1. BACKGROUND AND LITERATURE SURVEY

1.1. The Human eye

The Human eye is a complex organ that converts light into electrochemical impulses and transmits them to the brain where the signals are interpreted as images. The anterior part of the eye consists of the cornea, pupil, iris and lens. The pupil serves as an aperture which is adjusted by the iris to regulate the amount of light entering the eye. Both the pupil and iris are covered by the transparent cornea that acts as a major refractive component. Behind them is the crystalline lens held in place by suspensory ligaments that are attached to the ciliary body. Together with the crystalline lens, the cornea helps in focusing the light on retina at the posterior segment of the eye. The retina is comprised of various layers of nerve cells that transmit neurosensory light signals to the brain through the optic nerve. A tough white tissue called sclera covers the exterior of the eye. Alongside sclera is the choroid which is a vascular layer supplying nutrition to the whole eye. A jelly like substance called aqueous humor fills the anterior chamber between the lens and cornea, while vitreous humor fills the posterior chamber between the lens and retina. The balanced production and absorption of the two fluids control the intraocular pressure and maintains the shape of the eye (Bjorn et al., 2009; Gray et al., 1973).

1.1.1. Retinal morphology

The neuroretina, being a part of the central nervous system, is vital for vision as it receives the light and converts it into electrical impulses. It is the innermost tissue of the eye and has 10 distinct layers, the most important of which is the photoreceptors. Photoreceptors are of two types: rods and cones. In an adult retina, cones are mainly located in the fovea, the central part of retina which is responsible
for the high daylight or colour (photopic) vision. Whereas, the rods are abundant throughout the retina except for the fovea and mediate night (scotopic) vision (Dowling, 2012). When a photon hits a chromophore in a photoreceptor cell, a cascade of biochemical reactions starts leading to hyperpolarization of the cells. This cascade of reactions, known as the phototransduction, triggers signals that are transmitted from photoreceptors to the ganglion cells through various layers of the retina and finally reaches the optic nerve. Between the photoreceptor and ganglion cells, there are 2 layers, the outer plexiform layer (OPL) and inner plexiform layer (IPL) through which synaptic contacts are established. In the OPL, the rods and cones are connected to the vertically and horizontally oriented bipolar and horizontal cells respectively. While in the IPL, bipolar cells and ganglion cells form synapses that are regulated by the horizontally running amacrine cells (Masland, 1996). The retinal pigment epithelium (RPE), located in the distal part of retina, plays a significant role in the visual cycle and further maintains the structural integrity of retina by phagocytosis of the photoreceptor outer segment, shed by photoreceptors (Moyer, 1969). The only cells connecting the inner with the outer retinal surface, thus spanning the entire retina, are the Muller glial cells. Any structural or functional defect in the retinal layers would affect the visual signal processing, leading to visual impairment.

1.2. Inherited retinal degenerative disorders

Retinal dystrophies are degenerative disorders of the retina, exhibiting a wide range of clinical manifestations. In young people, hereditary retinal degeneration is the most frequent cause of severe vision deterioration or blindness (Nentwich and Rudolph, 2013). Hereditary retinal dystrophies can be broadly classified as developmental anomalies and progressive degenerative diseases. Examples of ocular
developmental anomalies include anophthalmia, coloboma, optic nerve hypoplasia etc where there is complete absence or underdevelopment of an ocular component (Taylor, 2007). Progressive degenerative disorders usually involve abnormal development of the retinal cells such as photoreceptors and RPE or defective signal transmission in the retina. Retinitis pigmentosa is the most common form, while Leber congenital amaurosis is the most severe form of progressive retinal dystrophies (Francis, 2006). Other conditions include cone dystrophy, Stargardt disease, cone-rod dystrophy, Best’s disease, achromatopsia etc. Although environmental factors increase the risk of retinal diseases, genetic influence is the major underlying molecular cause of inherited disorders (Sahel et al., 2015).

Genes involved in the hereditary retinal diseases can be broadly categorized into 4 groups: 1) genes associated with phototransduction 2) genes affecting the RPE or photoreceptor metabolism, 3) genes involved in the structural components of retina, and 4) genes affecting retina specific transcription factors (Veleri et al., 2015). Over the last few decades, extensive progress has been made in discovering genes and studying their genetic basis that lead to retinal diseases. Now, the primary focus has shifted towards deciphering the disease mechanisms and designing potential treatment strategies, especially gene therapy which has proven successful in some forms of hereditary blindness such as X-linked retinitis pigmentosa, Stargardt disease, Leber congenital amaurosis, choroideremia, Leber hereditary optic neuropathy and X-linked retinoschisis (XLRS) (Sahel et al., 2015).

1.2.1. Retinoschisis

Retinoschisis (RS) is a condition characterized by splitting of the retina’s neurosensory layer leading to progressive retinal degeneration. When the retina splits,
tiny cavities called cysts develop between the retinal layers. These cavities affect the structural organization of the retina which interrupts the signal transmission causing vision deterioration (Haas, 1898). There are two major forms of retinoschisis: Senile retinoschisis and juvenile X-linked retinoschisis.

The most common form is the senile retinoschisis also known as degenerative or acquired retinoschisis which is an idiopathic condition that affects both men and women. It usually occurs in middle aged or elderly individuals, although it can occur in early life (Byer, 1968). The other form, X-linked retinoschisis is rare, inherited and congenital affecting mostly young male (Pagenstecher, 1913).

Occasionally, a third category known as secondary retinoschisis may occur due to diabetic retinopathy, intraretinal hemorrhage, vitreoretinal traction, intraretinal exudation, or inflammation (Madjarov et al., 1995). In rare cases, retinoschisis may be seen associated with other ophthalmic disorders like Best disease, leukocoria, neovascular glaucoma and Coats' disease, which are distinctly different ocular entities manifesting either in the same eye or different eyes (Berinstein et al., 2001; Hung and Hilton, 1980; Silva et al., 2013; Wang et al., 2015).

1.2.2. Diagnostic tests to evaluate retinal function

To make an accurate diagnosis of a retinal disorder, number of diagnostic tests may need to be performed by an ophthalmologist. This clinical evaluation relies on the quantification of visual acuity, studying the retinal threshold and electrophysiological tests. Some of the state-of-the-art diagnostic equipments include optical coherence tomography (OCT), fluorescein or indocyanine green angiography, fundus photography, ultrasonography, multifocal and Ganzfeld electrotetinogram (ERG) etc (Mirshahi et al., 2011). The diagnosis of retinoschisis is usually made by
examining the fundus, analyzing the retinal morphology using OCT and assessing the electrical impulses by ERG.

1.2.2.1. Fundus photography: It is one of the most valuable tools where a specialized camera is used to take photographs of the inside of the eye. These photographs are used to document the appearance of retina and monitor the progression of a retinal disorder. It is a painless procedure; however the patients may experience a slight discomfort due to the bright flashes (Vail, 1963).

1.2.2.2. Optical coherence tomography: OCT is a diagnostic procedure that uses a computer to evaluate the interference patterns of light reflected from the interior side of the eye. It aids in studying the structural appearance of normal and abnormal retinas. Retinal OCT assessment resembles close to the in vivo histopathological studies of retina. High resolution spectral domain OCT aids in assessing the subtle structural changes in diseased retinas that can be made even before fundus abnormalities sets in (Meunier et al., 2014). Figure 1.1 depicts cross section of the normal human retina on OCT, delineating different layers.

Figure 1.1. OCT image of normal human retina. Ellipsoid layer represents the inner segments of photoreceptors. RNFL - Retinal Nerve Fiber Layer, RGC - Retinal Ganglion cells, IPL - Inner Plexiform layer, INL - Inner Nuclear Layer, OPL - Outer Plexiform Layer, ONL - Outer Nuclear Layer, ELM - External Limiting Membrane, RPE - Retinal Pigment Epithelium
1.2.2.3. Electroretinogram: Retinal responses elicited to a visual stimulus can be measured by ERG using a contact lens embedded with electrode that is worn by the patient. The response from the photoreceptors under both light and dark adapted conditions gives rise to a negative a-wave, while responses from the bipolar and muller cells give rise to a positive b-wave.

Normal waveforms represent normal visual processing of the retina, whereas diseased retina results in reduced peaks (amplitudes). Responses from retinal layers in the form of waveforms are measured for the amplitude intensities and implicit times to differentiate normal from abnormal retina. The a-wave amplitude is calculated from baseline of a-wave to a-wave trough, while the b-wave amplitude is calculated from the trough of a-wave to b-wave peak. The implicit times of a and b waves are measured from the time of flash to the wave peaks (Holopigian and Hood, 2003).

1.3. X-linked juvenile retinoschisis

The clinical features of X-linked juvenile retinoschisis were first described in two affected male siblings by Haas in 1898 and it was documented as X-linked in 1913 (Haas, 1898; Pagenstecher, 1913). Since then, the disorder has been known by many other terms such as congenital vascular veils, vitreous veils and congenital cystic retinal detachment (J A Magnus, 1951; Mann and Macrae, 1938). The term X-linked retinoschisis was introduced by Jager in 1953, where the word ‘schisis’ refers to ‘splitting’ in Greek (Jager, 1953).

1.3.1. Clinical presentation

X-linked retinoschisis (OMIM 312700) is a vitreoretinal disorder, characterized by spoke wheel pattern at the fovea and schisis within the retinal layers
resulting in visual deterioration in affected males. The disorder affects only the eye and does not involve any systemic abnormalities. The estimated worldwide prevalence of XLRS ranges from 1:5000 to 1:25000 (The Retinoschisis Consortium, 1998). Though XLRS is congenital, diagnosis is difficult in young males due to subtle fundus abnormalities at the initial stages. In general, XLRS is fully penetrant but the clinical features are quite variable especially in terms of schisis severity and peripheral retinal changes. As the disease progresses, secondary complications like retinal detachment and/or vitreous hemorrhage may occur (George et al., 1996, 1995a). Hence understanding the wide spectrum of phenotype in XLRS is critical so as to provide the patient with appropriate clinical management.

In contrast to other X-linked retinal dystrophies, female XLRS carriers usually show normal retinal function and morphology. Carrier women rarely exhibit retinal abnormalities and the disease phenotype varies greatly (Kim et al., 2007).

1.3.1.1. Diagnostic parameters: Diagnosis of XLRS is usually based on direct fundus visualization, optical coherence tomography and electroretinogram recordings. Fundus findings include one or more of the following features: spoke-wheel pattern, presence of foveal schisis, peripheral schisis, vitreous veils, retinal detachment etc (Figure 1.2 A) (Hotta et al., 2001; Kellner et al., 1990). OCT is an indispensable tool in identifying retinoschisis and to delineate the retinal layers involving schisis (Figure 1.2 B). Although splitting of the layers is expected to be evident in the inner and outer nuclear retina, different layers are found to be involved in XLRS (Chan et al., 2004; Muscat et al., 2001; Agnes B. Renner et al., 2008). The affected eyes might display normal a-wave but reduced inner retinal b-wave amplitudes in ERG indicating abnormal visual signal processing in the retina (Figure 1.2 C). A typical ERG pattern showing reduced b-wave amplitude accompanied with delayed implicit times is a
diagnostic feature of XLRS (Khan et al., 2001; Agnes B. Renner et al., 2008; Renner et al., 2006).

Figure 1.2. Representative clinical images and data of XLRS patient. (A) Fundus exhibiting spoke wheel pattern like schisis at the macula (indicated by arrow). (B) Optical coherence tomography showing splitting of the inner retinal layers. (C) Electroretinogram showing reduced waveforms of rod and cone responses, a negative b-wave pattern noted on standard combined response (circled).

1.3.1.2. Differential diagnosis: Some of the major differential diagnoses of XLRS include congenital stationary night blindness, retinitis pigmentosa, enhanced S-cone syndrome, Goldmann-Favre vitreoretinal degeneration etc., all of which share certain
clinical features of XLRS (Molday et al., 2012; Sikkink et al., 2007). In patients with peripheral retinoschisis, disorders with early retinal detachment such as Norrie disease, familial exudative vitreoretinopathy and peripheral vascular retinal abnormalities needs to be considered in differential diagnosis (Godel and Goodman, 1981; Hu et al., 2017). In patients with schisis involving only the fovea, early onset macular dystrophy and other forms of foveal retinoschisis have to be differentiated (Chen et al., 2006; Pérez Alvarez and Clement Fernández, 2002). Hence, the identification of foveal schisis in a male patient along with reduced b-wave pattern on ERG and a family history showing typical X-linked mode of inheritance makes the diagnosis of XLRS more clear.

1.3.2. Genetic basis of XLRS

XLRS is a genetic X-linked recessive disorder and hence only males are affected, while females are carriers of the disorder (Vainio-Mattila et al., 1969). It is a monogenic disorder caused by mutation in the retinoschisin 1 (RS1) gene. The gene was initially mapped to the short arm of the X chromosome between markers DXS418 and DXS999 (Alitalo et al., 1987; Warneke-Wittstock et al., 1998). Later, the precise molecular location of the gene was identified as Xp22.13 by positional cloning. The Human RS1 gene spans 32Kbp of the genomic DNA on the minus strand and is organized into six individual exons separated by five introns. It transcribes into a 3.1 kb mRNA product with two possible polyadenylation sites, which is then translated to a 224 amino acid protein called retinoschisin (RS1) (Figure 1.3) (Sauer et al., 1997).
1.3.3. RS1 expression and regulation

Across the mammalian species, expression of RS1 is restricted to the retina and pineal gland (Molday et al., 2001; Sauer et al., 1997; Takada et al., 2006). Additionally, nominal level of RS1 expression has also been detected in the uterus of Human females (Huopaniemi et al., 1999). RS1 immunolabeling in the retina was detected at the extracellular region of the inner segment of photoreceptors (rod and cone cells), bipolar cells and the two plexiform layers, while in the pineal gland, expression was confined to the pinealocytes. Expression of RS1 in the retina of mouse models resumes around Postnatal day 1, which is then maintained throughout adulthood (Molday et al., 2001; Takada et al., 2006).

Transcription factor, cone – rod homeobox (CRX) has been shown to regulate the expression of RS1 gene (Livesey et al., 2000). CRX binds to two highly conserved binding sites namely CRE1 and CRE3 in the RS1 promoter region and acts as a key factor driving the gene expression (Langmann et al., 2008). Two other components viz., leucine zipper protein (NRL) and orphan nuclear receptor (NR2E3) have been shown to have a regulatory effect on RS1 expression. Further, genome wide
expression profiling studies on knockout mouse models of CRX and NRL have revealed a complex retinal regulatory network controlled by CRX, NRL and NR2E3 (Mears et al., 2001; Yoshida et al., 2004). However, a recent research by Langmann and group have shown that NRL or NR2E3 do not exert any direct regulatory effect on the expression of RS1 gene (Langmann et al., 2008).

1.3.4. Structural organization of retinoschisin

Retinoschisin, the protein product of RS1 gene is a 24 KDa secretory protein (Sauer et al., 1997). It has three domains: N-terminus signal sequence (amino acids 1-21/23), a unique RS1 domain (amino acids 22/24-63) and a long discoidin domain (amino acids 64-219) with a C-terminal segment (amino acids 220-224) (Molday, 2007).

The processed mature monomer protein (201 amino acids) is linked by disulphide bonds to form the dimer which further assembles into an octamer linked by intermolecular as well as intramolecular disulphide bonds (Wu and Molday, 2003). Finally, two oligomers join to form a double octameric structure, which is the functionally active form of the protein (Figure 1.4) (Tolun et al., 2016). Retinoschisin has 10 crucial cysteine residues (4 in the RS1 domain and 6 in the discoidin domain) participating in intermolecular and intramolecular disulphide bond formation. The first class of Cysteine residues, Cys 63-Cys 219 and Cys 110-Cys 142 form intramolecular disulphide bridges that help in protein folding and secretion. The second class, Cys 59 and Cys 223 involve in intermolecular disulphide bond formation and helps in the oligomerization of RS1 protein. The third class of Cysteine residues, Cys 38, Cys 40, Cys 42 and Cys 83 have limited effect on the RS1 structure and secretion, but may contribute to protein stability (Wu and Molday, 2003).
1.3.4.1. **Leader or signal sequence:** The leader sequence has a stretch of polar residues at the N-terminal region, a core hydrophobic segment in the centre and a downstream stretch of polar residues (Martoglio and Dobberstein, 1998). The signal peptide guides the transit of newly synthesized RS1 into the endoplasmic reticulum (ER) where it is cleaved by signal peptidase resulting in a 23 KDa RS1 monomer (Wu et al., 2005). Analysis of RS1 signal sequence revealed two putative cleavage sites which results in the existence of 2 isoforms of RS1 protein with no apparent change in its overall charge. However, the biological significance of these 2 mature isoforms has not been determined yet (Vijayasarathy et al., 2006).

1.3.4.2. **RS1 domain:** The RS1 domain is located towards the upstream of the discoidin domain and lacks significant sequence similarity with other proteins. Since the RS1 domain harbours cysteine residues required for the formation of intermolecular and intramolecular disulphide bonds, it was suggested that this domain might play a crucial role in the oligomerization of RS1 (Wu et al., 2005; Wu and Molday, 2003). However, a recent study has shown that the RS1 domain is not
essential for the oligomerization of the protein (Tolun et al., 2016). Thus, the function of RS1 domain remains unclear.

1.3.4.3. Discoidin domain: This domain was first identified in Dictyostelium discoideum (Poole et al., 1981), which was subsequently found in a number of transmembrane and extracellular matrix proteins such as blood coagulation factors, lectins, cell surface tyrosine kinase receptors etc. These domains share moderate to high sequence homology and structural fold (Baumgartner et al., 1998; Kiedzierska et al., 2007). Proteins with discoidin domain are involved in migration, cell adhesion, signalling fertilization and development. The binding cavities and spike regions in this domain allow it to bind to a wide range of ligands (Ensslin and Shur, 2003; Raymond et al., 2009).

The most vital and functional part of retinoschisin protein is the discoidin domain that contributes to its adhesive property. To date, there is no X-ray crystallography or nuclear magnetic resonance structure generated for the discoidin domain of RS1. However, homology based models have been developed using C2 discoidin domain of factor V and VIII. Based on in silico studies, the RS1 discoidin domain has a core hydrophobic β-barrel structure comprised of eight β-strands. Three spikes protrude out from one end of the core structure with a small hairpin loop between β-strands five and six (Sergeev et al., 2010; Wu and Molday, 2003).

1.3.5. Mechanism of RS1 secretion

As the retinoschisin nascent polypeptide is released from the ribosome, its signal sequence interacts with the signal recognition particle (SRP) which in turn directs the complex to the cytoplasmic side of endoplasmic reticulum through its interaction with SRP receptor. The hydrophobic amino acids in the leader sequence
guide the transportation of polypeptide across the ER membrane through a channel complex known as the translocon. As the protein emerges from the translocon, the leader peptide is cleaved by signal peptidase present on the lumen side of the ER membrane. This nascent protein undergoes co-translational processing, folding and assembles into a native oligomeric conformation as shown in figure 1.5. After further processing in the Golgi complex, the post-Golgi transport vesicles fuse with the plasma membrane and release the functional protein into the extracellular environment (Molday et al., 2012). Though retinoschisin appears to undergo other post-translational modifications in the retina, comparing molecular weight (23 KDa) with mass and sequence analysis of the protein suggests lack of any glycosylation (Sauer et al., 1997).

Figure 1.5. Pictorial representation of retinoschisin synthesis and assembly in the endoplasmic reticulum.

Investigation on the secretion of retinoschisin under acute illumination effect revealed that RS1 expression and secretion were under circadian control. The mitogen-activated protein kinase (MAPK) extracellular-signal-regulated kinase (Erk), GTPase Ras and Calcium calmodulin kinase II pathways along with L-type voltage-
gated calcium channels (L-VGCC) were found to serve as part of circadian output pathway regulating the rhythmicity of RS1 secretion. The secretion level of RS1 was higher at night than during the day (Ko et al., 2008). Further, a recent study on the molecular mechanism of RS1 secretion reported that alterations in the F-actin cytoskeleton and microtubules are involved in the process of retinoschisin secretion (Kitamura et al., 2011).

1.3.6. Function of retinoschisin

RS1 is generally believed to function as a cell adhesion protein that helps in preserving retinal architecture and establishing proper synaptic connectivity. This is based on the knowledge gained from the retinal phenotype presented by RS1 knockout mouse models and XLRS patients, established role of discoidin domain in cell adhesion process and widespread expression of RS1 across the retina (Molday et al., 2001; Vijayasarathy et al., 2007; Weber et al., 2002). But, currently, there is no evidence demonstrating the participation of RS1 in direct cell-cell interaction. The other speculated role of RS1 is that it regulates the fluid balance between the intracellular and extracellular environment through membrane transporters and channels in retinal cells. The binding of RS1 to these ion channels may influence the fluid balance and in such case non-functional RS1 might result in extracellular fluid accumulation in the form of fluid filled cystic cavities (Molday et al., 2012). So, the exact molecular mechanism by which RS1 maintains the structural integrity of retina is not fully understood.

Apart from the retina, RS1 is known to express in the pineal gland and uterine tissues. But, the actual role of RS1 in these tissues has not been established yet. Though RS1 knockout mouse models show disorganised retinal layers, their pineal
glands remain intact, suggesting that RS1 might serve a different function in pineal gland (Takada et al., 2006). Furthermore, Huopaniemi et al proposed that retinoschisin might have a role in embryo implantation and survival since RS carriers exhibited skewed secondary sex ratio with the offspring (Huopaniemi et al., 1999).

1.3.7. Association of RS1 to the plasma membrane

The first evidence for membrane association of RS1 came from immunolabeling of RS1 on the membranes of in vitro cultured photoreceptors and bipolar cells, while cell permeabilization lead to the loss of RS1 membrane staining (Reid et al., 2003). Later, another study reported the detection of retinoschisin in purified photoreceptor membrane fractions as well as its association with the membranes of Weri-Rb1 cells (Wang et al., 2006). These findings were further supported by RS1 immunogold labelled sections of mouse retina showing distinct membrane localisation of RS1 on the inner segment of photoreceptor cells. Biochemical assays demonstrated the release of RS1 from the retinal membranes in the presence of ethylenediaminetetraacetic acid (EDTA) and egtazic acid (EGTA), while the presence of Ca$^{2+}$ and Mg$^{2+}$ ions prevented its dissociation. Protease digestion assay indicated that a major pool (80%) of RS1 was located on the exterior surface of the plasma membrane, while the rest 20% to 25% remained bound to the cytoplasmic surface (Vijayasarathy et al., 2007). Molecular modeling of RS-lipid interaction revealed 3 aromatic amino acids, Tyr89, Trp92 and Phe108 that form the membrane lipid anchoring sites by which RS1 binds to the negatively charged phospholipids (phosphatidylserine) in plasma membrane (Fraternali et al., 2003). Taken together, these data suggest that retinoschisin exists as a peripheral membrane protein bound by ionic forces.
1.3.8. Interacting partners of RS1

Studies on the molecular mechanism that anchor retinoschisin to the surface of photoreceptors and bipolar cells have revealed few candidate molecular components through which RS1 binds to the plasma membrane. The first retina-specific RS1 binding partner to be identified was Na/K ATPase (α3, β2 isoforms) and the sterile alpha and TIR motif-containing protein (SARM1), which actively maintains the membrane potential of the cell (Molday et al., 2007). In particular, the Na/K-ATPase subunits, ATP1B2 and ATP1A3 were found to be directly involved in anchoring RS1 to the membrane and contribute to its adhesive properties (Hilfinger et al., 2011). Further, SARM1 that inhibits innate immune signalling is known to interact with syndecan-2 which regulates the morphology of neuronal cells (Chen et al., 2011). This raises the possibility that RS1 binding might activate an intracellular signalling process that may maintain the retinal architecture.

RS1 was also found to physically interact with another ion channel L-type voltage calcium channels (L-VGCCa1). They are membrane permeable calcium ion channels that regulate numerous processes such as secretion, muscle contraction, neurotransmission and gene expression. RS1 mutant, R141G was found to deregulate the normal circadian rhythm and alter the gating kinetics of the ion channel. This suggests that RS1 may exert a regulatory influence upon the channel. Furthermore, RS1 has been shown to be essential in maintaining the synaptic localisation of this channel (Shi et al., 2009).

While research studies focused on detecting membrane binding partners of RS1, evidence for retinoschisin’s interaction with extracellular matrix have also been identified. Sahel et al found RS1 to form multimolecular complexes with β2 laminin
and αB crystallin. Interactions with αB crystallin are most likely to be intracellular, while association with β2 laminin occurs at the extracellular level. This suggests that RS1 may facilitate cell-matrix interactions (Steiner-Champliaud et al., 2006).

Another study on the interactions of retinoschisin revealed efficient binding of RS1 to galactose-agarose, but its physiological relevance is not known. Discoidin domain containing proteins are known to interact with diverse ligands and regulate various processes such as cell migration, cell adhesion, signalling and development. In particular, the discoidin protein from *D. discoidium* functions as lectins, interacting with galactose in order to mediate cell aggregation (Dyka et al., 2008).

1.3.9. *RS1* mutations

Currently, more than 250 unique disease-causing mutations have been reported in the *RS1* gene (Leiden Open Variation Database version 2.0). Approximately 80% of these are located in the coding region of discoidin domain (exons 4-6) and 20% of mutations occur in the leader sequence and RS1 domain (exons 1-3). Missense mutations constitute the most prominent class of pathogenic variants spread across the whole gene although significant percentage is observed in the discoidin domain. The other types of mutations include frameshift, nonsense, duplications, deletions, splice site and insertion/deletions that are randomly distributed throughout the protein (Molday et al., 2012).

Although new *RS1* mutations are being added to the repertoire every year, some pathogenic variations are recurrent. Most notably, mutation E72K has been more frequently reported in almost 66 unrelated families. Likewise, R102Q, R102W, R141C, P192S, R200C, and R213W are among the recurrently detected mutations. In
addition, residues E72, P192, R197, and R209 were found to be most affected indicative of mutational hotspots (The Retinoschisis Consortium, 1998).

1.3.10. Molecular mechanism of disease causation

A major category of XLRS causing \(RSI\) mutations affect protein folding and are retained within the cells. As a result, the cells lose their adhesive property leading to disruption of retinal architecture. In general, point mutations allow the translation of the full length protein but form aggregates and get accumulated within the ER (Figure 1.6). Such \(RSI\) mutations are spread across the whole protein, however, appear to be more clustered in the discoidin domain (The Retinoschisis Consortium, 1998). Any residual translation caused by nonsense codons or large deletions or frameshift mutations result in protein truncation and is rapidly degraded by the cell (Vijayasarathy et al., 2010a; T. Wang et al., 2002; Wang et al., 2006; Wu and Molday, 2003). 80-90% of these misfolded proteins are retrograde-transported from the ER to the cytoplasm where they are degraded by the ubiquitin-proteasome system, while the remaining 10-20% is degraded via the lysosomal degradation pathway (T. Wang et al., 2002). Most \(RSI\) mutants exhibit this molecular mechanism (Iannaccone et al., 2006; Sergeev et al., 2010; Vijayasarathy et al., 2010a; T. Wang et al., 2002; Wu and Molday, 2003). One of the other smaller categories of mutations are those found in the leader sequence (L12H and L13P) that prevent entry of nascent polypeptide into ER and are mislocalized to the cytoplasm generating a null phenotype (Figure 1.6) (Vijayasarathy et al., 2010a; T. Wang et al., 2002; Wu and Molday, 2003). While intracellular retention represents the predominant pathological mechanism, other \(RSI\) mutants which retain the secretion property have also been identified. A category of these mutants affects the residues mandatory for higher order \(RSI\) assembly. Therefore, these mutants (Cys 59 and Cys 223) show secretion, but do
not form the functional octamer, thereby causing XLRS (Figure 1.6) (Wu and Molday, 2003). Although most mutations fall into one of the 3 above discussed categories, mutant R141H (located in spike 3 of discoidin domain) appeared to fold, assemble into a native-like oligomeric conformation and was secreted out of the cell. Yet, the mutation was pathogenic and caused XLRS. It is suggested that the substitution might interfere with the protein’s inability to bind to its receptors or interact with its ligands (Dyka et al., 2008; Wang et al., 2006).

Figure 1.6. Picture depicting the effect of various point mutations on retinoschisin synthesis, folding and oligomerization.

In general, mutations at the cysteine residues form aberrant disulphide bridges leading to non-native folding of the protein. Disease causing mutations in the spike regions and core barrel structure that do not involve cysteine residues also resulted in misfolded proteins, probably by interfering with hydrophobic or hydrogen bonding interactions necessary for proper protein folding. XLRS patients with mutations that caused maximum structural damage (affecting cysteine residues or change in the hydrophobic core) showed a considerable reduction in the b/a ERG ratio with age (Sergeev et al., 2010). Based on the mechanisms exerted by various classes of RS1
mutants, Wang et al proposed three possible mechanisms which can lead to XLRS: “a) Mutations that interfere with RS1 secretion b) Mutations that interfere with RS1 oligomerization c) Mutations that allow secretion and octamerization but affect the function of the protein” (Wang et al., 2006). Thus XLRS disease phenotype is known to arise primarily from the loss of functional RS1 protein.

1.3.11. Components of intraschisis cavity fluid

It is well known that retinoschisis patients develop cavities within the retinal layers and in some cases there is fluid accumulation within these cavities, the source and mechanism of which is not known. To date, there are only very few reports on the characterization of this schisis fluid collected from retinoschisis patient’s eye during surgical intervention. One of the studies on an enucleated eye showed the presence of numerous extracellular filaments adjacent to the schisis cavities, while a recent study on surgically extracted schisis fluid reported the elevated levels of two proteins, Cystatin C and Tenascin C using high performance liquid chromatography (HPLC) analysis. They are protease inhibitors that protect the tissue during injury and pathological conditions. The presence of these proteins suggests that inflammation and tissue damage are the underlying pathologies in retinoschisis. One of the interesting findings of this report is that these two proteins were detected in a male RS patient with RS1 mutation as well as in a female RS patient without mutation; indicating that mutated RS1 might not be the sole reason for the intraretinal structural changes, but also due to some unknown mechanism which is yet to be explored (Drenser et al., 2007; Joshi et al., 2006).
1.3.12. Phenotype-genotype correlation

XLRS is a progressive disorder with extensive interfamilial as well as intrafamilial variation in disease phenotype (Li et al., 2007; Wang et al., 2015). There are several studies on phenotype-genotype correlation, reporting the effect of age and mutation type on visual acuity, ERG and macular pathology (Eksandh et al., 2000; George et al., 1996; Pimenides et al., 2005; Shinoda et al., 2000). There was no significant correlation between mutation type and disease severity although visual acuity was found to reduce with increasing age (Pimenides et al., 2005; Renner et al., 2008). However, patients with nonsense, splice site or frameshift RS1 mutations were found to exhibit electronegative ERG and delayed flicker response, while missense mutations carrying patients showed wide ranging ERG abnormalities (Bowles et al., 2011; Sergeev et al., 2010).

Though various correlation strategies and hypotheses were applied in diverse ethnicities with a large sample size, the relationship between phenotype and genotype of XLRS patients still remain unclear. Therefore, only close follow-up and raising awareness about the disorder are the only alternatives for early identification and treatment of vision-threatening secondary complications.

1.3.13. Animal models of retinoschisis and gene therapy

Various groups have generated mouse models of XLRS to study the effect of externally introduced functional RS1 gene using adeno-associated virus (AAV) (Min et al., 2005; Ou et al., 2015a; Takada et al., 2008; Zeng et al., 2004). The clinical phenotype of RS1 knockout mouse models closely mimic human XLRS patients with disorganised retina involving schisis and negative ERG pattern (Zeng et al., 2004). On subjecting the animal to gene replacement therapy, RS1 was found to express in
almost all layers of retina restoring ERG b-wave and improving the retinal architecture (Byrne et al., 2014; Kjellstrom et al., 2007).

The advantages of considering gene therapy as a viable treatment option for XLRS are: i) The disease progression is slow and hence there is convenience in treating the disease even in older patients (Janssen et al., 2008) ii) Very minimal immune response against the injected RS1 has been observed (Ye et al., 2015) iii) The high molecular plasticity of synapses in XLRS retina is favourable in restoring the b-wave post therapy (Ou et al., 2015b). At the same time, gene therapy carries its own risk factors and limitations such as the possible persistence of AAV in the brain, restricting RS1 expression to appropriate retinal layers, immunogenicity and oncogenicity elicited by the AAV vectors and the dominant negative effect of certain mutations that may hinder the efficacy of the therapy (Sieving et al., 1993). However, the advantages of the treatment outweigh any disadvantages as gene therapy offers the best hope to cure genetic disorders. Further, convincing results in rabbits and nonhuman primates concerning the safety issues have supported the advancement of gene therapy for XLRS to clinical trials (Bush et al., 2016; Marangoni et al., 2016).

1.3.14. Retinoschisis treatment and management

So far, there are no treatment options or medication available to permanently halt the natural progression of schisis formation in XLRS patients. Management strategies include surgical repair of retinal detachment or administering drugs such as topical dorzolamide or oral acetazolamide and the use of low-vision aids (Apushkin and Fishman, 2006; Ghajarnia and Gorin, 2007; Iordanous and Sheidow, 2013; Yu et al., 2012). These drugs are carbonic anhydrase inhibitors which apparently reduce central macular thickness and cystic cavities in retinoschisis patients by decreasing
the level of subretinal fluids, thereby improving visual acuity. But, withdrawal of
topical dorzolamide resulted in the reappearance of cavities, necessitating life-long
treatment (Apushkin and Fishman, 2006). Likewise, surgical interventions such as
laser photocoagulation or intraocular vitrectomy to flatten the schisis cavities are
prone to failure or risk of developing retinal detachment (Brockhurst, 1970; Turut et
al., 1989). Further, none of these approaches address the biological need for the
normal retinoschisin protein that is fundamental in maintaining the structural integrity
of the retina. For this reason, gene therapy may be a potential therapeutic choice in the
future. It is an emerging treatment which has been proven successful in RS1 animal
models and is currently in human clinical trials.

1.3.15. Genetic testing and counselling

Molecular diagnostic testing assists in establishing the correct clinical
condition especially in ambiguous cases where the diagnosis of XLRS is uncertain. It
involves collecting blood sample from patients, extracting genomic DNA and
analyzing it for mutations in the disease gene/genes. In the case of XLRS patients,
only the RS1 gene is examined as it a monogenic disorder. This process is followed by
genetic counselling where the family’s health history or pedigree is thoroughly
assessed. XLRS is inherited in an X-linked manner and carrier females have 50% chance of transmitting the RS1 mutation in each pregnancy; all males who inherit the
mutation will be affected as RS1 is fully penetrant (Sieving et al., 1993). Affected
males pass the mutation to all their daughters but not to their sons. During
counselling, the patient and family members are informed about the purpose and
results of genetic test along with guidance about possible next steps such as carrier
testing, prenatal diagnosis, educational support and low-vision aids. This might help
the patients in managing their lifestyle and plan for annual clinical evaluation to avoid
secondary complications (Chu et al., 2013; Kaplan et al., 1991; Kłosowska-Zawadka et al., 2005).