Early Synaptic Defects in *tulp1*"/"- Mice

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**PURPOSE.** Mutations in the photoreceptor-specific tubby-like protein 1 (*TULP1*) underlie a form of autosomal recessive retinitis pigmentosa. To investigate the role of Tulp1 in the photoreceptor synapse, the authors examined the presynaptic and postsynaptic architecture and retinal function in *tulp1"/"- mice.

**METHODS.** The authors used immunohistochemistry to examine *tulp1"/"- mice before retinal degeneration and made comparisons with wild-type (wt) littermates and retinal degeneration 10 (rd10) mice, another model of photoreceptor degeneration that has a comparable rate of degeneration. Retinal function was characterized with the use of electroretinography.

**RESULTS.** In wt mice, Tulp1 is localized to the photoreceptor synapse. In the *tulp1"/"-* synapse, the spatial relationship between the ribbon-associated proteins Bassoon and Piccolo are disrupted, and few intact ribbons are present. Furthermore, bipolar cell dendrites are stunted. Comparable abnormalities of postsynaptic membranes and the actin cytoskeleton.21 In *tulp1"/"- mice, the leading edge of the a-wave had normal kinetics in *tulp1"/"- mice but reduced gain in rd10 mice. The b-wave intensity-response functions of *tulp1"/"- mice are shifted to higher intensities than in wt mice, but those of rd10 mice are not.

**CONCLUSIONS.** Photoreceptor synapses and bipolar cell dendrites in *tulp1"/"- mice display abnormal structure and function. A malfunction of the photoreceptor synaptic ribbon is likely the cause of the dystrophy in bipolar cell dendrites. The association of early-onset, severe photoreceptor degeneration preceded by synaptic abnormalities appears to represent a phenotype not previously described. Not only is Tulp1 critical for photoreceptor function and survival, it is essential for the proper development of the photoreceptor synapse. (Invest Ophthal Vis Sci. 2009;50:3074–3085) DOI:10.1167/iovs.08-3190

The outer plexiform layer (OPL) of the vertebrate retina is where rod and cone photoreceptors make synaptic contact with second-order neurons of the inner nuclear layer (INL). Photoreceptors signal second-order neurons with the use of a graded synaptic output, whereby changes in stimulus intensity are conveyed by alterations in the tonic release of neurotransmitter at the active zone of the photoreceptor presynaptic membrane.3 In addition to this tonic release, the visual sensory system requires rapid transfer of information.9 At the photoreceptor synapse, hundreds of neurotransmitter-laden vesicles come in contact with the presynaptic membrane every second, liberating glutamate into the synaptic cleft. To support these requirements, photoreceptor terminals contain a highly specialized presynaptic apparatus, the ribbon synapse, which is thought to be a critical platform for the rapid transport, tethering, docking, and recycling of synaptic vesicles.1,3

Rod photoreceptor terminals typically contain a single ribbon, whereas larger cone terminals harbor multiple ribbons.3 Although many of the structural elements have been examined, the complete complement of their elements and how they interact to form functional synapses remain elusive. More insight into photoreceptor synaptic function is necessary for thorough understanding of the many disorders that affect vision.2,4

Retinitis pigmentosa (RP) refers to a large number of inherited retinal disorders characterized by photoreceptor degeneration. RP, which is genetically and phenotypically heterogeneous, affects more than 1 million persons worldwide.5,6 Mutations in *TULP1* underlie an early-onset, severe form of autosomal recessive RP.7–10 TULP1 is a member of the Tubby-like protein family, which includes TULP2, TULP3, and TUB.11 TUB and TULP3 are widely distributed throughout the central nervous system; expression of TULP1 and TULP2 is restricted largely to rod and cone photoreceptors and the testis, respectively.11–16 Although the complete role of TULPs in the central nervous system remains unclear, TULP proteins are known to play important roles in neuronal development and function. This is emphasized by the association of TUB and TULP1 mutations with neurosensory disease phenotypes.7–10,12,17

Tulp1 is expressed exclusively in photoreceptors, localizing to the inner segment (IS), connecting cilium, perikarya, and terminals.18–20 Although the function of Tulp1 remains elusive, there is evidence that Tulp1 plays a role in intracellular trafficking in the IS and at the photoreceptor synapse.19,20–22 Tulp1 is a cytoplasmic protein that associates with cellular membranes and the actin cytoskeleton.21 In *tulp1"/"- mice, photoreceptor cells degenerate, rod and cone opsins are mis-localized, and rhodopsin-bearing extracellular vesicles accumulate around the ellipsoid region of the IS.18 These defects indicate that Tulp1 may be involved in actin cytoskeletal dynamics such as protein transport from the IS to the outer segment (OS) through the connecting cilium.18,20–22

We recently showed that Tulp1 colocalizes with and binds to the neuronal-specific protein Dynamin-1 at the IS and the terminals of photoreceptors.22 Dynamin-1 is a GTPase that binds actin and regulates endocytosis, vesicle development, and movement at the trans-Golgi network, the plasma membrane, and the synaptic membrane.22–24 Consistent with a role for Tulp1 at the photoreceptor synapse, the b-wave component of the electroretinogram (ERG) generated by depolarizing bipolar cells (DBCs) is markedly reduced in young *tulp1"/"- mice.22

These results suggest that Tulp1 plays an important role in synaptic function and motivated us to look more closely at the
photoreceptor synapse. To accomplish this, we examined the architecture of the tulp1−/− synapse at postnatal day (P16), an age at which synaptic development is complete in wild-type (wt) mice and precedes photoreceptor degeneration in tulp1−/− mice.18 To ascertain whether alterations are specific to the tulp1−/− retina and are not merely a consequence of the degenerative process, we made parallel studies of the retinal degeneration 10 (rd10) mouse, which experiences a comparable rate of photoreceptor degeneration because of a point mutation in exon 13 of the β-subunit of the rod cGMP phosphodiesterase gene.26 Except for the comparable rate of degeneration, no reports have been published of early synaptic abnormalities associated with this model; thus, it presents a fitting control strain for synaptic-specific comparisons. Although mouse models with synaptic-specific mutations (such as Cadps1−/−, Cacna1f nob2, Cabp4−/−, and Bsn−/−) exhibit decreased b-waves, altered bipolar dendrites, and presynaptic abnormalities, they do not exhibit photoreceptor degeneration or rhodopsin mislocalization comparable to that observed in tulp1−/− mice.

In this study, we verified that Tulp1 is diffusely localized to the photoreceptor synapse and that its distribution overlaps that of several key synaptic proteins. Mice deficient in Tulp1 lack the tight spatial relationship between the ribbon-associated proteins, Bassoon and Piccolo, characteristic of wt animals. In addition, few intact ribbons are present in the OPL. We also report that dendrites of second-order neurons exhibit abnormalities at an early age in tulp1−/− mice. These structural defects are not apparent in rd10 animals. Although ERG a-waves of tulp1−/− mice are reduced in amplitude, they retain normal kinetics. In comparison, the tulp1−/− b-wave intensity-response function is desensitized, consistent with a functional defect at the photoreceptor output synapse. That similar synaptic and b-wave changes were not seen in rd10 mice indicates they are not generalized responses to photoreceptor degeneration. 

These results indicate that the absence of Tulp1 is associated with a synaptic malformation that precedes photoreceptor degeneration and most likely interferes with the proper development of postreceptor neurons. Taken together, this appears to represent a phenotype not previously described. These data indicate that Tulp1 plays an important role in photoreceptor synapse development and in photoreceptor function and survival.

**METHODS**

**Animals**

The generation of tulp1−/− mice has been described previously and is now maintained on a C57BL/6 background.18 Mice studied here were derived from mating tulp1−/− heterozygotes and were genotyped by PCR amplification of genomic DNA, as previously described.18 Homozygous rd10 breeders (B6.CXB1-Pde6b−/−/B6) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were euthanized by CO2 inhalation or by drug overdose; cervical dislocation followed. All experiments on animals were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic and were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

**Preparation of Retinal Sections**

Mouse eyes were prepared as previously described.22 Briefly, eyes were fixed in 4% paraformaldehyde in PBS for 60 minutes. After removal of the cornea and lens, the posterior pole was immersed through a graded series of sucrose solutions as follows: 10% for 1 hour, 20% for 1 hour, and 30% overnight. The posterior pole was embedded in OCT freezing medium, flash frozen on powdered dry ice, and immediately transferred to −80°C. Tissue was sectioned at 10-μm thickness with a cryostat (Leica, Wetzlar, Germany) at −30°C.

**Immunofluorescence Staining**

Retinal sections were blocked in 5% bovine serum albumin and 1% normal goat serum with 0.1% Triton X-100 for 1 hour before incubation with primary antibodies overnight at 4°C. Primary antibodies and dilutions were as follows: Tulp1, rabbit polyclonal Mtulp1N 1:250;20 Piccolo, rabbit polyclonal 1:500 (ab20664; Abcam Inc., Cambridge, MA); Bassoon, mouse monoclonal 1:500 (SAPT#F407; Assay Designs Inc., Ann Arbor, MI); Ribeye/CtBP2, mouse monoclonal 1:1500 (612044; BD Biosciences, San Jose, CA); protein kinase C-α (PKC), rabbit polyclonal 1:1000 (SC208; Santa Cruz Biotechnology Inc., Santa Cruz, CA); rhodopsin, mouse monoclonal 1:100 (B630N; Paul Hargrave, University of Florida, Gainesville, FL). After three washes in PBS, sections were incubated in fluorescent secondary antibodies at room temperature for 1 hour. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IglG and goat anti-mouse IglG and Alexa Fluor 594 goat anti-rabbit IgG and goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Sections were then rinsed three times in PBS and then rinsed lightly in 0.1× PBS before mounting with mounting medium with DAPI (Vectorshield; Vector Laboratories, Burlingame, CA). Sections were imaged with a fluorescence microscope (BX-61; Olympus, Tokyo, Japan) equipped with a charge-coupled device monochrome camera (Hamamatsu Photonics, Bridgewater, NJ). For the imaging of ribbon-associated synaptic proteins (Bassoon, Piccolo, and Ribeye), 2-μm Z-stacks were acquired with nearest-neighbor deconvolution, followed by a maximum intensity Z-axis projection (Slidebook software, version 4.2; Intelligent Imaging Innovations, Denver, CO).

**Electroretinography**

ERGs were recorded from tulp1−/−, tulp1+/-, and wt littermates and from rd10 homozygotes at P16. After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). Eyedrops were used to anesthetize the cornea (1% proparacaine HCl) and to dilate the pupil (1% mydriacyl, 2.5% phenylephrine HCl, 1% cyclopentolate HCl). Mice were placed on a temperature-regulated heating pad throughout the recording session. ERGs were recorded with a stainless steel electrode that made contact with the corneal surface through a thin layer of 0.7% methylcellullose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.3–1500 Hz), averaged, and stored with the use of a signal averaging system (UTAS E-3000; LKC Technologies, Gaithersburg, MD). ERGs were recorded to flash stimuli presented in an LKC Technologies ganzfeld that ranged in intensity from −3.6 to 2.1 log cd·s/m².

Amplitude of the a-wave was measured at 8 ms after flash presentation from the prestimulus baseline. Amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the prestimulus baseline. Implicit time of the b-wave was measured from the time of flash presentation to the b-wave peak.

The b-wave intensity-response function was analyzed using equation (1)

\[
R/R_{\text{max}} = 1/(1 + K^n)
\]

in which \( R \) represents the b-wave obtained to background intensity \( I \), \( R_{\text{max}} \) represents the amplitude asymptote, \( K \) represents the flash intensity required to generate a b-wave response that is \( 1/2 \) of \( R_{\text{max}} \), and \( n \) is a dimensionless slope parameter. As in humans, the b-wave intensity-response function in mice includes a high-intensity limb where the a-wave begins to precede the b-wave.30 We fit equation 1 only to data points that fell below this second limb.

We analyzed the leading edge of the dark-adapted a-waves obtained to high-intensity stimuli in terms of a modified form of the Lamb and Pugh model of rod phototransduction.31,32
in which $P_3$ represents the mass response of the rod photoreceptors, the amplitude of which is expressed as a function of flash energy ($i$) and time ($t$) after flash onset, $A$ represents the gain of phototransduction, $R_{mP3}$ represents the maximum response, and $td$ represents a brief delay.

**Dendritic Field Analysis**

Four PKC immunoreactive retinas from each genotype (tulp1-/-, wt, and rd10) were imaged under a fluorescence microscope (BX-60; Olympus). For analysis, individual images were imported into a program (Image-Pro PLUS, version 6.2; Media Cybernetics, Inc., Bethesda, MD) that measured the PKC immunoreactive dendrites. Only the portions of the dendrites that extended beyond the cell bodies of the bipolar cells of INL were measured (nuclei were verified by DAPI staining). Dendrite lengths were measured over the entire area of the images ($213 \mu m \times 162 \mu m$). Two images were taken from the superior or the inferior retina of each animal; one was taken from the central retina (within $200 \mu m$ of the optic nerve) or the peripheral retina (within $200 \mu m$ of the peripheral edge). Measurements were averaged per genotype and were reported as having been derived from the central or peripheral retina.

**RESULTS**

**Photoreceptor Synaptic Localization of Tulp1**

The retinal distribution of Tulp1 was examined in wt mouse sections at P16 (Fig. 1A). Consistent with previous observations, Tulp1 staining is strongest in the IS and OPL but is absent in the OS. In the outer nuclear layer (ONL) appeared to be located in the perikarya and did not overlap with the nuclei. Tulp1 staining in the OPL was diffuse and appeared to fill the entire terminal, and its colocalization with the presynaptic ribbon-associated protein Bassoon confirmed its presence in the photoreceptor synapse (Fig. 1B).

**Malformation of tulp1-/- Photoreceptor Synapses**

To characterize synaptic terminal architecture of tulp1-/- mice, we examined the distribution of synaptic proteins at ages before photoreceptor degeneration. Piccolo and Bassoon proteins have been implicated in the organization of the photoreceptor synapse, the functioning of the ribbon, and the trafficking of vesicles at the synapse. These proteins normally localize together at the presynaptic membrane with a horseshoe-like spatial arrangement. Figure 2A shows a wt mouse retinal section at P16 double-stained with antibodies to Piccolo (red) and Bassoon (green). Arrows highlight examples of the normal steric relationship between these two ribbon-associated proteins. A profoundly different appearance was noted in tulp1-/- mice at the same age, when the structure and distribution of both Piccolo and Bassoon were markedly abnormal (Fig. 2B). In contrast to the horseshoe-shaped ribbons typical of wt mice, Bassoon and Piccolo staining in the tulp1-/- retina appeared punctate, and normal ribbons were rarely seen. Moreover, even though the two proteins were in proximity, few terminals displayed any coupling between Piccolo and Bassoon. To determine whether these abnormalities were secondary to an early degenerative process, we also examined the rd10 mouse model in which the onset of degeneration occurred between P16 and P18, similar to that of tulp1-/- mice but caused by a different molecular defect. As shown in

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933246/ on 10/16/2017)
To examine more closely the coupling of Bassoon and Piccolo at P16, we generated three-dimensional surface plots from deconvoluted 2-μm Z-axis stacks of immunofluorescent images of wt and tulp1−/− OPLs (Figs. 2E, F). Deconvolution microscopy significantly reduces the blurring effect of reflected and emitted light associated with sources above or below a focal plane of interest. This results in images with increased resolution and markedly improved contrast, thus revealing structural information normally obscured in images generated by traditional methods.34,35 Implementing three-dimensional surface volume reconstruction then allows the viewing of relationships between objects in a three-dimensional landscape, further revealing associations normally not detected in two-dimensional microscopy. This analysis shows that Bassoon and Piccolo are often in immediate proximity with one another in the tulp1−/− retina (Fig. 2F; arrowheads indicate independent ribbon staining) but confirms that they are rarely spatially aligned into the normal horseshoe-shaped ribbon architecture typical of wt mice (Fig. 2E; arrows indicate coupling).

To examine whether normal terminal architecture is ever present in tulp1−/− mice, we analyzed the distribution of Bassoon and Piccolo at an earlier stage of ribbon development.25 In the wt retina, although the pattern of coupling between Bassoon and Piccolo is less robust at P13 than at P16, synaptic ribbons are clearly visible (Fig. 2G; arrows indicate coupling). In comparison, in the P13 tulp1−/− retina, the lack of coupling between Bassoon and Piccolo is already similar to that noted at P16, suggesting that normal ribbons may never form in the absence of Tulp1 (Fig. 2H).

We further examined synaptic terminal architecture using antibodies against Ribeye/Ctip2, a 120-kDa protein that constitutes the central scaffold of the ribbon and colocalizes with Piccolo and Bassoon.2,29,37 Figure 3A shows that the P16 wt OPL contains a multitude of distinct horseshoe-shaped Ribeye-positive ribbons (arrows). Although the P16 tulp1−/− retina contains few normal ribbons (Fig. 3B), a pattern similar to that of wt is seen in rd10 retinas at P16 (Fig. 3C) and P21 (Fig. 3D), before and after the commencement of retinal degeneration.

Given that ribbon-associated proteins exhibited structural abnormalities in the tulp1−/− retinas at P13, we wondered whether defects might also be noted in the ribbon itself at this early stage of development. Although the P13 wt retina contains many distinct and well-formed ribbons (Fig. 3E), fewer ribbons are seen in the P13 tulp1−/− retina (Fig. 3F). These results document distinct abnormalities in the young tulp1−/− retina that are not shared with another model of early-onset photoreceptor degeneration.

**Mislocalized Rhodopsin Not Sufficient to Induce Synaptic Ribbon Abnormalities**

Although rhodopsin is normally confined to the photoreceptor OS, it is distributed throughout tulp1−/− photoreceptors.18,20 It has been suggested that mislocalized rhodopsin causes photoreceptor degeneration, possibly because of an accumulation at the synapse.38 This hypothesis suggests that protein mislocalization may interfere with synaptic ribbon architecture. To determine the association between rhodopsin mislocalization and photoreceptor synaptic structure, we examined rhodopsin distribution in rd10 and tulp1−/− retinas at different ages. In the wt retina at P16, rhodopsin staining is confined to the OS (Fig. 4A). This is also the case in the rd10 retina at P16, before retinal degeneration (Fig. 4B). In comparison, rhodopsin is mislocalized to the IS, throughout the ONL, and within the pho-

**Figure 2.** Photoreceptor synaptic ribbon-associated proteins are abnormal in the tulp1−/− retina. Deconvolution-generated images of immunofluorescent localization of Bassoon (green) and Piccolo (red) in the OPL of mouse retinal sections. In the wt OPL at P16 (A), rd10 at P16 (C), and P21 (D), the horseshoe-like appearances of the photoreceptor synaptic ribbons are clearly visible. Arrows highlight the tight coupling between Bassoon and Piccolo, composing individual ribbons. In the tulp1−/− OPL at P16 (B), the ribbons appear to exhibit morphologic abnormalities, possibly indicating a structural ribbon defect. To best show the distinction between the proximity and coupling of Bassoon and Piccolo at P16, three-dimensional surface plots were generated from a portion of the 2-μm Z-stacks of wt (A) and tulp1−/− (B) retinas. In the wt retina (E), arrows point to the union of Bassoon and Piccolo, forming the compact horseshoe-shaped ribbons. In the tulp1−/− retina (F), both proteins are present and in immediate proximity; however, they are not spatially fixed, as they are in the wt retina, into the normal horseshoe-shaped ribbon formation (arrow points to the union of Bassoon and Piccolo; arrowheads point to the separate ribbon staining of Bassoon and Piccolo). Early in the development of the wt functional synapse (P13), the coupling of the two proteins is not as robust (G) compared with the more mature P16 wt OPL (A); however, individual synaptic ribbons can be observed (arrows show coupling). In the tulp1−/− OPL at P13 (H), the ribbons exhibit the same abnormalities as P16, possibly indicating that the deficits of the ribbon are present throughout development. Scale bar, 5 μm; gridlines, 10 μm.
In the P13 *tulp1"/-"* retina, however, DBC dendrites were difficult to differentiate from the DBC cell bodies, and branching was not observed (Fig. 5F). The presence of markedly reduced DBC dendrites early in the development of the *tulp1"/-"* retina suggested that normal dendrites were unlikely ever to form. In comparison, in the rd10 retina, it appeared that DBC dendrites first formed and then retracted in concert with the progressive photoreceptor degeneration, as previously noted.59

We quantified dendritic processes of DBCs identified by PKC labeling in wt, rd10, and *tulp1"/-"* mice at P16 at central and peripheral locations (Fig. 6). For each genotype, we examined four different PKC-immunoreactive retinas. Dendrite lengths were measured over the entire area of the image, averaged per genotype, and reported as deriving from the central retina (within 200 μm of the optic nerve) or peripheral retina (within 200 μm of the periphery). Figure 6A presents a low-power image of the inferior wt retina; PKC immunofluorescence is shown as white, and boxes indicate the central and peripheral locations examined. Figure 6 also shows representative higher power images of corresponding central (Figs. 6B, C) and peripheral (Figs. 6D, E) areas of wt and *tulp1"/-"* retinas. In each panel, the yellow vertical lines indicate the dendrite length. Average dendritic lengths (± SEM) are summarized in Figures 6F and 6G for central and peripheral locations, respectively. In the central retina, no significant differences in dendritic length were seen between wt and rd10 (12.93 μm ± 1.1 vs. 12.58 μm ± 0.8; *P* = 0.786). In the same region; however, average dendrite length was statistically significantly shorter in *tulp1"/-"* than in wt (7.29 μm ± 0.8 vs. 12.93 μm ± 1.1; *P* = 0.006) retinas. Similar results were seen in the peripheral retina (Fig. 6G), in which average dendrite length was also shorter in *tulp1"/-"* than in wt (5.46 μm ± 0.58 vs. 8.09 μm ± 0.2; *P* = 0.820) retinas. Thus, DBC dendrites were markedly abnormal at central and peripheral regions in P16 *tulp1"/-"* retinas, with an average reduction in DBC dendritic length of 33% to 44%. We observed shorter DBC dendrites in the peripheral retina, which we believe was related to the gradient of neurogenesis from the center to the periphery of the retina.40

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933246/) The central ribbon protein, ribeye, is abnormal in the *tulp1"/-"* retina. Deconvolution-generated images of immunofluorescent localization of Ribeye/CXBP2 (green) in the OPL of mouse retinal sections. In the wt OPL at P16 (A), rd10 at P16 (C), and P21 (D), the horseshoe-like appearance of the synaptic ribbons is distinct (arrows). This is also the case in the wt OPL early in development (E). In the *tulp1"/-"* OPL at P13 (F) and P16 (B), the ribbons appear to exhibit morphologic abnormalities. The ribbons change from the classic horseshoe shape to diffuse immunoreactive areas, further raising the possibility of a synaptic malformation in the *tulp1"/-"* retina. Scale bar, 5 μm.

To ascertain whether normal postsynaptic architecture might be noted earlier, we also examined P13 retinas using PKC staining. In the P13 wt retina, dendrite lengths were shorter and branching was sparser than in P16 retinas (Fig. 5E).

**Underdeveloped Bipolar Cell Dendrites in *tulp1"/-"* Mice**

To investigate the consequences of the photoreceptor synaptic malformation on postsynaptic elements, we examined *tulp1"/-"* retinas using antibodies against PKC, which labels rod DBCs and their respective dendrites.59 At P16, wt DBC dendrites have elongated processes that penetrate the OPL, and each termination has a high degree of arborization (Fig. 5A). In comparison, DBC dendrites of P16 *tulp1"/-"* mice are shorter and display less branching or complexity (Fig. 5B). On the other hand, DBC terminals of the P16 rd10 retina resemble those of wt (Fig. 5C). DBC dendritic retraction was, however, noted in the P21 rd10 retina, which resembled the P16 *tulp1"/-"* phenotype (Fig. 5D).

To ascertain whether normal postsynaptic architecture might be noted earlier, we also examined P15 retinas using PKC staining. In the P15 wt retina, dendrite lengths were shorter and branching was sparser than in P16 retinas (Fig. 5E).
played structural defects (Fig. 7B). In the son, panel) appeared to interact closely (right panel). In comparison, dendrites (left panel) and Bassoon-positive ribbons (middle panel) appeared to be apposed and confined to the OPL, providing at least a minimal platform for relaying signaling from photoreceptors to the inner retina is underdeveloped or malformed in tulp1−/− mice. Scale bars, 10 μm.

These data indicate that the structural platform for relaying signaling from photoreceptors to the inner retina is underdeveloped or malformed in tulp1−/− mice. To define the relationship between the presynaptic and postsynaptic photoreceptor elements, P16 retinas were labeled for PKC and Bassoon. In wt mice (Fig. 7A), PKC-positive DBC dendrites and presynaptic ribbons were, nevertheless, closely apposed and confined to the OPL, providing at least a minimal platform for photoreceptor-to-bipolar cell transmission.

Reduced and Less Sensitive ERG b-Wave in tulp1−/− Mice

We used dark-adapted ERG recordings to evaluate outer retinal function in the P16 mice studied here. From these recordings, we extracted parameters related to photoreceptor function (equation 2) and DBC activity (equation 1). Figure 8 presents representative responses obtained from wt, tulp1+/−, tulp1−/−, and rd10 mice to strobe flash stimuli presented after overnight dark adaptation (Fig. 8A) and summary intensity-response functions for the ERG a-wave (Fig. 8B) and b-wave (Fig. 8C). At P16, wt responses have distinct a- and b-waves, as expected from earlier reports, and indicate the presence of functional invaginating synapses between rod photoreceptors and rod DBCs.41 Responses of tulp1−/− mice were comparable to those of wt animals, as previously reported.18 In comparison to wt and tulp1−/− data, responses of tulp1−/− and rd10 mice were reduced in amplitude and had delayed b-wave implicit times.

We used equations 1 and 2 to examine the b-wave intensity-response function and the leading edge of the a-wave, respectively. There was no difference in any parameter between wt and tulp1−/− data. In comparison, the amplitude parameters \( R_{\text{max}} \) and \( R_{\text{mP3}} \) were significantly (\( P < 0.002 \)) reduced in tulp1−/− and rd10 mice. However, effects on the sensitivity parameters \( K \) and \( A \) were strain specific. Compared with wt or

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**Figure 5.** Bipolar cell dendrites are abnormal in the tulp1−/− retina. PKC immunofluorescent staining of rod bipolar cell bodies in the INL with processes extending into the OPL. Insets: high magnification, illustrating representative lengths and branching of the bipolar dendrites. In wt at P16 (A), the dendritic field is robust, with long processes penetrating the OPL and terminating with a high degree of arborization. This is in contrast to tulp1−/− at P16 (B), where the dendrites appear as short appendages and branching has been severely attenuated. rd10 retinas at P16 (C) and P21 (D) show some signs of disorganization of the bipolar layers; however, it is only at P21 that retraction of the dendrites results in lengths comparable to those of tulp1−/− at P16. In wt OPL at the early time point of P13 (E), dendrite lengths are shorter and branching is abbreviated compared with the wt at P16. However, in the tulp1−/− OPL at P13 (F), dendrites are difficult to differentiate from the bipolar cell bodies and branching is not observed, possibly indicating that the dendrites are poorly developed and never mature properly. Scale bars, 10 μm.

**Figure 6.** Bipolar cell dendritic field is attenuated in the tulp1−/− retina. (A) A montage of photomicrographs of the inferior mouse retina, immunostained for PKC. White boxes indicate the regions analyzed to quantify the dendritic field in P16 mice. Representative images of wt (B, D) and tulp1−/− (C, E) retinas analyzed from the central and peripheral regions, respectively. Yellow lines indicate the measurements of individual dendrites. The average dendrite length was statistically significantly shorter in both the central (F; \( P = 0.006) \) and the peripheral (G; \( P = 0.005 \)) regions in tulp1−/− retinas compared with the wt. Data bars are the average ± SEM for four mice. Scale bars, 10 μm.
indicating a reduction in phototransduction gain.

generate a b-wave. In comparison, the main departure from wt
K
along the
values of
rd10 response is substantially slower. Given this, it is not
synapses at the photoreceptor terminal. The association of

evidence that Tulp1 is required for the formation of normal

tulp1
A
and
B
Bassoon

PKC
A
wt:P16

B
tulp1−/−:P16

Merge

FIGURE 7. Relationship between pre-synaptic and postsynaptic elements show deficits in the tulp1−/− retina. Deconvolution-generated images of immunofluorescence of PKC (red) and Bassoon (green) in the OPL at P16 showing the relationship of the pre-synaptic and postsynaptic elements. In the wt OPL (A), long and branching dendrites stretch toward the photoreceptor terminals that are decorated with Bassoon-immunoreactive horse-shoe-shaped ribbons. In the tulp1−/− OPL (B), dendrites show shortened appendages with reduced branching, and Bassoon-immunoreactive ribbons appear punctate. However, the pre-synaptic and postsynaptic elements are still closely apposed. Scale bars, 10 μm.

Malformation of tulp1−/− Photoreceptor Synapses
In young tulp1−/− mice, two key ribbon-associated proteins, Piccolo and Bassoon, are rarely united into the horseshoe shape characteristic of the wt photoreceptor ribbon synaptic complex. The proteins are, however, situated close to one another and are confined to the OPL. These observations indicate that in the absence of Tulp1, Piccolo and Bassoon are able to arrive at their correct destinations but are unable to coordinate into the normal synaptic architecture. In addition, immunostaining for Ribeye showed that few intact ribbons were present in the tulp1−/− retina.

Before or during the height of photoreceptor degeneration, normal synaptic architecture is readily seen in the rd10 retina. This does not necessarily indicate that the observed synaptic defects are unique to the tulp1−/− retina, but it does make clear that generalized photoreceptor degeneration is not sufficient to induce synaptic alterations. Moreover, it appears that these abnormalities in ribbon-associated proteins are specific to mutations involving proteins that are critical for photoreceptor synapse formation. In general, these mice exhibit abnormalities in ribbon-associated proteins and dramatically diminished b-waves concomitant with altered DBC composition. An example of a mutation primarily affecting the synapse comes from a line of mice lacking Bassoon. In Bassoon-deficient mice (Bsn−/−), Ribeye and Piccolo staining is also punctate, but contrary to what occurs in the tulp1−/− retina, they still colocalize (GHG, unpublished data, 2008). The loss of Bassoon results in free-floating ribbons, splitting the presynaptic apparatus into the “ribbon-associated complex,” including Ribeye and Piccolo, and the “plasma membrane/arciform density-associated complex.” In tulp1−/− mice, the proteins affiliated with the ribbon-associated complex are in disarray; in Bsn−/− mice, the ribbon-associated complex is thought to be intact but not anchored to the presynaptic membrane. In both

tulp1+/− data, values of K were significantly elevated in
tulp1−/− mice but not in rd10 animals. This analysis confirms
the impression from Figure 8C that the rd10 b-wave intensity-
response function is shifted only downward from the wt or
tulp1+/− functions, whereas the tulp1−/− function appears to
be shifted down and to the right along the stimulus-intensity
axis.

Figure 8D plots the leading edge of the a-waves obtained to
a high-intensity stimulus for each genotype studied. Each trace
represents the average of all mice tested, after which the
a-wave trough was normalized to −1.0.42 In this representa-
tion, it is clear that there is substantial overlap between the
responses of wt, tulp1+/+, and tulp1−/− mice, whereas the
rd10 response is substantially slower. Given this, it is not
surprising that when equation 2 was fit to individual data, the
values of A obtained for tulp1−/− mice were not different from
those of wt or tulp1+/+ mice but were significantly (P <
0.001) decreased in rd10 mice.

By obtaining parameters that correspond to OS phototrans-
duction gain (A) and DBC sensitivity (K), we can begin to
evaluate signal transmission through the retina. Figure 8E plots
values of A and K for wt mice and the three mutant genotypes
studied here (tulp1+/+, tulp1+/−, and rd10), all compared
with the wt average. In this format, reduced phototransduction
gain, corresponding to lower values of A, fall below the wt
point, whereas reduced DBC sensitivity, corresponding to
higher values of K, fall to the right of the wt point. The
tulp1+/− data fall near wt in both dimensions, consistent with
normal photoreceptor and DBC function in heterozygotes. For
tulp1−/− mice, the main departure from wt is a rightward shift
along the K axis, indicating that more light is required to
generate a b-wave. In comparison, the main departure from wt
for rd10 mice is a downward shift to lower values of A,
indicating a reduction in phototransduction gain.

DISCUSSION
It is well established that mutations in TULP1 underlie a severe
form of photoreceptor degeneration.5–10 Herein, we present
evidence that Tulp1 is required for the formation of normal
synapses at the photoreceptor terminal. The association of
early-onset and severe photoreceptor degeneration with rho-
dopsin mislocalization, which is preceded by synaptic abnor-
malities, appears to represent a phenotype that has not been
described previously.

Malformation of tulp1−/− Photoreceptor Synapses
In young tulp1−/− mice, two key ribbon-associated proteins,
Piccolo and Bassoon, are rarely united into the horseshoe
shape characteristic of the wt photoreceptor ribbon synaptic
complex. The proteins are, however, situated close to one
another and are confined to the OPL. These observations
indicate that in the absence of Tulp1, Piccolo and Bassoon are able
to arrive at their correct destinations but are unable to coordi-
nate into the normal synaptic architecture. In addition, immu-
nostaining for Ribeye showed that few intact ribbons were
present in the tulp1−/− retina.

Before or during the height of photoreceptor degeneration,
normal synaptic architecture is readily seen in the rd10 retina.
This does not necessarily indicate that the observed synaptic
defects are unique to the tulp1−/− retina, but it does make
clear that generalized photoreceptor degeneration is not suf-
ficient to induce synaptic alterations. Moreover, it appears that
these abnormalities in ribbon-associated proteins are specific to
mutations involving proteins that are critical for photore-
ceptor synapse formation. In general, these mice exhibit ab-
normalities in ribbon-associated proteins and dramatically
diminished b-waves concomitant with altered DBC composition.
An example of a mutation primarily affecting the synapse
comes from a line of mice lacking Bassoon. In Bassoon-defi-
cient mice (Bsn−/−), Ribeye and Piccolo staining is also punctate,
but contrary to what occurs in the tulp1−/− retina, they still
colocalize (GHG, unpublished data, 2008).29 The loss of Bassoon
results in free-floating ribbons, splitting the presynaptic
apparatus into the “ribbon-associated complex,” including
Ribeye and Piccolo, and the “plasma membrane/arciform density-
associated complex.”29 In tulp1−/− mice, the proteins affil-
iated with the ribbon-associated complex are in disarray; in
Bsn−/− mice, the ribbon-associated complex is thought to be
intact but not anchored to the presynaptic membrane. In both
cases, we hypothesize that the capacity to signal second-order neurons would be greatly diminished. In addition to exhibiting attenuated dendrites similar to those in \textit{tulp1}^+/− mice, \textit{Bsn}^+/− mice develop ectopic photoreceptor terminals and sprouting dendritic projections in the ONL, a feature that makes them closely resemble two other presynaptic mouse mutants, \textit{Cabp4}^+/− and \textit{Cacna1fnob2}^+/−. Although it is not fully known why ectopic photoreceptor synapses form in these mutants, they all share an alteration in calcium channel function.\textsuperscript{27,28} To date, there is no link between Tulp1 and calcium movement. Moreover, the \textit{tulp1}^−/− phenotype does not involve ectopic sprouting, suggesting that the absence of Tulp1 may alter synaptic structure by a distinct mechanism. It is important to note that appreciable photoreceptor degeneration is not observed in \textit{Cabp4}^+/−, \textit{Bsn}^+/−, or \textit{Cacna1fnob2}^+/− mice.\textsuperscript{27–29} Taken together, these data indicate that the synaptic abnormalities and severe photoreceptor degeneration noted in \textit{tulp1}^−/− mice reflect distinct defects and indicate that Tulp1 plays multiple roles in photoreceptor cells.

**Mislocalized Rhodopsin Not Sufficient to Disrupt Synaptic Architecture in \textit{tulp1}^−/− Mice**

Rhodopsin mislocalization has been observed in numerous animal models of retinal degeneration. Regardless of the underlying cause, it is believed that the buildup of mislocalized rhodopsin throughout the photoreceptor induces pathologic changes that ultimately lead to cell death.\textsuperscript{38} It has been proposed that the primary pathologic change in photoreceptors is the alteration of structural and functional aspects of membranes because of the constant delivery and lack of removal of ectopic rhodopsin.\textsuperscript{38} It has been further postulated that the synapse would be particularly susceptible to "membrane crowding" by rhodopsin molecules, which would interfere with the highly regulated and dynamic processes of excytosis.
and endocytosis. This hypothesis raises the possibility that mislocalized rhodopsin in the tulp1−/− mice might disrupt the normal ribbon architecture, which is closely linked to the presynaptic membrane. In the rd10 retina, we noted that rhodopsin mislocalization occurs after the onset of degeneration, which is in agreement with the findings of a recent study. However, before and during retinal degeneration, the normal coupling between Piccolo and Bassoon is retained and the ribbons are intact. This finding indicates that mislocalized rhodopsin molecules are not sufficient to disrupt normal photoreceptor synaptic ribbon architecture and implicates the absence of Tulp1 in the synapse as the primary cause of ribbon malformation in tulp1−/− mice.

Underdeveloped Bipolar Cell Dendrites in tulp1−/− Mice

In many mutant mouse models, reduced inner retinal processes have been shown to occur secondarily to photoreceptor degeneration or anomalous development. Here we show that mice lacking Tulp1 have a reduced dendritic composition that precedes photoreceptor degeneration. Because photoreceptor presynaptic structural defects occur in the tulp1−/− retina, we hypothesize that there should be a reduction in the signal output potential. The attenuation of photoreceptor signaling has been previously linked to subsequent alterations in the adjacent neural layers of the retina. In tulp1−/− mice, there is a profound shortening of individual dendrites and a severe attenuation of dendrite branching in the central and the peripheral retina. The atrophy of postsynaptic dendritic processes has been observed in many models of retinal degeneration and has been termed retraction to denote the negative remodeling from an earlier developed state as a direct and downstream effect of photoreceptor cell death, regardless of the underlying genetic cause of retinal degeneration. This retraction response is thought to be initiated by a lack of signaling or trophic factors, no longer able to be delivered because of absent photoreceptor terminals.

However, in the tulp1−/− retina, shortened dendrite lengths and reduced branching are detected before photoreceptor cell death. Therefore, we conclude that the reduction of the DBC dendritic field in the tulp1−/− retina is a developmental consequence of an attenuated trophic and transmitter release that would be expected from a presynaptic malformation. This is in contrast to the rd10 retina, in which reduction in length and branching of bipolar dendrites are observed only after the commencement of photoreceptor cell death (P21). It is important to note that tulp1−/− DBC dendrites lengthen between P13 to P16. However, this developmental process never achieves a normal configuration. Given the abnormalities in rd10 mice at P21, we predict a further reduction from the modest DBC dendrites noted in tulp1−/− mice at later ages concomitant with maximal photoreceptor degeneration.

Two Distinct Functional Abnormalities in tulp1−/− Mice

ERG study results of young tulp1−/− mice indicate the presence of two functional defects. One defect occurs at the level of the photoreceptor OS, which generates the dark-adapted a-wave. Although photoreceptor loss is not evident at P16, the tulp1−/− a-wave is already reduced in amplitude. Despite the overall reduction of the tulp1−/− ERG a-wave, the leading edge of the a-wave had normal gain. This is readily seen when responses are normalized for overall amplitude. A similar conclusion applies to ERG a-waves obtained from P15 tulp1−/− mice. Although a comparable a-wave reduction is noted in P16 rd10 mice, this reduction appears to reflect the initial stages of rod degeneration and a lower phototransduction gain.

This latter result was confirmed by applying the same analysis to ERG a-waves obtained in another study from rd10 mice at P18 and P30. A second defect noted in tulp1−/− mice occurs at the level of b-wave generation, which, under these stimulus conditions, reflects the activity of rod DBCs subsequent to rod photoreceptor input. Reduced b-wave amplitude was expected in the presence of an a-wave reduction, and this result was noted in tulp1−/− and rd10 mice. However, our analysis showed that the b-wave intensity-response function was also shifted to the right along the intensity axis for P16 tulp1−/− mice but not for age-matched rd10 animals. Although fewer intensity settings were used, earlier ERG results obtained from P15 tulp1−/− mice show changes similar to those noted here for P16 tulp1−/− animals. We speculate that the overall amplitude reduction is associated with a decreased photoreceptor response and that the rightward shift, seen only in tulp1−/− mice, reflects the abnormalities in ribbon synapse architecture and DBC dendrite structure documented anatomically in these animals.

In conclusion, the absence of Tulp1 results in abnormalities that affect structure and function in multiple retinal sites. The photoreceptor degeneration and OS defects of tulp1−/− mice have been described, providing evidence that Tulp1 may function in the polarized transport of proteins at the apical end of the photoreceptor. We have shown that Tulp1 interacts with Actin and Dynamin-1, two proteins known to be critical in the cytoskeletal scaffold and involved in the molecular pathway of vesicular protein transport occurring from the inner segment to the outer segment and in vesicle cycling at terminals of photoreceptors. Interestingly, in tulp1−/− mice, Dynamin-1 staining in the synapse is greatly attenuated (GHG, unpublished data, 2008), raising the possibility that Tulp1 and Dynamin-1 are in the same pathway. Thus, Tulp1 may function in intracellular protein trafficking throughout the photoreceptor cell, and, in its absence, two distinct abnormalities at polar ends of the cell are highlighted. Here we provide evidence that photoreceptor ribbon synapses and DBC dendrites are also severely affected at an early age. These new findings indicate that Tulp1 is essential for photoreceptor cell survival and is required for the proper development of the photoreceptor synapse. It will be interesting to learn whether similar synaptic defects are present in human patients carrying TULP1 mutations.

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