Failure of Autophagy–Lysosomal Pathways in Rod Photoreceptors Causes the Early Retinal Degeneration Phenotype Observed in Cln6nclf Mice

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PURPOSE. Vision loss caused by photoreceptor death represents one of the first symptoms in neuronal ceroid lipofuscinosis, a condition characterized by accumulation of intracellular waste. Cln6nclf mice have a naturally occurring mutation in ceroid-lipofuscinosis neuronal (CLN) protein 6 and are a model of this disorder. In order to identify the effect intracellular waste (lipofuscin) accumulation plays in driving retinal degeneration, the time course of degeneration was carefully characterized functionally using the electroretinogram and structurally using histology.

METHODS. Cln6nclf and C57BL/6j, wild-type, mice were studied at postnatal day 18 (P18), P30, P60, P120, and P240, and retinal degeneration was correlated with changes in the retinal pigment epithelial (RPE) and neuronal autophagy–lysosomal pathways using super-resolution microscopy.

RESULTS. In Cln6nclf mice there was significant loss of rod photoreceptor function at P18, prior to photoreceptor nuclei loss at P60. In contrast, cone pathway function was not affected until P240. The loss of rod photoreceptor function correlated with significant disruption of the autophagy–lysosomal degradation pathways within photoreceptors, but not in the RPE or other retinal neurons. Additionally, there was cytosolic accumulation of P62 and undigested mitochondrial-derived, ATP synthase subunit c in the photoreceptor layers of Cln6nclf mice at P30.

CONCLUSIONS. These results suggest that rod photoreceptors have an increased sensitivity to disturbances in the autophagy–lysosomal pathway and the subsequent failure of mitochondrial turnover, relative to other retinal cells. It is likely that primary failure of the rod photoreceptors rather than the RPE or other retinal neurons underlies the early visual dysfunction that occurs in the Cln6nclf mouse model.

Keywords: retina, photoreceptor, rodent, autophagy, lysosome, ceroid-lipofuscinosis neuronal protein 6, CLN6, neuronal ceroid lipofuscinosis, NCL, neurodegeneration, super-resolution microscopy, electroretinogram

Neuronal ceroid lipofuscinoses (NCLs) are a group of inherited lysosomal storage disorders that lead to progressive neurodegeneration and are characterized by early vision loss, late-stage motor dysfunction, and premature death.1,2 The pathomorphologic hallmark of all NCLs is the accumulation of intracellular waste in the form of autofluorescent lipofuscin and ceroid lipopigments in nonmitotic cell types such as neurons.1,2 With a worldwide incidence of 1 in 100,000, mutations in a range of genes encoding ceroid lipofuscinosis neuronal proteins (CLN in humans; Cln in mice) cause various forms of the disease based on the age of onset and severity of disease progression (infantile, late infantile, juvenile, and adult).3-4 In particular, mutations in the CLN6 gene result in a late infantile variant form of NCL due to dysfunctional CLN6 protein. CLN6 has been suggested to play a significant role in the intracellular recycling process of the autophagy–lysosome pathway, modulating the regulation of lysosomal pH and lysosome-associated proteins.5-7 Despite this, the CLN6 protein does not directly colocalize with lysosome markers and is instead expressed at the endoplasmic reticulum where its function is currently unknown.5-7 Thus, the molecular mechanism of lysosomal failure that leads to severe neurodegeneration in patients with CLN6 mutations remains unclear.

The naturally occurring Cln6nclf mouse, which has a single nucleotide insertion in exon 4 of the Cln6 gene that induces a frameshift and a premature stop codon, provides a valuable tool for assessing the role of CLN6 in the neurodegenerative process.8,9 Studies of the ocular phenotype in the Cln6nclf mouse model reveal an early and progressive loss of photoreceptor cells in the outer nuclear layer (ONL), with relative preservation of the inner retina.10,11 Retinal function declines from 1 month of age, stemming from a decrease in rod photoreceptor function; however, the effect on the cone pathway function has not been assessed.10,11 Histologic...
investigation and work at the ultrastructural level show cytoplasmic inclusions and lysosomal accumulations in many different retinal cell types.\textsuperscript{15} In addition, activation of the local immune response (amoeboid microglia) and Müller cell gliosis are apparent.\textsuperscript{13} Despite this work, the mechanisms that lead to lipofuscin accumulation, early photoreceptor death, and ultimately vision loss in the Cln6\textsuperscript{-/-} mouse are unknown.

Intracellular accumulation of lipofuscin in the retinal pigment epithelium (RPE) has long been suspected to play a major role in a range of retinal degenerations, including age-related macular degeneration (AMD)\textsuperscript{12} and Stargardt’s disease.\textsuperscript{13} Failure of the autophagy-lysosomal pathway and accumulation of lipofuscin has been found to cause significant impairment in the RPE, resulting in photoreceptor dysfunction and subsequent vision loss in these diseases.\textsuperscript{14,15} This is also the case for other Cln mutants.\textsuperscript{16,17} Alternatively, lysosomal accumulations within the photoreceptors themselves may induce photoreceptor failure and retinal degeneration as has been suggested in the fly.\textsuperscript{18} As Cln6\textsuperscript{-/-} mice are characterized by deposits of lipofuscin in retinal neurons and the RPE, they provide a useful model for identifying which cell type is predominantly responsible for retinal degeneration. In the present study, the function of the autophagy-lysosomal pathway was investigated in RPE, photoreceptors, and inner retinal neurons in the Cln6\textsuperscript{-/-} mouse model to determine whether failure of the RPE or a primary photoreceptor dysfunction induces retinal degeneration. To investigate this, the time course of retinal degeneration was carefully characterized in Cln6\textsuperscript{-/-} mice with age and correlated with changes in the autophagy-lysosomal pathways in the RPE and retinal neurons using super-resolution microscopy. The results provide novel insights into the mechanism of lipofuscin accumulation in the retinal neurons and RPE as well as its effect on photoreceptor function and survival in Cln6\textsuperscript{-/-} mice.

**METHODS**

Animals

Cln6\textsuperscript{-/-} on a C57BL/6j background were obtained from the Jackson Laboratory (Stock No: 003605; Bar Harbour, ME, USA) and annually backcrossed to C57BL/6j mice obtained from the Animal Resources Centre (Canning Vale, WA, Australia). C57BL/6j (wild-type, WT) control mice were obtained from the Animal Resources Centre. Animals were housed at the University of Melbourne animal facility, under a 12:12-hour light:dark cycle with ad libitum access to food and water. Genotyping of the Cln6\textsuperscript{-/-} mice was performed as specified previously.\textsuperscript{19} All experiments and handling of animals were characterized in accordance with the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research as well as the institutional guidelines of The University of Melbourne Animal Ethics Committee (AEC) (Ethics ID 1513553). Experiments were completed on animals aged to postnatal day 18 (P18), P30, P60, P120, and P240.

Fundus Imaging

In vivo imaging was used to investigate ocular fundus appearance in WT and Cln6\textsuperscript{-/-} retinas at P30 to P240 using a Micron III Retinal Imaging Microscope (Phoenix Technology Group, Pleasanton, CA, USA). As previously described,\textsuperscript{20,21} light-adapted animals (n = 6) were deeply anesthetized by intraperitoneal injection of a mixture of ketamine (67 mg/kg; Prewet, Heatherton, VIC, Australia) and xylazine (13 mg/kg; Troy Laboratories, Glenndening, NSW, Australia). The corneal reflex was suppressed by applying 0.5% proparacaine hydrochloride (Alcaine; Alcon Laboratories, Frenchs Forest, NSW, Australia), while dilation of the pupils was facilitated by the topical application of 1% atropine sulfate (Alcon Laboratories) and 2.5% phenylephrine hydrochloride (Bausch&Lomb, Chatswood, NSW, Australia). Mice were positioned in a custom-made holder, the lens of the Micron III microscope was applied close to the cornea, and the fundus of each mouse was viewed and images were captured using the Micron III software (Phoenix Technology Group).

**Electroretinography to Assess Rod and Cone Pathway Function**

Retinal function of the rod and cone pathway of WT and Cln6\textsuperscript{-/-} mice was assessed at P18 (n = 7–10), P30 (n = 10), P60 (n = 8), P120 (n = 9–10), and P240 (n = 8–9) using a twin-flash electroretinogram (ERG). Animals were dark adapted overnight, deeply anesthetized, and the corneal reflex/dilation altered as above. Full-field responses were captured using custom-made Ag/AgCl electrodes, a custom-made Ganzfeld, and a commercial flash unit (Nikon SB900, Rhodes, NSW, Australia). Two 2.1 log cd.s/m\(^2\) full-field flashes were separated by an 0.8-second interstimulus interval ( ISI), to evoke mixed rod and cone (Flash 1, F1) and subsequent cone ERG responses (F2).\textsuperscript{22–24} The responses were amplified (gain ×5000; –3 decibel [dB] at 1 Hz and 1 kHz, ML152 BioAmp; ADInstruments, Bella Vista, NSW, Australia) and acquired at a 10-kHz sampling frequency over a 250-ms period (ML785 Powerlab/ 8sp amplifier; ADInstruments). Provision of the stimulus and recording of the ERG were coordinated by Scope software version 3.6.10 (ADInstruments).

For the analysis of ERG, the rod pathway response was generated by digital subtraction of the cone ERG from the mixed ERG signal.\textsuperscript{25,24} The rod pathway responses were modeled and waveform components representing the rod photoreceptor a-wave (PII), postphotoreceptor b-wave (PII), and oscillatory potentials (OPs, summed amplitudes of OPs 2-4) were assessed as previously described.\textsuperscript{25} OP1 and OP5 of the rod OPs were not included as they are often small and difficult to distinguish from system noise and so we included only OPs 2, 3, and 4 in our analysis as they could be consistently measured. Cones were identified as OPs present as a result of the twin-flash (F1, F2) protocol above. Other photoreceptor responses, the cone b-wave (PII), and OPs (summed OPs 1, 2, and 3) were assessed as previously described.\textsuperscript{25} Differences in genotype and age were analyzed in GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) for each rod and cone pathway ERG component using a 2-way ANOVA, including a Tukey multiple comparisons test, with \(P < 0.05\) considered significant.

**Electroretinography to Assess Rod Photoreceptor Recovery Kinetics**

The kinetics of rod photoreceptor recovery was assessed in P30, dark-adapted WT and Cln6\textsuperscript{-/-} mice (n = 11) using a modification of the twin-flash (F1, F2) protocol above. To assess changes in rod photoreceptor recovery rate, twin-flash ERG responses were recorded as described above and ISIs between F1 and F2 were varied between 0.8 and 4, 8, 16, 32, 64, 128, and 180 seconds, while intervals between F2 and F1 of the next recording were kept at 180 seconds. The F2/F1 ratios of photoreceptor a-wave amplitudes were calculated for each ISI, averaged, and plotted and the kinetics of photoreceptor recovery were analyzed using nonlinear regression (hyperbolic equation) in GraphPad Prism. Differences in genotype and ISI were analyzed using a 2-way ANOVA including a Tukey
Photoreceptor Failure in Cln6+/−/− Mice

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multiple comparisons test, with P < 0.05 considered significant.

Histology for Analysis of Retinal Layer Thickness

Retinal morphology and layer thickness were assessed in WT and Cln6+/−/− mice at P18, P30, P60, P120, and P240 (n ≥ 5 each group) using toluidine blue–stained resin sections as previously described.26 Mice were deeply anesthetized (as above) and killed by cervical dislocation. The eyes were enucleated and the posterior eye cups were fixed overnight in 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, and 0.01% calcium chloride in 0.1 M phosphate buffer, pH 7.4 (PB). Following fixation, eye cups were washed in PB and subsequently dehydrated in a series of methanol solutions (PB). Following fixation, eye cups were washed in PB and subsequently dehydrated in a series of methanol solutions (75%, 85%, 95%, and 100%) and acetone (100%) step. Dehydrated tissues were then incubated in a mixture of Epon resin (ProSciTech, Thuriningowa Central, QLD, Australia), embedded, and polymerized at 60°C overnight. Eye orientation was not accounted for. Semithin sections (1-μm thickness) of the eye cups were collected using an ultramicrotome (Reichert-Jung Ultracut S; Reichert, Depew, NY, USA) and stained with 1% toluidine blue. Sections were washed, mounted with resin, and sealed with a glass coverslip.

To determine the thickness of each retinal layer, bright-field images of central retinal resin sections were captured using a ×20 air objective and Tile Scan mode on a confocal laser scanning microscope (ISM 510 META; Carl Zeiss AG, Jena, Germany). Retinal layers were analyzed for one half of the retinal section, covering the area from optic nerve to the tip of the periphery, and then partitioned into the three sections, central, midperiphery, and periphery, based on the length of the covered area. Using a custom segmentation script for ImageJ v1.47 (National Institutes of Health, Bethesda, MD, USA), the thickness (μm) of the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), ONL, inner and outer photoreceptor segments (IS/OS), and total retina was obtained. Differences in genotype and age for each layer, were analyzed using a 2-way ANOVA including a Tukey multiple comparisons post test (GraphPad Prism, P < 0.05).

Immunohistochemistry and Super-Resolution Confocal Microscopy

Fluorescence immunohistochemistry as well as detection of autofluorescence was used to investigate various retinal cell types, cellular organelles, aggregated protein structures, and accumulated lipofuscin in the retinai of WT and Cln6+/−/− mice at P18, P30, P60, P120, and P240 (n ≥ 5 each group). For tissue collection and analysis of autophagy-lysosomal pathways, P30 animals were euthanized at the same time of the day (12 PM to 1 PM), to detect maximum levels of autophagy.27 After dissection, posterior eye cups were fixed for 30 minutes in 4% paraformaldehyde dissolved in PB, washed three times in PB, and incubated in a series of graded concentrations of sucrose (10%, 20%, 30% vol/vol in PB) for cryoprotection. For sectioning, the eye cups were embedded in OCT cryopreservation medium (Tissue-Tek O.C.T, Sakura; Torrance, CA, USA) and frozen. Sections were cut transversely at 14 μm (Leica CM1860 UV, Wetzlar, Germany), placed onto poly-L-lysine–coated slides (Menzel-Gläser, Braunschweig, Germany), and stored at −30°C until further use. For labeling, slides were defrosted, washed three times in PB, and incubated overnight with either a single antibody or a combination of primary antibodies (Table 1). Primary antibodies were diluted in antibody buffer (3% vol/vol normal goat serum, 1% wt/vol bovine serum albumin, 0.05% wt/vol sodium azide, 0.5% vol/vol Triton X-100 in PB). After incubation, sections were washed in PB and incubated for 90 minutes in a mixture of the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI; Life Sciences, Scoresby, VIC, Australia) and corresponding Alexa Fluor-conjugated secondary antibodies (Life Sciences) diluted in antibody buffer at 1:300 and 1:500, respectively. Sections were...
washed, covered with Dako mounting medium (Agilent Technologies, Santa Clara, CA, USA) and a glass coverslip.

Images were captured by a confocal laser scanning microscope (LSM 800, Carl Zeiss AG), using a ×20 air, ×60 oil, and ×63 oil immersion objective at a resolution of 2048 × 2048 pixels. Excitation lasers included a diode 40-nm laser with emission filter set 455/50; an Argon 488-nm laser with emission filter set 515/565; a DPSS 561-nm laser with emission filter set 615; and a HeNe 633-nm laser with emission filter set 615. After acquisition, all images collected in super resolution, Airyscan mode was deconvolved using the Airyscan processing function (ZEN software, Carl Zeiss AG). For comparison of WT and Cln6nclf tissue sections, all imaging settings were kept at the same levels. After scale bars were digitally added, images were adjusted for brightness, contrast, and black levels in Adobe Photoshop CC (Adobe Systems Incorporated, San Jose, CA, USA), keeping equal settings between WT and Cln6nclf mice for consistency.

Quantification of Müller Cell Number
To determine whether there was a change in Müller cell number, retina from WT and Cln6nclf mice at P240 (n = 6) were labeled with glutamine synthetase (GS; Table 1) and entire sections, which included the optic nerve head, imaged using a ×20 air objective and Tile Scan mode on the confocal. To count GS-positive cells, a line was drawn through the center of the IPL and a custom ImageJ-Microsoft Excel (Redmond, WA, USA) macro used to threshold and detect cell processes that crossed the line. Differences between genotype for GS counts were analyzed using one Student’s t-test (GraphPad Prism, P < 0.05).

Quantification of Autophagic Degradation
To assess the autophagy–lysosomal pathway in different retinal layers, WT and Cln6nclf retinae at P30 (n = 6) were taken and sections labeled using a combination of LC3B antibody (autophagosomes) and LAMP1 antibody (lysosomes; Table 1). For the individual retinal layers, the RPE, the ONL, and the INL, two super-resolution z-stack images per retina were collected from retinal layers, WT and Cln6nclf retinae at P30 (n = 6). Western blots were completed. WT and Cln6nclf retinae were collected from n = 5 animals at P30. To assess the effect of blocking autophagy–lysosomal degradation, for each animal, one retina was incubated in culture media with 50 μM chloroquine (autophagy–lysosomal blocker; Sigma-Aldrich Corp. (Cat. No. C6628-25G; St. Louis, MO, USA) and the other without, for 24 hours at 37°C in a humidified cell culture chamber with 5% CO2. The culture media consisted of Alpha minimum essential medium (HyClone, Cat. No. M5432-500ML; Sigma-Aldrich Corp.), 1% L-glutamine (Gibco, Cat. No. 25030081; ThermoFisher Scientific), 1% penicillin-streptomycin (Cat. No. 15140-148; Gibco), 10% fetal bovine serum (Cat. No. SH30071.03; In Vitro Technologies, Noble Park North, VIC, Australia). Western blots were completed as previously described.3 Briefly, retinae were placed in 30 μL homogenizing buffer and the cell contents lysed by sonication. Samples were spun at 7000g for 1 minute to pellet cell debris and the supernatant used for Western blot analysis. Protein samples (reducing conditions, samples not boiled) of 40 μg/lane were run on a 4% to 12% gradient Acrylamide-Bis Tris gel (Bolt Bis-Tris 4%-12%, 12 well, Cat. No. NW04122BOX; Thermofisher Scientific) along with a molecular weight marker (Chameleon Duo Prestained Protein Ladder; Cat. No. LCR-928-60000, Millenium Science, Malgruge, VIC, Australia). Proteins were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Transfer stack: PVDF iBlot Stack; Cat. No. IB24020; Thermofisher Scientific). After blocking, the LC3B antibody (1:500 dilution) or the P62/SQSTM1 antibody (1:500) was applied, along with an antibody against β-actin as a protein loading control (1:1000, Cat. No. PA121167; Thermofisher Scientific). After incubation in primary antibody overnight, membranes were washed and incubated with the secondary antibodies (IRDye 680LT Goat anti-rabbit IgG, Cat. No. LCR-926-68021; IRDye 800CW Goat anti-rabbit IgG, Cat. No. LCR-926-80021; IRDye 800CW Goat anti-mouse IgG, LCR-925-32210; Millenium Science). After washing, the membranes were imaged using an Odyssey CLX Infrared imaging system (Odyssey-Licor system, Millenium Science) and the same settings used for all blots. Band density was analyzed using Imagej and normalized to β-actin. LC3B results were expressed as a ratio of LC3B-II to LC3B-I, while P62 results were expressed as arbitrary fluorescence units. Differences between genotype and chloroquine treatment groups were analyzed using a 2-way ANOVA with a Tukey multiple comparisons test (GraphPad Prism, P < 0.05).

RESULTS
Cln6nclf Mice Show Accumulation of White Lesions in the Fundus From Postnatal Day 60
Representative fundus images were taken in vivo from P30, P60, P120, and P240 in Cln6nclf mice and compared to WT controls (Fig. 1). WT mice displayed a healthy fundus up to P240 (8 months of age; Fig. 1A). At P30, the fundus of Cln6nclf mice was similar to that of WT controls, showing normal pigmentation and vascular morphology (Fig. 1B). From P60 onward, Cln6nclf mice showed progressive accumulation of white fundus lesions (Fig. 1C), so that by P240 the entire retinal field displayed signs of discrete ocular lesions (Fig. 1D).

Retinal Function Is Impaired From Postnatal Day 18 in Cln6nclf Mice
Rod pathway responses of dark-adapted WT and Cln6nclf mice were assessed using a twin-flash ERG at P18, P30, P60, P120,
and P240 (Fig. 2). Representative rod pathway waveforms of WT (black line) and Cln6nclf mice (gray line) at P18 through P240 indicate a progressive loss of ERG amplitude from P18 in Cln6nclf mice (Fig. 2A). The individual ERG waveform components were modeled and the rod photoreceptor response (a-wave, PIII Rmax) was found to be significantly reduced in Cln6nclf mice from P18 (Fig. 2B). By P240 only a small, residual rod photoreceptor response was apparent in Cln6nclf mice when compared to the WT controls. Despite this dysfunction, the kinetics of rod photoreceptor recovery was not altered at P30 between Cln6nclf (gray circle) and WT mice (black square; Fig. 2C). As was seen for the rod photoreceptor response, the modeled b-wave (PII amplitude) reduced significantly from P18 in Cln6nclf mice relative to the WT response (Fig. 2D). To assess if the postphotoreceptor behavior in the b-wave (PII) of Cln6nclf mice was dependent on or independent from the decline of the photoreceptor response, the percentage loss of the modeled a-wave (PIII, gray circle) and b-wave (PII, gray square) was compared (Fig. 2E). At all ages investigated, the reduction in the postphotoreceptor response (b-wave, PII; Fig. 2B) and the summed cone OPs (Fig. 2C) were reduced.

This suggests a relative sparing of inner retinal function until later stages of degeneration.

Cone pathway function of WT (black) and Cln6nclf mice (gray) was investigated using a twin-flash ERG, and representative raw cone ERG waveforms at P18, P120, and P240 are shown (Fig. 3A). There were no changes in cone pathway function observed in Cln6nclf mice relative to WT controls until P240, when the cone postphotoreceptor response (b-wave, PII; Fig. 3B) and the summed cone OPs (Fig. 3C) were reduced.

Photoreceptor Layer Thickness Is Reduced From Postnatal Day 60 in Cln6nclf Mice, While the Inner Retinal Layers Are Preserved

With rod pathway function reduced from P18 in Cln6nclf mice, histologic analysis for retinal layer thickness for WT and Cln6nclf mice was undertaken to identify the retinal cell types affected. Retina from WT and Cln6nclf mice were fixed and prepared as semithin, toluidine blue–stained resin sections for analysis, and representative images from central retina are presented (Figs. 4A–I). There were no apparent differences in retinal layer thickness between WT and Cln6nclf mice at P18 (Figs. 4A, 4B) and P30 (Figs. 4C, 4D); however, from P60 onward there was progressive loss of the photoreceptor layers (ONL) in Cln6nclf mice (Figs. 4E, 4F). By P240, Cln6nclf mice had between two and three layers of photoreceptor nuclei in central regions (Fig. 4H), while some regions completely lacked photoreceptors in the midperipheral to peripheral eccentricities (Fig. 4I inset in Fig. 4H). Despite this outer retinal cell loss, there was no loss of inner retinal layers apparent in Cln6nclf mice at any age investigated (Figs. 4A–I).
FIGURE 2. Rod photoreceptor dysfunction precedes inner retinal functional losses in Cln6<sup>−/−</sup> mice. Rod pathway function was assessed in vivo using a twin-flash ERG (flash 2.1 log cd.s/m<sup>2</sup>) in WT and Cln6<sup>−/−</sup> mice at P18 through P240. (A) Representative rod pathway ERG waveforms are shown, highlighting loss of ERG amplitude from P18 onward in Cln6<sup>−/−</sup> mice (gray line) when compared with age-matched WT controls (black line). (B) Rod photoreceptor function (a-wave, PIII Rmax) is reduced in Cln6<sup>−/−</sup> mice from P18. (C) The time for the rod photoreceptor response to recover between twin flashes from the first (F1) to the second flash (F2) is not altered between WT and Cln6<sup>−/−</sup> mice at P30. (D) Rod postphotoreceptor function (b-wave, PII Rmax) is reduced in Cln6<sup>−/−</sup> mice from P18. (E) Both rod photoreceptor (PIII, circle) and photoreceptor (PII, square) amplitude are reduced in Cln6<sup>−/−</sup> mice when compared with WT at P30. (F) Rod outer plexiform layer (OP) amplitude is reduced in Cln6<sup>−/−</sup> mice from P18.
Retinal layer thickness was quantified in central, midperipheral, and peripheral regions for WT (black bars) and Cln6null mice (gray bars), and the data for central regions are presented in Figures 4J through 4N. Photoreceptor IS/OS progressively declined in thickness in Cln6null mice from P120 (Fig. 4J), while ONL thickness significantly reduced from P60 onward (Fig. 4K). In contrast, no significant changes were observed between WT and Cln6null mice in the remaining retinal layers at any age: INL (Fig. 4L), IPL (Fig. 4M), and GCL (Fig. 4N). Similar changes in Cln6null mice relative to WT controls were observed in midperipheral (Table 2) and peripheral eccentricities (Table 3). In summary, Cln6null mice undergo a progressive reduction in photoreceptors from P60, followed by loss of the inner and outer segments, while inner retinal neurons are preserved out to P240.

**Autofluorescent Debris Accumulates in Retinal Neurons and Müller Glia of Cln6null Mice**

Autofluorescent debris, lipofuscin, has been shown to accumulate in the retina of Cln6null mice during the retinal degeneration process; however, the retinal cell types affected have not been defined. Therefore, we investigated retinal lipofuscin accumulation in more detail by imaging autofluorescence in a range of spectra (Fig. 5) and subsequently in conjunction with markers for various retinal cell types (Fig. 6). In Figure 5, images of autofluorescence from unlabeled P240 WT and Cln6null mice in response to equal levels of laser excitation in a range of wavelengths (405, 488, 561, 633 nm) are shown. In the WT retina (Figs. 5A–D), mild levels of autofluorescence were apparent in the photoreceptor outer segments and to some extent in the outer plexiform layer, particularly when excited with the 405-nm (Fig. 5A; pseudo-colored blue), 488-nm (Fig. 5B; pseudo-colored green), and 561-nm lasers (Fig. 5C pseudo-colored red). In the Cln6null retina (Figs. 5E–H), autofluorescent deposits were apparent as discrete pockets throughout the retina, in all retinal layers, and were best imaged using the 633-nm laser (Fig. 5H; pseudo-colored magenta), which allowed good resolution of autofluorescence in neuronal cells. As excitation with the 633-nm laser produced the least autofluorescence in the WT photoreceptors and the best resolution of lipofuscin in the Cln6null retina, this wavelength was chosen for subsequent studies for colocalization with various retinal cell types.

**Representative images of autofluorescence and a range of markers for retinal cell types at P30 and P240 in WT and Cln6null mice are shown in Figure 6.** Cone photoreceptors...
were labeled using fluorescein-bound peanut agglutinin (PNA; green) and sections imaged for autofluorescence (Auto; 633-nm excitation; pseudo-colored red) and cell nuclei (DAPI, blue; Figs. 6A–D). WT mice at P30 and P240 (Figs. 6A, 6C) showed normal cone photoreceptor labeling and no autofluorescence (Figs. 6A, 6C, respectively). At P30, Cln6nclf mice exhibited similar PNA labeling to WT retina, and despite some rare autofluorescent deposits, there was no apparent colocalization with cone photoreceptors (Fig. 6B). However, by P240 autofluorescent debris was apparent in all retinal layers of Cln6nclf mice, including the RPE (Fig. 6D). Images were analyzed for retinal layer thickness changes between WT and Cln6nclf mice. (J) Inner segment (IS)/outer segment (OS) layer thickness was significantly reduced in Cln6nclf mice from P120. (K) ONL was significantly reduced in Cln6nclf mice from P60. (L–N) There were no changes in the inner retinal layers (L) INL, (M) IPL, or (N) GCL in Cln6nclf compared to WT mice. All data are expressed as mean ± SEM for n = 7 mice at each age. Data were analyzed by a 2-way ANOVA including a Tukey multiple comparisons test with P values < 0.05 considered significant (*). Scale bar: 20 μm.

Colocalization of calretinin and lipofuscin was absent in P30 Cln6nclf mice (Fig. 6F), autofluorescent deposits within intact amacrine and ganglion cells were apparent at P240 (Fig. 6H, arrow in magnified inset Hi). Retinal Müller cells were labeled using an antibody against glutamine synthetase (GS; green; Figs. 6I–J). At P240, Müller glia displayed normal morphology in WT retina (Fig. 6I); however, in Cln6nclf mice, isolated autofluorescent deposits were apparent within Müller cells adjacent to cell nuclei (Fig. 6J, arrow in magnified inset Ji). Despite this accumulation of autofluorescent debris, there was no effect on cell survival, as Müller cell number did not change between WT and Cln6nclf mice at this age (Fig. 6K). In summary, at P240, there was a distinct thinning of the photoreceptor layers in Cln6nclf mice; however, lipofuscin accumulation was apparent throughout the retina and had little adverse effect on retinal structure and cellular survival of the inner retinal cell types investigated, including amacrine cells, ganglion cells, and Müller glia.
Autophagy–Lysosomal Pathways Are Impaired in the Photoreceptor Layers but Not in the RPE or Inner Retina in Cln6<sup>mdf</sup> Mice at Postnatal Day 30

Disturbances in lysosomal degradation within the RPE have been implicated to play a role in the formation of lipofuscin and subsequent photoreceptor death of the Cln3 mouse model. To determine whether changes in the RPE, photoreceptors, or both contribute to photoreceptor loss in Cln6<sup>mdf</sup> mice, the autophagy–lysosomal pathways were assessed in WT and Cln6<sup>mdf</sup> mice at P30 (Fig. 7), a time when photoreceptors are dysfunctional but gross cellular structure is intact. Sections of this layer in the midperiphery and size of autophagosomes and lysosomes in the subcompartments of this layer in the Cln6<sup>mdf</sup> mice. In the INL, there were no distinct differences in LC3 or LAMP1 labeling between WT (Fig. 7G) and Cln6<sup>mdf</sup> mice (Fig. 7H).

LC3-positive autophagosomes and LAMP1-positive lysosome number (puncta number/area of region of interest, µm<sup>2</sup>) and average puncta size (µm<sup>2</sup>), as well as colocalization of autophagosomes and lysosomes, were quantified for each retinal layer in WT and Cln6<sup>mdf</sup> mice (Figs. 7I–L). The number of LC3-positive autophagosomes was significantly reduced in the photoreceptor layers, IS/OS, and ONL of Cln6<sup>mdf</sup> (gray) relative to WT mice (black), but no significant changes were apparent in the RPE or INL (Fig. 7I). Similarly, the number of LAMP1-positive lysosomes was not different in the RPE and INL between Cln6<sup>mdf</sup> and WT mice; however, there was a significant decrease in lysosome number in the IS/OS and an increase in number in the ONL of Cln6<sup>mdf</sup> (Fig. 7J). Colocalization of LC3-positive autophagosomes with LAMP1-positive puncta was significantly reduced in the photoreceptor layers, IS/OS, and ONL of Cln6<sup>mdf</sup> relative to WT mice, but there was no change in the RPE or INL of Cln6<sup>mdf</sup> at this stage (Fig. 7K). In addition, LC3-positive autophagosomes and LAMP1-positive lysosomes were significantly larger in the ONL of Cln6<sup>mdf</sup> relative to WT mice (Fig. 7L), but not in the other layers (data not shown). These data suggest that

### Table 2. Retinal Layer Thickness Measurements for the Midperiphery. Data Presented as Mean (µm) ± SEM for n = 5 or 6 Retinas From WT and Cln6<sup>mdf</sup> Mice of Each Postnatal Age

<table>
<thead>
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<th>Cln6&lt;sup&gt;mdf&lt;/sup&gt;</th>
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### Table 3. Retinal Layer Thickness Measurements for the Periphery. Data Presented as Mean (µm) ± SEM for n = 5 or 6 Retinas From WT and Cln6<sup>mdf</sup> Mice of Each Postnatal Age

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autophagy commencement and lysosomal degradation are reduced in the photoreceptor layers but not in the RPE or inner retina in the Cln6<sup>nc/e</sup> mouse at P30.

**LC3-II and P62 Accumulate in Cln6<sup>nc/e</sup> Retina and in WT Retina Treated With the Autophagy–Lysosomal Degradation Blocker Chloroquine at Postnatal Day 30**

To further investigate autophagy–lysosomal pathways at P30, retina from WT and Cln6<sup>nc/e</sup> mice were incubated overnight in culture media in the absence (−) and presence (+) of 50 μM chloroquine and the next day prepared for analysis by Western blot for LC3 (Figs. 8A, 8B) and P62 (Figs. 8C, 8D). Western blot bands from 2 of n = 5 eyes sampled for each group are presented as examples. In WT retina under control conditions, LC3-II expression was low relative to LC3-I; however, in the presence of the autophagy–lysosomal degradation blocker, chloroquine, LC3-II levels increased relative to LC3-I (Fig. 8A), and this change was significant (Fig. 8B; WT control versus WT chloroquine; *P < 0.05). In Cln6<sup>nc/e</sup> retina, LC3-II levels were consistently high relative to LC3-I (Fig. 8A) and were higher than in the WT control, and treatment with chloroquine had no further effect on this ratio (Fig. 8B; Cln6<sup>nc/e</sup> control versus WT control, *P < 0.05, and Cln6<sup>nc/e</sup> chloroquine versus WT chloroquine, *P < 0.05; Cln6<sup>nc/e</sup> control versus Cln6<sup>nc</sup> chloroquine, not significant *P = 0.27). Similarly, analysis of P62 showed that chloroquine enhanced deposition of debris tagged for autophagic degradation in WT retina (Fig. 8C), and this change was significant (Fig. 8D; WT control versus WT chloroquine, *P < 0.05). P62 expression levels were high in Cln6<sup>nc/e</sup> retina (Fig. 8C) relative to WT retina and were further increased by treatment with chloroquine (Fig. 8D; Cln6<sup>nc/e</sup> control versus WT control, *P < 0.05; Cln6<sup>nc/e</sup> control versus Cln6<sup>nc</sup> chloroquine, *P < 0.05). These data suggest that at P30, when cell loss in the retina is not yet significant, retinal autophagy–lysosomal degradation pathways are impeded but not completely blocked in Cln6<sup>nc/e</sup> mice.

**The Waste Aggregate Tag P62 Accumulates in the Cytosol, and the Mitochondrial Protein ATP Synthase Subunit C Accumulates in the Lysosomes of Photoreceptors in Cln6<sup>nc/e</sup> Mice at Postnatal Day 30**

As lysosomal degradation in the photoreceptor nuclear layer was disturbed in Cln6<sup>nc/e</sup> mice at P30, accumulation of undigested protein/waste aggregates and mitochondrial proteins was assessed histologically in WT and Cln6<sup>nc/e</sup> mice at P30 (Fig. 9). Aggregated cellular debris tagged for autophagic degradation, labeled by P62/SQSTM1 (green), was very low in WT photoreceptors and RPE as P62 is degraded in the autophagy process (Fig. 9A). In contrast, P62 accumulation was apparent in the cytosol of the photoreceptor layers in Cln6<sup>nc/e</sup> mice (Fig. 9B). In the RPE, P62 aggregates were occasionally apparent but were qualitatively infrequent, suggesting that like the LC3-LAMP1 assessment (Fig. 7), autophagy processes involving P62 are less affected in the RPE relative to the photoreceptors in Cln6<sup>nc/e</sup> mice at P30. Mitochondria and their degradation/recycling were also investigated (Figs. 9C–H). While there were no obvious changes observed over several images in the morphology, frequency, and distribution of mitochondria (MTCO1, green) in the photoreceptor layers between WT controls (Fig. 9C) and Cln6<sup>nc/e</sup> mice (Fig. 9D), there were changes in the degradation and recycling of mitochondria. The mitochondrial degradative product ATP synthase subunit C (ATPsyn; red) could not be detected in the WT retina, as like P62, it is readily digested in the autophagy–lysosomal recycling process (Fig. 9E). However, photoreceptors of Cln6<sup>nc/e</sup> mice showed accumulation of this mitochondrial protein (red), commonly colocalized with lysosomes (green; Fig. 9E, inset magnified in Fig. 9F). In the RPE of Cln6<sup>nc/e</sup> mice also, ATPsynthase subunit
C was found to accumulate relative to WT RPE (Figs. 9G, 9H; Cln6nclf), suggesting alterations in mitochondrial processing is an early and ubiquitous marker of cellular changes in ocular tissues in Cln6nclf mice.

**DISCUSSION**

Decline in vision caused by retinal accumulation of lipofuscin and photoreceptor death represents one of the first symptoms in infantile and childhood NCL forms.2,30 In the Cln6nclf mouse model of late-infantile NCL there was a significant loss of rod photoreceptor function at P18, prior to photoreceptor nuclei loss at P60. In contrast, postphotoreceptor rod and cone pathway function were not affected until P120 and P240, respectively. The loss of rod photoreceptor function correlated with significant disruption of the autophagy-lysosomal degradation pathways within photoreceptors (arrow). (H, Hi) At P240, Cln6nclf mice showed accumulation of autofluorescent debris in inner retinal neurons, including amacrine cells (arrow, Hi). (I-J) Retinae from (I) WT at P240 and (J) Cln6nclf mice at P240 were imaged for Müller glia (glutamine synthetase, GS, green), autofluorescence (red), and cell nuclei (DAPI, blue). (J, Ji) At P240, Cln6nclf mice showed accumulation of autofluorescent debris in Müller glia (arrow in Ji). (K) There were no changes in the number of GS-positive Müller glia between WT and Cln6nclf mice at P240. Data are expressed as mean ± SEM for n ≥ 5 mice and were analyzed by a Student’s t-test. Scale bar: 20 μm (A-J).

**FIGURE 6.** All retinal neurons and Müller glia accumulate autofluorescent debris in Cln6nclf mice. Transverse retinal sections from WT and Cln6nclf mice were collected for histologic analysis and imaged using confocal microscopy for autofluorescent debris within various retinal cell types. (A-D) Retinae from (A) WT at P30, (B) Cln6nclf mice at P30, (C) WT at P240, and (D) Cln6nclf mice at P240 were imaged for cone photoreceptors (PNA, green), autofluorescence (633-nm laser, pseudo-colored red), and cell nuclei (DAPI, blue). At P240, Cln6nclf mice showed accumulation of autofluorescent debris in all cell types, including cone photoreceptors (arrow). (E-H) Retinae from (E) WT at P30, (F) Cln6nclf mice at P30, (G) WT at P240, and (H) Cln6nclf mice at P240 were imaged for amacrine and ganglion cells (calretinin, CalR, green), autofluorescence (red), and cell nuclei (DAPI, blue). (H, Hi) At P240, Cln6nclf mice showed accumulation of autofluorescent debris in inner retinal neurons, including amacrine cells (arrow, Hi). (I-J) Retinae from (I) WT at P240 and (J) Cln6nclf mice at P240 were imaged for Müller glia (glutamine synthetase, GS, green), autofluorescence (red), and cell nuclei (DAPI, blue). (J, Ji) At P240, Cln6nclf mice showed accumulation of autofluorescent debris in Müller glia (arrow in Ji). (K) There were no changes in the number of GS-positive Müller glia between WT and Cln6nclf mice at P240. Data are expressed as mean ± SEM for n ≥ 5 mice and were analyzed by a Student’s t-test. Scale bar: 20 μm (A-J).
neurons underlies the early visual dysfunction that occurs in the Cln6nclf mouse model.

Lipofuscin accumulation within postmitotic cells is a key characteristic of NCL, and in humans, retinal deposits are apparent as reflective lesions in the fundus of infantile NCL patients. In Cln6nclf mice, white fundus lesions were apparent from 2 months of age (P60) and these increased in number across all quadrants of the eye with age. Currently, the origin of these fundus lesions in mice is unclear. Some studies suggest these types of lesions correlate with the accumulation of lipofuscin primarily in the RPE. Others study that infiltrating macrophages, which are autofluorescent, are responsible for this phenomenon. While resident retinal microglia are active by 1 month of age in Cln6nclf mice, fundus images have not been published previously. It is likely that in Cln6nclf mice, the fundus lesions represent a combination of both autofluorescent retinal macrophages and

**FIGURE 7.** At postnatal day 30, autophagy-lysosomal pathways are dysfunctional in the photoreceptor layers of Cln6nclf mice. Transverse retinal sections from P30 WT and Cln6nclf mice were collected for histologic analysis and imaged using confocal microscopy for autophagosomes (LC3B, red), lysosomes (LAMP1, green), and cell nuclei (DAPI, blue). (A, B) RPE layer from (A) WT and (B) Cln6nclf mice. (C, D) Photoreceptor OS/IS layers from (C) WT and (D) Cln6nclf mice. (E, F) Photoreceptor nuclei in the ONL from (E) WT and (F) Cln6nclf mice. (G, H) The INL from (G) WT and (H) Cln6nclf mice. (I) Quantification of LC3-positive puncta in each retinal layer for WT (black) and Cln6nclf mice (gray). (J) Quantification of LAMP1-positive puncta in each retinal layer for WT (black) and Cln6nclf mice (gray). (K) Quantification of colocalized LC3-positive puncta as a percentage of LAMP1-positive puncta in each retinal layer for WT (black) and Cln6nclf mice (gray). (L) The area of LC3-positive and LAMP1-positive puncta was assessed in each retinal layer and data for the outer nuclear layer are shown. Data are expressed as mean ± SEM for n ≥ 5 mice and were analyzed by a 2-way ANOVA, including a Tukey multiple comparisons test with P values < 0.05 considered significant (*). Scale bar: 20 μm (A–H).
cellular lipofuscin accumulation as has been shown to occur in other mouse models previously.\textsuperscript{20,21}

Histologic analysis showed that there was accumulation of autofluorescent lipofuscin within retinal neurons from P30 and that these deposits increased with age in \textit{Cln6\textsuperscript{nclf}} mice. These autofluorescent deposits were best imaged using 633-nm wavelength laser, suggesting their origin may be different from autofluorescent deposits were best imaged using 633-nm wavelength laser, suggesting their origin may be different from autofluorescent lipofuscin within retinal neurons from P30 and autofluorescent lipofuscin in the RPE and all retinal cell types (8 months), and even at this stage, cone numbers were still relatively plentiful in the central retina. Also, in the inner retina, while amacrine cell function was affected from P120, amacrine cell structure was intact up to P240 despite accumulation of lipofuscin in amacrine cells. This suggests a primary failure of the rod photoreceptors themselves or a failure of nonneural retinal support cells (RPE) as contributing to the retinal degeneration phenotype observed in \textit{Cln6\textsuperscript{nclf}} mice.

We directly addressed this question of whether the rod photoreceptor and/or RPE contribute to degeneration in the \textit{Cln6\textsuperscript{nclf}} model. Overall, the results of the autophagy–lysosomal pathway analysis suggest that a primary dysfunction within photoreceptor cells is the likely cause of rod photoreceptor failure of nonneural retinal support cells (RPE) as contributing to retinal degeneration observed in \textit{Cln6\textsuperscript{nclf}} mice.
Our functional and immunocytochemical data also support our autophagy–lysosomal pathway analysis suggesting that RPE dysfunction is unlikely to be the primary driver of rod photoreceptor death, as with RPE failure, both rod and cone photoreceptors might have been expected to deteriorate, and instead cone photoreceptor function remained relatively stable up until P240. Also, there were no early histologic changes in autophagy–lysosomal pathways in the RPE of Clan6nclf mice that might have contributed to rod photoreceptor dysfunction at P30. While we saw global retinal changes in the autophagy–lysosomal degradation process in Clan6nclf retina, one advantage of the histologic approach taken here, as opposed to total retinal or RPE protein quantifications using Western blot described herein and in previous studies, is that in this study the use of super-resolution microscopy allowed quantification of changes in autophagy on a cellular level. Thus, at P30, a time when photoreceptor nuclei remained but rods were dysfunctional, reductions in autophagosomes and autophagy–lysosomal fusion events were apparent specifically within photoreceptors and not other cell types, suggesting that this cell type is particularly sensitive to autophagy–lysosomal dysregulation. This suggestion is supported by findings in a mouse model in which autophagy is selectively blocked in rod photoreceptors by deletion of Atg5; this mouse shows a similar retinal degeneration phenotype to the Clan6nclf mouse, in terms of disruption of autophagy–lysosomal degradation and subsequent photoreceptor loss.

Further investigation of the photoreceptors at P30 showed that there was abnormal accumulation of P62 and the mitochondrial-derived protein, ATP synthase subunit C, in Clan6nclf mice. P62 plays an important role in tagging aggregated proteins for intracellular digestion and the antioxidant response, and it has been shown to accumulate in neurons of various NCL models. Accumulation of P62 in photoreceptors in Clan6nclf mice at P30 suggests that incomplete loading of autophagosomes and insufficient degradation of protein aggregates (aggrephagy) are occurring at this time. In addition, colocalization of ATP synthase subunit C with lysosomes was apparent in photoreceptors in Clan6nclf mice but not in WT mice. This phenomenon has been seen in the brain and other tissues of various models of NCLs, and suggests that failure of mitochondrial degradation (mitophagy) is occurring in Clan6nclf mice. As the retina and in particular, photoreceptors, have one of the greatest energy requirements of the entire body, they would be extremely sensitive to failures in energy production due to dysregulation of mitochondrial turnover. While it has been suggested that photoreceptor energy requirements are mostly glycolytic, photoreceptor inner segments are densely packed with mitochondria, perhaps to buffer calcium but also perhaps to meet high metabolic demands, such as maintaining photoreceptor depolarization, the photoreceptor dark current. Therefore, one reason that rod photoreceptors may be primarily affected in Clan6nclf mice may be failure of mitochondrial turnover, insufficient energy production, and reduced intracellular calcium buffering capacity. This begs the question why rod photoreceptors may be affected prior to cone photoreceptors. In the mouse, rod and cone photoreceptors have differences in mitochondrial structure and number and metabolism, which may afford cone photoreceptors protection against metabolic insults. Additionally, mouse cone photoreceptors have shorter outer segments, much larger inner segments, larger
axon diameter (1.4 μm in cones versus 0.35 μm in rods), and larger terminals,53 which may potentially provide additional cytoplasmic storage for lysosomal accumulations when compared with rods. It is possible that these metabolic and morphologic differences allow cones to be more resilient to autophagy-lysosomal dysfunction; however, more work is required to determine why rod pathway dysfunction precedes cone pathway dysfunction in Cln6nclf mice.

In conclusion and in summary, this study provides detailed evidence suggestive of primary photoreceptor failure and death as the likely mechanism underlying retinal degeneration in the Cln6nclf mouse model. Rod photoreceptor death likely occurs due to a significant decrease of autophagosome-lysosomal fusion events within photoreceptor cells and failure of lysosomal degradation, accompanied by early cytosolic accumulation of aggregated proteins. Furthermore, in comparison to other prevalent retinal diseases such as AMD and Stargardt’s disease, which are also characterized by lipofuscin accumulation, our findings in Cln6nclf mice show a different pathomechanism, as critical functions of the RPE were not affected early in the disease process in Cln6nclf mice.54–56 Future studies investigating therapies for retinal degeneration in Cln6nclf mice should therefore target photoreceptors to ameliorate or slow visual decline in this disorder.

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