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RESEARCH ARTICLE

Charged multivesicular body protein 4b forms complexes with gap junction proteins during lens fiber cell differentiation

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Abstract
Charged multivesicular body protein 4b (CHMP4B) is a core sub-unit of the endosomal sorting complex required for transport III (ESCRT-III) machinery that serves myriad remodeling and scission processes of biological membranes. Mutation of the human CHMP4B gene underlies rare forms of early-onset lens opacities or cataracts, and CHMP4B is required for lens growth and differentiation in mice. Here, we determine the sub-cellular distribution of CHMP4B in the lens and uncover a novel association with gap junction alpha-3 protein (GJA3) or connexin 46 (Cx46) and GJA8 or Cx50. Immunofluorescence confocal microscopy revealed that CHMP4B localized to cell membranes of elongated fiber cells in the outer cortex of the lens—where large gap junction plaques begin to form—particularly, on the broad faces of these flattened hexagon-like cells in cross-section. Dual immunofluorescence imaging showed that CHMP4B co-localized with gap junction plaques containing Cx46 and/or Cx50. When combined with the in situ proximity ligation assay, immunofluorescence confocal imaging indicated that CHMP4B lay in close physical proximity to Cx46 and Cx50. In Cx46-knockout (Cx46-KO) lenses, CHMP4B-membrane distribution was similar to that of wild-type, whereas, in Cx50-KO lenses, CHMP4B localization to fiber cell membranes was lost. Immunoprecipitation and immunoblotting analyses revealed that CHMP4B formed complexes with Cx46 and Cx50 in vitro. Collectively, our data suggest that CHMP4B forms plasma membrane complexes, either directly and/or indirectly, with gap junction proteins Cx46 and Cx50 that are often associated with “ball-and-socket” double-membrane junctions during lens fiber cell differentiation.

KEYWORDS
ball-and-socket junction, connexin 46, connexin 50, fiber cell, gap junction, lens

Abbreviations: B&S, ball and socket; CHMP, charged multivesicular body protein; DAPI, 4′,6-diamidino-2-phenylindole; ESCRT, endosome sorting complex required for transport; IFCM, immunofluorescence confocal microscopy; LFC, lens fiber cell; MVB/E, multivesicular body/endosome; PLA, proximity ligation assay; WGA, wheat germ agglutinin.

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1 | INTRODUCTION

The vertebrate lens is a transparent, highly refractive cellular structure that plays a critical role in anterior eye development and fine focusing of images onto the photosensitive retina. At the cellular level, the lens is surrounded by a basement membrane or capsule containing an anterior monolayer of epithelial cells that divide and terminally differentiate throughout life into highly elongated secondary fiber cells precisely organized into tightly packed, concentric layers or growth shells to form the refractive mass (nucleus and cortex) of the lens. Lens fiber cells (LFCs) display a flattened, hexagonal cross-section with two broad cell membrane surfaces that abut LFCs in adjacent growth shells and four narrow faces that border LFCs in adjacent radial cell columns. LFC differentiation is characterized by several coordinated processes—including cytoplasmic accumulation of crystallin proteins, plasma membrane and actin cytoskeleton remodeling, programmed organelle degradation, and core synctium formation that serve to establish and maintain a high refractive index and exquisite optical quality. Loss of lens transparency, or cataract(s), is often acquired with aging and, despite advances in surgical treatment, age-related cataract constitutes a leading cause of visual impairment (low vision and blindness) and a significant public healthcare burden worldwide.

Prominent during LFC membrane remodeling is the accumulation of intercellular gap junction channels, composed of gap junction α3 protein (GJA3) or connexin 46 (Cx46) and GJA8 or Cx50, which share 88% amino acid similarity and a similar 3D protein structure. Gap junction assembly involves oligomerization of six transmembrane connexin isoforms into homomeric (e.g., Cx46 or Cx50 isoforms) or heteromeric (e.g., Cx46 and Cx50 isoforms) hemi-channels, or connexons (i.e., hexamers), that dock in the extracellular space with connexons in neighboring cells to form homotypic (e.g., Cx46 or Cx50 connexons) or heterotypic (e.g., Cx46 and/or Cx50 connexons) intercellular channels with diverse gating properties that facilitate the cytoplasmic exchange of ions, small molecules, and fluids. Cryo-electron microscopy, mass spectroscopy, and molecular dynamics simulation studies of native Cx46 and Cx50, purified from sheep lenses, have demonstrated that they co-assemble into a mixture of heteromeric and/or heterotypic intercellular channels in an open-pore conformation that is stabilized, in part, by membrane–lipid interactions. LFC gap junctions are arranged in large (micron-sized) two-dimensional arrays or plaques particularly on the broad cross-sectional membrane faces and are often associated with so-called “ball-and-socket” (B&S) double-membrane junctions that interlock cortical LFCs. Loss-of-function studies in mice have shown that Cx46 and/or Cx50 are critical for lens growth, differentiation, and homeostasis, in part, by facilitating cell-cell adhesion and by providing an outflow pathway for the lens ion/fluid microcirculation system that is essential for maintaining the transparency and high refractive index of the avascular lens. Sequence variations in the genes coding for Cx46 and Cx50 have been associated with both rare forms of inherited cataracts and frequently acquired forms of age-related cataracts in humans (https://cat-map.wustl.edu), and gene-targeted mice that are homozygous for human Cx46 or Cx50 mutations develop cataracts associated with calcium bio-mineralization of the lens.

Charged multivesicular body proteins (CHMPs) comprise a phylogenetically conserved (Archaea-to-human) family of helical proteins (12 in humans and 11 in mice) that serve as core subunits of the endosomal sorting complex required for transport III (ESCRT-III) machinery, which facilitates myriad biological membrane remodeling and scission processes. These include, but are not limited to, multivesicular body/endosome (MVB/E) biogenesis, viral budding, cytokinetic (bridge) abscission, nuclear membrane sealing, plasma membrane and lysosome membrane repair, autophagy, mitophagy, and ciliogenesis. Structural studies have revealed that CHMP4B, or its conserved orthologs Snf7 (yeast) and Vps32 (C. elegans), can polymerize on cellular membranes with other ESCRT-III subunits to form spiral filaments that are recruited to the narrow necks of membrane bud-like structures in order to sever the vesicle bud and reseal the parent membrane. Like Cx46 and Cx50, sequence variations in the human gene for CHMP4B (CHMP4B) have been associated with inherited and age-related forms of cataracts in humans (https://cat-map.wustl.edu). Heterologous overexpression studies suggest that a mutant form of CHMP4B (p.D129V), underlying early-onset cataract, impairs viral budding and inhibits chromatin binding in transformed cell lines. In mice, germline knockout or homozygous mutation of CHMP4B is embryonic lethal, and conditional knock-down of CHMP4B in the lens resulted in severe inhibition of lens growth and fiber cell differentiation—consistent with the importance of the ESCRT-III machinery in membrane dynamics including cytokinesis. Beyond its association with human cataracts, little is known about CHMP4B function(s) in the lens. Here, we provide the first evidence, to our knowledge, that CHMP4B forms complexes with gap junction proteins Cx46 and Cx50, suggesting a novel role in lens cell membrane differentiation.
2 | MATERIALS AND METHODS

2.1 | Mice

C57BL6/J (B6J) mice (Stock no. 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Chmp4b-floxed (Chmp4b^f/f) mice were generated by homologous recombination (B6J background) and bred with mice transgenic for lens-specific Cre recombinase (MLR10-Cre, a generous gift from Dr. M.L. Robinson, Ohio State University) to produce a conditional knockdown of Chmp4b in the lens (Chmp4b-CKD) along with control (Chmp4b^f/f, Cre) mice as described. Mice were genotyped for Chmp4b-allele and Cre-transgene status by polymerase chain reaction (PCR) amplification of toe or ear biopsy genomic DNA using gene-specific primers (Table S1) as described. Cx46/Gja3 knock-out (KO) and Cx50/Gja8-KO mice were generated by homologous recombination, and Cx46 and Cx50 allele statuses were confirmed by PCR genotyping as described. All experimental mouse strains were maintained on the B6J genetic background to avoid a deletion mutation in the gene for lens-beaded filament structural protein 2 (Bfsp2) carried by certain inbred strains. Mice were humanely euthanized according to the American Veterinary Medical Association (AVMA) guidelines and the eyes were removed. All mouse studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University in compliance with the Institute for Laboratory Animal Research (ILAR) guidelines.

2.2 | CHMP4B antibody production and validation

A custom-synthesized, synthetic peptide from the carboxy terminus of CHMP4B was used to generate an affinity-purified polyclonal antibody (#PA0518) using standard immunological techniques in rabbits (Open Biosystems, Huntsville, AL). Antibody specificity was validated by using small interfering RNA (siRNA)-mediated knock-down of CHMP4B (Accell Human CHMP4B siRNA—SMARTpool, Dharmacon, Lafayette, CO) in cultured (37°C, 5% CO2) HEK-293T cells (CRL-3216, ATCC, Manassas, VA) according to the manufacturer’s instructions. Briefly, HEK293 cells (~50% confluent in 24-well plates) were transfected (72 h) with CHMP4B siRNA or negative-control (“scrambled”) siRNA (Accell Green Non-targeting, Dharmacon), each at 1 μM in 500 μL Accell Delivery Media, and then, refed with fresh culture media (Minimal Essential Media/MEM, 20% Fetal Bovine Serum/FBS Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Silenced cells were harvested (rinsed in PBS, detached in 0.02% EDTA, and centrifuged 1500g, 5 min) 24h later and cell pellets lysed by resuspension in 1% IGEPAL (C-630, Sigma-Aldrich, St. Louis, MO, USA). Lysate protein concentration was measured using the Non-Interfering Assay (G-Bioscience, St. Louis, MO) and 5 μg of soluble protein used for immunoblotting as described.

2.3 | Immunofluorescence confocal microscopy (IFCM)

Enucleated eyes were fixed (6h, 4°C) in 4% paraformaldehyde (PFA, #15710, Electron Microscopy Sciences, EMS, Hatfield, PA) diluted in phosphate-buffered saline (PBS, #P4417-100TAB, Sigma-Aldrich) and processed using standard formaldehyde-fixed paraffin-embedded (FFPE) serial section techniques. Single LFCs were gently dissected from the outer cortical region of fixed (4% PFA, 5h) lenses bisected along the optical axis, and then, dried onto polylysine-coated slides, permeabilized, and blocked as described. IFCM was performed using commercially available primary antibodies (Table S2) and species-appropriate Alexa-fluor conjugated secondary antibodies (Thermo Fisher Scientific), counterstaining of cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI; MilliporeSigma, Burlington, MA, USA) and cell membranes with rhodamine-based wheat germ agglutinin (WGA) conjugates (RL-1022, Vector Laboratories, Newark, CA, USA; CF 640R, Biotium, Freemont, CA, USA), followed by confocal scanning microscopy (FV1000, Olympus, Center Valley, PA, USA; Zeiss LSM 800 with Airyscan, Carl Zeiss, White Plains, NY, USA) as described. For fluorescence image co-localization analysis, dual-immunofluorescence signals from at least three images from six lens sections of three mice (≥18 images/genotype) or from one image from twelve single LFCs of three mice were analyzed using the Fiji distribution of open source ImageJ software and quantified using the Just Another Co-localization Plugin (JACoP) of the Bioimaging and Optics Platform (BIOP) with Otsu’s auto-threshold selection method to calculate Pearson’s correlation coefficient as described.

2.4 | In situ proximity ligation assay (PLA)

PLA was performed using the Duolink In Situ Detection Reagents Orange kit (DUO92106 Sigma-Aldrich) according to the manufacturer’s instructions and as described. Briefly, pre-blocked sections were incubated (16h, 4°C) with primary antibodies (Table S2) raised in different species
washed (2 × 5 min), re-incubated (1 h, 37°C) with anti-goat Plus and anti-rabbit Minus PLA probes, followed by ligation (30 min, 37°C), and amplification (100 min, 37°C) prior to fluorescence confocal microscopy (FV1000). For quantification, PLA positive signals from at least one image from six lens sections of three mice (≥6 images/genotype) were analyzed for particle number (average counts) using Fiji software. Non-specific background threshold and true particle size threshold were selected using control lens sections from Cx46-KO and Cx50-KO mice and wild-type mice using only one of the paired PLA antibodies.

2.5 | Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using the Pierce Classic IP kit (#26146, Thermo Fisher Scientific) with appropriate primary antibodies (Table S2) according to the manufacturer’s instructions and as described. Immunoblot analysis was performed with appropriate primary antibodies (Table S2) and IRDye-labeled secondary antibodies using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA) according to the manufacturer’s instructions and as described.

2.6 | Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistical significance ($p$ ± standard error (SE) of Pierson’s correlation coefficient.

3 | RESULTS

3.1 | CHMP4B localizes to LFC membranes

To determine the distribution of CHMP4B in the lens, we undertook immunofluorescence confocal microscopy (IFCM) using a synthetic peptide antibody that was validated for CHMP4B specificity by siRNA-mediated knockdown and immunoblotting techniques (Figure S1). Using this primary antibody for IFCM, we have localized CHMP4B specifically to the outer cortex (~50–100 μm depth from equatorial surface) of the wild-type mouse lens with intense, punctate, labeling mostly on the broad cross-sectional faces of secondary LFC membranes (Figure 1). By contrast, we have not observed CHMP4B localization to cell membranes of lens epithelial cells that often line the peripheral edges of lens sections suggesting that CHMP4B localization to

\[ \text{CHMP4B localization in the lens. Representative IFCM images of the wild-type mouse lens at P6 (A) and P21 (B-D) in the sagittal plane (A, B) and cross-section (C, D) showing CHMP4B (green) localization to LFC membranes, particularly on the broad faces (D). Cell membranes and nuclei were stained with WGA (red) and DAPI (blue), respectively. Scale bar: 100 μm (A), 30 μm (B, C), and 15 μm (D).} \]
cell membranes was associated with differentiation of LFCs from lens epithelial cells.

In order to further validate localization of CHMP4B to LFC membranes, we sought to generate Chmp4b-deficient lenses by crossing Chmp4b-floxed mice with transgenic, lens-specific MLR10-Cre mice that express Cre recombinase under the control of a modified αA-crystallin gene promoter. During lens development, MLR10-Cre expression commences in the lens vesicle and primary fiber cells at embryonic day 10.5 (E10.5) progressing to all lens epithelial cells and secondary fiber cells by E12.5. IFCM imaging confirmed localization of CHMP4B to LFC membranes in control (Chmp4b+/−; Cre) lenses at postnatal day 1 (P1) (Figure S2A). By contrast, there was a significant loss of CHMP4B membrane localization in Chmp4b-deficient lenses, particularly within the lens core or nucleus at P1 (Figure S2B–D). However, some CHMP4B-positive membrane labeling was observed toward the peripheral cortex of Chmp4b-deficient lenses at P1 (Figure S2B–D). In previous studies, we have shown that Chmp4b-deficient lenses display highly variable degrees of lens dysmorphology, including lens ablation, suggesting that MLR10-Cre deletion of Chmp4b was non-uniform or mosaic across the lens (at least on the B6J background)—consistent with a conditional knockout. We note that MLR10 transgenic mice lack Cre-recombinase protein in the lens epithelium at E15.5 and may exhibit incomplete recombination with some floxed alleles in the lens epithelium—raising the possibility that Chmp4b expression may recover, after conditional knockdown, to rescue nascent LFC differentiation. Regardless, these data suggest that CHMP4B function is required for lens growth and differentiation, in part, through localization to LFC membranes.

### 3.2 CHMP4B co-localizes with gap junction proteins Cx46 and Cx50 in the lens

Gap junctions composed of Cx46 and/or Cx50 are known to form large plaques, particularly on the broad cross-sectional surfaces of LFC membranes. In addition, both Cx46 and Cx50 gap junction plaques have been localized to B&S double-membrane junctions that interlock cortical LFCs. Since CHMP4B localization resembled that of gap junction plaques on LFC membranes, we performed dual-antibody IFCM to compare the distribution of CHMP4B with that of Cx46 and Cx50. Dual-antibody imaging suggested that CHMP4B predominantly co-localized with Cx46- and Cx50-containing gap junction plaques, particularly on the broad cross-sectional faces of LFC membranes where B&S junctions are also found (Figure 2). Quantification of the dual immunofluorescence signals using Pearson’s correlation coefficient (PCC) indicated that CHMP4B was approximately 70% co-localized with Cx46 and Cx50 in wild-type lenses (Figure 2G). In order to visualize CHMP4B and gap junction plaques “face-on” in the superficial plane, rather than in cross-section, we performed dual-antibody IFCM imaging of single LFCs isolated from the peripheral cortex of wild-type lenses. Figure S3 shows predominant co-localization of CHMP4B with large plaques of Cx46 (PCC = 0.57 ± SE 0.028) mostly on the broad faces of single LFC membranes. Mechanical disruption of gap junction plaques during isolation of single LFCs may have contributed to the reduced co-localization of CHMP4B and Cx46 compared to that observed in whole lens sections. Nevertheless, our data provide the first evidence, to our knowledge, that CHMP4B co-localizes with Cx46- and Cx50-containing gap junction plaques and B&S junctions found on LFC membranes.

### 3.3 CHMP4B forms complexes with gap junction proteins Cx46 and Cx50 in the lens and in vitro

Since the lens phenotypes of Cx46-KO and Cx50-KO mice are less severe than that of Chmp4b-deficient mice (Figure S2), we sought to model the effects of Cx46 and/or Cx50 loss of function on CHMP4B localization in the lens. In Cx46-KO lenses, CHMP4B was mostly localized to punctate “ladder-like” regions on the broad faces of LFC membranes similar to that of Cx46 in wild-type lenses (Figure 3A–F) and Cx50 in both wild-type and Cx46-KO lenses (Figure 4A–F). By contrast, in Cx50-KO lenses, CHMP4B exhibited diffuse cytoplasmic labeling (Figures 3G, I and 4G, I), whereas Cx46 displayed a more uniform membrane distribution in Cx50-KO lenses (Figure 3H, J) compared to the punctate pattern in wild type (Figure 3B, C)—consistent with a previous report. In Cx46 and Cx50 double-knock-out (Cx46/50-DKO) lenses, the diffuse CHMP4B labeling of the cytoplasm resembled that found in Cx50-KO lenses (Figures 3G, I, L, 4G, I, L). Quantification of the dual immunofluorescence signals using PCC confirmed that CHMP4B was ~70% co-localized with Cx50 in Cx46-KO lenses, similar to that in wild-type lenses, whereas CHMP4B exhibited minimal co-localization with Cx46 in Cx50-KO and Cx46/50-DKO lenses (Figures 3M and 4M). Overall, these observations suggest that in the absence of Cx50, CHMP4B had redistributed from its active (open) state—as a polymer on the membrane—to its auto-inhibited (closed) state—as a soluble monomer in the cytoplasm.

In order to estimate the physical distance between CHMP4B and gap junction proteins on LFC membranes, we performed the in situ PLA with primary antibodies
FIGURE 2  Co-localization of CHMP4B with gap junction proteins Cx46 and Cx50 in the lens. Representative IFCM images of CHMP4B (A, C, D, F), Cx46 (B, C), and Cx50 (E, F) distribution in cross-sections of the mouse lens (P28). White arrows indicate potential B&S structures rich in CHMP4B and Cx46 or Cx50 on the broad faces of LFC membranes. Cell membranes were stained with WGA (white) and nuclei with DAPI (blue). Scale bar: 5 μm. (G) Quantification of CHMP4B co-localization with Cx46 and Cx50 in wild-type lenses calculated using Pearson’s correlation coefficient. NS, not significant. Error bars represent SE.

FIGURE 3  CHMP4B and Cx46 distribution in Cx46-KO, Cx50-KO, and Cx46/Cx50-DKO lenses. Representative IFCM images of CHMP4B (A, C, D, F, G, I, J, L) and Cx46 (B, C, E, F, H, I, K, L) localization in lenses (P21) from wild-type (A-C), Cx46-KO (D-F), Cx50-KO (G-I), and Cx46/50-DKO (J-L) mice. Cell membranes were labeled with WGA (white) and nuclei with DAPI (blue). Scale bar: 10 μm. (M) Quantification of CHMP4B and Cx46 co-localization in wild-type, Cx46-KO, Cx50-KO, and Cx46/50-DKO lenses calculated using Pearson’s correlation coefficient. ***p ≤ .001. Error bars represent SE.
membrane proteins, including lens major intrinsic protein or aquaporin-0 (MIP/AQP0), catenin beta-1 (CTNNB1), and CHMP4B complexes with several other LFC proteins that are associated with connexin degradation. The ubiquitin-binding protein tumor susceptibility gene 101 (TSG101) has been shown to interact with the C-termini of several connexins including Cx43. Similarly, another ubiquitin-binding protein hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) along with TSG101 have been shown to be important for trafficking of ubiquitinated Cx43 to lysosomes for degradation. We note that Cx43 is expressed in lens epithelial cells that line the anterior surface of the lens but not in LFCs that form the refractive mass of the lens. 4 | DISCUSSION

CHMP4B serves as a core subunit of the ESCRT-III biomembrane remodeling and scission machinery and has been associated with inherited and age-related cataracts in humans and lens growth deficiency and dysmorphology in mice. In this study, we discovered that CHMP4B forms complexes with gap junction proteins Cx46 and Cx50 on LFC membranes (Figures 1–5) and in vitro (Figure 6). Furthermore, loss of Cx50—but not Cx46—in the lens resulted in redistribution of CHMP4B from LFC membranes to the cytoplasm (Figures 3–5) and loss of CHMP4B-Cx46 and CHMP4B-Cx50 complexes in vivo (Figure 6). At least a sub-population of CHMP4B on LFC membranes was found to lie in close proximity to Cx46 and/or Cx50 (Figure 5), suggesting possible protein–protein interactions. Although Cx50 has been reported to interact with MIP/AQP0, we did not find CHMP4B-MIP/AQP0 complexes in wild-type lenses (Figure S4). Whether or not CHMP4B interacts with Cx46 and Cx50 either directly and/or indirectly via an intermediate protein(s) remains to be determined. Overall, our data suggest that Cx50, but not Cx46, is required for CHMP4B complex formation on LFC membranes. Such complex formation among CHMP4B, Cx46, and Cx50 in the outer cortex of the mouse lens provides a spatiotemporal overlap with (1) the sub-capsular and cortical lens opacities observed in human CHMP4B-related cataract and (2) the formation of large gap junction plaques that are often associated with B&S double-membrane interlocking junctions on the broad cross-sectional surfaces of LFC membranes.

Although complex formation between the ESCRT-III protein CHMP4B and connexins on LFC membranes was unexpected, previous studies have identified two other ESCRT proteins that are associated with connexin degradation. The ubiquitin-binding protein tumor susceptibility gene 101 (TSG101) has been shown to interact with the C-termini of several connexins including Cx43. Similarly, another ubiquitin-binding protein hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) along with TSG101 have been shown to be important for trafficking of ubiquitinated Cx43 to lysosomes for degradation. We note that Cx43 is expressed in lens epithelial cells that line the anterior surface of the lens but not in LFCs that form the refractive mass of the lens.

CHMP4B and Cx50 distribution in Cx46-KO, Cx50-KO, and Cx46/50-DKO lenses. Representative IFCM images of CHMP4B (A, C, D, F, G, I, J, L) and Cx50 (B, C, E, F, H, I, K, L) localization in lenses (P21) from wild-type (A-C), Cx46-KO (D-F), Cx50-KO (G-I), and Cx46/50-DKO (J-L) mice. Cell membranes were labeled with WGA (white) and nuclei with DAPI (blue). Scale bar: 10 μm. (M) Quantification of CHMP4B and Cx50 co-localization in wild-type, Cx46-KO, Cx50-KO, and Cx46/50-DKO lenses calculated using Pearson’s correlation coefficient. ***p ≤ 0.001. NS, not significant. Error bars represent SE.
ESCRT-1 machinery, respectively, and sequential recruitment of ESCRT-0, ESCRT-I, and ESCRT-II, precedes that of the ESCRT-III machinery to the endosomal membranes before fusion with lysosomes. CHMP4B is known to play canonical roles in both of the lysosomal degradation pathways that contribute to gap junction/connexin degradation including Cx43 and Cx50. In the endo-lysosomal pathway, CHMP4B/ESCRT-III participates in the formation of intraluminal vesicles found in MVB/Es that subsequently fuse with lysosomes. Similarly, CHMP4B/ESCRT-III participates in several phagolyssosomal or autophagy pathways including the formation of MVB/Es that fuse with autophagosomes to form amphisomes prior to lysosome fusion in macroautophagy, endosomal and lysosomal microautophagy, and phagophore closure during mitophagy. Furthermore, CHMP4B deficiency in cortical neurons has been shown to result in accumulation of autophagosomes. However, it remains to be determined whether or not recruitment of CHMP4B to Cx46 and Cx50 gap junctions on LFC membranes is solely for the purpose of lysosomal degradation.

It is well established that Cx46 and Cx50 serve critical yet non-redundant functions in the lens. While lenses lacking either Cx46 or Cx50 developed nuclear
In summary, our data reveal novel complex formation between the ESCRT-III protein CHMP4B and gap junction proteins Cx46 and Cx50 on LFC membranes and in vitro. Further studies will be required to characterize the roles of (1) the endo-lysosomal versus autophagy pathways in Cx46 and Cx50 turnover and/or degradation, (2) direct versus indirect CHMP4B interactions with Cx46, Cx50, and/or other membrane-associated proteins (e.g., ESCRT-III proteins) in gap junction plaque formation, and (3) CHMP4B-Cx50-containing complexes in B&S double-membrane junction formation during LFC differentiation.

AUTHOR CONTRIBUTIONS
Yuefang Zhou and Alan Shiels conceived and designed the research; Yuefang Zhou and Thomas M. Bennett performed the research and acquired the data; Thomas W. White provided knock-out mice; Yuefang Zhou, Thomas M. Bennett, Thomas W. White, and Alan Shiels analyzed and interpreted the data. Alan Shiels wrote the first draft of the manuscript and all authors were involved in revising the final manuscript.

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DISCLOSURES
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the methods and/or Supplementary Material of this article.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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