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UBXN2A regulates nicotinic receptor degradation by modulating the E3 ligase activity of CHIP

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) containing the α3 subunit are known for their prominent role in normal ganglionic transmission while their involvement in the mechanisms underlying nicotine addiction and smoking-related disease has been emerging only in recent years. The amount of information available on the maturation and trafficking of α3-containing nAChRs is limited. We previously showed that UBXN2A is a p97 adaptor protein that facilitates the maturation and trafficking of α3-containing nAChRs. Further investigation of the mechanisms of UBXN2A actions revealed that the protein interacts with CHIP (carboxyl terminus of Hsc70 interacting protein), whose ubiquitin E3 ligase activity regulates the degradation of several disease-related proteins. We show that CHIP displays E3 ligase activity toward the α3 nAChR subunit and contributes to its ubiquitination and subsequent degradation. UBXN2A interferes with CHIP-mediated ubiquitination of α3 and protects the nicotinic receptor subunit from endoplasmic reticulum associated degradation (ERAD). UBXN2A also cross-talks with VCP/p97 and HSC70/HSP70 proteins in a complex where α3 is likely to be targeted by CHIP. Overall, we identify CHIP as an E3 ligase for α3 and UBXN2A as a protein that may efficiently regulate the stability of CHIP's client substrates.

Keywords

α3 Nicotinic subunit; CHIP; p97; UBXN2A; UBX domain; Proteasomal degradation

1. Introduction

Smoking is a worldwide problem that causes more than five million deaths each year [1]. The major addictive component in tobacco is nicotine, which binds to a broad population of nicotinic acetylcholine receptors (nAChRs) expressed in both the central and peripheral

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nervous systems and in many other tissues and organs [2]. Neuronal nAChRs are homo- or hetero-pentameric ligand-gated ion channels composed of α and β or α only-subunits [3]. Nicotine's ability to affect the properties and abundance of nAChRs contributes to the mechanisms behind the addictive properties of tobacco products. The α3 nAChR subunit is encoded by *CHRNA3,* which is part of the *CHRNA5–CHRNA3–CHRNB4* gene cluster located on chromosome 15 in humans and chromosome 9 in mice [4]. Single nucleotide polymorphisms in that gene cluster are associated with various aspects of nicotine dependence as well as cigarette-related health issues, including lung cancer, chronic obstructive pulmonary disease and peripheral arterial disease [5–12]. The levels of α5/α3/β4-containing nAChRs at the plasma membrane are highly regulated and may be different in disease vs. physiological conditions [13–14]. Overall, the information available of the mechanisms of α3-containing nAChR trafficking is limited. An early study showed that the long cytoplasmic loop of α3 – but not α5 – is important for the targeting of this nAChR subtype to the synapse during in vivo synapse formation [15]. Given the prominent role of the nAChR subunits encoded by *CHRNA5–CHRNA3–CHRNB4* in multiple aspects of dependence from nicotine and other addictive drugs, a better understanding of the principles that regulate the trafficking and maturation of α3-containing nAChRs can eventually shed light on the functional consequences of disease-related gene polymorphisms and guide pharmacotherapeutic approaches.

UBXN2A (also known as UBXD4) belongs to the UBXD family of proteins that contains the ubiquitin regulatory domain X (UBX) [16–18]. It was first detected by mass spectrometry techniques in p97 immunoprecipitates [16]. The p97 complex provides the driving force for the retro translocation of misfolded proteins from the ER to the cytoplasm [19–22]. p97 can recruit and bind many other regulatory proteins, including E3 ubiquitin ligases, isomerases, and co-chaperones [16]. Binding of UBXD family members to the p97 platform regulates the interaction between p97 and those numerous protein partners [23–26]. For example, UBXN7 (UBXD7) regulates the interactions between p97, the ubiquitin ligase CUL2, and one of CUL2 substrates, hypoxia-inducible factor 1 (HIF1) to affect HIF1 turnover [16]. UBXN1 (UBXD1), by regulating the binding of p97 to the adaptor protein Ufd1, affects the degradation of mutated CFTR [27]. Hence, based on the arrangement of their functional domains, UBXN proteins may provide spatial and temporal dimensions to p97 activity that result in either positive or negative modulation of ERAD function [17,28–32].

Our lab has demonstrated that UBXN2A can interfere with the proteasomal degradation of the α3 nAChR subunit by preventing its ubiquitination. The phenomenon leads to increased availability of α3 nAChR subunits for assembly into pentameric receptors that can be trafficked to the plasma membrane [18]. The ubiquitination process is conducted by a series of enzymatic processes involving a number of UPS-related enzymes, including E1, E2, and E3 ligases [33]. To date, which E3 ligase participates in the ubiquitination of the nAChR subunits encoded by the *CHRNA5–CHRNA3–CHRNB4* gene cluster is unknown.

CHIP is a co-chaperone of the 70-kDa molecular chaperones heat shock cognate 70 (Hsc70) and heat shock protein 70 (Hsp70) [34,35]. In addition to its co-chaperone function, CHIP displays intrinsic E3 ubiquitin ligase activity. This activity may either depend on, or be

independent from the molecular chaperones Hsp70/Hsp90, which assist in the loading of client proteins, including membrane proteins, to the UPS machinery [36,37]. Affinity-capture western blotting revealed that CHIP and Hsc70/Hsp70 associate with the p97 complex [38]. The present work shows that CHIP displays E3 ligase activity toward the α3 nAChR subunit and is found in a protein complex containing p97, α3, and UBXN2A. We also show that UBXN2A-mediated interference with CHIP's E3 ligase activity protects α3 from ERAD.

2. Materials and methods

2.1. Antibodies and reagents

The sources and the dilution of the various antibodies and reagents used in the present studies are listed in Table 1.

2.2. Plasmid constructs

Wild type (WT) and four truncated UBXN2A (WT: 1-259aa; ΔC: 1-196aa; SEP: 1-151aa; Linker: 152-196aa; UBX: 167-259aa) cDNAs were cloned into the pcDNA 3.1(Z+) vector with $(His)_6$ and TYG peptides tagged at the N-terminal. Primers pairs used for PCR are as listed below: WT (F1, R259), ΔC (F1, R196), SEP (F1, R151), Linker (F152, R196), UBX (F167, R259). The sequences of primers are listed in Table 2. $(His)_6$-tagged WT CHIP and its mutant H260Q cloned in pcDNA 3.1 vector were kindly provided by Dr. Tony Eissa (Baylor College of Medicine). shRNA against UBXN2A (shUBXN2A) and control, scramble shRNA in pSuper vector were designed as previously described [18].

2.3. Yeast two-hybrid screens

A bait encoding full length mouse UBXN2A cDNA was amplified by PCR and cloned into the GAL4 DNA binding expression vector pGBKT7 DNA-BD (Clontech). The bait was used to screen a mouse whole-brain cDNA library constructed in a pACT2 DNA-AD vector pre-transformed into the yeast strain Y187 (Clontech). The Y2H process was performed according to the manufacturer's instructions (Clontech).

2.4. Cell culture and transfection

PC12 cells (ATCC) and HEK293T cells (ATCC) were cultured as described previously [18]. Depending on the experiments, PC12 cells were used either in the undifferentiated or the differentiated state under NGF treatment. PC12 cells (70–75% confluent) were transiently transfected with expression plasmids using Lipofectamine 2000 (Invitrogen). To examine the effect of CHIP E3 ligase on α3 nAChR stability, differentiated PC12 cells were co-transfected with pcDNA-α3 expression plasmids (2 μg) and different amounts of pcDNA-$(His)_6$-CHIP plasmids (0, 0.4, 0.8, 1.6 μg). 48 h after transfection, cells were harvested and cell lysates were used for the desired assays.

Primary rat cortical neuron cultures were derived from the cortices of E18 Long-Evans rats. Cortex were dissected in Hank's saline and dissociated by trypsinization followed by mechanical dissociation. Cell suspensions were plated in serum-free Neuro-basal media with

B27 supplement (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml), and 2 mM glutamine on laminin- and poly-D-lysine treated coverslips.

2.5. Drug treatments

For p97 inhibition experiments, transfected, differentiated PC12 cells (48 h after transfection) were treated with DBeQ at a concentration of either 5 or 10 μM for 3 h. DMSO was used as vehicle treatment. Cells were then harvested for Western blotting analysis. To enhance E3 ligase–substrate interactions, differentiated PC12 cells were treated with the proteasomal inhibitor MG132 (10 μM) for 12 h before harvesting cells. Such MG132 concentration was previously reported by Wójcik et al. [39] to efficiently inhibit the proteasome complex at the ERAD level, leading to accumulation of substrates regardless of the status of the p97/E3 ligases complex. This strategy allowed us to monitor the α3 nicotinic subunit and its association with CHIP after its extraction from the ER. This nAChR population is different from the α3 population targeted by p97 at the ER level.

2.6. Animals and tissue harvesting

Male C57BL/6J mice (250–350 g) were anesthetized with a cocktail of xylazine, acepromazine, and ketamine, followed by decapitation under anesthesia. The prefrontal cortex was isolated in ice-cold PBS. Tissue homogenization was carried out in 500 μl of ice-cold homogenization buffer (0.32 M Sucrose, 10 mM HEPES buffer and 2 mM EDTA, pH 7.4) containing 1% protease inhibitor cocktail (Sigma). Homogenized tissues were solubilized by sonication and supernatants were used for Western blot analysis or immunoprecipitation assays. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with federal guidelines. Whole-brain samples from CHIP −/− mice and control wild-type littermates were kindly provided by Dr. Tony Eissa's lab (Baylor College of Medicine).

2.7. Immunoprecipitation and Western blot

Cells were lysed by sonication and harvested with lysis buffer containing Tris–HCl 20 mM, pH 7.2, EDTA 1 mM, NaN_3 1 mM, β-mercaptoethanol 1 mM, NP40 (0.1% v/v), glycerol (10% v/v) and 1% of tissue extracts protease inhibitor cocktail (Sigma). Cell extracts utilized in Western blot analyses or immunoprecipitation experiments were normalized for equal loading using a BCA Protein Assay Kit (Pierce Biotechnology). Immunoprecipitation experiments were conducted following a previously described protocol [18]. Immunoprecipitates were examined by SDS-PAGE followed by Western Blot analysis using the appropriate anti-bodies. In some experiments, we used p62 immobilized on agarose beads to enrich ubiquitinated proteins for WB analysis as previously described [18].

2.8. TALON metal pull down assay

HEK293T cells transiently cotransfected with $(His)_6$-empty vector and HA-UBXN2A or $(His)_6$-CHIP and HA-UBXN2A were harvested in lysis buffer 24 h after transfection. Cell lysates were subjected to pull down with TALON Cobalt affinity beads according to the manufacturer's protocol (Clontech). The pulled down proteins were analyzed with SDS-PAGE followed by Western blotting analysis with anti-His and anti-HA antibodies.

2.9. Iodixanol gradient analysis

The prefrontal cortex tissue from four C57BL/6J mice was homogenized in HEPES buffer containing 0.5% NP-40 (0.32 M sucrose,10 mM HEPES buffer, 2 mM EDTA, pH 7.4). After sonication, the samples were centrifuged at 1500 × *g* for 15 min and the supernatants were loaded onto the top of preformed, precooled iodixanol Optiprep gradient columns for linear iodixanol gradient fractionation at 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30% [40]. Samples were then centrifuged at 32,000 rpm for 18 h using a Beckman SW41i rotor at 4 °C. Twenty sequential, equal-volume fractions (500 μl) were collected from top to bottom with a Labconco Auto Densi-flow gradient collector (Labconco Corporation). Each fraction was analyzed by Western blotting for UBXN2A distribution. KDEL and GM130 were examined as ER/Golgi marker.

2.10. Confocal microscopy

Rat cortical neurons cultured on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% NP-40, followed by block with 10% donkey serum before overnight incubation at 4 °C with primary antibodies (UBXN2A/MAP2/neurofascin). Subsequently, neurons were incubated with fluorescence conjugated secondary antibodies for 1 h at room temperature (Donkey anti-rabbit Alexa 488 green, Donkey anti mouse Cy3 red and Donkey anti chicken blue). Nuclei were stained with 0.1% DAPI for 5 min. Cells were imaged using AxioVision confocal microscope (Carl Zeiss) at 63× with 10× objective lens.

2.11. Densitometric quantification of Western blot bands

Quantification of the intensity of Western blot bands was carried out using an automated digitizing system (UN-Scan-it gel, version 6.1). The digitized gel data (pixel total) were used to calculate the relative optical densities of each gel band for further statistical analysis.

2.12. Statistics

Statistical analysis was conducted using either the Student’s t test for normally distributed variables to determine the significant differences between control and treatments or by one-way ANOVA with Newman–Keuls post hoc tests, when appropriate. A p value of 0.05 or less was considered statistically significant. All data are reported as mean ± SEM.

3. Results

3.1. UBXN2A interacts with the E3 ligase CHIP

UBXN2A was originally isolated as a protein partner of the a3 nAChR subunit from a Y2H screen [18]. To further characterize the function of UBXN2A, we conducted an additional Y2H screen using full length UBXN2A cDNA as bait to screen a mouse brain cDNA library. The screen yielded several positive clones based on nutritional/enzymatic selection. The sequence of one of the positive clones matched that of the TPR motif of the E3 ubiquitin ligase CHIP (Fig. 1a). The physical interaction between UBXN2A and CHIP was confirmed by pull down experiments in HEK293T cells overexpressing HA-tagged UBXN2A and $(His)_6$ peptide-tagged CHIP (Fig. 1b).

3.2. CHIP regulates the ubiquitination and degradation of α3

Because we had previously shown that UBXN2A interferes with the proteasomal degradation of α3 [18], we explored the possibility of CHIP displaying E3 ligase activity toward α3. To test this hypothesis, we first examined the levels of ubiquitinated α3 in differentiated PC12 cells overexpressing CHIP or control, empty vector. Panel c in Fig. 1 shows that CHIP overexpression in this cell line significantly increased the levels of ubiquitinated α3, suggesting that CHIP ubiquitinates α3. We next examined the levels of ubiquitinated α3 in brain homogenates from control and CHIP−/− mice, and found significantly reduced levels of ubiquitinated α3 in the brain of CHIP−/− mice compared to their wild type littermates (Fig. 1d).

Because polyubiquitination by E3 ligase enzymes leads to proteasomal degradation of the substrate, we next investigated whether α3 ubiquitination by CHIP leads to increased α3 degradation. α3 and CHIP were co-transfected in differentiated PC12 cells with different amounts of plasmid encoding for $(His)_6$-CHIP and fixed amount of plasmid encoding for α3. Empty vectors were used to normalize all transfections for total amount of DNA. 48 h after transfection, the levels of α3 protein decreased with increased expression of $(His)_6$-CHIP in a dose-dependent manner (Fig. 2a). To determine whether the reduction in the levels of detected α3 protein depends on CHIP's E3 ubiquitin ligase activity, we examined the effect of the CHIP H260Q mutanton α3. The H260Q mutationabolishes the interaction of CHIPwith E2enzymes, thereby eliminating CHIP's E3 ligase activity [41]. Panel b in Fig. 2 shows that overexpression of H260Q CHIP did not significantly alter α3 levels, confirming that CHIP regulates α3 stability via its E3 ligase activity. To further validate the CHIP overexpression data, we examined α3 levels in brain homogenates from four pairs of CHIP−/− and wild typemice and found that α3 protein levels were consistently higher in CHIP−/− mice than in wild type, control mice (Fig. 2c). Based on the data in Figs. 1 and 2, we can conclude that CHIP is an E3 ligase that regulates the ubiquitination and degradation of α3 nAChR subunits. To further ascertain the specificity of CHIP's effects on α3, we also examined two other receptor proteins in CHIP−/− mice. Our data indicate that the levels of ubiquitinated β2, another nAChR subunit, were not changed in the same mouse brain lysates, suggesting that the detected CHIP ubiquitination activity toward the α3 nAChR subunit is specific (Fig. 2d). Moreover, the total protein levels of β2 subunit (Fig. 2e) and $GABA_A$ receptors (Fig. 2f) did not significantly change in brain homogenates of CHIP−/− vs. wild type littermates.

3.3. UBXN2A interferes with CHIP-mediated ubiquitination and degradation of α3

Given the seemingly opposite effects of CHIP and UBXN2A on the stability of α3, we investigated whether and how UBXN2A interferes with CHIP's ubiquitination activity over α3. First, CHIP/α3 interactions were examined by immunoprecipitation experiments conducted with anti-CHIP antibodies to precipitate α3 from lysates of differentiated PC12 cells in which UBXN2A was either overexpressed or knocked down. The proteasome inhibitor MG132 was added to the cell cultures to block the degradation of ubiquitinated proteins modified by E3 ligases. Western blot analyses showed that the amount of α3 co-precipitated with CHIP decreases when UBXN2A is overexpressed (Fig 3a and c). Conversely, greater amounts of α3 co-immunoprecipitate with CHIP when endogenous

UBXN2A expression is reduced by UBXN2A shRNA (Fig. 3b and c), suggesting that UBXN2A destabilizes the interaction between CHIP and α3.

To test the hypothesis that UBXN2A negatively regulates α3 ubiquitination by CHIP, we overexpressed CHIP+empty vector or CHIP+UBXN2A in differentiated PC12 cells. HA-tagged ubiquitin was cotransfected to facilitate the detection of ubiquitinated proteins. Panel d of Fig. 3 shows that CHIP overexpression increased the levels of ubiquitinated α3, but co-expression of UBXN2A with CHIP attenuated the phenomenon, indicating that UBXN2A interferes with CHIP's ubiquitination toward α3. The same cell lysates were examined for total levels of α3 and the results confirmed that, even in this system, CHIP overexpression decreases α3 protein levels, while overexpression of UBXN2A together with CHIP prevents CHIP-mediated degradation of α3 (Fig. 3e).

3.4. UBXN2A and CHIP oppositely regulate α3 at the ERAD level and UBXN2A promotes the formation of a p97/α3 complex

The UBXN protein family is one group of p97 cofactors that, together with p97 and other protein partners, regulate the ubiquitination state of many ERAD substrates [28,42–44]. It has been shown that muscle-type nAChRs are subjected to ERAD regulation during their biosynthetic process [45] and neuronal nAChRs might be regulated through a similar pathway. Therefore, we postulated that UBXN2A, as a p97 cofactor, and CHIP, as an E3 ligase for α3, could co-regulate the abundance of α3 at the ERAD level. We used anti-CHIP antibodies to immunoprecipitate CHIP protein interacting complexes from differentiated PC12 cell lysates and examined whether those complexes contain UBXN2A, α3, andthe ERAD component, p97. As shown in Fig. 4a, we found that those three proteins can be co-immunoprecipitated with CHIP, suggesting that they may be present in one complex. This result was confirmed by a reciprocal immunoprecipitation experiment with anti-p97 antibodies that also demonstrated that UBXN2A, CHIP and α3 can be detected in p97 immunoprecipitates (Fig. 4b).

To further confirm that CHIP requires p97 to regulate α3 at the ERAD level, we treated CHIP-transfected, differentiated PC12 cells with DBeQ, a p97 inhibitor [46], and examined whether CHIP can affect α3 levels when p97 is inhibited. As expected, p97 inhibition by DBeQ treatment blocked CHIP-mediated degradation of α3 (Fig. 4c), suggesting that CHIP regulates α3 stability at the ERAD level.

We finally investigated whether UBXN2A is critical for the formation of the p97/α3 complex by examining p97/α3 interactions in differentiated PC12 cells transfected with UBXN2A shRNA. Fig. 4d shows that UBXN2A knock down (verified as in panel 4e) significantly decreases the interaction between p97 and α3, and suggests that UBXN2A is necessary for the interaction of α3 with the p97 platform.

3.5. UBXN2A and p97 are associated with a proteasome complex at the ER–Golgi intermediate space

We originally reported that in PC12 cells, UBXN2A is enriched at the ER/Golgi compartments [18]. To further characterize the subcellular localization of UBXN2A in neuronal tissues, prefrontal cortex homogenates from C57BL/6J mice were subjected to a 9-

layer iodixanol gradient centrifugation followed by collection of 20 fractions from the top to the bottom of the column [40]. Collected fractions were analyzed by Western blotting analysis. Fig. 5a shows that UBXN2A co-sediments with the ER marker KDEL and the *cis*-Golgi marker GM130, suggesting that in neuronal cells, UBXN2A is mainly localized at the ER/Golgi compartments, similar to the enriched localization of p97 near the ER lumen [47]. To determine whether UBXN2A distributes in additional cytoplasmic compartments in neurons, we conducted another biochemical fractionation experiment that allows the separation of proteins from different membrane compartments such as ER/Golgi, synaptosomal and vesicular membrane compartments based on different centrifugation speeds [48,49]. The separation procedure is illustrated in the diagram shown in Fig. 5b. Our results confirmed that UBXN2A is enriched in ER/Golgi fractions in neuronal cells, but not in vesicular membrane or synaptosomal membrane fractions (Fig. 5b, left panel). The fractionation data were corroborated by immunoprecipitation experiments in neuronal cell lysates with antibodies against ERGIC53 (Fig. 5c). ERGIC53 is a protein known to cycle between the ER, ERGIC, and Golgi, that was recently shown to interact with p97 and UBXD1, another member of the UBXD family of proteins [50]. Finally, immunofluorescence staining of neurons revealed that UBXN2A mainly distributes in neuronal somata and some specific regions such as dendritic bifurcations and the axon initial segment (Fig. 5d). The function of UBXN2A at the latter two sites remains unknown. Enriched staining of UBXN2A near the nucleus is consistent with its ER/Golgi compartmentalization.

The p97 complex interacts with several members of the ubiquitin superfamily to control proteasomal degradation at the ERAD level in a protein-specific, temporally regulated fashion [51]. Experiments were conducted on 26S proteasomes purified from the prefrontal cortex of C57Bl/6J to examine whether UBXN2A and p97 are associated with the proteasomal complex [52]. Fig. 5e indicates that both p97 and UBXN2A can be detected in partially purified proteasomes, while GM130, a Golgi marker protein used as control, cannot be detected in such precipitates. These data suggest that the detected association of UBXN2A and p97 with the proteasome complex is specific. Overall, these data validate the association of UBXN2A with the p97 complex next to the ER lumen [53] and its regulatory role within the p97-containing complex [54].

3.6. UBXN2A interacts with p97 via the UBX domain while the interaction with α3 requires the SEP domain

Pull down experiments showed that His-tagged UBXN2A can pull down endogenous p97 from mouse prefrontal cortex homogenates (Fig. 6a). To determine which UBXN2A domain is engaged in the UBXN2A/p97 interaction, we generated constructs expressing either full length or truncated UBXN2A forms. Constructs containing cDNA for the SEP (Shp, eye-closed, p47) domain, the UBX domain, or the linker between the SEP and the UBX domains were cloned into the pcDNA 3.1 vector with $(His)_6$ and a TYG peptide tags at N-terminals (Fig. 6b). Constructs were expressed in PC12 cells and the cell lysates were subjected to pull down experiments to examine the interaction between WT/truncated UBXN2A and p97. The results show that only WT UBXN2A and the truncated form containing the UBX domain are able to pull down p97 from the PC12 cell lysates (Fig. 6c). Hence, the UBX domain is

necessary and sufficient for the interaction between p97 and UBXN2A. To further examine which domain of UBXN2A is responsible for the interaction with α3, we conducted immunoprecipitation experiments with anti-TYG antibodies in the same cells lysates used for the experiment in Fig. 6c and examined the presence of α3 in the TYG-UBXN2A complex. The results show that the SEP domain of UBXN2A is necessary for the interaction with α3 (Fig. 6d).

4. Discussion

The present work examined the regulation of α3 nAChR ubiquitination by UBXN2A, a member of the largest group of p97 cofactors [16,17]. p97, a homohexameric member of the AAA ATPase family, is regulated by a large number of cofactors that coordinate the recruitment of specific, typically ubiquitinated substrates, to the p97 complex. UBXN1, UBXN7, and UBXN8 have been shown to interact with the p97 platform through their UBX domain [16]. In general, UBX domains are thought to interact with the N-terminal domain of p97 and we were able to confirm that UBXN2A, similar to most other members of the UBXN family, uses the UBX domain for the interaction with p97. In addition, we showed that the SEP domain is required for the interaction with one of its client proteins, the α3 nAChR subunit.

The interaction of p97 with its cofactors controls the fate of the substrate, often by influencing its ubiquitination state. Such interactions either channel the substrates for degradation or extract deubiquitinated substrates to the cytosol [17,55–57]. Y2H experiments gave the first clue that CHIP might be a partner of UBXN2A and a series of studies established that CHIP, UBXN2A and α3 can be found in the same protein complex. The role of UBXN2A in such complex was revealed by the experiments showing that UBXN2A interferes with CHIP's ability to ubiquitinate α3 by affecting the interaction of the nAChR subunit with the E3 ligase. The fact that p97 is also present in the macromolecular complex containing UBXN2A, CHIP, and α3, and that the proteasome is also found in that complex (see also [58,59]), strongly suggests that UBXN2A regulates CHIP's activity on its substrates at the interface between p97 and the UPS. CHIP is a cytosolic protein implicated in the degradation of a variety of chaperone-bound proteins [35,58,60–62] and therefore it is conceivable that CHIP/UBXN2A interactions occur while α3 is being extracted from the ER into the cytosol by p97. This notion is supported by the fact that CHIP-mediated α3 degradation can be blocked by the p97 inhibitor DBeQ. Other – yet unidentified – E3 ligases, which likely reside in the ER, are also expected to participate in α3 ERAD. One example of such cooperative action is provided by the sequential ubiquitination of ER-anchored hemoproteins by ER-resident ubiquitin ligases and CHIP [63,64]. Multiple rounds of ubiquitination are probably required for extraction from the ER and following proteasomal degradation [65]. A first round of ubiquitination is necessary for recognition by, and binding to the p97 complex [66]. Partial de-ubiquitination by p97-associated deubiquitinating enzymes (DUBs) may be necessary for threading of the substrate into the central pore of the p97 complex and dislocation from the ER [67]. The extracted protein subsequently undergoes a second round of ubiquitination (by either ER-associated or cytosolic E3 ligases) followed by proteasomal degradation [67,68]. Because UBXN2A overexpression reduces the interaction of CHIP for α3 and UBXN2A knockdown reduces

the interaction of α3 for p97, UBXN2A seems to have a definite role in the steps that lead to proteasomal degradation after ER extraction (Fig. 7). At the same time, we cannot rule out the involvement of UBXN2A in other ERAD mechanisms. For example, UBXN2A could also interact with ER-associated E3 ligases or prevent recruitment of additional adaptor proteins necessary for ER extraction. While interfering with CHIP's ligase activity, UBXN2A could also recruit de-ubiquitinating enzymes to trim ubiquitin off α3. That would also result in a reduction of α3 ubiquitination and proteasomal degradation. There are other known proteins that inhibit ERAD. For example, SVIP (small p97/VCP-interacting protein) functions as an endogenous inhibitor of ERAD by uncoupling p97 from the retrotranslocation channel, Derlin 1, and by inhibiting substrate targeting to the E3 ligase gp78. Similar to UBXN2A, SVIP is found in a protein complex containing a ubiquitin ligase (gp78) and p97, and its overexpression leads to decreased ubiquitination and degradation of the client protein CD3δ. SVIP's mechanism of action seems to depend on the competition for the binding to p97, as both the E3 ligase gp78 and SVIP contain a p97/VCP-interacting motif (VIM) [69]. The UBX/UBA-containing protein SAKS1 also inhibits ERAD and p97-dependent degradation by binding ubiquitinated substrates and protecting them from the DUB activity necessary for efficient ERAD [28].

Other proteins have been shown to interfere with CHIP's E3 ligase activity toward its substrates, including two members of the BAG (BCL2-associated athanogene) family of proteins, BAG-2 and BAG-5 [70,71], and HspB1 [72]. However, to our knowledge, UBXN2A is the first member of its family to be found to interfere with CHIP-dependent ubiquitination.

Besides identifying the first E3 ligase for a neuronal nAChR subunit and providing information on nAChR regulation at the ERAD, our work shows that UBXN2A binds to CHIP. It has been reported that the ubiquitin ligase activity of CHIP, through autoubiquitination, leads to conformational changes that alter its affinity to its partner proteins [73]. Hence, this discovery has broad implications as UBXN2A mechanisms of action may impinge on the degradation of other CHIP's substrates. CHIP E3 ligase is involved in the turnover of many disease-related proteins, including Alzheimer's disease-related protein tau and APP; the breast cancer-related protein, ErbB2; and tumor suppressor PTEN and p53 [74–77]. UBXN2A might interfere with the degradation of those proteins and could become a target of future research related to the pathophysiology of several human disorders.

In summary, UBXN2A emerges as the first UBX-containing protein that negatively regulates ERAD by inhibiting substrate targeting to the E3 ligase CHIP. Originally, our lab identified UBXN2A as a protein partner of the α3 nAChR subunit that can positively regulate plasma membrane levels and stability of α3-containing nAChRs [18]. The present work provides significant insight into how UBXN2A affects that process and opens the door to research into the potential differences in the trafficking of α3 proteins encoded by disease-associated CHRNA3 gene variants. Such type of information isimportant as nAChRs comprising the α3 subunit have been implicated in several human disorders, including drug addiction [78–84], Alzheimer's disease [85–87], lung cancer [88–91], chronic obstructive pulmonary disease (COPD) [12,15,92–94] and hypertension in smokers [95]. Furthermore,

the rare disorder, megacystis, microcolon, intestinal hypoperistalsis syndrome (MMIHS), which is associated with mutations in the ACTG2 gene, encoding gamma-2 smooth muscle actin [96], is also accompanied by severely reduced levels of α3-containing receptors [97]. Given that mice null for the α3 nAChR subunit display several symptoms observed in MMIHS [98], it is tempting to speculate that UBXN2A-related mechanisms might also contribute to this disease. [96]

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Abbreviations

GABA	gamma-aminobutyric acid receptor
HSC70	70-kDa heat shock cognate protein
HSP70	70 kDA heat shock protein
nAChRs	nicotinic acetylcholine receptors
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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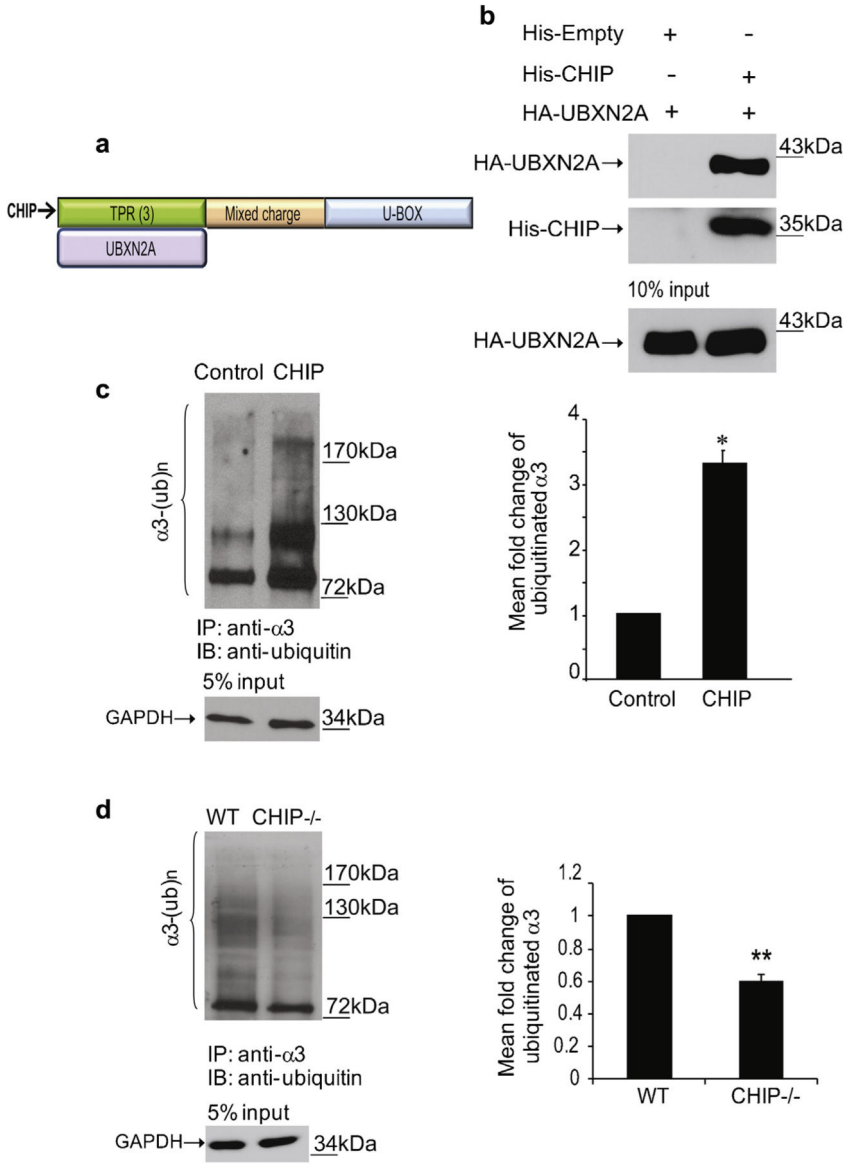

Fig. 1.

UBXN2A interacts with the E3 ligase CHIP, and CHIP mediates α3 nAChR subunits ubiquitination. (a) A Y2H screen using full length mouse UBXN2A cDNA as bait revealed that UBXN2A interacts with the tetratricopeptide repeat (TPR) domain of CHIP. The TPR domain mediates CHIP–Hsc70–Hsp70 and Hsp90 interaction whereas two other domains of CHIP i.e., U-box and mixed charge with predicted coiled-coil region mediate the E3 ubiquitin ligase and dimerization activities of CHIP, respectively. A set of nutritional selection and β-GAL assays were performed to confirm the identified interactions. The cartoon shows the location of CHIP's domains and where UBXN2A binds to CHIP according to the Y2H screen. (b) HA-UBXN2A can be detected in the $(His)_6$-CHIP samples pulled down from lysates of HEK293 cells co-transfected with His-CHIP and HA-UBXN2A, but not in the pull-down samples from lysates of HEK cells co-transfected with HA-UBXN2A and $(His)_6$-empty vector. (c) Differentiated PC12 cells were homogenized

and subjected to immunoprecipitation with anti-α3 antibodies followed by SDS-PAGE and immunoblotting with anti-ubiquitin antibodies. These experiments showed that CHIP overexpression (right lane, left panel) significantly increases the levels of ubiquitinated α3 in this cell culture system ($n = 3$ separate cultures, $*p < 0.02$). (d) We also examined how lack of CHIP affects α3 ubiquitination in mice null for the E3 ligase and their littermate controls. The levels of ubiquitinated α3 in brain homogenates from CHIP −/− mice were significantly lower than those from wild-type littermates ($n = 5$ mice/group, $**p < 0.001$).

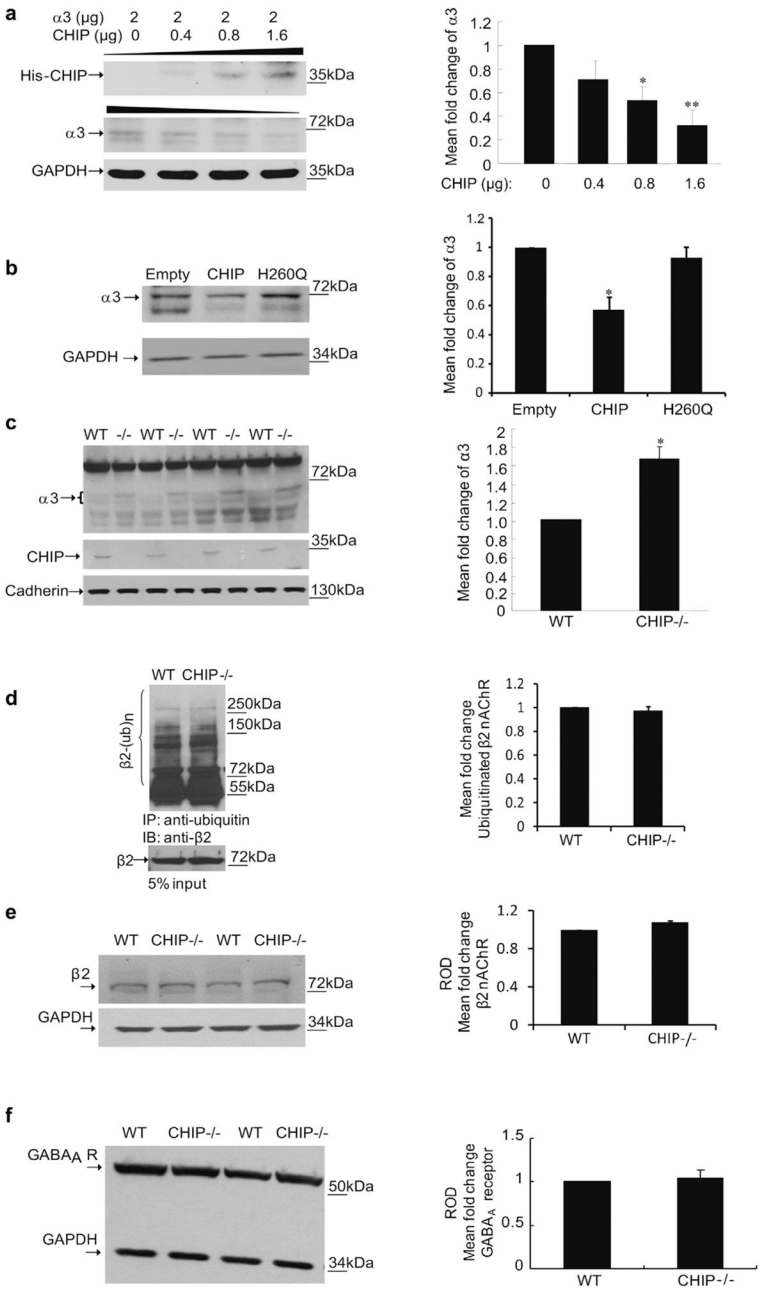

Fig. 2.

CHIP decreases the stability of the α3 nAChR subunit. (a) The levels of α3 protein decreased in correspondence with increasing expression of CHIP in differentiated PC12 cells co-transfected with pcDNA-α3 expression plasmids and different amounts of pcDNA-$(His)_6$-CHIP plasmid ($n = 3$ separate cultures, $*p < 0.02$, $**p < 0.005$). (b) In differentiated PC12 cells, overexpression of the H260Q CHIP mutant, which is devoid of E3 ligase activity, had no significant effect on α3 stability compared to wild-type CHIP overexpression ($n = 4$ separate experiments, $*p \leq 0.02$). (c) The levels of α3 are significantly higher in the brain homogenates of CHIP −/− mice than those of wild type mice ($n = 4$ mice/

group, *p <0.02). (d and e) We also examined the levels of β2 nAChR subunits in brain homogenates of CHIP−/− and wild type mice and found that the levels of both ubiquitinated (panel d, n = 4 mice/group, p > 0.5) and total (panel e, n = 3 mice/group, p > 0.05) β2 protein were not different between the two mouse groups. (f) Finally, as an example of another ligand-gated ion channel, we measured the levels of $GABA_A$ receptor proteins and found no significant changes in control vs. CHIP −/− brains (n = 4 mice/group, p > 0.5). ROD, relative optical density.

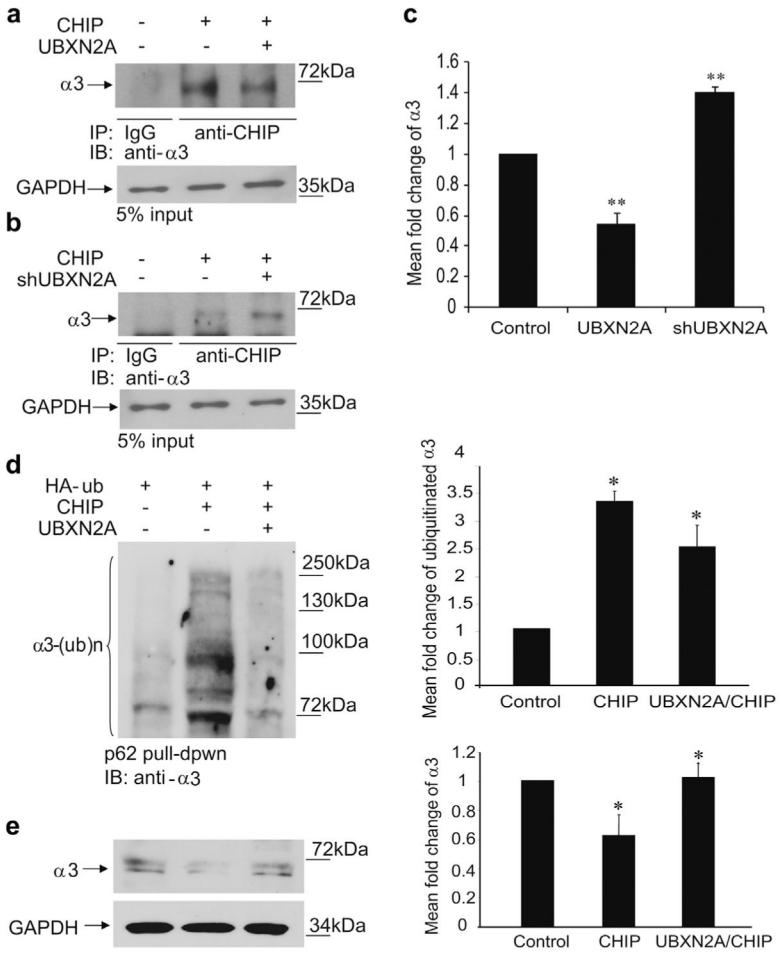

Fig. 3.

UBXN2A affects CHIP/α3 interactions and interferes with CHIP-mediated α3 nAChR ubiquitination and degradation. (a and c). The interaction between CHIP and α3 was investigated in differentiated PC12 cells. PC12 cells were co-transfected with either CHIP +empty vector or CHIP + UBXN2A followed by immunoprecipitation experiments with anti-CHIP antibodies. UBXN2A overexpression weakened α3/CHIP interactionsin differentiated PC12 cells ($n = 3$ separate experiments, $^{**}p < 0.01$). (b and c). Similar immunoprecipitation experiments were conducted to examine the interaction between CHIP and α3 when UBXN2A levels were reduced using UBXN2A shRNA. The interaction between CHIP and α3 was significantly enhanced upon UBXN2A knock-down ($n = 4$ separate experiments, $^{**}p < 0.01$). (d and e) Levels of ubiquitinated α3 were measured in cell lysates of differentiated PC12 cells co-transfected with CHIP, CHIP + UBXN2A or corresponding empty vectors. The experiments revealed that UBXN2A overexpression inhibits CHIP-mediated ubiquitination of α3 (panel d, $n = 4$ separate experiments, $^{*}p < 0.05$ CHIP vs. CHIP + UBXN2A), resulting in higher levels of total α3 protein (panel e, $n = 4$ separate experiments, $^{*}p < 0.05$ CHIP vs. CHIP + UBXN2A.

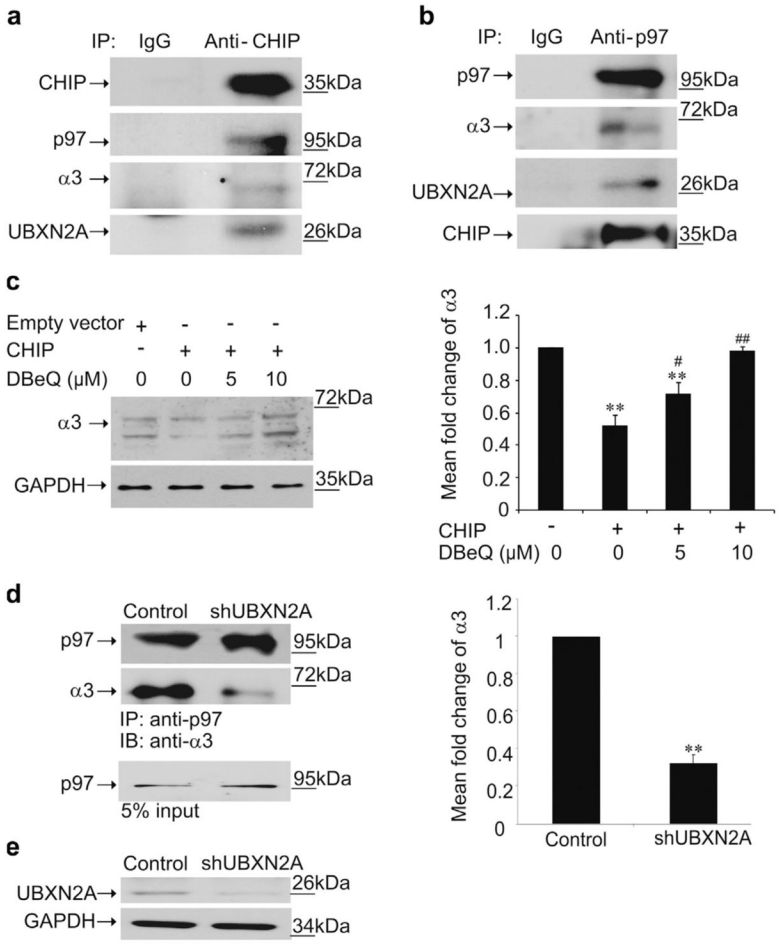

Fig. 4.

UBXN2A is found in a complex with CHIP/p97/α3 and facilitates the formation of p97/α3 complexes. (a) Immunoprecipitation experiments conducted on differentiated PC12 cell lysates using anti-CHIP antibodies revealed the presence of a complex containing CHIP, p97, α3 and UBXN2A. (b) UBXN2A, CHIP and α3 were also detected in p97 immunoprecipitates from differentiated PC12 cell lysates. (c) Differentiated PC12 cells transiently transfected with CHIP were treated with DBeQ, a potent and selective p97 inhibitor, followed by SDS-PAGE and probing with anti-α3 antibodies. The experiments showed that DBeQ treatment significantly blocked CHIP-mediated α3 degradation ($n = 4$ separate experiments, CHIP vs. Control, $^{**}p < 0.001$; CHIP + DBeQ (5 μM) vs. CHIP + no treatment, $\#p < 0.02$, CHIP + DBeQ (10 μM) vs. CHIP + no treatment, ##p <0.001). (d) The amount of α3 that co-immunoprecipitated with p97 was reduced by UBXN2A knockdown ($^{**}p < 0.001$, $n = 3$ separate experiments). (e) UBXN2A expression was effectively reduced in dPC12 cells transfected with pSuper vector-based UBXN2A shRNA. GAPDH was probed as loading control.

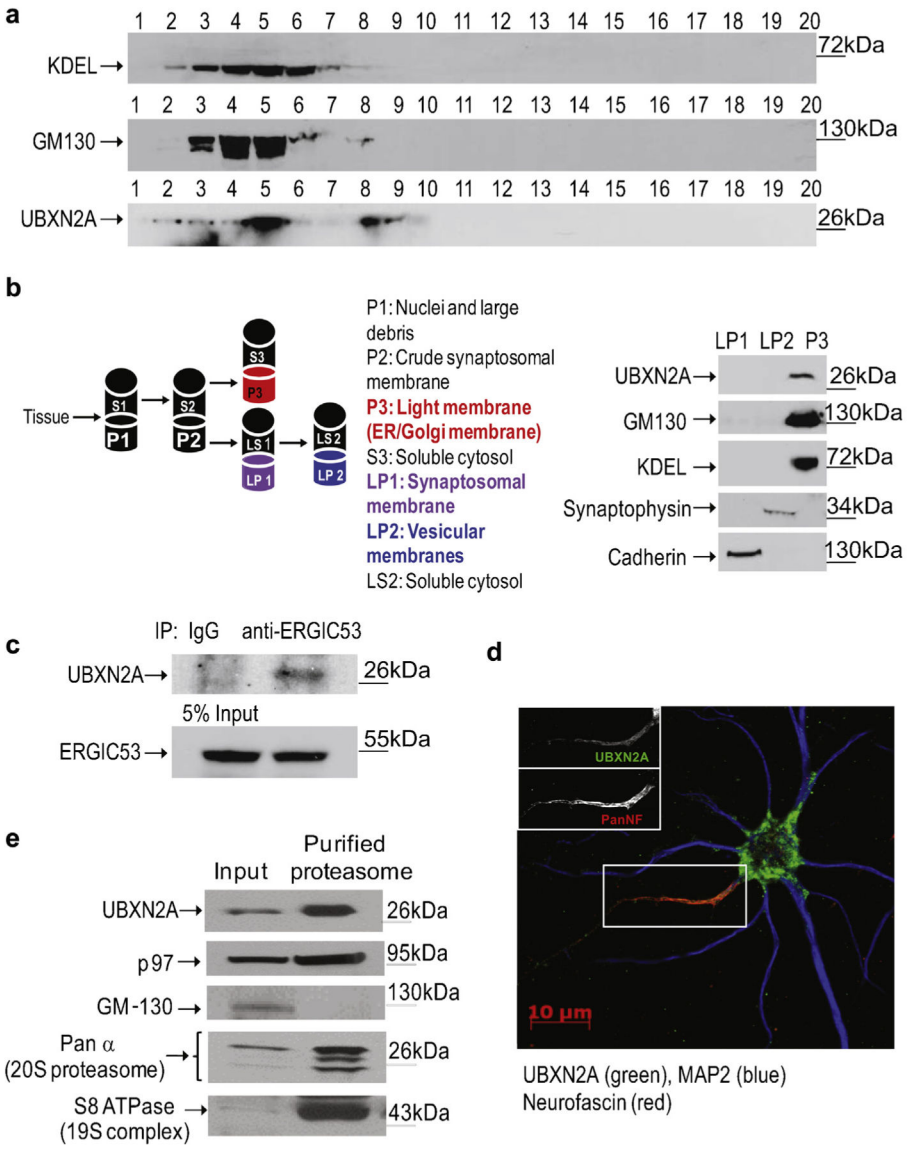

Fig. 5.

UBXN2A/p97 complexes are associated with the proteasome mega complex at the ER–Golgi intermediate space. (a) Fractions of mouse prefrontal cortex homogenates subjected to iodixanol gradient centrifugation were analyzed by Western blot analysis. UBXN2A co-sediments with KDEL (an ER, *cis*-Golgi marker) and GM130 (a *cis*-Golgi marker), suggesting that UBXN2A can be found in ER–Golgi compartments in neuronal tissues. (b) The right panel displays the strategy we used forthe subcellular fractionation of membrane proteins from mouse PFC tissue. That approachfurther showed that UBXN2A is enriched in ER/Golgi membrane fractions, but not in synaptosomal or vesicular membrane fractions (right panel). (c) IP experiments using anti-ERGIC53 antibody showed that UBXN2A co-immunoprecipitates with ERGIC53, indicating that UBXN2A may exist in the ER/Golgi intermediate compartment. (d) Immunofluorescence staining of rat hippocampal neurons revealed that the majority of UBXN2A localizes in the soma while some is present at

dendritic bifurcations and axon initial segments. UBXN2A (green), MAP2 (blue) and neurofascin (red). (e) UBXN2A and p97 associate with the 26S protesome complex purified from mouse prefrontal cortex. Anti-Pan α and anti-S8 ATPase antibodies were used to detect components of the 26S proteasome. The *cis*-Golgi marker, GM130 could not be detected in the purified proteasome sample, confirming the successful isolation of partially purified proteasomal complexes.

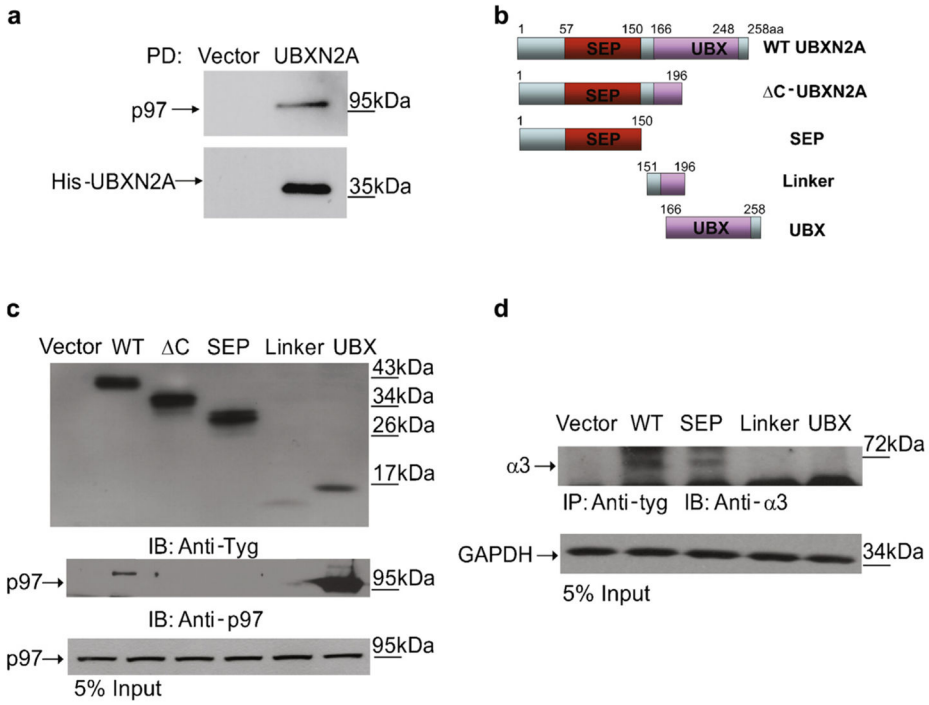

Fig. 6.

UBXN2A interacts with p97 via the UBX domain while it interacts with α3 nAChR via the SEP domain. (a) A pull down experiment conducted on mouse PFC homogenates using in vitro-expressed, $(His)_6$-tagged UBXN2A showed that p97 can be pulled down by UBXN2A. (b) Schematic diagrams of wild type and several truncated UBXN2A cDNAs cloned into a $(His)_6$-Tyg peptide tagged pcDNA 3.1 vector. (c) Talon metal affinity resin pulldown experiments conducted in differentiated PC12 cell lysates expressing each construct listed in panel b showed that only WT UBXN2A and truncated UBXN2A containing the UBX domain can pull down p97. These results confirm that UBXN2A interacts with p97 by UBX domain. (d) Immunoprecipitation experiments conducted with anti-Tyg antibodies from the same cell lysates used for the experiment inpanel c showed that only WT UBXN2A and the SEP domain truncated UBXN2A interact with α3, suggesting that UBXN2A interacts with α3 via the SEP domain.

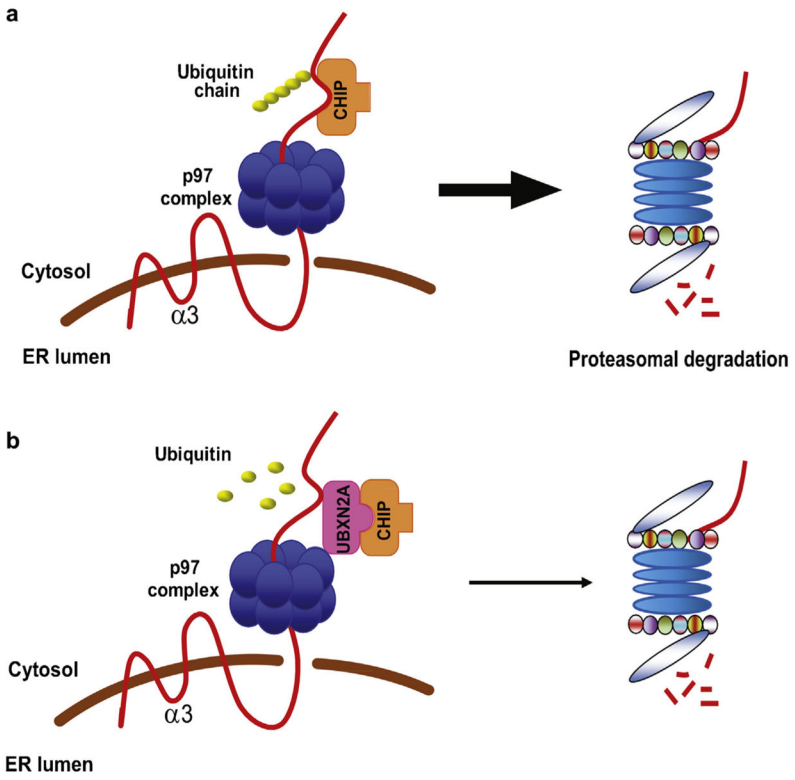

Fig. 7.

UBXN2A alters CHIP-mediated α3 nAChR subunit ubiquitination and ERAD by interfering with CHIP's E3 ligase activity. The cartoondepicts a model for the interaction between CHIP and α3. (a) CHIP, as an E3 ligase and co-chaperone, ubiquitinates the α3 subunit. Upon retro-translocation into the cytosol by the p97 complex, α3 undergoes proteasomal degradation. (b) UBXN2A protects α3 from CHIP-mediated ubiquitination and degradation by binding to CHIP and reducing CHIP's affinity for α3. The result is less ubiquitination and ultimately, less degradation of α3.

Table 1

Antibodies and reagents used in the experiments.

Antibodies	Company	Dilution
Anti-α3 (rabbit)	Santa Cruz Biotechnology	1:200
Anti-CHIP (mouse)	Santa Cruz Biotechnology	1:1000
Anti-p97 (mouse)	Abcam	1:5000
Anti-Pan α1,2,3,5,6 and 7 subunit of 20S proteasome	Biomol	1:1000
Anti-β2 subunit of 20S proteasome	Biomol	1:1000
Anti-cadherin receptor (mouse)	Sigma	1:10000
Anti-HA (mouse)	Invitrogen	1:1000
Anti-KDEL (mouse)	Assay Designs	1:1000
Anti-GM130 (mouse)	BD Biosciences	1:1000
Anti-ERGIC-53 (mouse)	Santa Cruz Biotechnology	1:1000
Anti-TYG (mouse)	Gift from Dr. John Mayer (University of Nottingham)	1:1000
Anti-UBXN2A (rabbit)	Generated by Pacific Immunology	1:500
Anti-ubiquitin	Enzo Life Science	1:1000
Anti-synaptophysin	Provided by Dr. Christian Rosenmund lab (Baylor College of Medicine)	1:5000
Anti-MAP2 (chicken)	Provided by Dr. Matt Rasband lab (Baylor College of Medicine)	1:1000
Anti-neurofascin (mouse)	Provided by Dr. Matt Rasband lab (Baylor College of Medicine)	1:500
Goat anti-mouse IgG HRP	Santa Cruz Biotechnology	1:4000
Mouse anti-rabbit IgG HRP	Santa Cruz Biotechnology	1:4000
Alexa Fluor 488 donkey anti-rabbit IgG	Invitrogen	1:1000
Cy3 552 donkey anti-mouse IgG	Jackson Immunoresearch laboratories	1:1000
AMCA-conjugated goat anti-chicken IgG	Jackson Immunoresearch laboratories	1:1000
MG132	Enzo Life Science	
DBeQ	Sigma-Aldrich	
UBXN2A recombinant protein	Abnova	
Hsp70 (human) recombinant protein	Enzo Life Science	
CHIP (human) recombinant protein	Alexis Biochemicals	
GST-Ubiquitin	Enzo Life Science	
Ubiquitin	Enzo Life Science	
CHRNA3 recombinant protein	Abonova	
Ubiquitin activating enzyme E1	Enzo Life Science	
UbcH5b (human) recombinant	Enzo Life Science	
P62-derived UBA domain, agarose conjugate	Enzo Life Science	

Table 2

Primers used to make full length or truncated forms of UBXN2A.

Primers	Sequences
F1	5′AAG GAA AAA AGC GGC CGC CAT GAA AGA AGT AGA TAA TCT TGA CAG 3′
F152	5′AAG GAA AAA AGC GGC CGC CAA AGC AAA GAG TGT TGA AG 3′
F167	5′AAG GAA AAA AGC GGC CGC CGT TTC ACT GAA CAA CTT GG 3′
R151	5′AAG GAA AAA AGC GGC CGC AGA AAC GAT TCT TGG TGT AG 3′
R196	5′AAG GAA AAA AGC GGC CGC CCT ATG AGA AAC ATT AAA TC 3′
R259	5′AAG GAA AAA AGC GGC CGC TCA AAG TTT TCT AAA AGG CTC GGC AG 3′