



# Connexin mutants and cataracts

Eric C. Beyer<sup>1\*</sup>, Lisa Ebihara<sup>2</sup> and Viviana M. Berthoud<sup>1</sup>

<sup>1</sup> Department of Pediatrics, University of Chicago, Chicago, IL, USA

<sup>2</sup> Department of Physiology and Biophysics, Rosalind Franklin Health Sciences University, North Chicago, IL, USA

## Edited by:

Stefan Dhein, Universitätsklinik  
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## \*Correspondence:

Eric C. Beyer, Department of  
Pediatrics, University of Chicago, 900  
East 57th Street, KCBD 5152,  
Chicago, IL 60637, USA.  
e-mail: ecbeyer@uchicago.edu

The lens is a multicellular, but avascular tissue that must stay transparent to allow normal transmission of light and focusing of it on the retina. Damage to lens cells and/or proteins can cause cataracts, opacities that disrupt these processes. The normal survival of the lens is facilitated by an extensive network of gap junctions formed predominantly of connexin46 and connexin50. Mutations of the genes that encode these connexins (*GJA3* and *GJA8*) have been identified and linked to inheritance of cataracts in human families and mouse lines. *In vitro* expression studies of several of these mutants have shown that they exhibit abnormalities that may lead to disease. Many of the mutants reduce or modify intercellular communication due to channel alterations (including loss of function or altered gating) or due to impaired cellular trafficking which reduces the number of gap junction channels within the plasma membrane. However, the abnormalities detected in studies of other mutants suggest that they cause cataracts through other mechanisms including gain of hemichannel function (leading to cell injury and death) and formation of cytoplasmic accumulations (that may act as light scattering particles). These observations and the anticipated results of ongoing studies should elucidate the mechanisms of cataract development due to mutations of lens connexins and abnormalities of other lens proteins. They may also contribute to our understanding of the mechanisms of disease due to connexin mutations in other tissues.

**Keywords:** connexin46, connexin50, cataract, lens, gap junction

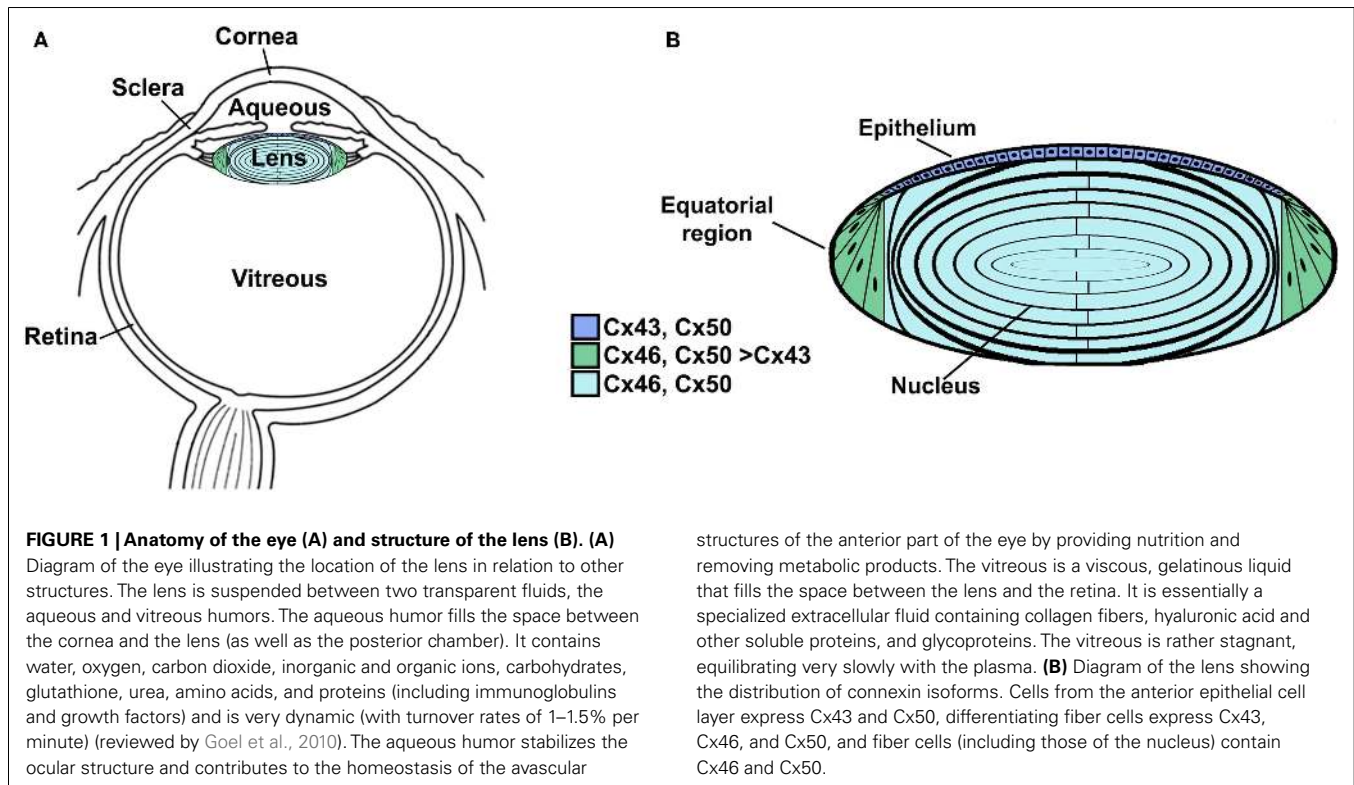
## THE LENS AND CATARACTS

The lens is a transparent organ whose main function is to transmit light and focus it on the retina. It sits suspended between two clear fluids (the aqueous humor and the vitreous) and has no direct blood supply (**Figure 1A**). The lens is comprised of two cell types: epithelial cells that form a single layer along the anterior surface and fiber cells that form the bulk of the organ (**Figure 1B**). At the lens equator, epithelial cells differentiate into fiber cells, a process that involves cell elongation and loss of nuclei and organelles. This differentiation process occurs throughout the lifespan of the organism. Lens fiber cells contain very high concentrations of small soluble proteins called crystallins. These proteins act as chaperones and increase the refractive index (but do not interfere with transparency). Mature fiber cells have limited metabolic activities, are non-dividing, and must survive for the lifespan of the organism. Most of the metabolic, synthetic, and active transport machinery in the lens is localized to surface (nucleated) cells (Mathias and Rae, 2004).

A cataract is a cloudiness or opacity in the lens. It may cause a decrease in vision and may eventually lead to blindness. This disease has substantial public health consequences, because cataracts are the leading cause of blindness worldwide (Resnikoff et al., 2004). Even in those countries where cataractous lenses are surgically removed and replaced with prosthetic intraocular lenses, this eye pathology has a major financial impact. Therefore, many efforts have been devoted to determine the factors that lead to cataract formation and to develop treatments to prevent their

formation. Cataracts can be subdivided according to their anatomical location within the lens (e.g., cortical, nuclear, sub-capsular), their appearance (e.g., total, pulverulent), and most commonly by a combination of these two parameters (e.g., nuclear pulverulent). They can also be subdivided according to their etiology (e.g., congenital, disease-related, or age-related). The specific biochemical and structural changes associated with cataract formation are diverse, but a common biochemical change is the presence of high molecular weight insoluble protein aggregates (Moreau and King, 2012).

Because the lens does not have a direct blood supply, the nutrients for the organ all derive from the fluids in which it is suspended. Specifically, the aqueous humor (which is dynamically produced from the plasma and subsequently resorbed) provides the main source for inorganic and organic ions, carbohydrates, glutathione, amino acids, and oxygen. The aqueous humor is also the repository for metabolites and carbon dioxide produced by lens cells. Ions and nutrients reach cells in the interior through an internal “circulation” in which flow of ions and water drives the movement of solutes throughout the organ. A model of this circulation has been developed based on surface currents recorded from lenses (Robinson and Patterson, 1982; Parmelee, 1986; Mathias et al., 1997) and measurements of hydrostatic pressures at different depths within the lens (Gao et al., 2011). In this model, current carried by ions (and associated water and solutes) enters the lens along the extracellular spaces at the anterior and posterior poles, it crosses fiber cell membranes in the lens interior, and it flows back to the surface



at the equator (through a cell-to-cell pathway) (Mathias et al., 2007, 2010). The hydrostatic pressure gradient also drives water flow toward the exterior (Gao et al., 2011). The lens circulatory system provides a pathway for internal fiber cells to obtain essential nutrients, remove potentially toxic metabolites, and maintain resting potentials (Goodenough, 1979; Piatigorsky, 1980). Thus, fiber cell survival and the maintenance of transparency depend on the function of epithelial cells and on communication between lens cells (Goodenough, 1992).

### LENS GAP JUNCTIONS AND CONNEXINS

Intercellular communication among the cells of the lens is facilitated by an extensive network of gap junctions. Gap junctions are membrane specializations that contain clusters of intercellular channels that are permeable to ions and small solutes ( $\leq 1$  kDa). Lens fiber cells share ions and small metabolites through gap junction channels, and consequently behave as a functional syncytium (Goodenough et al., 1980; Mathias and Rae, 1989). Epithelial and fiber cells contain morphologically and physiologically distinct gap junctions (Rae and Kuszak, 1983; Miller and Goodenough, 1986).

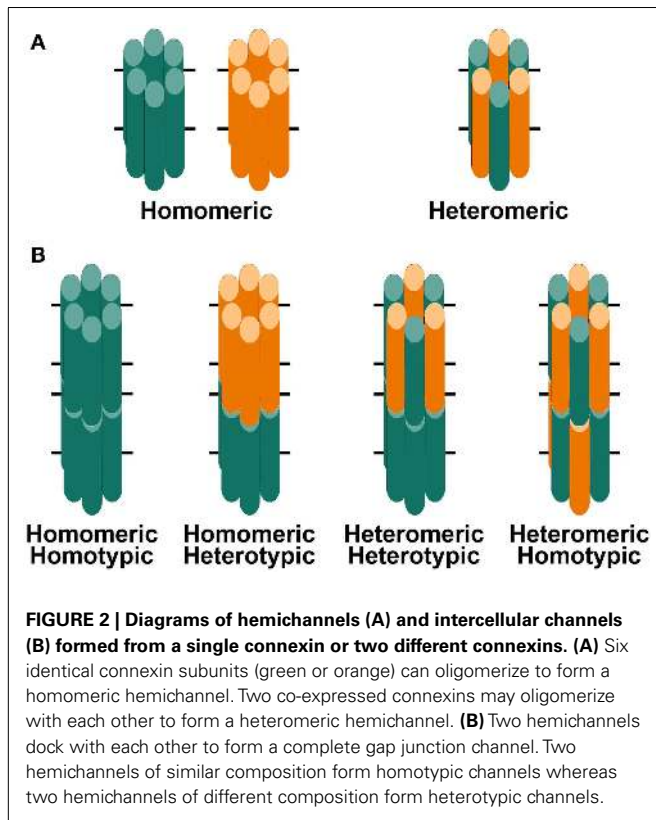
Gap junction channels are oligomeric assemblies of members of a family of related proteins called connexins (Cx) (Beyer and Berthoud, 2009). Connexins contain four transmembrane domains connected by two extracellular loops and one cytoplasmic loop. The amino and carboxyl termini of the polypeptide are in the cytoplasm. Six connexins oligomerize to form a connexin hemichannel which traffics to the plasma membrane of one cell where it can dock with another hemichannel from an adjacent cell to form a complete gap junction channel (Figure 2). Channels formed by diverse connexins differ in physiological properties

including unitary conductance, permeability, gating, and regulation by different protein kinase-dependent pathways (reviewed in Harris, 2001, 2007; Saez et al., 2003). Thus, the regulation of intercellular communication and the permeation of different molecules in different regions of the lens are determined by the repertoire of connexins expressed.

Three connexins have been identified in the lens with somewhat overlapping expression patterns (Figure 1B). Cx43 is expressed in lens epithelial cells (Musil et al., 1990), but its expression is turned off as epithelial cells in the equatorial region differentiate into fiber cells. Cx50 is also expressed in epithelial cells (TenBroek et al., 1994; Dahm et al., 1999; Rong et al., 2002). Cx46 and Cx50 become abundantly expressed in the differentiating cells and are the two most abundant connexins in lens fiber cells (Paul et al., 1991; White et al., 1992). Cx46 and Cx50 co-localize at gap junction plaques and can form mixed hexamers (Paul et al., 1991; Jiang and Goodenough, 1996). (Heteromeric mixing is illustrated schematically in Figure 2).

Transcripts for a fourth connexin, Cx23, have been detected in the zebrafish embryo lens (Iovine et al., 2008) and in the mouse lens (Puk et al., 2008). Cx23 has been implicated in fiber cell differentiation, because fiber cells in mice expressing a missense mutation of Cx23 did not appear to elongate properly (Puk et al., 2008). Lens cells from other mammalian species may also express Cx23, since it has been identified as an expressed sequence in mRNA from whole eyes or lenses<sup>1</sup>. However, Cx23 transcript

<sup>1</sup>Accession numbers for *Oryctogalus cuniculus* EST from eye: EB384661.1, EB384662.1; *Bos taurus* EST from lens: EG705869.1; *Macaca mulatta* EST from lens: EG703836.



was not detected in RNA isolated from human lenses (Sonntag et al., 2009). Moreover, while Cx23 protein has been detected in proteomic studies of mouse lens membrane proteins (Bassnett et al., 2009), it was not detected in human samples (Wang et al., 2013). Therefore, Cx23 is not included in **Figure 1B** nor considered further in this review.

### MOUSE STUDIES IMPLICATE CONNEXINS IN CATARACTOGENESIS

The importance of gap junction-mediated lens intercellular communication for the maintenance of lens transparency has been substantiated by a number of genetic studies in mice. Targeted deletion of either Cx46 or Cx50 results in the development of cataracts in homozygous (but not heterozygous) null mice (Gong et al., 1997; White et al., 1998). The Cx50-null mice have a milder cataract than the Cx46-null mice (Gerido et al., 2003), but Cx50-null mice exhibit microphthalmia and smaller lenses (Gong et al., 1997; White et al., 1998; Rong et al., 2002). The onset of the cataract phenotype in Cx50 knock-out mice occurs within the first post-natal week (White et al., 1998; Rong et al., 2002) whereas the cataracts in Cx46-null mice are visible by the third week of age (Gong et al., 1997). The solubility of some crystallins is decreased in both Cx50- and Cx46-null mice. Double knock-out mice lacking both the Cx46 and Cx50 genes show dense lens opacities that are far more extensive than those observed in either the Cx46 or Cx50 single null mice (Xia et al., 2006).

Transgenic mice over-expressing Cx50 also develop severe cataracts (Chung et al., 2007). This finding suggests that any

significant alteration of connexin levels in these cells (either absence or a major increase) may lead to cataract formation.

The role of Cx43 in normal lens function is uncertain. Lenses of prenatal or newborn Cx43-null mice appear normal and transparent ((White et al., 2001) and Berthoud and Beyer, unpublished observations), but Gao and Spray (1998) have observed ultrastructural abnormalities in these lenses. The long-term effects of the loss of Cx43 in the lens cannot be determined in these mice, because global deletion of Cx43 results in neonatal lethality (Reaume et al., 1995). However, the lenses of animals with a conditional deletion of Cx43 are transparent and develop normally through at least 6 months of age, even though intercellular transfer of neurobiotin and Lucifer yellow among epithelial cells is decreased (DeRosa et al., 2009).

The cataract trait in several mutant mouse strains has been mapped to the lens connexin loci. The *No2* mouse carries a missense mutation within the coding region of Cx50 resulting in a change of amino acid residue 47 from aspartate to alanine (Cx50D47A) and develops congenital cataracts (Favor, 1983; Steele et al., 1998); these cataracts are less severe in heterozygous than in homozygous animals. Mice carrying a Cx50 mutation at amino acid residue 64 (changing from valine to alanine, Cx50V64A) exhibit dominantly inherited cataracts (Graw et al., 2001). Another mouse with cataracts, *lop10*, carries a missense mutation at amino acid residue 22 of Cx50 (Cx50G22R) (Chang et al., 2002). Both cataracts and microphthalmia have been observed in mice with the semi-dominant mutation, Cx50R205G (Xia et al., 2012).

### CONNEXIN MUTATIONS AND CONGENITAL CATARACTS IN HUMANS

Mutations in lens connexins have been associated with human disease. Missense and frame shift mutations of the Cx46 and Cx50 genes have been identified in members of families with inherited cataracts of various different phenotypes. These mutants and their associated cataract phenotypes are summarized in **Table 1** (Cx46) and **Table 2** (Cx50). Other than the few exceptions noted in the tables, nearly all of the cataracts are inherited as autosomal dominant traits. The positions of these mutations in relation to the membrane topology of these connexins are shown in **Figure 3**.

Mutations in Cx43 have been associated with oculodentodigital dysplasia, a disease which is only rarely accompanied by cataracts. Therefore, Cx43 mutations are not considered in this review.

Our laboratories have been interested in determining the biochemical, cell biological and physiological abnormalities in the behaviors of these human mutants. We have expressed different cataract-associated connexin mutants using *in vitro* expression systems, by transfection of communication- and connexin-deficient mammalian cells and by microinjection of *in vitro* transcribed connexin cRNAs into *Xenopus* oocytes. We have identified several different abnormalities (as illustrated by different examples in **Table 3**). In this paper, we will review some of these findings and consider their implications for understanding cataract pathogenesis. The data summarized will primarily derive from the human connexin mutant experiments performed in our laboratories.

Typically, we have performed functional and cellular screening tests in parallel. These initial studies are designed to test whether a mutant construct induces a level of intercellular conductance

**Table 1 | Human Cx46 mutants linked to cataract formation.**

Position and alteration	Disease phenotype		Reference
	Cataract appearance	Other features	
G2D	Nuclear pulverulent and posterior polar		Yao et al. (2011)
D3Y	Zonular pulverulent		Addison et al. (2006)
L11S	“Ant-egg”		Hansen et al. (2006)
T19M	Posterior polar		Santhiya et al. (2010)
V28M	Variable		Devi et al. (2005)
F32L	Nuclear pulverulent		Jiang et al. (2003)
R33L	Finely granular embryonal		Guleria et al. (2007a)
V44M			Zhou et al. (2010), Bennett and Shiels (2011)
W45S	Nuclear		Ma et al. (2005)
D47N	Nuclear		Yang et al. (2011)
P59L	Nuclear punctate		Bennett et al. (2004)
N63S	Zonular pulverulent		MacKay et al. (1999)
R76G	Total		Devi et al. (2005)
R76H	Lamellar nuclear opacity with surrounding pulverulent nuclear opacities; lamellar with moderate opacity of the fetal nucleus and Y-shaped condensations in the anterior suture	Dominant inheritance with incomplete penetrance	Burdon et al. (2004), Hansen et al. (2009)
T87M	“Pearl box”		Guleria et al. (2007b)
G143R	Coppock-like		Zhang et al. (2012a)
P187L	Homogeneous zonular pulverulent		Rees et al. (2000)
P187S	Nuclear pulverulent		Ding et al. (2011)
N188I	Nuclear coralliform		Zhang et al. (2012b)
N188T	Nuclear pulverulent		Li et al. (2004)
F206I	Embryonal nuclear		Wang and Zhu (2012)
fs380	Zonular pulverulent		MacKay et al. (1999)

When the descriptions of the cataract phenotype differed in different reports, both are listed, but separated by a semi-colon. All cataracts were inherited as dominant traits.

above that seen in untransfected cells or water-injected *Xenopus* oocytes and whether the construct leads to the formation of gap junction plaques. Plaque formation is identified as immunoreactive connexin that localizes along appositional membranes with a punctate distribution (examples are shown for wild type Cx46 and Cx50 in **Figures 4** and **5**).

### CONNEXIN MUTANTS WITH ABNORMALITIES OF CELLULAR BIOSYNTHESIS OR DEGRADATION

The most frequently observed phenotype is a cataract-associated connexin mutant that does not induce a significant intercellular conductance and forms very few or no gap junction plaques. Examples include Cx50R23T, Cx50D47N, Cx50P88S, Cx50P88Q, and Cx46fs380 (Berthoud et al., 2003; Minogue et al., 2005; Arora et al., 2006, 2008; Thomas et al., 2008) (**Table 3** and **Figures 4** and **5**). Among these mutants, Cx50R23T rarely forms small plaques (Thomas et al., 2008), while Cx46fs380 never forms them (Minogue et al., 2005). These differences likely reflect variations in the severity of the trafficking defects and the specific mechanisms involved.

For many of the mutants that do not form plaques, immunoreactive connexin localizes within the cytoplasm. Co-localization

studies using antibodies directed against compartments of the protein biosynthetic/secretory pathway have shown that the mutant connexins are contained within the ER, ERGIC, and/or Golgi apparatus (e.g., Cx50D47N and Cx46fs380) (Minogue et al., 2005; Arora et al., 2008). The connexin within these subcellular compartments likely represents mutant protein that has been retained within the export pathway due to misfolding and/or incomplete/improper oligomerization. The interpretation that some of the mutant connexins (e.g., Cx50D47N, Cx50P88Q, Cx50P88S) are misfolded is supported by the presence of gap junction plaques at the plasma membrane after incubation of expressing cells under conditions that should promote protein folding (reduced temperatures or chemical chaperone treatment) (Berthoud et al., 2003; Arora et al., 2006, 2008).

The cytoplasmic retention of a cataract-linked mutant has been explored in detail for Cx46fs380. This mutant contains a frame shift that causes a change in reading frame such that the connexin contains an abnormal C-terminal sequence. We have shown that a two amino acid motif (FF) within the abnormal polypeptide is responsible for its localization within the ERGIC and Golgi (Minogue et al., 2005). This motif has been identified as a trafficking signal in other proteins.

**Table 2 | Human Cx50 mutants linked to cataract formation.**

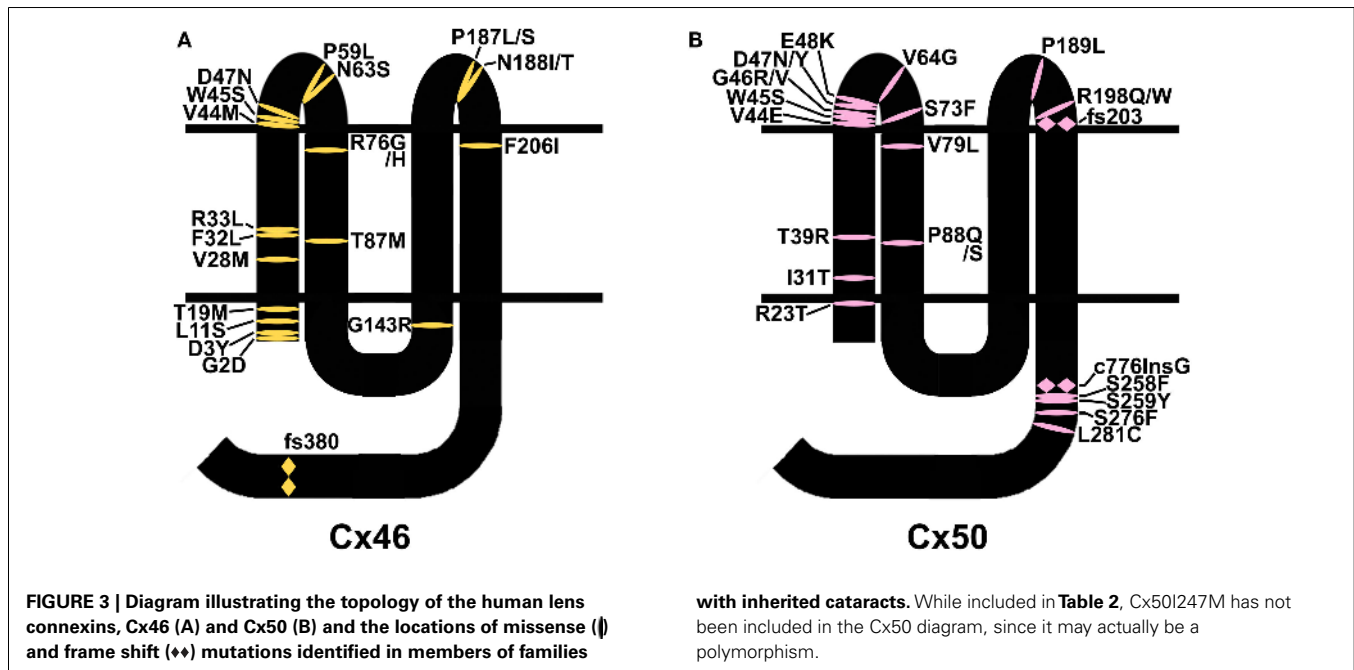
Position and alteration	Disease phenotype		Reference
	Cataract appearance	Other features	
R23T	Nuclear		Willoughby et al. (2003)
I31T	Fetal and embryonic nuclear		Wang et al. (2009)
T39R	Complete	Microcornea and iris hypoplasia	Sun et al. (2011)
V44E	Total	Microcornea and variably associated with myopia	Devi and Vijayalakshmi (2006)
W45S	Jellyfish-like		Vanita et al. (2008b)
G46R	Complete	Microcornea	Sun et al. (2011)
G46V	Total		Minogue et al. (2009)
D47N	Nuclear pulverulent		Arora et al. (2008), Wang et al. (2011)
D47Y			Lin et al. (2008)
E48K	“Zonular nuclear” pulverulent		Berry et al. (1999)
V64G	Nuclear		Ma et al. (2005)
S73F	Dense and “star-shaped,” various locations in the nucleus or the poles		Hansen et al. (2009)
V79L	“Full moon” with Y-sutural opacities		Vanita et al. (2006)
P88Q	Lamellar pulverulent; balloon-like with Y-sutural opacities		Arora et al. (2006), Vanita et al. (2008a)
P88S	Zonular pulverulent		Shiels et al. (1998)
P189L	Star-shaped nuclear opacity with a whitish central core	Microcornea	Hansen et al. (2007)
R198Q		Microcornea and variably associated with myopia	Devi and Vijayalakshmi (2006)
R198W		Microcornea without microphthalmia	Hu et al. (2010)
fs203	Total	Recessive inheritance. Associated with nystagmus and amblyopia	Ponnam et al. (2007)
I247M	Zonular pulverulent	May be a polymorphism rather than a disease-causing mutation	Polyakov et al. (2001), Graw et al. (2009)
c776InsG	Triangular cataract	Recessive inheritance	Schmidt et al. (2008)
S258F	Nuclear		Gao et al. (2010)
S259Y			Hansen et al. (2009)
S276F	Pulverulent nuclear		Yan et al. (2008)
L281C	Lamellar/zonular		Kumar et al. (2011)

When the descriptions of the cataract phenotype differed in different reports, both are listed, but separated by a semi-colon. All cataracts were inherited as dominant traits, except as noted.

Cx50P88S is an interesting mutant that does not form gap junction plaques when expressed by itself. It has a cytoplasmic localization, but little of the protein is localized within compartments of the biosynthetic/secretory pathway. Rather, this mutant forms cytoplasmic inclusions (1–5/cell) of 0.6–2.7  $\mu\text{m}$  in diameter (Berthoud et al., 2003). Similar inclusions are observed after transfection of several different cell lines. The Cx50P88S accumulations are very long lived (as compared to the turnover of the wild type protein in these expression systems) and correspond to closely apposed circular or semicircular membrane stacks that likely originate from the rough endoplasmic reticulum (Berthoud et al., 2003; Lichtenstein et al., 2009). Some of the Cx50P88S inclusions co-localize with components of the autophagic degradation pathway, and their turnover is altered by interventions that affect

autophagy. Thus, the persistence of the Cx50P88S accumulations likely results from insufficient degradation capacity of constitutive autophagy (Lichtenstein et al., 2011). We have also studied another cataract-associated mutant of this connexin at the same position, Cx50P88Q. Although this mutant forms some cytoplasmic inclusions like Cx50P88S, the glutamine substitution seems to have less severe consequences than the serine substitution, since many cells expressing Cx50P88Q contain immunoreactive connexin that co-localizes with markers for the ER or Golgi (Arora et al., 2006).

Additional kinds of cellular and biochemical abnormalities may be predicted for some of the identified cataract-associated mutants. For example, phosphorylation events have been implicated in regulation of many aspects of the connexin life cycle and



physiology (reviewed by Solan and Lampe, 2005; Moreno and Lau, 2007). Two Cx50 mutants, Cx50S258F and Cx50S259Y, alter amino acids that are phosphorylated in the wild type protein as shown in a proteomic study of human lens membrane proteins (Wang et al., 2013); however, the cellular and physiological behaviors of these mutants have not yet been studied. In the *Lop10* mouse, expression of the Cx50 mutant reduces the abundance of phosphorylated forms of Cx46, and the Cx46 alterations may contribute to the cataracts in these animals (Chang et al., 2002). The carboxyl termini of both Cx46 and Cx50 are sensitive to cleavage by calpains (Kistler and Bullivant, 1987; Lin et al., 1997; Jacobs et al., 2004). When expressed in heterologous systems, the resulting truncated Cx50 forms channels with reduced function (DeRosa et al., 2006) and sensitivity to intracellular pH (Lin et al., 1998; Xu et al., 2002). It is likely that some of the mutants within the C-terminal region of the connexin may interfere with this cleavage.

These studies allow prediction of some consequences of mutant connexin expression in the lens. All of the mutants with this general phenotype (loss of function due to a severe reduction in the number or complete absence of gap junctions) should reduce intercellular communication between lens fiber cells, regardless of their different mechanisms of retention/accumulation. In the lens, interactions of these mutants with other lens fiber cell proteins (including wild type connexins) might alter their trafficking or function as well. The mutants that are retained within the ER might cause ER stress and might lead to stimulation of the unfolded protein response. A study of the Cx50G22R and Cx50S50P mutant mice concluded that the unfolded protein response might be a contributing factor to the cataracts in these animals (Alapure et al., 2012). The stable cytoplasmic inclusions formed by some mutants (e.g., Cx50P88S) may cause light scattering and act as nucleation particles for accumulation/aggregation of other proteins.

### CONNEXIN MUTANTS WITH ABNORMALITIES OF CHANNEL BEHAVIOR

The gap junction conductance between a pair of cells depends on the number of channels, their open probabilities, and their single channel conductances. The trafficking mutants discussed above are complete (or near complete) loss of function mutants, because they effectively have reduced the number of intercellular channels.

There are other mutants that form non-functional channels (e.g., Cx50W45S, Cx46D3Y, and Cx46L11S) which make abundant gap junction plaques, but have no gap junction channel activity when expressed by themselves (Tong et al., 2011, 2013) (Table 3 and Figure 3). These mutants effectively have an open probability of zero (or no unitary conductance).

Because mutant lens connexins can oligomerize with wild type Cx46 and/or Cx50 to form gap junction channels, several of the cataract-associated mutant lens connexins have been studied for their ability to alter the function of co-expressed wild type lens connexins. Some of these mutants (e.g., Cx46N63S, Cx46fs380) do not inhibit the function of either Cx46 or Cx50 (Pal et al., 2000). Other mutants (e.g., Cx50P88S, Cx50P88Q, Cx50W45S, Cx50E48K, Cx46D3Y, Cx46L11S), decrease the junctional conductance supported by their wild type counterparts (Pal et al., 1999; Arora et al., 2006; Banks et al., 2009; Tong et al., 2011, 2013). In the case of Cx50P88S, a single mutant subunit is sufficient to inhibit function of a full gap junction channel (Pal et al., 1999). Some lens connexin mutants that inhibit their homologous wild type counterparts do not act as strong dominant negative inhibitors of the other lens fiber cell connexin. For example, Cx46D3Y and Cx46L11S inhibit wild type Cx46, but they do not block wild type Cx50 (Tong et al., 2013). In addition, the function of certain lens connexin mutants such as Cx46D3Y is partially rescued by heterotypically pairing cells expressing the mutant connexin with cells expressing wild type lens connexins (Tong et al., 2013). HeLa

Table 3 | Examples of cataract-associated lens connexin mutants with different cellular or physiological abnormalities.

Mutant	Intercellular channel function	Plaque formation	ER/Golgi localization (without plaques)	Accumulation at other cellular sites	Hemichannel function	Effects on co-expressed wild type connexin
						<b>Cx50</b>
Cx50D47N	None	Absent	Yes	No		No effect
Cx46fs380	None	Absent	Yes	No	None	No effect
Cx50R23T	None	Very rare		No		Inhibition
Cx50P88Q	None	Absent	Yes	Yes		Weak inhibition
Cx50P88S	None	Absent	No	Yes		Inhibition
Cx50W45S	None	Normal	No	No	None	Inhibition
Cx46N63S	None				Reduced and altered sensitivity to divalent cations	No effect
						<b>Cx46</b>
Cx46D3Y	None	Normal	No	No	Reduced	Hemichannels with altered properties
Cx46L11S	None	Normal	No	No	None	Hemichannels with altered properties
Cx50G46V	Normal	Normal	No	No	Increased	Increased hemichannel function

Properties of mutants were all determined for each connexin when expressed by itself, except for the co-expression results. Blanks represent parameters that have not been tested.

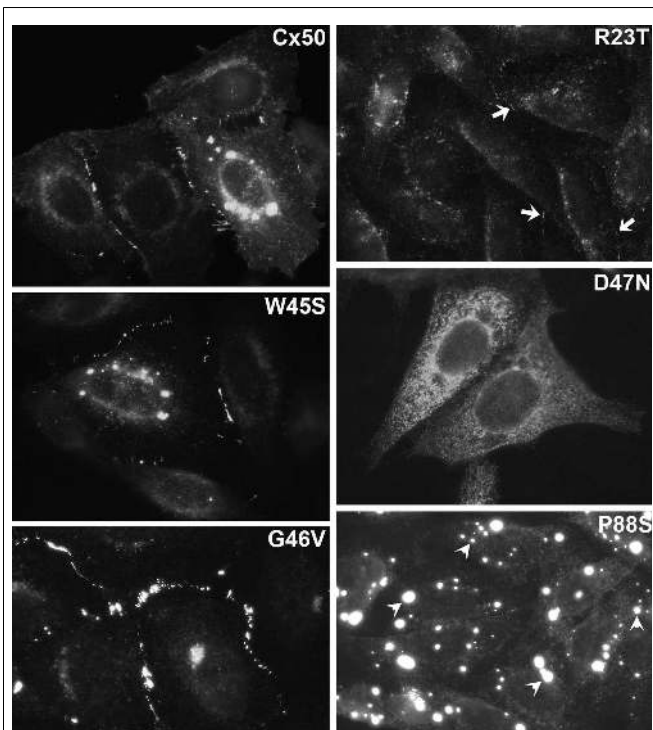
cells expressing an EGFP fusion protein of Cx46D3Y have been reported to show increased intercellular transfer of Lucifer yellow or ethidium as compared to cells expressing wild type Cx46-EGFP (Schlingmann et al., 2012).

The direct link between loss of connexin function and cataracts is an active area of research. The studies of Matthias and colleagues (reviewed in Matthias et al., 2010) have established that loss of function of either Cx46 or Cx50 (as observed in “knock-out” mice) reduces coupling conductance between lens fiber cells. Reductions of intercellular communication would be anticipated to decrease the circulation of gap junction permeant molecules (including water, ions, and metabolites) between lens cells. Indeed, Gao et al. (2011) observed that reductions in gap junction channels in genetically manipulated mice resulted in a proportional decrease in the gradient of intracellular hydrostatic pressure from the center to the periphery of the lens. One hypothesis links connexin function, calcium levels and proteolysis of crystallins to cataracts based on studies of the Cx46-null mice. These animals have nuclear cataracts associated with generation of a cleaved form of  $\gamma$ -crystallin and significant amounts of NaOH-insoluble  $\alpha$ -,  $\beta$ -, and  $\gamma$ -crystallins in the lens nucleus (Gong et al., 1997). Cx46-null mice also have elevated levels of calcium in their lens nuclear regions (Baruch et al., 2001; Gao et al., 2004) and increased activation of calcium-dependent protease activity, likely due to the calpain3 isozyme, Lp82 (Baruch et al., 2001). Moreover, cataract formation is delayed in Cx46-null mice that are also deficient in the gene (*Capn3*) encoding this protease (Tang et al., 2007). However, the mechanism linking Cx46 function to cataracts in humans may be somewhat different, since *CAPN3* mRNA expression has only been identified in skeletal muscle in people (Fougerousse et al., 1998).

Connexin mutants could also affect parameters such as voltage gating, sensitivity to intracellular pH or channel permeability that would lead to altered function, but not necessarily complete loss of function. As summarized in Table 4, Cx46 and Cx50 exhibit some differences in many of these physiological properties. Studies of mouse connexins have identified mutants (like Cx50S50P) that alter the voltage-dependent gating properties of co-expressed Cx46 or Cx50 (DeRosa et al., 2007). Interestingly, mouse Cx50S50P is also a potent inhibitor of co-expressed Cx43, suggesting that intercellular communication may be interrupted in lens epithelial cells where these two connexins are co-expressed (DeRosa et al., 2009). Another mouse mutant, Cx50R205G, blocks the gap junction channel function of co-expressed wild type Cx50, but only affects the gating of Cx46 channels (Xia et al., 2012).

## CONNEXIN FUNCTIONS BEYOND INTERCELLULAR COMMUNICATION

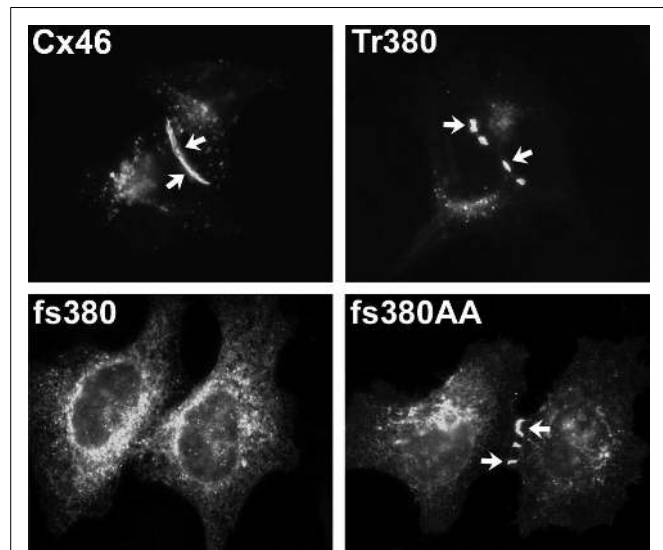
In addition to forming intercellular channels, connexins can also form functional hemichannels that induce large, relatively non-selective conductances in single plasma membranes. These conductances are caused by permeation through “undocked” single connexons. This phenomenon has been best demonstrated in primary cultures of various cells and in expression systems. One of the most dramatic examples is the large permeabilities induced by expression of Cx46 in single *Xenopus* oocytes that can be gated by modulation of extracellular concentrations of divalent cations



**FIGURE 4 | Immunofluorescent localization of wild type Cx50 and of different cataract-associated Cx50 mutants (R23T, W45S, D47N, G46V, and P88S) after their expression by transfection of HeLa cells.** Similar to wild type Cx50, W45S and G46V show abundant localization in a punctate distribution along appositional membranes corresponding to gap junction plaques. The abundance of plaques is very reduced for R23T, but small spots at appositional membranes are occasionally observed. D47N and P88S show no localization consistent with gap junction plaque formation. D47N is found in a reticular, cytoplasmic distribution. P88S localizes in intensely fluorescent cytoplasmic inclusions. Reproduced and adapted from Berthoud et al. (2003), Arora et al. (2008), Thomas et al. (2008), and Tong et al. (2011).

including calcium (Paul et al., 1991). Because Cx46 hemichannels are mechanosensitive, it has been proposed that their opening plays a physiological role during lens accommodation (Bao et al., 2004). Opening of such hemichannels has been demonstrated in isolated mouse lens fiber cells (Ebihara et al., 2010). The ability of some mutant lens connexins to form functional hemichannels has been assessed. Unlike wild type Cx46, many of the cataract-associated Cx46 mutants do not form functional hemichannels (e.g., Cx46L11S, Cx46fs380). Others exhibit a reduced ability to form them (e.g., Cx46D3Y, Cx46N63S) (Pal et al., 2000; Tong et al., 2013). Thus, entry of sodium and calcium into lens cells expressing these mutants would be impaired if Cx46 hemichannels serve as conduits for these ions in the normal lens.

Cataract-associated mutants may also form hemichannels with altered properties (like gating or charge selectivity) as compared with the wild type connexin. For example, Cx46N63S forms hemichannels with increased sensitivity to the extracellular concentration of magnesium ions (Ebihara et al., 2003). Cx46D3Y forms hemichannels that have altered charge selectivity and voltage gating (Tong et al., 2013).



**FIGURE 5 | Immunofluorescent localization of wild type Cx46, the cataract-associated mutant Cx46fs380 (fs380), Cx46 truncated after amino acid 379 (Tr380) and Cx46fs380 with the FF motif replaced by AA (fs380AA) in transfected HeLa cells.** Wild type Cx46 localizes in an intense, linear distribution along appositional membranes as expected for large gap junctions, but such staining is absent for Cx46fs380 which is only found in a cytoplasmic distribution. The cytoplasmic retention must be due to the abnormal sequence in the carboxyl terminus of Cx46fs380, since its removal by truncation (Tr380) restores gap junction formation. Similarly, gap junction formation is restored when the FF motif in Cx46fs380 is replaced with two alanines (fs380AA). Reproduced and adapted from Minogue et al. (2005).

A very striking alteration of hemichannel properties is exemplified by Cx50G46V, a mutant found in a patient with total cataract (Minogue et al., 2009). This mutant forms gap junction plaques and supports intercellular communication normally. However, unlike wild type Cx50, Cx50G46V has a greatly increased ability to form functional hemichannels (Minogue et al., 2009; Tong et al., 2011). Expression of this mutant increases the proportion of apoptotic cells and causes cell death (Minogue et al., 2009) (Figure 6), suggesting that opening of the hemichannels would also cause severe cell damage *in vivo*. This cytotoxicity appears dominant, since co-expression of Cx50G46V with wild type Cx46 or Cx50 also decreases cell (oocyte) viability (Tong et al., 2011). Connexin mutants with enhanced hemichannel activity may cause fiber cell death through a complex sequence of events including loss of membrane potential, disruption of transmembrane ion gradients, and entry of calcium ions, leading to activation of intracellular proteases and decreased metabolic activity.

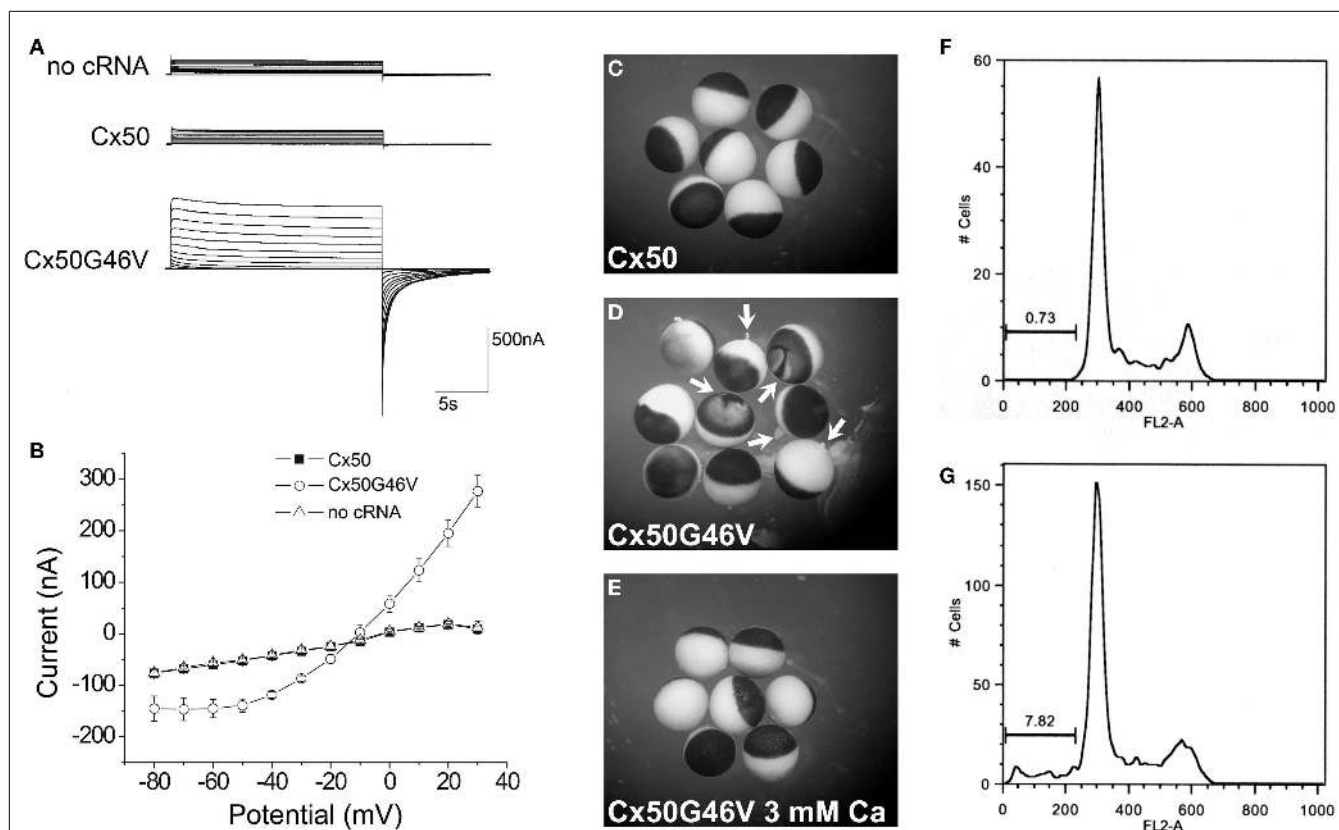
Connexins may also be involved in functions not directly associated with their channel forming ability. Cx50 is required for normal growth of the lens. As mentioned above, mice that are null for Cx50 (but not those null for Cx46) have smaller lenses (Gong et al., 1997; White et al., 1998). The decreased lens size appears to result from reduced proliferation of lens epithelial cells, especially during the first few days of postnatal life (Sellitto et al., 2004). It has been speculated that this proliferative deficiency is due to a connexin function other than intercellular exchange of



**Table 4 | Physiological properties of wild type Cx50 and Cx46.**

Property	Cx50	Cx46	Reference
Unitary gap junctional channel conductance	~220 pS	~150 pS	Srinivas et al. (1999), Hopperstad et al. (2000)
Ionic selectivity	Cations > anions	Cations > anions	Srinivas et al. (1999), Trexler et al. (2000)
Dye permeability	DAPI, NB >> LY	DAPI >> LY	Srinivas et al. (1999), Trexler et al. (2000), Thomas et al. (2008)
Sensitivity to transjunctional voltage	++	+	Ebihara and Steiner (1993), White et al. (1994), Srinivas et al. (1999), Hopperstad et al. (2000)
Sensitivity to intracellular pH	+	+	Eckert (2002), Xu et al. (2002)
Sensitivity to activated MAPK	++	-	Shakespeare et al. (2009)
Ability to form functional hemichannels	+	+++	Paul et al. (1991), Ebihara and Steiner (1993), Beahm and Hall (2002)

Except for the final row, the properties refer to those of intercellular channels formed of each connexin as determined from studies in expression systems. pS, picoSiemens; DAPI, 4,6-diamidino-2-phenylindole; NB, Neurobiotin; LY, Lucifer yellow; MAPK, Mitogen-activated protein kinase.



**FIGURE 6 | The Cx50G46V mutant induces large hemichannel currents, cytotoxicity, and apoptosis. (A)** Hemichannel currents elicited in response to a series of voltage pulses in control *Xenopus* oocytes (no cRNA) or oocytes injected with cRNA encoding wild type Cx50 or Cx50G46V. The mutant induces much larger currents than wild type Cx50 or control oocytes. **(B)** Steady-state current-voltage relationships in control (no cRNA), wild type Cx50, and Cx50G46V cRNA-injected oocytes. The mutant induces large outward currents that activate on depolarization. **(C–E)** *Xenopus* oocytes were injected with similar amounts of Cx50 **(C)** or Cx50G46V cRNA **(D,E)** and incubated in modified Barth's solution containing 1 mM Ca<sup>2+</sup> **(C,D)** or 3 mM Ca<sup>2+</sup> **(E)** overnight at 18°C. Oocytes injected with Cx50G46V cRNA showed obvious discoloration, membrane disruption, and leakage of yolk

(arrows in **D**) when incubated in modified Barth's solution containing 1 mM Ca<sup>2+</sup> **(D)** while oocytes injected with Cx50 cRNA showed no apparent detrimental changes when studied under identical conditions **(C)**. The rate of cell death of the Cx50G46V cRNA-injected oocytes was significantly reduced by increasing the external calcium concentration from 1 to 3 mM **(E)**. **(F,G)** Graphs show the results of cell cycle analysis of propidium iodide-stained HeLa cells induced to express wild type Cx50 **(F)** or Cx50G46V **(G)**. This analysis revealed a substantial population of cells in the sub-G1 fraction, a marker for apoptosis, in cells expressing Cx50G46V but not in cells expressing Cx50 implying that expression of this mutant increased the proportion of apoptotic cells. Reproduced and adapted from Minogue et al. (2009).

ions and small molecules, since introduction of Cx46 into the Cx50 locus does not restore normal lens growth to Cx50-null mice (Martinez-Wittinghan et al., 2004). Over-expression of Cx50 also leads to a decrease in lens size (Chung et al., 2007); thus, the proper level of Cx50 expression is required for normal lens development and growth.

The growth and differentiation promoting properties of Cx50 (and the roles of different portions of the molecule) have been further analyzed. Expression of the chicken Cx50 ortholog (including non-functional mutants) or a chicken Cx46 chimera containing the Cx50 carboxyl terminus promotes differentiation of chicken lens cells in culture (Gu et al., 2003; Banks et al., 2007). The mechanism for this “non-channel”-induced effect of Cx50 has not been clarified, but it has been suggested that it involves interactions of its carboxyl terminus with other cellular proteins to modulate intracellular signaling critical for lens cell differentiation. The major intrinsic protein, aquaporin0, is among the lens proteins that can interact with lens connexins (Yu and Jiang, 2004; Yu et al., 2005).

It is plausible that some human Cx50 mutants affect protein interactions or differentiation promoting properties involved in the normal development of the lens and other eye structures. In some of the families with inherited cataracts, affected individuals have small lenses. Moreover, in some pedigrees, the cataracts are associated with additional abnormalities including microcornea, iris hypoplasia, and myopia (see **Tables 1** and **2**).

## CONCLUSIONS AND PERSPECTIVES

In conclusion, there are many different derangements in the cellular/biochemical behavior and function of the Cx46 and Cx50 mutants that have been linked to cataract formation.

As discussed, the most common abnormality caused by a mutation is improper trafficking and reduced gap junction plaque formation. Such mutations occur at positions throughout the connexin polypeptide, likely because deleterious substitutions of many different residues may cause misfolding (e.g., Cx50D47N). Many identified mutations involve substitutions of amino acids near the amino terminus or within the first transmembrane domain (**Figure 3**). Since these regions contribute to the channel pore, mutants within them (if targeted properly to the plasma membrane) would be expected to cause functional alterations (e.g., Cx46L11S). The extracellular loops are the other regions with a large number of mutations. Since the structure of these domains is critical for docking between hemichannels and for gating of hemichannels, it would be expected that mutants in this region would affect function if the mutant connexin is appropriately targeted to the plasma membrane (e.g., Cx50G46V).

Elucidating the abnormalities produced by many of these mutations will help to understand the pathogenesis of cataracts and of other diseases caused by mutations in other members of the connexin family. Indeed, reduced formation of gap junctions

leading to loss of function is also one of the most commonly observed abnormalities of disease-associated Cx26, Cx32, and Cx43 mutants (reviewed by Laird, 2010; Abrams and Scherer, 2012; Scott et al., 2012; Xu and Nicholson, 2012).

The cataracts associated with Cx46 and Cx50 mutants are predominantly inherited as autosomal dominant traits. This is similar to the inheritance pattern of oculodentodigital dysplasia caused by Cx43 mutants, but differs from Cx32 and most Cx26 mutants which cause disease in a recessive fashion. In some cases, the mutant connexin acts as a “dominant-negative” inhibitor of the function of the co-expressed wild type protein (like Cx50P88S) (Pal et al., 1999). But, other lens connexin mutants show little or no inhibition of the function of the wild type counterparts when studied in expression systems (**Table 3**). The absence of cataracts in mice that are heterozygous-null for Cx46 or Cx50 (Gong et al., 1997; White et al., 1998) implies that haplodeficiency of these connexins in mice is not sufficient to cause disease. Thus, there must be additional abnormalities conferred by the mutations that lead to lens opacities. Some of these other abnormalities such as formation of cytoplasmic inclusions or gain of hemichannel function have been considered in this review.

It is noteworthy that there are significant differences in the anatomical locations or appearances of the cataracts caused by different lens connexin mutations and among individuals within a single pedigree or in different pedigrees with the same mutation (**Tables 1** and **2**). Some of the phenotypic dissimilarities between different mutants likely reflect the variety of altered cellular/biochemical mechanisms, since mutants may disrupt different protein–protein interactions and affect different processes. The phenotypic differences among people with the same mutant allele imply that there are other genes that contribute to the phenotype. This conclusion is supported by the strain-dependent differences in severity of the cataracts in Cx46- and Cx50-null mice (Gong et al., 1999; Gerido et al., 2003). The differential severities of the cataracts in Cx46-null mice may partially result from differences in expression of HSP27/25 and ERp29 between strains (Hoehenwarter et al., 2008).

Finally, we can anticipate further progress in the elucidation of the mechanisms involved in cataract formation based on the characterization of additional mutants in expression systems and through the study of corresponding mutants using animal models. Eventually, these studies will contribute to the development of therapeutic approaches to prevent the formation or progression of cataracts due to connexin mutations, and perhaps those due to other genetic or non-genetic causes.

## ACKNOWLEDGMENTS

This work was supported by National Institute of Health grants RO1 EY08368 (to Eric C. Beyer) and RO1 EY10589 (to Lisa Ebihara).

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Received: 15 February 2013; accepted: 26 March 2013; published online: 15 April 2013.
- Citation:* Beyer EC, Ebihara L and Berthoud VM (2013) Connexin mutants and cataracts. *Front. Pharmacol.* 4:43. doi: 10.3389/fphar.2013.00043
- This article was submitted to *Frontiers in Pharmacology of Ion Channels and Channelopathies*, a specialty of *Frontiers in Pharmacology*.
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