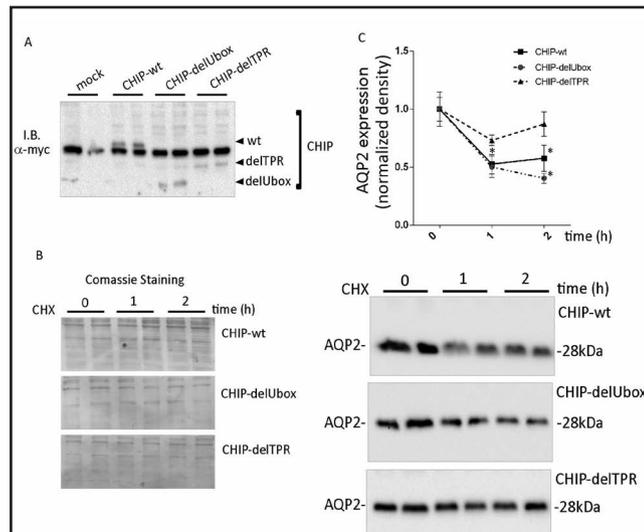
**Fig. 10.** HSP70 binding affinity was higher for AQP2-S256D-S261A. (A) M1 cells were transfected with pCB6-hAQP2, pCB6-hAQP2-S256A, pCB6-hAQP2-S256D, pCB6-hAQP2-S261A, pCB6-hAQP2-S261D, pCB6-hAQP2-S256DS261D, and pCB6-hAQP2-S256DS261A. 48 h after transfection, co-immunoprecipitation experiments were performed. (B) Statistical analysis of data (means  $\pm$  SEMs) revealed that point mutations at AQP2-S261 either with D or with A did not alter the interaction ability of AQP2 with HSP70. Double mutations at S256 and S261 with D, reduced this protein association similarly to what observed in cells expressing the AQP2-S256D mutant (n=3, \*P< 0.01). In contrast, double mutations at S256 with D and S261 with A (AQP2-S256D-S261A) increased the HSP70/AQP2 interaction (n=3, \*P<0.01).

*The ubiquitin-protein ligase activity of CHIP is not required for AQP2 degradation: involvement of the E3 ligase MDM2*

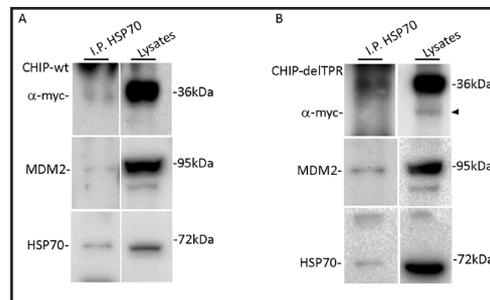
To assess whether CHIP participates directly in the degradation of AQP2, MCD4 cells were transiently transfected either with constructs coding for wild-type CHIP (CHIP-wt), or with CHIP-delUbox, lacking the catalytic domain, or with CHIP-delTPR, which is deficient in the TPR domain needed for the interaction with chaperone proteins as HSP70. All proteins were expressed (Fig. 11A). AQP2 expression at 1 and 2 hours after CHX incubation was evaluated by Western Blotting (Fig. 11B). The loading control by Coomassie staining is also shown (Fig. 11C). Surprisingly, in cells expressing CHIP-delUbox, AQP2 degradation was similar to that measured in cells transfected with CHIP-wt (Fig. 11D) indicating that the ubiquitin ligase activity of CHIP is not required for AQP2 degradation. In contrast, expression of CHIP-delTPR resulted in a relevant attenuation of AQP2 degradation underlining the crucial role of CHIP association with HSP70 to determine the fate of AQP2. Based on these findings, it was hypothesized that HSP70, anchored to the C-terminal domain of CHIP, tethers other E3-ligases. The E3 ligases Ubr, Cul5, Parkin, TRIM32 and MDM2 can complex with HSP90/HSP70, targeting client proteins for degradation [44-46]. MDM2 plays an important role in renal physiology and pathophysiology [47-49]. To test the hypothesis that CHIP can complex with HSP70 and MDM2, co-immunoprecipitation experiments were performed (Fig. 12). Specifically, MCD4 cells were co-transfected with CHIP-wt and MDM2 and cell lysates were incubated with antibodies specific for HSP70. Immunocomplexes were precipitated with protein-A Sepharose and analyzed by immunoblotting with antibodies specific for  $\alpha$ -myc, to detect CHIP, and MDM2; HSP70 antibodies were used as control (Fig. 12A). Alternatively, co-immunoprecipitation assays were carried out in cells co-expressing CHIP-delTPR and MDM2 (Fig. 12B). We observed that only CHIP-wt can complex with HSP70 recruiting MDM2 in the same interactome (Fig. 12A). In contrast, CHIP-delTPR loses the ability to interact with HSP70 that still tethers MDM2 (Fig. 12B). To clarify the involvement of MDM2 in AQP2 degradation, MCD4 cells were transfected either with CHIP-wt or co-transfected with CHIP-wt and MDM2-wt. Alternatively, cells were co-transfected with CHIP-wt and MDM2-delRING lacking the catalytic domain. AQP2 abundance was evaluated at 1 and 2 hours after CHX treatment (Fig. 13A). Immunoblot analysis (Fig. 13B) revealed that in cells expressing CHIP-wt, AQP2 degradation occurred similarly to that measured in cells co-transfected with CHIP-wt and MDM2-wt. In contrast, in cells expressing MDM2-delRING the reduction in AQP2 expression, observed within 1 hour of CHX incubation, was significantly attenuated. Together these findings suggest that MDM2 is the E3 ligase committed to promote AQP2 degradation in CHIP expressing cells.



**Fig. 11.** CHIP dependent AQP2 degradation in cells expressing CHIP-delTPR, the CHIP mutant unable to bind HSP70/HSP90 (A) CHIP mutants expression in MCD4. MCD4 cells were transfected with CHIP-wt, CHIP-delTPR, deficient in the TPR (tetra tripeptide) repeat, or CHIP-delUbox, lacking the catalytic domain. Cell lysates were subjected to immunoblot using antibodies against c-Myc. (B) MCD4 cells were transfected with CHIP-wt and its mutants and left under basal condition or incubated with 100 µg/ml cycloheximide (CHX) for indicated time. Cells were lysed and equal amounts of protein (30 µg/lane) were separated by gel electrophoresis and immunoblotted with anti-AQP2 antibody. The loading control by Coomassie staining is shown. Immunoreactive signals were semiquantified by densitometry indicating that expression of CHIP-delTPR resulted in a significant decrease of AQP2 degradation compared to cells expressing CHIP-wt and CHIP-delUbox.



**Fig. 12.** CHIP complexes with HSP70 and MDM2 to promote AQP2 degradation. MCD4 cells were co-transfected with CHIP-wt and MDM2 (A) or CHIP-delTPR and MDM2 (B). 48 h after transfection, cells were lysed and incubated with antibodies specific for HSP70. Immunocomplexes were precipitated with protein-A Sepharose and analyzed by immunoblotting with antibodies anti-myc or anti-MDM2; anti-HSP70 antibodies were used as control. Only CHIP-wt can complex with HSP70 recruiting MDM2 in the same interactome (n = 6). In contrast, CHIP-delTPR lost the ability to interact with HSP70 that still tethers MDM2 (n = 6).



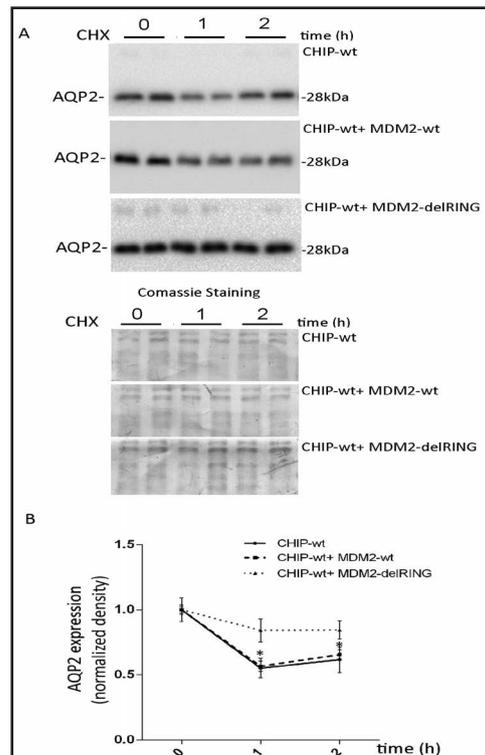
## Discussion

AQP2 expression is mainly controlled by vasopressin-dependent changes in protein abundance which is in turn regulated by AQP2 ubiquitylation and degradation. However the proteins involved in these processes are largely unknown. Here, we provide new findings regarding the potential role of the E3 ligases CHIP and MDM2 in AQP2 regulation.

Interestingly, AQP2 contains, within its C-terminal, the motif SXS, which is needed for direct interaction with the E3 ligase CHIP [34]. Consistent with this observation, AQP2 interacts with the E3-ligase CHIP in renal tissue (Fig. 1). However, point mutation at S231 with A, within the SXS motif, in AQP2 did not alter the association between CHIP and AQP2 in renal collecting duct MCD4 cells (data not shown) likely indicating that accessory proteins may modulate this binding.

CHIP is only one player of multichaperone complexes consisting of several other chaperones. In the kidney, the thiazide-sensitive cotransporter, NCC, co-immunoprecipitates with multiple chaperones and co-chaperones such as HSP70, HSP90, HSP40, CHIP and HOP. These bindings are crucial for cellular fate of NCC [38]. CHIP overexpression controls stability and promotes the degradation of several target proteins as the apoptosis suppressor (DDIAS), the potassium channel Kv1.5, the receptor tyrosine kinase AXL and several others [50-52]. We found here that overexpression of CHIP in MCD4 cells significantly decreased AQP2 abundance within one hour after CHX incubation. It cannot be excluded, however, that

**Fig. 13.** MDM2-induced AQP2 degradation in cells expressing CHIP-wt. (A) MCD4 cells were transfected with CHIP-wt alone, CHIP-wt and MDM2-wt or CHIP-wt and MDM2-delRING. After transfection cells were treated with 100 µg/ml cycloheximide (CHX) for 1 or 2 h and cell lysates were subjected to immunoblot with anti-AQP2 antibodies. The loading control by Coomassie staining is shown. (B) Densitometric analysis and statistical studies revealed that in CHIP-wt expressing cells, AQP2 degradation occurred similarly to that measured in cells co-transfected with CHIP-wt and MDM2-wt (n = 6). In contrast, in MDM2-delRING expressing cells, AQP2 reduction observed within 1 hour of CHX treatment was significantly attenuated (n = 6).



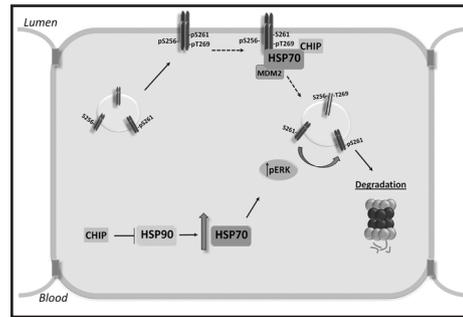
at long term compensatory mechanism may also play a role in controlling AQP2 abundance and stability. Equal amount of proteins were loaded under any conditions and Coomassie staining was used as loading control in the Western Blotting, as GAPDH abundance significantly changed in cells expressing CHIP-wt and mutants (not shown).

GAPDH indeed is wrongly considered a good housekeeping marker used to normalize mRNA and proteins as GAPDH expression is also modulated and can change under different physiological cellular conditions [53]. Client proteins of CHIP can be targeted either to lysosomes or to proteasomes for degradation [50, 54]. While short-chain ubiquitylation increases AQP2 endocytosis and lysosomal degradation [22], polyubiquitylation targets AQP2 to proteasomes, in fact MG132 treatment increased the stability of AQP2 by impairing its proteasomal degradation [23]. In the present study, we found that overexpression of CHIP reduces the abundance of AQP2 through proteasomal pathway, as MG132 completely abolished the CHIP-dependent decrease of AQP2 expression (Fig. 4).

HSP70 and HSP90 are members of multiprotein complexes playing a role in modulating protein folding and stability [19]. CHIP, as co-chaperone, can switch HSP70/HSP90 complex from folding to protein degradation [35]. It has been reported that increased expression of CHIP activates the stress pathway by inducing the nuclear translocation of HSF-1, and the stimulation of HSE containing stress responsive promoters, like HSP70 [36]. In line with these findings, we have found that the expression of CHIP results in a significant increase in HSP70 abundance (Fig. 5). Chemical inhibition of HSP90, with 17AAG, also increased HSP70 expression [39, 55], that can sequester the clients released by the inactive HSP90 and trigger their degradation. Inhibition of HSP90 with 17AAG reduces AQP2 expression level in MCD4 cell not transfected with CHIP (mock). Conversely, no relevant effect on AQP2 abundance was observed in CHIP-wt cells indicating that overexpression of CHIP includes a selective downregulation of HSP90, secondary to the upregulation of HSP70.

Exposure of cells to different stresses leads to upregulation of heat shock proteins and the activation of the ERK signal pathway [41, 56]. Interaction between CHIP and ERK5 has been shown and mediates cardio-protective effects [57]. The ERK cascade plays a central role in vasopressin secretion and signaling. During dehydration vasopressin release is paralleled by increased phospho-ERK in VP-ergic termini of the pituitary [58]. In the kidney, vasopressin stimulation causes ERK activation as ERK inhibition with U0126 impaired the vasopressin-induced AQP2 expression [59]. In contrast, more recent data showed that vasopressin reduces the level of ERK phosphorylation which has been shown to phosphorylate AQP2 at S261 [42, 60]. The reason of this discrepancy is unclear even though the decrease in the phosphorylation, at sites recognized by the MAP kinase, might

**Fig. 14.** Schematic model. Expression of CHIP, inhibiting HSP90, increases proteasomal degradation of AQP2 and HSP70 abundance. Increased HSP70 level, secondary to CHIP expression, promotes ERK signaling which in turn increases AQP2 phosphorylation at S261. Phosphorylation of AQP2 at S256 and T269 are instead downregulated. Since expression of CHIP-delUbox, displaying a loss of E3 ligase activity, still induced AQP2 degradation, we hypothesized that CHIP does not cause AQP2 ubiquitination and degradation itself, but represents a possible anchoring protein for MDM2 E3 ligase, which binds HSP70 and appears to be committed to AQP2 degradation.



support the finding, demonstrating that vasopressin reduces ERK activity [42]. Here, the increase of ERK phosphorylation in cells expressing CHIP, is paralleled by an increase in the phosphorylation of AQP2 at S261, which was attenuated when cells were incubated with a specific inhibitor of ERK.

Phosphorylations of AQP2 at the C-terminal domain play multiple roles in regulating the fate of AQP2. Previous studies revealed that the increase in pS261 was paralleled by an increase of AQP2 ubiquitylation [30]. Phosphorylation at S256 stimulates the vasopressin-dependent translocation of AQP2 vesicles to the plasma membrane where it is retained by increased phosphorylation at S/T269. In the present study, we show that overexpression of CHIP alters the dynamic of AQP2 phosphorylations under basal conditions. Besides an increase in pS261, immunoblotting analysis revealed a significant decrease of pS256 and pT269 compared to cells not expressing CHIP (Fig. 9). In the absence of hormonal stimulation AQP2 can constitutively traffic. In fact, under basal condition, the signal-induced proliferation-associated 1 like 1 (Sipa1L1) binds the intracellular PDZ domain of AQP2 stimulating its endocytosis. Stimulation with vasopressin abolished this interaction reducing AQP2 endocytosis, thereby increasing the accumulation of AQP2 at the plasma membrane [61]. AQP2 also binds the signal-induced proliferation-associated gene-1 (SPA-1) and this association in turn plays a role in modulating the cellular distribution of AQP2, as in SPA-1 deficient mice AQP2 fails to translocate to the luminal side of the collecting ducts under fluid restrictions [62]. Therefore, phosphorylations and interaction with accessory proteins are crucial to control the trafficking of AQP2. Nevertheless, it has been shown that constitutive recycling of AQP2 is not associated with phosphorylation at any C-terminal residues [63]. It is not clear, however, whether phosphorylation may modulate AQP2 interaction with proteins involved in endocytosis. Mutations of S256 with D or with A equally impair the interaction of Hsc70 and AQP2 [21]. In this respect, we evaluated the involvement of phosphorylation at S256 and S261 on AQP2 association with HSP70. In cells expressing CHIP, we found that point mutations at S256 with D (AQP2-S256D; AQP2-S256D-S261D) decrease the interaction of HSP70 and AQP2. In contrast, the double mutant at S256 with D and S261 with A (AQP2-S256D-S261A) displays a higher ability to associate with HSP70. Phosphorylation at S256 is determinant for AQP2 accumulation at the plasma membrane as AQP2-S256D, AQP2-S256D-S261D, AQP2-S256D-S261A always located at the apical plasma membrane regardless of hormonal stimulation [30, 64].

Time course of vasopressin-dependent AQP2 phosphorylation showed that S256 is the first phosphorylation event occurring after vasopressin stimulation. S261 becomes dephosphorylated with a  $t_{1/2}$  of 10, 6 minutes [11] suggesting that just after few minutes of vasopressin action, AQP2 can be poly-phosphorylated at S256, S261 and S/T269. The temporal succession of phosphorylation at the C-terminal of AQP2 indicates that dephosphorylation of S261 occurs after S/T269 phosphorylation [65]. Thus, dephosphorylation at S261 may promote the association of AQP2 with the endocytotic machinery and accessory proteins such as HSP70. In this study, MCD4 cells were not stimulated with vasopressin or forskolin, nevertheless overexpression of CHIP altered the dynamic of AQP2 phosphorylations that probably generates a specific phospho-isoform of AQP2, which is preferentially targeted

to HSP70/CHIP degradative pathway. Unexpectedly, the present data reveal that CHIP is not directly committed to reduce AQP2 abundance since deletion of the catalytic domain, the UBox, did not change AQP2 expression compared to CHIP-wt expressing cells. It may be assumed that CHIP-delUBox can form dimers with endogenous CHIP resulting in the formation of a functional dimeric complex. However, transfection with CHIP-delTPR attenuates the CHIP-induced AQP2 downregulation, underlying the importance of TPR domain which tethers chaperones. It cannot be excluded, however, the existence of a CHIP dependent signaling at the endoplasmic reticulum where HSP70/HSP90 protein folding occurs. Importantly, it has been shown that CHIP can switch HSP70/HSP90 complexes from protein folding to protein degradation [35]. In this regard, HSP70/HSP90 can associate with other E3 ubiquitin ligase proteins such as MDM2. Consistently, co-immunoprecipitation analysis confirms that CHIP-delTPR does not complex with HSP70 and MDM2 which instead is involved in decreasing AQP2 abundance in CHIP-wt cells. MDM2 is the major negative regulator of p53. Several studies, however, demonstrated that MDM2 plays multiple roles in modulating transduction cascades as NF- $\kappa$ B and cAMP/PKA signaling [66, 67]. MDM2 can ubiquitylate beta-arrestin-sequestered PDE4D5 [23, 30, 44, 56], that is involved in controlling vasopressin-regulated water reabsorption. Rolipram, a selective inhibitor of PDE4D5 activated AQP2 shuttle and thus the osmotic water permeability [68]. A likely hypothesis is that MDM2 participates in controlling the cAMP degrading system. On the other hand, deletion of MDM2 in renal tubular cells associates with strong kidney injury and extensive tubular dilation [67]. Interestingly, renal tubular dysfunctions were also observed in AQP2-null mice and it was postulated that this complex phenotype might be associated with a defect in the interaction between AQP2 and integrins [69]. Whether MDM2 controls this interaction remains to be explored.

To conclude, we report here that expression of CHIP increased proteasomal degradation of AQP2 (Fig. 14). This event occurs through the inhibition of HSP90, the upregulation of ERK/HSP70, which binds the AQP2, when it is simultaneously phosphorylated at S256 and dephosphorylated at S261. Moreover, the present data indicate that HSP70 tethers the E3 ligase MDM2 which is directly involved in the AQP2 degradation. These data might contribute to better understand the molecular mechanisms underlying the actions of molecules or hormones such as ATP and dopamine, counteracting vasopressin response and targeting AQP2 for degradation [30]. Indeed, these findings might be of clinical relevance to human diseases characterized by overexpression or abnormal stimulation of CHIP signaling [70].

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## Disclosure Statement

The authors declare not to have any conflicts of interest disclosure

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