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X-linked juvenile retinoschisis: Clinical diagnosis, genetic analysis, and molecular mechanisms

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ABSTRACT

X-linked juvenile retinoschisis (XLRS, MIM 312700) is a common early onset macular degeneration in males characterized by mild to severe loss in visual acuity, splitting of retinal layers, and a reduction in the b-wave of the electroretinogram (ERG). The *RS1* gene (MIM 300839) associated with the disease encodes retinoschisin, a 224 amino acid protein containing a discoidin domain as the major structural unit, an N-terminal cleavable signal sequence, and regions responsible for subunit oligomerization. Retinoschisin is secreted from retinal cells as a disulphide-linked homo-octameric complex which binds to the surface of photoreceptors and bipolar cells to help maintain the integrity of the retina. Over 190 disease-causing mutations in the *RS1* gene are known with most mutations occurring as non-synonymous changes in the discoidin domain. Cell expression studies have shown that disease-associated missense mutations in the discoidin domain cause severe protein misfolding and retention in the endoplasmic reticulum, mutations in the signal sequence result in aberrant protein synthesis, and mutations in regions flanking the discoidin domain cause defective disulphide-linked subunit assembly, all of which produce a non-functional protein. Knockout mice deficient in retinoschisin have been generated and shown to display most of the characteristic features found in XLRS patients. Recombinant adeno-associated virus (rAAV) mediated delivery of the normal *RS1* gene to the retina of young knockout mice result in long-term retinoschisin expression and rescue of retinal structure and function providing a 'proof of concept' that gene therapy may be an effective treatment for XLRS.

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1. Introduction

X-linked juvenile retinoschisis (XLRS, MIM 312700) is a relatively common early onset retinal degenerative disease that affects males early in life. Characteristic features include mild to severe loss in central vision, radial streaks arising from foveal schisis, splitting of inner retinal layers in the peripheral retina, and a negative electroretinogram (ERG) arising from a marked reduction in b-wave amplitude (George et al., 1995; Tantri et al., 2004). Disease progression and severity is highly variable even within families. During the course of the disease, secondary complications including retinal detachment and vitreous hemorrhage can occur leading to a poor outcome. Female carriers are asymptomatic although detailed clinical examination can reveal minor retinal abnormalities (Kim et al., 2007).

XLRS was first thought to arise as a result of inherited defects in Müller cells. This was based on the b-wave response which was originally thought to directly involve Müller cells and histological examination of retina tissue from deceased patients showing filamentous material merging with Müller cell membrane and splitting of the nerve fiber layer (Condon et al., 1986; Gass, 1999; Miller and Dowling, 1970; Yanoff et al., 1968). Identification of the gene responsible for XLRS in 1997 by Sauer et al. (1997) and the subsequent analysis of gene and protein expression in the retina, however, have directly implicated photoreceptors and bipolar cells, and not Müller cells, in the disease process (Grayson et al., 2000; Molday et al., 2001; Mooy et al., 2002; Reid et al., 1999). The *RS1* gene encodes a 24 kDa discoidin-domain containing protein which is secreted as a homo-oligomeric complex (Sauer et al., 1997; Wu et al., 2005). This complex binds tightly to the surface of photoreceptors and bipolar cells where it helps to maintain the cellular organization of the retina and structure of the photoreceptor-bipolar synapse.

Over the past 15 years significant progress has been made in understanding XLRS at a clinical, genetic, molecular, and cellular level. To date 191 different mutations in the *RS1* gene are known to cause XLRS. Protein expression studies have provided insight into the mechanisms by which specific mutations affect the expression, structure and secretion of retinoschisin and lead to a pathogenic state. Mice deficient in retinoschisin have been developed and used to obtain insight into the role of retinoschisin in retina structure, function and pathology. Finally, the delivery of the normal *RS1* gene to knockout mice deficient in endogenous retinoschisin has resulted in significant restoration of retinal structure and function. In this chapter, we review our current knowledge of retinoschisin and its role in XLRS pathology from a clinical, genetic and molecular perspective.

2. Clinical findings of congenital XLRS

2.1. Clinical manifestations

XLRS was first described in 1898 in two affected brothers by the Austrian ophthalmologist Josef Haas (Haas, 1898). Since then, XLRS has been shown to be one of the more frequent inherited retinal disorders affecting macular function in males with an estimated prevalence ranging between 1:5000 to 1:20,000 (George et al., 1995). The name derives from an internal splitting of the retina mostly affecting the temporal periphery of the fundus. This peripheral retinoschisis occurs in less than 50% of affected individuals, whereas foveal involvement is present in all affected patients. Foveal involvement is usually associated with moderate visual loss. Therefore, XLRS is frequently diagnosed prior to school age suggesting a juvenile onset. Several cases of severe retinoschisis have been described in the first year of age suggesting that XLRS indeed is present at birth (Lee et al., 2009; Prasad et al., 2006; Renner et al., 2008; Sieving, 1998). These severe cases as well as the absence of acute visual loss in the majority of cases indicates that the onset of XLRS is congenital, but the diagnosis is delayed because small infants are not affected in their daily tasks by moderate visual loss.

Multiple studies reporting clinical features of XLRS in a series of families have been reported world-wide (Apushkin et al., 2005b; Atchaneeyasakul et al., 2010; Eksandh et al., 2000; Forsius et al., 1963; George et al., 1995, 1996; Hewitt et al., 2005; Kellner et al., 1990; Lesch et al., 2008; Pimenides et al., 2005; Renner et al., 2008; Riveiro-Alvarez et al., 2009; Shinoda et al., 2000; Shukla et al., 2007; Simonelli et al., 2003; Vainio-Mattila et al., 1969; Xu et al., 2011). The penetrance of XLRS is almost complete but clinical expression is highly variable (Sieving, 1998). In our series of 100 XLRS patients the manifestations ranged from almost complete retinoschisis at the age of 3 months in both eyes to normal visual acuity with mild pigmentary macular abnormalities and a negative full-field electroretinogram (ERG) (Kellner et al., 1990; Renner et al., 2008). The expression of the disease is usually symmetrical in both eyes, however, a marked asymmetry of visual function can be present especially in cases where additional complications occur (Tantri et al., 2004). Visual acuity is reduced to 20/100 in most patients although it may vary greatly. On ophthalmoscopy, foveal retinoschisis presents as a spoke-wheel pattern and peripheral retinoschisis as a sharply delineated detachment of the inner retinal sheet usually limited to the periphery or mid-periphery (Fig. 1). Peripheral retinoschisis may extend from the periphery to the macula including the fovea in some cases; in rare instances marked retinoschisis might involve nearly the complete retina. If the inner

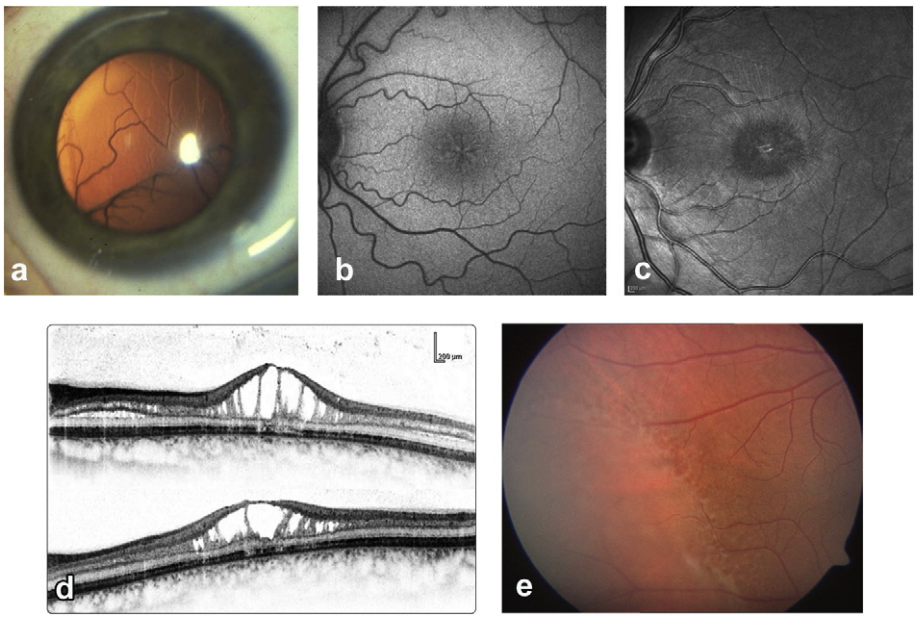

Fig. 1. Retina imaging of individuals with XLRS. (a) Severe retinoschisis involving almost the complete retina observed in a boy 3 months of age carrying a *RS1*-gene p.R213W mutation. (b) Fundus autofluorescence indicating a spoke-wheel pattern due to overlying foveal retinoschisis in a 12 year old boy carrying a *RS1*-gene p.R102W mutation. (c) Near-infrared image of foveal retinoschisis in a 21 year old patient carrying a *RS1*-gene p.D126_L127delinsE mutation. (d) Spectral domain optical coherence tomography in a 22 year old male carrying a *RS1*-gene p.W96G mutation. The upper scan shows marked retinoschisis in different retinal layers. The lower scan after 3 months of local application of dorzolamide shows a smaller foveal retinoschisis cavity and absence of perifoveal retinoschisis cavities. (e) Color image of peripheral retinoschisis in the lower temporal quadrant of the right eye in a 20 year old male carrying a *RS1*-gene p.R209H mutation.

sheet of the schisis degenerates, the retinal vessels may remain running free through the vitreous cavity presenting as so-called vitreous veils. If additional breaks occur in the outer sheet of the schisis, a retinal detachment may occur.

In the majority of patients, the disease either shows no or minimal progression (Apushkin et al., 2005b; Kellner et al., 1990; Kjellstrom et al., 2010; Roesch et al., 1998). Around the age of 30 years, the macular alterations may change from the characteristic spoke-wheel pattern to unspecific mild retinal pigment abnormalities. In some cases, severe visual loss with increased age has been described (George et al., 1996) although incidence data are not available due to the lack of extended long-term follow-up studies.

2.2. Clinical diagnosis

Young boys are often presented for evaluation of bilaterally reduced visual acuity without acute visual loss. Sudden visual loss rarely occurs and is often associated with complications like vitreous hemorrhage or retinal detachment (Lee et al., 2009; Prasad et al., 2006). Hyperopia is a frequent finding.

The hallmark of XLRS is the presence of a spoke-wheel pattern in the macula on high magnification ophthalmoscopy in patients younger than 30 years of age (Fig. 1b and c). The spoke-wheel pattern varies in severity and might be difficult to detect or exclude especially in young boys with limited cooperation even by experienced clinicians (Kellner et al., 1990; Sieving, 1998). In patients older than 30 years the foveal retinoschisis is replaced by unspecific mild retinal abnormalities. Peripheral retinoschisis, mostly in the lower temporal quadrant, is an important diagnostic sign but is present in less than 50% of affected patients (Fig. 1e). Additional phenomena, e.g. the Mizou phenomenon or white flecks, have been observed in few cases of XLRS (Hotta et al., 2001; Vincent et al., 2009).

Optic coherence tomography (OCT) has changed the diagnostic approach for XLRS (Apushkin et al., 2005a; Chan et al., 2004; Muscat et al., 2001; Prenner et al., 2006; Renner et al., 2008; Stanga et al., 2001; Urrets-Zavalia et al., 2007) and nowadays spectral domain OCT (SD-OCT) is the major diagnostic technique for this disease (Fig. 1d). Even in an uncooperative child a single scan of the foveal area is sufficient to detect signs of XLRS and to distinguish XLRS from other differential diagnosis associated with visual loss in young boys (Dhingra and Patel, 2010; Eriksson et al., 2004; Renner et al., 2008). SD-OCT revealed that the area of retinoschisis markedly extends beyond the ophthalmoscopically visible spoke-wheel pattern and includes the macular area up to the vascular arcades (Gerth et al., 2008; Gregori et al., 2009; Yu et al., 2010). This novel imaging tool is also important for follow-up examinations and has resolved the long-standing histologic debate in which retinal layer the retinal splitting occurs (Condon et al., 1986; Manschot, 1972; Yanoff et al., 1968). Indeed, cystoid changes may involve various retinal layers from the retinal nerve fiber layer to the nuclear layer (Gerth et al., 2008; Gregori et al., 2009; Yu et al., 2010). Thinning of the retinal nerve fiber layer has been reported in a series of XLRS patients (Genead et al., 2010). In older patients SD-OCT might show absence of retinoschisis, retinal thinning and epiretinal membranes that increases the difficulty to differentiate from other forms of macular dystrophy (Menke et al., 2011).

Fundus autofluorescence (FAF) is frequently used for the differential diagnosis of retinal dystrophies. XLRS alterations of the foveal FAF are most likely due to the altered light transmission in the area of retinoschisis and are a characteristic sign (Fig. 1b), although the diagnosis should be validated by SD-OCT (Renner et al., 2008). Fluorescein angiography may reveal retinal pigment epithelial alterations in older males; but does not contribute to the differential diagnosis of XLRS.

For several decades the ERG was the major diagnostic technique for XLRS. The characteristic ERG sign is a so-called 'negative' ERG elicited by a bright flash of light in the dark-adapted retina in which the a-wave is larger than the b-wave in contrast to the normal findings. Usually, the light adapted responses show an amplitude

reduction as well. The origin of the retinal dysfunction is an abnormality in the ON- and OFF-pathways on the level of the bipolar cells (Khan et al., 2001). Detailed evaluation of macular dysfunction with the multifocal electroretinogram (mfERG) has demonstrated a widespread cone dysfunction as well (Piao et al., 2003; Sen et al., 2010). A 'negative' ERG can be associated with various retinal disorders (Koh et al., 2001; Renner et al., 2006); however, in young males the only major differential diagnosis is congenital stationary night blindness (CSNB) (Bradshaw et al., 2004). The easier and much faster application of SD-OCT compared to ERG has diminished the diagnostic role of the ERG for XLRS. In addition, earlier reports on ERG in XLRS were limited to patients with clinically manifest XLRS. Recent studies selectively including patients carrying *RS1* mutations have shown that the ERG response is more variable than previously expected (Eksandh et al., 2005; Renner et al., 2008; Sieving et al., 1999). Most patients present with an a-wave relatively larger than the b-wave, resulting in a reduced b/a-ratio, however, a 'negative' ERG with a b-wave smaller than the a-wave is present only in about 50% of the patients and a relative normal ERG does not exclude the diagnosis of XLRS. Nevertheless, an ERG is valuable for differential diagnosis in unexplained visual loss especially in older patients (Koh et al., 2001; Renner et al., 2006; Sobaci et al., 2007).

Other tests of retinal function, e.g. electro-oculography, color vision or visual fields, are of limited diagnostic value. Color vision is variably abnormal due to affection of the macula and absolute scotoma which is present in the area of peripheral retinoschisis.

2.3. Findings in carriers

In contrast to other X-linked inherited retinal dystrophies, e.g. retinitis pigmentosa or choroideremia, female carriers usually have normal retinal function and rarely present with retinal abnormalities. Only 2/9 obligate carriers of XLRS had functional abnormalities in the mfERG (Kim et al., 2007). Female carriers have rarely been reported with retinal abnormalities or visual loss (Gieser and Falls, 1961; Mendoza-Londono et al., 1999; Rodriguez et al., 2005; Saldana et al., 2007; Wu et al., 1985). Retinal alterations and ERG abnormalities were variable. Six affected woman were in fact homozygous carriers of disease mutations (Ali et al., 2003; Forsius et al., 1963; Saleheen et al., 2008).

2.4. Complications

Vitreous hemorrhages or retinal detachment complicate the clinical course of XLRS in approximately 5% of all affected males and most frequently develop in the first decade of life. Vitreous hemorrhage mostly clears spontaneously and only rarely requires vitreous surgery to avoid amblyopia. Results of retinal detachment surgery are of limited benefit even with advanced surgical techniques (Rosenfeld et al., 1998). Macular holes secondary to XLRS have rarely been reported (Brasil et al., 2011; Shukla et al., 2006).

2.5. Differential diagnosis

In patients with marked peripheral retinoschisis, disorders with early onset retinal detachments have to be considered in differential diagnosis. These include X-linked Norrie syndrome (NS), in which complete retinal detachment is present at birth and visual function is nearly absent. NS is associated with mutations in the Norrie disease gene on Xp11.4 (Berger et al., 1992; Chen et al., 1992). A less severe variant of NS is the X-linked familial exudative vitreoretinopathy (FEVR), also associated with mutations in the Norrie disease gene. Peripheral vascular retinal abnormalities are present in a variable degree, which may lead to retinal detachment. Similar alterations can be observed in autosomal dominant FEVR (Criswick-Schepens syndrome) and can be associated with mutations in frizzled-4 (FZD4) (Robitaille et al., 2002), *LRP5* (Toomes et al., 2004), and *TSPAN12* (Nikopoulos et al., 2010). The ophthalmoscopic features in both forms of FEVR are distinct from XLRS. Incontinentia pigmentii (Bloch-Sulzberger syndrome) can present with early onset retinal detachment. This syndrome, however, is a lethal condition in males and is not a differential diagnosis for XLRS. Other forms of congenital or juvenile retinal detachment, e.g. following trauma or retinopathy of prematurity, can usually be excluded by the patient's history.

In XLRS patients with involvement limited to the fovea other forms of foveal retinoschisis or early onset macular dysfunction have to be differentiated. Foveal retinoschisis has rarely been reported as an apparent autosomal recessive trait in families with predominantly affected females (Chen et al., 2006; Cibis, 1965; Lewis et al., 1977; Perez Alvarez and Clement Fernandez, 2002). A male patient with features of XLRS but without a *RS1*-mutation has been reported (Hayashi et al., 2004). In Enhanced S-Cone Syndrome (alias Goldmann-Favre syndrome), which is associated with mutations in the *NR2E3* gene on 15q23 (Haider et al., 2000), a foveal retinoschisis may be present. The ERG is quite different from the one typical for XLRS and allows one to distinguish the two disorders (Sohn et al., 2010). Rare syndromes may present with foveal retinoschisis, they can be differentiated by presence of other syndrome associated pathologies (Ayala-Ramirez et al., 2006; Phadke et al., 2011). The SD-OCT serves to differentiate other causes of early onset macular dysfunction, e.g. CSNB, cone dysfunction or macular dystrophies.

2.6. Treatment options

In most cases, treatment of XLRS is limited to the prescription of low-vision aids. Recently, a marked reduction of retinoschisis at the posterior pole was reported following local application of 2% dorzolamide (Apushkin and Fishman, 2006; Genead et al., 2010; Ghajarnia and Gorin, 2007). In about half of the eyes this treatment is associated with improvement of visual acuity. Not all patients show a response to this treatment and in some a response is only seen after several months of application. The response to treatment is independent of the genotype (Khandhadia et al., 2011; Walia et al., 2009). When the retinoschisis worsened under therapy, discontinuation and later retreatment might be beneficial (Thobani and Fishman, 2010). The value of long-term treatment over many years still has to be established. At present a treatment trial with dorzolamide is recommended and should be monitored with visual acuity and SD-OCT. Long-term application should be based on the response to treatment in the individual patient.

Pars-plana vitrectomy is indicated in vitreous hemorrhage without spontaneous resolution to avoid amblyopia or when retinal detachment occurs (Ferrone et al., 1997; Wu et al., 2007). Although in a few patients vitreoretinal surgery has been performed to release vitreoretinal traction (Ikeda et al., 2008; Trese and Ferrone, 1995), in general prophylactic treatment of retinoschisis either by laser or vitreoretinal surgery cannot be recommended due to possible severe long-term complications including retinal detachment (Kellner et al., 1990; Sobrin et al., 2003).

3. Genetics of XLRS

3.1. Gene identification and gene structure

Following the first tentative assignment of the *RS1* gene to Xg blood group markers on the short arm of the human X-chromosome (Wieacker et al., 1983), a refined mapping of the disease locus was gradually achieved to an approximately 1000 kb interval on

Xp22.13 flanked by DNA markers DXS418 and DXS999 (Alitalo et al., 1987; Gal et al., 1985; Huopaniemi et al., 1997; Van de Vosse et al., 1996; Warneke-Wittstock et al., 1998). Within this interval, the *RS1* gene was finally identified by positional cloning based on retina-specific expression of the transcript and segregation analysis of functional DNA variants with the disease in several large multi-generation XLRS pedigrees (Sauer et al., 1997).

The *RS1* gene spans 32.4 kb of genomic DNA and is organized in six exons and five intervening regions. The 3.1 kb mRNA translates into a precursor protein of 224-amino-acids, termed retinoschisin. It consists of a 23-amino-acid N-terminal signature characteristic for cellular proteins processed through the secretory pathway (Sauer et al., 1997; Wu and Molday, 2003) and a discoidin homology domain, first described in discoidin I from the slime mold *Dictyostelium discoideum* and since found in many extracellular and membrane proteins (Poole et al., 1981).

3.2. RS1 expression and its regulation

Across mammalian species, *RS1* mRNA and protein expression is specifically found in the retina (Gehrig et al., 1999b; Molday et al., 2001; Reid et al., 1999; Sauer et al., 1997) and pineal gland (Takada et al., 2006), with both organs of a common neuro-ectodermal origin. In the retina, prominent immunolabeling of retinoschisin is consistently observed at the extracellular surfaces of the inner segments of rod and cone photoreceptors, most bipolar cells as well as the two plexiform layers (Fig. 2) (Grayson et al., 2000; Molday et al., 2001; Reid et al., 2003; Takada et al., 2004). In the pineal gland, *RS1* expression is confined to pinealocytes and absent from pineal glia (Takada et al., 2006), the latter finding consistent with the findings in the retina, where retinoschisin is also absent from homologous Müller glial cells (Grayson et al., 2000; Molday et al., 2001; Takada et al., 2004).

In postnatal eye development of the mouse, measurable *RS1* mRNA expression resumes around P1 reaching adulthood levels between P5 and P7 which is then maintained throughout adulthood (Weber and Kellner, 2007). Comparably, the developing rat retina reveals weak retinoschisin immuno-labeling at P6 within the neuroblastic zone while the staining intensity of the outer retina increases over time with intense staining of the newly formed inner segment layer at P10 (Molday et al., 2001). Adult pattern labeling in the rat is reached around P12. These findings suggest that across mammalian species the sustained expression of retinoschisin is required in adulthood and is likely essential for maintenance of retinal integrity (Weber et al., 2002).

First insight into *RS1* gene regulation was gained from mice deficient for cone-rod homeobox (CRX) (Livesey et al., 2000) and neural retina leucine zipper protein (NRL) (Mears et al., 2001), emphasizing a critical role of these transcription factors for retinal expression. Another component of a complex regulatory network of *RS1* expression in the retina is the orphan nuclear receptor NR2E3 (Blackshaw et al., 2001; Corbo et al., 2007; Hsiau et al., 2007; Livesey et al., 2000; Yoshida et al., 2004). While CRX is a nuclear protein essential for general photoreceptor maturation for both rods and cones (Chen et al., 1997; Furukawa et al., 1999), NRL and NR2E3 have specific roles in rod photoreceptor maturation and suppression of cone proliferation (Cheng et al., 2004; Haider et al., 2001; Mears et al., 2001).

A core proximal promoter encompassing nucleotides −177 to +32 was shown to be sufficient to drive basal *RS1* gene activity in the retina with two evolutionarily conserved CRX binding sites, CRE1 (−26/−23) and CRE3 (−58/−55), stimulating *RS1* transcription (Langmann et al., 2008). In human, an upstream CpG island was found to exert strong cis-acting effects on *RS1* expression in vitro and in vivo (Kraus et al., 2011). A second CRX-bound region strongly conserved between human and mouse was identified in the first intron of *RS1* with multiple CRX sites and likely modulates basal promoter activity (Kraus et al., 2011).

3.3. Spectrum of RS1-associated mutations

To date, a total of 191 unique variants have been reported in the *RS1* gene to be associated with the XLRS phenotype (Leiden Open Variation Database, LOVD version 2.0, Build 31; http://grenada.lumc.nl/LOVD2/eye/home.php?select_db=RS1). Of these, 155 (81%) are nucleotide substitutions resulting in amino acid changes, alterations of splice site sequences or activation of cryptic splice sites. In addition, there are 25 (13%) deletions, 5 (3%) duplications, 3 (1.5%) insertions and 3 (1.5%) insertion/deletions. Judging by the nature of the unique variants, about 40% are expected to represent true null alleles (i.e. nonsense mutations or frameshift mutations) and thus should not produce a functional retinoschisin protein.

The most prominent class of unique variants constitute the missense mutations (100 of 191 unique variants). This type of mutation affects all regions of the protein although a significant clustering is observed within the discoidin domain (85 of 191). The

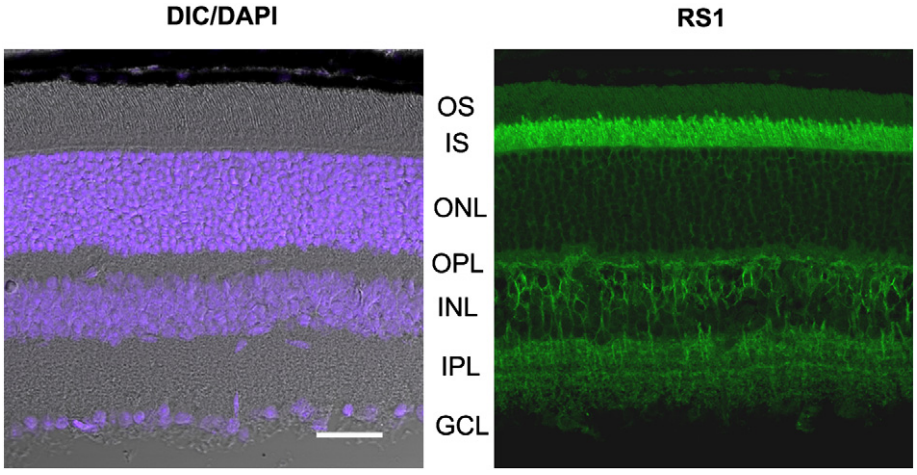

Fig. 2. Light micrographs of an adult mouse retina immunolabeled for retinoschisin. Left: Differential interference contrast (DIC) image of a mouse retina stained with DAPI to show the nuclear layers. Right: Immunofluorescence image of the same section showing retinoschisin distribution (green) in the retinal cell layers. Intense immunofluorescence staining is observed in the inner segment layer with more moderate staining in the outer nuclear, outer plexiform, inner nuclear and inner plexiform layers. 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 − 20 μm.

remaining types of mutations combined (i.e. frameshift, nonsense, splice site and in-frame deletion mutations) are randomly distributed along the protein. This suggests that the discoidin domain is most crucial for *RS1* function with strong constraints on defined amino acid residues.

While XLRS families often segregate unique disease mutations (e.g. Leu69Pro or Asn104Lys), a number of sequence alterations are recurrent. Most notably, the missense mutation Glu72Lys has been reported 66 times while mutations Arg102Trp, Arg102Gln, Arg141Cys, Pro192Ser, Arg200Cys, and Arg213Trp were found in 17 or more apparently unrelated families, respectively. In addition, residues such as Glu72, Pro192, Arg197, and Arg209 were found to be affected by at least 4 different amino acid substitutions. These commonly altered amino acid residues could be indicative of mutational hotspots and/or functionally or structurally important moieties within the mature retinoschisin protein.

4. Mouse models for X-linked juvenile retinoschisis

To date, three independent mouse lines targeting *Rs1h*, the murine ortholog of the human *RS1* gene (Gehrig et al., 1999b), have been reported, all resulting in deficiency of the endogenous murine retinoschisin (Jablonski et al., 2005; Weber et al., 2002; Zeng et al., 2004). Disruption of the *Rs1h* gene was accomplished by targeting exon 1 (Zeng et al., 2004) or exon 3 (Weber et al., 2002) with a neomycin resistance (neo^r) expression cassette or an in-frame lacZ-neo^r expression cassette, respectively. An ENU-induced mutagenesis resulted in a T > C substitution in intron 2 of the *Rs1h* gene at donor splice site position +2 (Jablonski et al., 2005). This latter mutation activates an alternative splice donor site 11 bp further downstream of intron 2 and results in the expression of two novel retinoschisin isoforms both of which encode a premature stop codon close to the site of the original mutation.

Predicting a convergent effect of *Rs1h* targeting on retinoschisin protein expression in the knockout lines and the splice site mutation, it is not surprising that the retinal phenotypes reveal striking similarities in the three mouse models (Jablonski et al., 2005; Weber et al., 2002; Zeng et al., 2004). Prominent morphological features include disruption of retinal layers accompanied by displaced photoreceptor nuclei and schisis of the inner nuclear layer of the retina, as well as disruption of synaptic structures in the outer plexiform layer (see Fig. 7). OCT has confirmed that microcystic cavities observed using histological techniques are present in vivo (Xu et al., 2009). These studies further suggest that the observed cavities are filled with fluid. Overall, the diseased murine retina in hemizygous males shows a dramatic and progressive loss of photoreceptor cells starting at postnatal day 14. Functionally, the morphological pathology correlates well with an attenuation of the b-wave in the scotopic ERG response while at the same time the a-wave appears mostly regular. This electrodiagnostic feature, known as the 'negative' ERG response in human patients (Karpe, 1945; Weleber and Francis, 2006) points to an inner retinal transmission deficit and, more specifically, to severe impairment of bipolar cell-associated pathways. Overall, the structural and functional changes observed in the retinoschisin-deficient mice closely mimic human XLRS patients making these mutant mice excellent models to further study functional aspects of normal and mutant retinoschisin and to develop potential therapeutic treatments for XLRS.

5. Structural features of retinoschisin

Retinoschisin, also known as RS1, is a highly conserved 23 kDa extracellular protein (Sauer et al., 1997). All mammalian proteins for which the complete sequence is available including human, horse, mouse, rat, bovine, rabbit and canine are 224 amino acids in length and share 95–97% sequence identity. Orthologs of lower vertebrates tend to be slightly longer and show a lower degree of sequence identity. For example, chicken retinoschisin contains 225 amino acids and is 81% identical to human retinoschisin whereas zebrafish (*Danio rerio*) and frog (*Xenopus laevis*) contain 230 and 237 amino acids, respectively, and are about 73% identical.

Retinoschisin is organized in four distinct regions. These include a 23 amino acid N-terminal leader or signal sequence, a dominant 157 amino acid discoidin domain, a 39 amino acid Rs1 domain upstream of the discoidin domain, and a 5 amino acid C-terminal segment (Molday, 2007; Sauer et al., 1997).

5.1. Discoidin (DS) domain

The discoidin (DS) domain, also known as the F5/8 type C domain, was first identified in the discoidin 1 protein of *D. discoideum* (Poole et al., 1981). It was subsequently found in a wide variety of eukaryotic and prokaryotic transmembrane and extracellular proteins either as a single entity or as tandem repeats often in combination with other extracellular domains (Baumgartner et al., 1998; Kiedzierska et al., 2007). Some types of proteins which contain DS domains are blood coagulation factors (Factor V and Factor VIII), enzymes (galactose oxidase and sialidase), lectins (discoidin I and II and hemocytin), cell surface tyrosine kinase receptors (discoidin receptor I and II), and proteins involved in cellular adhesion, fertilization, migration, signaling, and development (SEDI also known as milk fat globule-EGF factor, neurexin IV, neuropilin, and others).

DS domains are typically composed of approximately 150 amino acids and share a moderate to high degree of sequence similarity and protein structural fold. Cysteine residues commonly mark the beginning and end of the DS domain.

High resolution structures for a number of DS domains have been determined by X-ray crystallography and nuclear magnetic resonance (Carafoli et al., 2009; Fuentes-Prior et al., 2002; Lee et al., 2003; Lin et al., 2007; Macedo-Ribeiro et al., 1999; Pratt et al., 1999; Vander Kooi et al., 2007). They all have a protein fold similar to that first observed for the crystal structure of the D1 domain of galactose oxidase (Baumgartner et al., 1998; Ito et al., 1994). The core DS domain is organized as a compact β sandwich or distorted barrel consisting of a 5-stranded antiparallel β-sheet (β1, β2, β7, β4, β5) packed against 3-stranded antiparallel β-sheet (β6, β3, β8) (Fig. 3). At one end, short segments connect the β-strands and a disulphide bridge links the N and C-terminal cysteine residues together to further stabilizing the structure. At the opposite end spikes or loops extend from the core β-barrel structure. Two or more of these spikes form a groove or cavity which serves as the recognition site for interaction of the DS domain with its ligand. In the case of blood coagulation Factors V and VIII, the spikes contain a complement of hydrophobic amino acids which can insert into the plasma membrane of blood platelets and endothelial cells (Fuentes-Prior et al., 2002; Macedo-Ribeiro et al., 1999; Pratt et al., 1999). A ring of charged residues within the hydrophobic groove enables the DS domains of Factor V and VIII to selectively interact with the polar head group of phosphatidylserine as a key step in the blood coagulation cascade.

Spike regions and the binding cavity of different DS domains vary widely in amino acid sequence. This sequence variation appears to allow DS domains to bind a wide range of ligands including lipids, carbohydrates and proteins. The DS domain of SED1 or milk fat globule (MFG), like Factor V and VIII, binds acidic phospholipids including phosphatidylserine on the membrane surface of sperm and zona pellucida as an essential step in fertilization (Ensslin and Shur, 2003; Raymond et al., 2009). Discoidin

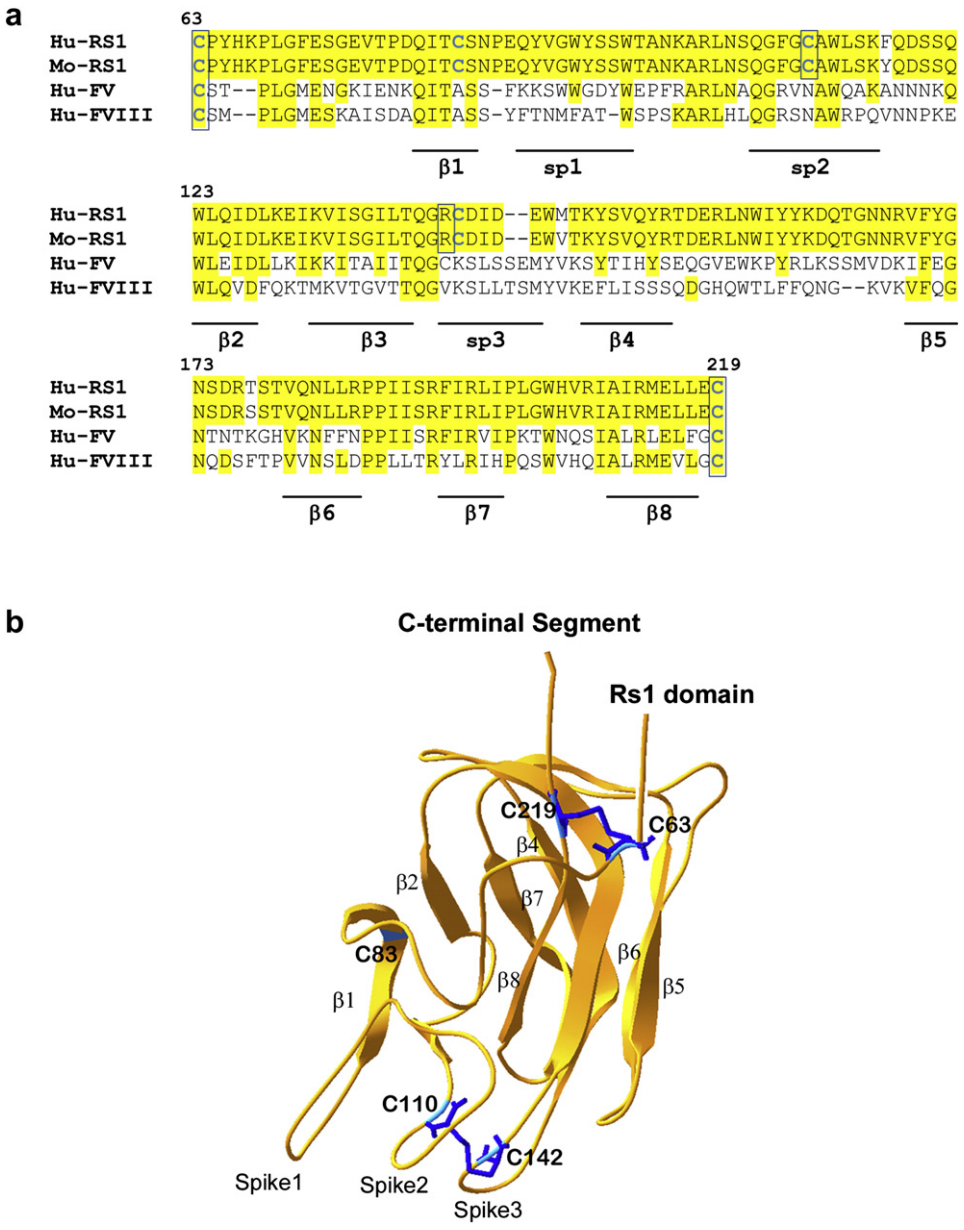

Fig. 3. Sequence alignment and structural homology model of retinoschisin discoidin domain. (a) Alignment of the discoidin domain sequences of human (Hu-RS1) and mouse (Mo-RS1) retinoschisin with D2 discoidin domain sequences of human Factor V (Hu-FV) and human Factor VIII (Hu-FVIII). Numbering is for retinoschisin; yellow shows amino acid identity; β-strands and spike regions are shown below sequences; cysteine residues involved in disulphide bonds are outlined. (b) Homology model of the discoidin domain of retinoschisin obtained using Factor V as a template. Cysteine residues including those involved in intramolecular disulphide bonds are shown in blue. Modified from Wu and Molday (2003).

domain receptors 1 and 2 (DDR1 and DDR2) are tyrosine kinase receptors. The extracellular DS domains of these receptors bind collagen and initiate receptor phosphorylation which in turn regulates a variety of cellular processes including cell proliferation, adhesion and migration (Carafoli et al., 2009; Vogel et al., 1997, 2006). Neuropilin, a cell surface receptor which regulates a variety of developmental processes including cell adhesion, angiogenesis, and neuronal migration binds semaphorin and VEGF (Gu et al., 2003; Vander Kooi et al., 2007). Finally, DS domains of some lectins, sialidases and chitobiases bind various glycoconjugates (Baumgartner et al., 1998; Mathieu et al., 2010). The ligands for most DS domains, however, have yet to be determined. In contrast to the spike regions, the amino acid sequence of the core β-barrel structure of the DS domain is more highly conserved consistent with its role as a scaffold for the hypervariable spikes (Fig. 3a).

A high resolution structure of the retinoschisin DS domain has yet to be determined. However, homology models have been generated using the C2 discoidin domains of Factor V (39% sequence identity) and Factor VIII (33% sequence identity) as templates (Fraternali et al., 2003; Sergeev et al., 2010; Wu and Molday, 2003). In these models the DS domain of retinoschisin is predicted to have a core hydrophobic distorted β-barrel structure consisting of 8 β-strands (Fig. 3b). Three prominent spikes protrude from one end of the core structure with a shorter hairpin loop between β-strands 5 and 6 (Wu and Molday, 2003). There are 5 cysteine residues within the DS domain (Fig. 3a). The conserved cysteine residues at the beginning (C63) and end (C219) are modeled with a disulphide bridge as has been found for corresponding cysteine residues in other eukaryotic DS domains (Carafoli et al., 2009; Lee et al., 2003; Macedo-Ribeiro et al., 1999).

Two additional cysteine residues, one in spike 2 (C110) and another in spike 3 (C142), are in close proximity to each other and have been modeled to join through a disulphide bridge. Mass spectrometric analyses of tryptic peptides from nonreduced retinoschisin together with mutagenesis studies support the existence of a C110-C142 disulphide bond (Wu and Molday, 2003; Wu et al., 2005). The C63-C219 and C110-C142 intramolecular disulphide bonds are crucial for the proper folding and stability of retinoschisin since substitution of any of these residues with a serine results in a misfolded protein which is retained in the cell by the quality control system of the ER (Wu and Molday, 2003). Furthermore, genetic screening has revealed that missense mutations involving cysteine residues at positions 110, 142, or 219 (C110Y, C142W, C219 R/G) are responsible for XLRS (Hiriyanna et al., 1999). A missense mutation in C63 is also likely to cause XLRS although to date patients with such a mutation have not been reported.

An additional cysteine residue is present at position 83 within the retinoschisin DS domain. The corresponding position is occupied by alanine in the DS domain of Factor V and VIII and most other DS containing proteins. Mass spectrometry of nonreduced bovine retinoschisin treated with N-ethylmaleimide prior to protease digestion together with cysteine mutagenesis studies support the view that C83 is buried within the core DS structure in its reduced state (Wu et al., 2005).

5.2. Rs1 domain and the C-terminal segment

The region upstream of the DS domain lacks significant sequence similarity to other proteins in the databases and hence has been called the Rs1 domain (Molday, 2007). It contains four conserved cysteine residues, three of which (C38, C40, C42) are within a conserved KACKCDCQ motif. A fourth cysteine Cys59 is located just prior to the start of the DS domain. A stretch of 5 highly conserved amino acids lies just downstream of the DS domain at the carboxyl terminal end of retinoschisin. This segment contains a conserved cysteine (C223) at the penultimate position of retinoschisin which is preceded by a conserved lysine residue.

The RS1 domain and C-terminal segment play a crucial role in the oligomerization state of retinoschisin (Fig. 4). Site directed mutagenesis studies have implicated C59 and C223 in the formation of intermolecular disulphide bridges between adjacent retinoschisin subunits (Wu and Molday, 2003; Wu et al., 2005). These disulphide bonds are required for homo-octameric complex formation since elimination of these linkages through serine substitutions abolishes the complex even under nondenaturing conditions (Wu et al., 2005). The C40 residue participates in an additional intermolecular disulphide bridge that links two retinoschisin subunits together as dimers within the octameric complex (see below). The role of C38 and C42 and their possible involvement in disulphide bonding remains to be determined experimentally.

5.3. Leader or signal sequence

Most proteins which are destined to be secreted from cells contain a short N-terminal sequence known as a leader or signal sequence or peptide (Blobel and Dobberstein, 1975; Gierasch, 1989; Martoglio and Dobberstein, 1998; Walter and Johnson, 1994). The signal sequence is synthesized off of free ribosomes. As the signal sequence emerges from the ribosome, it interacts with the signal recognition particle (SRP) which in turn directs the complex to the cytoplasmic surface of the endoplasmic reticulum (ER) through its interaction with the SRP receptor. The nascent polypeptide is then transported across the ER membrane through a channel complex known as the peptide translocation complex or translocon. As the peptide emerges from the translocon, the signal peptide is cleaved

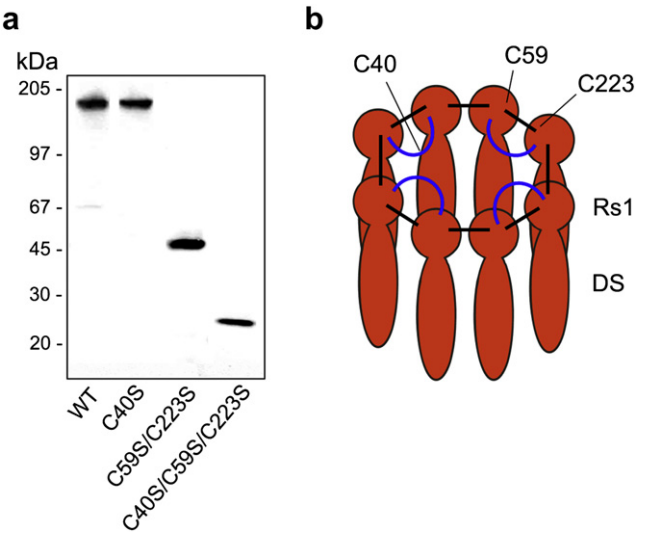

Fig. 4. Oligomeric structure of retinoschisin. (a) SDS gels of wild-type (WT) and mutant retinoschisin proteins run under nonreducing conditions. WT and C40S mutant run as a 186 kDa octamer; C59S/C223S mutant runs as a dimer; and C40S/C59S/C223S runs as a monomer. (b) Working model for the organization of retinoschisin subunits into an octameric complex via disulphide bonding. The octameric structure is maintained by C59–C223 disulphide bonds. Within the octamer, four dimers are maintained by disulphide bonding involving C40. Rs1 domain and discoidin domain (DS) and carboxyl terminal segment (Ct) are indicated. Modified from Wu et al. (2005).

by a signal peptidase present on the lumen side of the ER membrane and the nascent polypeptide chain undergoes cotranslational processing and folding into a native-like conformation (Fig. 5a). In some cases it can subsequently undergoes subunit assembly before emerging from the ER into transport vesicles. After further processing in the Golgi network, the post-Golgi transport vesicles fuse with the plasma membrane and release the protein into the extracellular environment.

A signal sequence has three characteristic features (Martoglio and Dobberstein, 1998). It has an N-terminal stretch of polar residues with a net positive charge, a central core hydrophobic segment of 6–15 amino acids, and a downstream segment of polar residues often containing glycine or proline residues and small uncharged polar residues at positions −3 and −1 from the site of cleavage.

When retinoschisin was first cloned in 1997 (Sauer et al., 1997), the N-terminal 23 amino acid was found to have the structural features of a cleavable signal sequence. N-terminal sequencing of purified bovine retinoschisin by Edman degradation and tryptic peptide analysis by mass spectrometry have confirmed that cleavage does occur following serine at position 23 to generate a 201 amino acid mature protein (Wu et al., 2005). Subsequent studies of mouse retinoschisin, however, have revealed the existence of another signal peptidase cleavage site at position 21 (Vijayasarathy et al., 2006). The physiological significance of the two mature isoforms of retinoschisin has not been established.

5.4. Oligomeric structure of retinoschisin

Although retinoschisin is synthesized as a ~23 kDa protein, it is assembled in the ER and secreted from cells as complex composed of four disulphide dimers within a disulphide-linked homo-octameric complex (Fig. 4). Evidence for this complex has come from biochemical analysis of retinal membrane extracts and cellular and secreted fractions of cultured Weri retinoblastoma cells and HEK293 cells expressing wild-type (WT) and mutant retinoschisin (Molday et al., 2001; Wu et al., 2005). Under disulphide reducing

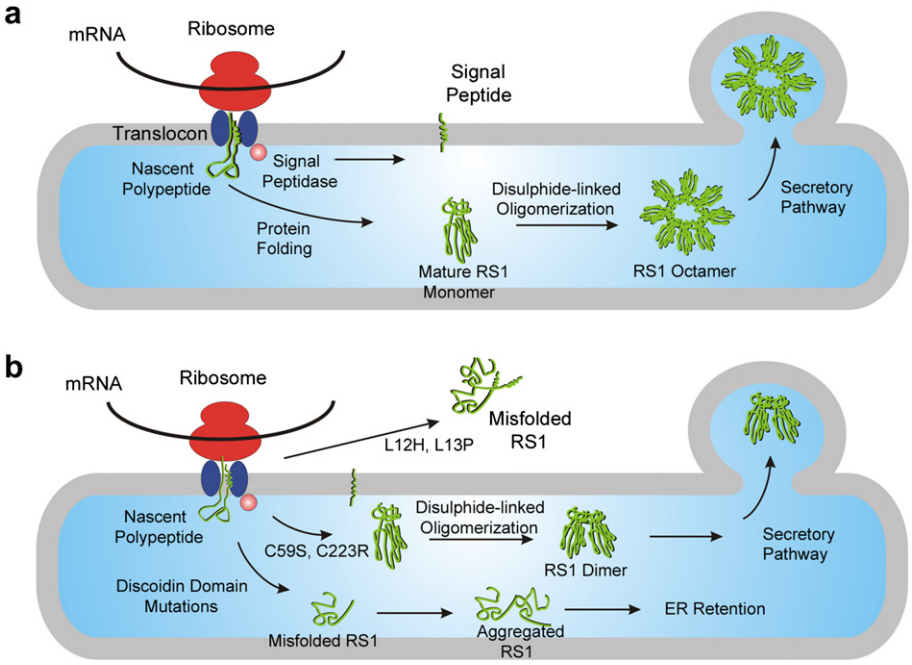

Fig. 5. Diagram depicting the effect of various disease-causing missense mutations on retinoschisin synthesis, protein folding/ER retention, and subunit oligomerization. (a) Wild-type retinoschisin synthesized off of ribosomes associated with the ER membrane is threaded through the translocon and the signal sequence is cleaved by a signal peptidase in the ER lumen to produce the mature folded retinoschisin (RS1) polypeptide. RS1 assembles into a disulphide-linked octameric complex which is exported from cells via the secretory pathway (not shown). (b) Mutations in the signal sequence (L12H and L13P) prevent the insertion of the nascent polypeptide chain into the translocon of the ER resulting in a misfolded polypeptide localized to the cytoplasm where it is rapid degraded by the proteosome. Mutations in the discoidin (DS) domain enable the nascent polypeptide chain to be transported into the ER lumen, but the protein fails to fold into a native conformation and as a result is retained in the ER. Cysteine mutations (C59S and C223R) in the regions flanking the DS domain result in relatively normal protein synthesis, folding, and disulphide-linked dimerization, but fail to further oligomerize into an octameric complex. As a result the retinoschisin dimers are secreted from cells, but are non-functional due to their failure to form disulphide-linked octamers.

conditions retinoschisin in these preparations migrates on SDS polyacrylamide gels as a 23 kDa protein in agreement with its size predicted from its amino acid sequence. In contrast, under nonreducing conditions retinoschisin and the C40S mutant run as a 186 kDa complex (Fig. 4a). The retinoschisin C38S/C40S/C42S triple mutant also runs predominantly as a 186 kDa complex under nonreducing conditions, but in addition the monomer and intermediate oligomers corresponding to the dimer through heptamer are visible providing strong evidence that the 186 kDa protein consists of 8 subunits (Wu et al., 2005).

Cysteine residues which form intermolecular disulphide bonds responsible for the homo-octameric structure were first identified by site-directed mutagenesis (Wu and Molday, 2003; Wu et al., 2005). Substitution of the cysteine at position 59 in the RS1 domain and/or the cysteine at position 223 in the C-terminal segment with serine prevents the formation of the 186 kDa octameric complex (Fig. 4a). Instead, these mutants migrate as dimers indicating that one or more additional disulphide bridge involving different cysteine residues are responsible for dimer formation. Cysteine at position 40 in the RS1 domain has been implicated in dimer formation since the C59S/C223S/C40S triple mutant migrates as a monomer under both reducing and nonreducing conditions. A working model which encompasses these observations is illustrated in Fig. 4b.

Disulphide-linked homo-octamerization appears to play an essential role in the function of retinoschisin as an extracellular protein since mutations which disrupt the complex i.e. C59S and C223R, are known to cause XLRS. The oligomeric nature may be important in increasing the binding affinity of retinoschisin for its receptor on cell surfaces. This is supported in studies which have shown that wild-type homo-octameric retinoschisin efficiently binds to galactose-agarose whereas the dimeric mutant (C59S/C223S) exhibits weak binding. Most DS domain containing proteins have either multiple DS domains within the polypeptide chain or assemble as oligomers suggesting that multiple DS domains are needed to enhance binding to their ligands.

6. Binding of retinoschisin to photoreceptor and bipolar cell surfaces

Retinoschisin expressed and secreted primarily from photoreceptor and to a lesser extent bipolar cells bind strongly and specifically to the surface of these cells as revealed by immunocytochemical and biochemical studies (Molday et al., 2001; Reid et al., 2003; Takada et al., 2004). In order to gain insight into the function of retinoschisin in the retina, a number of studies have been directed toward identifying the molecular component(s) which anchor retinoschisin to the surface of photoreceptor and bipolar cells through its DS domain. Three candidates have emerged from these studies.

6.1. Na/K ATPase-SARM1 complex

Na/K ATPase is a P-type ATPase which plays an essential role in generating Na and K gradients across the plasma membrane (Blanco and Mercer, 1998; Jorgensen et al., 2003; Kaplan, 2002). It consists of two principal subunits, α or A subunit of 112 kDa and β or B subunit of 40–60 kDa. An additional FXYD protein of approximately 10 kDa is present in some tissue specific Na/K ATPases. There are four isoforms of the α catalytic subunit (α1–α4) encoded by the *ATP1A1-4* genes and four β-isoforms (β1–β4) encoded by the *ATP1B1-4* genes. The α subunit consists of 10 membrane spanning segments with the N and C termini and the large catalytic domain located on the cytoplasmic side of the

membrane and only short loops joining the transmembrane segments on the extracellular side of the membrane. The β subunit contains a single transmembrane segment and a relatively large, highly glycosylated extracellular domain. This subunit plays a crucial role in the proper folding and cellular trafficking of Na/K ATPase and is required for generating a functional protein complex. Earlier studies directed toward mapping the various Na/K ATPase isoforms in the mouse retina showed that Na/K ATPase (α3/β2) is a major isoform in the retina and is restricted to photoreceptors and bipolar cells (Wetzel et al., 1999). The β2 subunit of Na/K ATPases in neuronal cells has been identified as the AMOG (Adhesion Molecule On Glia) protein implicated in neural–glia interactions (Antonicek et al., 1987; Gloor et al., 1990; Magyar et al., 1994; Mink et al., 2001; Molthagen et al., 1996). Hence, this subunit not only functions as an accessory subunit for the catalytic subunit of Na/K ATPase, but is also involved in cell adhesion.

SARM1, the sterile alpha and TIR motif-containing 1 protein, is an intracellular protein that plays a role in innate immunity as a negative regulator of Toll-like receptor 3 (TLR3) (Carty et al., 2006; Chen et al., 2011; Mink et al., 2001; O'Neill and Bowie, 2007). It has also been implicated in the regulation of neuronal cell morphology through its interaction with syndecan-2 (Chen et al., 2011).

When a detergent solubilized retina extract was applied to a solid support consisting of a highly specific retinoschisin monoclonal antibody coupled to Sepharose, the α3 and β2 subunits of Na/K ATPase, and SARM1, were found to be major components which co-immunoprecipitated with retinoschisin as revealed by both mass spectrometry and western blotting (Molday et al., 2007). Immunofluorescence labeling studies confirmed the colocalization of retinoschisin and Na/K ATPase (α3, β2) to photoreceptor and bipolar cells in the mouse retina. Immunofluorescence microscopy also indicated that SARM1 was present in photoreceptors and bipolar cells.

Friedrich et al. (2011) have subsequently used knockout mice and heterologous protein expression in HEK293 cells to study the interaction of retinoschisin with Na/K ATPase. Both retinoschisin and Na/K ATPase expression were severely reduced in the retinas of Atp1b2$^{-/-}$ mice deficient in β2 subunit of Na/K ATPase (Magyar et al., 1994). Exogenously added retinoschisin bound to retinal membranes of retinoschisin-deficient *Rs1h*$^{-/Y}$/*Atp1b2*$^{+/-}$ mice which express the Na/K ATPase (α3/β2) isoform, but failed to bind membranes from *Rs1h*$^{-/Y}$/*Atp1b2*$^{-/-}$ mice deficient in the β2 isoform (Friedrich et al., 2011). Furthermore, retinoschisin was found to specifically bind to membrane extracts from HEK293 cells co-expressing the α3 and β2 subunits of Na/K ATPase, but not membranes from nontransfected cells or cells expressing only one of the subunits.

Taken together, these studies provide strong support for the role of Na/K ATPase in anchoring retinoschisin to the surface membranes of photoreceptors and bipolar cells. The site of interaction of retinoschisin with Na/K ATPase remains to be determined. However, based on structure-function analysis of Na/K ATPase and studies showing that the β2 subunit (AMOG) is involved in neural–glial interactions in neural tissue (Gloor et al., 1990), the extracellular domain of β2 subunit of the Na/K ATPase complex most likely contains the binding site for retinoschisin (Friedrich et al., 2011; Molday et al., 2007). Previous studies have shown that retinoschisin binds to modified galactose residues, a property that can be used to purify retinoschisin (Dyka et al., 2008). Hence, it is possible that the site of interaction could be one or more of the N-glycans of the β2 subunit. Alternatively the polypeptide chain of the β2 subunit may contain the binding site.

Since SARM1 co-immunoprecipitates and co-localizes with retinoschisin and the Na/K ATPase in retina, SARM1 has been suggested to be a component of the retinoschisin-Na/K ATPase complex (Molday et al., 2007). However, it remains to be determined whether SARM1 acts as a signaling molecule to modulate photoreceptor and bipolar cell homeostasis or is only a passive member of the complex. Analysis of the retina of a SARM1 knockout mouse would provide insight into the possible role of SARM1 in retinal structure and function.

6.2. Phosphatidylserine (PS)

Phosphatidylserine (PS) is an acidic phospholipid principally localized to the cytoplasmic leaflet of cell membranes and in particular the plasma membrane. In some instances, the asymmetrical distribution of PS is compromised through cell injury leading to the exposure of PS on the outer leaflet of cells. This can trigger such cellular events as phagocytosis or protein binding. A number of discoidin-domain containing proteins have been shown to bind PS, with blood coagulation Factor V and Factor VIII being the most-well studied (Ortel et al., 1992a, 1992b; Zwaal et al., 1998). In resting platelets and endothelial cells, PS is found exclusively on the inner leaflet of the cell surface membrane. Upon injury to blood vessels a series of reactions occurs that leads to the loss in PS membrane asymmetry as part of the platelet/endothelial activation process. This allows Factor V and Factor VIII to bind to PS on the surface of the activated cells through their DS domains and initiate the blood coagulation cascade (Fuentes-Prior et al., 2002).

Sequence similarity between retinoschisin and Factor V together with early homology modeling and molecular dynamics first led to the suggestion that PS may serve as the ligand for retinoschisin (Fraternali et al., 2003). Sieving and colleagues have pursued this idea experimentally showing that a bacterial expressed recombinant protein corresponding to the DS domain of retinoschisin bound to immobilized acidic phospholipids and in particular PS (Vijayasarathy et al., 2007). They subsequently showed that retinoschisin interacts with PS containing planar lipid bilayers in the presence of Ca^{2+} as revealed by atomic force microscopy (Kotova et al., 2010). These studies used lipid mixtures containing varying amounts of PS down to a molar composition of 12%, a concentrate which resembles the PS composition of synaptic vesicles. However, since the external surface of most cells typically contains extremely low levels of PS due to PS asymmetry generated by aminophospholipid flippases (Folmer et al., 2009), the physiological significance of the interaction of retinoschisin to these model membranes is unclear and needs further clarification.

Two independent groups have investigated the binding of retinoschisin to PS-containing lipid vesicles derived from a mixture of synthetic phospholipids or retinal lipids (Friedrich et al., 2011; Molday et al., 2007). In both studies, retinoschisin failed to bind these PS containing membranes even in the presence of Ca^{2+}. Hence, it remains to be determined if PS mediates the specific binding of retinoschisin to photoreceptor and bipolar cell surfaces or alternatively adsorbs to negatively charged lipid surfaces under certain conditions through weak ionic interactions.

6.3. L-type voltage-gated calcium channels

L-type voltage-gated calcium channels play a crucial role in a number of cellular process including muscle contraction, cell motility, cell division and synaptic transmission (Hosey et al., 1996). They consist of 4 subunits, α1, β, α2 and δ, with α1 being responsible for most of the electrophysiological and pharmacological properties of the channel and the other accessory subunits being important for channel stability and modulation of the gating and kinetics. L-type voltage-gated channels play a crucial role in photoreceptor-bipolar neurotransmission. In mammalian retina, rod and cone photoreceptors express the CACNA1F (α1F) subunit encoded by the *CACNA1F* gene (Morgans, 2001; Morgans et al., 2005). In addition,

cone photoreceptors express the CACNA1D (α1D) subunit. Mutations in the CACNA1F are known to cause incomplete congenital stationary night blindness (CSNB2) associated with a loss in b-wave amplitude of the ERGs (Bech-Hansen et al., 1998; Shi et al., 2009; Strom et al., 1998).

Shi et al. (2009) have recently examined the interaction of retinoschisin with L-type voltage-gated calcium channels from chick retina. In these studies, an anti-retinoschisin antibody was found to co-immunoprecipitate an L-type voltage-gated channel with retinoschisin. Using a mammalian two-hybrid assay, they further showed that retinoschisin binds within a 500 amino acid N-terminal region of chicken CACNA1D gene, which is highly conserved in human CACNA1D and CACNA1F subunits (Shi et al., 2009). On the basis of these studies, they have suggested that retinoschisin plays an important role in the retention of L-type voltage calcium channels in photoreceptor presynaptic membranes and is indirectly involved in photoreceptor-bipolar transmission. The possible interaction of retinoschisin with these channels is intriguing since it may explain the loss in ERG b-wave amplitude which is observed in patients with XLRS and CSNB2. However, the distribution of the retinoschisin and L-type channels differ significantly in the retina. Whereas retinoschisin is widely distributed in photoreceptor and bipolar cells as previously discussed, the CACNA1D and CNCNA1F channels are restricted to the outer and inner plexiform layers (Morgans, 2001; Morgans et al., 2005). Hence, if the interaction of retinoschisin with these channels can be confirmed by additional studies, it would suggest that retinoschisin likely has multiple membrane binding partners.

7. Possible function of retinoschisin in the retina

The function of retinoschisin in the retina is not well understood. However, it has been generally suggested that retinoschisin functions as a cell adhesion protein to maintain the cellular organization of the retina and the structural integrity of the photoreceptor-bipolar synapse. This is based on the finding that XLRS patients and *Rs1h* knockout mice exhibit a highly disorganized retinal architecture and the existence of the DS domain which has been implicated in cell adhesion processes. However, to date there is no direct evidence that retinoschisin interacts with other components of the extracellular matrix or participates in direct cell–cell interactions to stabilize the structure of the retina.

Another possible role of retinoschisin may be to help regulate the fluid balance between the intracellular and extracellular environment particularly within the photoreceptor and bipolar cell layers. Regulation of cell volume and tissue osmotic homeostasis is a complex process involving numerous membrane transporters and channels including the NaK ATPase. One can speculate that the binding of retinoschisin to the NaK ATPase may directly affect the activity of this pump in controlling ion gradients and consequently fluid balance and tissue osmolarity. Alternatively, the binding of retinoschisin to the NaK ATPase may influence fluid balance indirectly through an intracellular signaling pathway. In such a case loss of functional retinoschisin could cause fluid accumulation in the extracellular environment in the form of fluid-filled cystic cavity as observed in the retina of XLRS patients and *Rs1h* knockout mice by OCT and histology (Eriksson et al., 2004; Renner et al., 2008; Weber et al., 2002; Xu et al., 2009). The cystic cavities could in turn disrupt the organized layers of the retina causing a dysfunction of the photoreceptor-bipolar synapse. Indeed, this may also explain the effect of dorzolamide, a carbonic anhydrase inhibitor, in reducing schisis cavities in some XLRS patients as observed by OCT (Apushkin and Fishman, 2006). It also may explain why retina splitting and cystic cavities are sometimes seen in layers of the retina which are removed from cells expressing retinoschisin. Additional studies are needed to test this model and further define the role of retinoschisin in retinal cell physiology.

8. Molecular and cellular mechanisms underlying XLRS

As discussed above, up to 40% of the disease-causing mutations are nonsense or frameshift mutations which are predicted to result in the absence of a full-length retinoschisin protein. Any residual translation would likely result in an unstable truncated polypeptide that would be rapidly degraded in the cell. Hence, the disease phenotype for such mutations is expected to arise from a complete deficiency of retinoschisin. Over 50% of disease-causing mutations, however, are missense mutations which allow the translation of the full-length mutant protein. A number of studies have been directed toward determining how various disease-associated missense mutations affect retinoschisin expression, subcellular localization, and protein structure (Vijayasarathy et al., 2010; Wang et al., 2002; Wu and Molday, 2003). Three principal mechanisms have been defined all of which produce a non-functional protein (Fig. 5). This is generally consistent with studies that have found little or no significant correlation between genotype and phenotype in XLRS patients (Eksandh et al., 2000; Sergeev et al., 2010; Shinoda et al., 2000).

8.1. Mutations in the DS domain cause protein misfolding and retention in the ER

A number of studies have been carried out to assess the effects of *RS1* disease-linked missense mutations in the DS domain on retinoschisin structure and function (Curat et al., 2001; Iannaccone et al., 2006; Sergeev et al., 2010; Vijayasarathy et al., 2010; Walia et al., 2009; Wang et al., 2002; Wu and Molday, 2003). In one study, Curat et al. (2001) analyzed mutations G70S, R102W, W112C, G140R, G178D, P193L, P203L, R213W and E215Q at homologous positions of the human discoidin receptor 1 (DDR1). In this chimeric receptor system, some residues affected receptor phosphorylation, while others influenced both collagen-binding and receptor activation.

In another experimental approach, Wang et al. (2002) and Wu and Molday (2003) expressed wild-type and mutant retinoschisin in culture cells and compared their expression, biochemical properties, and secretion from cells. Whereas the wild-type protein was efficiently expressed and secreted from cells, the majority of the proteins with disease-associated mutations in the DS domain (e.g. G70S, E72K, R102W, G109R, G109E, C110Y, W112C, R141C, C142W, D143V, R182C, P203L, R213W, C219R) were severely misfolding and retained in the endoplasmic reticulum (ER) by the quality control system of cells (Ellgaard et al., 1999). Mutations which result in either the addition or removal of cysteine residues in particular were found to induce the formation of aberrant disulphide bridges leading to significant protein aggregation within the ER of the cells as analyzed on nonreducing SDS polyacrylamide gels (Wu and Molday, 2003). Disease-causing mutations in the core barrel structure and spike regions which do not involve cysteine residues also caused protein misfolding most likely by interfering with specific hydrophobic, hydrogen bonding, and/or electrostatic interactions essential for proper protein folding. The large number of disease-associated missense mutations in the retinoschisin DS domain which result in protein misfolding and ER retention indicates that this domain is a finely tuned structure that cannot accommodate many amino acid substitutions (Fig. 6). The finding that these mutants are expressed but not secreted from cells suggests that the disease phenotype arises primarily from the loss in functional protein. Any stress resulting from the unfolded protein response (Nakatsukasa and Brodsky, 2008) appears to have little if any influence on the disease phenotype (Vijayasarathy et al., 2010).

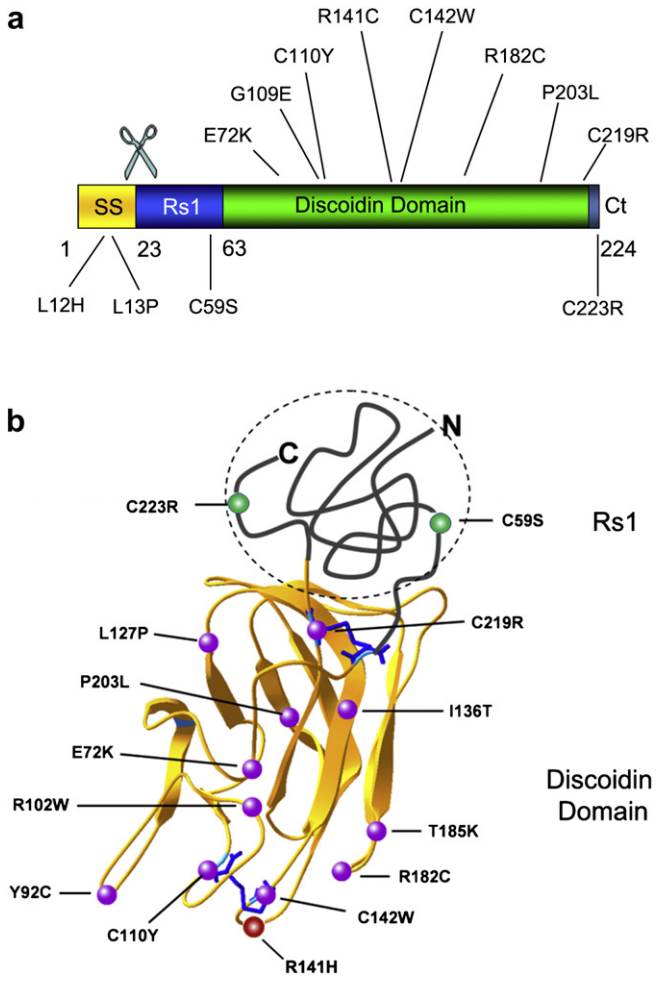

Fig. 6. Location of selected missense mutations within retinoschisin structure. (a) A linear diagram showing the organization of retinoschisin into it various domains along with selected disease-associated missense mutations (SS – cleavable signal sequence; Rs1 domain; discoidin domain; and Ct – C-terminal segment). (b) Selected missense mutations are shown within a model of the mature retinoschisin subunit. The structure of the Rs1 domain and C-terminal segment is not known and drawn as lines within a dashed ellipse with mutations shown in green spheres. The discoidin domain structure is based on the homology modeling shown in Fig. 3b with mutations shown in blue spheres with the exception of R141H which is shown as a red sphere.

8.2. Cysteine mutations in Rs1/C-terminal regions cause defective oligomerization

To date two disease-linked missense mutations (C59S and C223R) have been found in the regions flanking the DS domain (Gehrig et al., 1999a; Hiriyanna et al., 1999). Neither mutation significantly interferes with protein folding and secretion from cells. Instead, they prevent the assembly of retinoschisin into a homo-octameric complex (Fig. 5b) (Wu and Molday, 2003). These studies highlight the importance of the octameric structure in the function of retinoschisin as an extracellular protein. Disease-causing missense mutations in other residues of the RS1 domain and C-terminal segment have yet to be found.

8.3. Mutations in the signal sequence cause abnormal protein synthesis and localization

Disease-causing missense mutations in the 23-amino-acid signal sequence of RS1 (L12H, L13P) cause severely reduced protein expression and mislocalization within cells (Hiriyanna et al., 1999; Vijayasarathy et al., 2010; Wang et al., 2002; Wu and Molday, 2003). These mutations most likely disrupt the α-helical conformation of the leader sequence thereby preventing the nascent polypeptide chain from inserting into the ER membrane during protein biosynthesis (Fig. 5b). As a result the protein is mislocalized to the cytoplasm where it is rapidly degraded by the proteasomal degradation pathway (Wang et al., 2002; Wu and Molday, 2003).

8.4. Other pathogenic mechanisms

Although most disease-associated missense mutations fall into one of the three mechanisms discussed above and illustrated in Fig. 5, at least one disease-associated mutation may cause XLRS by a different mechanism. The R141H mutant is secreted from cultured cells as a disulphide associated octamer and exhibits similar galactose binding properties as wild-type retinoschisin (Dyka and Molday, 2007; Dyka et al., 2008; Wang et al., 2006). The R141H mutation located in spike 3 of the DS domain (Fig. 6b) appears to enable retinoschisin to fold and assemble into a native-like octameric complex which is secreted from cells, but this substitution may interfere with the ability of retinoschisin to bind to its cognate receptor on retinal cell surfaces. Alternatively, this mutant may bind to its receptor, but serve as an antagonist with respect to retinoschisin function. Finally, it is possible that retinal cells differ sufficiently from culture cells in their chaperones and ER quality control system such that the R141H mutant is retained in retinal cells, but not in culture cells. Indeed, differential effects of some mutations on the folding and secretion of proteins have been observed in different cell types. For example, a small amount of the R141G mutant has been found to be secreted from transfected COS-7 cells, whereas secretion of this mutant was not detected in transfected HEK293 cells (Dyka and Molday, 2007; Wang et al., 2002). Wang et al. (2006) have also reported that small amounts of several other disease-associated mutants (F108C, R182C, H207Q and R209H, and C219G) are secreted from COS-7 cells, but it is unclear whether such mutants are functionally active.

9. Gene therapy in mouse models of XLRS

Gene therapy is a viable approach to prevent or slow vision loss in inherited retinal degenerative diseases (Dinculescu et al., 2005; Liu et al., 2010; Smith et al., 2009). For retinal degeneration caused by complete or partial loss in protein function, the delivery of the normal gene to cells harboring the defective gene is often sufficient to restore protein function and halt or at least slow retinal degeneration. Most studies have employed recombinant adeno-associated viral (rAAV) vectors to deliver the gene of interest to photoreceptor or RPE cells (Liu et al., 2010; Smith et al., 2009). These vectors have a number of advantages. They are nontoxic and nonimmunogenic when administered by subretinal injections, come in a variety of serotypes which differ in specific cell transfection efficiencies, effectively transduce nondividing cells including photoreceptors and RPE cells, and display long-term gene expression. Gene replacement strategy has proven successful in animal models for a number of recessive retinal degenerative diseases caused by mutations in genes expressed in retinal pigment epithelial cells (RPE) or photoreceptors (Acland et al., 2001; Alexander et al., 2007; Boye et al., 2010, 2011; Kong et al., 2008; Min et al., 2005; Pawlyk et al., 2005). Recently, Phase I/II clinical trials have shown that AAV-mediated gene therapy is safe and can restore some vision in individuals with autosomal recessive Leber congenital amaurosis type 2 (LCA2) associated with mutations in RPE65 (Bainbridge et al., 2008; Cideciyan et al., 2008; Jacobson et al., 2011; Maguire et al., 2009, 2008).

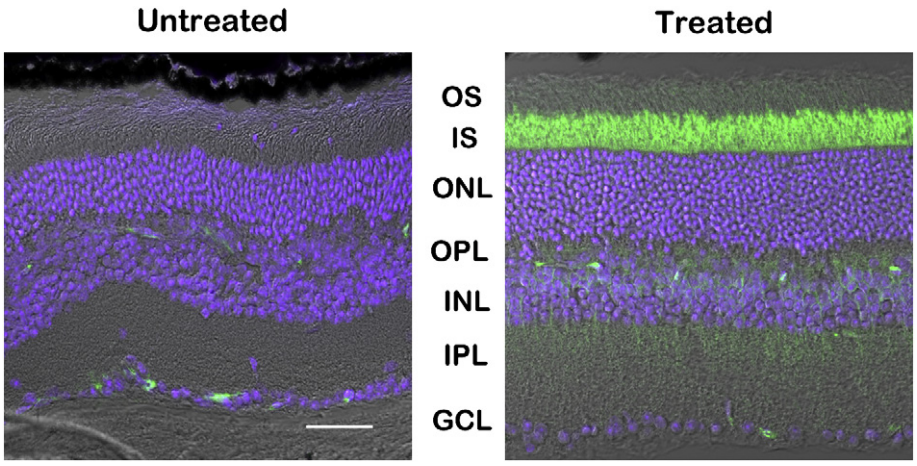

Fig. 7. Retinoschisin expression and retinal structure in a *Rs1h* knockout mouse treated with rAAV containing the human *RS1* cDNA under control of an upstream mouse opsin promoter (rAAV-mOps-RS1). The right eye of a 14 day old *Rs1h* knockout mouse was injected subretinally with rAAV-mOps-RS1 (Treated); the left eye was not injected and served as a contra lateral control (Untreated). One year post-treatment, the mouse was sacrificed and the both the untreated and treated retinas were immunolabeled for retinoschisin and visualized by confocal scanning microscopy. The untreated eye showing no retinoschisin expression was highly disorganized with a merging of the inner and outer nuclear layers and disrupted outer plexiform layer. The treated eye showed retinoschisin expression and distribution comparable to that of a wild-type retina (see Fig. 2) and significant improvement in retinal structure and photoreceptor survival as determined by the thickness of the outer nuclear layer (ONL). Bar – 20 μm.

Since XLRS is a recessive disease caused by the loss in retinoschisin function, gene replacement therapy has been considered as a potential treatment for this disease. Furthermore, since retinoschisin functions as an extracellular protein, beneficial treatment is not necessarily restricted to the transfected cell expressing the replacement gene, but can encompass a wider area due to the spread of the secreted protein from the site of expression. Two independent groups have investigated the potential of gene replacement therapy to restore retinal structure and function and slow retinal degeneration in *Rs1h* knockout mice as an animal model for XLRS (Janssen et al., 2008; Kjellstrom et al., 2007; Park et al., 2009; Min et al., 2005; Takada et al., 2008; Zeng et al., 2004).

In one study, the normal recombinant AAV serotype 5 containing the human *RS1* cDNA under the control of the mouse photoreceptor-specific mouse opsin promoter (AAV5-mOPs-RS1) was injected into the subretinal space of the right eyes of 14-day old *Rs1h* knockout mice with the untreated left eyes serving as controls (Janssen et al., 2008; Min et al., 2005). A gradual increase in retinoschisin expression was observed by immunofluorescence microscopy over the initial 12 week post-injection period. After this initial period, immunostaining of retinoschisin was seen throughout most of the treated retina with a distribution similar to that observed for wild-type mice. In particular, intense staining was observed in the inner segment layer and more moderate staining in the outer nuclear layer (ONL) and outer plexiform layer (OPL) of the outer retina and inner nuclear layer (INL) and inner plexiform layer (IPL) of the inner retina (Fig. 7). Retinoschisin was absent in the untreated control retina of the *Rsh1* knockout mouse as expected. Although *RS1* gene expression was restricted to photoreceptors due to the mouse opsin promoter, the presence of retinoschisin in other layers of the retina indicated that the secreted protein was able to spread to other parts of the retina.

Retinoschisin expression in the treated eyes of *Rsh1* mice coincided with a significant improvement in the structure and function of the retina (Janssen et al., 2008; Min et al., 2005). The treated retina showed well-organized retina layers, the absence of cystic cavities within the bipolar cell layer, preservation of scotopic and photopic ERGs, and stabilization of the ONL thickness characteristic of sustained photoreceptor cell survival (Fig. 7). The untreated retina on the other hand showed the characteristic features of the advanced disease including disorganization of the retinal layers, loss in scotopic and photopic ERS response, and progressive degeneration of rod and cone photoreceptor cells. Importantly, retinoschisin expression and distribution together with improved structure and function of the retina following a single subretinal injection persisted for the lifetime of the mouse, i.e. >19 months (unpublished results). However, rescue of retina structure and function was dependent on the age at which the mice were treated with older mice showing significantly reduced therapeutic benefit (Janssen et al., 2008).

Sieving and coworkers have achieved similar rescue of retinal structure and function in *Rsh1* knockout mice using a different although related approach (Kjellstrom et al., 2007; Takada et al., 2008; Zeng et al., 2004) In their studies, rAAV2 containing the mouse *Rs1h* cDNA under the control of the general CMV promoter was administered to knockout mice of various ages by intravitreal injections. Significant improvement in ERGs, retinal structure and photoreceptor cell survival was observed with the most evident success achieved when injections were carried out on 14 day old mice. Retinoschisin expression together with improved retinal structure and function persisted for over 15 months. Detailed structural analysis also showed an improved OPL structure which correlated with enhanced ERGs. Although the inner limiting membrane of wild-type mice appears to limit penetration of rAAV2 into the retina by intravitreal injections, this barrier appears to be compromised in the *Rs1h* knockout thereby allowing infiltration of rAAV2 into the retina. The retinal cell type(s) which expressed and secreted retinoschisin, however, was not determined in this study. More recently, Park et al. (2009) have administered rAAV8 containing the mouse *Rs1h* cDNA under the control of a 3.5 kb human retinoschisin promoter to *Rs1h* knockout mice by intravitreal injections. This approach was also reported to yield strong retinoschisin expression and secretion and significant rescue of retinal structure and function.

These studies indicate that the delivery of the normal *RS1* gene to retinal cells which do not express retinoschisin can promote long-term rescue of retinal structure and function and greatly slow photoreceptor degeneration. However, many XLRS patients harbor missense mutations which allow the expression of the mutated protein. Accordingly, wild-type retinoschisin expressed in these cells by gene delivery could interact with the endogenously expressed mutant retinoschisin diminishing the therapeutic effect

of gene therapy. This has been studied by analyzing protein expression, cellular localization and secretion of wild-type retinoschisin co-expressed with various disease-linked missense mutants in culture cells (Dyka and Molday, 2007; Gleghorn et al., 2010). These studies indicate that wild-type retinoschisin undergoes protein folding, subunit assembly, and secretion largely independent of the misfolded mutants. Hence, delivery of the normal gene to XLRS patients expressing the mutant protein may not be a problem although ideally this should be investigated further in photoreceptors cells.

In considering future XLRS gene therapy clinical trials, both routes of vector administration (subretinal and intravitreal) are effective and feasible, although intravitreal injections may be the optimal route of administration due to the fragile nature of the retina of XLRS patients. However, it will be important to determine if AAV8 or other AAV serotypes can efficiently penetrate the retina of XLRS patients following intravitreal injections as demonstrated in *Rs1h* knockout mice.

10. Conclusions and future directions

Since XLRS was first reported over a century ago, significant progress has been made in the clinical diagnosis, genetic analysis, and molecular and cellular basis for the disease. A wide assortment of diagnostic tools has enabled clinical ophthalmologists to accurately identify and monitor the disease progression. Clinical diagnosis of XLRS has been greatly improved through the application of SD-OCT to noninvasively image splitting and disruption of the retinal layers. Following the initial discovery of the gene responsible for XLRS, international teams of molecular geneticists have identified over 190 different disease-causing mutations in the *RS1* gene, most of which lead to null alleles or non-functional protein. Immunocytochemical and biochemical studies have shown that retinoschisin is expressed in retinal neurons early in development and secreted as a disulphide-linked oligomeric protein complex which specifically binds to the surface of photoreceptors and bipolar cells. A number of molecular mechanisms by which missense mutations cause XLRS have been elucidated. These include aberrant retinoschisin synthesis, protein misfolding, and defective subunit assembly. Hemizygous *Rs1h* knockout mice deficient in retinoschisin have been generated and shown to exhibit many of the characteristic features found in XLRS patients including cystic cavities, disorganization of the photoreceptor-bipolar synapse, reduction in b-wave of the ERG, and progressive loss in rod and cone photoreceptor cells. Delivery of the *RS1* gene to *Rs1h* knockout mice using recombinant AAV has resulted in a remarkable improvement in retinal structure and function offering hope that this or related approaches may lead to future clinical trials for XLRS and benefit individuals affected with the disease.

Although significant progress has been made in understanding XLRS, there are still many important unresolved issues that require further study. The high resolution structure of retinoschisin remains to be determined to validate and extend biochemical analyses. The molecular mechanisms by which a deficiency in retinoschisin results in the disorganization of the photoreceptor-bipolar cell synaptic structure and function and the formation of fluid-filled cystic cavities require further study. Controversies regarding the molecular nature of the components that interact with retinoschisin need to be resolved to more fully understand the role of retinoschisin in retinal cell biology and physiology. Finally, gene replacement studies successfully carried out on hemizygous *Rs1h* knockout mice need to be extended to large animals to optimize the route of delivery and site of injections prior to initiation of human clinical trials.

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