Supplementary Material for J. C. Gilliam, et al., Three-Dimensional Architecture of the Rod Sensory Cilium and its Disruption in Retinal Neurodegeneration

Supplemental Figures

Figure S1. Rootletin-Associated Structures in the Rod IS, (Related to Figure 1)  
(A) Northern blot of total RNA from multiple mouse tissues (20 μg) probed with 25 ng of radiolabeled rootletin cDNA. Ethidium bromide stained 18S ribosomal bands are shown for loading control.  
(B) Immunostaining of retina wholemount with anti-rootletin antibodies.  
(C) Projection of a tomogram slice (~30 nm thick) through the rod IS region showing a single mitochondrion anchored to the isolated ciliary rootlet. Connecting cilium (CC), basal bodies (BB), rootlet (R), and mitochondrion (MT) are visible. Bar = 0.2 μm.  
(D) Tomogram slice projection of mitochondria membrane fragments anchored to the ciliary rootlet. Connecting cilium (CC), basal bodies (BB), rootlet (R), and mitochondria fragments (MT) are visible. Bar = 0.2 μm.  
(D') Expanded view of the mitochondria fragment (MT) found in the boxed region in (D). Mitochondria fragments are identified by the F1F0-ATPase complexes (arrowheads) in the membrane bilayer. Bar = 0.2 μm.  
(E) Segmentation of the rod IS region. Cross-section slices (red lines) through the CC (E1), axoneme basal body (E2) and proximal basal body (E3) along the z-axis (proximal-to-distal direction indicated by white arrows) are shown [right]. Bar = 0.1 μm.  
(F) Tomogram slice projections (~5 nm thick) of microtubule-bound transport vesicles show electron-dense boundaries formed by phosphate head groups. (E-F) All bars = 50 nm.  
(G) Tomogram slice projections (~5 nm thick) of rootlet-bound particles (arrows) show low-contrast boundaries, which are uncharacteristic of lipid-based vesicles.

Movie S1. Structures of the Rod Inner Segment, (Related to Figure 1)  
This movie shows the segmentation of a reconstructed tomogram displaying the 3D organization of the structures and membranes within the inner segment, including microtubules (green, blue, red), axoneme filaments (pink), rootlet (brown), plasma membrane (gray), endoplasmic reticulum (blue), membranes (magenta), and ribosomes (yellow).

Figure S2. Basal Body-Associated Structures in the Rod Inner Segment,  
(Related to Figure 2)  
(A-B) Multiple tomographic slices through a 3D reconstruction of a single cell are shown as raw data [top] or segmentation [bottom]. Highlighted structures are indicated in both panels by color-matched arrowheads.  
(A) Transition fibers (red) originate from basal body triplets. Densities identified as transition fibers are indicated (red arrowheads).
(B) Filaments originating from the IS (red) interact with vesicles (yellow) and ciliary membrane filaments (blue). Distal appendages (green) extend from the basal body to the IS filaments (red). All bars = 0.1 μm.

(C-E) Multiple tomogram slices through a 3D reconstruction of the axoneme focusing on the top (C), central (D), and bottom (E) regions of the axoneme. The connecting cilium (CC), basal bodies (BB), rootlet (R) are visible. (C-E) Bars = 0.2 μm.

(D) The central filament is indicated in the X-Y plane (black arrowheads).

(F) Cross section slice through the basal body along the X-Z axis. The central position of the filament is indicated (black arrowhead).

(G) Segmentation of the basal body showing the spatial distribution of the A-microtubules (green), B-microtubules (blue), C-microtubules (red), and the central filament (yellow, black arrowhead). (F-G) Bars = 50 nm.

Movie S2 Filaments and Vesicles Associated with the Basal Bodies in the Inner Segment, (Related to Figure 2 and Figure S2)
This movie shows tomographic slices and segmentation of the structures present in the IS and the CC along the z-axis of the 3D reconstruction. Part 1 indicates the locations of the transition fibers (red), vesicle (yellow), and plasma membrane (gray) within the tomogram. Part 2 indicates the locations of the IS filaments (red), distal appendages (green), membrane filaments (blue), and vesicles (yellow) with respect to the plasma membrane (gray).

Figure S3 Filament Structures of the Connecting Cilium (Related to Figure 3)
(A-B) Multiple tomographic slices through a 3D reconstruction of a single cell are shown as raw data [top] or segmentation [bottom]. Highlighted structures are indicated in both panels by color-matched arrowheads. Colors correspond to filaments of the connecting cilium (red), microtubules (green, blue), and low-density particles (cyan). All bars = 0.1 μm.

Movie S3. Structures of the Connecting Cilium, (Related to Figure 3).
This movie shows tomographic slices and segmentation of the axoneme and structures within the CC along the z-axis of the 3D reconstruction. Part 1 indicates the locations of lumenal particles (cyan) within the central compartment of the axoneme. Part 2 indicates the location of a single lumen particle (cyan) bound to microtubules (green, blue) by actin-like filaments (red).

Figure S4. Rhodopsin Labeling in the Connecting Cilium Membrane (Related to Figure 4)
(A and A’) Tomogram slice projections from a 3D reconstruction through the center (A) or the plasma membrane surface (A’) of the CC from an unlabeled wildtype rod photoreceptor.
(B and B’) Tomogram slice projections from a 3D reconstruction through the center (B) or the plasma membrane surface (B’) of the CC from a wildtype rod photoreceptor that has been labeled with concanavalin A-ferritin to indicate rhodopsin present in the membrane. All bars = 0.1 μm.

Movie S4. Concanavalin-A Ferritin Labeling of Rhodopsin N-termini in the Connecting Cilium (Related to Figure 4). This movie shows tomographic slices along the z-axis of a 3D reconstruction where rhodopsin has been labeled by electron-dense ferritin. The measured distribution of ferritin reveals the random distribution of rhodopsin in the CC plasma membrane.

Figure S5. Nascent Disks contained within the OS Plasma Membrane, (Related to Figure 5)
(A) Conventional electron micrograph of a stained rod OS showing nascent disks (black arrowhead) enclosed by the OS plasma membrane (white arrowhead).
(B) Tomogram slice projection from a 3D reconstruction of the OS, showing the repeating features of the disk membranes. Tomogram was reconstructed at 1.7 nm/pixel Bar = 0.3 μm.
(C) Fourier transform (computed diffraction pattern) spectrum (from (B)) showing bright spots corresponding to repetitive features of the original tomogram projection.
(D) Spectrum identical to (C) indicating the line used for a density plot profile of the Fourier intensities.

Figure S6. IS of Crocc<sup>-/-</sup> Rods Contain Electron-dense, Crystalloid Granules (Related to Figure 7)
(A) A panel of consecutive tomogram slice projections (each ~30 nm thick) of a single electron-dense granule (arrowhead) may indicate stress conditions in Crocc<sup>-/-</sup>, but not wildtype rod IS regions. Basal bodies (BB). All bars = 100 nm.

Supplemental Experimental Procedures

Northern blotting
Total RNA was isolated from multiple mouse tissues that were homogenized in cold TRI Reagent (Ambion). Fresh eyes were hemisected at the ora serrata to remove the anterior segment; retinas were carefully peeled from eyecups and immediately frozen on dry ice. RNA (20 μg per tissue) was resolved using 1% agarose gels containing 0.25 M formaldehyde and transferred overnight to BrightStar positively charged nylon membranes (Ambion). Rootletin cDNA was amplified from reverse-transcribed retina mRNA template using the following
primers: 5'-TCCTTCAACACCTACTTCAGCAGCG-3' and 5'-CTGCTCAGGAGCTCTGGCA-3'. Rootletin cDNA probe was generated by random-prime labeling with [α-32P] dCTP (6000 Ci/mmol) using the DECAprime II kit and hybridized at 10˚C for 15 hr at 42°C in ULTAhyb (Ambion). Stringency washes using 2 x SSC/0.1% SDS and 0.5 x SSC/0.1% SDS were applied at 60°C, followed by washes using 0.1 x SSC/0.1% SDS applied at 42°C prior to exposing membranes to phosphorscreens. Phosphorscreens were imaged on a Typhoon image scanner (GE Healthcare).

Immunostaining of Sectioned or Wholemount Retinas
Prior to sectioning, Retinas were separated from the eyecup and fixed in 4% paraformaldehyde in Ringer's buffer (10 mM HEPES (pH 7.4), 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 0.02 mM EDTA) for 3 hours at 4°C. Retinas were washed in Ringer's buffer and embedded in 5% buffered agarose prior to vibratome sectioning. Sections (150 μm thick) were collected and pre-blocked for 6 hours at 4˚C in 10% donkey serum, 5% BSA, 0.5% fish gelatin, 0.1% Triton X-100 in Ringer's buffer before addition of primary antibody. For wholemounts, retinas were separated from the eyecup and placed, unfixd, in Ringer's buffer containing 10% donkey serum, 5% BSA, 0.5% fish gelatin, 0.1% saponin. Commercial goat-anti-rootletin (C-20, SC-67824, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied to retinas or vibratome sections in blocking buffer overnight at 4˚C at a final concentration of 1.0 μg/mL. After three washes using Ringer's buffer, Alexa 488 conjugated antibodies were applied to tissues for 1 hr at room temperature in blocking solution. Confocal images were acquired using a Zeiss laser scanning microscope LSM 510 (Carl Zeiss Microimaging, Inc.)

Labeling of Isolated Rod Outer Segments
Rod OS were isolated as described from dark adapted retinas under infrared illumination. Rod OS were pre-blocked unfixd, in Ringer's buffer containing 10% donkey serum, 5% BSA, 0.5% fish gelatin, 0.1% saponin. Commercial goat-anti-rootletin (C-20, SC-67824, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and 1D4 anti-rhodopsin antibody were applied to OS in blocking buffer for 1 hr at room temperature. After three washes using Ringer's buffer, Alexa 488 and Alexa 546 conjugated antibodies were applied to OS for 1 hr at room temperature in blocking solution before confocal images were collected.

Electron Microscopy of Ultra-Thin Sections
Eyes were fixed for 15 min in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Eyes were hemisected at the ora serrata to remove the anterior segment and lens, and fixed for 24 hours in the same fixative at 4°C. Eyecups were subsequently rinsed in 0.1 M cacodylate, post-fixd in 1% osmium tetroxide in 1 M sodium phosphate buffer for 2 hours. Eyecups were rinsed several times in distilled water and dehydrated in a graded ethanol series prior to epon embedding. Eye cups were incubated in two 2 hour changes of a 1:1 mixture of Epon resin and acetone, followed by overnight incubation in pure Epon.
resin in block molds. Sections (1 μm thick) were cut and stained with toluidine blue. Ultrathin sections (80 nm thick) were cut and mounted on 100 mesh copper grids and stained with 2% uranyl acetate and Reynold’s lead citrate. Electron micrographs were captured in a Zeiss CEM 902 electron microscope on Kodak (Rochester, NY) 4489 EM film.
Figure S1
Figure S4