Published in final edited form as:

Bios. 2018 June ; 89(2): 58–64. doi:10.1893/0005-3155-89.2.58.

Localization of CHMP2B in postnatal *rd1* mouse retina

Fadi Assaf, Ameair Abu Irqeba, and Judith Mosinger Ogilvie

Department of Biology, Saint Louis University, St. Louis, Missouri 63103

Abstract

Retinitis pigmentosa is the most common form of inherited blindness in humans. A well-studied model of the disease is the *rd1* mouse, characterized by a loss of function mutation in the catalytic β subunit of the phosphodiesterase 6 (Pde6) holoenzyme involved in phototransduction within rods and cones. The period of photoreceptor degeneration in the *rd1* mouse occurs during postnatal days 10–21. In previous work, only Pde6 β and vesicular-trafficking protein Prenylated Rab Acceptor 1 (PRA1) have been found to be consistently downregulated during the first ten days following birth. In a yeast-two-hybrid assay conducted by our lab, PRA1 was shown to interact with Charged Multivesicular Body Protein 2B (CHMP2B), an endosomal sorting protein that has been implicated in several neurodegenerative diseases, such as frontotemporal dementia and amyotrophic lateral sclerosis. We investigated whether CHMP2B is mislocalized in the *rd1* mouse. Immunohistochemical labeling of CHMP2B was done in both postnatal wild type and *rd1* mouse retinas. Prior to the onset of degeneration, CHMP2B immunolabeling was weaker in *rd1* retinas, particularly in the developing photoreceptor synaptic layer, compared to wild type. Furthermore, staining of CHMP2B in wild type photoreceptors peaked at postnatal day 12, while CHMP2B staining in *rd1* retinas was diffuse and disorganized. In conclusion, these findings show that proper localization of CHMP2B is disrupted in *rd1* photoreceptors. Further studies are needed to investigate possible roles for CHMP2B in endocytic activity that is vital to photoreceptor maintenance, as well as differentiation, and development in mouse photoreceptors.

Keywords

photoreceptor; retinitis pigmentosa; retinal degeneration; development; *Mus musculus*; vesicular trafficking; immunohistochemistry

Introduction

Retinitis pigmentosa (RP) is the world's leading cause of inherited blindness (Hartong et al., 2006). The name refers to a group of inherited eye diseases that lead to the degeneration of rod photoreceptors. Over 100 different gene mutations have been linked to RP, resulting in variation in age of onset and severity of the disease (Daiger et al., 2007; Daiger et al., 2008). The *rd1* mouse is a well-studied animal model of an early-onset form of the disease, characterized by rapid degeneration of rod photoreceptors, with cell death beginning at postnatal day 10 (P10), during photoreceptor morphogenesis (LaVail and Sidman, 1974).

The *rd1* mutation occurs on the β subunit of the phosphodiesterase complex (Pde6 β), a downstream regulator of phototransduction. Rods of *rd1* mice are completely degenerated by P21, when retinal development is complete in the wild type (wt) retina. However, the specific pathway through which degeneration is initiated is not well understood.

Vesicular trafficking, a process responsible for transporting proteins into, out of, and within the cell, breaks down at the Golgi level in photoreceptors of *rd1* mice (Dickison, et al., 2012; Farber, 1994). The potential role of vesicular trafficking defects in photoreceptor degeneration in the *rd1* retina remains unexplored. In 2012, our lab published a study which showed that in the postnatal days (P2–P8) leading up to degeneration, Pde6 β and Prenylated Rab Acceptor 1 (PRA1) were the only two proteins confirmed to be differentially expressed at all time points examined. Both genes were downregulated, which was expected for Pde6 β with a loss of function mutation in the gene. Because PRA1 plays a role in vesicular trafficking, its downregulation may be involved in the mechanisms that lead to photoreceptor death in the *rd1* retina. Our lab also conducted a split-ubiquitin yeast two-hybrid assay to identify proteins that interact with PRA1. This screen showed that PRA1 binds to a protein called Charged Multivesicular Body Protein 2B (CHMP2B) (Abu Irqeba and Ogilvie, 2013).

CHMP2B is involved in the endocytic pathway, a specific type of vesicular trafficking (Urwin et al., 2010). CHMP2B participates in the ESCRT-III complex, an element of the endosomal transport system involved in recycling and degradation of cell surface receptors, as well as packing ubiquitinated proteins into intraluminal vesicles (Filimonenko, et al., 2007). The endocytic pathway is responsible for transporting substances within the cell for degradation in the lysosome via multivesicular bodies (Luzio et al., 2010). The specific role of CHMP2B in trafficking within the retina is still unknown. However, mutations in CHMP2B and disruptions in trafficking have been implicated in several other neurodegenerative diseases. Specifically, two mutations in CHMP2B, both predicted to result in C-terminal truncation of the protein, have been identified in families with frontotemporal dementia (Skibinski et al., 2005; van der Zee et al., 2008). Point mutations in CHMP2B have also been associated with amyotrophic lateral sclerosis (Parkinson et al., 2006). *In vitro* studies have demonstrated that depletion of ESCRT subunits or overexpression of CHMP2B mutant protein can inhibit autophagic degradation and result in protein aggregates that are associated with neurodegenerative disease (Filimonenko, et al., 2007; Urwin et al., 2010).

Because PRA1 and CHMP2B have been shown to interact, we hypothesized that downregulation of PRA1 in the *rd1* retina may contribute to dysfunctional CHMP2B, which past studies have associated with neurodegeneration (Filimonenko, et al., 2007; Lee, et al., 2007; Urwin, et al., 2010). In this study, our aim is to investigate whether the localization of CHMP2B is disrupted in the *rd1* retina, which would be consistent with our hypothesis.

Material and methods

Animals

The animals used in this study were wt and *rd1* mice, both on a C57Bl/6 background, obtained from Jackson Laboratories, and bred in-house. Mice were housed in a vivarium

under standard conditions with a 12:12 light:dark cycle; food and water were provided *ad libitum*. All experiments on mice followed the National Institutes of Health Guidelines on Laboratory Animal Welfare and protocols approved by the Saint Louis University Institutional Animal Care and Use Committee. All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cryostat tissue preparation

To collect tissue samples for immunohistochemistry, four mice were euthanized from each of two mouse strains (wt and *rd1*) at each of seven postnatal days (P6, P8, P10, P12, P15, and P21). Animals less than 14 days of age were euthanized by decapitation. Older animals were euthanized by a single intraperitoneal injection of 100 mg/kg pentobarbital. Both eyecups were dissected in cold phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer. Eyecups were rinsed in 0.1 M phosphate buffer three times on ice, followed by incubation in 30% sucrose at 4°C overnight for cryoprotection. Eyecups were then incubated in a 1:1 mixture of 30% sucrose and Optimal Cutting Temperature compound (OCT, EMS, Hatfield, PA) for 1 hour at room temperature, followed by incubation in 100% OCT compound for another hour. Eyecups were then flash frozen in OCT compound. Twelve micron tissue sections were cut on a Leica CM 1850 cryostat and three sections were placed on each gelatin-subbed slide. Slides were stored for approximately two weeks in a -20°C freezer with desiccant until primary and secondary antibody staining.

Immunohistochemistry

In order to determine protein localization, slides from at least two different retinas from each mouse strain and each age were incubated in 4% paraformaldehyde for 1 minute. Slides then were placed into Coplin jars containing PBS and rinsed three times for 15 minutes each. Subsequently, sections were blocked with 2% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.3% Triton X-100 in PBS for 20 minutes, followed by incubation with rabbit anti-CHMP2B polyclonal antibody (1:200, Abcam, Cambridge, MA) in PBS with 0.3% Triton X-100 and 2% NDS at 4°C overnight.

Slides were subsequently washed in PBS for three 10-minute periods, followed by blocking for 20 minutes and staining with goat anti-rabbit Alexa Fluor 555 secondary polyclonal antibody (ThermoFisher Scientific) diluted 1:50 in blocking solution for 1 hour in the dark. Next, slides were washed for three 20 minutes periods, coverslipped with Vectashield media (Vector Laboratories, Burlingame, CA), and sealed with nail polish. For immunohistochemistry controls, the same procedures were followed except that PBS with 0.3% Triton X-100 and 2% NDS was substituted for the primary antibody. Slides were imaged using a Zeiss Axioskop 2 mot plus fluorescent microscope with the AxioCam MRm camera and Axiovision software. Imaging was performed on at least three sections from each age that included regions near the optic nerve.

Results and discussion

The mouse retina is immature at birth. By P6, it is comprised of three nuclear layers separated by two plexiform layers. Photoreceptors are located in the outer nuclear layer (ONL). At this age, they have begun to differentiate an apical inner segment (IS) region, with axons extending basally to the outer plexiform layer (OPL) where they form synapses on bipolar cell dendrites. Bipolar cells are located in the inner nuclear layer (INL) and send processes to the inner plexiform layer (IPL) where they synapse onto ganglion cells, the retinal output neurons that send axons to the brain. Interneurons located in the INL also send processes through the OPL and IPL. At all ages examined, CHMP2B labeling is apparent in the IPL, with no detectable difference between wt and *rd1* retinas. Since the mutant gene in *rd1* retinas is only expressed in photoreceptor cells, no difference is expected in the inner retina.

At P6, CHMP2B immunofluorescence is seen in the OPL of both wt and *rd1* retinas, however, the staining is much weaker in the *rd1* OPL (Fig. 1). This may signify a developmental event that is absent or delayed in the *rd1* mutation. For instance, there may be a decrease in turnover of proteins at the synapse. If membrane or secreted molecules are not being taken up by the cell in an appropriate manner through the ESCRT-III pathway, this may be leading to an accumulation of inappropriate proteins within the cytosol or cell membrane. The removal of proteins with aberrant structures has been found to be essential to the maintenance of homeostasis in non-dividing neural cells. Specifically, mice lacking genes required for autophagy in the central nervous system exhibit behavioral defects and die within 28 weeks of birth (Komatsu et al., 2006). Thus, it would be interesting to determine whether inappropriate autophagy may be a contributing factor to the deterioration of the photoreceptors in *rd1* mice, as is characteristic of some other neurodegenerative diseases (Filimonenko, 2007).

From P6 to P10, the pattern of CHMP2B in the ONL becomes more punctate in the wt retina, as would be predicted for a protein localized within the ESCRT-III pathway. However, in the *rd1* retina, the ONL label appears diffuse and disorganized compared to wt retinas during this developmental period. Despite an observed increase of CHMP2B staining in the *rd1* ONL between P6 and P10, the relative expression appears weaker than in wt mice at the same ages. In the wt P10 retina, a distinct accumulation of CHMP2B punctate immunoreactivity is observed in the position of photoreceptor synapses in the OPL. This staining pattern is reduced in the *rd1* retina at P10, which may indicate disorganization at the synapse of *rd1* photoreceptors. The diffuse label in *rd1* photoreceptors may point to problems with the trafficking of the protein to its appropriate location within the cell, which, in turn, could reinforce the degenerative cascade of events. Mislocalization of CHMP2B is consistent with past studies that have associated improper trafficking with *rd1* retinas (Dickison et al., 2012).

By P12, wt photoreceptors begin to grow outer segments (OSs), specialized sensory cilia that extend from the apical IS. Correlating with this time point, a strong spike in CHMP2B labeling is seen in wt photoreceptors, specifically in the IS, consistent with a possible role for CHMP2B in this process (Fig. 2). A heightened intensity of CHMP2B staining is seen in

wt P12 retinas compared to earlier and later time points and also in comparison to *rd1* P12 retinas where the localization is diffuse with a few large aggregates rather than punctate. Beyond P12, the CHMP2B expression in the *rd1* photoreceptors appears to become more disorganized, as would be expected during their degeneration. As degeneration continues in P15 and P21 retinas, the localization pattern within photoreceptors continues to appear disorganized.

The preliminary results presented here were limited by the inaccessibility of a confocal microscope. A thorough analysis of CHMP2B localization will require confocal imaging to confirm the observations reported here. Future studies may also investigate the expression of CHMP2B at embryonic stages of development. Co-localization studies between CHMP2B and proteins known to be downregulated in the developing *rd1* retina, such as PRA1 and Pde6 β (Dickison et al., 2012), would elucidate whether the proteins interact in the photoreceptors and if such an interaction is compromised in the *rd1* mouse. To determine whether autophagy-related failures contribute to degeneration, the levels of ubiquitination on these proteins may also be measured. Experiments that investigate these targets using large data sets such as RNA sequencing at the mRNA level or mass spectroscopy at the protein level may be more revealing of the molecular pathway that triggers degeneration, as demonstrated by research on Parkinson's disease (Gehrke et al., 2010). This study presents preliminary data consistent with our hypothesis that downregulation of PRA1 in the *rd1* retina may contribute to dysfunctional CHMP2B, providing justification and direction for future studies.

Acknowledgments

This study was supported by grants from the National Institute of Childhood Health and Human Disease (R15HD064269, JMO); Fight for Sight (FA); Beaumont Faculty Development Fund (JMO), Presidents Research Fund (JMO), Knoedler Award (FA), Saint Louis University; and NSF MRI Award 0421383.

References

- Abu Irqeba A and Ogilvie JM (2013). COPI Coated Vesicular Trafficking in Photoreceptors is Differentially Regulated in the *rd1* Retina. *Invest. Ophthalmol. Vis. Sci* 54, 3738. [PubMed: 23557734]
- Daiger SP, Bowne SJ, and Sullivan LS (2007). Perspective on genes and mutations causing retinitis pigmentosa. *Arch. Ophthalmol* 125, 151–158. [PubMed: 17296890]
- Daiger SP, Sullivan LS, Gire AI, Birch DG, Heckenlively JR, and Bowne SJ (2008). Mutations in known genes account for 58% of autosomal dominant retinitis pigmentosa (adRP). *Adv. Exp. Med. Biol* 613, 203–209. [PubMed: 18188946]
- Dickison VM, Richmond AM, Abu Irqeba A, Martak JG, Hoge SC, Brooks MJ, Othman MI, Khanna R, Mears AJ, Chowdhury AY, Swaroop A, and Ogilvie JM (2012). A role for prenylated rab acceptor 1 in vertebrate photoreceptor development. *BMC Neuroscience* 13, 152. [PubMed: 23241222]
- Farber DB, Flannery JG, and Bowes-Rickman C (1994). The *rd* mouse story: seventy years of research on an animal model of inherited retinal degeneration. *Prog. Ret. Eye Res* 13, 31–64.
- Filimonenko M, Stuffers S, Raiborg C, Yamamoto A, Malerød L, Fisher EM, Isaacs A, Brech A, Stenmark H, and Simonsen A (2007). Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J. Cell Biol* 179, 485–500. [PubMed: 17984323]
- Gehrke S, Imai Y, Sokol N, and Lu B (2010). Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature* 466, 637–41. [PubMed: 20671708]

- Hartong DT, Berson EL, and Dryja TP (2006). Retinitis pigmentosa. *The Lancet* 368, 1795–1809.
- Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, and Tanaka K (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441, 880–4. [PubMed: 16625205]
- LaVail MM, and Sidman RL (1974). C57BL/6J Mice with inherited retinal degeneration. *Archives of Ophthalmology* 91, 394–400. [PubMed: 4595403]
- Lee JA, Beigneux A, Ahmad ST, Young SG, and Gao FB (2007). ESCRT-III dysfunction causes autophagosome accumulation and neurodegeneration. *Curr. Biol* 17, 1561–7. [PubMed: 17683935]
- Luzio JP, Gray SR, and Bright NA (2010). Endosome-lysosome fusion. *Biochem. Soc. Trans* 38, 1413–6. [PubMed: 21118098]
- Parkinson N, Ince PG, Smith MO, Highley R, Skibinski G, Andersen PM, Morrison KE, Pall HS, Hardiman O, Collinge J, Shaw PJ, Fisher EM; MRC Proteomics in ALS Study; FReJA Consortium (2006). ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). *Neurology* 67, 1074–1077. [PubMed: 16807408]
- Skibinski G, Parkinson NJ, Brown JM, Chakrabarti L, Lloyd SL, Hummerich H, Nielsen JE, Hodges JR, Spillantini MG, Thusgaard T, Brandner S, Brun A, Rossor MN, Gade A, Johannsen P, Sorensen SA, Gydesen S, Fisher EM, Collinge J (2005). Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nature Genetics* 37, 806–8. [PubMed: 16041373]
- Urwin H, Authier A, Nielsen JE, Powell C, Froud K, Malcolm DS, Holm I, Johannsen P, Brown J, Fisher EM, van der Zee J, Bruyland M, Freja C, Van Broeckhoven C, Collinge J, Bradner S, Futter C and Issacs AM (2010). Disruption of endocytic trafficking in frontotemporal dementia with CHMP2B mutations. *Human Molecular Genetics* 19, 2228–38. [PubMed: 20223751]
- van der Zee J, Urwin H, Engelborghs S, Bruyland M, Vandenberghe R, Dermaut B, De PT, Peeters K, Santens P, De Deyn PP, Fisher EM, Collinge J, Isaacs AM, and Van Broeckhoven C (2008). CHMP2B C-truncating mutations in frontotemporal lobar degeneration are associated with an aberrant endosomal phenotype in vitro. *Human Molecular Genetics* 17, 313–22. [PubMed: 17956895]

Figure 1.

CHMP2B immunofluorescence is decreased in *rd1* retina compared to wt prior to onset of degeneration. During this developmental period, CHMP2B staining in the ONL of wt retinas appears punctate (arrows, top row), while CHMP2B staining in *rd1* retinas is diffusely distributed throughout processes in the ONL (arrows, bottom row). Four mice from each age and genotype were harvested and at least three sections from each were examined. Representative images are shown. P = postnatal, wt = wild type, IS = inner segment, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Bar in lower right = 10 microns.

Figure 2.

CHMP2B immunofluorescence is disorganized in *rd1* retina after onset of degeneration. An increase in CHMP2B staining with many distinct punctae is seen in inner segments of wt retinas at P12 (arrows, upper row), correlating with genesis of outer segments, whereas diffuse staining with a few large aggregates (arrows, bottom row) are seen in *rd1* inner segments at P12. Four mice from each age and genotype were harvested and at least three sections from each were examined. Representative images are shown. P = postnatal, wt = wild type, OS = outer segment, IS = inner segment, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Bar in lower right = 10 microns.