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THE UBIQUITIN-PROTEASOME PATHWAY, OXIDATIVE STRESS AND AGE-RELATED MACULAR DEGENERATION:

THE GOOD, THE BAD AND THE UGLY

Universidade de Coimbra

2008

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para prestação de provas de Doutoramento em Ciências Biomédicas

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Chapter 1. Introduction

1.1 The human retina

The anatomy of the human retina, as well as that of other non-human primates, gives priority to high transparency and photoreceptor density. The retinal supply and oxygenation are sparse compared with the rate of oxygen consumption. Phototransduction and neural signaling are energy-consuming processes and have presumably developed in response to an evolutionary pressure for high-performance vision. The arrangement and anatomy of human retina is built around this critical function of high-acuity, high-performance vision.

The human retina is approximately 0.2 mm thick, and has an area of approximately 1100 mm^2. Each retina possesses about 200 million neurons. The optic disc, where neuronal cells merge to form the optic nerve, is the only area of the retina that is "blind" as it lacks photoreceptors. It appears as an oval white area of 3 mm^2. Temporal to this disc is the macula. At its center is the fovea, a pit that is most sensitive to light and is responsible for the sharp central vision. Humans possess only one fovea. Around the fovea extends the central retina for about 6 mm and then the peripheral retina. The edge of the retina is defined by the *ora serrata*. The length from one *ora* to the other (or macula), the most sensitive area along the horizontal meridian is about 3.2 mm.

The retina consists of three major types of cells: neurons, glial cells and blood vessels and most, if not all, of these cell types are affected to some degree in many retinal diseases including age-related macular degeneration. Retina is primarily a neuronal tissue. Indeed, neurons and glial cells comprise about 95%of the retinal mass. The glial cells of the retina, Müller cells and astrocytes, serve as support cells for the neurons and blood vessels.

The whole retina is frequently described as having ten layers (Figure 1), which are:

1. The internal limiting membrane.
2. The *stratum opticum*, or nerve fiber layer (NFL).
3. The ganglion cell layer.
4. The inner plexiform layer (IPL).

5. The inner nuclear layer (INL).
6. The outer plexiform layer (OPL).
7. The outer nuclear layer (ONL).
8. The external limiting membrane.
9. The layer of rods and cones.
10. The retinal pigment epithelium (RPE).

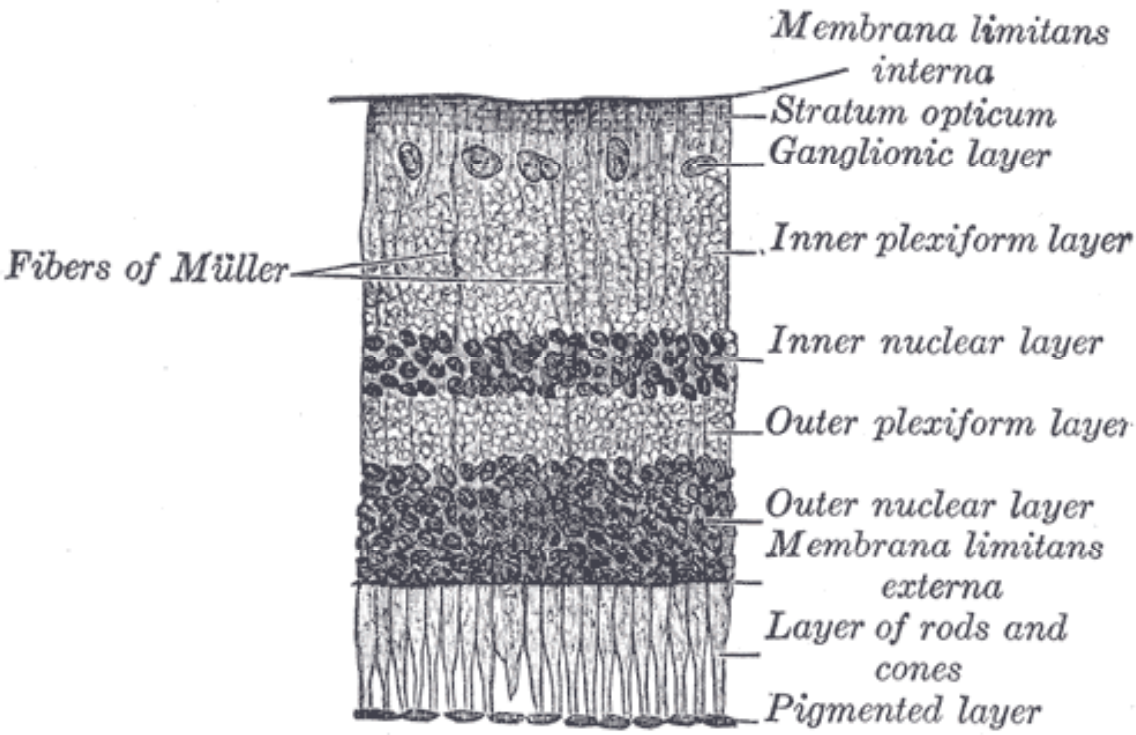

Figure 1 – Cross section of the human retina. A scheme of a retinal cross section showing the different retinal cells is shown (from Wolff, 1976).

1.1.1 Photoreceptor cells

The photoreceptor cells of the retina consist of the rods and cones. These are the primary neurons in the visual pathway. The photoreceptors differentiate longitudinally into two major divisions that extend from their cell body: the inner and outer segments. The inner segment contains the metabolic apparatus, while the outer segment's major function is the conversion of light into neuroelectrical energy. Histologically, the photoreceptors are classified into two types: rods and cones. The cones are present predominantly in the foveal region, whereas the rods are more

common at the periphery of the retina. Rods are responsible for sensing motion, contrast and brightness, while cones are necessary for color vision, fine detail perception and spatial resolution. In order to maintain the photoreceptor excitability, the outer segments are shed from the photoreceptors, allowing a constant renewal of photoreceptor outer segments (POS) (Young, 1967; Young and Droz, 1968; Young and Bok, 1969; Young, 1976; Bok, 1993; Nguyen-Legros and Hicks, 2000; Strauss, 2005). Cell bodies of rods and cones are connected to specialized synaptic terminals known as spherules and pedicles, respectively.

1.1.2 Bipolar cells

The bipolar cells in the retina constitute the second retinal neuron, connecting the photoreceptors to the ganglion cells. With their cell bodies in the INL, bipolar cells extend from the outer to the inner plexiform layer. In the foveal region, bipolar cells may receive input from just one cone, while in the periphery of the retina one bipolar cell can receive stimuli from up to 50-100 rods.

1.1.3 Horizontal cells

The horizontal cells receive their name because of the very broad horizontal extension of their processes. The two different types of horizontal cells, small field and large field, share the common anatomical feature of having their dendrites synapse only with cone pedicles while their axons synapse only with rod spherules. Each rod receives input from at least two different horizontal cells. Although the horizontal cells lie primarily in the outer lamina of the INL, they are present in almost all areas of the retina. However, they are absent in the fovea and parafoveal regions.

1.1.4 Amacrine cells

The amacrine cell bodies are located in the innermost zone of the INL. There are none in the fovea, while in the retinal periphery they constitute the majority of cells in the internal lamina of the INL. A considerably variety of types have been identified in the human retina. These have been named stratified and diffuse, the chief

criterion of differentiation being in the mode of arborization of their single process. Stratified subpopulations of amacrine cells have different connections within the retinal circuits, playing different modulatory roles in the physiological output of the retina (Strettoi and Masland, 1996).

1.1.5 Ganglion cells

The ganglion cell bodies form the distinct layer that lies external to the nerve fiber layer and internal to the IPL. All the axons from the ganglion cells merge to form the optic nerve, which has been shown to have approximately the same number of medullated nerve fibers, as there are ganglion cells. While ganglion cells form a six- to eight-cell-thick layer at the macula, there are no ganglion cells at the fovea or at the optic nervehead.

There are many forms of ganglion cells. All share the basic function of transmitting a signal from the bipolar cell to the lateral geniculate body. Variations among the ganglion cells are based on size, degree of arborization, spread of their dendritic processes into the bipolar synaptic field and their pattern of synaptic connections with amacrine cells. These anatomical differences among ganglion cells have been shown to correlate with their electrophysiologic functional specificity.

1.1.6 Müller cells

The Müller cells are the principal glial cells that maintain the structure of the retina. They are the largest cells in the retina, and with their shape similar to columnar epithelial cells, they extend all the way from the external to the internal limiting membrane. The Müller cells form tight junctions on contact both with other Müller cells and with neural cells. Here, the Müller cells form the external limiting membrane by a continuous even row of *zonulae adherentes*. This row of junctions constitutes a barrier to the passage of metabolites in and out of the retina.

1.1.7 Astrocytes

Although the Müller cells constitute the majority of retinal glial cells, the astrocytes (astroglia) are widely dispersed between the vasculature and the neurons. Morphological and cytoplasmic features of these cells allow them to be categorized into fibrous astrocytes, protoplasmic astrocytes and lemnocytes (Hogan and Feeney, 1963). Their pedicles are believed to constitute an important functional component of the blood-retinal barrier.

1.1.8 Microglia

The microglia distributes itself primarily to follow the two main capillary plexuses of the retina – one near the horizontal cells and the other near the amacrine cells. However, other microglial cells are found in all layers. It is not known whether these scavenger cells are present in the normal retina. It is possible that they enter the retina only after damage. Rather than playing a structural role, as does other glia, microglial cells play an active role in phagocytosis and destruction of phagocytozed material. In addition to this, microglial cells release proinflammatory mediators in response to a variety of stresses (Krady *et al.*, 2005). The microglia has, therefore, an important immunomodulatory role in the retina.

1.1.9 Retinal vasculature

The retina is unique in having the highest oxygen consumption per unit weight of any tissue in the human body. In fact, as Otto Warburg first reported in 1928, when normalized for its weight, the retina has an oxygen consumption ~50% higher than the brain or kidney (Warburg, 1928). To meet this metabolic demand, the retina has two separate circulatory systems. While the choroidal circulation nourishes its outer third, the inner two-thirds receive nutrition from the retinal circulation. These two systems have distinctly different anatomical and physiologic attributes. While the choroidal circulation is a high-flow and variable-rate circulation with free transfer of metabolites of all sizes between it and the surrounding tissues, the retinal circulation is a lower but more constant flow system with a higher rate of oxygen extraction. The

choroidal circulation functions as both a nutritive and cooling system for the eye, while the retinal circulation serves only the nutritive function for the inner two-thirds of the retina (Jakobiec, 1982).

Most of the oxygen consumption in the retina occurs in photoreceptors. One drawback of this vascular arrangement, at least on a timescale of decades, is that it constitutively exposes the retinal pigment epithelium and the distal outer segments to an oxygen concentration roughly equivalent to that found in arterial blood (Linsenmeier and Steinberg, 1986). This results in the accumulation of oxidative and photooxidative damage, the latter reflecting the unique vulnerability of the retina as the only neural tissue that is exposed directly to light and hence to chemically reactive oxygen species (ROS) generated by light absorption (Rattner and Nathans, 2006).

1.1.10 The retinal pigment epithelium (RPE)

The RPE is a monolayer of pigmented cells and forms part of the blood-retinal barrier (BRB) (Bok, 1993; Rizzolo, 1997; Marmorstein, 2001). The apical membrane of the RPE faces the photoreceptor outer segments, whereas its basolateral membrane faces Bruch's membrane, a semi-permeable exchange barrier that separates the RPE from the choroid (Strauss, 2005).

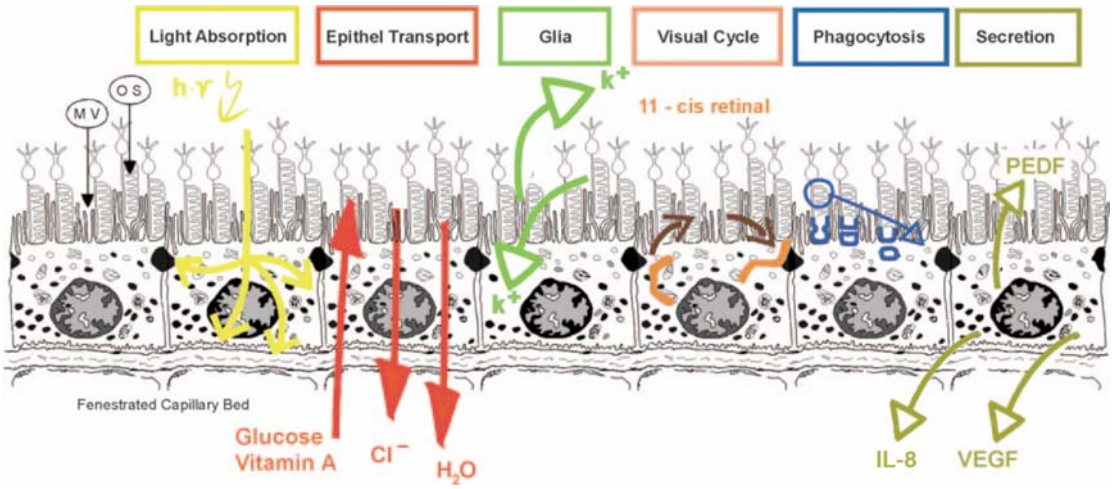

Figure 2 - Summary of retinal pigment epithelium (RPE) functions. PEDF, pigment epithelium-derived growth factor; VEGF, vascular epithelium growth factor; Epithel, epithelium. Adapted from (Strauss, 2005).

The RPE is essential for visual function, providing multiple functions that support normal photoreceptor function (Figure 2). In fact, the RPE and photoreceptors are together considered a functional unit. Mutations in genes that are expressed in the RPE, such as MerTK or RPE65, can lead to photoreceptor degeneration. On the other hand, mutations in genes expressed in photoreceptors, such as ABCR, can lead to degenerations of the RPE (Strauss, 2005).

The RPE pigmentation absorbs the light energy focused by the lens on the retina (Bok, 1993; Boulton and Dayhaw-Barker, 2001; Strauss, 2005).

The RPE transports ions, water, and metabolic end products from the subretinal space to the blood and takes up nutrients such as glucose, retinol and fatty acids from the blood, delivering them to photoreceptors (Strauss, 2005).

The RPE also plays an important role in the visual cycle of retinal. Photoreceptors lack *cis-trans* isomerase function for retinal and are unable to reisomerize all-*trans*-retinal, formed after photon absorption, back into 11-*cis*-retinal. To maintain the photoreceptor excitability, retinal is transported to the RPE, reisomerized to 11-*cis*-retinal and transported back to photoreceptors (Baehr *et al.*, 2003; Besch *et al.*, 2003; Thompson and Gal, 2003; Strauss, 2005). Furthermore, the voltage-dependent ion conductance of the apical membrane enables the RPE to stabilize ion composition in the subretinal space, which is essential for the maintenance of photoreceptor excitability (Steinberg *et al.*, 1983; Steinberg, 1985; Strauss, 2005). Another function in the maintenance of photoreceptor excitability is the phagocytosis of shed photoreceptor outer segments (Bok, 1993; Strauss, 2005). Photoreceptors are exposed to intense levels of light. This leads to accumulation of photo-damaged proteins and lipids. In addition, retinal itself can generate photo-oxidative radicals (Strauss, 2005). Thus, the concentration of light-induced toxic substances inside the photoreceptors increases over time (Beatty *et al.*, 2000; Strauss, 2005). To maintain the excitability of photoreceptors, the POS undergo a constant renewal process (Young, 1967; Young and Droz, 1968; Young and Bok, 1969; Young, 1976; Bok, 1993; Nguyen-Legros and Hicks, 2000; Strauss, 2005). In this renewal process, POS are newly built from the base of outer segments, at the cilium. The tips of the POS that contain the highest concentration of radicals, photo-damaged proteins and lipids are shed from the photoreceptors (Strauss, 2005). Through coordinated POS tip shedding and formation of new POS, a constant length of the POS is maintained. Shed POS are phagocytozed by the RPE. In the RPE, shed POS

are digested and important molecules, such as retinal or docosahexaenoic acid, are recycled and delivered back to photoreceptors in a manner comparable to the visual cycle (Bibb and Young, 1974; Strauss, 2005). The process of disk shedding and phagocytosis must be tightly coordinated between the RPE and the photoreceptors. A failure in this regulation would result in POS that are either too long or too short (Strauss, 2005).

In addition to these functions, the RPE is known to produce and to secrete a variety of growth factors, as well as factors that are essential for maintenance of the structural integrity of the retina and choriocapillaris (Witmer *et al.*, 2003; Strauss, 2005). The RPE produces factors that support survival of photoreceptors and ensure a structural basis for optimal circulation and supply of nutrients. This secretory activity of the RPE also plays an important role in establishing the immune privilege of the eye by secreting immunosuppressive factors (Streilein *et al.*, 2002; Strauss, 2005).

Altogether, RPE cells perform a variety of complex functions in the retina. Therefore, it is not surprising that deregulation of the RPE has a critical impact on retinal function and may lead to loss of visual function and loss of vision.

1.2 Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in the western world in the population over 60 years of age (Beatty *et al.*, 2000; Rattner and Nathans, 2006), and its prevalence is likely to rise as a consequence of increasing longevity (Beatty *et al.*, 2000).

AMD is characterized by degenerative changes in the macula, the central region of the retina bearing the highest concentration of photoreceptors. Accordingly, this degeneration of the macula leads to loss of central vision. The earliest clinical manifestation and pathological feature of AMD is the formation of drusen, extracellular deposits of glycoproteins, lipids, and cellular debris located between Bruch's membrane and the RPE (Ambati *et al.*, 2003a; Rattner and Nathans, 2006; Patel and Chan, 2008). A few small drusens can be found in healthy individuals over the age of 50, but the presence of large or numerous drusen confers significant risk for AMD (Klein *et al.*, 1992; Klein *et al.*, 1997).

Clinically and histologically, AMD is generally classified into two major subtypes: dry AMD and wet AMD (Figure 3). Dry AMD progresses more slowly and

manifests with drusen, geographic atrophy of RPE, and photoreceptor dysfunction and degeneration. Wet AMD, on the other hand, is more debilitating and often follows after dry AMD. The key feature of wet AMD is choroidal neovascularization (CNV), the growth of new blood vessels from the choroid into the region underlying the RPE or extending past the RPE into the subretinal space and retina. This choroidal neovascularization can lead to leakage of blood into the subretinal space, which, along with RPE atrophy and photoreceptor degeneration, leads to vision loss (Ambati *et al.*, 2003a; Rattner and Nathans, 2006; Patel and Chan, 2008).

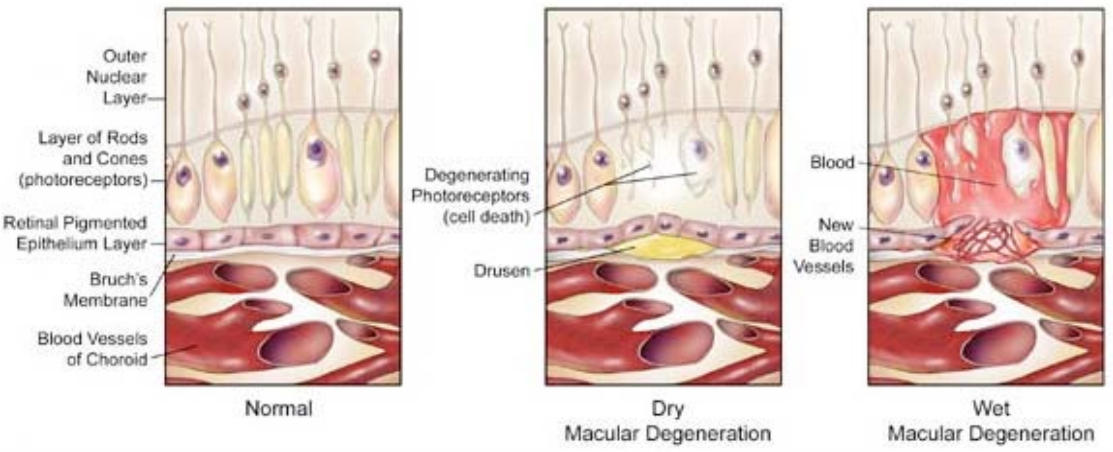

Figure 3 – Comparison between the dry and wet forms of Age-Related Macular Degeneration (AMD) (adapted from *National Eye Institute*, *National Institutes of Health*[1]).

Risk factors for AMD include smoking, body mass index, diet, and light exposure. A number of studies have linked genetics, oxidative stress, RPE senescence, hypoxia, and many other factors to AMD. Still, the exact etiology and pathogenesis of the disease remain largely unclear (Taylor *et al.*, 1990; Beatty *et al.*, 2000; Ambati *et al.*, 2003a; Clemons *et al.*, 2005; Scholl *et al.*, 2007)

[1] http://www.nei.nih.gov

1.2.1 Oxidative stress and age-related macular degeneration

Oxidative stress, which refers to cellular damage caused by reactive oxygen species (ROS), has been implicated in many diseases, including age-related macular degeneration (Beatty *et al.*, 2000; Liang and Godley, 2003). ROS include free radicals, hydrogen peroxide, and singlet oxygen.

An increasing body of literature indicates that development of AMD is related to dysfunction of the RPE (Boulton *et al.*, 2004; Zarbin, 2004; Zhou *et al.*, 2005). This dysfunction may be related with the higher susceptibility of the RPE to oxidative stress. In fact, a variety of studies suggest that RPE cells can be readily damaged following exposure to an oxidative insult (Beatty *et al.*, 2000; Liang and Godley, 2003).

The retina is an ideal environment for the generation of ROS for several reasons. The oxygen consumption by the retina is much higher than by any other tissue. Physiologically, RPE cells phagocytose and digest POS. This unique phagocytic function of RPE provides an additional oxidative burden since the shed outer segments are extremely rich in polyunsaturated fatty acids (PUFA), which can be readily oxidized and which can initiate a cytotoxic chain-reaction, producing an abundance of ROS. In fact, the process of phagocytosis by the RPE is itself an oxidative stress and results in the generation of ROS (Beatty *et al.*, 2000; Liang and Godley, 2003).

Age-related accumulation of lipofuscin in RPE is another source of oxidative stress. Lipofuscin is a mixture of non-degradable protein-lipid aggregates derived from the ingestion (phagocytosis) of POS (Sparrow and Boulton, 2005). A2E is the major fluorophore of lipofuscin and acts as a photosensitizer to generate ROS inside the cells upon exposure to blue light (Rozanowska *et al.*, 1995; Rozanowska *et al.*, 1998; Sparrow and Boulton, 2005).

Consistent with a role of oxidative stress in the development of AMD, several studies indicate that exposure of RPE cells to oxidative stress results in an increase of angiogenic cytokines and growth factors, as well as in phenotypes associated with development of AMD (Higgins *et al.*, 2003; Schlingemann, 2004; Zhou *et al.*, 2005; Kannan *et al.*, 2006).

To cope with these toxic oxygen species, the RPE has three lines of defense (Boulton and Dayhaw-Barker, 2001). The first line is absorption and filtering of light.

The RPE contains a complex composition of various pigments that are specialized to absorb different wavelengths (Beatty *et al.*, 1999; Beatty *et al.*, 2000; Beatty *et al.*, 2001). General light absorption occurs via melanin in melanosomes. Photoreceptors also absorb light and contain important pigments such as the carotenoids lutein and zeaxanthin (Handelman *et al.*, 1988; Handelman *et al.*, 1992; Bone *et al.*, 1997; Landrum *et al.*, 1999). These pigments have a protective function by absorbing blue light (Beatty *et al.*, 1999; Beatty *et al.*, 2000; Beatty *et al.*, 2001). The second line of defense is made by antioxidants, both enzymatic and nonenzymatic. For example, the RPE contains high amounts of superoxide dismutase (Newsome *et al.*, 1990; Miceli *et al.*, 1994; Frank *et al.*, 1999) and catalase (Miceli *et al.*, 1994; Tate *et al.*, 1995). The RPE also accumulates carotenoids, such as lutein and zeaxanthin (Beatty *et al.*, 2000; Beatty *et al.*, 2001), ascorbate, α-tocopherol, and β-carotene (Beatty *et al.*, 2000), which are nonenzymatic antioxidants. In addition, RPE cells contain glutathione and melanin, which itself can function as an antioxidant. The third line of defense is the cell's physiological ability to repair damaged DNA, lipids and proteins (Strauss, 2005).

However, with increasing age, the RPE antioxidant capability appears to diminish. In fact, an age-dependent reduction in α-tocopherol, one of the most important antioxidants, and melanosomes has been reported in the RPE (Feeney, 1978; Feeney-Burns *et al.*, 1984; Friedrichson *et al.*, 1995). Thus, it is likely that aging RPE cells are more susceptible to oxidative damage (Liang and Godley, 2003). In support of this hypothesis, recent studies have shown that mice deficient in Cu, Zn-superoxide dismutase, an antioxidant enzyme, display features typical of AMD in humans (Imamura *et al.*, 2006; Justilien *et al.*, 2007; Dong *et al.*, 2009).

1.2.2 Inflammation and age-related macular degeneration

Emerging pathological evidence indicates that many age-related diseases such as AMD, atherosclerosis, arthritis and certain types of cancer are associated with an inflammatory component (Lavrovsky *et al.*, 2000; Zarbin, 2004; McGeer *et al.*, 2005; Chung *et al.*, 2006; Donoso *et al.*, 2006; Moshfeghi and Blumenkranz, 2007). The activation of redox-sensitive transcription factors may be involved in triggering the

expression of proinflammatory cytokines, thus providing a link between oxidative stress and inflammation upon aging (Lavrovsky *et al.*, 2000; Chung *et al.*, 2006).

The complement system is a central part of innate immunity and in its normal setting is aimed at recognizing and eliminating invading microorganisms. The complement system is divided into three main pathways: classical, lectin and alternative. The classical pathway is activated largely by immune complexes (antibody-antigen complex); the mannose-binding lectin pathway is activated primarily by mannose and *N*-acetyl glucosamine residues that are abundant on bacterial cell surfaces and the alternative pathway is initiated by a variety of activating substances including microbial surfaces and polysaccharides. The activation of these pathways results in a proinflammatory response including generation of a membrane attack complex (MAC), which mediates cell lysis, release of chemokines to attract neutrophils and macrophages to the site of damage, and enhancement of capillary permeability (Walport, 2001a, 2001b; Patel and Chan, 2008). A deregulation of the complement, particularly the alternative pathway, results in defective recognition of microorganisms (or other toxic agents) and leads to an accumulation of toxic activation products (Zipfel *et al.*, 2006).

Several studies have suggested a role for the complement system in AMD. Components of the complement, such as C5 and MAC consisting of C5b-9, have been identified in drusen from human eyes, including eyes of patients with AMD (Mullins *et al.*, 2000). In addition, C3a and C5a have been localized to drusen, RPE cells and Bruch's membrane in human AMD (Nozaki *et al.*, 2006; Patel and Chan, 2008). Other studies have demonstrated the presence of immune complexes, complement, and/or complement regulatory proteins in drusen and RPE (Hageman *et al.*, 1999; Johnson *et al.*, 2000; Johnson *et al.*, 2001; Crabb *et al.*, 2002; Patel and Chan, 2008).

Complement factor H (CFH) is a negative regulator of the complement system. CFH acts by inhibiting several steps of the alternative pathway and by promoting degradation of activated complement components (Alsenz *et al.*, 1985; Patel and Chan, 2008). CFH binds heparin on cell surfaces to prevent complement-mediated damage to heparin and the cells bearing them. It also binds C-reactive protein (CRP) to inhibit CRP-mediated activation of the alternative pathway in response to damaged tissue (Rodriguez de Cordoba *et al.*, 2004; Prosser *et al.*, 2007; Patel and Chan, 2008). Recent studies have shown that polymorphisms in CFH and complement Factor B (CFB), a positive regulator of the alternative pathway of the

complement, are strongly associated with increased risk to develop AMD (Edwards *et al.*, 2005; Hageman *et al.*, 2005; Haines *et al.*, 2005; Klein *et al.*, 2005; Gold *et al.*, 2006), highlighting the role of the complement in the pathogenesis of this disease.

Interestingly, a recent report suggests that oxidative stress in RPE can trigger the activation of the complement (Zhou *et al.*, 2006). Moreover, complement activation is associated with enhanced expression of interleukin-8 (IL-8) (Fukuoka and Medof, 2001; Fukuoka *et al.*, 2003). IL-8 is an important inflammatory and angiogenic cytokine (Brat *et al.*, 2005) and its increased expression has also been reported in RPE cells that were fed with oxidized POS (Higgins *et al.*, 2003), supporting the link between oxidative stress and inflammation. The increased expression and secretion of IL-8 may account, at least in part, for the inflammatory reactions during development of AMD (Higgins *et al.*, 2003; Kalayoglu *et al.*, 2005).

In addition, there is further evidence, including animal models of disease, for the involvement of inflammation in the pathogenesis of AMD. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine involved in macrophage infiltration into sites of inflammation (Oppenheim *et al.*, 1991). In RPE, this chemokine has been suggested to play a role in drusen clearance (Duvall and Tso, 1985). A recent study showed that deficiency in MCP-1 or its cognate receptor Ccr-2 causes AMD-like features in mice, including accumulation of lipofuscin and drusen beneath the RPE, photoreceptor atrophy and choroidal neovascularization (CNV) (Ambati *et al.*, 2003b). The impaired macrophage recruitment results in the accumulation of complement components, such as complement regulatory proteins and IgG in the retina. Because deposition of complement-related proteins and IgG precedes the accumulation of drusen and lipofuscin, it is likely that the AMD-like pathology is due, at least in part, to complement activation and immune complex deposition (Ambati *et al.*, 2003b).

On the other hand, several studies have demonstrated accumulation of macrophages in histologic specimens from patients with AMD, especially in regions of RPE atrophy, breakdown of Bruch's membrane and CNV (Lopez *et al.*, 1991; Dastgheib and Green, 1994; Penfold *et al.*, 2001; Grossniklaus *et al.*, 2002). This macrophage accumulation may serve a pathological role in AMD (Kamei *et al.*, 2007).

Therefore, it is still quite unclear whether macrophages play a pathologic or a restorative/adaptive role in AMD.

1.3 The Ubiquitin Proteasome Pathway

The ubiquitin- proteasome pathway (UPP) is a major proteolytic pathway present in virtually every eukaryotic cell where it regulates vital biological and physiological processes. Cellular processes such as cell division, differentiation, signal transduction, quality control and protein trafficking are all regulated to some extent by the UPP (Glickman and Ciechanover, 2002; Ciechanover, 2003; Shang and Taylor, 2004; Welchman *et al.*, 2005). The importance of UPP in normal physiology is tremendous and disruption of components of UPP has been implicated in a great variety of human diseases, including age-related diseases such as Alzheimer's disease (Hope *et al.*, 2003), Parkinson's disease (Dawson and Dawson, 2003), diabetic retinopathy (Fernandes *et al.*, 2006) and cataract (Jahngen-Hodge *et al.*, 1992a; Shang *et al.*, 1997a; Shang *et al.*, 2001b; Dudek *et al.*, 2005).

There are numerous forms of ubiquitin and ubiquitin-like proteins present in the cells. In fact, more than 10 ubiquitin-like modifiers have been identified to date (Kerscher *et al.*, 2006). Ubiquitin was described as "Darwin's phosphate" (R. Hampton, FASEB meeting 2001, Vermont, USA) and the total number of genes involved in ubiquitin and ubiquitin-like reactions is comparable to the number of genes involved in phosphorylation/dephosphorylation reactions (Lorick *et al.*, 2005). Although this was initially recognized as a selective degradation pathway, more recent evidence showed that UPP has a number of non-degradative functions (Welchman *et al.*, 2005) that have a significant role in numerous biological processes and that are also implicated in a number of human diseases.

1.3.1 Ubiquitin-dependent Protein Degradation

Degradation of a protein by the UPP involves two discrete and successive steps: first, the substrate is tagged by covalent attachment of multiple ubiquitin molecules. In a second stage, the polyubiquitinated protein is degraded by the 26S proteasome complex with the release of free ubiquitin that can be used in subsequent ubiquitination cycles (Pickart, 2001a; Glickman and Ciechanover, 2002). Removal of ubiquitin from substrates prior to degradation is also a regulated process that is carried out by deubiquitinating enzymes (Pickart, 2001a; Glickman and Ciechanover, 2002). Ubiquitin is a small peptide of 76 aminoacids, which is highly conserved from yeast

to humans. Indeed, there are only three aminoacid substitutions between yeast and human ubiquitin (P19S, E24D, A28S). This makes ubiquitin the most highly conserved protein identified in eukaryotes so far (Ozkaynak *et al.*, 1984).

Conjugation of ubiquitin to substrates proceeds via a three-step mechanism. Initially, the ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent reaction to generate a high-energy thiol ester intermediate. In the second step, one of a variety of ubiquitin conjugating enzymes (E2) transfers the activated ubiquitin moiety from the E1 to the substrate that is generally associated to one of a member of the ubiquitin ligase family (E3) (Figure 4). In some cases, ubiquitin can be transferred from the E2 to the E3 and subsequently from the E3 to the substrates (Pickart, 2001a; Glickman and Ciechanover, 2002). This sequence of reactions is repeated until a chain of typically four or more ubiquitins is attached to the substrate (Beal *et al.*, 1996b; Pickart, 1997b; Lam *et al.*, 2002b). The most common and best-characterized fate of a polyubiquitinated protein is its translocation to a large proteolytic complex, the 26S proteasome, where it is degraded. Although this is the canonical view of the system and indeed, the process responsible for the degradation of numerous cytosolic proteins, it should be emphasized that sometimes only one ubiquitin is attached to the protein in one or more aminoacid residues. This results in the production of a monoubiquitinated protein. Proteins can be monoubiquitinated in different residues resulting in the formation of a multi-monoubiquitinated protein (Hicke and Dunn, 2003; Haglund and Dikic, 2005). In most cases the final destination of such proteins is not degradation but rather targeting to specific subcellular compartments, including the endocytic pathway (Hicke and Dunn, 2003; Haglund and Dikic, 2005).

The formation of a polyubiquitinated protein is generally initiated by the formation of an isopeptide bond between the C-terminus of the last glycine residue of ubiquitin (G76) and the $\varepsilon\tilde{-}NH_2$ group of an internal lysine residue in the substrate, to generate a covalent isopeptide bond (Glickman and Ciechanover, 2002). Less frequently, ubiquitin can be conjugated to the terminal NH_2 group of the substrate (Ciechanover and Ben-Saadon, 2004).

The subsequent addition of other ubiquitin molecules involves frequently (but not always) the formation of a peptide bond between the G76 at the C-terminus of a newly activated ubiquitin and the lysine 48 of the previously attached ubiquitin molecule. This sequence of reactions leads to the formation of polyubiquitin chains of

different sizes (Pickart, 1997b). The polyubiquitin chain is, then, recognized by the 26S proteasome and the substrate is degraded. As the substrate is unfolded and enters the pore of the catalytic chamber of the 20S proteasome, deubiquitinating enzymes present in the 19S caps of the proteasome remove polyubiquitin chains from the substrates, thus regenerating free ubiquitin (DeMartino and Slaughter, 1999; Pickart and Cohen, 2004; Demartino and Gillette, 2007).

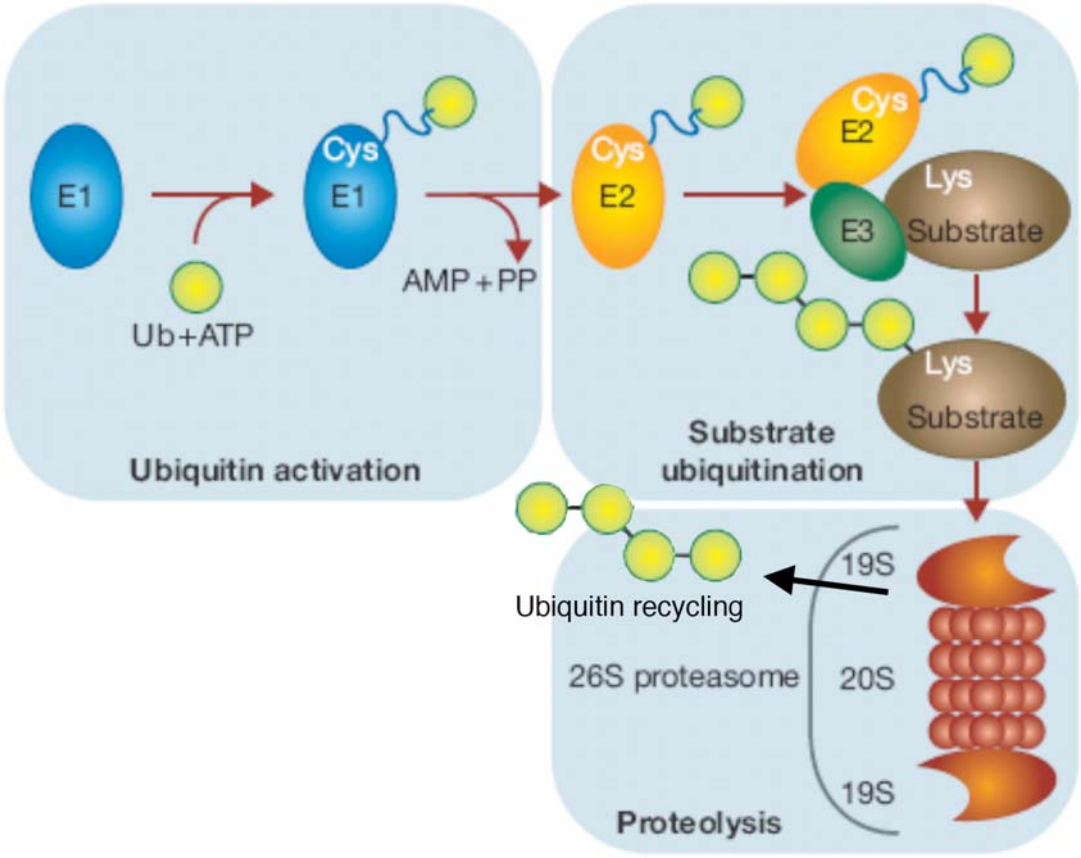

Figure 4 - The ubiquitin–proteasome pathway (UPP). A cascade of enzymatic reactions leads to ubiquitination of lysine residues of the substrate. Adapted from (Meusser *et al.*, 2005).

Ubiquitin contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and all of them can be used for chain formation, resulting in ubiquitin chains of different lengths and shapes *in vitro* and *in vivo* (Johnson *et al.*, 1995; Peng *et al.*, 2003; Pickart and Fushman, 2004; Kim *et al.*, 2007; Ikeda and Dikic, 2008). Whereas Lys48-linked polyubiquitin chains are well established as a signal for targeting substrates to the 26S proteasome (Glickman and Ciechanover, 2002; Pickart and Fushman, 2004), Lys63-linked chains are involved in

nonproteolytic events, such as DNA repair, signal transduction and receptor endocytosis in vivo (Pickart and Fushman, 2004; Haglund and Dikic, 2005; Chen and Sun, 2009). Nevertheless, some studies suggest that Lys63-linked polyubiquitin chains may support proteasomal degradation *in vitro* (Hofmann and Pickart, 2001; Kim *et al.*, 2007; Saeki *et al.*, 2009). However, the *in vivo* significance of these observations is still poorly understood. Much less is known about the roles of unconventional polyubiquitin chains linked through Lys6, Lys11, Lys27, Lys29 or Lys33. Polyubiquitin chains linked via Lys6, Lys11 or Lys48 all bind to the proteasomal subunit Rpn10/S5a with similar affinities (Baboshina and Haas, 1996), suggesting that these chains may play a role in proteasomal degradation. In fact, recent studies imply that Lys11-linked chains are involved in proteasome-dependent protein degradation of cell cycle regulators (Kirkpatrick *et al.*, 2006; Jin *et al.*, 2008) and endoplasmic reticulum-associated degradation (ERAD) substrates (Xu *et al.*, 2009). Lys6 linkages catalyzed by the BRCA1/BARD1 E3 may regulate DNA repair (Nishikawa *et al.*, 2004). Both Lys27 and Lys33 may be built during stress response by U-box-type E3 ligases (Hatakeyama *et al.*, 2001). Lys29-linked chains may be important in ubiquitin fusion degradation (Johnson *et al.*, 1995).

The specificity and selectivity of the UPP was unclear for many years and some specific aspects of the system selectivity still remain to be elucidated. It is currently accepted that the high selectivity of the system is primarily the result of the presence of specific signals in substrate proteins and the action of both ubiquitin ligases (E3s) and related ancillary proteins (Glickman and Ciechanover, 2002). In most instances, substrates are not constitutively nor directly recognized by ligases. In some cases, the ligase must be activated by undergoing some posttranslational modification to yield an active form that recognizes the substrates. In other instances, it is the substrate that must undergo specific modifications that renders it a target for ubiquitination (Glickman and Ciechanover, 2002). Ubiquitin ligases are therefore key players in determining the system specificity. The first E3s to be identified were very diverse and apparently unrelated. The first big family of E3s to be recognized presented a 350-aminoacid residue sequence *H*omologous to *E*6-AP (E6-Associated Protein) *C*arboxyl *T*erminus and are currently known as HECT domain E3s (Huibregtse *et al.*, 1995). This domain contains a conserved cysteine residue to which the ubiquitin moiety is transferred from E2s (Scheffner *et al.*, 1995). The first ligase of the HECT domain family to be identified was E6-AP. E6 is the oncoprotein of the

high-risk human papillomaviruses (HPVs). The E6 oncoprotein binds to the cellular ubiquitin ligase E6-AP and targets the tumour suppressor protein p53 for rapid degradation (Scheffner *et al.*, 1993) rendering cells more susceptible to tumour development. In the absence of the viral ancillary protein, E6-AP targets other cellular proteins for degradation. However, the physiological relevance of these interactions remains to be shown (Scheffner and Staub, 2007). Mutations in E6-AP are implicated in diseases such as Angelman syndrome, a severe form of mental and motor retardation (Kishino *et al.*, 1997). Other important members of the HECT domain family of ubiquitin ligases include NEDD4, Npi1/Rsp5, Smurf1 and Smurf2 (Scheffner and Staub, 2007).

Virtually all ubiquitin ligases, or E3s, that do not belong to the HECT domain family, belong to the large family of RING (*R*eally *I*nteresting *N*ew *G*ene) finger proteins (Glickman and Ciechanover, 2002; Pickart, 2004). The mammalian RING finger domain family is very large and it is currently accepted that many of its members are, indeed, E3s (Lorick *et al.*, 1999). RING fingers consist of domains of approximately 70 aminoacid residues containing a pattern of conserved cysteine and histidine residues that bind zinc and stabilize the typical globular conformation of these proteins (Lovering *et al.*, 1993). Interestingly, it is the spacing of zinc ligands, rather than any primary sequence, that is conserved in the RING finger family, suggesting that RING fingers are likely to function as structural elements in a complex, rather than presenting catalytic activity (Pickart, 2001a). Indeed, it was shown that unlike HECT domain E3s, RING fingers proteins do not have a catalytic function in the ubiquitination process (Glickman and Ciechanover, 2002). Apparently, RING fingers function as scaffolds that bring other proteins together including E2s and substrates, thus facilitating ubiquitin transfer (Zheng *et al.*, 2000). The RING finger E3s can form either single or multisubunit proteins of various sizes and composition. The multisubunit RING finger family of E3s is often subdivided into three major groups: APC (*A*naphase *P*romoting *C*omplex), SCF (*S*kp1-*C*ul1-*F*-box protein) and CBC (*C*ul2-Elongin *B*-Elongin *C*) (Petroski and Deshaies, 2005). The architecture and organization of E3s of the SCF and CBC group is similar in many ways. In both cases, a central 100-residue RING finger protein called Rbx1 acts as a scaffold that strongly interacts with a subunit belonging to the cullin protein family (Cul1/Cdc53 for the SCF and Cul2 in CBC) (Pickart, 2001a; Petroski and Deshaies, 2005). Rbx1 also assists in recruiting the cognate E2s. The APC has a central RING

finger protein and displays a core ligase activity in conjunction with the ubiquitin conjugating enzymes Ubc4 or UbcH10 (Tang *et al.*, 2001). This ubiquitin ligase is involved in degradation of cell cycle regulators such as cyclins and securin (Page and Hieter, 1999; Peters, 2006).

The single subunit members of the RING finger E3s family recognize the ubiquitination signals in their specific substrates through domains that are structurally distinct from the RING finger. For the multisubunit RING finger E3s, substrate recognition is delegated in a different protein of the complex. For example, in the SCF E3s, substrate specific F-box proteins are recruited to SCF complexes through the adaptor protein Skp1which recognizes the eponymous F-box motif (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Deshaies, 1999; Pickart, 2001a). The SCF family is usually involved in the degradation of signal- and cell cycle-induced phosphorylated proteins (Deshaies, 1999). The SOCS-box protein, like the F-box protein in the SCF complex, is a variable component of the CBC complex and is thought to play a role in substrate recognition. VHL is a SOCS-box protein that was identified as the product of a gene that is frequently mutated in individuals with von Hippel–Lindau disease(Seizinger *et al.*, 1988). The protein pVHL is recruited to the complex through interactions with the heterodimeric adaptor Elongin B/C (Pickart, 2001a). This complex is involved in degradation of HIF-1α (Maxwell *et al.*, 1999; Kamura *et al.*, 2000; Ivan *et al.*, 2001b; Jaakkola *et al.*, 2001a). The existence of substrate-binding (F-box), cullin and adaptor protein families (Skp, elongins) together with other data indicates that E3 specificity can be reprogrammed, among other factors, by changing the identity of the substrate recognition subunit (Pickart, 2004; Petroski and Deshaies, 2005).

Examples of monomers or homodimers that contain both the RING finger domain and the substrate binding site in the same molecule include Mdm2 (that targets p53 for proteasomal degradation) (Lorick *et al.*, 1999; Boyd *et al.*, 2000; Geyer *et al.*, 2000), Ubr1/E3α (Reiss and Hershko, 1990; Kwon *et al.*, 1998), Parkin(Shimura *et al.*, 2000) and c-Cbl (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999).

A third family of ubiquitin ligases, distinct from the HECT and RING families, has recently been identified: the U-box family (Hatakeyama and Nakayama, 2003). Proteins belonging to this family contain a domain of ~70 aminoacids (the U-box domain), similar to the RING finger, but lack the zinc-binding domain(Aravind

and Koonin, 2000). These proteins are able to mediate ubiquitination in conjunction with E1 and E2 and in the absence of other E3 components (Hatakeyama *et al.*, 2001), suggesting that they possess ubiquitin ligase activity. This family of ubiquitin ligases has been implicated in the quality control system that underlies the cellular stress response to the intracellular accumulation of abnormal proteins (Hatakeyama and Nakayama, 2003).

1.3.2 Diversity of degradation signals in the UPP

Recognition of a protein substrate depends not only on the specificity of E3s and ancillary proteins, but also on intrinsic signals present in the substrate that determine its half-life and its susceptibility to ubiquitination and subsequent proteolysis (Ravid and Hochstrasser, 2008). In the late 80s, a set of short-lived substrates of the UPP was identified that contained specific aminoacids in its N-terminus. This led to the idea that the stability of a protein was largely determined by the nature of its N-terminus aminoacid (Bachmair *et al.*, 1986; Pickart, 2004). This rule, for protein susceptibility to ubiquitin-dependent degradation, is still known as the N-end rule. Subsequent studies revealed that, for some substrates, there are elements that are both necessary and sufficient to determine its ubiquitination (Varshavsky, 1997; Pickart, 2004). These elements that were eventually found in different locations in the protein structure are called degrons and can consist of various aminoacid motifs or sequences.

Recognition by the N-end rule involves direct binding of the substrates to the ubiquitin ligase E3α/Ubr1 (Varshavsky *et al.*, 2000). E3 α/Ubr1 has two N-end rule recognition sites: one for substrates with basic aminoacids at the NH_2 terminal and another one for substrates with hydrophobic residues at the NH_2 terminal (Reiss *et al.*, 1988; Kwon *et al.*, 1998). A third site in the ligase is involved in targeting non-N-end rule substrates.

One of the first aminoacid sequences to be identified in substrates that render them prone to UPP-dependent degradation was the destruction box found in mitotic cyclins and many other substrates of the APC (Glotzer *et al.*, 1991; Deshaies, 1999; Koepp *et al.*, 1999; Page and Hieter, 1999). The destruction box is a short sequence, RXALGXIXN, where the arginin and leucine residues are key determinants of specificity. This sequence, in the primary structure of the protein, appears to be

sufficient to recruit the appropriate E3s and to promote degradation of substrates. However, it also appears that the signal conformation may play a role in the efficient binding of E3s to substrates (Pickart, 2001a). Over the years, it also became apparent that phosphorylation is a common signal that targets proteins for degradation. Many of such proteins contain the so-called PEST regions. More recently, however, it became clear that, although such proteins are often degraded by the ubiquitin system, the PEST regions provide phosphorylation sites to regulate the accessibility of the degradation signals.

Physiological recognition of degrons is not trivial and is subjected to a complex and highly coordinated sequence of events and interactions. Interactions between an E3 and a degron can be modulated by a variety of mechanisms, including posttranscriptional modifications, that serve to link ubiquitination to other cellular events (Deshaies, 1999; Deshaies and Ferrell, 2001). For example, the cell cycle needs to be precisely regulated and this involves the phosphorylation of Cyclin-Dependent Kinases (CDKs) that is required to trigger ubiquitin-dependent degradation of CDK regulators. These regulators include (but are not limited to) the mammalian G1 cyclins D and E and the mammalian CDK inhibitor $p27^{KIP1}$ (Glickman and Ciechanover, 2002). Other regulatory proteins that need to be phosphorylated prior to ubiquitination include the transcriptional regulators IκBα and β-catetin (Kornitzer and Ciechanover, 2000), as well as the NF-κB precursor p105 (Orian *et al.*, 2000; Heissmeyer *et al.*, 2001).

Another important example of the posttranscriptional modification of a protein, that is particularly relevant in the context of neovascularization associated with AMD, is the degradation of the Hypoxia Inducible Factor-1 (HIF-1α). Indeed, degradation of HIF-1α occurs continuously under normoxic conditions and requires prior oxygen-dependent hydroxylation of two specific proline residues that triggers recognition by a cullin-based E3 that has the von-Hippel Lindau (VHL) tumour suppressor protein as its specificity subunit (Ivan *et al.*, 2001b; Jaakkola *et al.*, 2001a).

1.3.3 Degradation of ubiquitinated proteins

The second major step in the UPP consists on the recognition and degradation of ubiquitinated substrates by the proteasome. The proteasome holoenzyme, also referred to as the 26S proteasome, is a 2.5 MDa complex made up of two copies. Each copy comprises, at least, 32 different subunits that are highly conserved among all eukaryotes. The overall structure can be divided into two major subcomplexes, the 20S complex or core particle (CP) that contains the proteolytic activity and a regulatory particle (RP) also referred to as 19S cap complex or PA700 in mammals and μ particle in *D. melanogaster* (Glickman *et al.*, 1999; Gorbea *et al.*, 1999; Pickart and VanDemark, 2000) (Figure 5). The 20S particle is a cylindrical structure composed of four stacked heptameric rings. In yeast and higher eukaryotes, the rings are formed by 14 distinct subunits designated by α or β. The active sites reside within the β subunits, which provide the catalytic N-terminal threonine residues. However, only three of the seven different β subunits have free N-terminal threonines, which means that the proteasome will present a total of 6 active sites (Groll *et al.*, 1997). The active sites are buried in a central chamber isolated from the external solvent. Access to the catalytic core is very restricted and substrates must be unfolded to fit to an axial pore of 13 Aº in diameter (Pickart and VanDemark, 2000). The compartmentalization of the active sites inside a restricted chamber prevents indiscriminate degradation of proteins and confers some selectivity to the system. However, it is the 19S regulatory particle that confers both selectivity and specificity to the 26S proteasome activity. Biochemically, the 20S core presents only ATP-independent peptidase activity. However, the assembled 26S proteasome presents a variety of activities and functions including polyubiquitin chain recognition and binding, nucleotidase activity, isopeptidase activity, unfoldase and endopeptidase activity (Groll *et al.*, 1997; Glickman *et al.*, 1999). Thus, binding of the 19S regulatory particle to the 20S proteasome confers selectivity and specificity of the proteasome towards ubiquitinated proteins.

Indeed, ubiquitinated substrates need to be recognized, at a first stage, by specific protein subunits present in the 19S cap. Subsequently, the protein needs to be unfolded and fed to the catalytic chamber of the proteasome. As substrates are translocated through the central pore of the 20S core particle, ubiquitin is removed

and recycled for further use in other ubiquitination cycles (Glickman and Ciechanover, 2002; Pickart, 2004; Pickart and Cohen, 2004). These and other activities are all located in specific subunits present at the 19S caps and many of them require ATP that is used up by the ATPases present in these regulatory subunits.

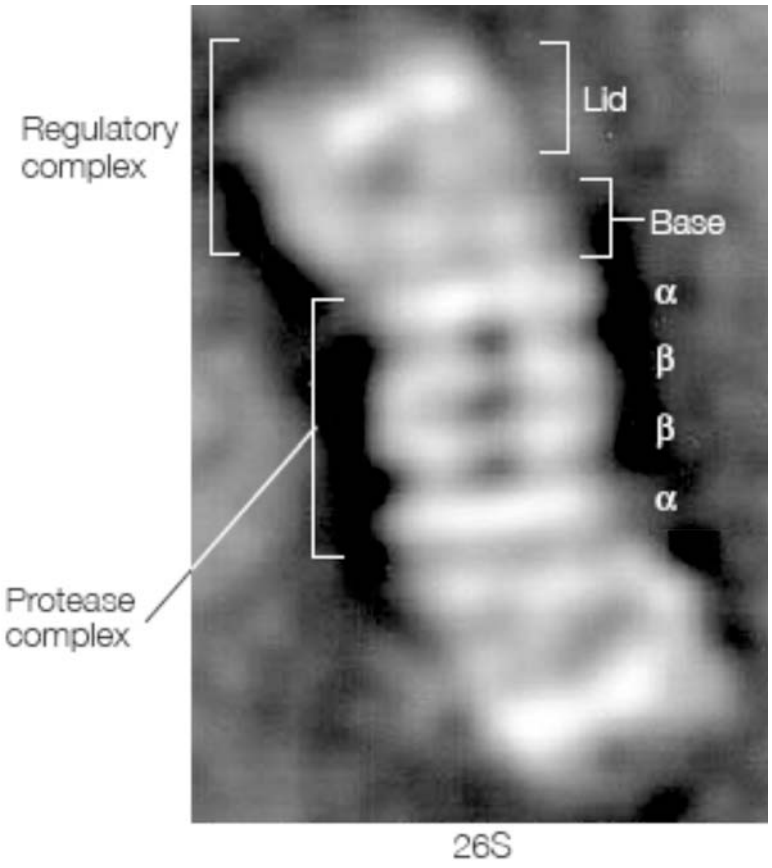

Figure 5 - Electron-microscopy image of a eukaryotic proteasome. Adapted from (Pickart and Cohen, 2004).

1.3.4 UPP and signal transduction

In addition to a well established signal for protein degradation, ubiquitin is now recognized as an important regulator in many cellular processes, including (but not limited to) gene transcription, DNA repair and replication, virus budding, endocytosis and signal transduction (Haglund and Dikic, 2005; Welchman *et al.*, 2005; Kirkin and Dikic, 2007; Chen and Sun, 2009).

Virtually all the signal transduction pathways in the cell are regulated by ubiquitin to some extent. Some of these pathways include the nuclear factor κB (NF-

κB) (Chen, 2005; Chen and Sun, 2009), HIF-1α (Ivan *et al.*, 2001a; Jaakkola *et al.*, 2001b), the mitogen-activated protein kinase (MAPK) pathway (Laine and Ronai, 2005), the Notch pathway (Bray, 2006), the Wnt pathway (Logan and Nusse, 2004; Angers *et al.*, 2006), and the transforming growth factor-β (TGF-β) pathway (Glasgow and Mishra, 2008), among others.

Two of these pathways, the NF-κB and HIF-1α pathways, are particularly relevant in the context of AMD. In fact, the altered activity of these transcription factors may underlie some of the pathophysiological changes associated with the development and progression of the disease, such as CNV and the increased inflammatory response.

1.3.4.1 UPP and activation of NF-κB

NF-κB is critical in the processing of a variety of proinflammatory signals, including the up-regulation of numerous cytokines (interleukin-1, 6, 8 and tumour necrosis factor - TNF), proangiogenic factors such as VEGF, inducible enzymes such as cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), cell adhesion molecules (intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1), stress proteins, anti-apoptotic factors (Bcl-2, survinin) and immune system receptors (Baldwin, 1996).

NF-κB is a generic term for a dimeric transcription factor formed by the hetero- or homodimerization of subunits belonging to a family of five members. Three of these proteins (p65, RelB and c-Rel) contain transactivation domains and two other proteins, p50 and p52, are expressed as the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). These precursors require posttranslational processing and do not contain transactivation domains. In fact, the UPP is also involved in the limited processing of the precursor protein p100 to yield the active subunit p52 (Chen, 2005; Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). In quiescent cells, NF-κB is located in the cytoplasm in an inactive form bound to a labile inhibitor molecule called IκBα, or other structurally related proteins (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008).

NF-κB can be activated in response to a variety of different stimuli including viruses, growth factors, antigens, radiation and chemotherapeutic drugs. However, the best-studied mechanism of activation of NF-κB involves the response to the proinflammatory interleukin-1β (IL-1β) (Figure 6).

The various pathways that lead to activation of NF-κB appear to converge in the phosphorylation of IκB by a cascade of complexes that include TAK1 (TGF-β-Activated Kinase-1) and the IκB Kinase complex (IKK). Activation of these kinases results in phosphorylation of IκB (Karin and Ben-Neriah, 2000). The phosphorylated motif docks with a specific ubiquitin ligase complex, resulting in conjugation of ubiquitin to IκBα (Carter *et al.*, 2005). Polyubiquitinated IκBα is then degraded by the 26S proteasome, NF-κB is released and can translocate to the nucleus where it activates some of the genes described above (Maniatis, 1999; Karin and Ben-Neriah, 2000) (Figure 6). The IKK complex is composed of three subunits: α, β and γ. It is the subunit β (IKKβ) that is responsible for IκB phosphorylation and NF-κB activation in response to proinflammatory stimuli, whereas IKKα targets distinct cellular substrates in an alternative pathway for NF-κB induction (Carter *et al.*, 2005).

The molecular events that activate the kinases that phosphorylate IκB, targeting its degradation, are fairly complex and often involve different molecules that respond to different stimuli (Scheidereit, 2006; Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). For example, when TNF-α binds to TNF receptor-1, the receptor oligomerizes and associates with a complex that contains the ubiquitin ligase TRAF2 (TNF-Receptor-Associated Factor-2). Working in association with the heterodimeric E2 Ubc13-MMS2, TRAF2 attaches Lys63-linked polyubiquitin chains to the receptor interacting protein (RIP) (Meylan and Tschopp, 2005). This triggers the TAK1 complex to bind to the membrane-receptor complex through the Lys63-linked polyubiquitin chains and results in IκBα phosphorylation and its subsequent ubiquitination (Sun and Chen, 2004).

In addition to its well-established role in targeting IκBα for degradation via Lys48-linked polyubiquitin chains, the UPP also regulates the NF-κB pathway at multiple stages through nonproteolytic functions. In fact, as mentioned above, the assembly of Lys63-linked polyubiquitin chains regulates several steps in the activation of NF-κB by serving as a signal to recruit proteins harbouring ubiquitin-binding domains, thereby bringing together ubiquitinated proteins and ubiquitin

receptors, ultimately leading to IκBα degradation and NF-κB activation (Chen and Sun, 2009).

In addition, a recent study highlights the importance of linear polyubiquitination in NF-κB activation (Tokunaga *et al.*, 2009). The authors found that the LUBAC (linear ubiquitin chain assembly complex) ligase complex, composed of two RING finger proteins, conjugates a head-to-tail-linked polyubiquitin chain to NEMO, leading to NF-κB activation (Tokunaga *et al.*, 2009) .

The switching off of this signalling cascade depends, among other factors, on deubiquitination. The importance of deubiquitination of proteins in switching off this signalling cascade is well illustrated by the human tumour condition cylindromatosis. Cylindromatosis is due to a mutation in the tumour suppressor CYLD, which cleaves Lys63-linked polyubiquitin chains from TRAF2 (Wilkinson, 2003). More recently, the protein A20 has emerged as a dual-catalysis enzyme that down-regulates the NF-κB system (Wertz *et al.*, 2004). A20 cleaves the Lys63-linked polyubiquitin chains from RIP and adds Lys48-linked polyubiquitin chains to RIP, which results in its proteasomal degradation. In addition to deubiquitination, the termination of NF-κB activity can also be achieved by degradation of its subunits. In fact, it was recently reported that PDLIM2 (PDZ and LIM domain 2) negatively regulates NF-κB activity, acting as a nuclear ubiquitin E3 ligase that promotes p65 polyubiquitination and its subsequent degradation by the proteasome (Tanaka *et al.*, 2007).

There are a number of compounds that were shown to interfere with the activity of NF-κB in cells. These include antioxidants, glucocorticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), cytokines, peptides inhibiting the nuclear localization of NF-κB and proteasome inhibitors such as MG132, PS-341 and PS-519. Stabilization of NF-κB by proteasome inhibitors, for example, results in the blockade of NF-κB activation and prevents the actions of NF-κB within the cells (Palombella *et al.*, 1994). An alternative method of controlling NF-κB activity is via the modulation of phosphorylation of the DNA-binding subunits that can regulate the interactions between the transcription factor and regulatory proteins (Schmitz *et al.*, 2001).

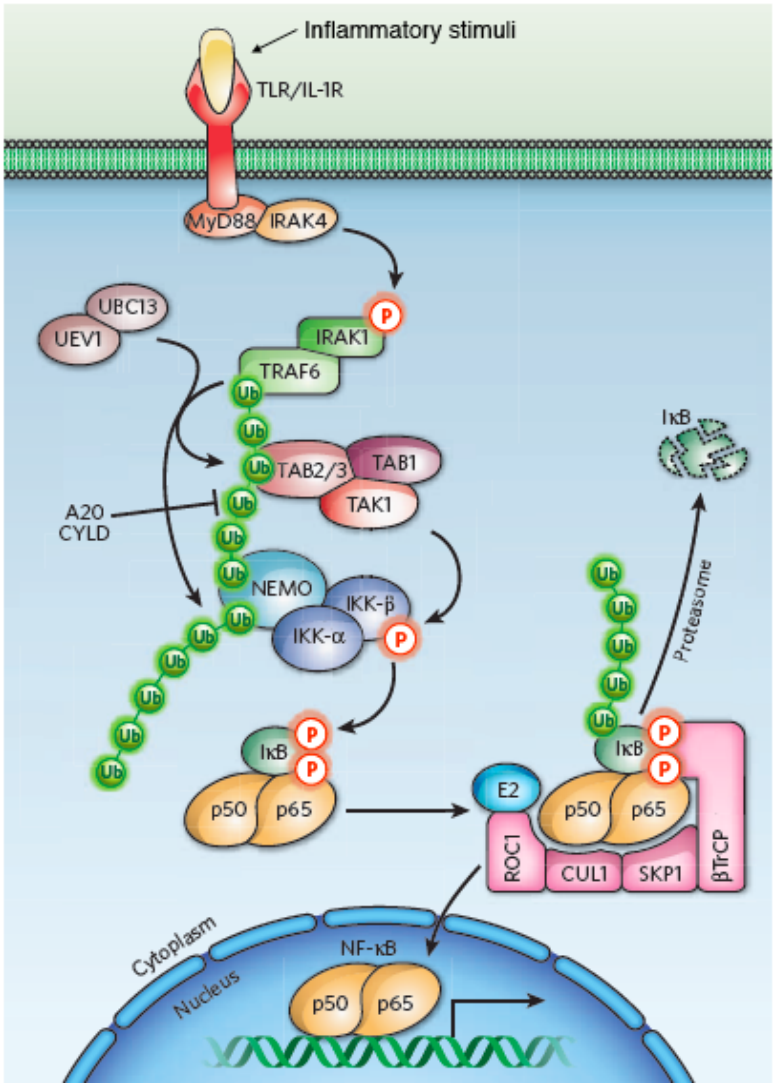

Figure 6 – Regulation of the NF-κB signalling pathway. Adapted from (Bhoj and Chen, 2009).

1.3.4.2 UPP and degradation of HIF-1α

HIF is an oxygen-dependent transcription factor that plays a crucial role in the cell response to hypoxia (Pugh and Ratcliffe, 2003). This transcription factor induces the expression of more than 60 proteins involved in angiogenesis, glycolysis, glucose transport, erythropoiesis and others (Zagorska and Dulak, 2004).

HIF is an αβ-heterodimer, first recognized as a DNA-binding factor that mediates hypoxia-induced activity of the erythropoietin 3' enhancer (Semenza and Wang, 1992; Wang *et al.*, 1995). Both HIF-α and HIF-β subunits exist as a series of isoforms encoded by distinct genetic loci. HIF-1β subunits are constitutive nuclear

proteins, whereas HIF-α subunits are induced by hypoxia. Among three HIF-α isoforms, HIF-1α and HIF-2α appear closely related and each one is able to interact with hypoxia response elements (HREs) to induce transcriptional activity (Pugh and Ratcliffe, 2003). In contrast, HIF-3α appears to be involved in negative regulation of the response, through an alternatively spliced transcript termed inhibitory PAS domain protein (Makino *et al.*, 2001; Pugh and Ratcliffe, 2003). The abundance of HIF-1α subunits is mainly regulated by the rate of degradation. Under non-hypoxic (normoxia) conditions, HIF-1α is subject to oxygen-dependent prolyl hydroxylation (Ivan *et al.*, 2001b; Jaakkola *et al.*, 2001a), which is required for binding of the von Hippel-Lindau tumour suppressor protein (VHL), the recognition component of an ubiquitin ligase complex, which targets HIF-1α for ubiquitin-dependent proteasomal degradation (Maxwell *et al.*, 1999) (Figure 7). HIF-1α contains two sites for hydroxylation, Pro402 and Pro564, within the oxygen-dependent degradation (ODD) domain and each site contains a conserved LXXLAP motif (Masson *et al.*, 2001a).

Ubiquitination of HIF-1α, as of most proteins, requires primarily formation of a polyubiquitin chain through lysine 48 of ubiquitin. Subsequently, the polyubiquitinated HIF-1α needs to be translocated to the 26S proteasome for degradation. Under hypoxic conditions, oxygen becomes limiting for prolyl hydroxylase activity (Epstein *et al.*, 2001) and ubiquitination of HIF-1α is inhibited (Sutter *et al.*, 2000). As a result, HIF-1α accumulates, dimerizes with HIF-1β, and activates transcription of target genes, including VEGF (Pugh and Ratcliffe, 2003).

Although the classic model (described above) indicates that HIF-1α ubiquitination and subsequent degradation only occurs under normoxic conditions, recent studies challenge this model by suggesting that HIF-1α can be ubiquitinated and degraded by the 26S proteasome even under hypoxic conditions (Cheng *et al.*, 2007; Koh *et al.*, 2008). Interestingly, hypoxia induces SUMOylation of HIF-1α, which promotes its binding to VHL, through a proline hydroxylation-independent mechanism, leading to its ubiquitination and degradation (Cheng *et al.*, 2007).

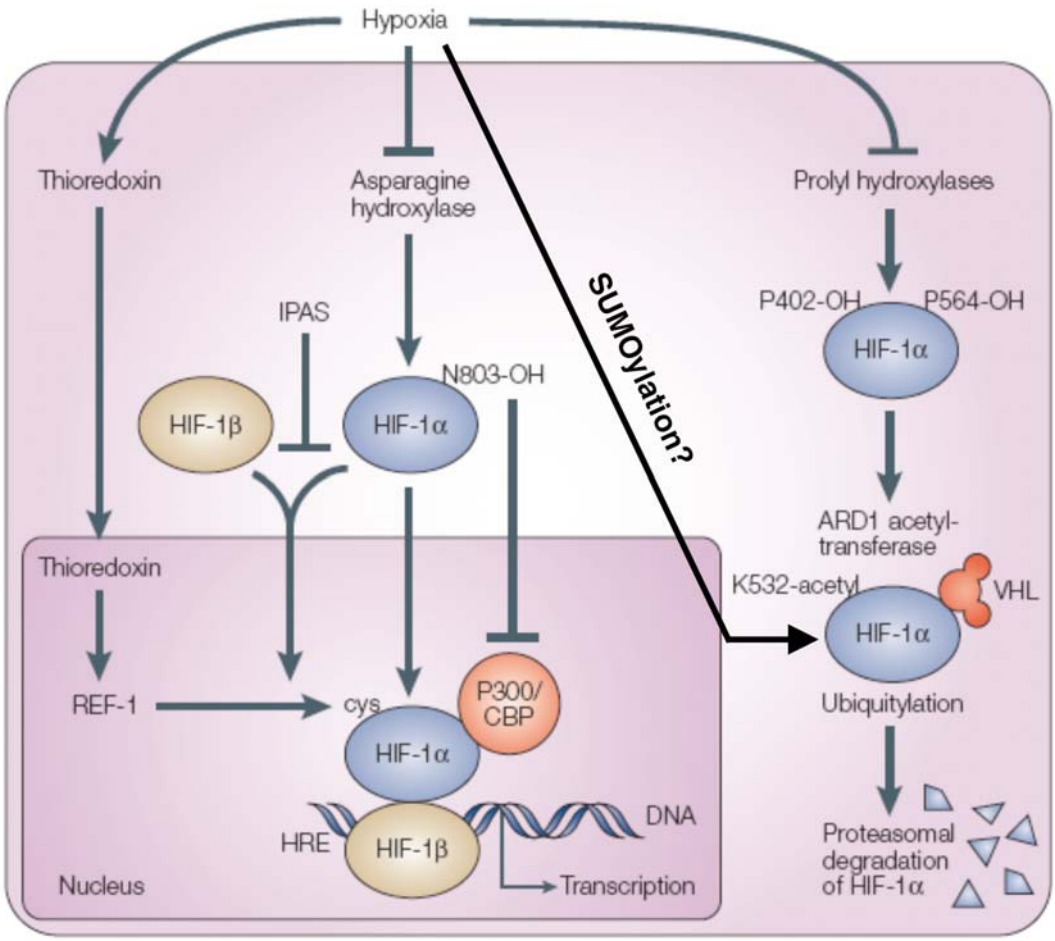

Figure 7 - Mechanisms of HIF-1α regulation under aerobic and hypoxic conditions. Adapted from (Giaccia *et al.*, 2003).

The role of SUMOylation in the regulation of HIF-1α stability and activity under hypoxia remains controversial. Although SUMOylation has recently been shown to constitute a signal for ubiquitination and degradation of HIF-1α (Cheng *et al.*, 2007), previous studies implicate SUMO in the activation of transcription through HIF-1α stabilization (Bae *et al.*, 2004; Carbia-Nagashima *et al.*, 2007).

Further studies are therefore required to clarify this issue and to elucidate the multiple regulatory pathways that control HIF-1α under hypoxia.