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Ubiquitin-mediated internalization of Connexin43 is independent on the canonical endocytic tyrosine-sorting signal

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Running head: Ubiquitin-mediated Cx43 internalization

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The abbreviations used are: Cx, connexin; Eps15, epidermal growth factor receptor substrate 15; GJIC, gap junction intercellular communication; Ub, Ubiquitin; UBD, ubiquitin-binding domain.

Abstract

Gap junctions are specialized cell-cell contacts that provide direct intercellular communication between eukaryotic cells. The tyrosine-sorting signal (YXXØ), present on amino acids 286-289 of Cx43, has been implicated in the internalization of the protein. In recent years, ubiquitination of Cx43 has also been proposed to regulate gap junction intercellular communication, however, the underlying mechanism and molecular players involved remain elusive. Here we demonstrate that ubiquitinated Cx43 is internalized through a mechanism that is independent on the YXXØ signal. Indeed, expression of a Cx43-Ub chimera was shown to drive the internalization of a mutant Cx43 in which the YXXØ-motif was eliminated. Immunofluorescence, cycloheximide-chase and cell surface protein biotinylation experiments demonstrate that oligomerization of Cx43-Ub into hemichannels containing wild type Cx43 or mutant Cx43Y286A is sufficient to drive the internalization of the protein. Furthermore, the internalization of Cx43 induced by Cx43-Ub was shown to depend on its interaction with Eps15.

Keywords: Connexin43, Gap Junction, Ubiquitination, Tyrosine-sorting signal, Endocytosis, Eps15

Introduction

Gap junctions (GJ) are specialized areas of the plasma membrane that directly connect adjacent cells, allowing for the transfer of small metabolites, ions and second messengers. Two adjacent cells must supply one hemichannel each to form a functional gap junction channel. Each hemichannel, or connexon is, in turn, composed of six connexin subunits. The function of gap junctions in cell and tissue biology is of the utmost importance, and not surprisingly, gap junction intercellular communication is present in nearly every mammalian cell type [1, 2]. In vertebrates, connexins play critical roles in embryonic development, coordinated contraction of excitable cells, tissue homeostasis, normal cell growth and differentiation. Cx43, a member of the alpha connexin family, is the most widely expressed connexin, being found in tissues as diverse as the heart, lens, retina, skin, brain, kidney and bone marrow [3, 4].

The extent of gap junction intercellular communication (GJIC) is directly related to the number and function of gap junction channels present at the plasma membrane. Although GJIC can be regulated by the gating of the channel pore, connexin degradation also plays an important role in this process. Indeed, Cx43 has a markedly short half-life, of less than 5

hours, when compared to other plasma membrane proteins [3, 5, 6]. Gap junctions are not static entities, but rather exist in a constant state of degradation and renewal. Newly synthesized connexin hemichannels are constantly being added to the periphery of existing gap junction plaques while older portions are internalized from the centre of the plaque [7]. In contrast to most plasma membrane proteins, which are internalized through single membrane vesicles, both membrane sides of a gap junction plaque are internalized by one of two adjacent cells, as large double membrane vesicular structures termed annular gap junctions [8-10].

Both the lysosome and the proteasome have been implicated in regulating Cx43 turnover [5, 11-15]. While degradation of Cx43 by the lysosome has long been established, the role of the ubiquitin-proteasome system in Cx43 turnover is still poorly understood. Initial studies suggested that the proteasome could be directly involved in connexin degradation [15]. However, more recently it was suggested that the proteasome acts by regulating the stability of Cx43 at the plasma membrane [14, 16]. For example, a model has been proposed in which proteasome-dependent degradation of a putative Cx43 binding protein regulates Cx43-ZO-1 interaction, which in turn regulates Cx43 internalization [16].

It is well established that in mammalian cells, ubiquitin acts as a signal for the internalization and sorting of a variety of cargo receptors, thus acting as a link between cargo and components of the endocytic and sorting machinery [17-20]. However, to promote the internalization and intracellular sorting of membrane proteins, ubiquitin needs to be recognized by ubiquitin receptors, such as Eps15, Epsin and Hrs, that contain ubiquitin-binding domains which recognize ubiquitinated proteins and sort them along the endocytic pathway [17, 21]. Significantly, some of these ubiquitin receptors are often themselves monoubiquitinated [22-25].

Several studies have also shown that Cx43 is a substrate for ubiquitination [15, 26-28]. For example, data obtained by immunofluorescence and immunogold labelling, shows that ubiquitin is localized at gap junction plaques [29]. Moreover, treatments that induce Cx43 internalization also induce ubiquitination of the protein [26, 27]. Furthermore, the E3 ubiquitin ligase Nedd4 was shown to interact with Cx43 [30], and we have consistently shown that Nedd4 mediates the ubiquitination of Cx43, through a mechanism that requires the Proline-rich motif (PY) of Cx43 [28]. We also demonstrated that Eps15 binds to ubiquitinated Cx43 through its ubiquitin-interacting motifs (UIMs) and that this interaction is, at least in part, required for the internalization of Cx43 [28].

Altogether, these studies suggest a role for ubiquitin in mediating the internalization of Cx43. However, Thomas et al. showed that Cx43 internalization is primarily regulated by the tyrosine-sorting signal (YXXØ), present on amino acids 286-289 of its carboxyl-terminal, that mediates the internalization of membrane proteins by interacting directly with subunits of the endocytic adaptor AP2 [31, 32], which is associated with clathrin-mediated endocytosis [33, 34]. Consistently, inhibition of clathrin-mediated endocytosis [27, 35, 36] or the depletion of AP2 [35] were both shown to inhibit Cx43 internalization. Indeed, mutation of Val289, a critical amino acid for the YXXØ signal, led to a 3.5 fold increase in the steady-state pool of Cx43, while mutation of Pro283, a critical amino acid of the PY-motif present on amino acids 283-286 of Cx43, had a more moderate effect leading only to a 1.7 fold increase. Furthermore, mutation of Tyr286, a critical amino acid for both motifs, displays no additive effect [37].

Although several reports have suggested that ubiquitin may be implicated in gap junction internalization, direct evidence of ubiquitin-dependent internalization/degradation of Cx43 has not been clearly demonstrated. To better understand the molecular mechanisms that control the internalization of Cx43 we used a Cx43-Ub chimera as a tool to directly incorporate ubiquitin signals into Cx43-containing hemichannels. Furthermore, the importance of the YXXØ signal, which is considered to mediate the constitutive internalization of Cx43, for the ubiquitin-mediated internalization of the protein was also evaluated by mutating Tyr286 on Cx43. Results obtained in this study show, for the first time, that incorporation of ubiquitin into Cx43-containing channels induces their internalization and degradation. Furthermore, this internalization induced by ubiquitin was shown to be

independent of the YXXØ motif present on Cx43.

Experimental

Antibodies and Reagents

The rabbit anti-Cx43 polyclonal antibody, obtained from Zymed (CA, USA) was raised against a peptide corresponding to a segment of the third cytoplasmic domain (carboxyl-terminal portion) of rat Cx43 (Cat No 71-0700). The mouse anti-Cx43 monoclonal antibody obtained from Zymed (CA, USA) was raised against a cytoplasmic sequence located near the carboxyl-terminal of rat Cx43 (Cat No 13-8300). The mouse anti-c-myc antibody, obtained from Zymed (CA, USA) was raised against a 32 amino acid synthetic peptide (residues 408-439) derived from the carboxyl-terminal of the human c-myc protein (Cat No 13-2500). The rabbit polyclonal antibodies against GFP (ab290) were obtained from Abcam (Cambridge, UK). The mouse monoclonal antibodies against the V5 tag were obtained from Invitrogen (Paisley, UK). Polyclonal antibodies against Hrs (M-79) and Eps15 (H-896) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal P4D1 antibodies against ubiquitin were obtained from Covance (CA, USA). Monoclonal antibodies against β -actin (AC-15), cycloheximide and DAPI were obtained from Sigma.

Cell culture and transfections

The retinal pigment epithelium cell line ARPE-19 (LGC Promochem, Teddington, UK) was cultured in Ham's F12/DMEM (1:1) supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) and GlutaMax (1x). The cardiomyocyte cell line HL-1, kindly provided by Dr. William C Claycomb (LSU Health Sciences Center, New Orleans, LA, USA), was cultured in Claycomb medium supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin), 0.1 mM norepinephrine and GlutaMax (1x). The kidney fibroblast-like Cos-7 cell line and the human embryonic kidney (HEK) 293A and 293 STAR cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). All cell lines were maintained at 37°C under 5% CO₂. Transient transfection of cells was performed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations.

Plasmid constructions

Cx43 cDNA was cloned into a modified pENTR GFP C2 vector [38]. Site-directed mutagenesis was performed to generate the Cx43Y286A mutant from Cx43 cDNA. Site-directed mutagenesis was performed to generate the UbK48R(AA) mutant from ubiquitin cDNA. The myc tag and UbK48R(AA) were then subcloned into a pCMV7 vector containing Cx43 to generate the myc-Cx43-UbK48R(AA) mutant (Cx43-Ub). Correct mutagenesis was verified by sequencing the constructs. Plasmids expressing GFP-Cx43, V5-Cx43 and GFP-Cx43Y286A were generated by cloning the appropriate cDNA into pENTR vectors containing either GFP or the V5 tag. To generate the mCherry-Cx43-UbK48R(AA) construct, Cx43-UbK48R(AA) cDNA was subcloned into pcDNA ENTR BP mCherry C1. pcDNA ENTR BP mCherry C1 was constructed using the backbone of pcDNA6.2-GW/EmGFP-miR (Invitrogen) using the following strategy. pcDNA ENTR BP was generated by excising EmGFP/miR from pcDNA6.2-GW/EmGFP-miR with Dral/XhoI restriction enzymes and inserting a polylinker. pcDNA ENTR BP GFP C1 was generated based on a previous vector by inserting GFP, excised from pEGFPC3 (Clontech), with NheI/HindIII restriction enzymes. In order to obtain pcDNA ENTR BP myc mCherry C1, the myc mCherry tag was amplified by PCR using pCMV myc mCherry as a template [39] and cloned using NheI/BglII. Mouse Rab1a, Rab5a and Rab7a cDNA were amplified from AtT20 cells by RT-PCR and cloned into a pENTR GFP C2 vector [40]. Plasmids expressing Hrs-HA were kindly provided by Dr.

Silvie Urbé (University of Liverpool, UK). Plasmids expressing Eps15 and Eps15 Δ UIM were kindly provided by Dr. Simona Polo (University of Milan, Italy).

Lentiviral vector production and cell transduction

The lentiviral vector stocks were produced by lipofectamine transfection of HEK293 STAR cells. Briefly, HEK293 STAR cells were cotransfected with the vector plasmid (pLenti6 prom CMV V5 C1 Cx43), the lentiviral packaging constructs pMD2-G, pMDLg/pRRE, and pRSV-rev. The viruses were collected from the cell culture supernatants 3 days after transfection by centrifugation. To create the HEK293A cell line stably expressing V5-Cx43, cells were transduced with lentiviral vector pLenti6 prom CMV V5 C1 Cx43. After 1 day, 8 μ g/ml blasticidin was added to select the transduced cells.

siRNA-mediated knockdown

siRNA targeting Eps15 (s4773 (CAGCAUUCUUGUAAACGGAtt) or s4774 (CUACCUUACUAGCCCAUAUtt)) were obtained from Ambion (Silencer Select Pre-designed siRNA). Prior to transfection, cells were grown until they reached 40-50% confluency. siRNA was complexed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations, and added to the cells medium to a final concentration of 20 nM. Eps15 knockdown was achieved by transfecting twice at intervals of 24 hours, and experiments were performed 24 hours after the second transfection. Controls were transfected with a non-targeting control sequence obtained from Ambion.

Immunoprecipitation and Western blotting

Cells were rinsed with PBS at 4°C, resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodoacetamide, and incubated on ice during 10 min. The samples were then centrifuged at 10,000g for 10 min, and the supernatants used for immunoprecipitation. Briefly, protein A was incubated with polyclonal antibodies directed against Cx43 or GFP. Non-specific antibodies were used as controls. Incubations proceeded for 1 h, at 4 °C, followed by incubation with supernatants, for 3 h at 4 °C. The samples were then centrifuged and the protein A-sepharose sediments washed 3 times in an appropriate washing buffer (500 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3), resuspended in Laemmli buffer and denatured at 100 °C, for 5 min. For Western blot analysis of the immunoprecipitated proteins, samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with appropriate antibodies. Inputs represent about 5% of the total amount of protein in the lysates before immunoprecipitation.

Triton X-100 extraction

The detergent solubility assay with 1% Triton X-100 was performed essentially as described previously by others [41]. Cells were resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF and 10 mM iodoacetamide. Samples were then ultracentrifuged at 100,000g for 50 min and the supernatant recovered (Triton X-100 soluble fraction). The detergent-insoluble pellets were resuspended in lysis buffer supplemented with 0.1% SDS (Triton X-100 insoluble fraction) and sonicated. Laemmli buffer was then added to the Triton X-100 soluble and insoluble fractions and denatured at 100 °C for 5 min before SDS-PAGE analysis.

Immunofluorescence and Triton X-100 extraction of cells *in situ*

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). The samples were then washed with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS and blocked with goat serum (1:10) for 20 min prior to incubation with primary antibodies. Incubation with primary monoclonal antibodies against V5 (Invitrogen), c-myc (Zymed) and polyclonal antibodies against Hrs proceeded for 1 h at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 1 h at room temperature. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent (Calbiochem). All solutions were made in 0.2% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls, primary antibodies were omitted. The images were collected by confocal microscopy using a Zeiss LSM 710.

For the extraction of the detergent soluble fraction of cells *in situ*, cells were processed essentially as described previously by others [42]. Briefly, cells were incubated in PBS supplemented with 1 mM CaCl₂, 1 mM MgCl₂ and protease inhibitor cocktail (Roche) for 45 min at 4°C in the presence or absence of 1% Triton X-100. The dishes were swirled gently and intermittently. Following incubation, cells were fixed and imaged by confocal microscopy..

Biotinylation of cell surface proteins

Cos-7 cells grown on 60 mm culture dishes were rinsed twice with 5 ml of ice-cold PBS containing 0.5 mM MgCl₂ and 1 mM CaCl₂, followed by the addition of 1.5 ml of the same ice-cold solution containing 1 mg/ml of freshly added Sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA). After 30 min at 4 °C, to stop subcellular trafficking, the medium was discarded and the plates were washed three times with PBS containing 0.5 mM MgCl₂, 1 mM CaCl₂ and 100 mM glycine. The cells were scraped in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, containing 1% Triton X-100, 0.5% DOC, 0.1% SDS and supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodoacetamide, pH 7.5). After 15 min on ice the cells were sonicated and the homogenates were centrifuged at 14,000 rpm, for 10 min. Samples were then transferred to 1.5 ml Eppendorf microfuge tubes containing 100 µl of Neutravidin beads (Pierce, Rockford, IL, USA). After 2 h incubation at 4 °C under agitation, the beads were washed four times with RIPA buffer. The final pellets were resuspended in 75 µl 2x Laemmli buffer and denatured at 100 °C, for 5 minutes. The beads were pelleted and the solubilized proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies directed against Cx43.

Statistical analysis

Data present in this manuscript are representative of at least three independent experiments. Data are expressed as means ± S.D. Comparison between groups was performed using a Student's *t* test.

Results

The fusion protein Cx43-Ub oligomerizes into the same connexons as Cx43.

Although it was previously demonstrated that Cx43 is ubiquitinated, the role of ubiquitination in Cx43 internalization still remains elusive. We hypothesized that the incorporation of ubiquitinated Cx43 into connexin hemichannels would be sufficient to trigger the internalization of non-ubiquitinated Cx43 present in the same hemichannels. Several studies have demonstrated that ubiquitination of various plasma membrane proteins is both necessary and sufficient to target them for internalization, in a process mediated by endocytic adaptors containing ubiquitin binding domains (UBDs). A common strategy to evaluate the role of ubiquitin in the internalization of membrane proteins involves the overexpression of a chimeric protein formed by the membrane protein fused to an ubiquitin molecule [43-47]. Therefore, to evaluate the role of Ub in Cx43 internalization and

degradation we constructed a chimeric Cx43 protein in which an ubiquitin molecule was fused in frame to the carboxyl-terminal of Cx43 (Cx43-Ub). To prevent this fusion protein from being conjugated to other proteins by the ubiquitination machinery, the two terminal glycines were mutated to alanine. Furthermore, to prevent the chimeric protein from being recognized by the ERAD machinery and degraded by the proteasome, Lys48 was also mutated to Arg, to prevent the extension of a Lys48 polyubiquitin chain on the fused ubiquitin. Lastly, to allow the specific detection of this chimeric protein through immunofluorescence, a myc tag was fused in frame to the amino-terminal of Cx43, completing the myc-Cx43-Ub(K48R) chimera, hereafter abbreviated to Cx43-Ub. To first evaluate the expression levels of Cx43 and Cx43-Ub, cDNA encoding these proteins was transfected into Cos-7 cells and the presence of each of the proteins determined by immunoprecipitation and Western blot. As shown in Figure 1A, the presence of a slower migrating band, with a molecular weight of about 50 KDa, that is recognized both by Cx43 and ubiquitin antibodies, is compatible with the expression of the chimeric protein.

Transport of Cx43 to the plasma membrane requires its prior oligomerization into connexons, a process that occurs in the trans-Golgi Network (TGN). To investigate whether Cx43-Ub oligomerizes into connexons with wild type Cx43, thus forming heteromeric hemichannels, Cos-7 cells were co-transfected with varying quantities of plasmids encoding Cx43-Ub and a construct in which GFP was fused in frame to the amino-terminal of Cx43 (GFP-Cx43). Although this GFP-Cx43 fusion protein does not form functional gap junction channels [48], it is delivered to the plasma membrane and has been used to study the trafficking of Cx43 [49]. Polyclonal antibodies directed against GFP were then used to selectively immunoprecipitate the GFP-Cx43 chimera. As depicted in Figure 1B, monomeric Cx43-Ub precipitates only with the GFP antibodies when co-expressed with GFP-Cx43, suggesting that Cx43-Ub oligomerizes into the same connexons as GFP-Cx43. Moreover, as the amount of expressed GFP-Cx43 increases, the levels of Cx43-Ub that co-immunoprecipitate with the protein also increase. These results suggest that at least part of Cx43-Ub is capable of forming heteromeric connexons with Cx43.

To investigate the subcellular distribution of hemichannels containing Cx43-Ub, Cos-7 cells were co-transfected with either cDNA encoding GFP-Cx43 and the myc-tagged Cx43-Ub or with cDNA encoding V5-Cx43 and mCherry-tagged Cx43-Ub. Cells were subsequently fixed and stained with monoclonal antibodies directed against the myc or V5 tags, and imaged by confocal microscopy. Results depicted in Figure 2A show that in both cases there is an extensive colocalization of the two proteins, mainly in large intracellular vesicles, thus reinforcing that they are being incorporated into the same hemichannels. In addition, some perinuclear localization of both proteins is also observed. These results show that the pattern of colocalization of Cx43 with Cx43-Ub is maintained when using either tagged construct.

To evaluate whether the Cx43-Ub construct traffics properly, being delivered to the plasma membrane and subsequently internalized, we used time-lapse imaging acquisition, using HEK293 cells transiently transfected with GFP-Cx43 and mCherry-Cx43-Ub. Vesicles carrying mCherry-Cx43-Ub and GFP-Cx43, were observed to be moving, being transported to the plasma membrane, where they are presumably incorporated into previously existing plaques. In addition, vesicles containing mCherry-Cx43-Ub can be seen rapidly leaving the plasma membrane.

To ascertain that the Cx43-Ub construct is incorporated into gap junction plaques, Cos-7 cells co-transfected with GFP-Cx43 and mCherry-Cx43-Ub were permeabilized *in situ* with Triton X-100 to solubilize non-gap junctional Cx43, as Cx43 present at gap junction plaques is known to be insoluble in Triton X-100 [41, 42]. As shown in Figure 2B, following detergent extraction, most of the intracellular vesicles containing GFP-Cx43 and mCherry-Cx43-Ub disappear. However, both GFP-Cx43 and mCherry-Cx43-Ub can still be localized at the plasma membrane at cell-cell contact sites, presumably at gap junction plaques. Furthermore, Triton X-100 extraction on cells transfected with either Cx43 or myc-Cx43-Ub reveal that Cx43-Ub is found in Triton X-100 insoluble fractions, corresponding to protein incorporated into gap junction plaques (Figure 9C). The accumulation of a slower migrating band of Cx43 in Triton X-100 insoluble fractions is consistent with the preferential presence

of phosphorylated Cx43 at gap junction plaques.

Expression of the fusion protein Cx43-Ub induces the internalization and degradation of Cx43.

Having established that Cx43-Ub incorporates into Cx43-containing hemichannels, we attempted to evaluate the effect of the presence of ubiquitin moieties upon Cx43 channels. First, we investigated the effect of Cx43-Ub overexpression upon the stability of Cx43 in cells that endogenously express Cx43. For this purpose, we used two cell lines that endogenously express detectable amounts of Cx43: a retinal pigment epithelium cell line (ARPE-19) and a cardiomyocyte cell line (HL-1). The half-life of endogenous Cx43, either in the presence or absence of Cx43-Ub, was determined by a cycloheximide (CHX)-chase assay. Following transfection with plasmids encoding Cx43-Ub, protein synthesis was inhibited with 50 μ g/ml of CHX and cells were collected at several time points. Samples were then analysed by Western blot using polyclonal antibodies directed against Cx43 and the density of the detected bands was measured and plotted in a graph. The results presented in Figure 3 show that the overexpression of Cx43-Ub results in a decrease of the half-life of endogenous Cx43 in both cell lines: from around 4 hours to 2.5 hours in ARPE-19 cells (Figure 3A) and from around 5 hours to less than 2 hours in HL-1 cells (Figure 3B). Similar results were obtained when Cx43 was co-expressed with Cx43-Ub in Cos-7 cells. As shown in Figure 4A, the presence of Cx43-Ub results in a decrease in Cx43 half-life from 2.5 hours to 1 hour.

It was previously shown for various membrane proteins that the conjugation of ubiquitin may act as a triggering signal for their internalization. Although the data presented above suggests that the incorporation of Ub signals into Cx43 hemichannels increases the turnover rate of non-ubiquitinated forms of the protein, this effect may not be correlated with an upregulation of Cx43 internalization. It was previously described that newly synthesized Cx43 that does not reach the plasma membrane can be transported to lysosomes [50]. Additionally, ubiquitination of transmembrane proteins can also signal trafficking from the TGN to the endolysosomal system [51]. Thus, the fusion of an ubiquitin moiety to the carboxyl terminal of Cx43 may signal its direct transport from the TGN to the lysosome. Therefore, we decided to perform biotinylation experiments in order to establish whether fusion of ubiquitin to Cx43 alters the stability of Cx43 at the plasma membrane. To determine the half-life of Cx43 at the plasma membrane, transfected Cos-7 cells were first treated with 50 μ g/ml of CHX during different time frames before being subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were then precipitated with Neutravidin beads, followed by Western blot analysis with antibodies directed against Cx43. Results presented in Figure 4B show that the co-expression of Cx43-Ub induces a reduction in the surface stability of wild type Cx43 from 1 hour to roughly 40 minutes.

To investigate the effect of the Cx43-Ub chimera on the subcellular localization of Cx43, Cos-7 cells were transfected with GFP-Cx43, either in the presence or absence of Cx43-Ub, after which the cells were fixed and stained with antibodies directed against the myc tag, and imaged by immunofluorescence microscopy. When GFP-Cx43 is expressed alone (Figure 5A, upper panel), extensive plasma membrane plaques can be found along cell-cell interfaces. However, when Cx43-Ub is co-expressed with the GFP-Cx43 chimera (Figure 5A, lower panels) there is a substantial decrease in the size of GFP-Cx43 containing plaques. These results were further confirmed using a stably transfected HEK293A cell line that expresses V5-Cx43. The effect of Cx43-Ub was evaluated by transfecting this cell line with plasmids encoding mCherry-Cx43-Ub. Cells overexpressing GFP-Cx43 were used as controls. The results presented in Figure 5B show that the overexpression of mCherry-Cx43-Ub results in the accumulation of V5-Cx43 at intracellular vesicles positive for mCherry-Cx43-Ub, and concomitantly, a decrease of V5-Cx43 localization at the plasma membrane. However, overexpression of GFP-Cx43 does not significantly alter the subcellular localization pattern of V5-Cx43. Interestingly, in cells that present lower levels of Cx43-Ub, it is possible to detect the presence of Cx43-Ub at the plasma membrane, colocalizing with V5-Cx43.

However, the amount of Cx43 at the plasma membrane is always lower in plasma membrane regions that are in direct contact with cells overexpressing Cx43-Ub. Taken together, the immunofluorescence data and the biotinylation data strongly suggest that the presence of ubiquitin in Cx43-containing channels induces the internalization and subsequent degradation of gap junctions.

To further reinforce that internalized Cx43-Ub follows the endocytic pathway en route to its degradation in the lysosome we decided to evaluate whether Cx43-Ub could be found in intracellular vesicles positive for several markers of endocytic compartments. The small Rab GTPases are known to regulate the intracellular trafficking of membrane proteins between intracellular compartments and also regulate transport to and from the plasma membrane [52]. For example, vesicle traffic from the endoplasmic reticulum (ER) to the TGN is modulated by Rab1 [52]; traffic from the plasma membrane to early endosomes is regulated by the small GTPase Rab5 [53], while Rab7 is involved in trafficking from early to late endosomes [54] and from late endosomes to lysosomes [55]. To characterize the intracellular trafficking of Cx43-Ub, cells co-expressing V5-tagged Cx43, mCherry-tagged Cx43-Ub and either GFP-tagged Rab1a, Rab5a or Rab7a were analysed by confocal microscopy. Results presented in Figure 6 show that both connexins and Rab1a can be found colocalized in the perinuclear region, presumably in the ER and TGN. Furthermore, data in Figure 6 also shows an accumulation of Cx43 and Cx43-Ub in intracellular vesicles containing Rab5a or Rab7a. Furthermore, when each connexin protein is expressed alone with each individual Rab protein, wild type Cx43 and Cx43-Ub can still be found in vesicles positive for each Rab protein (data not shown). Recently it was demonstrated that the ESCRT-0 component Hrs modulates the trafficking of ubiquitinated Cx43 from the early endosome to the lysosome [56]. As such, we also evaluated whether Cx43-Ub could also be found in intracellular compartments positive for Hrs using cells co-expressing V5-tagged Cx43, mCherry-tagged Cx43-Ub and Hrs-HA. As depicted in Figure 6 (lower panels), both connexins could also be found in intracellular vesicles positive for Hrs. Similar results were obtained when each connexin was expressed alone with Hrs (data not shown). Taken together, these results suggest that Cx43-Ub traffics alongside Cx43 through the secretory pathway, the plasma membrane and through the endocytic pathway before presumably being degraded in the lysosome. Importantly, when expressed alone, Cx43-Ub appears to have the same trafficking pattern as wild type Cx43.

Ubiquitin triggers the internalization of an endocytic-impaired mutant of Cx43.

Cx43 contains within its carboxyl-terminal a putative tyrosine-based sorting signal domain corresponding to the consensus sequence YXXØ (where X is any amino acid and Ø is a hydrophobic amino acid). It was previously shown that mutation of Tyr286, which is critical for the sorting function of the YXXØ motif on Cx43, increases the half-life of the protein, induces its accumulation at the plasma membrane and increases intercellular communication [37], suggesting that the YXXØ motif is the prime determinant of the stability of Cx43 at the cell surface. Thus, we decided to investigate the role of ubiquitin as an internalization signal in channels containing an endocytic defective mutant of Cx43. Besides its critical role as a part of the YXXØ domain on Cx43, Tyr286 is also an integral part of the PY-motif on Cx43, which is important for the interaction and ubiquitination of Cx43 by Nedd4. Therefore, we first generated a Cx43 mutant in which the tyrosine at position 286 was replaced with an alanine (Cx43Y286A), and investigated whether this mutation impaired Cx43 ubiquitination. Cos-7 cells were transfected either with the wild type or the mutated form of Cx43, after which the proteins were selectively immunoprecipitated using polyclonal antibodies against Cx43. The levels of ubiquitinated protein were then determined by Western blot using antibodies directed against ubiquitin. The results presented in Figure 7 consistently show that the Cx43Y286A mutant is less ubiquitinated when compared to wild type Cx43. Taken together with the previous data showing that ubiquitin induces the internalization and degradation of Cx43, it is possible to suggest that the increased stability of the Cx43Y286A mutant may not

only be due to a defect in the tyrosine-based sorting signal, but also due to an ubiquitination defect.

Since the internalization of Cx43 occurs mainly through a mechanism that depends on the tyrosine-based sorting signal, and mutations on this motif are known to strongly stabilize Cx43 at the plasma membrane, we hypothesized that if YXXØ-driven internalization of Cx43 is impaired, ubiquitin would act as an internalization signal, triggering the endocytosis of this Cx43Y286A mutant. As with the wild type form of Cx43, we first evaluated the effect of Cx43-Ub on Cx43Y286A half-life. Consistent with what was observed by Thomas et al. [37], mutation of Tyr286 on Cx43 increased its half-life from roughly 2.5 hours to 5.5 hours (compare Figure 8A with Figure 4A), thus reinforcing the importance of the YXXØ sorting motif for Cx43 internalization. However, co-expression of Cx43-Ub with Cx43Y286A markedly reduced its half-life from 5.5 hours to only 3 hours (Figure 8A).

To further investigate the effect of incorporating Cx43-Ub into Cx43Y286A-containing hemichannels, the cell surface half-life of Cx43Y286A was determined when expressed alone or together with Cx43-Ub, by cell surface protein biotinylation. Results presented in Figure 8B show that mutation of Tyr286 on Cx43 induces a 2-fold increase in the half-life of Cx43 at the cell surface, further reinforcing the importance of this residue for Cx43 internalization (compare Figure 8B with Figure 4B). Similarly to what was observed for Cx43 surface half-life, co-expression of Cx43-Ub induces a significant reduction in the surface stability of the Y286A mutant from 2 hours to less than 1 hour. Consistent with the results obtained with the biotinylation assays, the subcellular distribution of the Cx43Y286A mutant is also affected by the presence of Cx43-Ub. Indeed, using a construct in which GFP was fused to the amino-terminal of Cx43Y286A (GFP-Cx43Y286A), we show that this construct localizes to the plasma membrane where it appears to form large gap junction plaques, when compared to cells expressing GFP-Cx43 (compare the top panel of Figure 5A with the top panel of Figure 8C). Furthermore, and as demonstrated for GFP-Cx43, the incorporation of Cx43-Ub into GFP-Cx43Y286A hemichannels results in a decrease of GFP-Cx43Y286A at the plasma membrane (Figure 8C, lower panels). Data obtained with this Cx43Y286A mutant is particularly important since the main internalization pathway of Cx43, that requires an intact YXXØ sorting motif, is impaired. Furthermore, and considering that this mutant also displays impaired ubiquitination (Figure 7), the substantial increase in the internalization of Cx43Y286A can be largely attributed to the ubiquitin signal present on the Cx43-Ub chimera.

Internalization of Cx43 triggered by Ubiquitin is dependent on the interaction with Eps15.

We have previously shown that Eps15 interacts with ubiquitinated Cx43 and that Eps15 depletion resulted in the accumulation of both Cx43 at the cell surface and gap junction plaques [28]. Having shown that the expression of a chimeric Cx43 fused to ubiquitin induces the destabilization of gap junction plaques and reduces the cell surface stability of non-ubiquitinated Cx43, we attempted to ascertain if this results from an increased interaction with Eps15. To address this question, Cos-7 cells were transfected with Eps15 together with either wild type Cx43 or Cx43-Ub. Cx43 was then selectively immunoprecipitated using polyclonal antibodies against Cx43, followed by Western blot and probing with antibodies against Eps15 (Figure 9A). The results obtained clearly show that the levels of Eps15 co-immunoprecipitated with anti-Cx43 antibodies is higher in cells expressing Cx43-Ub when compared to cells expressing the wild type form of the protein.

To assess whether the UIM of Eps15 is required for interaction with Cx43-Ub at the gap junction plaques, we performed Triton X-100 extraction experiments in Cos-7 cells overexpressing either Eps15 or a mutant form of the protein where its ubiquitin interacting domains (UIM) domains were deleted (Eps15ΔUIM). The results obtained show that the Eps15 mutant (Eps15ΔUIM) interacts neither with Cx43 nor Cx43-Ub, demonstrating that similarly to what we observed previously for wild type Cx43 [28], the UIM domains of Eps15 are required for the interaction with Cx43-Ub (Figure S1A in the supplemental data). In addition, Cx43-Ub present in gap junction plaques (Triton X-100 insoluble fraction) displayed

increased interaction with Eps15 when compared to the Triton X-100 soluble fraction (compare lane 6 with lane 8 of the upper panel of Figure S1B in the supplemental data).

To demonstrate that Eps15 is indeed important for Cx43-Ub-triggered internalization of Cx43, we decided to evaluate the levels of Cx43-Ub at the plasma membrane following the depletion of endogenous Eps15. Cos-7 cells were first transfected with siRNA targeted against Eps15. Following 24 hours, cells were then transfected with either wild type Cx43, Cx43-Ub or both. After another 24 hours, cells were subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were isolated with Neutravidin beads and the samples analysed by Western blot using polyclonal antibodies directed against Cx43. Given that Eps15 was previously shown to be important for the internalization of Cx43, and that Cx43 ubiquitination was shown to be important for Eps15 recognition of the protein, we predicted that the depletion of Eps15 would lead to the accumulation of Cx43-Ub at the plasma membrane. Indeed, data depicted in Figure 9B confirms this hypothesis as Eps15 depletion induced the accumulation of both Cx43 and Cx43-Ub at the plasma membrane.

As our biotinylation procedures do not allow for the analysis of Cx43 proteins present at gap junction plaques, we performed Triton X-100 extraction experiments to distinguish between junctional and non-junctional Cx43 [41]. Cos-7 cells were first transfected with siRNA targeted against Eps15. Following 24 hours, cells were transfected either with wild type Cx43 or with Cx43 and Cx43-Ub. After another 24 hours, cells were subjected to Triton X-100 extraction. Subsequently, the Triton X-100 soluble and insoluble fractions were analysed by Western blot with polyclonal antibodies directed against Cx43. As depicted in Figure 9C, Cx43-Ub can be readily observed in the Triton X-100 insoluble fractions, indicating that the protein can be integrated into gap junction plaques. Furthermore, depletion of Eps15 leads to the accumulation of both wild type Cx43 and Cx43-Ub in both the Triton X-100 soluble and insoluble fractions. This effect however is more pronounced for Cx43-Ub in the Triton X-100 soluble fraction. Additionally, analysis of Triton X-100 insoluble fractions from cells subjected to a cycloheximide-chase revealed that the co-expression of Cx43 and Cx43-Ub leads to a significant decrease in the levels of Cx43 present at gap junction plaques after 6 hours, when compared to cells expressing only Cx43 (compare lane 6 with lane 12 of Figure S1C in the supplemental data).

Discussion

Several studies have demonstrated that the internalization of Cx43 follows the canonical clathrin-dependent endocytosis, mediated by the tyrosine-sorting signal present on the carboxyl-terminal of Cx43 [27, 35-37]. Although it was previously shown that Cx43 is ubiquitinated, the role of this posttranslational modification on gap junction internalization and degradation has never been clearly demonstrated. The results presented in this paper show, for the first time, that the expression of a chimeric Cx43-Ub protein is sufficient to trigger the internalization of wild type Cx43, suggesting that ubiquitination is likely to signal the internalization and subsequent degradation of Cx43. Moreover, we demonstrate that the internalization triggered by ubiquitin is independent on the endocytic YXXØ sorting signal present at the carboxyl-terminal of Cx43. Using CHX-chase assays we showed that expression of Cx43-Ub decreases the half-life of both Cx43 and Cx43Y286A by about 50%. Consistently, the half-life of the two forms of Cx43 at the plasma membrane also decreases in the presence of Cx43-Ub. Data obtained with the Cx43Y286A mutant is particularly important since the main internalization pathway of Cx43, that requires an intact YXXØ sorting motif, is impaired. For this mutant, ubiquitination would constitute the main signal to trigger its internalization from the plasma membrane.

In the present work we use a chimeric Cx43 protein fused to ubiquitin as a tool to directly incorporate ubiquitin moieties into Cx43 hemichannels and gap junction plaques. Although we did not investigate whether this Cx43-Ub chimera forms functional gap junction channels, data presented here supports the notion that this chimera can be used as a tool to evaluate the role of Cx43 ubiquitination in modulating the intracellular trafficking of the protein. First, we show that Cx43-Ub can oligomerize with wild type Cx43, as suggested by the co-

immunoprecipitation experiments where Cx43-Ub specifically co-precipitates with Cx43. Previous studies have demonstrated that the presence of tags on the carboxyl-terminal of Cx43 may negatively affect Cx43 internalization, resulting in the stabilization of the protein at the plasma membrane [16, 57]. However, in our current study, we show that the presence of ubiquitin at the carboxyl-terminal of Cx43 leads to a destabilization of the channels. Triton X-100 extraction experiments, which have been previously described to separate gap junctional Cx43 into Triton X-100 insoluble fractions [41, 42], demonstrate that Cx43-Ub is incorporated into gap junction plaques. Immunofluorescence experiments also show that not only does Cx43-Ub colocalize with Cx43, but that both proteins colocalize within intracellular vesicles positive for several markers of the endocytic pathway (Rab5, Rab7 and Hrs), thus suggesting that both proteins have reached the plasma membrane before being incorporated in endocytic vesicles. Importantly, Cx43-Ub and wild type Cx43 can also be found within these same vesicles when expressed alone. Although the majority of Cx43-Ub is found in intracellular vesicles, Cx43-Ub can also be found colocalized with Cx43 at the plasma membrane. The difficulty in visualizing Cx43-Ub at the plasma membrane through fluorescence microscopy is probably due to the low half-life of the chimera at the plasma membrane. Taken together, this data suggests that Cx43-Ub is trafficked through similar pathways as the wild type form of the protein. Although this Cx43-Ub chimera does not represent a naturally lysine-ubiquitinated Cx43, we present evidence suggesting that the chimera functions similarly to endogenously ubiquitinated Cx43. We have previously demonstrated that Cx43 ubiquitination was required for its interaction with the UIM domains of the endocytic adaptor Eps15 and that the depletion of Eps15 led to the accumulation of Cx43 at the plasma membrane [28]. Here we show that Cx43-Ub displays increased interaction with Eps15 when compared to wild type Cx43. Additionally, we also show that Eps15 depletion leads to the accumulation of wild type Cx43 and of Cx43-Ub at the plasma membrane and at gap junction plaques. These observations show that the internalization of Cx43-Ub is dependent on similar mechanisms as those used by endogenously ubiquitinated Cx43.

Although monoubiquitination was initially suggested to be the signal that targets plasma membrane proteins for internalization and degradation, more recently it was demonstrated that, at least in mammals, plasma membrane proteins usually undergo polyubiquitination and multimonoubiquitination, rather than monoubiquitination. To trigger the internalization of plasma membrane proteins, ubiquitin moieties have to be recognized by endocytic adaptors containing UBDs. Consistently, it was recently shown that UBD domains have a rather low affinity for monoubiquitin [45, 46]. Instead, UBD domains were shown to have a higher affinity for Lys63-linked polyubiquitin chains. Additionally, Barriere et al. showed that the monoubiquitination of several subunits in an oligomeric membrane protein, but not the monoubiquitination of a single monomeric protein, could also function as an internalization signal. It was suggested that multiple monoubiquitin moieties in close spatial proximity, either due to the multimonoubiquitination of a single protein or due to the monoubiquitination of several subunits in an oligomeric complex, would provide UBD domains with multiple interaction points, generating a strong recognition signal [45]. Data obtained by confocal microscopy shows that in cells expressing low levels of Cx43-Ub, this construct can be found at the plasma membrane. We thus hypothesize that a critical threshold of ubiquitin moieties at gap junction plaques must be reached to trigger Cx43 internalization. Based on this assumption the Cx43-Ub chimera would induce the internalization of Cx43 by directly increasing the ubiquitin-dependent internalization signal present at these channels. Indeed, biotinylation experiments demonstrated that Cx43-Ub expression destabilizes Cx43 present at the plasma membrane, increasing its internalization. However, data presented in this paper does not allow to clarify the type of ubiquitination (monoubiquitination, multimonoubiquitination or polyubiquitination) involved in Cx43 internalization. Indeed, the formation of Lys63-linked chains on the Ub moiety present in the chimera is still permitted, therefore we cannot exclude the hypothesis that the increased internalization and degradation of Cx43 in the presence of Cx43-Ub is due to the formation of Lys63-linked chains. Similarly, the accumulation of multiple monoubiquitin moieties on the same protein is

also still permitted, and therefore, may account for the increased degradation of Cx43 in the presence of Cx43-Ub.

Several authors have shown that Cx43 is a substrate for ubiquitination and that a small population of ubiquitinated Cx43 can be found at the plasma membrane within gap junction plaques [15, 29]. Although for some receptors ubiquitin is not required for internalization, but rather for signalling intracellular sorting to lysosomes, this does not appear to be the case of Cx43. Data in this study with the Cx43Y286A mutant, together with reports by Thomas et al. [37], show that constitutive internalization of Cx43 occurs mainly through the canonical AP2 dependent clathrin-mediated endocytosis. However, the presence of ubiquitin in both wild type and mutant Cx43 hemichannels accelerates the internalization and degradation of the proteins. Leithe et al. have shown that phosphorylation of Cx43 induced by epidermal growth factor or 12-O-tetradecanoylphorbol 13-acetate treatment leads to Cx43 ubiquitination and internalization [26, 27]. Furthermore, data from Leykauf et al. shows that phosphorylated Cx43 has a higher binding affinity for the ubiquitin ligase Nedd4 and that Nedd4 depletion leads to the accumulation of Cx43 at the plasma membrane [30]. We have previously demonstrated that Nedd4 mediated ubiquitination of Cx43 is important for the subsequent interaction of the protein with Eps15, and that this interaction occurred through the UIM domains of Eps15. Depletion of Eps15 also led to the accumulation of Cx43 at the cell surface [28]. Accordingly, we show here that siRNA depletion of Eps15 stabilizes both Cx43 and Cx43-Ub at the plasma membrane.

Although the main constitutive internalization pathway of Cx43 occurs through the canonical AP2 dependent clathrin-mediated endocytosis, ubiquitin may play an important role in regulated signal-induced gap junction internalization. Therefore, we propose a model in which under basal conditions Cx43 internalization occurs through a pathway that does not depend on ubiquitin, while the rapid internalization of Cx43 in response to changes in the cellular environment may require the prior ubiquitination of the protein.

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Figure legends

Figure 1. Cx43 and Cx43-Ub oligomerize into the same connexons. (A) Lysates of Cos-7 cells transfected with either Cx43 or myc-Cx43-Ub were immunoprecipitated with polyclonal antibodies directed against Cx43 and the precipitates were analysed by Western blot using monoclonal antibodies directed against Cx43 (upper panels) or ubiquitin (lower panels). The Cx43-Ub construct is readily detectable with anti-ubiquitin antibodies. (B) Cos-7 cells were co-transfected with varying quantities of plasmids encoding GFP-Cx43 and myc-Cx43-Ub (values represent the mass of plasmid DNA used). Lysates were immunoprecipitated with polyclonal antibodies directed against GFP and the precipitates analysed by Western blot using monoclonal antibodies directed against Cx43. Monomeric Cx43-Ub is selectively co-precipitated with GFP-Cx43.

Figure 2. Cx43-Ub colocalizes with Cx43. (A) Cos-7 cells were either transfected with GFP-Cx43 together with myc-Cx43-Ub (upper panels) or with V5-Cx43 together with mCherry-Cx43-Ub (lower panels). Cells were then fixed and stained with monoclonal antibodies directed against the myc or V5 tags. Cx43 and Cx43-Ub display extensive colocalization, further reinforcing that they oligomerize into the same connexons. Small arrows indicate intracellular vesicles where Cx43 and Cx43-Ub colocalize. The large arrow highlights a cell expressing high levels of both proteins, displaying increased colocalization. Scale bars, 100 μ m. (B) Cos-7 cells were co-transfected with GFP-Cx43 together with mCherry-Cx43-Ub. In situ Triton X-100 extraction was then performed prior to cell fixation (lower panels). Controls were incubated without Triton X-100 (upper panels). mCherry-Cx43-Ub can be found at sites of cell-cell contact following Triton X-100 extraction, suggesting its presence in gap junction plaques. Arrows indicate Cx43-Ub present at points of cell-cell contact. Scale bars, 100 μ m.

Figure 3. Expression of the Cx43-Ub chimera reduces the half-life of endogenous Cx43. (A) ARPE-19 cells transfected with myc-Cx43-Ub, were incubated with 50 μ g/ml cycloheximide for 0, 2 and 4 hours. Lysates were then analysed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the endogenous Cx43 bands was measured and plotted in a graph (Squares = non-transfected cells; Triangles = transfected cells). Expression of Cx43-Ub reduced the half-life of endogenous Cx43 from 4 to 2.5 hours in ARPE-19 cells. (B) HL-1 cells transfected with myc-Cx43-Ub, were incubated with 50 μ g/ml cycloheximide for 0, 2, 4 and 6 hours. Lysates were then analysed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the endogenous Cx43 bands

was measured and plotted in a graph (Squares = non-transfected cells; Triangles = transfected cells). Expression of Cx43-Ub reduced the half-life of endogenous Cx43 from 5 to less than 2 hours in HL-1 cells. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$).

Figure 4. Ubiquitination of Cx43 reduces its half-life and cell surface stability. (A) Cos-7 cells transfected with Cx43 or with Cx43 and myc-Cx43-Ub, were incubated with 50 $\mu\text{g/ml}$ cycloheximide for 0, 1, 2 or 4 hours. Lysates were then analysed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43; Triangles = Cx43 + Cx43-Ub). Co-expression of Cx43 with Cx43-Ub reduced its half-life from 2.5 hours to 1 hour. (B) Cos-7 cells transfected with Cx43 either alone or together with myc-Cx43-Ub, were incubated with 50 $\mu\text{g/ml}$ cycloheximide for 0, 1 or 2 hours. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were then analysed by Western blot using polyclonal antibodies against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43; Triangles = Cx43 + Cx43-Ub). Co-expression of Cx43 with Cx43-Ub reduced its surface half-life from 1 hour to 40 minutes. The values are the average of three individual experiments \pm SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$).

Figure 5. Expression of Cx43-Ub reduces the levels of Cx43 present at the plasma membrane. (A) Cos-7 cells were transfected with GFP-Cx43 (upper panel) or with GFP-Cx43 and myc-Cx43-Ub (lower panels) and then fixed and stained with monoclonal antibodies against myc (lower panels). Nuclei were stained with DAPI. Co-expression of Cx43-Ub induces the internalization of gap junction plaques. Scale bars, 100 μm . (B) HEK293A cells stably expressing V5-Cx43 were transfected with either GFP-Cx43 (upper panels) or mCherry-Cx43-Ub (lower panels) and then fixed and stained with monoclonal antibodies against V5. While GFP-Cx43 colocalizes with V5-Cx43 at gap junction plaque sites (arrows), cells expressing mCherry-Cx43-Ub (marked with asterisks) display lower levels of Cx43 at cell-cell contact sites. Scale bars, 40 μm .

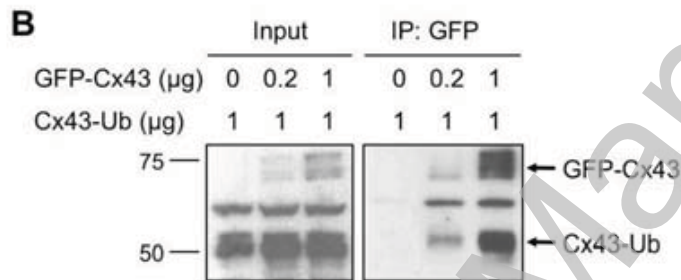
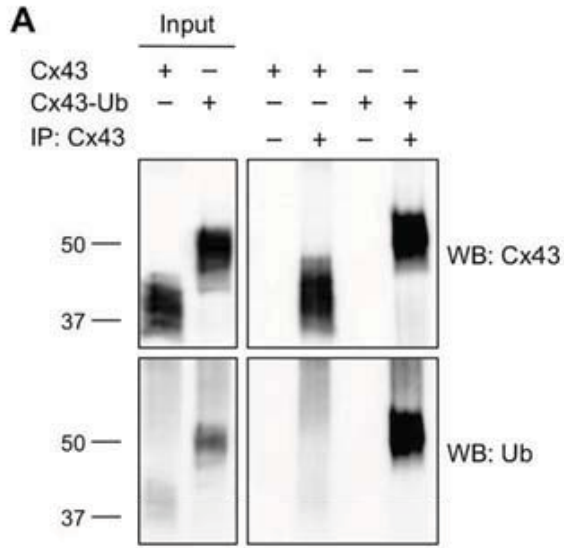
Figure 6. Cx43 and the Cx43-Ub chimera colocalize with markers of endosomal vesicles. Cos-7 cells were co-transfected with V5-Cx43, mCherry-Cx43-Ub and either GFP-Rab1a, GFP-Rab5a, GFP-Rab7a or Hrs-HA. Cells were then fixed and stained with monoclonal antibodies directed against V5 (all panels) and polyclonal antibodies directed against Hrs (lower panels) before being imaged by confocal microscopy. Cx43 and Cx43-Ub can be found colocalized with Rab1a mainly at perinuclear regions (arrowheads), while colocalization with the endosomal markers Rab5a, Rab7a and Hrs occurs mainly in intracellular vesicles (arrows). Scale bars, 100 μm .

Figure 7. Mutation of Tyr286 on Cx43 impairs its ubiquitination. Lysates from Cos-7 cells, transfected with either wild type or mutant Cx43 (Cx43Y286A), were immunoprecipitated with polyclonal antibodies against Cx43. Immunoprecipitates were then analysed by Western blot and probed with monoclonal antibodies against Cx43 or ubiquitin (P4D1). Cx43Y286A is substantially less ubiquitinated than wild type Cx43.

Figure 8. Ubiquitination reduces the half-life and cell surface stability of the endocytic-impaired mutant Cx43Y286A. (A) Cos-7 cells transfected with Cx43Y286A either alone or together with myc-Cx43-Ub, were incubated with 50 $\mu\text{g/ml}$ cycloheximide for 0, 2, 4 and 6 hours. Lysates were then analysed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43Y286A; Triangles = Cx43Y286A + Cx43-Ub). Co-expression of Cx43Y286A with Cx43-Ub reduced its half-life from 5.5 to 3 hours. (B) Cos-7 cells transfected with Cx43Y286A

either alone or together with myc-Cx43-Ub, were incubated with 50 μ g/ml cycloheximide for 0, 1 and 2 hours. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were then analysed by Western blot using polyclonal antibodies against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43Y286A; Triangles = Cx43Y286A + Cx43-Ub). Co-expression of Cx43Y286A with Cx43-Ub reduced its surface half-life from 2 to 1 hour. The values are the average of three individual experiments \pm SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$). (C) Cos-7 cells were transfected with GFP-Cx43Y286A either alone (upper panel) or together with myc-Cx43-Ub (lower panels) and then fixed and stained with monoclonal antibodies against myc (lower panels). Nuclei were stained with DAPI. Co-expression of Cx43-Ub induces the internalization of the large gap junction plaques formed by Cx43Y286A. Scale bars, 100 μ m.

Figure 9. Internalization of Cx43 induced by Cx43-Ub depends on the interaction with Eps15. (A) Cos-7 cells were co-transfected with Eps15 and either Cx43 or myc-Cx43-Ub. Lysates were immunoprecipitated with polyclonal antibodies against Cx43 and the samples analysed by Western blot using polyclonal antibodies against Eps15 (upper panels) or monoclonal antibodies against Cx43 (lower panels). Eps15 displays increased interaction with Cx43-Ub when compared to Cx43. (B) Cos-7 cells were transfected with Cx43 and/or myc-Cx43-Ub alone or together with siRNA for Eps15. A non-targeting siRNA sequence was used for controls. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were analysed by Western blot using polyclonal antibodies against Cx43. Inputs were also analysed with polyclonal antibodies against Nedd4 and monoclonal antibodies against actin. Cx43 and Cx43-Ub both accumulate at the plasma membrane in cells depleted of Eps15 when compared to control cells. (C) Cos-7 cells were transfected with Cx43 and/or myc-Cx43-Ub alone or together with siRNA for Eps15. A non-targeting siRNA sequence was used for controls. Following detergent extraction, the Triton X-100 soluble and insoluble fractions were analysed by Western blot using polyclonal antibodies against Cx43 or Eps15 (S = Triton X-100 soluble fraction; I = Triton X-100 insoluble fraction). The Cx43-Ub construct is present in the Triton X-100 insoluble fraction, suggesting that it is present in gap junction plaques. Furthermore, both Cx43 and Cx43-Ub accumulate in both fractions when Eps15 is depleted.

**Figure 1**

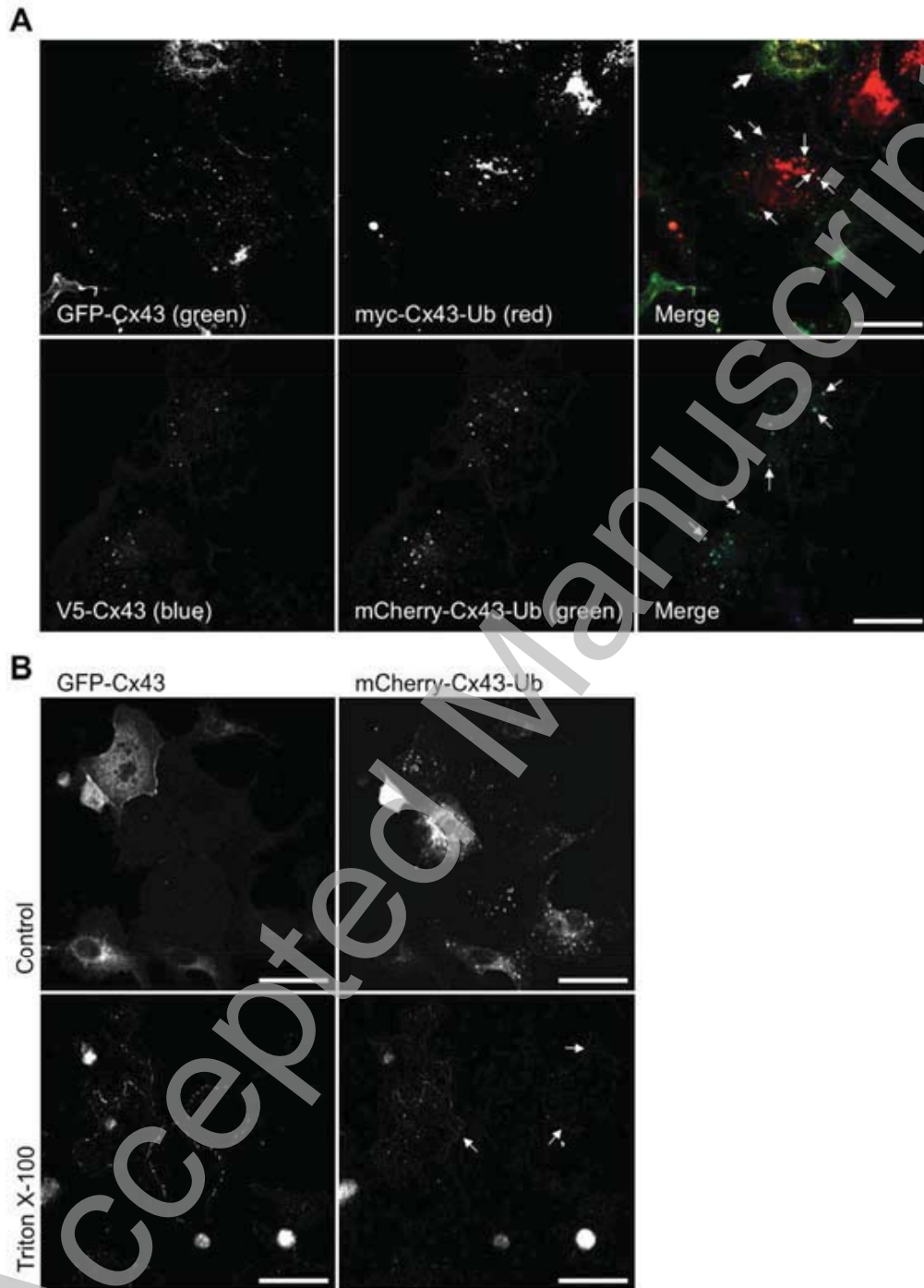
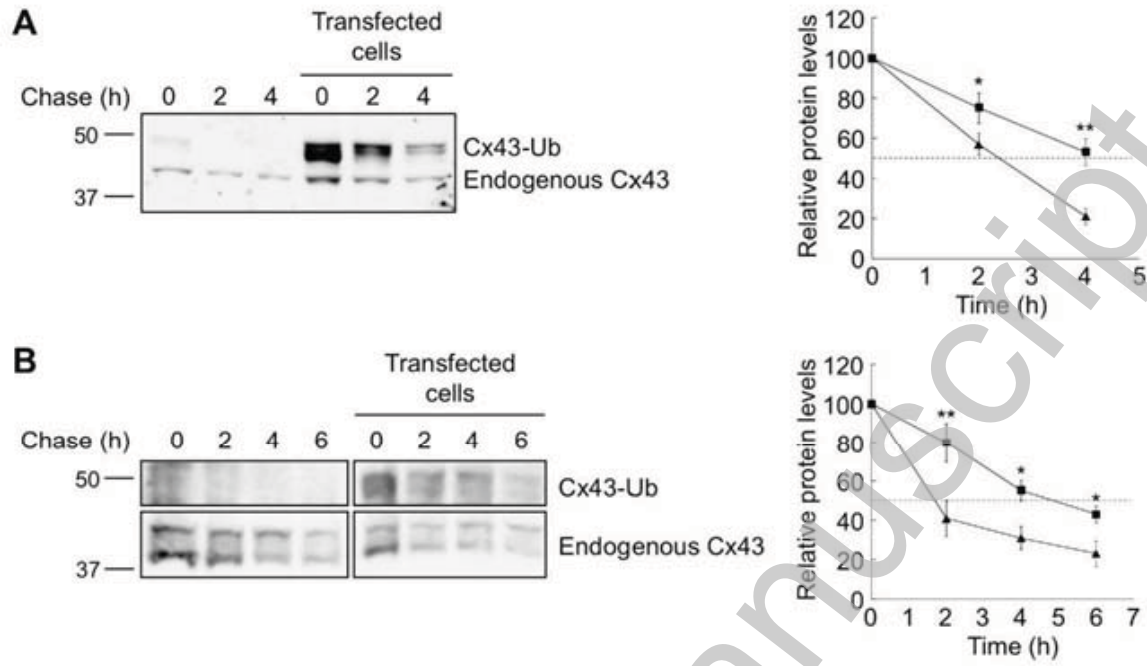
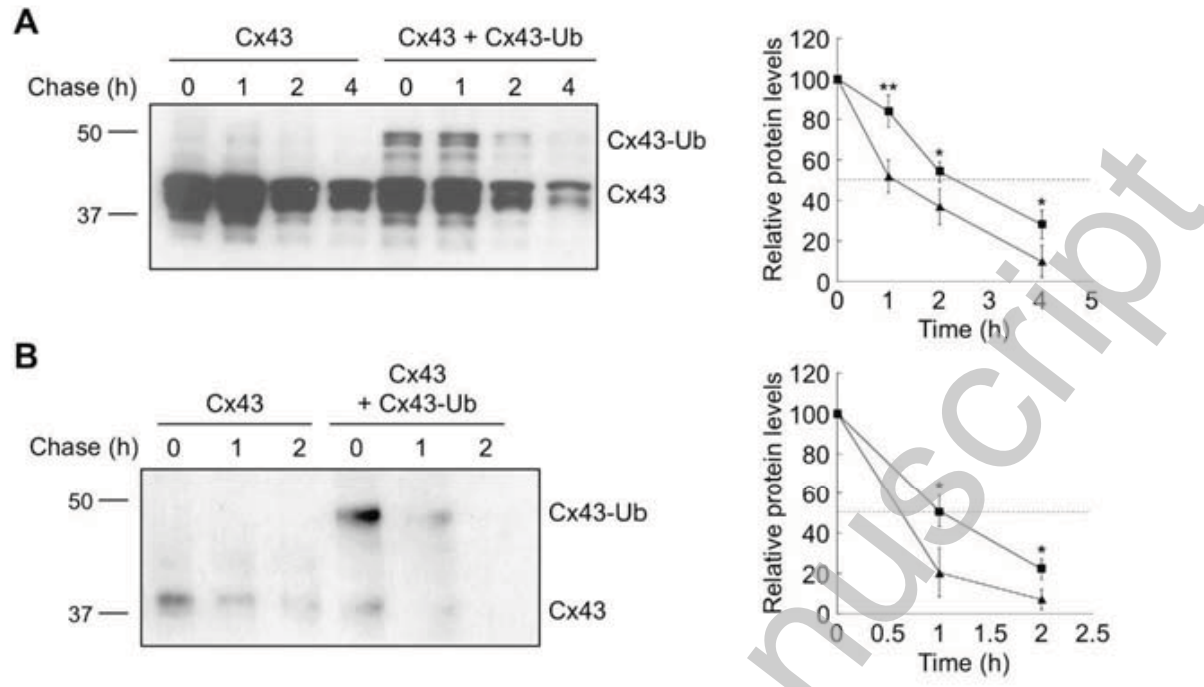


Figure 2

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**Figure 3**

**Figure 4**

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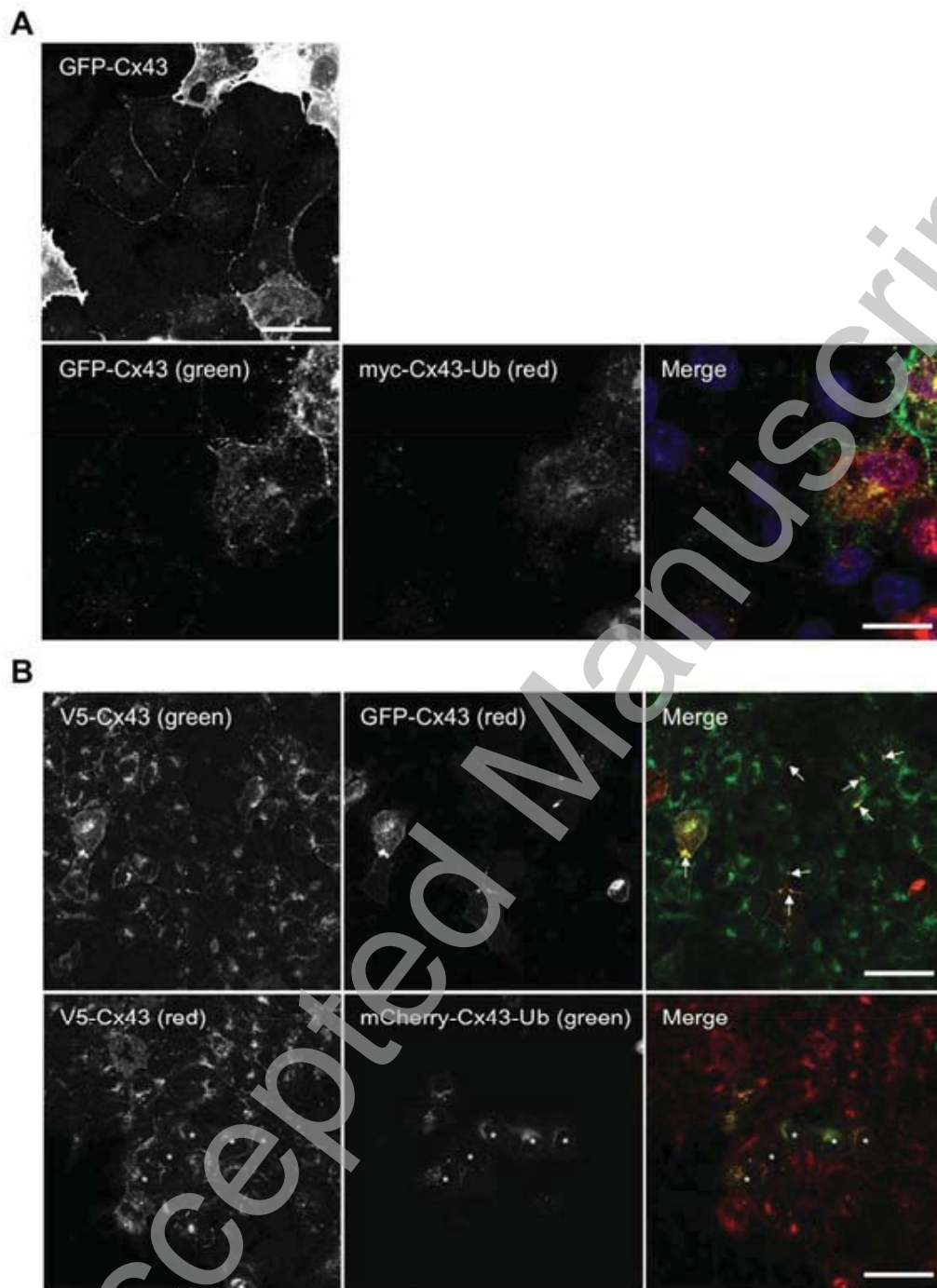


Figure 5

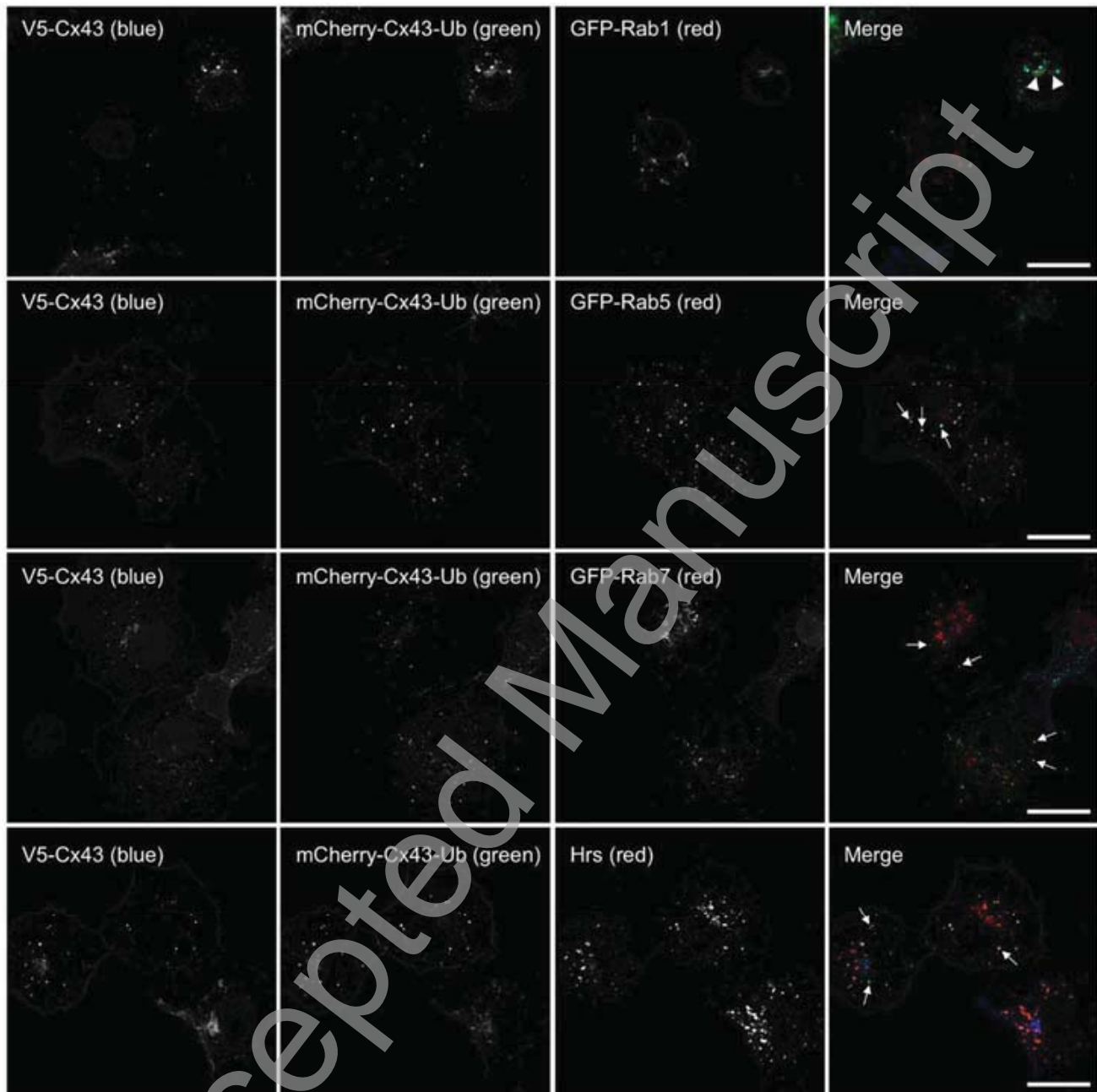
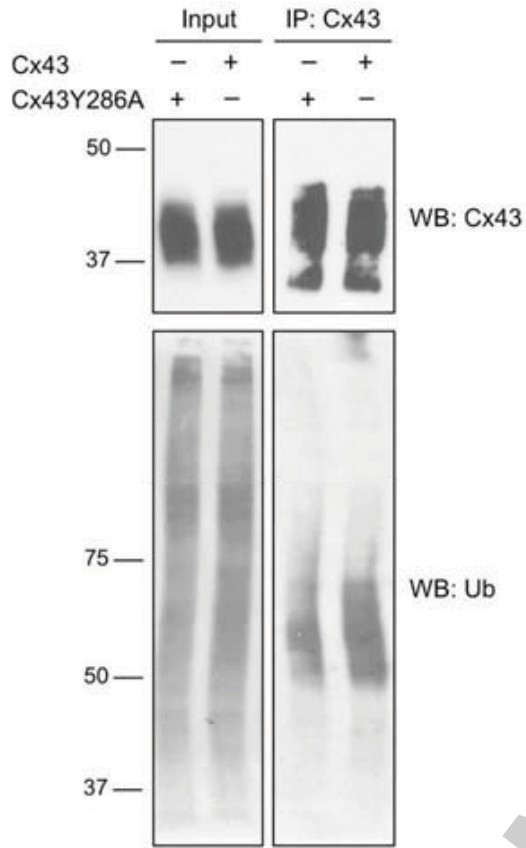
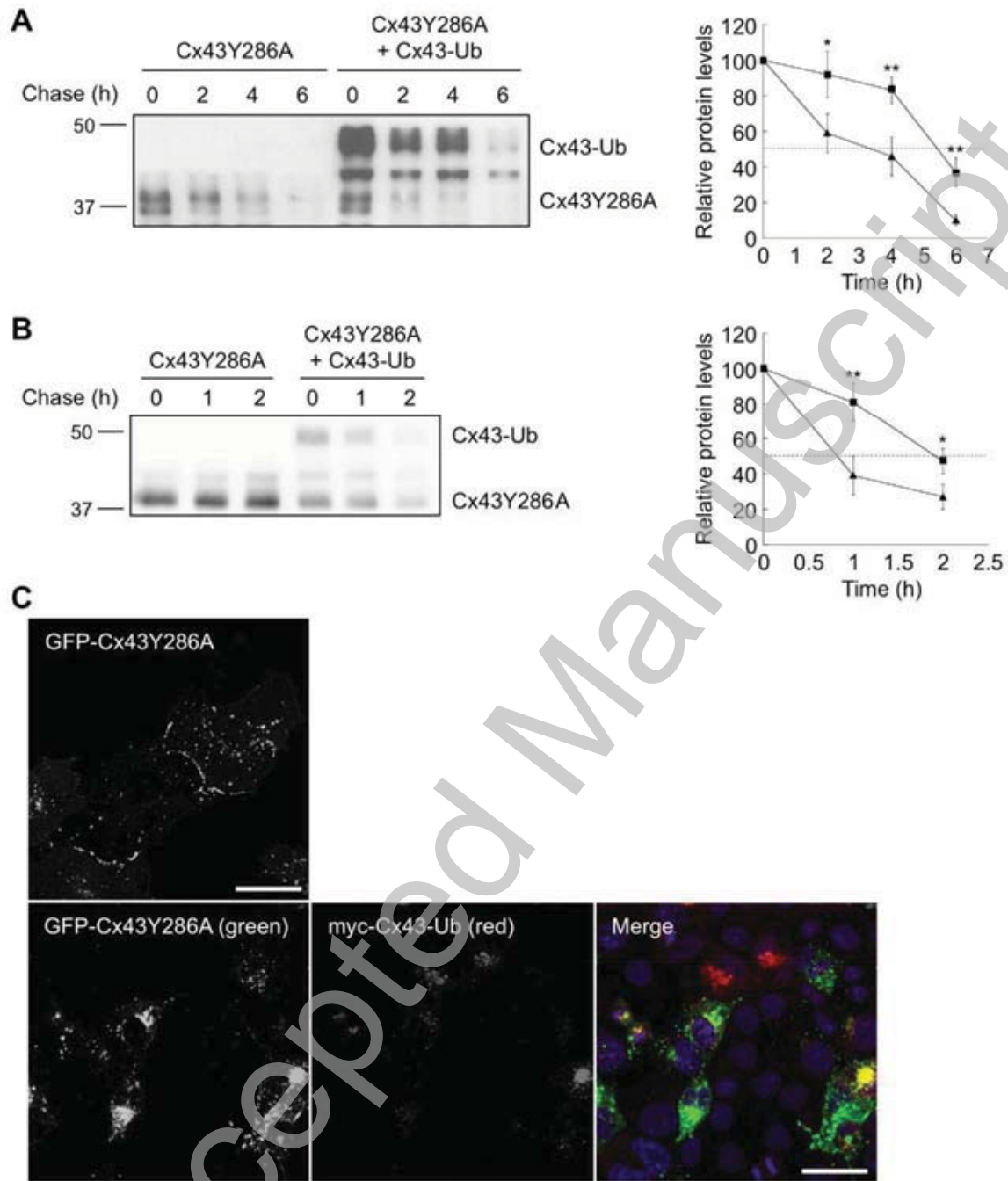


Figure 6

**Figure 7**

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**Figure 8**

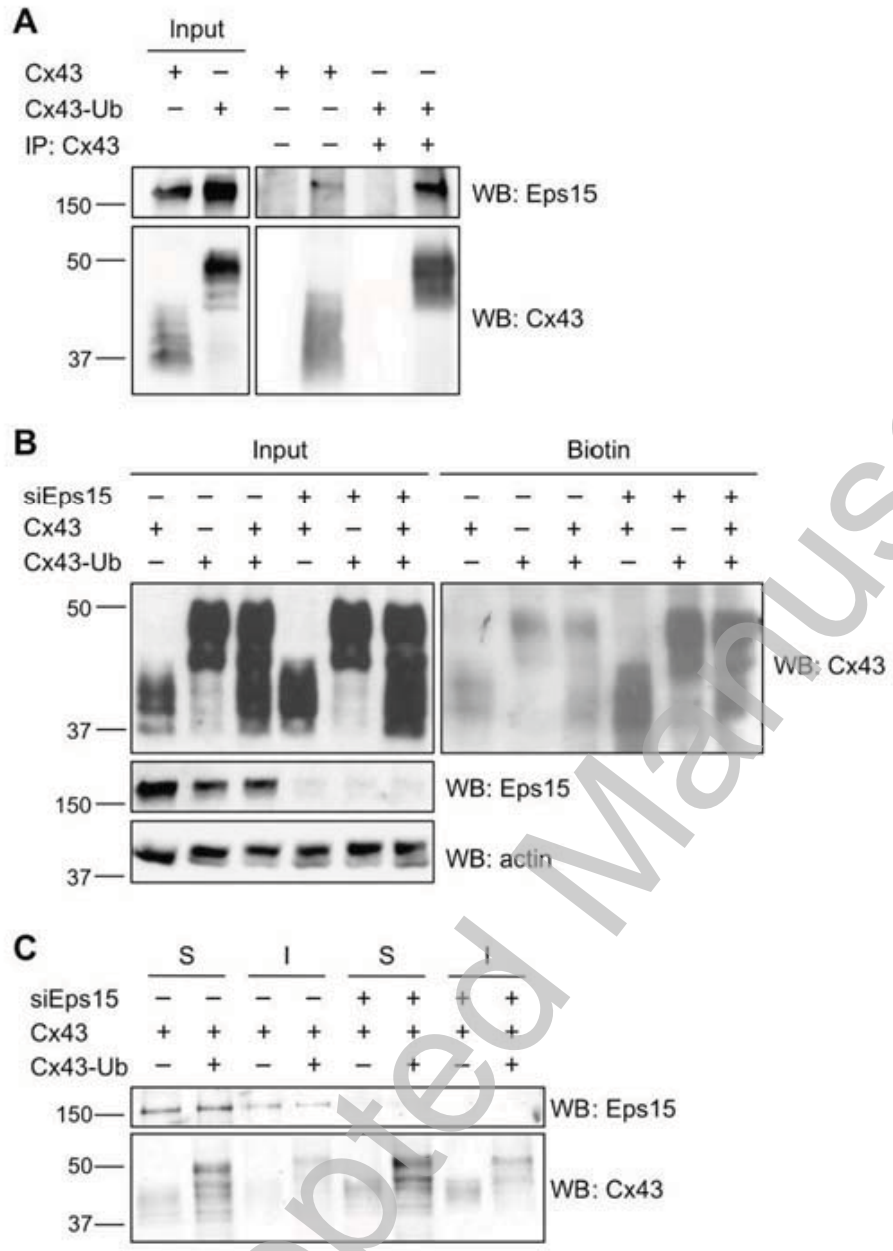


Figure 9

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