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Autophagy regulating kinases as potential therapeutic targets for age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of central vision loss in the elderly in the developed countries. The number of AMD patients will double during the next decades due to increasing number of aged people. Chronic oxidative stress, inflammation and accumulation of protein-rich deposits both in the retinal pigment epithelium lysosomes and under the retinal pigment epithelium herald the onset of AMD. The disease can be divided into dry and wet AMD forms. The dry form of the disease is more prevalent accounting for up to 90% of all cases. Continued intraocular injections are the current treatment strategy to prevent progression of wet AMD. It is a major challenge to develop new drugs that could prevent or at least ease the symptoms of the increasing population of AMD patients. Since AMD pathology is clearly associated with accumulated protein deposits, the autophagy clearance system might represent a potential future therapeutic target for AMD as is thoroughly discussed here.

Age-related macular degeneration as a degenerative eye disease

Age-related macular degeneration (AMD) is a complex disorder of the eye and the primary cause of irreversible blindness among elderly people in the developed countries [1]. The disease affects the macula enriched with cones in the central area of the retina. AMD has a multifactorial etiology; it results in a progressive loss of fine and colour vision. Globally, 50 million individuals are affected by AMD with the direct cost estimated at US\$255 billion [2,3]. In the USA alone, 1.8 million individuals are afflicted by AMD and the number is estimated to double over the coming decades [4].

AMD is characterized by degeneration of the macular **retinal pigment epithelium** (RPE) cells, Bruch's membrane and choriocapillaris [5]. AMD etiology is known to be complex, involving both a strong genetic component and several environmental risk factors such as aging, smoking, obesity, arteriosclerosis, hypertension and hypercholesterolemia [6,7]. AMD has a progressive character and it may develop into a dry (non-exudative) or wet (exudative) form. Both forms share certain common clinical features – primarily, the degeneration of RPE that is characterized by either hypo- or hyper-pigmentation of the cells and the accumulation of extracellular drusens between the RPE and the inner collagenous layer of the Bruch's membrane (**FIGURE 1**) [8]. The dry form of the disease is more prevalent and it accounts for up to 90% of all cases.

In this form, most patients experience a slow, gradual but progressive and inexorable decline in central vision over a period of months or years. Geographic atrophy (GA) is an advanced form of dry AMD being characterized by extensive loss of the RPE, as well as its neighboring photoreceptors (PR) and choriocapillaris and it is associated with severe central visual loss. In some cases, the dry form may be converted into the wet form, in which the visual loss is abrupt, occurring over a time frame of days or weeks. Choroidal neovascularization sprouting into the retina is a diagnostic sign of wet AMD. Central visual loss is due to photoreceptor damage resulting from RPE degeneration, RPE detachment, retinal swelling, accumulation of retinal exudates, choroidal neovascularization, sub-retinal hemorrhaging and, finally, fibrovascular disciform scarring [8].

To date, no effective cure is available for dry AMD, although antioxidants and omega-fatty acids have been shown to have some preventive properties in certain AMD patient groups [9–11]. VEGFs are potent regulators of angiogenesis in the wet AMD form, and these proteins can be targeted in therapy via their inhibitors: ranibizumab, bevacizumab and VEGF Trap [12]. Large, multicenter, randomized clinical trials have been conducted exploring the safety and efficacy of anti-VEGF treatments [13–16]. However, the need for lifelong intraocular injections are not attractive for patients and, furthermore, these drugs only slow the progression of visual loss. Therefore,

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Figure 1. Schematic and photograph presentation of dry and wet age-related macular degeneration. Drusens (white arrows) are present in both age-related macular degeneration (AMD) types, but neovascularization (red arrow) from choriocapillaris is a diagnostic sign for wet AMD. Usually, hemorrhages (black arrow) are observed in wet AMD. AMD: Age-related macular degeneration; BM: Bruch's membrane; Ch: Choriocapillaris; PR: Photoreceptors; RPE: Retinal pigment epithelium.

new drugs are needed to prevent or at least slow development of the most severe AMD forms.

AMD pathogenesis essentially involves a chronic oxidative stress, increased accumulation of lipofuscin in the lysosomes of RPE cells and extracellular drusen formation and chronic inflammation in response to many hereditary and environmental risk factors. The capacity to prevent the accumulation of cellular cytotoxic protein aggregates and the ability to remove them via **autophagy** decline in aged cells suffering degenerative changes, particularly in postmitotic RPE cells. This article examines autophagy as a possible therapeutic target, that is, finding ways to enhance aggregate clearance and prevent RPE cell degeneration and AMD development.

Central role of RPE in AMD pathogenesis

Degeneration of the RPE cells is one of the most important hallmarks of AMD (**FIGURE 1**) [17]. Clinically, it can be observed by pigment dispersion, accumulation of intracellular autofluorescent lysosomal lipofuscin and extracellular drusen deposits. In postmitotic cells such as the RPE cells, the increased oxidative stress during the aging process may be lethal [8,18–22]. The RPE cells constitute a polygonal monolayer between the choroidal capillaries and the rod and cones in the neurosensory retina. They have

many important and crucial functions such as transport of metabolites and nutrients between photoreceptors and choriocapillaris, absorption of light energy, production of growth factors for photoreceptors, regulation of homeostasis of the ionic environment, phagocytosis of the retinal outer segments, regulation of the vitamin A-based visual cycle and the creation of the blood–retinal barrier [23].

Constant oxidative stress is a well-known inducer of the protein misfolding and aggregation and this phenomenon is considered to underlie the development of many age-related degenerative diseases including AMD [7]. Ultimately, the pathogenesis of AMD is strongly associated with oxidative stress, resulting in RPE cell damage and degeneration due to the presence of reactive oxygen species (ROS) [8,22,24]. The macula is a region with a high metabolic rate, continuously occurring redox reactions and a high partial pressure due to the adjacent layer of choriocapillaris [25]. High levels of polyunsaturated fatty acid lipids derived from retinal outer segments and the extensive exposure to light increase retinal oxidative stress. In young RPE cells, there are many functional protective mechanisms including a molecular chaperone repair system, antioxidant production and active proteolytic machines, particularly those participating in autophagy [8]. However, in aged RPE cells, the constant exposure to photo-oxidation-generated ROS and phagocytosis of the lipid-rich outer segments expose RPE cells to exceptionally high oxidative stress leading to impaired proteolysis and lipofuscin accumulation in lysosomes [7,8,26]. The routine phagocytosis of consumed photoreceptor outer segments and their degradation in lysosomes are two of the critical maintenance functions performed by RPE in order to preserve vision. Lipofuscin includes the fluorophore *N*-retinylidene-*N*-retinylethanolamine, which is a compound that disturbs lysosome membrane stability and inhibits mitochondrial respiration. Subsequently, it is able to promote the unfolding of intracellular proteins [27–30]. In addition to protein damage and aggregation, the amount of oxidized phospholipids is known to be elevated in the macula region of AMD patients [31,32]. These findings highlight the important role of oxidative stress in the pathogenesis of AMD.

Regulation of autophagy by mTOR

Autophagy, literally, ‘self eating’, is an intracellular degradation process that clears long-lived proteins, aggregated proteins, lipid droplets and

Key Terms

Age-related macular degeneration: Retinal disease that usually affects older adults and results in a loss of fine and color vision in the center of the visual field.

Retinal pigment epithelium: Pigmented layer of the retina between the neurosensory retina and choroid. It is predominantly retinal pigment epithelium cells that primarily degenerate in age-related macular degeneration.

Autophagy: Catabolic process involving the degradation of a cell's own components through the lysosomal machinery.

organelles from the cytoplasm in all eukaryotic cells [33]. It is activated as an adaptive response to environmental stress (e.g., nutrient deprivation, hypoxia, oxidative stress, exposure to xenobiotics) to promote cell survival through the recycling of precursors (free fatty acid, amino acids, nucleotides) derived from the degradation of endogenous cellular components (**FIGURE 2**) [34,35]. The autophagy process begins with the formation of isolation membranes called omegasomes that enlarge into phagophores. The phagophores then elongate and engulf a portion of the cytosol to form mature double membrane autophagosomes that can engulf portions of cytoplasm containing oligomeric protein complexes and organelles. The autophagosomes fuse with lysosomes and their contents are then degraded by lysosomal enzymes. A failure of autophagy in aged postmitotic cells, including RPE cells, can result in the accumulation of aggregate-prone proteins, cellular degeneration and, finally, cell death [8,33–36]. Pharmacological induction of autophagy can enhance the clearance of intracytoplasmic aggregate-prone proteins and ameliorate cellular and tissue pathology [37]. Autophagy, generally, can be viewed as a housekeeping mechanism, and it has crucial significance for the maintenance of normal cellular homeostasis [33–35].

Several autophagy-related proteins (Atg) co-ordinate the autophagosome formation that are composed of four subgroups: the Atg/ULK complex; the cPtdIns3K/VPs34 complex Atg12–Atg5–Atg16L and Atg8/LC3 conjugation system; and conserved Atg9/mAtg9 transmembrane proteins [38–44]. Atg9 regulates the early phase of autophagosome creation apparently by collecting part of the membranes required for membrane extension. In response to inhibition of mTOR (see later), the ULK complex regulates PtdIns3K complex leading to phagophore formation from which autophagosomes are generated. The Atg12–Atg5–Atg16L conjugation system contributes to the phagophore membrane elongation. LC3-I is transformed to the PE-conjugated form (LC3-II), which is inserted into the membrane of the autophagosome [45,46]. P62/sequestosome protein binds to LC3-II and acts as facilitator of the degradation of ubiquitinated proteins, thereby linking proteasomal and lysosomal clearance systems [47,48]. Finally, the created autophagosomes fuse with lysosomes forming autolysosomes after which lysosomal enzymes are released to degrade the engulfed cytoplasmic material.

There is a growing body of evidence suggesting that regulation of the signaling pathways involved in autophagy could well provide novel pharmaceutical therapeutic strategies for the prevention or treatment of human diseases [49,50]. AMD is of particular interest, since autophagy impairment has been shown in the AMD patient material and RPE cell culture models [51–53]. The classical pathway that regulates autophagy acts through the mTOR, a protein kinase that plays a key role as an energy, nutrient, growth factor, stress and redox sensor to increase protein synthesis and to decrease autophagy (**FIGURE 2**) [54,55]. mTOR is a ubiquitously expressed, 289-kDa serine/threonine kinase belonging to the PI3K-related kinase family. The mTOR consists of the central regulatory catalytic core of two functionally distinct multiprotein complexes, mTORC1 and mTORC2. Rheb, Raptor, PRAS40-P, mLST8 and Deptor are regulatory units for mTORC1, while Sin1, Rictor, mLST8 and Protor are the corresponding units for mTORC2 [56]. Rapamycin, an anti-fungal and immunosuppressant agent, binds to its intracellular receptor FKBP12, which becomes attached to mTOR and interferes with its signalling [55]. Recent findings have revealed that the rapamycin-induced mTORC1 inhibition and activation of autophagy can slow the aging process in mice (**FIGURE 2**) [56]. At present, there is convincing evidence that the modulation of mTORC1 function may be a potential target for the development of therapeutics for neurodegenerative diseases including AMD [55–59]. Another way to enhance autophagy is in a mTOR-indirect way, that is, the process regulated by inositol-1,4,5-triphosphate levels. The mTOR-independent autophagy induction is targeted by many drugs such as lithium, sodium valproate, carbamazepine, verapamil and trehalose [55,60].

Interestingly, RPE degeneration has been associated with increased sensitivity and enhanced activity of mTORC1 in experimental AMD studies [61,62]. Rapamycin, an inhibitor of the mTORC1, prevents the development of harmful AMD-related aging signs in RPE cells. It has been shown that mTOR regulates the detrimental dedifferentiation and hypertrophy of RPE cells exposed to oxidative stress, while rapamycin treatment can prevent these effects and preserve photoreceptor functions [61]. Moreover, rapamycin has been observed to inhibit choroidal neovascularisation by inhibiting the function of VEGF-A in animal models [62,63]. Co-culture assays of RPE and endothelial cells revealed

Key Term**Sirtuins or Sir2 proteins:**

Group of proteins that have either histone deacetylase or monoribosyltransferase activities.

Figure 2. Overview of the autophagy signaling pathways. Oxidative stress, reactive oxygen species production and hypoxia activates AMPK and autophagy clearance. In young cells, this process is working highly effectively, but during aging autophagy clearance is estimated to be suppressed and this leads to a detrimental aggregation of waste products and on to the encountered cellular degeneration in age-related macular degeneration. AICAR, metformin and rapamycin induce autophagy by activating AMPK and/or inactivating mTOR, suppressor of autophagy.

AICAR: 5-aminoimidazole-4-carboxamide ribonucleoside.

that rapamycin is an effective VEGF inhibitor and it can reduce sprouting of endothelial cells [64]. The expression of mTOR has been shown to be higher in the central part of retinal layer, which might be one explanation account for greater protein aggregation in aging macula [8,65].

Regulation of autophagy by AMPK

AMPK is a master sensor of energy and redox status that maintains cellular energy homeostasis. In addition, AMPK regulates autophagy, mitochondrial biogenesis and disposal, cell polarity, cell growth and proliferation. The heterotrimeric protein AMPK has α , β and γ subunits [66]. The α subunit is the catalytic subunit with β and the γ being the regulatory subunit is; however, in mammals, each subunit has many subtypes that are expressed at different intensities depending on the tissue [67]. For example, the catalytic α subunit comprises the $\alpha 1$ and $\alpha 2$ isoforms, β subunit has the $\beta 1$ and $\beta 2$ isoforms, and γ subunit has $\gamma 1$, $\gamma 2$ and

$\gamma 3$ isoforms. The α subunit of AMPK is phosphorylated by several upstream kinases, such as CaMKK β , LKB1 and TAK1 [68–70]. The β subunit provides myristoyl- and carbohydrate-binding sites and gathers α and γ subunits together [71,72]. Interestingly, degraded oligosaccharides, compounds that are estimated to be involved in RPE aging and AMD development, can suppress AMPK activity [73,74]. The γ subunit of AMPK is a central sensor to detecting the AMP:ATP ratio in cells [67].

As discussed previously, autophagy is switched off by TORC1. AMPK directly phosphorylates multiple components in the mTORC1 pathway. For instance, AMPK inhibits mTORC1 through phosphorylation of TSC2 and Raptor (Figure 2) [55]. Activation of the ULK1 and 2 is the final step prior to autophagosome formation. It has been recently shown that ULK1 and ULK2 form stable complexes with AMPK [75] and ULK1 is a phosphorylation target at multiple sites for AMPK [76,77].

AMPK is classically activated by energy depletion and hypoxia. Moreover, a variety of chemicals including the adenosine analogue 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), biguanides (metformin and phenformin), glycolysis inhibitors (2-deoxy-D-glucose) and metabolic poisons (oligomycin and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) have been used to investigate the role of AMPK in the regulation of the mTORC1 pathway (FIGURE 2).

Qin and Rodriques [78] showed that 4-hydroxynonenal (HNE)-induced lipid peroxidation could evoke RPE cell dysfunction and thus it decreased cell viability in conjunction with the downregulation of basal activity of AMPK. Interestingly, AMPK α 1 sensitized RPE cells to the detrimental effects of lipid peroxidation, while AMPK α 2 exerted a protective effect. Thus, selective modulation of AMPK α activity may benefit patients with retinal degeneration associated with RPE cell atrophy. AICAR was observed to protect RPE cells in response to oxidative stress [79]. Moreover, AMPK-induced autophagy protected RPE cells from TRAIL-induced cell death [80].

Regulation of autophagy by sirtuins

Sirtuins have beneficial effects against age-related diseases. They modulate many major biological pathways, such as stress responses, protein aggregation and inflammatory processes, that are involved in age-related diseases. To date, sirtuins have been extensively studied in the context of the age-related pathologies. They are special focusses of interest in drug development for neurodegenerative diseases. Sirtuins are evolutionarily conserved proteins and their name derives from the *Sir2* gene of *Saccharomyces cerevisiae* [81]. Mammals have seven sirtuins (SIRT1–7) and have usually been categorized as NAD⁺-dependent class III histone deacetylase proteins in the histone deacetylase family [81]. Originally, acetylated histones H1, H3 and H4 were found to serve as physiological substrates for the sirtuins. Subsequently, several non-histone proteins have also been shown to be modulated by sirtuin enzymes [81]. SIRT1, 2, 3 and 5 couple protein substrate deacetylation and NAD⁺ cleavage to produce 2'-O-acetyl-ADP-ribose, which together with its nonenzymatically formed derivative, 3'-O-acetyl-ADP-ribose, is involved in the formation of a transcriptionally silent and functionally heterochromatic state [81,82]. SIRT 4 and 6 catalyze the ADP-ribosylation of proteins, although SIRT6 can also act as a deacetylase [81,83,84].

Moreover, along with deacetylation [85], SIRT5 is also capable of modulating protein functions via demalonylation and desuccinylation [86,87].

SIRT1 is the best-studied mammalian sirtuin [88,89]. By deacetylating transcription factors, cofactors, chromatin proteins and the components of the DNA repair machinery, SIRT1 can regulate many significant cellular functions that also play a role in several metabolic, inflammatory, cardiovascular and neurodegenerative diseases, as well as in cancer [81,90]. SIRT1 is well known for its role in increasing the longevity caused by caloric restriction [91]. Since it can deacetylate several crucial transcription factors and co-factors, such as p53, FoxO proteins, and NF- κ B, it regulates several factors involved in cell's stress resistance and housekeeping [89,91].

In conjunction with SIRT1, 5'AMPAMPK is another enzyme sensing effectively diminished cellular energy states as discussed previously. In order to restore the energy balance, AMPK activates many catabolic processes to generate ATP and it also downregulates the ATP-consuming anabolic processes that are not urgently needed for survival [92,93]. It has become evident that SIRT1 and AMPK are able to stimulate each other (FIGURE 2). When there is a reduced energy state, LKB1 plays a principal role in the phosphorylation and, thus, in the activation of AMPK [94]. AMPK has been reported to activate SIRT1 by increasing the level of the SIRT1 substrate, NAD⁺ [95]. This may be mediated by Nampt, which catalyzes the conversion of nicotinamide to NAD⁺ [95]. On the other hand, it has been shown that SIRT1 can activate AMPK by deacetylating its regulator protein LKB1 [96]. As an example of their shared targets, SIRT1 by deacetylating, and AMPK by phosphorylating activate the transcription cofactor PGC-1 α , which promotes the mitochondrial biogenesis and function [97,98].

Recently, more information has started to accumulate also about other sirtuins. The mitochondrial SIRT3 protein has proved to be another sirtuin that can be activated by diet and exercise [99]. In addition to the CREB transcription factor, SIRT3 activates PGC-1 α through AMPK [99–101]. With the help of ERR- α , PGC-1 α is also capable of activating SIRT3 [102,103]. SIRT3 activates AMPK by deacetylating LKB1 [104] or by modulating the AMP/ATP ratio [99]. Factors mediating the AMP/ATP regulation have been postulated to include ATP synthase (mitochondrial F1 complex) α/β subunits, UQCRH protein and AceCS2 [105,106]. Similar to SIRT1, SIRT3 also deacetylates the transcription factor FoxO3,

which activates antioxidant genes and, thereby, reduces the intracellular levels of ROS [107]. Moreover, SIRT1 and SIRT3 are able to deacetylate the tumor suppressor protein p53 [108,109]. Hyperacetylation of p53 induces growth arrest and apoptosis in cells [105], whereas deacetylation promotes cell cycle-dependent autophagocytosis [110,111]. Mutual interactions with AMPK, PGC-1 α and other intracellular molecules connect sirtuins into an interesting ensemble, which increases longevity and cell survival via several signaling pathways including those associated with the induction of autophagy. Therefore, they might represent a target for drugs to treat neurodegenerative diseases including AMD.

Future perspective

AMD is the most important cause of blindness in individuals over 60 in western countries. AMD is a multifactorial disease resulting from a complex interaction of genetic and environmental factors, age is the only widely recognized risk factor. The socio-economic impact of the disease is enormous, if we take into account the high cost of the available antiangiogenic therapy, the numerous medical visits that it requires and suffering imposes on the patients. One major public health challenge is to find some kind of effective primary preventive measure to combat AMD or to develop more effective drugs so that dry AMD patients could also be treated. To date, there are only recommendations for modifying the risk

factors of AMD and only wet AMD patients can be treated and even then only with repeated intravitreal injections that merely slow progression of disease. In recent years, data on mechanisms that regulate autophagy have enormously increased and dysfunctions in these processes are certainly one central component of many neurodegenerative diseases including AMD. However, at the same time, it has been appreciated that autophagy might represent an important therapy target. Particularly, autophagy-regulating kinases AMPK and mTOR both interesting in themselves and as promising targets for preventing RPE cell degeneration and AMD development.

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Executive summary

Age-related macular degeneration as a degenerative eye disease

- Age-related macular degeneration (AMD) is the most common reason for central vision loss in the elderly in the developed countries.
- The number of patients will double during the next decades.
- The disease is categorized into dry and wet AMD forms.
- Only wet AMD patients can be treated but these only account for approximately 10% of all AMD cases.

Central role of retinal pigment epithelium in AMD pathogenesis

- Retinal pigment epithelium degeneration is a primary sign of AMD development.
- Lysosomal lipofuscin accumulation reflects the AMD severity level.
- Extracellular drusens are hallmarks of AMD development.

Regulation of autophagy by mTOR

- Rapamycin inhibits mTOR and activates autophagy.
- Rapamycin prevent detrimental AMD signs in experimental models.

Regulation of autophagy by AMPK

- 5-aminoimidazole-4-carboxamide ribonucleoside induces AMPK and autophagy.
- 5-aminoimidazole-4-carboxamide ribonucleoside protect retinal pigment epithelium cells from oxidative stress.

Regulation of autophagy by Sirtuins

- SIRT1 and SIRT3 interact with AMPK and regulates autophagy.
- Sirtuins are longevity factors.

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