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# A cataract-causing connexin 50 mutant is mislocalized to the ER due to loss of the fourth transmembrane domain and cytoplasmic domain<sup>☆</sup>

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## ABSTRACT

Mutations in the eye lens gap junction protein connexin 50 cause cataract. Earlier we identified a frameshift mutant of connexin 50 (c.670insA; p.Thr203AsnfsX47) in a family with autosomal recessive cataract. The mutant protein is smaller and contains 46 aberrant amino acids at the C-terminus after amino acid 202. Here, we have analysed this frameshift mutant and observed that it localized to the endoplasmic reticulum (ER) but not in the plasma membrane. Moreover, overexpression of the mutant resulted in disintegration of the ER-Golgi intermediate compartment (ERGIC), reduction in the level of ERGIC-53 protein and breakdown of the Golgi in many cells. Overexpression of the frameshift mutant partially inhibited the transport of wild type connexin 50 to the plasma membrane. A deletion mutant lacking the aberrant sequence showed predominant localization in the ER and inhibited anterograde protein transport suggesting, therefore, that the aberrant sequence is not responsible for improper localization of the frameshift mutant. Further deletion analysis showed that the fourth transmembrane domain and a membrane proximal region (231–294 amino acids) of the cytoplasmic domain are needed for transport from the ER and localization to the plasma membrane. Our results show that a frameshift mutant of connexin 50 mislocalizes to the ER and causes disintegration of the ERGIC and Golgi. We have also identified a sequence of connexin 50 crucial for transport from the ER and localization to the plasma membrane.

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## 1. Introduction

Cataract is a visible opacity in the eye lens leading to partial or complete loss of transparency. It accounts for a major proportion of impaired vision and blindness worldwide. Congenital cataract is the leading cause of reversible blindness in childhood [1,2] and inherited cataracts account for 8–25% of congenital cataracts [2]. It is genetically heterogeneous and mutations in several classes of candidate genes including crystallins and connexins have been linked to the disease [2]. The eye lens is a transparent, avascular organ that transmits and focuses light energy on the retina. The human lens has three main

parts, viz., lens capsule, lens epithelium and lens fibres. During the process of lens development towards the stage of maturation, human lens undergoes denucleation and degradation of various cytoplasmic organelles in the lens fibre cells to establish and maintain lens transparency [3,4]. The lens has developed an extensive cell to cell communication system via gap junction channels so that the cells in the central region can communicate with the cells on the surface. This is necessary for the exchange of ions and metabolites in the avascular lens, thus crucial for maintaining lens homeostasis and transparency [4].

Gap junctions are transmembrane channels formed by connexins allowing the exchange of small molecules and ions ( $M_r \leq 1000$  Da) between two closely apposed neighbouring cells [5]. Each connexin sub-unit consists of four  $\alpha$ -helical transmembrane domains (M1–M4), two extracellular loops (E1 and E2), one cytoplasmic loop (CL) between second and third transmembrane domain and cytoplasmic amino terminal (NT) and carboxy terminal (CT) domains [6,7]. Six such connexin sub-units assemble to form a functional membrane hemichannel, also called connexon. Different connexin isoforms interact selectively with each other resulting in the assembly of homo-oligomeric or hetero-oligomeric connexons [7,8]. When two cells are

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Abbreviations: Cx50, connexin 50; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; TGN, trans-Golgi network

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closely apposed, connexon in one cell docks with its counterpart in the apposing cell forming a gap junction. Connexins are synthesized in the endoplasmic reticulum (ER) and are oligomerized in the ER, Golgi or *trans*-Golgi network, depending on the type of connexin, to form connexons [8–10]. These connexons finally move to the plasma membrane in vesicular structures along microtubules. The domain requirements for targeting or transport of connexins to the plasma membrane are not clear. Connexins are ubiquitously expressed in mammalian cells and in humans 21 different connexin genes are reported so far, mutations in which cause various diseases including cataracts [1,7,11]. In the eye, three lens specific connexins exist where *GJA1*(Cx43) is specific to lens epithelial cells, *GJA3*(Cx46) in lens fibre cells and *GJA8*(Cx50) in both lens epithelium and fibre cells [7]. Mutations in connexin 50 (Cx50) occur in all parts of the gene [7,12–23] and no correlation has been detected between the mutation location and cataractogenesis. Majority of the mutations known cause autosomal dominant cataracts and only two mutations of recessive inheritance are reported [6,18,20,21]. Studies using knockout mouse models showed that the deletion of Cx50 results in significantly reduced size of lens and eye (microphthalmia) in addition to mild nuclear cataract [24].

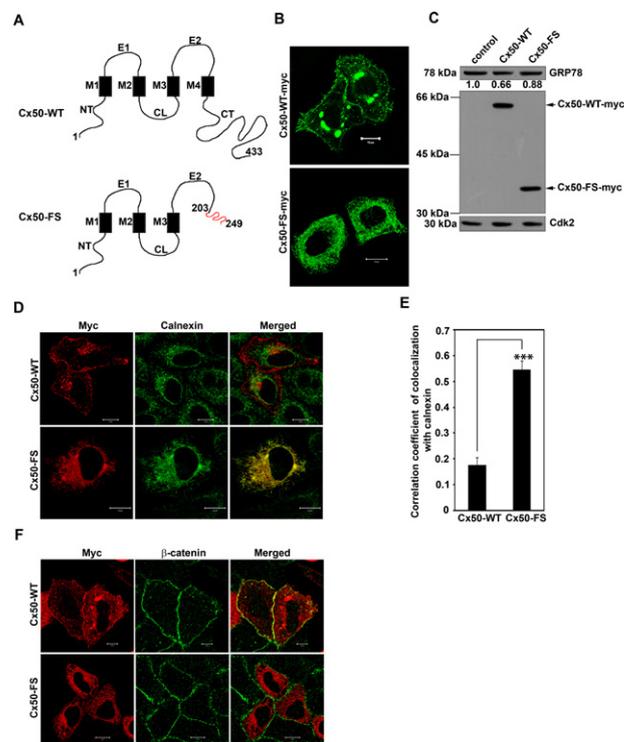
The mutant of Cx50 studied here was reported earlier by us in a South Indian family with autosomal recessive cataract (c.670insA; p.Thr203AsnfsX47) [20]. This mutation is a single base insertion in the codon 203, which is in the second extracellular domain, leading to frameshift and translation of 46 aberrant amino acids followed by truncation. This causes loss of fourth transmembrane domain and intracellular carboxy terminal domain. The schematic of wild type connexin 50 (Cx50-WT) and the frameshift mutant (Cx50-FS) are shown in Fig. 1(A).

Here, we have analysed subcellular localization and consequence of expression of this frameshift mutant. We observed that the mutant protein is localized mainly in the ER and does not go to the plasma membrane. We also explored the mechanism of this altered localization by analysing the localization of various deletion mutants. Our results show that the aberrant sequence generated by frameshift is not responsible for altered sub-cellular localization. We also show that 4th transmembrane domain and a membrane proximal region of C-terminal cytoplasmic domain are important for localization of connexin 50 to the plasma membrane. The frameshift mutant causes disruption of protein trafficking and breakdown of organelles of the early secretory pathway. Our results suggest that defective transport and localization of the frameshift mutant to the plasma membrane is likely to cause loss of connexin 50 function leading to recessive cataract.

## 2. Materials and methods

### 2.1. Expression vectors

The study protocol had been approved by the institutional review board of the L.V. Prasad Eye Institute where the subjects were recruited after complete ophthalmic examination. Blood samples were obtained after informed written consent from the participants. Human wild type connexin 50 (Cx50) and Cx50-FS mutant were amplified from the DNA isolated from the patient's blood samples using gene specific primers with *EcoRI* and *HindIII* sites in the forward and reverse primers, respectively. The amplified product was cloned in the *EcoRI* and *HindIII* sites of pcDNA 3.1/myc-His(-) A vector with myc and His tag at the 3'-end of the cDNA. Cx50 was also cloned in the *EcoRI* and *XhoI* sites of pEGFP-N1 vector with GFP tag at the 3'-end of the cDNA. Similar to Cx50, deletion constructs Cx50-202, Cx50-230 and Cx50-294 were also cloned in the *EcoRI* and *HindIII* sites of the pcDNA 3.1/myc-His(-) A vector with myc and His tag at the 3'-end of the cDNA. GFP-TC48 has been described previously [25].



**Fig. 1.** Sub-cellular localization of wild type and the frameshift mutant of connexin 50. (A) Line diagram depicting the domain structure of wild type connexin 50 (Cx50) and its frameshift mutant (Cx50-FS). (B) HeLa cells grown on coverslips were transfected with either Cx50-myc or Cx50-FS-myc constructs and were fixed 25 h post transfection. Cells were stained with anti-myc mouse monoclonal antibody and analysed by confocal microscopy. (C) Western blot showing expression of Cx50 and Cx50-FS constructs. Cdk2 was used as a loading control. (D) Cx50-myc and Cx50-FS-myc transfected cells were stained with anti-myc antibody (red) and calnexin rabbit polyclonal antibody (an ER marker) (green) and colocalization was observed by confocal microscopy. Colocalization of the frameshift mutant with calnexin was shown by merging of red with green showing yellow colour in the merged image panel. Scale bar: 10  $\mu$ m. (E) The graph shows the correlation coefficients of colocalization of Cx50 and Cx50-FS with calnexin. The data shown here is the mean  $\pm$  SD of 80 cells with each construct. \*\*\* $p$  < 0.001. (F) HeLa cells transfected with Cx50 and Cx50-FS-myc constructs were immunostained with anti-myc antibody (red) and  $\beta$ -catenin mouse monoclonal antibody (green) and analysed by confocal microscopy. Colocalization of the Cx50 and  $\beta$ -catenin at the cell junctions can be seen by bright yellow colour at the appositional membranes whereas Cx50-FS showed intracellular localization and no colocalization with  $\beta$ -catenin at the plasma membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 2.2. Antibodies

Calnexin, anti-myc (rabbit polyclonal and mouse monoclonal), mouse monoclonal  $\beta$ -catenin, rabbit polyclonal TGN-38 and rabbit polyclonal cdk2 antibodies were from Santa Cruz Biotechnology. Mouse monoclonal anti-ERGIC-53 antibody was obtained from Enzo Lifesciences. The rabbit polyclonal anti-Giantin antibody was from Covance. The rabbit polyclonal anti-Sec24C antibody was a gift from Dr. William E. Balch (Scripps Research Institute). The mouse monoclonal  $\beta$ -cop antibody was from Sigma. The mouse monoclonal GAPDH antibody was from Millipore. Secondary antibodies Cy-3 conjugated anti-mouse and anti-rabbit IgG were from Amersham. Alexa-488 and Alexa-633 conjugated anti-mouse and anti-rabbit IgGs and Alexa-594 conjugated anti-rabbit IgG were from Molecular Probes. HRP conjugated anti-mouse and anti-rabbit IgGs were from Amersham.

### 2.3. Cell culture and transfections

Hela cells were grown as monolayers in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Transient transfections in Hela cells were performed using Qiagen column (Qiagen, Valencia, CA) purified plasmids and Lipofectamine PLUS or Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. For immunofluorescence staining, cells grown on coverslips were transfected with the required plasmids. For Cos-1 cells transfections were done with cationic lipid DHDEAB as described [26,27].

### 2.4. Indirect immunofluorescence and confocal microscopy

Indirect immunofluorescence was carried out essentially as described previously [27,28]. Cells grown on coverslips were transfected with required plasmids and after 25 h fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. These cells were washed with PBS three times and permeabilized for 6 min with 0.5% Triton-X-100 and 0.05% Tween-20 prepared in PBS at room temperature. After three washes with PBS, the permeabilized cells were incubated with 2% BSA in PBS for 1 h at room temperature to prevent non-specific antibody binding. Cells were then incubated in required dilution of primary antibody in 2% BSA for 2 h at room temperature or overnight at 4 °C depending on the antibody. Unbound antibodies were removed by washing with PBS and cells were stained with secondary antibody for 1 h. After washing with PBS again, stained cells were mounted with Vectashield mounting medium (Vector labs, Molecular Probes) containing DAPI.

For studying colocalizations, stained coverslips were observed under LSM 510 Meta Confocal Microscope and images were captured using 63X/1.4 NA oil immersion objectives. Similar parameters of image capture were used for analysis of cells belonging to a particular experiment. Colocalizations were determined by observing the staining pattern and two middle sections were projected to observe colocalization using LSM 510 (version 3.2) software. Colocalizations were quantitated by calculating Pearson's correlation coefficients using the LSM 510 software.

### 2.5. Co-immunoprecipitation

Hela cells plated in 35-mm dishes were transfected with required plasmids, overexpressed for 30 h, lysed in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, 1.0% Triton-X-100, 1 mM PMSF, 5 mM EDTA, protease inhibitor cocktail (Roche Biochemicals) for 20 min at 4 °C. Lysates were collected and centrifuged at 10000 g for 14 min at 4 °C. The supernatant was used for immunoprecipitation with 2 µg of c-myc rabbit polyclonal antibody or 2 µg of normal rabbit IgG control antibody and incubated for 8 h at 4 °C. The complexes were precipitated using protein A/G Plus agarose beads (Santa Cruz Biotechnology), washed and lysed in 20 µl of 3X SDS-sample buffer (180 mM Tris-HCl pH 6.8, 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol, 0.01% bromophenol blue). The samples were then subjected to SDS-PAGE followed by western blotting.

### 2.6. SDS-PAGE and western blotting

The proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-ECL from Amersham) using semi-dry transfer apparatus (Amersham). Western blot analysis was done as described [25].

### 2.7. Statistical analysis

Statistical significance of differences among experimental groups was assessed by Student's *t* test for populations with unequal variance. *P* values less than 0.05 were considered statistically significant. The level of significance was shown at 3 levels, viz., \*0.05, \*\*0.01 and \*\*\*0.001.

## 3. Results

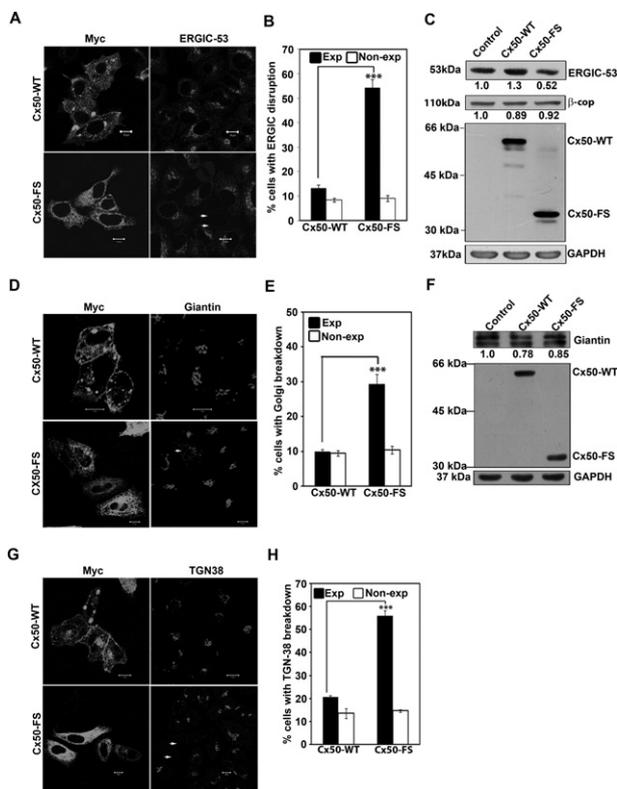
### 3.1. Subcellular localization of mutant connexin 50

Earlier studies have shown that wild type Cx50 is localized to the plasma membrane and the appositional membranes [15,23]. To study the sub-cellular localization of the frameshift mutant, Hela cells were transfected with either the plasmid expressing wild type connexin 50 (Cx50-myc) or its frameshift mutant (Cx50-FS-myc). After 25 h of expression, the cells were fixed and stained with myc antibody and analysed by confocal microscopy. As previously known, the wild type protein localized to the plasma membrane, to the appositional membranes of two juxtaposed cells and also to distinct cytoplasmic foci (Fig. 1(B)). In contrast, Cx50-FS mutant protein did not localize to the plasma membrane but it showed distinct tubular/reticular pattern in the cytoplasm, which resembled the characteristic ER staining in a cell (Fig. 1(B)). The expression of Cx50 and Cx50-FS constructs was also checked by western blot with myc antibody (Fig. 1(C)). To determine whether Cx50-FS mutant localized to the ER, Hela cells expressing Cx50-FS mutant were co-stained for the ER marker, calnexin. Cx50-FS mutant showed colocalization with calnexin, which suggested that Cx50-FS mutant was present predominantly in the ER (Fig. 1(D)). Quantitative analysis of colocalization was carried out by calculating correlation coefficients of colocalization. Compared to wild type connexin 50, the frameshift mutant showed significantly more colocalization with calnexin (Fig. 1(E)). Unlike wild type connexin 50, the frameshift mutant rarely formed foci or aggregates. Plasma membrane localization of wild type Cx50 was confirmed by co-staining of transfected cells with β-catenin. In normal epithelial cells, β-catenin is found at the plasma membrane where it provides a mechanical linkage between cell-to-cell junctional proteins [29,30]. Cx50-WT showed colocalization with β-catenin in the plasma membrane whereas Cx50-FS did not (Fig. 1(F)).

Some proteins show misfolding or mislocalization at 37 °C but this defect can be rescued at lower temperature. However, the frameshift mutant of connexin 50 showed no localization to the plasma membrane even when transfected cells were kept at 30 °C (Fig. S1).

### 3.2. Frameshift mutant overexpression causes disintegration of the ERGIC and breakdown of the Golgi

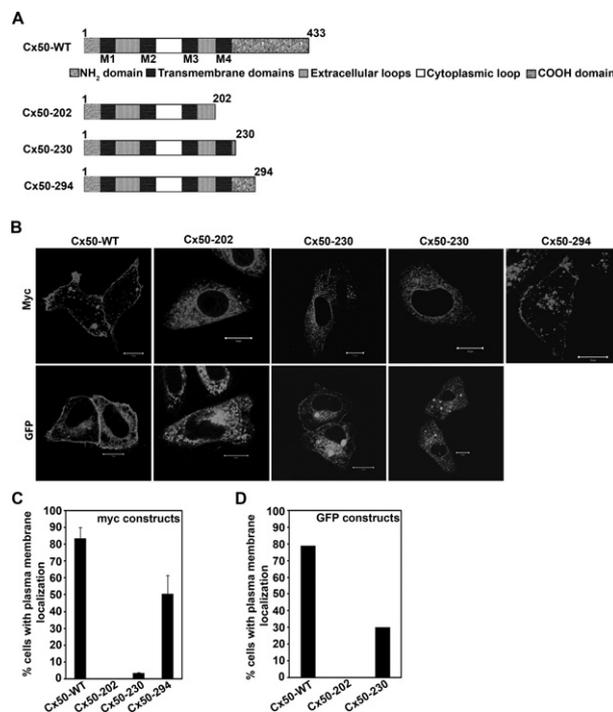
As we observed that Cx50-FS mutant localized to the ER, we were interested to see if it also localized to the ER-Golgi intermediate compartment (ERGIC) and Golgi. Hela cells were transfected with either Cx50 or Cx50-FS mutant and after 25 h of expression the cells were fixed and immunostained with myc antibody and ERGIC-53 (marker for the ERGIC) antibody or giantin (marker for the Golgi) antibody or TGN-38 (marker for trans Golgi network), and analysed using confocal microscopy. Surprisingly, we observed that overexpression of Cx50-FS mutant caused disintegration of the ERGIC in 53% of the transfected cells, whereas only 13% of the cells overexpressing wild type connexin 50 showed disintegration of the ERGIC (Fig. 2(A) and (B)). GFP transfected cells showed disintegration of the ERGIC in 12% of the cells (figure not shown). Moreover, overexpression of Cx50-FS mutant led to 48% decrease in ERGIC-53 protein level, as analysed by western blotting, whereas Cx50 expression caused some increase in ERGIC-53 protein level (Fig. 2(C)). In the cells where ERGIC-53 staining was unaffected, the frameshift mutant showed some colocalization



**Fig. 2.** The frameshift mutant causes disruption of the ERGIC and Golgi. Hela cells transfected with Cx50 and Cx50-FS constructs were fixed after 25 h of transfection and immunostained with anti-myc and ERGIC-53 (A) antibody or Giantin (D) antibody or TGN-38 (G) antibody. The analysis was done by confocal microscopy. Scale bar: 10  $\mu$ m. (B) Graph showing percentage of cells with ERGIC disruption in cells expressing (exp) or not expressing (non-exp) connexin 50 constructs. \*\*\* $p < 0.001$ . (E) Graph showing percentage of cells with Golgi breakdown. \*\*\* $p < 0.001$ . (H) Graph showing percentage of cells with TGN breakdown. \*\*\* $p < 0.001$ . The results shown in B, E and H are the means  $\pm$  SD of six experiments. A total of 900 cells were counted with each construct. Hela cells plated in 35 mm dishes were transfected with a control plasmid or Cx50-myc or Cx50-FS myc. After 48 h of transfection, cell lysates were made and subjected to western blotting with ERGIC-53,  $\beta$ -COP and myc antibodies (C) or Giantin and myc antibodies (F). GAPDH was used as a loading control in both the blots.

with ERGIC-53 (Fig. S2(A) and (B)), which was only marginally better than that seen with wild type connexin 50. Overexpression of Cx50-FS mutant caused breakdown of the Golgi in 29% of expressing cells while Cx50 expressing as well as non-expressing cells showed breakdown of the Golgi in 10% of cells (Fig. 2(D) and (E)). GFP transfected cells showed breakdown of the Golgi in 10% of the cells (figure not shown). Overexpression of Cx50-FS did not affect giantin protein levels as analysed by western blotting (Fig. 2(F)). In the cells with intact Golgi staining, the frameshift mutant showed weak colocalization with the Golgi marker (Fig. S2(C) and (D)). On staining with TGN-38, cells overexpressing Cx50-FS mutant showed breakdown of TGN in 55.85% of cells whereas 20.6% of cells overexpressing Cx50 showed TGN breakdown. Non-expressing cells showed breakdown in 13–14% of the cells (Fig. 2(G) and (H)). To determine whether the disruption of the ERGIC and Golgi by Cx50-FS mutant is a cell type specific phenomenon, similar experiments were done in Cos-1 cell line. Overexpression of Cx50-FS showed disruption of the ERGIC in 39.3% cells while Cx50 showed disruption in 16.75% cells (Fig. S3(A) and (B)). Cx50-FS caused breakdown of the Golgi in 25.5% of expressing cells while Cx50-WT showed breakdown in 10.6% of expressing cells (Fig. S3(C) and (D)).

Since we observed that overexpression of the frameshift mutant caused disruption of the ERGIC and Golgi, the effect of this mutant

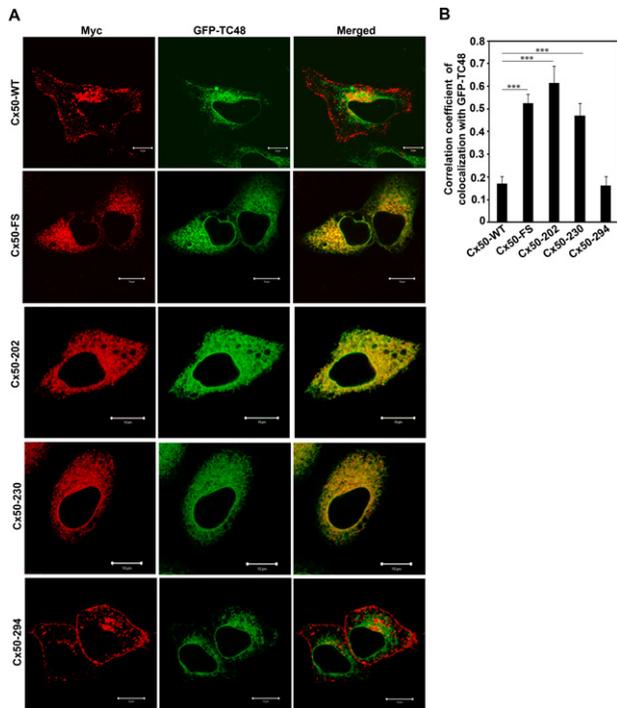


**Fig. 3.** Subcellular localization of deletion constructs of connexin 50 in HeLa cells. (A) Schematic of wild type connexin 50 and its deletion constructs. The transmembrane domains are shown as M1–M4. (B) HeLa cells transfected with myc and GFP tagged wild type and various deletion constructs of Cx50 were fixed after 25 h of transfection. Cells were stained with anti-myc antibody and subcellular localization was studied by confocal microscopy. Scale bar: 10  $\mu$ m. (C) Bar diagram showing percentage of cells with plasma membrane staining in cells expressing various myc constructs. The graph shows mean  $\pm$  SD of 150 cells with each construct. (D) Bar diagram showing percentage of cells with plasma membrane staining in cells expressing Cx50-GFP, Cx50-202-GFP and Cx50-230-GFP. A total of 60 cells with each construct were used for the graph.

on staining pattern of COPII and COPI vesicles was examined. COPII coated vesicles are anterograde transport vesicles carrying protein cargo from the ER to the ERGIC [31,32], whereas COPI vesicles are involved in retrograde transport from the Golgi to the ERGIC or from the ERGIC to the ER or directly from the Golgi to the ER [33]. While we observed a disturbed pattern of Sec24C staining (COPII vesicles and ER exit site marker) in 16% of the cells by overexpression of the Cx50-FS mutant (Fig. S4(A) and (B)), no significant effect was seen in the staining pattern of  $\beta$ -COP (COPI vesicles marker) (Fig. S4(C)). Wild type connexin 50 did not show any effect on the staining pattern of either Sec24C or  $\beta$ -COP (Fig. S4(A)–(C)).

### 3.3. Aberrant sequence does not cause defective localization of mutant Cx50

The results so far showed that the frameshift mutant is retained in the ER and it does not move to the plasma membrane. What makes this mutant protein to remain in the ER? Is it due to the extra 46 amino acids that are generated by the frameshift? To test this possibility we generated a mutant of connexin 50 lacking this extra sequence and it consists of 1–202 amino acids of the normal Cx50 (Cx50-202). This mutant did not show localization to the plasma membrane and showed ER-like localization pattern like the frameshift mutant (Fig. 3(A) and (B)). Like Cx50-FS (Fig. 4(A) second row and (B)), Cx50-202 also showed good colocalization with overexpressed TC48 tyrosine phosphatase (Fig. 4(A) third row and (B)), which localizes predominantly in the ER [25,34]. This suggested that the aberrant sequence generated by the frameshift is not responsible for ER localization or lack of transport of the frameshift mutant to the plasma membrane.

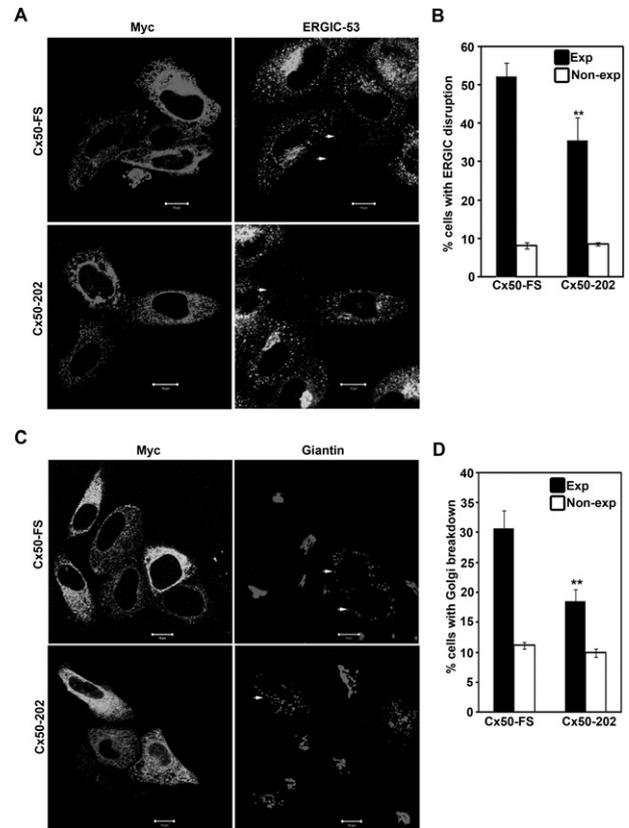


**Fig. 4.** Colocalization of connexin 50 and its deletion mutants with GFP-TC48. (A) HeLa cells plated on coverslips were cotransfected with GFP-TC48 and Cx50-myc or the deletion mutants. After 25 h the cells were fixed, stained with anti-myc antibody and analysed by confocal microscopy. Colocalization was seen by merging of green (GFP-TC48) with red (connexin 50 constructs) giving yellow colour in the merged image panel. Scale bar: 10  $\mu$ m (B) Bar diagram showing correlation coefficient of colocalization between GFP-TC48 and Cx50 constructs. The graph shows mean  $\pm$  SD of 40 cells with each construct for calculation of correlation coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We next analysed the role of aberrant sequence of the frameshift mutant in causing the breakdown of ERGIC and Golgi. For this purpose we examined the effect of overexpression of deletion construct (1–202) of connexin 50 on the ERGIC and Golgi. As compared to the frameshift mutant the deletion mutant caused significantly less breakdown of the ERGIC and Golgi (Fig. 5(A)–(D)). These results suggest that the aberrant sequence present in the frameshift mutant contributes to the breakdown of ERGIC and Golgi.

#### 3.4. Identification of domains required for localization of connexin 50 to the plasma membrane

The frameshift mutant lacks the 4th transmembrane domain and the C-terminal cytoplasmic domain. Since aberrant sequence does not seem to cause mislocalization of the frameshift mutant we hypothesized that the loss of 4th transmembrane domain or the loss of cytoplasmic domain may be responsible for altered localization of the frameshift mutant. A deletion construct of connexin 50, which has all four transmembrane domains (M1–M4) (1–230 amino acids) (Cx50-230-myc) was made. This construct showed localization to the plasma membrane in only about 3% of the cells (Fig. 3(A)–(C)) and the staining in the plasma membrane was not as prominent as seen with the wild type protein. The remaining cells showed intracellular staining predominantly in the ER, as shown by colocalization with TC48 (Fig. 4(A) fourth row and (B)). On the other hand the Cx50-230-GFP construct showed plasma membrane localization in about 30% of the cells (Fig. 3(B) and (D)). The staining pattern in the remaining cells was similar to that of Cx50-FS mutant protein but contained large intracellular foci observed in the case of Cx50. These results suggest that



**Fig. 5.** Effect of Cx50-202 deletion mutant on the ERGIC and Golgi. HeLa cells were transfected with Cx50-FS-myc and Cx50-202-myc constructs. After 25 h of transfection the cells were double immunostained with anti-myc and ERGIC-53 (A) or anti-myc and Giantin (C). The analysis was done by confocal microscopy. Scale bar: 10  $\mu$ m. (B) Bar diagram showing percentage of cells with ERGIC disruption in cells expressing (exp) or not expressing (non-exp) connexin 50 constructs. \*\* $p$  < 0.01. (D) Bar diagram showing percentage of cells with Golgi breakdown. The graphs (B, D) show mean  $\pm$  SD of six experiments and a total of 900 cells were scored. \*\* $p$  < 0.01.

the fourth transmembrane domain is essential for localization of Cx50 to the plasma membrane or retention in the plasma membrane. However, it appears that the C-terminal cytoplasmic domain contributes to efficient localization of connexin 50 to the plasma membrane.

A deletion mutant of mouse connexin 50 consisting of 1–290 amino acid (corresponding to 1–294 amino acids of human connexin 50) has been shown to localize to the plasma membrane, like the wild type protein [35]. This truncation occurs naturally in the cells by calpain [36]. This indicated that the entire C-terminal cytoplasmic domain may not be necessary for localization of Cx50 to the plasma membrane. Therefore, we made a deletion construct encompassing 1–294 amino acids of human connexin 50 (Cx50-294). This construct showed efficient localization to the plasma membrane in 50–60% cells (Fig. 3(A)–(C)) and did not show colocalization with overexpressed TC48 (Fig. 4(A) fifth row and (B)) similar to Cx50 (Fig. 4(A) first row and (B)). These results suggest that membrane proximal cytoplasmic domain (amino acids 230–294) is essential for efficient localization of Cx50 to the plasma membrane.

#### 3.5. Effect of deletion mutants on transport and localization of connexin 50 to the plasma membrane

Our deletion analysis showed that the construct Cx50-202 does not localize to the plasma membrane and it remains predominantly in the ER. This defective localization may be due to a defect in its transport from the ER or anchoring in the plasma membrane. A transport

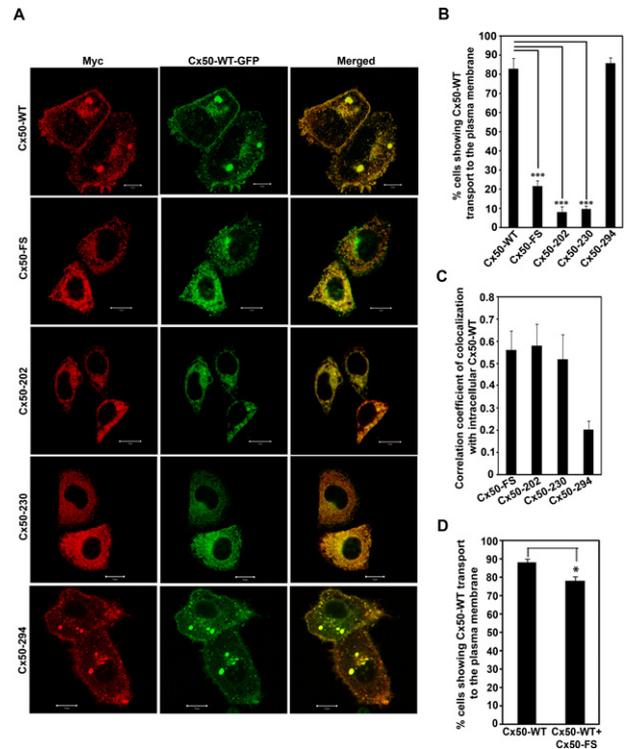
defective deletion mutant may inhibit the transport of full length connexin 50. We examined this possibility by coexpressing myc-tagged deletion constructs with GFP tagged full length connexin 50 in the ratio of 2.5:1. Coexpression of deletion mutant, Cx50-202, resulted in inhibition of plasma membrane localization of full length connexin 50 (Fig. 6(A) third row and (B)). Quantitative analysis showed that in 80% of the cells coexpressing deletion mutant, the Cx50-GFP did not show any staining in the plasma membrane and accumulated inside the cell. The intracellular Cx50-GFP showed strong colocalization with the deletion mutant 1–202, possibly in the ER (Fig. 6(A) third row and (C)). The other deletion construct Cx50-230 that encompasses transmembrane domains M1–M4 also inhibited the localization of connexin 50 to the plasma membrane (Fig. 6(A) fourth row and (B)). However, the deletion construct Cx50-294 that shows good membrane localization, did not inhibit localization of full length connexin 50 to the plasma membrane (Fig. 6(A) last row and (B)). These results suggest that the deletion constructs of connexin 50 that are defective in localization to the plasma membrane also cause inhibition of transport of Cx50 to the plasma membrane; therefore, these mutants (Cx50-202 and Cx50-230) are likely to be defective in their transport from the ER to the plasma membrane. The plasma membrane localization of Cx50-294 was examined by costaining the Cx50-294 transfected cells with  $\beta$ -catenin. While Cx50-294 showed good colocalization with  $\beta$ -catenin at the junctional membranes, Cx50-202 and Cx50-230 did not show any colocalization with  $\beta$ -catenin at the plasma membrane and showed intracellular localization (Fig. S5).

Since the frameshift mutant of connexin 50 is localized in the ER, we examined the affect of coexpressing the frameshift mutant on the localization of Cx50. Coexpression of the frameshift mutant resulted in inhibition of localization of Cx50 to the plasma membrane in most of the cells (Fig. 6(A) second row and (B)). However when the frameshift mutant and Cx50 were coexpressed in 1:1 ratio, there was only a marginal effect in localization of Cx50 to the plasma membrane (Fig. 6(D)).

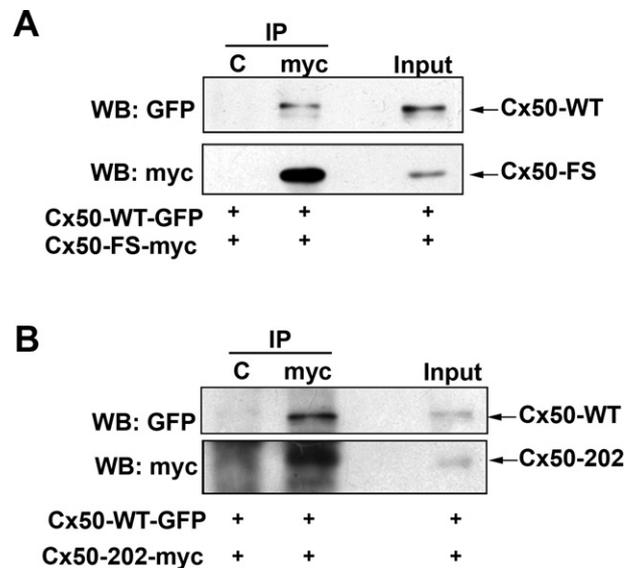
Connexin polypeptides interact with each other to form homo or hetero-oligomers in the ER before their transport to the plasma membrane. The ability of the frameshift mutant to interact with wild type Cx50 was examined by coimmunoprecipitation. The frameshift mutant was able to coimmunoprecipitate wild type connexin 50 (Fig. 7(A)). The deletion mutant Cx50-202 was also able to coimmunoprecipitate wild type Cx50 (Fig. 7(B)). These results show that even though the Cx50-FS and Cx50-202 form a complex with Cx50, these proteins do not get co-transported to the plasma membrane along with Cx50. Instead, these mutants inhibit the transport of Cx50 to the plasma membrane.

**4. Discussion**

Most of the mutants of connexin 50 that cause cataract are dominant and recessive mutants are rare [6,18]. The frameshift mutant of Cx50 studied here is a recessive mutant that causes congenital cataract [20]. The results presented here suggest that this mutant is a loss of function mutation because it does not reach plasma membrane where wild type connexin 50 performs its function as gap junction and hemichannel. This mutant has 46 aberrant amino acids after codon 202 that are generated due to frameshift. However, the aberrant sequence generated by frameshift does not seem to be responsible for its improper localization because deletion of aberrant sequence resulted in a protein that, like the frameshift mutant, did not reach plasma membrane and localized predominantly in the ER. The frameshift mutant, when co-expressed with Cx50, was not able to reach the plasma membrane even though it forms a complex with the wild type connexin 50. The inability to localize at the plasma membrane may be due to its defective transport or due to defective retention in the plasma membrane. However, complete absence of the frameshift mutant from the plasma membrane, and inhibition of anterograde



**Fig. 6.** Effect of deletion constructs on plasma membrane localization of full length Cx50. (A) HeLa cells were cotransfected with 1:2.5 ratio of plasmids expressing Cx50-GFP and Cx50-myc or Cx50-FS-myc or Cx50-202-myc or Cx50-230-myc or Cx50-294-myc and fixed after 25 h of transfection. Cells were immunostained with anti-myc antibody and analysed by confocal microscopy to check the effect of overexpression of the deletion mutants on plasma membrane localization of Cx50. (B) Bar diagram showing the percentage of cells with plasma membrane localization of Cx50 in the presence of various mutants of Cx50. The values in the bar diagram are mean  $\pm$  SD of four experiments. A total of 300 cells were used with each construct. \*\*\* $p < 0.001$ . (C) Bar diagram showing the correlation coefficient of colocalization of Cx50-GFP (intracellular) with indicated mutants of Cx50. The graph shows mean  $\pm$  SD of 40 cells with each construct. (D) Bar diagram showing the percentage of cells with plasma membrane localization of Cx50 in the presence of Cx50-FS at a ratio of 1:1. \* $p < 0.05$ . The graphs show mean  $\pm$  SD of four experiments. A total of 300 cells were counted.



**Fig. 7.** The frameshift mutant as well as Cx50-202 construct interact with the wild type connexin 50. HeLa cells were cotransfected with Cx50-GFP and Cx50-FS-myc (A) or Cx50-GFP and Cx50-202-myc (B) in the ratio of 1:2.5. After 30 h of overexpression, cell lysates were prepared and immunoprecipitation was carried out using anti-myc antibody or control IgG antibody. The samples were later subjected to western blotting with the indicated antibodies. Input is 5% lysate.

transport of wild type connexin 50, suggest that this mutant is likely to be defective in transport to the plasma membrane.

While examining the colocalization of the frameshift mutant with markers of ERGIC and Golgi, we observed that this mutant caused breakdown of the ERGIC and Golgi in some of the cells. Earlier studies have already shown that transfection per se causes disruption of the ERGIC and Golgi [37,38], but the breakdown observed with the frameshift mutant is highly significant compared to that observed with wild type or control transfections. The aberrant sequence seems to play some role in breakdown of the Golgi and ERGIC because deletion of aberrant sequence resulted in less breakdown of these organelles. The breakdown of Golgi may be due to inhibition of anterograde transport because this mutant inhibits transport of Cx50-WT to some extent. One possible explanation for transport inhibition is that Cx50-FS may be interacting, directly or indirectly, with a component of transport machinery, thereby affecting its function. This hypothesis is supported by our observation that Cx50-FS expression disrupts Sec24c staining in 16% cells. Sec24c is present in the ER exit site and also in the COPII vesicles. Sec23/24 heterodimer is a structural compartment of COPII and is also involved in the recruitment of cargo (by direct interaction).

It is somewhat surprising that in spite of causing breakdown of the ERGIC and Golgi, and inhibiting the localization of normal connexin 50 to the plasma membrane, the frameshift mutant causes recessive cataract. Since at lower level of expression, the frameshift mutant showed only a marginal effect on localization of Cx50-WT to the plasma membrane, the effect may not be enough to cause cataract in heterozygous condition. Another possible explanation for recessive phenotype of this frameshift mutant is that its effect on the ERGIC and Golgi are not severe enough to cause cell death or cataract. However, the available data do not rule out the possibility that this mutant in heterozygous condition contributes to some extent to cataract development in the adults.

Connexins have been studied extensively with respect to their structure, oligomerization and function as gap junctions or hemichannels in the plasma membrane. Transport of connexins to the plasma membrane occurs through the ER-Golgi mediated secretory pathway [9,10]. Deletion analysis has shown that connexin 32 polypeptide extending from N-terminus past M3 is required for oligomerization and assembly with full length connexin 32, and M4 and C-terminal cytoplasmic domain are not required for this process [10,39]. The role of carboxy terminal region of connexins have been analysed in gap junction function and plaque formation [39–42]. Cx50 interacts with ZO-1 and contains a PDZ domain-binding motif at the extreme C-terminus [40,43]. The interaction of this motif with ZO-1 are required for targeting of Cx50 to the gap junction plaques and formation of active channels [40]. However, with respect to localization of a deletion construct (1–290 amino acids) to the plasma membrane, the results of Chai et al. [40] are apparently contradictory to the observation of DeRosa et al. who showed that a deletion mutant of mouse connexin 50 (1–290 amino acids), corresponding to 1–294 amino acid of human Cx50, localizes to the plasma membrane [35]. Our results show that fourth transmembrane domain and a portion of cytoplasmic domain extending up to amino acid 294 are required for localization of human connexin 50 to the plasma membrane. Those deletion constructs of Cx50 that are defective in localization to plasma membrane are retained in the ER and also inhibit localization of full length Cx50 to plasma membrane, suggesting therefore, that these are defective in transport.

What is the role of M4 in localization or transport? One possibility is that it is required for proper assembly of Cx50 into transport competent complex. Alternatively, the M4 may be involved in interaction with a transport protein. These two possibilities are not mutually exclusive. The C-terminal cytoplasmic domain extending up to amino acid 294 may be involved in retention of connexin 50 in the plasma membrane, or like M4, it may be involved in facilitating transport

from the ER to the plasma membrane.

In conclusion, our results show that a cataract-causing frameshift mutant of connexin 50 is defective in its localization to the plasma membrane and shows ER localization possibly due to defective transport from the ER. This is likely to result in loss of function of connexin 50 leading to recessive phenotype of congenital cataract associated with this frameshift mutant. We have identified a role for the fourth transmembrane domain and a sequence in the adjacent cytoplasmic domain in localization of connexin 50 to the plasma membrane. The M4 is likely to be involved in facilitating the transport of Cx50 from the ER, whereas the adjacent cytoplasmic region may be facilitating the transport or anchoring of the protein in the plasma membrane.

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## Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.11.005.

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