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Ability of a Connexin Chaperone to Rescue Oculodentodigital Dysplasia Mutant Connexin 43

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Abstract

Ability of a Connexin Chaperone to Rescue Oculodentodigital Dysplasia Mutant Connexin 43

By Huyen Tran T. Pham

Connexin 43 (Cx43) is a widely expressed gap junction protein responsible for forming multimeric channels enabling communication between two adjacent cells. Mutations in the GJA1 gene, which encodes for Cx43, can lead to physiological pathology as well as functional impairment at the cellular level. Oculodentodigital dysplasia (ODDD) is an autosomal dominant disorder resulting from mutations in the GJA1 gene. Characterization of the ODDD mutants G60S in the first extracellular loop, G138R in the intracellular loop, and R202H in the second extracellular loop indicates that most Cx43 proteins carrying these mutants localized in the endoplasmic reticulum (ER) or Golgi complex. Treatment of HeLa cells transiently transfected with ODDD mutants with the chemical chaperone 4-phenylbutyrate modulated Cx43 expression. However, trafficking of the mutant proteins were not altered by 4-PBA. Overexpression of the molecular chaperone ERp29, localized in the ER, was able to rescue the transport of G60S to the plasma membrane, but not the G138R or R202H mutants. These results suggest a differential effect of ERp29 on Cx43 mutants that may depend on the site of the mutation.

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LIST OF ABBREVIATIONS

4-PBA: 4-phenylbutyrate
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
CT: carboxyl terminus
Cx: Connexin
Cx43: Connexin 43
DMEM: Dulbeco's modified Eagle medium
DTT: dithioreitol
EGFP: enhanced green fluorescent protein
EL1: first extracellular loop
EL2: second extracellular loop
ER: endoplasmic reticulum
ERGIC: ER-Golgi intermediate complex
ERp29: endoplasmic reticulum protein 29
GJA1: gap junction protein, alpha 1
GS: goat serum
HeLa: Human cervical carcinoma cells
HRP: horseradish peroxidase
IL: intracellular loop
MOI: multiplicity of infection
NT: amino terminus
ODDD: oculodentodigital dysplasia
PBS: phosphate buffered saline
ROS: rat osteoblastic cells
TGN: trans Golgi network
TMD: transmembrane domain
WT: wildtype
(ZO)-1: zonula occludens protein 1

INTRODUCTION

Connexins (Cx) are gap junction proteins that enable direct connection between cells which allows for cellular communication and exchange of ions and molecules through formation of intercellular channels (Koval, 2008). Currently twenty-one human connexin genes have been identified. They are expressed by most cells of the body, which usually contain more than one isoform.

Connexins are named according to their predicted molecular weight. Connexin 43 (Cx43), which has a molecular mass of 43 kD, is the most commonly expressed and extensively studied connexin protein. Connexins can also be classified according to α or β subgroup based on amino acid homology (Koval, 2008). Cx43 belongs to the α family of gap junction proteins, thus the gene encoding for the protein is called gap junction protein, alpha 1 (GJA1).

Structure and Assembly of Connexins

The connexin protein consists of four transmembrane domains (TMD), two extracellular loops (EL1 and EL2), one intracellular loop (IL), and an amino (NT) and carboxyl terminus (CT) in the intracellular domain (Figure 1). There is considerable sequence conservation of the extracellular loops and transmembrane domains among connexin proteins. In contrast, the cytoplasmic loop and C-terminus exhibit the most variation in sequence and size.

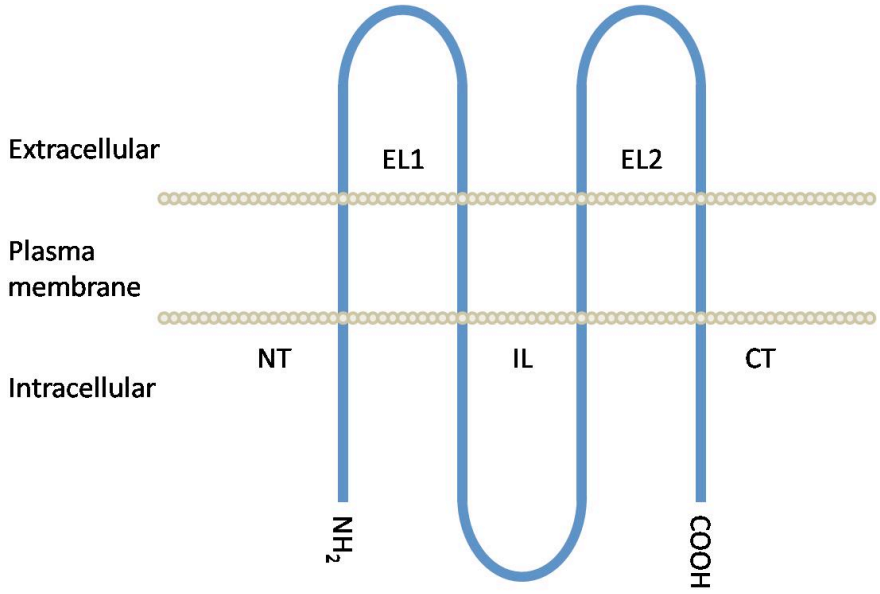

Figure 1. Structure of connexin proteins. Gap junction proteins consist of four transmembrane domain, amino (NT) and carboxyl terminus (CT) in the intracellular space, two extracellular loops (EL1 and EL2), and one intracellular loop (IL). The CT and IL exhibit the most sequence divergence whereas EL1, EL2, and NT are the most conserved.

A connexin channel consists of two hexameric hemichannels formed between two adjacent cells. The hemichannel in one cell is transported to the plasma membrane where it docks with the hemichannel of the opposite cell. Thus, each cell provides half of a functional gap junction. At sites where intercellular communication most frequently occurs, the channels tend to cluster together to form gap junction plaques. These plaques result in an array of aqueous channels with a diameter of 2 nm (Laird, 2006). In some cases, gap junctions can be made up of more than one type of connexin, such as the combination of Cx43 and Cx46. These channels, in which the hemichannels are composed of two different types of connexins, are known as heteromeric connexins (Koval, 2008).

Transmembrane proteins typically assemble in the endoplasmic reticulum (ER). For most connexins, oligomerization occurs before contact with the plasma membrane, though not necessarily in the ER. Different types of connexins assemble in different cellular compartments. Studies indicate that Cx43 remain as a monomer in the ER whereas Cx32 forms hexamers in the ER or ER-Golgi intermediate complex (ERGIC). Cx43 oligomerization is likely to occur later in the trans Golgi network (TGN) (Koval, 2008).

Connexin43 Pathophysiology

Mutations in connexin proteins has been associated with at least ten human diseases, including chromosome-X-linked Charcot-Marie-Tooth disease, oculodentodigital dysplasia (ODDD), and various skin diseases (Laird, 2010). Of particular interest is the autosomal dominant disorder ODDD, caused by defects in Cx43 proteins. The typical phenotype of ODDD is characterized by ophthalmic, dental, and craniofacial defects such as microcornea, narrow nose, cleft palate, syndactyly, and enamel hypoplasia. It also affects brain and bone development and in some cases, can play a role in causing congenital cardiovascular disease (W. A. Paznekas, et al., 2003).

At the genotypic level, ODDD is caused by mutations in the gene encoding for Cx43, GJA1. To date there are currently 62 known alterations to the DNA sequence, the majority of which occur in the first two-thirds of the coding region (Laird, 2010; W. Paznekas, et al., 2009). Most amino acid changes occur before residue 192 in the first half of the Cx43 protein. Mutations are equally divided between conserved extracellular

and transmembrane domain of the protein and the more divergent intracellular regions (W. Paznekas, et al., 2009).

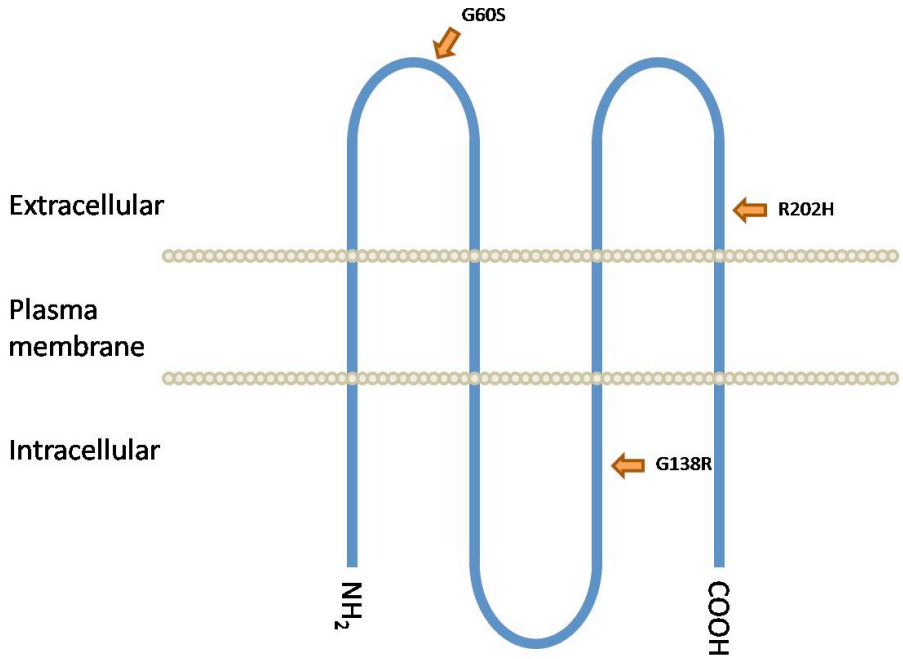

Figure 2. Location of mutant Cx43 amino acid substitutions G138R and R202H, located in the intracellular loop and second extracellular loop, respectively. The mouse mutant G60S is in the first extracellular loop.

Mutations can fall in regions important for maintaining function. For example, residues 58-60, 73-76 of EL1 and 201-206 of EL2 has been shown to play a role in forming gap junction channels. Coincidentally, ODDD mutations have also been found within the same region, located at residues 58, 59, 74, 76, 201, and 202. The intracellular loop can serve as a binding site for connexin-interacting proteins or the C-terminus of the connexin protein itself (W. Paznekas, et al., 2009). This study will examine the ODDD mutants G60S, G138R, and R202H (Figure 2). G138R and R202H are both amino acid

substitutions found in the intracellular loop and second extracellular loop, respectively. G60S mutant occurs in the first extracellular loop and is derived from a mouse mutant model (Flenniken, et al., 2005).

Not all mutants exert the same impact and the effects can vary. Some mutants may disrupt the ability to form plaques whereas others may affect secretory pathway trafficking (Laird, 2006). It is possible that mutations can interfere with the assembly process and result in misfolding, thereby affecting delivery to the plasma membrane. Several mutations have been found to cluster in the ER or TGN and fail to be delivered to their proper location. Fewer channels are formed, leading to a decrease in cellular communication.

Chaperones and Mutant Transmembrane Proteins

The assembly, function, and trafficking of connexin protein is a highly regulated process requiring interactions with many proteins. Cx43, like other connexin proteins, is subject to regulation by protein kinases, phosphatases, scaffolding proteins such as zonula occludens (ZO)-1, and cytoskeletal proteins (Laird, 2010). Among these are molecular chaperones that have the potential to mitigate defective Cx43.

The retention of connexins in intracellular compartments could be alleviated with chemical chaperones, which enhances a cell's ability to handle misfolded proteins. This is best exemplified in the case of the histone deacetylase inhibitor 4-phenylbutyrate (4-PBA) in cystic fibrosis cells (CF). Mutations in the gene that encodes for CF transmembrane conductance regulator (CFTR) causes the cell to express a defective

form of the protein, the most common being ΔF508 CFTR (Andersson & Roomans, 2000). Cells expressing the mutated version cannot transport the protein to the plasma membrane, leading to accumulation of ΔF508CFTR in the ER. However, treating these cells with 4-PBA enables the mutant CFTR to be properly trafficked to the plasma membrane (Andersson & Roomans, 2000). Recent studies also indicate that 4-PBA may regulate Cx43 oligomerization and transportation. Overexpression of Cx43 can cause intracellular plaques to form. In such cases, cells treated with 4-PBA were able to compensate for Cx43 overexpression (Sarma, 2008).

One possible mechanism for 4-PBA to mitigate protein trafficking is by influencing the expression levels of regulatory proteins, such as endoplasmic reticulum protein 29 (ERp29) (Suaud, et al., 2008). Like its namesake, ERp29 is a 29kD molecular chaperone associated in the ER. It is involved in facilitating the trafficking and folding of several proteins, including Cx43. Interaction of ERp29 with monomeric Cx43 confers stability and enables timely oligomerization (Das, et al., 2009). ERp29-deficient rat osteoblastic (ROS) cells have been shown to accumulate Cx43 intracellularly (Das, et al., 2009). This suggests the potential for chemical and molecular chaperones to rescue mutant Cx43 proteins whose ability to traffic and oligomerize may be impaired.

In this study, we examined whether 4-PBA or ERp29 has the ability to restore proper trafficking of the ODDD Cx43 mutants G60S, G138R, and R202H. HeLa cells transfected with the respective mutants and treated with 4-PBA or EGFP-ERp29 lentivirus were analyzed for Cx43 expression and localization patterns. Results suggested that 4-PBA did not have a significant effect on reducing intracellular localization.

However, HeLa cells overexpressing ERp29 were able to increase Cx43 trafficking to the plasma membrane for G60S mutants but not G138R or R202H.

MATERIALS AND METHODS

Antibodies and Reagents

Rabbit anti-Cx43 was from Sigma-Aldrich. Polyclonal rabbit anti-ERp29 was purchased from ABR. Monoclonal mouse anti-Cx43 was from Chemicon Fluorescent and horseradish peroxidase-conjugated (HRP) secondary antibodies were from Jackson ImmunoResearch Laboratories. Triton X-100 was from Roche Molecular Biochemicals.

Cell Culture

Human cervical carcinoma (HeLa) cells were cultured in Dulbeco's modified Eagle medium (DMEM) with L-glutamine supplemented with 5% fetal bovine serum (Atlanta Biologicals), 100 IU/ml penicillin, 100 μg/ml streptomycin. Transfected HeLa cells were maintained in the same medium as described above with the addition of 0.5 mg/ml Geneticin (G-418; Invitrogen).

Transfection

Before transfection, HeLa cells were placed in Opti-MEM (Invitrogen) with 4% FBS containing no antibiotics. Media was replaced with serum-free Opti-MEM before transiently transfecting HeLa cells with mutant G60S, G138R, R202H, and wildtype

Cx43 using Lipofectamine (Invitrogen) at 37°C. 4 μg of purified plasmid DNA were used for the 35-mm dishes and 8 μg were used for 60-mm dishes.

4-PBA Treatment

After HeLa cells were transfected with mutant Cx43 for 24 hours, the media was changed to selective DMEM containing 4mM 4-PBA per dish. Cells were analyzed 24 hours after treatment.

Lentiviral Vector Transduction

Following transfection with Cx43 mutants, HeLa cells were maintained and passed in selective DMEM. The transfected cells were replated at 4 x 10^5 per dish in selective DMEM containing lentivirus encoding for the enhanced green fluorescent protein tagged ERp29 (EGFP-ERp29). The cells were infected at 20 multiplicity of infection (MOI) for 48 hours before analysis.

Immunoblot Protein Analysis

Cells were placed in phosphate buffered saline (PBS; Sigma) with NaF, $NaVO_4$, and protease inhibitor (1 complete mini tablet/ 50 ml PBS; Roche). The scraped cells were centrifuged at 15000 rpm for 5 min. The cell pellets were resuspended in the same solution described above, sonicated, and analyzed for total protein using a protein assay kit (Bio-Rad). Samples were suspended in 2X sample buffer with 50mM dithioreitol (DTT) and 15 μg were loaded per lane on a 10% SDS-polyacrylamide gel. The samples were then transferred to Immobilon membranes (Millipore), blocked for at least an hour

at room temperature in PBS with 5% non-fat milk and 0.1% Tween. Membranes were incubated with antibodies described above for one hour, washed, and incubated with the appropriate HRP secondary antibody. Signals were visualized using enhanced chemiluminescence protocol. Total protein was normalized to β-actin.

Immunofluorescence

HeLa cells transfected with EGFP-ER29 lentivirus were fixed in 2% paraformaldehyde for 10 minutes at room temperature, followed by incubation for 10 min in 1M glycine dissolved in PBS. HeLa cells were washed with PBS twice before being fixed/permeabilized with MeOH:acetone (1:1). After rinsing with PBS three times, the cells were placed in PBS + 0.5% Triton X-100, PBS + 0.5% Triton X-100 + 2 % goat serum (GS). Cells were incubated with primary antibodies diluted in PBS/GS for 1 hour, washed with PBS/GS, and then labeled with secondary antibodies for 1 hour. After rinsing with PBS/GS and PBS three times each, cells were mounted in MOWIOL. Proteins were visualized using an X-70 microscope system (Olympus). Cells treated with 4-PBA followed the same protocol, except without paraformaldehyde fixation and incubation in glycine.

RESULTS

To determine the effects of 4-PBA on expression levels of Cx43, cells transiently transfected with mutant Cx43 were treated with 4mM of 4-PBA for 24 hr and analyzed

via immunoblot (Figure 3, A and B). Untreated cells were used as a control. In cells not treated with 4-PBA, there were more G60S and G138R mutants expressed compared to wildtype (WT) .The R202H mutant had comparable levels of expression as WT Cx43. Administration of 4-PBA did not change Cx43 protein levels for HeLa cells transfected with wildtype Cx43 or the R202H mutant. Results show that Cx43 protein levels for G60S and G138R mutants decreased to levels similar to the wildtype after 4-PBA treatment (Figure 3, C).

In order to examine whether 4-PBA affected trafficking of mutant proteins, transiently transfected cells were immunolabeled and analyzed using immunofluorescence microscopy (Figure 3, D – K). Untreated G60S (Figure 3E), G138R (Figure 3F), and R202H mutants (Figure 3G) all localized in the intracellular space, although a few were able to dock at the plasma membrane. Wildtype Cx43 localized at the cell surface, with some subgroups exhibiting intracellular localization (Figure 3, D). For all three mutants, results indicated that treatment with 4-PBA did not alter trafficking of the mutant proteins (Figure 3, I – K). Localization of Cx43 WT to the plasma membrane was unchanged by 4-PBA (Figure 3H).

To examine the effects of ERp29 on Cx43 expression, HeLa cells transfected with ODDD mutants and wildtype Cx43 were passed and kept in selective media before infection with lentiviral vectors containing EGFP-tagged ERp29 for 48 hours. Cells were assessed for Cx43 and ERp29 expression by immunoblot analysis (Figure 4, A – C).

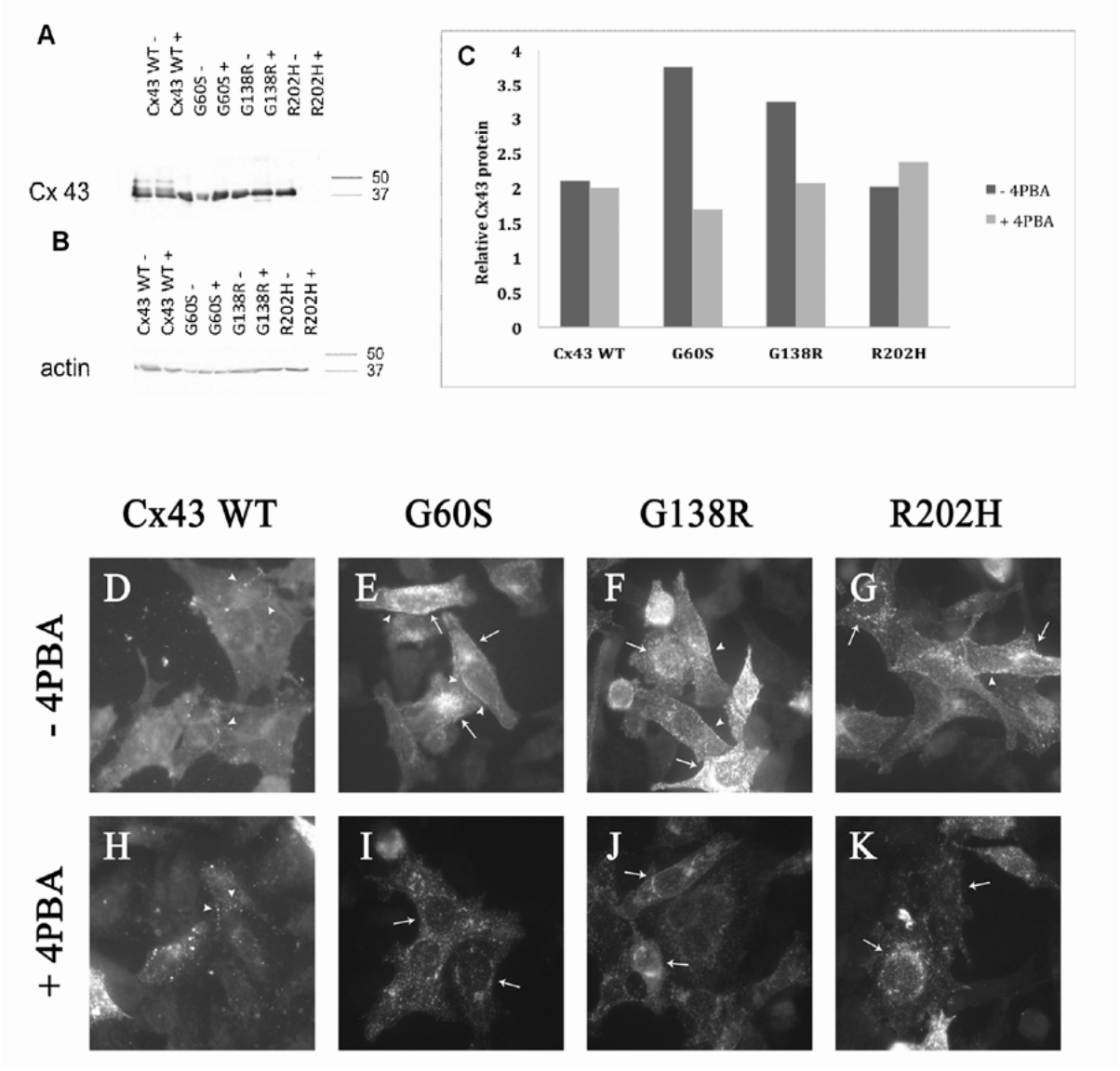

Figure 3. Expression and localization of wildtype and mutant Cx43 proteins. HeLa cells transiently transfected with Cx43 WT, G60S, G138R, and R202H for 24 hours followed by 24 hour-treatment with 4 mM 4-PBA were analyzed for expression of Cx43 protein by Western immunoblot. Membranes were probed with antibodies recognizing Cx43 (A) and β-actin (B). Quantification of Cx43 protein relative to actin indicated that administration of 4-PBA decreased Cx43 expression of G60S and G138R to levels comparable to wildtype, but had no effect on R202H, which was not overexpressed (C). Immunfluorescence images of untreated cells showed that mutant Cx43 proteins primarily localized in the intracellular space (arrows) while some subgroups docked to the plasma membrane (arrowhead) (E - G). Cx43 WT localized to the cell surface (D). The G60S, G138R, and R202H mutants treated with 4-PBA remained localized in intracellular compartments (I, J, K, respectively). Trafficking of Cx43 were unaltered by 4-PBA and could be transported to the cell surface (H).

Results confirmed the expression of EGFP-ERp29 by mutant and wildtype cells, indicated by the band near 50 kD (Figure 4B). Total ERp29 levels increased dramatically for Cx43 WT, G60S, G138R, and R202H mutants transfected with EGFP-ERp29 (Figure 4E). Cx43 expression levels increased slightly when ERp29 is overexpressed for the G138R mutant but not for the wildtype, G60S, or R202H mutants (Figure 4, D). A band for Cx43 was not seen for the HeLa cells transiently transfected with wildtype Cx43, suggesting that cDNA encoding for Cx43 had been lost.

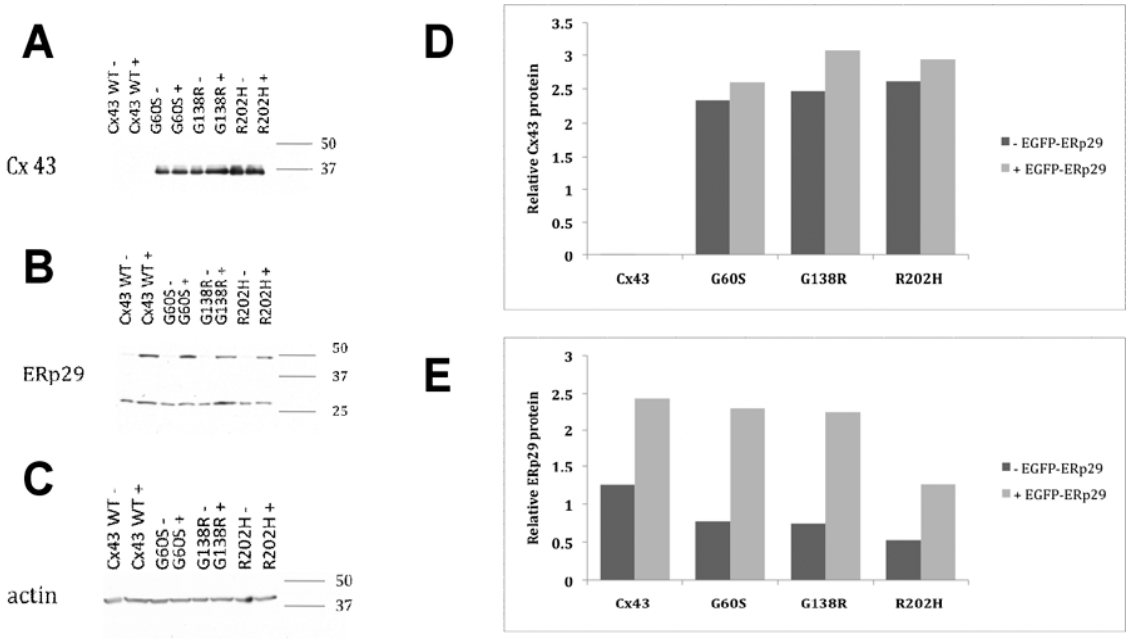

Figure 4. Expression of wildtype and mutant Cx43 proteins after transfection with EGFP-ERp29 lentivirus. HeLa cells transfected with Cx43 WT, G60S, G138R, and R202H were cultured and passed in selective media before tranfection with EGFP-ERp29 lentivirus. After 48 hours, cells were harvested and immunoblotted for Cx43 (A) and ERp29 (B). Protein expression levels were normalized to β-actin (C). Western immunoblotting showed that Cx43 remained unaltered (D) despite overexpression of ERp29 after treatment (E). Dark columns denote untreated cells and lighter columns represent cells transfected with EGFP-ERp29 lentivirus.

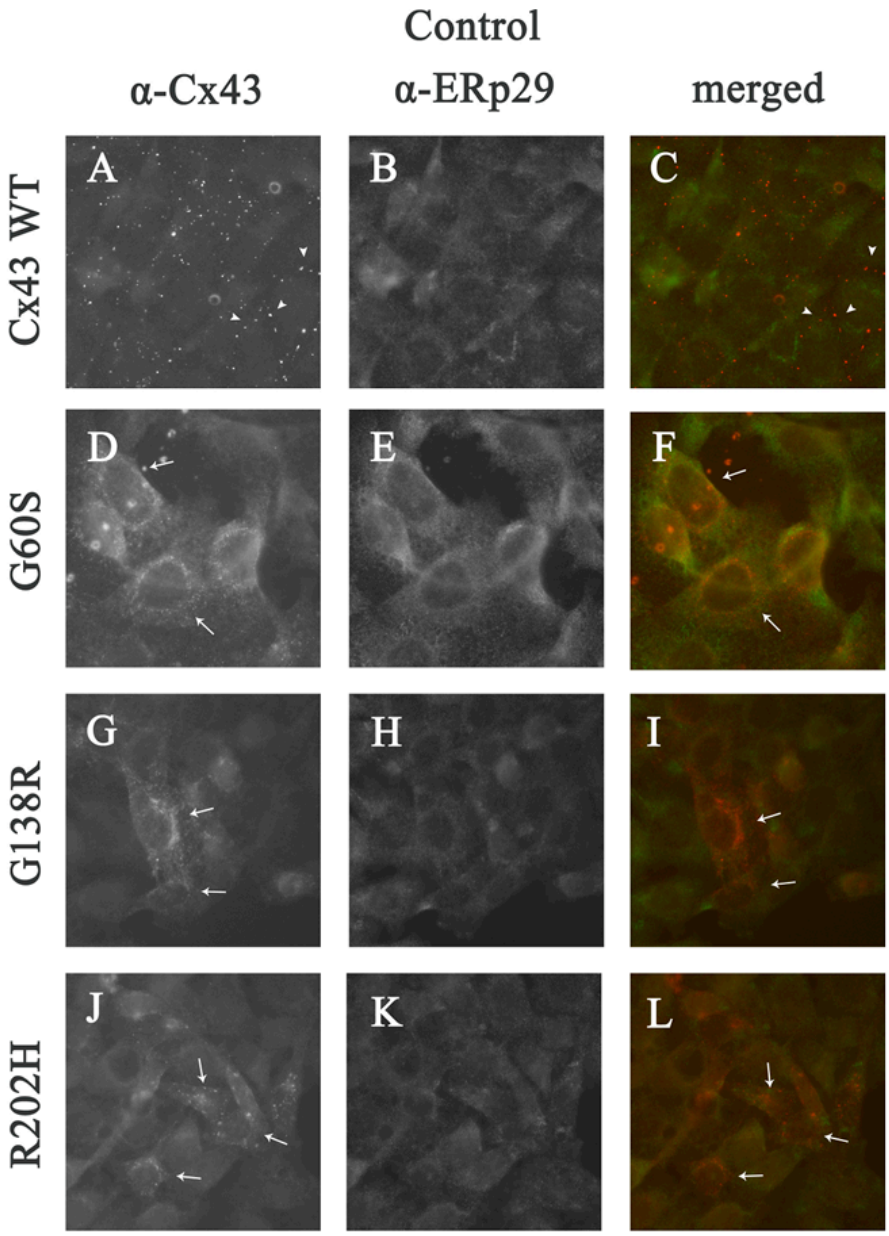

Figure 5. Immunofluorescence images of untreated cells. HeLa cells were double-labeled with Cx43(A, D, G, J) and ERp29 (B, E, H, K). Merged images are in C, F, I, and L, with Cx43 shown in red and ERp29 in green. All Cx43 mutants primarily localized in the ER or Golgi complex (arrows) (D – L). HeLa cells stably expressing WT Cx43 were transported to the plasma membrane (arrowhead) (A-C).

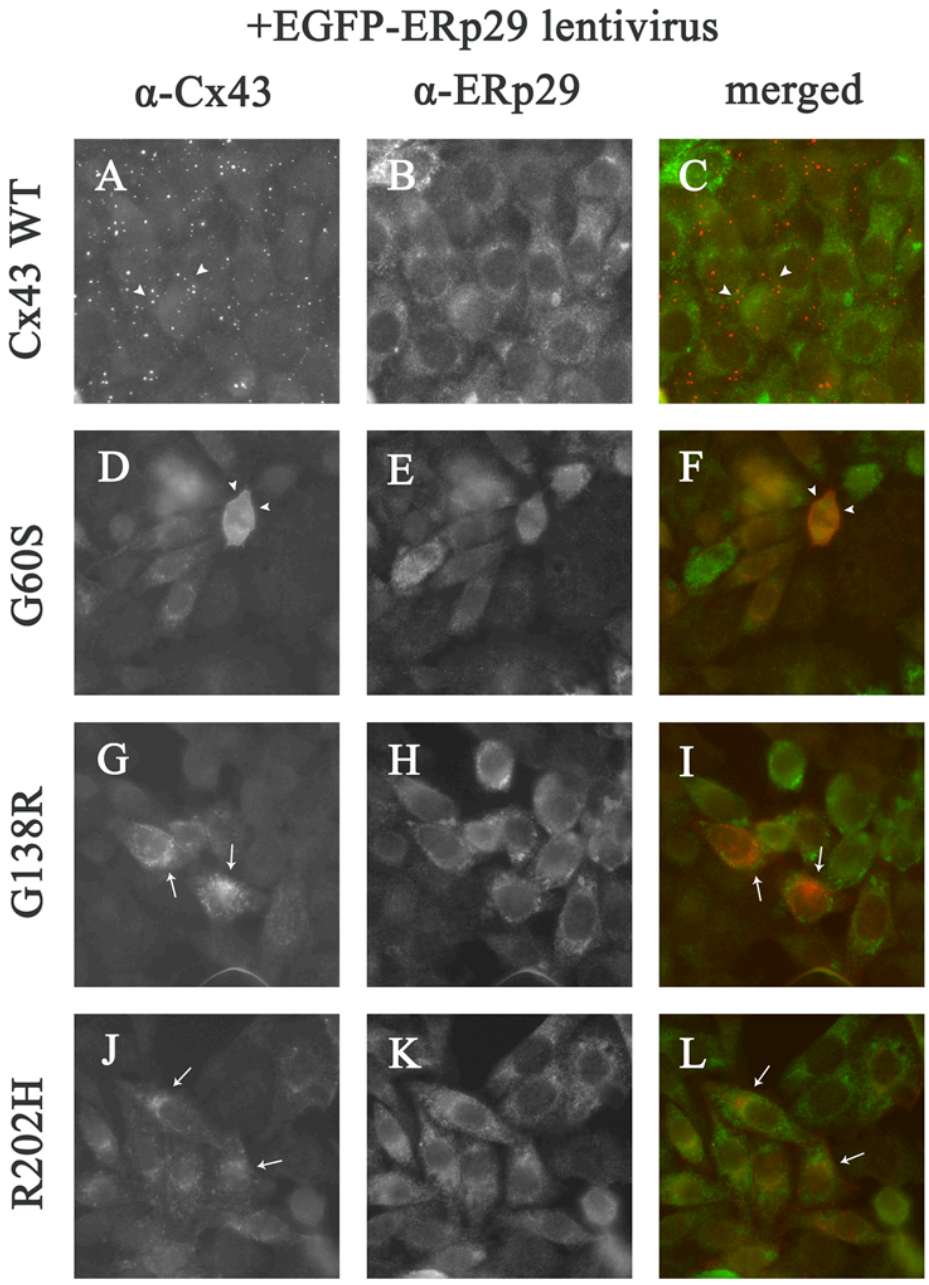

Figure 6. Immunostained images of cells overexpressing ERp29. HeLa transfected with Cx43 WT, G60S, G138R, and R202H were cultured and maintained in selective media before transfection with lentivirus vector containing EGFP-ERp29 for 48 hours. Cx43 were trafficked to the plasma membrane in HeLa cells stably expressing wildtype Cx43 (arrowhead) (A – C). G60S mutants were also transported to the plasma membrane following treatment (D – F). Localization patterns of the G138R and R202H mutants did not change with ERp29 overexpression. Mutant Cx43 were retained in the intracellular space (arrows) in the same manner as untreated cells (G, I, J, L).

Cx43 localization visualized using immunofluorescence microscopy for cells transfected with mutant Cx43 and maintained under selective pressure were similar to the transiently transfected cells. Stably transfected Cx43, used as a control, trafficked to the plasma membrane both with and without ERp29 overexpression (Figure 5, A – C and Figure 6, A – C). Cx43 with the G60S mutation was confined to the intracellular space under control conditions (Figure 5, D – F). After G60S mutant cells were transfected with the vector containing EGFP-ERp29, gap junction plaques were able to form at the plasma membrane (Figure 6, D – F). Both Cx43 proteins with the G138R (Figure 5, G – I) and R202H (Figure 5, J – L) mutations localized predominantly at the ER when only endogenous ERp29 were expressed. The addition of EGFP-ERp29 was not able to rescue the G138R (Figure 6, G – I) or R202H Cx43 mutants (Figure 6, J – L) to the plasma membrane.

DISCUSSION

Characterization of the three different ODDD mutants G60S, G138R, and R202H showed different localization patterns compared to the wildtype Cx43 protein. Although mutant Cx43 transiently transfected into GJIC-deficient HeLa cells were all able to traffic to the plasma membrane, the degree of plaque formation at the cell surface was much lower than wildtype Cx43. Consistent with results found by Shibayama et al. (2005), the R202H mutants localized primarily in the intracellular space. However, a few of the R202H mutants were able to simultaneously accumulate in intracellular compartments and traffic to the plasma membrane. This is similar to results found by McLachlan, et al.

(2005), but the extent of intracellular localization was greater here (Figure 3G and Figure 5L). Cells expressing the G60S and G138R mutations also exhibited similar patterns. Most of the mutant Cx43 proteins were retained intracellularly while a small subpopulation was transported to the cell surface. Expression of the Cx43 mutants G60S and G138R were much higher compared to wildtype. Roscoe et al. (2005) also noted a similar finding for the G138R mutant.

Treatment with the chemical chaperone 4-PBA was able to modulate Cx43 levels of the G60S and G138R mutants to levels similar to the wildtype, but had no effect on the R202H mutant which was not expressed in excess. Previous work on 4-PBA also indicates that it can compensate for overexpressed Cx43 (Sarma, 2008). However, results from this experiment suggest that 4-PBA is not able to alter localization of the G60S, G138R, and R202H mutants. Trafficking of Cx43 may rely on other factors not regulated by 4-PBA. In addition, because 4-PBA is a histone deacetylase inhibitor, it may influence other unidentified proteins whose effects on Cx43 trafficking and folding are not yet known.

The ability of 4-PBA to influence protein folding and secretion may be due to its influence on cellular levels of ERp29. ERp29 has been shown to stabilize monomeric wildtype Cx43 (Das, et al., 2009). Here, we show that overexpression of ERp29 by inducing the expression of EGFP-ERp29 was able to rescue the G60S, but not the G138R and R202H mutants to the plasma membrane. The differential effects of ERp29 on Cx43 mutant localization may be explained by binding site preference. Little is known about ERp29 binding sites with Cx43, but it has been suggested to occur at the second extracellular loop, where the R202H mutation is located (Das, et al., 2009). Mutations in

this conserved region may prevent proteins like ERp29 from binding with Cx43. Point mutations in the EL2 region (F199L, R202E, and E205H) have also been unable to localize to the plasma membrane (Olbina & Eckhart, July 2003). This raises the possibility that the second extracellular loop plays a crucial role in enabling Cx43 interaction with proteins, which consequently affects trafficking and oligomerization.

Investigating the binding sites for ERp29 can help elucidate the mechanism of this chaperone and its impact on mutant Cx43 proteins. In addition, the effects of ERp29 on mutant Cx43 can be explored further by conducting functional assays to see whether it can restore channel permeability. Future studies can also include conducting this experiment on cells stably expressing transfected Cx43 mutants in order to ensure uniform protein expression levels and formation of gap junctions.

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