Published in final edited form as:

Exp Eye Res. 2016 May ; 146: 283–288. doi:10.1016/j.exer.2016.03.025.

Connexin23 deletion does not affect lens transparency

Viviana M. Berthoud^a, Peter J. Minogue^a, Joseph I. Snabb^a, Yulia Dzhashvili^b, Layne A. Novak^c, Rebecca K. Zoltoski^c, Brian Popko^b, and Eric C. Beyer^a

^aDepartment of Pediatrics, University of Chicago, Chicago, Illinois

^bDepartment of Neurology, University of Chicago, Chicago, Illinois

^cIllinois College of Optometry, Chicago, Illinois

Abstract

While connexin46 (Cx46) and connexin50 (Cx50) are crucial for maintaining lens transparency and growth, the contributions of a more recently identified lens fiber connexin, Cx23, are poorly understood. Therefore, we studied the consequences of absence of Cx23 in mouse lenses. Cx23-null mice were generated by homologous Cre recombination. Cx23 mRNA was abundantly expressed in wild type lenses, but not in Cx23-null lenses. The transparency and refractive properties of Cx23-null lenses were similar to wild type lenses when examined by darkfield microscopy. Neither the focusing ability nor the light scattering was altered in the Cx23-null lenses. While both Cx46 and Cx50 localized to appositional fiber cell membranes (as in wild type lenses), their levels were consistently (but not significantly) decreased in homozygous Cx23-null lenses. These results suggest that although Cx23 expression can influence the abundance of the co-expressed lens fiber connexins, heterozygous or homozygous expression of a Cx23-null allele does not alter lens transparency.

Keywords

connexin; gap junction; cataract; knockout mouse

1. Introduction

Gap junction subunit proteins (connexins, Cx) are critical for maintaining homeostasis and transparency in the lens (Beyer et al., 2013). Several connexins are predominantly expressed in the vertebrate lens. Rodent lens fiber cells express Cx23, Cx46 and Cx50 (Bassnett et al., 2009; Paul et al., 1991; Puk et al., 2008; White et al., 1992). Connexins form intercellular channels that facilitate the circulation of water, ions and solutes within the avascular lens (Mathias et al., 2010). They can also form “hemichannels” connecting the cytoplasm and extracellular space (Beyer and Berthoud, 2014).

Address correspondence to: Viviana M. Berthoud, Department of Pediatrics, University of Chicago, 900 East 57th St., KCB-5, Chicago, IL 60637, Telephone: (773) 834-2115, FAX: (773) 834-1329, vberthou@peds.bsd.uchicago.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

There is extensive evidence demonstrating the importance of Cx46 and Cx50 for the lens. Mice containing homozygous disruptions of the genes encoding either Cx46 (*Gja3*) or Cx50 (*Gja8*) develop cataracts (Gong et al., 1997; White et al., 1998). Cx50-null animals also have small lenses. Mutations of both of these genes have been linked to congenital cataracts in rodents and humans (reviewed in Beyer et al., 2013).

In contrast, Cx23 and its role in the lens have been much less extensively studied. Cx23 was discovered more recently than the other lens connexins (Gustincich et al., 2003). The Cx23 gene (*Gje1*) has a unique structure among connexins. While the coding region of most of the other connexins is encoded by a single exon, the coding region of Cx23 is encoded by 3 exons (Sonntag et al., 2009). Exon 1 of *Gje1* encodes 13 amino acids of the intracellular N-terminus, exon 2 encodes the last 9 amino acids of the N-terminus through the first extracellular loop and exon 3 encodes the second transmembrane domain through the intracellular C-terminus (Sonntag et al., 2009) (Fig. 1Aa). Although the connexin nomenclature was standardized a few years ago (reviewed in Beyer and Berthoud, 2009), and *Gje1* was assigned to the gene encoding Cx23, the literature published earlier includes some confusing usage of the gene and protein names. The Cx23 encoding gene was termed *Gjfl* in a manuscript published before nomenclature standardization (Puk et al., 2008). *Gje1* was sometimes used to denote the gene encoding Cx29 (Yang et al., 2005); but, in the standardized nomenclature, the Cx29 gene is *Gjc3*.

Cx23 expression has been detected in RNA prepared from adult mouse lens (but not in RNA prepared from various other adult mouse tissues) (Puk et al., 2008; Sonntag et al., 2009). The microphthalmic mouse (*Aey12*) has a point mutation in the coding region of the Cx23 gene and severe problems with lens fiber elongation (Puk et al., 2008). Proteomics studies of adult mouse lenses show that Cx23 is a component of lens fiber cell membranes with an abundance similar to Cx46, but less than Cx50 (Bassnett et al., 2009). During embryonic development, Cx23 transcripts are initially detected in the posterior region of the lens vesicle, then at the tips of the elongating primary fiber cells, and subsequently in the epithelium and elongating fibers of the equatorial region (Puk et al., 2008).

To examine the importance of Cx23 for the lens, we generated a Cx23-null mouse by targeted deletion of exon 2 of *Gje1*. In the current study, we evaluated the consequences of Cx23 deletion on lens appearance and on the co-expressed lens fiber connexins (Cx46 and Cx50). The data demonstrate that absence of Cx23 expression does not cause severe problems for lens transparency.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Thermo Scientific (Pittsburgh, PA) unless otherwise specified.

2.2. Generation of Cx23-null mice

ES cells containing a targeted conditional allele (*Gje1^{tm1a}*(EUCOMM)Hmgu) were obtained from EUCOMM. Chimeric mice were produced by injection of these ES cells into

C57BL/6J blastocysts at the Transgenic Mouse and Embryonic Stem Cell Facility of the University of Chicago. Cx23-null mice were generated by crossing mice carrying the *Gje1^{tm1a}(EUCOMM)Hmgu* allele with MOX-*Cre* mice, which express Cre in all embryonic tissues. Exposure to Cre deleted the promoter-driven *neo* cassette and critical exon 2 of the *Gje1^{tm1a}(EUCOMM)Hmgu* allele to generate a *LacZ*-tagged deletion allele (Skarnes et al., 2011) (Fig. 1Ab and Ac). Then, males were mated to C57BL/6J females to produce heterozygotes, which were subsequently mated to each other to produce homozygous Cx23-null mice.

All animal procedures conformed to the National Institutes of Health guide for the care and use of laboratory animals and followed the Institutional Animal Care and Use Committee guidelines from the University of Chicago.

Experiments were performed on animals of both sexes.

2.3. Genotyping

Mouse genomic DNA was isolated from tail biopsies (Sambrook and Russel, 2001) using PCR grade proteinase K and RNaseA (Roche Diagnostics Corporation, Indianapolis, IN). Then, a DNA aliquot was subjected to PCR using Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA) and Cx23 primers (sense: 5' TTAACCGTGAACCTTGACCAT 3'; antisense: 5' CTGTAGAAGAAGAACTCTGAG 3') (1 and 2 in Fig. 1Ab) or LacZ primers (sense: 5' CTATCCCATTACGGTCAATC 3'; antisense: 5' GCCATAAAGAACTGTTACC 3') (3 and 4 in Fig. 1Ab).

2.4. Real time PCR

Lens RNA was prepared from 4 week-old mice with the miRNeasy Mini Kit (Qiagen, Valencia, CA) using a glass-glass homogenizer. Complementary DNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen). Then, an aliquot of the cDNA was subjected to real time PCR (RT-qPCR) using Fast SYBR® Green Master Mix (Life Technologies, Grand Island, NY) and Cx23 primers (sense: 5' TCAGCTGTGACCCAGACAAG 3'; antisense: 5' CATGCAGCATACAGGTGGA 3') (5 and 6 in Fig. 1Ab) or LacZ primers (sense: 5' TTCAACATCAGCCGCTACAG 3'; antisense: 5' CGTCGATATTCAGCCATGTG 3') (7 and 8 in Fig. 1Ab). The reaction was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Only primer sets with efficiencies within the 95% confidence interval were considered acceptable and used for these studies. Melting curves were run for each primer set to exclude sets that produced primer dimers. All samples were normalized to equal RNA concentrations.

2.5. Immunofluorescence and immunoblotting

Connexins were detected by immunofluorescence and immunoblotting as previously described using anti-Cx46, anti-Cx50 and three different anti-Cx23 antibodies (Berthoud et al., 2013). One anti-Cx23 antibody (Sigma-Aldrich) was raised against a peptide corresponding to 26 of the 36 amino acids in the second extracellular loop of the predicted human Cx23, which shares 65% sequence identity with the mouse Cx23 including a stretch

of 8 consecutive identical amino acids. A second commercially available antibody (Abgent) was raised against amino acid residues 178–205 (including 14 amino acids of the predicted fourth transmembrane domain and the cytoplasmic C-terminus of human Cx23), which share 64% sequence identity with mouse Cx23. An antibody produced by our group was raised against the N-terminus of mouse Cx23. HeLa cells were transiently transfected as previously described (Minogue et al., 2005) using a Myc-DDK-tagged pCMV6-Cx23 plasmid (OriGene, Rockville, MD). Protein concentrations were determined using the BioRad Protein Assay (BioRad, Hercules, CA) based on the Bradford dye-binding procedure (Bradford, 1976). Equal amounts of protein were loaded per lane (20 µg for Cx50 immunoblots and 100 µg for Cx46 immunoblots). The intensities of the immunoreactive bands were quantified by densitometry using Photoshop CS3 Extended (Adobe Systems, Inc., San Jose, CA), and are reported as percentages of the values determined in wild type samples. Graphs were generated using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA). Statistical analysis was performed using Student's paired t-test.

2.6. Light microscopy and optical quality analyses

Darkfield photomicrographs of mouse lenses from different genotypes were obtained using a Zeiss Stemi-2000C dissecting scope (Carl Zeiss, München, Germany) under identical illumination conditions. To evaluate the refractive properties, mouse lenses were photographed against a 200-mesh electron microscopy grid as previously performed by Shiels et al. (2007). Helium neon laser scan analysis was performed as described in Kuszak et al. (1991) to determine back vertex distance (BVD, a measure of spherical aberration) and BVD variability (sharpness of focus). An average of 40 data points were collected for each lens analyzed. Data are presented as mean ± S.E.M; the number of animals used is indicated in parenthesis. SigmaPlot Systat Software (San Jose, CA) was used to perform a one way ANOVA looking for differences between the genotypes with $p < 0.05$ set for level of significance.

3. Results

3.1. Generation of mice with targeted deletion of Cx23 exon 2

To assess deletion of the targeted exon 2 of *Gje1* and its replacement by a *LacZ* coding sequence in the Cx23-null mice, we performed PCR using genomic DNA templates and a set of primers located in the intronic regions flanking exon 2 of *Gje1* (primers 1 and 2 in Fig. 1Ab) or a set of primers within the coding region of *LacZ* (primers 3 and 4 in Fig. 1Ab). Some samples contained bands corresponding to both Cx23 (408 bp) and LacZ (473 bp) amplicons as expected for heterozygous mice (Fig. 1B). Other samples contained only the Cx23 or the LacZ amplicons as expected for wild type and for homozygous animals, respectively (Fig. 1B).

Levels of Cx23 transcripts were assessed by real time PCR (RT-qPCR) using cDNA reverse transcribed from lens RNA. These experiments detected Cx23 mRNA in lens RNA from wild type mice; they also showed that levels of Cx23 transcripts were decreased in heterozygotes and absent in homozygous Cx23-null mice (Fig. 1C). Parallel RT-qPCR experiments showed that *LacZ* transcripts were present in homozygous and heterozygous

Cx23-null mice, whereas they were absent from wild type animals (Fig. 1D). As expected, higher concentrations of *LacZ* transcripts were detected in homozygous than in heterozygous Cx23-null animals.

We attempted to assess levels and distribution of Cx23 by immunological methods using three different anti-Cx23 antibodies. Unfortunately, this proved impossible, since all of these antibodies recognized multiple bands of differing molecular mass in lens homogenates, and the immunoblot patterns did not differ between genotypes, i.e., they did not detect specific bands (data not shown). In addition, the antibodies did not specifically recognize the protein in transiently transfected HeLa cells.

3.2. Cx23 deletion does not affect body size

Homozygous Cx23-null mice appeared normally viable, active, and phenotypically indistinguishable from their heterozygous or wild type littermates (of the same sex); they had no differences in appearance or size. The ratio of *Gje1*^{+/+}:*Gje1*^{+/-}:*Gje1*^{-/-} mice was 1.0:2.5:1.2 (n = 284; p = 0.29), which is not significantly different from the expected Mendelian ratio of 1:2:1.

3.3. Cx23 deletion does not severely affect lens transparency, focusing ability or refraction

To determine the effects of deletion of *Gje1* expression in the lens, we examined lenses under darkfield illumination. Lenses from our wild type mice (maintained on a C57BL/6J background) appeared grossly normal and transparent (Fig. 1E). However, as previously observed for the C57BL/6 strain (Pettan-Brewer and Treuting, 2011), most animals contained a few (usually 5–7) very small (variable in size, but typically < 25 µm) punctate opacities in the nuclear region, which could hardly be photographed (Fig. 1E). Similar to wild type, lenses from heterozygous and homozygous Cx23-null mice contained a few small, opalescent puncta in the nuclear region (Fig. 1E). Thirty or more mice of each mutant genotype (including both sexes) were examined at ages from 4 to 45 weeks, but they did not contain major cataracts at any age examined. Additionally, heterozygous and homozygous Cx23-null lenses were similar in size to wild type lenses. Thus, no obvious differences were observed in the appearance, size, and transparency of the heterozygous and homozygous Cx23-null lenses compared with those from wild type mice.

To test whether Cx23 affected the optical quality of the lens, we assessed the refractive properties of these lenses by photographing a 200-mesh electron microscope grid through wild type and homozygous Cx23-null lenses. The square pattern of the grid could be focused sharply through both wild type and homozygous Cx23-null lenses without distortions or deformations (Fig. 1F). The slight deformations at the periphery of the lens were considered normal, because they were present in both wild type and homozygous Cx23-null lenses (Fig. 1F).

In addition, we analyzed the optical quality of wild type and Cx23-null lenses by laser scanning. Because this technique involves measurement of the deflection of a laser beam impacting different regions through the lens, it may be more sensitive to slight changes in the focusing ability of the lens. Examples showing the similarity of the laser scan profiles

through wild type and Cx23-null heterozygous and homozygous lenses are shown in Fig. 1G. Since lenses from all genotypes had small punctate cataracts in the central region, it is likely that deviations from the average BVD resulted from the laser beam impacting one of these small opacities. The BVD averaged 1.76 ± 0.27 mm ($n = 4$) in wild type lenses, 1.89 ± 0.11 mm ($n = 6$) in heterozygous Cx23-null lenses and 2.00 ± 0.39 mm ($n = 4$) in homozygous Cx23-null lenses. The BVD variabilities (or S.E.M. of individual points to the average BVD) were 0.31 ± 0.14 , 0.28 ± 0.06 and 0.21 ± 0.06 in wild type, heterozygous Cx23-null and homozygous Cx23-null lenses, respectively. The BVD and the BVD variability were not significantly different among the different genotypes ($p = 0.822$ for BVD and 0.765 for BVD variability). The relationships between BVD or BVD variability and sex were not examined.

3.4. Deletion of Cx23 does not affect Cx46 and Cx50 localization, but can affect their levels

To determine whether absence of expression of Cx23 affected the distributions of Cx46 or Cx50, we performed immunofluorescence staining of lens sections from wild type and homozygous animals using specific antibodies against these connexins. Sections were also labeled with TRITC-conjugated phalloidin to delineate cell borders and with DAPI to stain the nuclei. No differences in the localization of immunoreactive Cx46 or Cx50 were detected in these samples (Fig. 2A and B). Moreover, the distributions of filamentous actin and of nuclei appeared similar.

To test whether deletion of Cx23 led to changes in Cx46 and/or Cx50 levels (as compensation for the lack of Cx23) without affecting their localization, we performed immunoblots on total lens homogenates from wild type mice, and from heterozygous and homozygous Cx23-null animals. Unexpectedly, levels of Cx46 and Cx50 were consistently decreased in homozygotes compared with wild type levels (on average by 38% and 22%, respectively) (Fig. 2C and D), but these differences were not statistically significant ($p = 0.093$ for Cx46 and $p = 0.157$ for Cx50). Levels of Cx46 were decreased in some but not all heterozygotes, whereas levels of Cx50 were slightly increased in some but not all heterozygous Cx23-null mice (Fig. 2C and D). These experiments were not specifically designed to study the influence of sex on connexin levels. However, some immunoblot experiments contained all male or all female samples, and showed similar results and variabilities (data not shown).

4. Discussion

In this study, we have found that mice with targeted deletion of Cx23 exon 2 did not have major general systemic defects. This finding was not unexpected, since expression of *Gjrl* is almost completely restricted to the eye (Puk et al., 2008; Sonntag et al., 2009). However, we were surprised that the lack of Cx23 expression did not lead to cataracts, because *Aey12* mice (which carry a missense mutation in Cx23) have small eyes, small lenses and polar lens opacities (Puk et al., 2008).

In contrast to the normal appearing, transparent lenses of our homozygous Cx23-null animals, mice with targeted deletion of either of the other lens fiber cell connexins (Cx46

and Cx50) develop substantial cataracts (Gong et al., 1997; Rong et al., 2002; White et al., 1998). The absence of substantial cataracts in Cx23-null mice could not be due to the age of analysis, because we did not observe increased opacification of the lens even in older Cx23-null mice. Moreover, the Cx23-null animals did not have more subtle defects in focusing or light scattering, because an electron microscopy grid was sharply focused through them, and they showed similar spherical aberration and sharpness of focus compared to wild type lenses. Taken together, these results suggest that although Cx23 may play a role in the mouse lens, its expression is not required for lens transparency.

Cx23 transcripts have not been found in cDNA prepared from primates or from the human lens. Moreover, sequence analysis of primate genomes suggests inactivation of the Cx23 gene (Sonntag et al., 2009). Thus, it is possible that because Cx23 is not absolutely required for lens transparency, its expression was lost during evolution of primates.

We considered the possibility that the main role of Cx23 in the mouse lens could be the formation of functional hemichannels. When exogenously expressed in HeLa cells, mouse Cx23 does not form intercellular channels, but both Zebrafish and mouse Cx23 form hemichannels (Iovine et al., 2008; Sonntag et al., 2009). Cataracts linked to Cx46 or Cx50 abnormalities might sometimes be due to abnormalities of connexin hemichannels, since Cx46 readily forms hemichannels and since a cataract-linked Cx50 mutant (Cx50V44A) has decreased hemichannel activity (but normal intercellular channel activity) (Zhu et al., 2014). However, the data of Ebihara et al. (2011) suggest that Cx23 does not make a significant contribution to hemichannel currents in mouse fiber cells, since these authors could detect calcium-sensitive hemichannel currents in wild type and Cx50-null fiber cells, but not in cells isolated from double Cx46 and Cx50 knockout animals. Cx46 hemichannels have been proposed to participate in lens accommodation (Bao et al., 2004). However, the mouse lens lacks accommodation, because of its rigidity and the absent (or less developed) ciliary muscle (Woolf, 1956; Treuting et al., 2012). Thus, it is unlikely that accommodation is the major role of Cx23 when it is absent in the accommodating human lens, but present in the non-accommodating mouse lens.

Interestingly, the absence of lens Cx23 may affect the co-expressed connexins. Levels of Cx46 and Cx50 in homozygous Cx23-null mice were consistently reduced, but the differences were variable enough that they were not statistically significant. In addition, the localization of Cx46 and Cx50 along the broad sides of fiber cells was not affected. It should not be surprising that the decreases in Cx46 and Cx50 levels did not result in obvious changes in immunofluorescence intensity, since lens gap junction plaques contain a large number of closely packed gap junction channels. The fiber cells of all Cx23-null lenses examined apparently contained adequate levels of Cx46 and Cx50 to maintain transparency. Previous studies have shown that heterozygous mice with targeted deletion of Cx46 or Cx50 (containing ~50% of the normal Cx46 or Cx50 levels without affecting the level of co-expressed connexin) do not develop cataracts (Gong et al., 1998; Rong et al., 2002), implying that more severe decreases in Cx46 or Cx50 levels are necessary for cataract formation.

It is possible that Cx23 enhances the stability of the other lens fiber connexins, especially Cx46, whose levels decreased in homozygous animals to a larger extent than those of Cx50. Decrease in levels of a connexin after targeted deletion of a co-expressed connexin is not without precedent. Deletion of Cx32 decreases levels of the co-expressed Cx26 (without affecting its transcript levels) in liver (Nelles et al., 1996). Additionally, deletion of Cx30 in astrocytes leads to loss of Cx32 on the oligodendrocyte side of astrocyte-oligodendrocyte gap junctions, almost complete loss of Cx26 in cerebral leptomeninges and downregulation of Cx26 mRNA (Lynn et al., 2011).

The absence of an evident cataract phenotype in Cx23-null lenses is in stark contrast with the phenotype observed in the *Aey12* mice (which carry the Cx23R32Q mutation). Expression of one Cx23R32Q-encoding allele is sufficient to retard lens fiber cell elongation, while mutation of both alleles arrests eye development at the lens vesicle stage (Puk et al., 2008). Considering that different allelic variants of other lens genes like *Pax6* (Favor et al., 2008) may produce a wide range of phenotypic severities, we suggest that the Cx23-null allele behaves like a hypomorph, whereas the Cx23R32Q allele behaves as a dominant negative. Likely, expression of the Cx23 mutant protein had toxic effects on the lens (absent in animals with a null mutation) that contributed to slowed cell proliferation and impaired differentiation. This hypothesis is consistent with the observation that most of the cataracts linked to connexin mutants are inherited as autosomal dominant traits, while mice that are heterozygous null for Cx46 or Cx50 have normal lenses. Thus, expression of a mutant lens fiber cell connexin can be far more deleterious than haploinsufficiency (reviewed in Beyer et al., 2013), implying that many lens connexin mutants have toxic effects.

Acknowledgments

This work was supported by National Institutes of Health Grants RO1 EY08368 (ECB) and NS067550 (BP). The authors thank Linda Degenstein, technical director of the Transgenic Mouse and Embryonic Stem Cell Facility of the University of Chicago, who was instrumental in the generation of the Cx23-null mice.

References

- Bao L, Sachs F, Dahl G. Connexins are mechanosensitive. *Am J Physiol Cell Physiol.* 2004; 287:C1389–C1395. [PubMed: 15475518]
- Bassnett S, Wilmarth PA, David LL. The membrane proteome of the mouse lens fiber cell. *Mol Vis.* 2009; 15:2448–2463. [PubMed: 19956408]
- Berthoud VM, Minogue PJ, Yu H, Schroeder R, Snabb JJ, Beyer EC. Connexin50D47A decreases levels of fiber cell connexins and impairs lens fiber cell differentiation. *Invest Ophthalmol Vis Sci.* 2013; 54:7614–7622. [PubMed: 24204043]
- Beyer, EC.; Berthoud, VM. The family of connexin genes. In: Harris, A.; Locke, D., editors. *Connexins: A Guide*. New York: Humana Press; 2009. p. 3-26.
- Beyer EC, Berthoud VM. Connexin hemichannels in the lens. *Front Physiol.* 2014; 5:20. [PubMed: 24575044]
- Beyer EC, Ebihara L, Berthoud VM. Connexin mutants and cataracts. *Front Pharmacol.* 2013; 4:43. [PubMed: 23596416]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
- Ebihara L, Tong JJ, Vertel B, White TW, Chen TL. Properties of connexin 46 hemichannels in dissociated lens fiber cells. *Invest Ophthalmol Vis Sci.* 2011; 52:882–889. [PubMed: 20861491]

- Favor J, Gloeckner CJ, Neuhäuser-Klaus A, Pretsch W, Sandulache R, Saule S, Zaus I. Relationship of Pax6 activity levels to the extent of eye development in the mouse, *Mus musculus*. *Genetics*. 2008; 179:1345–1355. [PubMed: 18562673]
- Gong X, Baldo GJ, Kumar NM, Gilula NB, Mathias RT. Gap junctional coupling in lenses lacking $\alpha 3$ connexin. *Proc Natl Acad Sci U S A*. 1998; 95:15303–15308. [PubMed: 9860964]
- Gong X, Li E, Klier G, Huang Q, Wu Y, Lei H, Kumar NM, Horwitz J, Gilula NB. Disruption of $\alpha 3$ connexin gene leads to proteolysis and cataractogenesis in mice. *Cell*. 1997; 91:833–843. [PubMed: 9413992]
- Gustincich S, Batalov S, Beisel KW, Bono H, Carninci P, Fletcher CF, Grimmond S, Hirokawa N, Jarvis ED, Jegla T, Kawasaki Y, LeMieux J, Miki H, Raviola E, Teasdale RD, Tominaga N, Yagi K, Zimmer A, Hayashizaki Y, Okazaki Y. RIKEN GER Group; GSL Members. Analysis of the mouse transcriptome for genes involved in the function of the nervous system. *Genome Res*. 2003; 13:1395–1401. [PubMed: 12819138]
- Iovine MK1, Gumpert AM, Falk MM, Mendelson TC. Cx23, a connexin with only four extracellular-loop cysteines, forms functional gap junction channels and hemichannels. *FEBS Lett*. 2008; 582:165–170. [PubMed: 18068130]
- Kuszak JR, Sivak JG, Weerheim JA. Lens optical quality is a direct function of lens sutural architecture. *Invest Ophthalmol Vis Sci*. 1991; 32:2119–2129. [PubMed: 2055702]
- Lynn BD, Tress O, May D, Willecke K, Nagy JJ. Ablation of connexin30 in transgenic mice alters expression patterns of connexin26 and connexin32 in glial cells and leptomeninges. *Eur J Neurosci*. 2011; 34:1783–1793. [PubMed: 22098503]
- Mathias RT, White TW, Gong X. Lens gap junctions in growth, differentiation, and homeostasis. *Physiol Rev*. 2010; 90:179–206. [PubMed: 20086076]
- Minogue PJ, Liu X, Ebihara L, Beyer EC, Berthoud VM. An aberrant sequence in a connexin46 mutant underlies congenital cataracts. *J Biol Chem*. 2005; 280:40788–40795. [PubMed: 16204255]
- Nelles E, Bützler C, Jung D, Temme A, Gabriel HD, Dahl U, Traub O, Stümpel F, Jungermann K, Zielasek J, Toyka KV, Dermietzel R, Willecke K. Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci U S A*. 1996; 93:9565–9570. [PubMed: 8790370]
- Paul DL, Ebihara L, Takemoto LJ, Swenson KI, Goodenough DA. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. *J Cell Biol*. 1991; 115:1077–1089. [PubMed: 1659572]
- Pettan-Brewer C, Treuting PM. Practical pathology of aging mice. *Pathobiol Aging Age Relat Dis*. 2011; 1:7202.
- Puk O, Löster J, Dalke C, Soewarto D, Fuchs H, Budde B, Nürnberg P, Wolf E, de Angelis MH, Graw J. Mutation in a novel connexin-like gene (*Gjfl*) in the mouse affects early lens development and causes a variable small-eye phenotype. *Invest Ophthalmol Vis Sci*. 2008; 49:1525–1532. [PubMed: 18385072]
- Rong P, Wang X, Niesman I, Wu Y, Benedetti LE, Dunia I, Levy E, Gong X. Disruption of *Gja8* ($\alpha 8$ connexin) in mice leads to microphthalmia associated with retardation of lens growth and lens fiber maturation. *Development*. 2002; 129:167–174. [PubMed: 11782410]
- Sambrook J, Russell DW. *Molecular Cloning a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2001.
- Shiels A, King JM, Mackay DS, Bassnett S. Refractive defects and cataracts in mice lacking lens intrinsic membrane protein-2. *Invest Ophthalmol Vis Sci*. 2007; 48:500–508. [PubMed: 17251442]
- Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AF, Bradley A. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature*. 2011; 474:337–342. [PubMed: 21677750]
- Sonntag S, Söhl G, Dobrowolski R, Zhang J, Theis M, Winterhager E, Bukauskas FF, Willecke K. Mouse lens connexin23 (*Gje1*) does not form functional gap junction channels but causes enhanced ATP release from HeLa cells. *Eur J Cell Biol*. 2009; 88:65–77. [PubMed: 18849090]

- Treuting, PM.; Wong, R.; Tu, DC.; Phan, I. Special senses. In: Treuting, PM.; Dintzis, SM., editors. *Comparative Anatomy and Histology: A Mouse and Human Atlas*. Vol. Chapter 21. Academic Press; 2012. p. 395-417.
- White TW, Bruzzone R, Goodenough DA, Paul DL. Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol Biol Cell*. 1992; 3:711–720. [PubMed: 1325220]
- White TW, Goodenough DA, Paul DL. Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts. *J Cell Biol*. 1998; 143:815–825. [PubMed: 9813099]
- Woolf D. A comparative cytological study of the ciliary muscle. *Anat Rec*. 1956; 124:145–163. [PubMed: 13302815]
- Yang JJ, Liao PJ, Su CC, Li SY. Expression patterns of connexin 29 (GJE1) in mouse and rat cochlea. *Biochem Biophys Res Commun*. 2005; 338:723–728. [PubMed: 16236250]
- Zhu Y, Yu H, Wang W, Gong X, Yao K. A novel GJA8 mutation (p.V44A) causing autosomal dominant congenital cataract. *PLoS One*. 2014; 9:e115406. [PubMed: 25517998]

Fig. 1.

Lenses of Cx23-null mice are transparent and have similar characteristics as wild type lenses. A. Diagram illustrating the strategy for deletion of Cx23 exon 2. (a) Region of mouse chromosome 10 containing the *Gje1* (Cx23) gene. (b) Targeted conditional allele. (c) *LacZ*-tagged null allele generated by *Cre* recombination. Exons (E1, E2 and E3) are represented by light gray rectangles with the untranslated regions depicted in a lighter gray. The locations of the genotyping primers (short black bars 1–4 in [b]) and RT-qPCR primers (short gray bars 5–8 in [b]) are indicated. (B) Gel containing electrophoresed PCR products using the Cx23 or the *LacZ* genotyping primers and genomic DNA from wild type (+/+), heterozygous (+/-) and homozygous (-/-) mice showing the presence of the 408 bp amplicon of wild type Cx23 (Cx23) in the samples from wild type and heterozygous animals and of the 473 bp amplicon of *LacZ* on samples from heterozygous and homozygous mice.

The migration positions of the 1000, 500 and 200 bp DNA standards are indicated on the right. (C and D) Graphs show the RT-qPCR amplification curves for Cx23 exon 2 and *LacZ* obtained using total RNA from 4 week-old wild type (+/+), heterozygous (+/-) and homozygous (-/-) mice and Cx23 (C) or LacZ (D) primers. The data are presented as Rn vs. cycle number. The small increase in Rn observed after the 38th cycle in wild type samples is non-specific; this product has a different melting temperature than the transgenic *LacZ* amplicon. (E) The lenses from wild type (+/+), and Cx23-null heterozygous (+/-) and homozygous (-/-) mice at 5 weeks of age were photographed using darkfield illumination. Lenses from wild type and Cx23-null mice appear indistinguishable; all show a few very small nuclear opacities. Bar, 497 μ m. (F) Images were obtained by photographing lenses from 5 week-old wild type (+/+) and Cx23-null homozygous (-/-) mice against an electron microscopy grid. The patterns appear indistinguishably sharp without distortions. Bar, 477 μ m. (G) Representative laser scan profiles from wild type (+/+), heterozygous (+/-) and homozygous (-/-) Cx23-null mouse lenses. The pathway of the laser beam (lines) and the data points (+) represent the focal point of each beam. The average BVD (white asterisk) was calculated from averaging the value of each of these data points, whereas the scatter of the points around the average is the variability in the BVD. There was no difference between the three genotypes.

Fig. 2.

Cx46 and Cx50 distributions and levels in Cx23-null lenses. (A and B) Confocal images show the distributions of immunoreactive Cx46 (a–d) and Cx50 (e–h) in cross sections from lenses of wild type (+/+) and homozygous (–/–) mice at 4 weeks of age. The localization of Cx46 or Cx50 (green) is shown alone (a, c and e, g, respectively) or in combination (b, d and f, h, respectively) with the fluorescent signals from filamentous actin (Phalloidin, red) and nuclei (DAPI, blue). Bar, 20 μ m. (C and D) Immunoblots show the levels of immunoreactive Cx46 (C) and Cx50 (D) in total lens homogenates from 9 week-old wild type (+/+), and

Cx23-null heterozygous (+/-) and homozygous (-/-) littermates. Aliquots containing equal amounts of proteins were loaded per lane (20 µg for Cx50 immunoblots and 100 µg for Cx46 immunoblots). The graphs show the densitometric values of the bands obtained in independent experiments expressed as percentages of the values obtained in wild type animals (n = 4 for wild type and heterozygous mice; n = 3 for homozygotes). The short horizontal lines indicate the average value for each genotype.