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Iron homeostasis and toxicity in retinal degeneration

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Abstract

Iron is essential for many metabolic processes but can also cause damage. As a potent generator of hydroxyl radical, the most reactive of the free radicals, iron can cause considerable oxidative stress. Since iron is absorbed through diet but not excreted except through menstruation, total body iron levels build up with age. Macular iron levels increase with age, in both men and women. This iron has the potential to contribute to retinal degeneration.

Here we present an overview of the evidence suggesting that iron may contribute to retinal degenerations. Intraocular iron foreign bodies cause retinal degeneration. Retinal iron buildup resulting from hereditary iron homeostasis disorders aceruloplasminemia, Friedreich's Ataxia, and panthothenate kinase associated neurodegeneration cause retinal degeneration. Mice with targeted mutation of the iron exporter ceruloplasmin have age-dependent retinal iron overload and a resulting retinal degeneration with features of age-related macular degeneration (AMD). *Post mortem* retinas from patients with AMD have more iron and the iron carrier transferrin than age-matched controls.

Over the past ten years much has been learned about the intricate network of proteins involved in iron handling. Many of these, including transferrin, transferrin receptor, divalent metal transporter 1, ferritin, ferroportin, ceruloplasmin, hephaestin, iron regulatory protein, and histocompatibility leukocyte antigen class I-like protein involved in iron homeostasis (HFE) have been found in the retina. Some of these proteins have been found in the cornea and lens as well. Levels of the iron carrier transferrin are high in the aqueous and vitreous humors. The functions of these proteins in other tissues, combined with studies on cultured ocular tissues, genetically engineered mice, and eye exams on patients with hereditary iron diseases provide clues regarding their ocular functions.

Iron may play a role in a broad range of ocular diseases, including glaucoma, cataract, AMD, and conditions causing intraocular hemorrhage. While iron deficiency must be prevented, the therapeutic potential of limiting iron induced ocular oxidative damage is high. Systemic, local, or topical iron chelation with an expanding repertoire of drugs has clinical potential.

I. Introduction

Iron is essential for life, but can produce toxic reactive oxygen species. Enzymes from the citric acid cycle, succinate dehydrogenase and aconitase, are iron-dependent. Iron is also a critical component of cytochromes a, b, and c, cytochrome oxidase, and the iron-sulfur complexes of the electron transport chain, making iron essential for the production of adenosine triphosphate

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(ATP) (Wigglesworth and Baum, 1988; Poss and Tonegawa, 1997). Iron is also required for activity of ribonucleoside reductase, the rate-limiting enzyme of the first metabolic reaction committed to DNA synthesis (Wigglesworth and Baum, 1988). In the CNS, additional demands on iron arise from myelogenesis and myelin maintenance by oligodendrocytes, which have higher iron relative to other CNS cells (LeVine and Macklin, 1990; Morris et al., 1992). Iron deficiency in children results in auditory defects from disruption of myelin (Roncagliolo et al., 1998), and demyelinating diseases such as multiple sclerosis are associated with defects in cellular iron homeostasis (Drayer et al., 1987). Iron is also a necessary cofactor for the synthesis of neurotransmitters, dopamine, norepinephrine, and serotonin, and disruption of iron homeostasis may be involved in Parkinson's disease and/or mood disorders (Youdim, 1990).

In the retina, iron is particularly important for the visual phototransduction cascade. Photoreceptor cells are constantly shedding and synthesizing their outer segments containing disc membranes. Photoreceptors thus depend highly on iron-containing enzymes including fatty acid desaturase for synthesis of lipids used in generating new disc membranes (Schichi, 1969). Additionally, iron is an essential cofactor for the enzyme guanylate cyclase, which synthesizes cGMP, the second messenger in the phototransduction cascade (Yau and Baylor, 1989). RPE65, the isomerohydrolase found in the microsomal membrane of the RPE responsible for catalyzing the conversion of all- trans-retinyl ester to 11-cis-retinol in the visual cycle, is an iron-containing protein that is also dependent on iron for its isomerohydrolase activity (Moiseyev et al., 2005).

While iron is necessary for retinal function, excess iron can be harmful. Free Fe^{2+} participates in the Fenton reaction by catalyzing the conversion of hydrogen peroxide to the hydroxyl radical, the most reactive of reactive oxygen species. Hydroxyl radicals are extremely reactive, causing lipid peroxidation, DNA strand breaks, and degradation of biomolecules (Halliwell and Gutteridge, 1984), and have been implicated in the pathogenesis of Alzheimer's and other CNS diseases (Smith et al., 1997). Particularly in the photoreceptors, where there is a high oxygen tension and high concentration of easily oxidized polyunsaturated fatty acids, iron must be carefully regulated to provide necessary iron levels without causing oxidative damage.

1.1 General iron homeostasis

In general, iron is taken up by most tissues through transferrin-mediated uptake following binding to the transferrin receptor. Most non-heme iron in the circulation is bound to transferrin, an 80kDa protein capable of binding two molecules of ferric (3+) iron with high affinity (Baker and Morgan, 1994). Adults normally have approximately 3 mg of circulating iron, with transferrin binding sites only approximately 30% saturated. As transferrin cannot diffuse across the blood-brain barrier, cells that comprise this barrier must import the iron and transfer it to neural tissue. At a cell surface, iron-laden transferrin binds to transferrin receptor, and this complex is internalized through clathrin-coated pits into endosomes. Iron is released by the acidity of the low pH endosome (Sipe and Murphy, 1991) and is exported from the endosome for use, storage, or export by the cell. Transferrin bound to its receptor is then recycled to the cell surface (Hunt and Davis, 1992), where the extracellular pH triggers release of apotransferrin (Dautry-Varsat et al., 1983), which can then accept another two molecules of ferric iron.

Intracellular iron is safely sequestered by ferritin, a multimeric shell of approximately 450 kDa consisting of 24 polypeptide subunits surrounding a cavity capable of accommodating up to 4,500 iron atoms (Aisen et al., 2001). Human ferritins comprise a tissue-specific ratio of two types of ferritin subunits designated H for heavy (21 kDa) (or heart, because of its abundance in heart) and L for light (19.5 kDa) (or liver, because of liver abundance). H-ferritin is a ferroxidase, and iron oxidation is thought to facilitate its incorporation into the ferritin core. Increased H-ferritin reduces the level of intracellular free iron and increases cellular resistance

to oxidative stress (Cozzi et al., 2000; Goralska et al., 2001). L-ferritin is 50% identical to H-ferritin at the amino acid level and promotes iron incorporation inside the ferritin cavity but lacks ferroxidase function (Levi et al., 1994).

Most of the metabolically active iron in the cell is processed in the mitochondria, which contains its own mitochondrial ferritin (MtF), encoded by a gene distinct from the H- or L-ferritin genes (Levi et al., 2001). While MtF has been shown to possess ferroxidase activity, its function is unclear. MtF increases have been observed in the mitochondria of iron-overloaded sideroblasts in sideroblastic anemia, suggesting that MtF may protect mitochondria from iron-induced oxidative damage (Cazzola et al., 2003).

Iron that is not utilized or stored by the cell may be exported by the transport protein ferroportin (also known as MTP-1 or IREG-1) (Donovan et al., 2000; Abboud and Haile, 2000; McKie et al., 2000). Iron is exported by ferroportin in its ferrous state and must be oxidized to be accepted by circulating transferrin. Oxidation of ferrous iron is believed to be accomplished by ferroxidases, ceruloplasmin and hephaestin, making them important for iron export. Ceruloplasmin is a copper binding protein, which contains over 95% of copper found in plasma and whose characteristic low levels in Wilson's disease are caused by decreased stability of ceruloplasmin resulting from failure of copper incorporation into ceruloplasmin. The only known ceruloplasmin homolog, hephaestin, has recently been identified as the disrupted gene product in the *sex-linked anemia* mouse, which has decreased serum iron and elevated enterocyte iron resulting from failure of hephaestin to facilitate iron export from enterocytes to the circulation (Vulpe et al., 1999). Hephaestin has 50% homology to ceruloplasmin and has ferroxidase activity. Unlike ceruloplasmin, which is present as a secreted plasma protein and a glycosylphosphatidylinositol (GPI)-anchored protein (Patel and David, 1997), hephaestin is present only as a membrane-bound protein.

The opposing requirements and toxicities of iron are managed by an iron-responsive mechanism of post-transcriptional regulation of key iron-handling proteins (Hentze and Kuhn, 1996). This regulation allows individual cells to regulate iron uptake, sequestration, and export according to their iron status. Iron-regulatory proteins (IRPs) register intracellular iron status and, in cases of intracellular iron deficiency, bind to iron-responsive elements (IREs) on the mRNA of the regulated protein. Binding of IRPs to the IRE of ferritin, which lies on the 5' portion of ferritin mRNA, sterically obstructs efficient translation, resulting in decreased ferritin levels in iron-deficiency. In contrast, binding of IRP to the IRE of transferrin receptor, which lies on the 3' end of the transferrin receptor mRNA, protects the mRNA from degradation, resulting in increased transferrin receptor in iron-deficiency. In cases of iron-sufficiency, during which cells would want to increase iron storage and decrease iron import, loss of translational obstruction leads to increased ferritin levels, while loss of protection from degradation leads to decreased transferrin receptor mRNA (Rouault, 2002). Iron-responsive regulation of ferroportin, whose IRE lies on the 5' end of its mRNA, is differentially regulated in different cell types; in liver, iron depletion results in ferroportin downregulation, while iron depletion in duodenum results in increased ferroportin (Abboud and Haile, 2000).

There are two IRPs in the mammalian cytosol, IRP-1 and IRP-2, which share protein sequence homology but differ in mode of regulation. IRP-1 contains an iron-sulfur cluster, and in cellular iron-sufficiency, IRP-1 accumulates in the cytosol unbound to IREs and functions as a cytosolic aconitase, which interconverts citrate and isocitrate. In iron-depletion, oxidative degradation of the sulfur cluster occurs, and the iron-free IRP-1 binds to IREs to regulate ferritin, transferrin receptor, and ferroportin (Rouault and Klausner, 1996; Beinert and Kiley, 1996). IRP-2 lacks an iron-sulfur cluster but similarly binds IREs during cellular iron-depletion. During iron sufficiency, IRP-2, which binds iron, undergoes iron-dependent oxidation followed by

proteasomal degradation such that IRP-2 is absent in iron-replete cells (Guo et al., 1995; Iwai et al., 1998).

2. Iron import into the retina

2.1 Transferrin and transferrin receptor

The retina is isolated from the bloodstream by blood-retinal barriers. The retinal pigment epithelium (RPE) and the neuroretinal vasculature form independent barriers, the intercellular tight junctions of which prevent intercellular diffusion, thereby protecting both sides of the retina from the systemic circulation.

Ferric iron is carried in the bloodstream in association with a protein, transferrin (Baker and Morgan, 1994). Transferrin, with iron, is endocytosed into cells following binding to the cell surface transferrin receptor. Transferrin receptor has been detected by immunohistochemistry in the ganglion cell layer, inner nuclear layer, outer plexiform layer, the inner segments of photoreceptors, the RPE, and the choroid (Yefimova et al., 2000).

Transferrin is also found in the retina (Yefimova et al., 2000). Transferrin mRNA expression was detected by *in situ* hybridization in the RPE cell layer, indicating that the RPE is the main site of transferrin synthesis. Immunohistochemistry for transferrin revealed label in the RPE and in the inner and outer segments of photoreceptors, as well as in the inner retina. Transferrin may carry iron from the RPE to the photoreceptors via a Tf-TfR-dependent mechanism (Yefimova et al., 2000).

Human RPE cells contain transferrin receptors with a monomeric molecular mass of 93kDa (Hunt et al., 1989). Cell surface transferrin receptors are located on both the basolateral and apical surfaces of RPE cells. Transferrin receptor mediated endocytosis occurs on both surfaces, suggesting that there may be bidirectional flow of iron through the RPE.

Iron bound to transferrin in the choroidal circulation has been shown to be taken up by high-affinity transferrin receptors at the basolateral surfaces of RPE cells. From there, iron is transported to the apical surfaces of RPE cells where it is released to the neural retina. It is proposed that the likely mechanism for transport from the basolateral surface of RPE cells to the apical surface occurs via endocytosis followed by release of iron from the endosome. After circulating iron-transferrin complexes bind to transferrin receptors in the basolateral surfaces of RPE cells, the complex is internalized into a low pH endosome where iron can dissociate from transferrin. Iron leaves the endosome and enters a cytoplasmic pool of ferritin-bound iron where it can be processed for further use or can bind to ferritin; it is free iron from this cytoplasmic pool that is secreted from the apical surface of cells, from which it can bind to apo-transferrin on the apical side of the RPE (Hunt and Davis, 1992).

This iron-transferrin complex may then be taken up by transferrin receptors on the photoreceptor inner segments. The photoreceptor inner segments of adult rat retinas are immunopositive for transferrin receptor (Yefimova et al., 2000), which likely bind and internalize iron-transferrin complexes in the photoreceptor matrix.

Aqueous (Tripathi et al., 1990; Yu and Okamura, 1988) and vitreous humor (Hawkins, 1986) also contain substantial amounts of transferrin, suggesting that they may provide a route for iron delivery to ocular cells. Experiments with intravitreal injection of labeled fucose or tyrosine in the vitreous suggest that at least part of the vitreous transferrin is synthesized locally in the eye (Laicine and Haddad, 1994).

In vitro, using a bovine retinal epithelial cell culture system (BRECs), Burdo and colleagues provide support for iron transport across the blood-retinal barrier by both transcytosis of Tf-

bound iron and endocytosis of Tf-bound iron followed by the removal of iron from Tf within endosomes (Burdo et al., 2003). They found that the relative frequencies of transcytosis versus endocytosis of Tf-iron complexes depended on the iron status of the epithelial cells. When epithelial cells were iron overloaded, the amount of non-transferrin bound iron transported decreased dramatically, suggesting a mechanism by which iron uptake into the retina is decreased when the retina has adequate iron. The authors also demonstrated increased secretion of apo-Tf in conditions of iron overload, possibly serving as a protective mechanism for neighboring cells.

2.2 Divalent Metal Transporter-1

Divalent Metal Transporter-1 (DMT1) is a proton symporter that moves one atom of ferrous iron and a proton in the same direction (Gunshin et al., 1997). In the gut, DMT1 is localized on the apical surface of intestinal epithelial cells and transports dietary free iron, upon reduction from its ferric to ferrous state, from the luminal surface of the small intestine into the enterocytes (Rouault and Cooperman, 2006). In most other cells, DMT1 is localized on endosomes and serves to transport iron from the endosome into the cytoplasm.

In the brain, immunohistochemistry demonstrated high levels of DMT1 in the vascular and ependymal cells of normal rat brain sections (Burdo et al., 2001). While it is unclear whether DMT1 is intracellular, basal, or apical, the presence of DMT1 in vascular endothelial cells and astrocytic glial end feet separating the blood and the brain suggests that DMT1 may be involved in iron transport to and/or from the brain. It has been shown that Belgrade rats with a mutation in DMT1 have a hypochromic, microcytic anemia and less iron in their brains (Burdo et al., 1999), indicating that DMT1 may be involved in iron transport to the brain.

In the retina, we were able to detect strong DMT1 immunolabel in rod bipolar cell bodies, rod bipolar cell axon termini, horizontal cell bodies, and photoreceptor inner segments (Fig. 1). Whether retinal DMT1 serves to export iron from cells, export iron from endosomes, or import iron is unknown. Studies on the retinas of Belgrade rats with a DMT1 mutation may help to elucidate the function of DMT1 in the retina.

2.3 Dexras

Dexas1, a 30kDa G-protein in the Ras subfamily, can be induced by the activation of glutamate-NMDA receptors to signal the uptake of iron in the brain (Cheah et al., 2006). The activation of glutamate-NMDA receptors stimulates neuronal nitric oxide synthase (nNOS), which binds to CAPON, and delivers nitric oxide (NO) to Dexas1, causing the S-nitrosylation of Dexas1. Downstream, Dexas1 interacts with Peripheral Benzodiazepine Receptor Associated Protein (PAP7), which binds to DMT1 to induce iron uptake. NMDA neurotoxicity has been hypothesized to result from increased iron uptake following induction of this signaling cascade, and selective chelation of intracellular iron in brain cultures protects against NMDA neurotoxicity.

3. Iron Storage

3.1 Ferritin

Iron in cells is primarily stored in cytosolic ferritin, a highly conserved protein consisting of 24 subunits of heavy (H) and light (L) ferritin (Aisen et al., 2001). A ferritin molecule can hold as many as 4500 iron molecules in the ferric state in its central core. H-ferritin has ferroxidase activity allowing it to rapidly accumulate Fe^{3+} in its central core (Aisen et al., 2001) while L-ferritin does not (Levi et al., 1994).

The distribution of iron and ferritin has been characterized in the adult rat retina. Proton-induced x-ray emission demonstrated the largest amounts of heme and non-heme iron in the inner segments of photoreceptors, the RPE, the choroid, the inner nuclear layer, and the ganglion cell layer (Yefimova et al., 2000). Iron was present in lesser amounts in the outer segments of photoreceptors. Interestingly, the regions with the most iron coincided with the regions of greatest ferritin label. Immunohistochemistry for L- and H-ferritin in the rat revealed the strongest label in the inner segments of photoreceptors, the RPE, the choroid, inner nuclear layer, and the ganglion cell layer. Unlike iron distribution, however, L- and H-ferritin label in the outer segments of photoreceptors was also strong.

We have found that in lightly fixed retinas from BALB/c and C57Bl/6 mice, L- and H-ferritin antibodies labeled the photoreceptor inner segments, outer plexiform layer, inner nuclear layer cell bodies, and the innermost inner plexiform layer near the ganglion cell layer (Fig. 2, panels A and D) (Hahn et al., 2004a). In the inner nuclear layer, much of the label was found to be in rod bipolar cells, confirmed by double labeling L-ferritin (Fig. 2, panels A–C) or H-ferritin (Fig. 2, panels D–F) with a marker for rod bipolar cells, PKCa. The axon terminals of these rod bipolar cells had a punctate label (Fig. 2, panels C and F) immunopositive for L- and H-ferritin, suggesting that ferritin may play a role in the transport or storage of iron in the pre-synaptic terminal. In the RPE, L- and H-ferritin label was faint (Fig. 2, panels A and D), even in the non-pigmented BALB/c RPE. But RT-PCR detected L- and H-ferritin mRNAs in the RPE of C57Bl/6 mice in two separate experiments, one from a single 6-month-old C57Bl/6 mouse and another from 3 mice aged 3, 5, and 7 months.

Ferritin levels are regulated by iron regulatory proteins (IRPs) (Hentze and Kuhn, 1996). IRPs negatively regulate ferritin levels by binding to ferritin mRNA in iron-depleted cells and sterically obstructing translation. In an IRP-deficient mouse, ferritin levels would be expected to increase because of the absence of IRP to hinder translation of ferritin mRNA.

Immunohistochemistry for L-ferritin on IRP deficient mouse retinas between 9–12 months of age revealed increased label (Fig. 3) compared to wild-type mouse retinas, demonstrating that ferritin levels in the retina are IRP-regulated (Hahn et al., 2004a).

3.2 Ferritin in corneal epithelial cells

Although ferritin is a cytoplasmic molecule in most cells, the heavy chain of ferritin has been observed in avian corneal epithelial cells as a developmentally regulated nuclear protein. The properties and structure of nuclear ferritin in corneal epithelial cells resembles that of cytoplasmic ferritin in other cell types. Since corneal epithelial cells are constantly exposed to ultraviolet (UV) light, nuclear ferritin likely functions to sequester iron and thereby reduce oxidative damage to DNA from UV light. A nuclear transport protein, termed ferritoid, has been identified that binds to ferritin in the cytoplasm and carries it into the nucleus (Linsenmayer et al., 2005 for a review).

3.3 Mitochondrial ferritin

Mitochondrial ferritin (MtF) is a recently identified form of ferritin most similar to H-ferritin and localized to the mitochondria (Levi et al., 2001). We detected MtF by immunohistochemistry in the photoreceptor inner segments (Fig. 4A) and diffusely throughout the inner retina (Hahn et al., 2004a). To confirm mitochondrial localization, anti-MtF was co-labeled with an antibody specific for the ATPase in Complex V of the electron transport chain of mitochondria. Label co-localized to the inner segment ellipsoids, the location of the mitochondria, but not the inner segment myoid (Fig. 4, panels D–F).

4. Iron Export

4.1 Ceruloplasmin

Ceruloplasmin is a multicopper oxidase with ferroxidase activity. By oxidizing iron from its Fe^{2+} to its Fe^{3+} state, ceruloplasmin can function as an antioxidant, since Fe^{2+} catalyzes the generation of free radicals via the Fenton Reaction (Osaki, 1966). Additionally, by oxidizing iron from its Fe^{2+} to Fe^{3+} state, ceruloplasmin facilitates iron export, since only ferrous iron is known to be exported across the plasma membrane, but only ferric iron can be taken up in the extracellular space by transferrin (Osaki et al., 1966); it is believed that extracellular ferroxidases including ceruloplasmin are important in the ferroxidation which is thus necessary for efficient export to occur, probably through the generation of an ion gradient (Sarkar et al., 2003).

There are two known forms of ceruloplasmin: the membrane-anchored glycosyl phosphatidyl inositol (GPI) linked form (Patel and David, 1997) and the secreted form. The GPI-linked and secreted forms are produced by alternate splicing (Patel et al., 2000). The secreted form is the predominant form made by the liver while the GPI-linked form is the predominant form found in the brain (Patel et al., 2000).

We have detected both forms of ceruloplasmin in the mouse retina using reverse transcription PCR (RT-PCR) (Chen et al., 2003). The secreted form of ceruloplasmin mRNA was also detected in the RPE of a normal 6-month-old C57BL/6 mouse (Fig. 5A, top panel) (Hahn et al., 2004b). RT-PCR also detected ceruloplasmin mRNA in ARPE-19 cells (Fig. 5A, bottom panel), a spontaneously immortalized human RPE cell line.

Western analysis has shown that ceruloplasmin is present in the normal retina in significant quantities (Chen et al., 2003). Ceruloplasmin protein was found in mouse and human retina and in rMC-1 cells (Fig. 5B), a rat Müller glial cell line (Hahn et al., 2004b). Immunohistochemistry confirmed these results, demonstrating that ceruloplasmin protein is located diffusely throughout the retina (Chen et al., 2003). Evidence for labeling specificity was provided by pre-adsorption with purified human ceruloplasmin protein. The strongest ceruloplasmin label in mouse retinas was in Müller cells. Ceruloplasmin is also present in the aqueous, vitreous and retina from a normal human eye, as demonstrated by Western analysis.

Ceruloplasmin is upregulated after light damage (Chen et al., 2003). Ten week old male BALB/c mice were exposed to white light for seven hours and their retinal ceruloplasmin levels examined at three different time points: immediately after light exposure (t_0), 8 hours after light exposure (t_8), and 28 hours after light exposure (t_{28}). Control mice were exposed to seven hours of room light before euthanasia. TUNEL positive photoreceptor nuclei were found at t_0 , t_8 , and t_{28} in the light-exposed but not in the control group. Ceruloplasmin levels were studied using Western analysis, immunohistochemistry, and quantitative PCR (Q-PCR). Western analysis showed higher ceruloplasmin levels in t_0 , t_8 , and t_{28} retinas than no light control retinas. Immunohistochemistry similarly showed increased ceruloplasmin label throughout the retina following photic injury at all three time points, with the strongest label in Müller cells. Labeling of whole mouse eyes showed detectable ceruloplasmin in the vitreous of t_{28} eyes, suggesting that some of the increased ceruloplasmin in the retina following light damage may be secreted into the vitreous. Q-PCR for the secretory form of ceruloplasmin produced a ceruloplasmin amplification product in the exponential amplification phase that was present three cycles earlier in the t_0 light damaged retinas than in control retinas, indicating an eight-fold increase in ceruloplasmin mRNA at t_0 that was sustained through t_8 and t_{28} . It is likely that this upregulation of ceruloplasmin following light damage serves to protect the eye. Both the direct antioxidant function of ceruloplasmin in reducing reactive ferrous iron levels as well

as the facilitation of iron export may be important protective features in a response to light-induced and other oxidative stresses.

Ceruloplasmin is upregulated in other pathologic conditions as well. Levin and Geszvain demonstrated that ceruloplasmin is upregulated in the rat retina after optic nerve crush (Levin and Geszvain, 1998). Total RNA from rat retinas 1 and 4 days after intraorbital optic nerve crush was subjected to the differential display technique, which detected a single upregulated band. The band was reamplified and cloned, and shown to be ceruloplasmin by sequencing. In situ hybridization localized ceruloplasmin mRNA to the inner nuclear and ganglion cell layers, with increases after optic nerve crush. Immunofluorescence detected ceruloplasmin protein in sporadic cells within the nerve fiber layer of untreated retinas. After optic nerve crush, ceruloplasmin was found by immunohistochemistry in more nerve fiber layer cells as well as in cells in the ganglion cell layer and inner nuclear layer.

Ceruloplasmin is additionally upregulated in glaucomatous human and monkey retinas (Miyahara et al., 2003; Farkas et al., 2004). In monkeys, glaucoma was induced by repeated argon laser photocoagulation of the trabecular meshwork. After 30 days, retinas were isolated and analyzed by human microarray chips, real-time PCR analysis, and immunohistochemical studies for gene expression changes. Ceruloplasmin was among the upregulated genes, and immunohistochemical analysis localized ceruloplasmin to Müller cells. In human glaucoma, immunohistochemistry demonstrated increased levels of ceruloplasmin in the nerve fiber layer, ganglion cell layer, and inner plexiform layer compared to human control retinas.

Ceruloplasmin has also been shown to be upregulated in the diabetic rat retina (Gerhardinger et al., 2005). Gene expression profiling with the GeneChip Rat Genome oligonucleotide array identified ceruloplasmin as a differentially upregulated gene in streptozotocin-induced diabetic Müller cells, which was confirmed by Northern and Western analyses. Ceruloplasmin is upregulated in the aqueous and vitreous of rabbit eyes with endotoxin-induced ocular inflammation (McGahan and Fleisher, 1986). Ceruloplasmin is additionally upregulated in lens epithelial cells after oxidative stress (Li et al., 2004), as demonstrated by exposure of immortal murine lens epithelial cells to the oxidant tert-butyl hydroperoxide.

4.2 Hephaestin

Another multicopper ferroxidase, hephaestin (Heph), is a close homolog to ceruloplasmin with 50% amino acid sequence identity (Vulpe et al., 1999). Vulpe and colleagues identified hephaestin through characterization of the sex-linked anemia (Sla) mouse, containing a Heph mutation that severely decreases its ferroxidase activity. Hephaestin is a membrane-bound protein that mediates the uptake of iron from the intestine into circulation. Similar to ceruloplasmin, Heph facilitates iron export through its ferroxidase activity, which allows for efficient loading of ferric iron to transferrin. In the Sla mouse, reduced Heph ferroxidase activity resulted in reduced export of iron from intestinal epithelial cells, yielding an anemic mouse.

Mouse and human RPE cells express hephaestin mRNA and contain hephaestin protein (Hahn et al., 2004b). As with ceruloplasmin, hephaestin mRNA has been detected in mouse RPE and in a human ARPE-19 cell line by RT-PCR (Fig. 5A). Hephaestin protein, like ceruloplasmin, has also been found in mouse and human retina and in rMC-1 cells, a rat Müller glial cell line, by Western analysis (Fig. 5B). Hephaestin immunolocalizes to Müller cells, to both the cell bodies within the inner nuclear layer and to the cell processes extending radially through the retina (Fig. 5E). The greatest levels of hephaestin were found in the Müller endfeet next to the internal limiting membrane (Fig. 5G). In the RPE, hephaestin immunofluorescence is weak (Fig. 5D), although still greater than in a secondary antibody-only control (Fig. 5C), consistent with the presence of hephaestin mRNA in the RPE.

Combined deficiency of ceruloplasmin and hephaestin results in age-dependent retinal pigment epithelium and retinal iron accumulation, demonstrating the importance for these ferroxidases in facilitating iron export from retinal cells (Hahn et al., 2004b). Perls' staining for iron on *Cp*^{-/-}*Heph*^{-Y} retinas older than five months showed a granular label in the RPE (Fig. 6, panels C and D), and DAB enhancement of the Perls' label demonstrated iron in the photoreceptor outer segments as well (Fig. 6E). DAB-enhanced Perls' stain of a 4-week-old *Cp*^{-/-}*Heph*^{-Y} retina, however, did not detect iron. We were also unable to detect iron accumulation in mice deficient in either ceruloplasmin or hephaestin at any age. Perls' staining of *Cp*^{-/-} (Fig. 6B) and *Heph*^{-Y} retinas, like WT retinas (Fig. 6A), did not produce detectable Perls' label even at nine months.

Atomic absorption spectrophotometry of dissected sclera/choroid/RPE and neural retina (without RPE) has been performed to quantify the iron differences among 7 month old mice deficient in *Cp* or *Heph* (Hahn et al., 2004b). *Cp*^{-/-}*Heph*^{-Y} sclera/choroid/RPE had a significant 4.2 fold increase in iron compared to non-*Cp*^{-/-}*Heph*^{-Y} sclera/choroid/RPE, and *Cp*^{-/-}*Heph*^{-Y} retinas had a significant 3.6- fold increase in iron compared to non-*Cp*^{-/-}*Heph*^{-Y} retinas. Iron differences among WT, *Cp*^{-/-}, and *Heph*^{-Y} tissues were small.

Electron micrographs of five to six month old *Cp*^{-/-}*Heph*^{-Y} retinas showed electron-dense vesicles in the RPE (Hahn et al., 2004b). These vesicles were likely lysosomes or endosomes, which were sometimes fused with melanosomes (Fig. 6G). These vesicles contained high iron content, as demonstrated by electron dispersive X-ray spectroscopy (EDX); these electron dense vesicles had greater than four times more iron than wild type melanosomes or other cytoplasmic structures or even *Cp*^{-/-}*Heph*^{-Y} organelles in the RPE but outside of these vesicles.

Along with increases in iron, *Cp*^{-/-}*Heph*^{-Y} mice also had increased ferritin levels (Hahn et al., 2004a). Ferritin translation is regulated by intracellular iron levels through iron-regulatory proteins, which increase ferritin mRNA translation when intracellular iron levels are high (Rouault, 2002). Immunohistochemistry revealed that 5- to 6- month-old *Cp*^{-/-}*Heph*^{-Y} retinas had stronger H- and L- ferritin label than WT, *Cp*^{-/-}, and *Heph*^{-Y} retinas (Fig. 7) (Hahn et al., 2004a). Labeling patterns were the same across all genotypes. H- and L-ferritin were present in rod bipolar cell termini in the inner plexiform layer. Additionally, L-ferritin was found in the inner sections of the outer plexiform layer and the RPE (Fig. 7, panels D-F). H- ferritin label was strongest in the photoreceptor inner segments and axons in the outer part of the outer plexiform layer (Fig. 7, panels A-C).

Cp^{-/-}*Heph*^{-Y} mice also had increased mitochondrial ferritin (MtF) levels (Hahn et al., 2004a). In contrast to cytosolic H- and L-ferritin, MtF has not been shown to be IRP-regulated. But MtF was increased in the photoreceptor inner segments of *Cp*^{-/-} (Fig. 4B) and *Cp*^{-/-}*Heph*^{-Y} mice (Fig. 4C) (Hahn et al., 2004a). In *Cp*^{-/-} mice, MtF colocalized with a mitochondria-specific antibody to the inner segment ellipsoid but not the inner segment myoid (Fig. 4, panels D-F).

4.3 Ferroportin and hepcidin

In 2000, three groups independently identified the *ferroportin* gene (*SLC40A1*). Donovan and colleagues identified the zebrafish *ferroportin* in a screen for anemic animals (Donovan et al., 2000). The mammalian orthologs for *ferroportin* were identified by McKie et al. using a subtractive cloning screen of cDNA from hypotransferrinemic mice and by Abboud and Haile from a cDNA library constructed from mRNA that binds to iron regulatory protein 1 (Abboud and Haile, 2000; McKie et al., 2000). Ferroportin (Fpn) is expressed in the placenta, intestine, reticuloendothelial macrophages, hepatocytes, lung, brain, and retina (Abboud and Haile,

2000; Burdo et al., 2001; Dentchev et al., 2005; Donovan et al., 2000; Hahn et al., 2004a; McKie et al., 2000; Yang et al., 2002).

Evidence suggests that Fpn functions with the ferroxidases ceruloplasmin (Cp) and hephaestin (Heph) to export ferrous iron out of cells and convert it to ferric iron (Harris et al., 1999; Vulpe et al., 1999). When expressed in *Xenopus* oocytes, Fpn can increase the export of iron from oocytes (Donovan et al., 2000; McKie et al., 2000). This effect is enhanced by addition of ceruloplasmin to the medium (McKie et al., 2000). Ceruloplasmin also facilitated iron export from macrophages, but only when tissue culture oxygen levels reflected tissue oxygen levels rather than atmospheric oxygen levels (Sarkar et al., 2003). Fpn and Cp co-immunoprecipitate from astrocyte lysate and iron efflux is reduced in astrocytes from *Cp*^{-/-} mice (Jeong and David, 2003). Furthermore, stable expression of Fpn in J774 cells, a macrophage cell line, increases iron efflux after erythrophagocytosis (Knutson et al., 2005). Conditional knockout of mouse *ferroportin* in villus enterocytes has demonstrated that the protein functions as the major, if not only, iron exporter from the duodenum to the circulation in mammals (Donovan et al., 2005).

Mutations in human *ferroportin* (*SLC40A1*) lead to autosomal dominant Type IV Hereditary Hemochromatosis (Ferroportin disease). Clinically, Ferroportin disease is grouped into two distinct phenotypes – one group presents with normal serum ferritin levels, low to normal transferrin saturation, and iron accumulation in macrophages, while the other group presents with high transferrin saturation and parenchymal iron overload (Pietrangelo, 2004 for a review). Molecular analysis of Fpn containing human mutations demonstrates that the mutant Fpn either acts as a dominant negative that is mis-localized within the cell or fails to be internalized and degraded in response to ferroportin's ligand, hepcidin (De Domenico et al., 2005).

Hepcidin (hepc) was identified as a 20 or 25 aa protein found in human plasma and urine and produced by the liver (Krause et al., 2000; Park et al., 2001). Hepcidin was subsequently shown to have anti-microbial activity, and its expression is upregulated by infection, inflammation, and iron (Nicolas et al., 2002b; Pigeon et al., 2001). Upstream stimulatory factor 2 knockout mice have impaired hepc gene expression and accumulate iron in the liver and pancreas, while mice overexpressing hepc have severe neonatal iron deficiency anemia, most likely due to impaired placental iron transport (Nicolas et al., 2001; Nicolas et al., 2002a). The 25 aa form of hepc has been shown to induce the internalization and lysosomal dependent degradation of exogenous Fpn and decreased iron export in J774 stably expressing Fpn (Knutson et al., 2005; Nemeth et al., 2004).

In the mouse retina Fpn is localized to RPE, photoreceptor inner segments, the inner and outer plexiform layers, and the ganglion cell layer (Fig. 8A) (Hahn et al., 2004a). In the RPE the immunoreactivity is primarily basolateral (Fig. 8C), suggesting that Fpn functions to facilitate export, along with Cp and Heph, from the RPE into the choroidal vasculature. In response to iron overload in the retina of *Cp*^{-/-}*Heph*^{-/-} mice, Fpn immunoreactivity increases (Fig. 9), presumably due to increased translation. Like ferritin, ferroportin levels are regulated through IRP-mediated steric obstruction of ferroportin translation. Immunohistochemistry of IRP deficient mouse retinas revealed increased ferroportin label in the inner segments compared with wild type retinas (Fig. 10). *Cp*^{-/-}*Heph*^{-/-} RPE, which are iron-overloaded, demonstrate an increase in both apical and basolateral membrane Fpn immunoreactivity (Fig. 9, panels D–F) presumably through down-regulation of IRP in the setting of iron overload.

5. Iron homeostasis in lens epithelial cells

Lens epithelial cells exhibit similarities to retinal cells in their metabolism of iron. Lens epithelial cells in culture make and secrete Tf (McGahan et al., 1995) and ceruloplasmin

(Harned et al., 2006). In cultured lens epithelial cells with iron overload, ceruloplasmin and transferrin synergistically increased the flow of iron out of cells, resulting in decreased iron incorporation into ferritin. Under physiologic conditions, however, ceruloplasmin increased iron incorporation into ferritin (Harned et al., 2006). Cytosolic extracts of iron-replete cultured lens epithelial cells were loaded with ^{59}Fe -Tf and electrophoresed. Two pools of ^{59}Fe , a ferritin pool and a low molecular weight pool of unknown composition, were found. In cells treated with ceruloplasmin, the ferritin pool was significantly increased. In these cultured lens epithelial cells, iron-overload increased ferritin levels eight- fold, while addition of the iron chelator, Desferal, decreased ferritin levels (McGahan et al., 1994).

6. Disruption of iron homeostasis and oxidative damage

Increased intraocular iron has been found to cause oxidative damage to the retina. Adult C57Bl/6 mice given intravitreal injections of FeSO_4 demonstrated increased superoxide radicals in photoreceptor inner segments (Rogers et al., 2007). A higher concentration of injected FeSO_4 also caused the outer border of the outer nuclear layer to become irregular, suggesting photoreceptor damage. Lipid peroxidation of photoreceptors also occurred, as demonstrated by immunohistochemistry for 4-hydroxynonenal (HNE). Ultimately, injecting FeSO_4 resulted in retinal degeneration. TUNEL staining one day after FeSO_4 injection revealed apoptosis in the outer edge of the outer nuclear layer, which increased at two days after FeSO_4 injection. At 14 days after injection, the outer nuclear layer appeared to have thinned although measurement by image analysis did not show a significant reduction. Consistent with the apoptosis observed at the outer edge of the outer nuclear layer, Campochiaro recently provided evidence of increased cone sensitivity to intravitreal iron injection (Campochiaro, 2007).

Ceruloplasmin deficient mice also exhibit retinal degeneration. 18- month-old *Cp*^{-/-} mice retinas showed mild degeneration in the inner nuclear layer (Patel et al., 2002). Magnification of the inner nuclear layer showed condensed chromatin and dark cytoplasm in the cells.

We have found that ceruloplasmin and hephaestin deficient mice, which demonstrated age-dependent iron accumulation, have a retinal degeneration (Hahn et al., 2004b). In the retinas of 6- to 9- month-old *Cp*^{-/-}*Heph*^{-/Y} mice, RPE cells in up to 75% of a histologic section across the entire retina were severely hypertrophic (Fig. 11C). Electron microscopy of these hypertrophic RPE cells revealed excessive accumulation of phagosomes and lysosomes, most likely containing undigested outer segments (Fig. 11G). There were also a few focal areas of RPE hyperplasia and necrosis (Fig. 11, panels D and E) with local photoreceptor loss and subretinal neovascularization. People with a deficiency in ceruloplasmin resulting from the recessive disease aceruloplasminemia also have retinal iron accumulation with retinal degeneration (see below).

Disruption in iron homeostasis between the retina and RPE may cause iron overload. In Royal College of Surgeons rats with a mutation of the receptor tyrosine kinase gene *Mertk*, the RPE is unable to phagocytose shed outer segments, leading to a layer of undigested outer segment tips in the subretinal space (Yefimova et al., 2002). This layer disrupts normal RPE-photoreceptor functions, such as the normal diffusion of transferrin (Tf). Non-heme iron was found to build up in this debris layer in a time-dependent manner with photoreceptor degeneration, while transferrin levels in the photoreceptor layer were diminished. Photoreceptor loss starts at postnatal day 20 and is significantly increased one month later. In this model, the disruption of normal RPE-photoreceptor interactions leads to an iron homeostasis disorder, which may ultimately contribute to retinal degeneration.

7. Retinal disorders resulting from abnormal retinal iron metabolism

Several recent studies suggest that abnormal retinal iron metabolism may promote a variety of retinal disorders. These include ocular siderosis either from intraocular foreign bodies or from intraocular hemorrhage. Retinal degeneration has also been observed in hereditary disorders resulting in iron overload, including aceruloplasminemia, hereditary hemochromatosis, pantothenate kinase associated neurodegeneration (formerly Hallervorden-Spatz Disease), and Friedreich's Ataxia. Recently, evidence suggests that iron overload may also play a role in the pathogenesis of age-related macular degeneration (AMD). Antioxidants and iron chelators may be of benefit in the treatment or prevention of these retinal disorders.

7.1 Siderosis

Ocular siderosis is a sight threatening condition resulting from intraocular iron deposition. The most common etiology is a retained metallic foreign body (Cibis, 1959). In cases of retained intraocular foreign bodies (IOFB), siderosis bulbi may occur anywhere between 18 days to 8 years after ocular injury.

The clinical findings include iris heterochromia, pupillary mydriasis, cataract formation (anterior subcapsular), lens discoloration, retinal detachment, retinal arteriolar narrowing, and retinal pigment epithelium clumping and atrophy. A secondary glaucoma may also occur if there is involvement of the trabecular meshwork and Schlemm's canal (Cibis et al., 1959; Talamo et al., 1985; Sneed, 1988). Fluorescein angiography (FA) in ocular siderosis has shown localized capillary non-perfusion (Shaikh and Blumenkranz, 2001). Electroretinography (ERG) results vary depending on the stage of the disease (Knave, 1969). In general, ERG testing may initially show an increased a-wave and b-wave amplitude, but as the siderosis progresses, there is a gradual decrease in amplitude as progressive degeneration of the rods and cones occurs.

Cibis and colleagues examined pathologic specimens of patients who had ocular siderosis and hemosiderosis (Cibis et al., 1959). Sequelae of intraocular iron overload observed in the posterior segment included contraction bands in the vitreous body and at the inner surface of the retina, proliferation and obliteration of blood vessels, retinal detachment, and degeneration of the retina. Anterior segment pathology showed destruction of the trabecular meshwork and sclerotic changes within Schlemm's canal, the latter being a possible cause of secondary glaucoma. Cytosolic changes were seen in the endothelial cells of intraocular blood vessels, cornea, and trabecular meshwork as well as in the epithelial cells of the ciliary body, lens, the retina at the ora serrata, and the RPE. Lastly, increased iron was demonstrated in several forms. Iron was seen within ferritin or in hemosiderin giving a coarse, granular appearance histologically.

Animal models of siderosis have shown both histopathologic and functional changes in the retina. Masciulli and colleagues studied retinal changes after injecting iron-containing solutions into the vitreous of squirrel monkeys (Masciulli et al., 1972). Ophthalmoscopy showed geographic patches of retinal whitening and RPE disruption. Dose-dependent ERG changes were seen within the first few hours with diminished or absent signals with higher doses of iron.

In another animal model based on insertion of solid iron foreign bodies into the rabbit vitreous, degeneration of the outer nuclear layer and RPE was observed 10 days after foreign body insertion (Declercq et al., 1977). ERG measurements also showed a decrease in both the a- and b-wave amplitudes under both scotopic and photopic conditions.

7.2 Subretinal Hemorrhage

Vision loss may follow subretinal hemorrhage. Subretinal blood within the macula may cause vision loss in a number of diseases including age-related macular degeneration (AMD), myopic degeneration, angioid streaks, and ocular histoplasmosis. Gillies and Lahav studied patients with intra and subretinal hemorrhage and found that hemorrhage size and ability of the tissue to clear the blood were significant factors in amount of visual acuity lost (Gillies and Lahav, 1983).

Possible mechanisms of vision loss include direct iron toxicity to the photoreceptors, iron toxicity or mechanical damage to the RPE, cellular migration and proliferation in the subretinal space, proliferation of fibrovascular membrane, or separation of photoreceptors from the RPE (Gillies and Lahav, 1983). Injection of fresh autologous blood into the subretinal space of albino rats resulted in progressive degeneration of the photoreceptor cells with edematous changes seen as early as day 1 leading to near total destruction by day 7 (Glatt and Machemer, 1982). Perls' staining at day 7 demonstrated iron accumulation. Rabbit eyes injected with autologous blood into the subretinal space also had iron accumulation (Bhisitkul, R., unpublished). Perls' staining of rabbit eye sections following injection with autologous blood demonstrated iron accumulation in the RPE, in large cells within the photoreceptor outer segment matrix, most likely macrophages, and in photoreceptor outer segments with strongest label directly overlying the subretinal hemorrhage with fading further away from the site of blood accumulation (Fig. 12). *In vitro*, oxyhemoglobin produces lipid peroxidation in retinal tissue (Ito et al., 1995), and oxyhemoglobin in subretinal blood may damage the retina after release from red blood cells. The hemoglobin-binding protein hemopexin may serve as a protective mechanism against the mediated retinal toxicity. RPE cells bind and internalize the heme-hemopexin complex from the retina and thus facilitate clearance of sub or intra-retinal blood (Hunt et al., 1996). Treatment with the iron chelator, deferoxamine (DFO), has been shown to reduce retinal toxicity from autologous subretinal blood as measured by ERG in albino rabbits (Youssef et al., 2002). High subretinal iron concentration, perhaps resulting from subretinal hemorrhage, is associated with worse visual prognosis in patients undergoing surgery for stage V retinopathy of prematurity (Trese, 1986).

7.3 Hereditary iron overload

Four clinical entities of hereditary iron overload have been associated with pathologic changes in the retina: aceruloplasminemia, hereditary hemochromatosis, pantothenate kinase associated neurodegeneration, and Friedreich's ataxia.

7.3.1 Aceruloplasminemia—Aceruloplasminemia is a rare adult-onset autosomal recessive disease caused by mutations in the ceruloplasmin gene on chromosome 3q (Harris et al., 1995). Patients with aceruloplasminemia have impaired iron export from certain tissues, since ceruloplasmin facilitates iron export by converting it from Fe^{2+} to Fe^{3+} , the form of iron that can be loaded onto transferrin. The retina, brain, and pancreas are iron overloaded and the clinical triad of aceruloplasminemia is retinal degeneration, dementia, and diabetes (Yamaguchi et al., 1998). Two cases of aceruloplasminemia in the eye have been reported, one involving a Japanese patient and the other a Caucasian patient, both 56 years old at the time of examination.

In the case of the Japanese patient, ophthalmoscopic examination revealed yellowish discoloration of the fundus of both eyes (Yamaguchi et al., 1998). In the midperipheral fundus, yellowish opacities could be seen over grayish atrophic RPE cells. The posterior pole was homogeneously yellow. Fluorescein angiography (FA) of the posterior pole showed RPE atrophy resulting in window defects in the midperipheral fundus. The yellowish opacities seen by ophthalmoscopy caused blocked fluorescence on the FA. Electrooculography showed a low

standing potential and a reduced response to light bilaterally, demonstrating impaired RPE function. Electroretinogram with single white-flash stimulation was also subnormal with lowered a- and b-wave amplitudes. The best corrected visual acuity was 20/20 in both eyes.

In contrast, the case of the Caucasian patient is dominated by macular degeneration (Dunaief et al., 2005). Subretinal lesions, similar to drusen found in patients with AMD, were found in the macula. These yellowish-white, round or oval lesions, which began to develop at age 47, became smaller and more centrifugally spread out over the course of 9 years. Ophthalmoscopic examination revealed RPE depigmentation in the macula of both eyes and fluorescein angiography demonstrated RPE atrophy indicated by transmission defects. The patient also had yellow pingueculae in each eye, which were found to contain patches of iron-overloaded epithelial cells detected by a Perls' Prussian blue stain after a biopsy. The patient's corrected visual acuity was also 20/20 in both eyes.

While the retinal histopathology of human aceruloplasminemia has not been reported, mice deficient in ceruloplasmin and hephaestin showed age-dependent retinal degeneration (Hahn et al., 2004b). By 6–9 months, the RPE cells of ceruloplasmin and hephaestin deficient mice were severely hypertrophic. Electron microscopy of these RPE cells showed increased phagosomes and lysosomes, and sub-RPE deposits of wide-spaced collagen (Fig. 11H). There were also a few focal areas of RPE hyperplasia and necrosis with local photoreceptor loss and subretinal neovascularization.

7.3.2 Hemochromatosis—There are two types of hemochromatosis: primary and secondary hemochromatosis. Primary, or hereditary, hemochromatosis is the most common form. It is characterized by excessive iron accumulation in the liver, heart, and pancreas (Pietrangelo, 2006). Most patients with hereditary hemochromatosis have a mutation in the histocompatibility leukocyte antigen class I-like protein involved in iron homeostasis (HFE) gene product (Feder et al., 1996). The HFE protein normally forms a stable complex with the transferrin receptor, lowering the affinity of the receptor for transferrin (Feder et al., 1998). But patients with mutations in the HFE gene form less of these complexes and have more transferrin binding to transferrin receptors, resulting in more iron take up into tissues. Other forms of hereditary hemochromatosis involve mutations in ferroportin, hemojuvelin (HJV), and transferrin receptor 2 (TfR2) (Pietrangelo, 2005). The most common of these, “the ferroportin disease,” reduces iron export from cells (Pietrangelo, 2004). Most of these mutations affect hepcidin transcription and lead to hepcidin deficiency (Nemeth and Ganz, 2006).

Roth and Foos reported on three cases with hereditary hemochromatosis (Roth and Foos, 1972). They found iron in the peripapillary retinal pigment epithelium, ciliary epithelium, and sclera. In two of the patients, there was also drusen formation, the clinical hallmark of AMD. HFE is expressed in the mouse retina and RPE (Dunaief, 2006; Martin et al., 2006), suggesting that it may play a role in local control of retinal iron homeostasis. HFE knockout mice have retinal degeneration, supporting the importance of this gene in retinal function (Gnana J, et al. *IOVS* 2007;47:ARVO E-Abstract 2061).

Secondary hemochromatosis, or acquired hemochromatosis, results from iron intake in the form of multiple transfusions, such as in patients with sickle cell anemia or thalassemia. These diseases are associated with retinal abnormalities, sometimes including RPE atrophy or angiod streaks. It is difficult to separate the effects of intraocular hemorrhage, chelation therapy, and iron overload, but it is possible that retinal iron levels are elevated in these patients. Alternatively, retinal iron levels may be unaffected, if local control of iron homeostasis in the retina can prevent excess iron accumulation despite systemic iron overload. Retinal iron quantification and localization in *post mortem* eyes are needed to resolve these issues.

7.3.3 Friedreich's Ataxia (FRDA)—Friedreich's ataxia is an autosomal recessive neurodegenerative disorder characterized by progressive ataxia. In most patients with FRDA, the gene encoding the mitochondrial protein frataxin contains a mutation that leads to the progressive accumulation of iron in the mitochondria. While most cases of FRDA in the eye have shown a progressive late-onset optic neuropathy that rarely leads to severe vision loss, a recent case in a 59-year-old woman with FRDA documented severe optic neuropathy with rapid-onset catastrophic vision loss (Porter et al., 2007). Color images of the fundus showed a pale optic disk and scattered, fleck-like, yellowish deposits throughout the macula. These yellowish deposits autofluoresce and are likely to contain lipofuscin, which is also found in patients with AMD. A pigmentary retinopathy has also been associated with FRDA.

7.3.4 Pantothenate kinase associated neurodegeneration (PKAN)—Pantothenate kinase associated neurodegeneration, formerly known as the Hallervorden-Spatz syndrome, is an autosomal recessive neurodegenerative disorder characterized by iron accumulation in the brain (Koeppen and Dickson, 2001). Patients with PKAN have mutations in the pantothenate kinase 2 (PANK2) gene (Zhou et al., 2001). PANK2 belongs to a family of essential regulatory enzymes, pantothenate kinases. These enzymes produce phosphopantothenate, which condenses with cysteine in the production of coenzyme A. In patients with PKAN, phosphopantothenate is deficient, leading to the buildup of cysteine, which binds iron, potentially explaining iron accumulations in the brains of these patients. The regions of the brain that have the most iron normally are also those that are affected most severely by PKAN, namely the medial globus pallidus and the substantia nigra pars reticulata. In tissues where CoA is in the highest demand, CoA depletion and defective membrane biogenesis is likely to occur. In the retina, the rod photoreceptor cells continually generate membrane discs and the deficit of CoA may account for the retinopathy seen in patients with PKAN.

Clinical features of PKAN include childhood onset, dystonia, choreoathetosis, rigidity and spasticity, tremor, and dementia or psychomotor retardation (Koeppen and Dickson, 2001). The globus pallidus and the substantia nigra pars reticulatae have a brownish discoloration as a result of local iron accumulation. In the retina, a pair of dizygotic twins with PKAN showed pigmentary degeneration at age 7½ (Newell et al., 1979). These children had attenuated arterioles, hyperpigmented foveas, diffusely depigmented posterior poles, and bone corpuscle pigmentation. There was also subretinal accumulation of yellowish-white globular masses at the peripheral fundus. In the boy, the yellowish-white masses were located beneath choroidal vessels. In the girl, some of these masses were coated with pigment, giving the fundus a reticular appearance. Ophthalmoscopy of another 10 year old girl with PKAN showed a flecked retina, bone-spicule formation, and a bull's eye annular maculopathy (Luckenbach et al., 1983). The flecks and macular annulus corresponded to melanolipofuscin-containing macrophages. Nearby RPE cells were hypertrophic and contained membrane-bound aggregates composed of melanolipofuscin. The bone spicule pattern found by ophthalmoscopy corresponded to melanin pigment in RPE cells found around the retinal blood vessels. Light microscopy revealed total loss of photoreceptor outer segments and near total loss of inner segments throughout the retina. A mouse with knockout of PANK2 also exhibits photoreceptor degeneration, providing a model of the retinopathy in PKAN (Kuo et al., 2005).

7.3.5 Age-related macular degeneration—Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in developed nations in people age 65 and older (Klein et al., 1995; Leibowitz et al., 1980). Drusen are seen in early stages of the disease. As the disease progresses, large areas of geographic atrophy can develop in the dry or non-exudative form of AMD; choroidal neovascularization develops in the wet or exudative form. Oxidative stress and free radical damage have been implicated in the pathogenesis of AMD (Beatty et al., 2000; Zarbin, 2004). In a large clinical trial, patients with dry AMD given dietary supplements of antioxidants and zinc had reduced progression to advanced AMD (AREDS,

2001), suggesting that oxidative stress is somehow involved in its pathogenesis. The source of oxidants, however, is unknown.

Iron may be a source of oxidants in AMD. AMD-affected maculas (n=10) had more iron than healthy age-matched maculas (n=9), as demonstrated by an enhanced Perls' Prussian blue stain on sections of the optic disk and macula followed by computer-assisted analysis of digital images to quantify the stain (Hahn et al., 2003). Iron was found in the RPE and Bruch's membrane of early AMD, geographic atrophy, and exudative AMD (Fig. 13). Some sections were also treated with deferoxamine to determine whether the iron was chelatable. Comparisons of deferoxamine-treated sections to adjacent nonchelated sections showed that some of this iron in RPE and Bruch's membrane was chelatable (Fig. 14).

Iron overload was detected not only in the RPE but also in the photoreceptor layer of a 72-year-old white male post-mortem macula with advanced geographic atrophy (Dentchev et al., 2005). Perls' stain revealed iron in the photoreceptor and internal limiting membrane of the donor macula with geographic atrophy while no iron was detected in the neurosensory retina of 9 elderly donors with normal maculas. The macula of the eye with geographic atrophy also had elevated levels of ferritin and ferroportin in the photoreceptors and along the internal limiting membrane whereas the normal maculas were only weakly labeled when stained with anti-ferritin and anti-ferroportin.

While iron accumulation is observed in AMD retinas, it is unclear whether iron is involved in AMD pathogenesis or is simply a byproduct of AMD pathology; however, several lines of evidence suggest that iron directly contributes to AMD pathogenesis. First, *Cp-/-Heph-/Y* mice with retinal iron overload developed subsequent retinal degeneration with some features of AMD (Hahn et al., 2004b). These features included sub-RPE wide-spaced collagen that is normally found in association with drusen in humans, RPE lysosomal inclusions, and RPE death. As in AMD, *Cp-/-Heph-/Y* mice retinas exhibited focal photoreceptor degeneration, likely the result of preceding RPE death. *Cp-/-Heph-/Y* mice also developed subretinal neovascularization, which occurs in the progression of AMD to its wet form. Whereas subretinal neovascularization in AMD originates from the choroid and occasionally from the retinal circulation, the source of neovascularization in *Cp-/-Heph-/Y* mice is most often from the retinal vasculature but sometimes from the choroid (King, C., unpublished). Second, a patient with retinal iron overload as a result of aceruloplasminemia had early onset subretinal lesions similar to drusen found in patients with AMD (Dunaief et al., 2005). Third, transferrin is upregulated in patients with AMD (Chowers et al., 2006). Microarray analysis and quantitative real-time RT-PCR on *post mortem* retinas obtained from patients with AMD and retinas without AMD from donors of comparable ages detected a 3.5-fold increase in transferrin mRNA levels in non-neovascular AMD retinas compared to retinas without AMD and a 2.1-fold increase in neovascular AMD retinas compared to retinas without AMD. Western blot analysis showed that transferrin protein levels were elevated 2.1-fold in AMD retinas, and immunohistochemistry revealed more transferrin label in AMD retinas, especially in the photoreceptors, Müller cells, and drusen. Fourth, iron levels in the retina increase with age (Hahn et al., 2006). Eyes from younger donors (less than 35 years old) and older donors (older than 65 years old) were dissected and separated into the retina and RPE/choroid. Atomic absorption spectrophotometry of these components demonstrated a significant increase in iron levels in the retina in older versus younger individuals. There was no increase detectable in the RPE/choroid. Finally, iron is a potent catalyst of oxidative stress, and is known to cause photoreceptor/RPE toxicity experimentally and in human siderosis. Although siderosis causes pan-retinal degeneration and not drusen, geographic atrophy or CNV, this variability may reflect differences in the route of iron delivery and spatial and temporal patterns of iron accumulation.

8. Potential Therapeutics

Given that age-related macular degeneration may be caused by iron-mediated oxidative damage, it is reasonable to assume that antioxidants and iron chelators may be effective in reducing the occurrence and progression of AMD. While the Age-Related Eye Disease Study has shown that supplemental zinc, vitamin C, vitamin E, and β -carotene can provide a protective effect on AMD progression, it is likely that additional antioxidants may further prevent or slow the progression of AMD. Since iron is one of the most potent generators of oxidative damage through production of hydroxyl radicals in the Fenton reaction, and since the antioxidants used in the AREDS study may not quench all of the hydroxyl radical produced by iron, it is possible that iron chelators will prove a useful adjunct to AREDS vitamins. Several reports suggest that iron chelation may play a role in the treatment of a number of neurological diseases such as Alzheimer's disease and Parkinson's disease, Huntington's disease and Friedreich's Ataxia (Zheng et al., 2005; Richardson, 2004). It is plausible that iron chelation may also be useful in retinal disease associated with iron overload.

However, there are many challenges with using clinically-available iron chelators to prevent and treat retinal degeneration. Ideally, an iron chelator should selectively bind iron and not other biologically important divalent metals such as Zn^{2+} (Liu and Hider, 2002). In addition, an effective iron chelator must reach its target sites at a sufficiently high level; the chelator must be able to be absorbed in sufficient quantity through the gastrointestinal tract, the blood-brain barrier (BBB), or in the case of the retina, the blood-retina barrier. In order to successfully penetrate the blood-brain/blood-retinal barrier, an iron chelator must possess appreciable lipid solubility (Kalinowski and Richardson, 2005) and small molecular size, ideally below 500 Daltons (Maxton et al., 1986). An ideal iron chelator should additionally be uncharged in order to cross the BBB effectively (Richardson et al., 1990; Aouad et al., 2002).

Until recently the only iron chelator in widespread clinical use in the United States was deferoxamine B (DFO), and despite being a relatively effective iron chelator for the treatment of transfusional iron overload, it has many notable limitations. The drug is an inefficient iron chelator; typically only 5% or less of the drug administered promotes iron excretion (Bergeron et al., 2002). In addition, because the drug is poorly absorbed by the gastrointestinal system, and its elimination from the body is rapid, effective DFO treatment requires subcutaneous (SC) or IV administration for 9 to 12 hours for 5 or 6 days each week (Lee et al., 1993; Pippard, 1989). Therefore, for patients requiring chronic treatment, chelation with DFO is costly, inefficient, cumbersome, and unpleasant. Perhaps more significantly, there can be some rare but potentially serious side effects associated with DFO administration, including pulmonary toxicity, bony changes, growth failure, and promotion of *Yersinia enterocolitica* infections (Tenenbein et al., 1992; Brill et al., 1991; De Virgiliis et al., 1988).

DFO treatment can also sometimes result in retinotoxicity, making it a dubious candidate for the treatment of AMD or other ocular diseases (Olivieri et al., 1986). Haimovici *et al.*, describe macular and peripheral pigmentary changes, as well as reduction in retinal function as evidenced by decreases in ERG amplitude and EOG light-peak to dark-trough ratios (Haimovici et al., 2002). Patients who are treated with DFO should thus be followed closely with routine ophthalmologic consultation; FA, ERG and EOG testing may be more helpful in identifying early and more widespread retinal injury than fundus examination alone.

More recently, several other iron chelators have been put into clinical use, including deferiprone (L1) and deferasirox (Exjade). Deferiprone has the advantage of being orally active and has been shown to be a more efficient iron chelator than DFO in removing cardiac iron, the cause of most of the mortality in transfusional iron overload (Anderson et al., 2002). A recent report demonstrates the ability of L1 to decrease brain iron in patients with Friedreich's

Ataxia (Boddaert et al., 2007). This result suggests that L1 may similarly decrease retinal iron levels. Development of rare but serious side-effects of L1 may be avoided by careful monitoring. These include hepatic fibrosis, agranulocytosis, neutropenia, and arthropathy (Olivieri et al., 1986; Cohen et al., 2003; Ceci et al., 2002). The cause of deferiprone-related side effects is not currently known, but it may be due to the fact that deferiprone is a bidentate iron chelator. At low concentrations, bidentate iron chelators can actually facilitate the formation of free-radicals from the formation of incomplete iron chelator:iron 1:1 and 2:1 complexes (Hershko et al., 2005). Since three molecules of deferiprone are required to completely remove iron from the labile pool, low levels of deferiprone can leave iron incompletely chelated and may cause the unbound portion of iron to be an even more effective catalyst for the generation of free radicals. Given that side effects may be avoidable by careful monitoring and the evidence for efficacy in Friedreich's Ataxia, L1 may be a promising candidate for the long-term reduction of iron for the prevention or treatment of AMD, and its potential to reduce retinal degeneration due to iron-mediated oxidative stress will be studied in animal models.

Deferasirox (Exjade) is a novel iron chelator that has just been recently approved for clinical use in patients with iron overload due to blood transfusion. Deferasirox is orally active and has an extended half-life, allowing for once-daily oral dosing (Vanorden and Hagemann, 2006). Due to its relatively small size (MW 373.4), it is well absorbed and shows a two- to fivefold increased potency over deferoxamine for the mobilization of iron from tissue both *in vitro* and *in vivo* (Galanello et al., 2003). Current data show deferasirox to be as effective an iron chelator as subcutaneous deferoxamine, which is the current drug of choice for chronic iron overload patients (Piga et al., 2002). Clinical trials so far have shown deferasirox to have minimal side effects, with nausea, abdominal pain, diarrhea, and skin rash being the most serious and common side effects (Maxton et al., 1986). Deferasirox is another promising candidate for the long-term reduction of iron for the prevention or treatment of AMD, and its potential to reduce retinal degeneration due to iron-mediated oxidative stress will be studied in animal models.

Another potentially therapeutic iron chelator with interesting properties is salicylaldehyde isonicotinyl hydrazone (SIH). This chelator has excellent cell permeability because of its lipophilicity. It has thus far proven non-toxic in animals (Klimtova et al., 2003). SIH can protect cultured cardiomyocytes from oxidative stress induced death at concentrations 1000 fold lower than DFO (Simunek et al., 2005). In our experiments with cultured RPE cells, SIH affords nearly complete protection to ARPE-19 cells against hydrogen peroxide toxicity (Amado D, et al. *IOVS* 2006;47:ARVO E-Abstract 2081) and can even protect the cells against insults that are not oxidants (Lukinova, N., Dentchev, T., and Dunaief J.L., unpublished). We plan to extend these preliminary *in vitro* studies, then test the ability of SIH to protect ceruloplasmin/hephaestin deficient mice from their iron overload induced retinal degeneration.

9. Future directions

Iron is necessary for energy production through one electron transfer but is also a potent generator of oxidative damage. Many of its molecular chaperones have been identified, and their levels are tightly controlled by iron itself. A number of iron's handling proteins have been found in the retina, including transferrin, transferrin receptor, DMT-1, ferritin, ferroportin, ceruloplasmin and hephaestin. The challenge now is to determine how these proteins regulate the flow of iron into and out of the retina and among intracellular compartments. New information on these issues will come from polarized cell culture systems and conditional knockout mice targeting individual iron homeostasis genes in specific retinal cell types.

Evidence has implicated iron as a contributing factor in AMD. It is possible that iron induced oxidative damage is an early step in AMD pathogenesis, with inflammation, complement

deposition, and in some patients, neovascularization coming later. Iron may potentiate complement activation, as it has been shown in vitro to activate complement factor V (Vogi et al., 1991). Elevated iron levels, or even normal iron levels could be a significant contributor to oxidative stress. Preclinical models will determine which iron chelators can protect the retina against which range of insults. Ultimately, the only definitive test of iron's role in AMD would be a clinical trial, should preclinical studies show efficacy. It will be important to monitor closely for retinal toxicity and to avoid chelation of all iron, as some is needed for continued retinal function. Fortunately, it is the loosely bound, chelator-accessible intra- and extracellular iron that is most likely to cause oxidative damage. If this pool can be kept in check without depleting iron from enzymes, then chelator toxicity can be minimized.

People with elevated retinal iron levels may be at increased risk for AMD. Development of a non-invasive approach for iron measurement would be beneficial. Those with hereditary hemochromatosis resulting from mutations in HFE, ferroportin or hepcidin may have increased retinal iron levels and increased risk of AMD. A systematic study of the incidence of AMD in hemochromatosis patients, or conversely, a genetic survey of AMD patients for mutations in these genes will help address this issue.

Another approach to protection against the harmful effects of iron is to limit body (including retinal) iron stores. Iron stores throughout the body, including the macula (Hahn et al., 2006) increase with age in men and in post-menopausal women. This accumulation likely occurs because iron is ingested but not excreted. These iron stores are not necessarily safe, and may contribute to age-related disease (Sullivan, 2004). Harmless practices including limitation of red meat consumption and periodic blood donation will serve to limit iron stores and could reduce the risk of age-related disease including AMD. Iron accumulation has been associated with shortened lifespan in *Drosophila* (Massie et al., 1985), and prevention of this accumulation has been associated with lengthened lifespan (Massie et al., 1993). We have initiated studies on the effect of iron limitation on mouse lifespan and age-related disease, to determine whether this may one day prove a reasonable approach in people.

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Fig. 1. BALB/c WT immunostained with DMT1 (red). Strong DMT1 label can be seen in the rod bipolar cell bodies, rod bipolar cell termini, horizontal cell bodies, and photoreceptor inner segments. Scale bar represents 50 μ m.

Fig. 2.

H- and L- ferritin are present in normal retina in rod bipolar cells. Normal retina labeled for H- ferritin (red; **A**) and L- ferritin (red; **D**). Nuclei are counterstained with DAPI (blue). Both ferritin subunits have punctate label in the inner IPL, the region adjacent to the ganglion cell layer (GCL). This punctate label is characteristic of the synaptic terminals of rod bipolar cells, labeled with anti-PKCα (green), which colocalizes (gold) with both H-ferritin (**B–C**) and L-ferritin (**E–F**). This colocalization is best seen in high power images of these synaptic terminals which are positive for both PKCα and L- ferritin (arrowheads, inset **F**).

Fig. 3.

IRP1+/-IRP2-/- retinas have increased L-ferritin. Fluorescence photomicrographs of age matched wild type and *IRP1+/-IRP2-/-* retinas immunolabeled for L-ferritin (red), counterstained for nuclei with DAPI (blue), and imaged under identical exposure parameters. L- ferritin is increased in the *IRP1+/-IRP2-/-* inner retina, including bipolar cell synapses in the IPL, as well as in the OPL and inner segments. Scale bars represent 50 μ m.

Fig. 4.

Mitochondrial ferritin is present in normal inner segments and is increased in *Cp*^{-/-} and *Cp*^{-/-Heph-/Y} retinas. Fluorescence photomicrographs of age matched wild type, *Cp*^{-/-}, and *Cp*^{-/-Heph-/Y} retinas immunolabeled for mitochondrial ferritin (A–C) and imaged under identical exposure parameters. *Cp*^{-/-} retinas (B) have increased mitochondrial ferritin (red) in the inner segments (IS) compared to wild type (A), and *Cp*^{-/-Heph-/Y} retinas (C) have further increased mitochondrial ferritin. The label in the *Cp*^{-/-} inner segments (E) excludes the inner segment myoid (arrowhead, D–F) and colocalizes with a mitochondria-specific antibody (green) to the inner segment ellipsoid (D,F), suggesting mitochondrial localization of MtF. Nuclei are labeled with DAPI (blue). Scale bars represent 50 μ m.

Fig. 5. Cp and Heph mRNA and proteins are present in normal RPE and retina within Müller cells. (A) Bands from ethidium bromide-stained agarose gels corresponding to RT-PCR amplification products of the indicated mRNAs from dissected C57BL/6 murine RPE and cultured ARPE-19 cells. In all cases, the amplification product was of the expected size. (B) Western analysis of Cp and Heph in dissected C57BL/6 murine retinas (mRet) human retinas (huRet), cultured ARPE-19 cells, and cultured rMC-1 cells. Purified human Cp (labeled Cp) was used as control. (C) Control BALB/c retina labeled with a Cy3-conjugated donkey anti-rabbit secondary antibody. (D) Normal BALB/c retina immunolabeled for Heph. (E–G) Normal BALB/c retina double-labeled with anti-Heph and anti-CRALBP, a marker for Müller glia. A single red exposure reveals anti-Heph immunoreactivity (E). A single green exposure shows anti-CRALBP immunoreactivity (F). Double exposure shows yellow colocalization of Heph and CRALBP (G). Scale bars: 50 μ m.

Fig. 6.

Adult (6-month-old) *Cp*^{-/-}*Heph*^{-/-} RPE and photoreceptors accumulate iron. (A-C) 6-month-old WT (A), *Cp*^{-/-} (B), and *Cp*^{-/-}*Heph*^{-/-} (C) retinas Perls' stained for iron (blue) and counterstained with hematoxylin/eosin. (D) High magnification of Prussian blue Perls' label in 6-month-old *Cp*^{-/-}*Heph*^{-/-} RPE. (E and F) Light photomicrographs of 6-month-old *Cp*^{-/-}*Heph*^{-/-} (E) and WT (F) retinas after DAB enhancement (brown) of Perls' stain. (G-H) Electron micrographs of RPE from 6-month-old *Cp*^{-/-}*Heph*^{-/-} (G) and WT (H) eyes. Only the *Cp*^{-/-}*Heph*^{-/-} RPE (G) contains electrodense vesicles (*) sometimes fused with melanosomes.

Fig. 7. *Cp*^{-/-}*Heph*^{-/-}*Y* retinas have increased ferritin. Fluorescence photomicrographs of 6-month-old WT, *Cp*^{-/-}, and *Cp*^{-/-}*Heph*^{-/-}*Y* retinas immunolabeled for H-ferritin (A–C) and L-ferritin (D–F) and imaged by using identical exposure parameters. Scale bars: 50 μ m.

Fig. 8.

Ferroportin is present in normal retina at high levels in the Müller cell endfeet and RPE. Normal BALB/c retina immunolabeled with omission of primary antibody (**A**) localizes to Müller endfeet near the ILM, in photoreceptor inner segments (IS), and in the RPE, as demonstrated by the co-label with CRALBP (green), a marker for Müller cells and RPE (**B,C**). Ferroportin in the RPE excludes its apical microvilli, the green only label indicated with an asterisk (“*”) in **C** and in the inset of **C**, which shows a high power image of RPE co- labeled with ferroportin and CRALBP. Ferroportin is also present in a punctate pattern throughout the inner retina.

Fig. 9. *Cp*^{-/-Heph}^{-Y} retinas have increased ferroportin. Fluorescence photomicrographs of age matched wild type, *Cp*^{-/-}, and *Cp*^{-/-Heph}^{-Y} retinas immunolabeled for ferroportin (red) and imaged under identical exposure parameters. Nuclei were counterstained with DAPI (blue). Differences between wild type (A) and *Cp*^{-/-} (B) were subtle, but there is a clear increase in ferroportin label in the Müller endfeet of the *Cp*^{-/-Heph}^{-Y} retina (C). The punctate ferroportin label throughout the IPL is also increased in the *Cp*^{-/-Heph}^{-Y} retina. In order to optimally detect differences in ferroportin in the pigmented RPE, it was necessary to pre-bleach sections (D-F). Equivalently bleached retinas immunolabeled with ferroportin and imaged with equivalent exposure parameters reveals a robust increase in the *Cp*^{-/-Heph}^{-Y} RPE of ferroportin, which localized to both the apical and basolateral surfaces of the RPE (demarcated with brackets). Scale bars represent 50 μm.

Fig. 10. *IRP1+/-IRP2-/-* retinas have increased ferroportin. Fluorescence photomicrographs of age matched wild type and *IRP1+/-IRP2-/-* retinas immunolabeled for ferroportin (red), counterstained for nuclei with DAPI (blue), and imaged under identical exposure parameters. Ferroportin label is increased in the *IRP1+/-IRP2-/-* inner retina including Müller endfeet. Scale bars represent 50 μm.

Fig. 11.

Nine- month-old *Cp*^{-/-}*Heph*^{-/Y} mice have retinal degeneration. (A) Light photomicrograph of WT retina. (B and C) *Cp*^{-/-}*Heph*^{-/Y} retina has focal patches of hypertrophic RPE cells in some areas (B) and confluent hypertrophic RPE cells in other areas (C). (D) In an area of RPE hyperplasia (demarcated by arrowheads), *Cp*^{-/-}*Heph*^{-/Y} retinas have local photoreceptor degeneration [demarcated by arrows in the outer nuclear layer (ONL)] and subretinal neovascularization (red *). (E) In an area of hypertrophic, hyperplastic (area demarcated by arrowheads) RPE cells, a necrotic RPE cell also observed by electron microscopy (Left Inset) is present. Within the area of RPE hyperplasia, there is local photoreceptor thinning and subretinal neovascularization (red *) visible as small vessels containing erythrocytes (Right

Inset). The hyperplastic RPE have formed a localized cyst (Cy). (F) Electron micrograph of WT RPE. Br, Bruch's membrane; AM, apical microvilli; OS, photoreceptor outer segments. (G) Electron micrograph of *Cp*^{-/-}*Heph*^{-/Y} RPE overloaded with phagosomes and lysosomes containing photoreceptor outer segments at various stages of digestion. Some of these lysosomes (*) contained multilamellar structures characteristic of outer segment membranes (*Inset*). (H) Electron micrograph of *Cp*^{-/-}*Heph*^{-/Y} deposits between RPE and Bruch's membrane containing wide-spaced collagen (*). (Scale bars: A–E, 50 μm; F and G, 2 μm; H, 500 nm.)

Fig. 12.

Perls' Prussian blue staining of rabbit eye section injected with autologous blood into the subretinal space. Label can be seen in photoreceptor outer segments with the strongest label at the site of subretinal injection with fading further away from the site of blood accumulation. Scale bar represents 50 μm .

Fig. 13.

Photomicrographs of increased Perls-positive iron in age-related macular degeneration (AMD)-affected retinas compared with healthy retinas. Insets show Perls Prussian blue-stained retinal pigment epithelium (RPE) and Bruch's membrane (Br) in the same region as the larger figure of an adjacent periodic acid-Schiff (PAS)-hematoxylin-stained section. The PAS-hematoxylin-stained sections were unbleached, while sections shown in the insets were bleached as indicated. ONL indicates outer nuclear layer; PR, disorganized photoreceptors. A, Healthy macula has no Perls-3,3'-diaminobenzidine (DAB) stain after bleaching (inset). The remaining melanin in the RPE is identical to the DAB-hydrogen peroxide-only control (not shown), consisting of incompletely bleached endogenous RPE and not Perls-DAB signal. B, Nonexudative (Non-ex) AMD-affected, geographic atrophic macula with severe photoreceptor loss, RPE atrophy, and sub-RPE deposits. Bruch's membrane and sub-RPE deposits (sub) are positive for iron (inset). The RPE cells are unpigmented because endogenous RPE melanin has been bleached, but the Richardson (methylene blue/azure II) counterstain identifies RPE cells (Rs). C, Exudative AMD-affected retina adjacent to a fibrovascular scar in the macula. There is severe photoreceptor loss with RPE atrophy and thickened BR. The RPE contains iron detectable by the Perls Prussian blue stain without DAB enhancement (inset). Scale bars indicate 50 μm.

Fig. 14.

Photomicrographs of chelatable and nonchelatable iron accumulation in unbleached age-related macular degeneration (AMD)-affected maculas but not healthy maculas. Large panels show maculas stained with periodic acid–Schiff (PAS)-hematoxylin, and insets show adjacent sections unstained or stained with Perls-3,3'-diaminobenzidine (DAB) with or without prior deferoxamine chelation. ONL indicates outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; CNV, choroidal neovascularization. Note that red blood cells are positive for Perls-DAB stain, as expected. A, Healthy macula with minimal Perls-DAB signal observed in the retinal pigment epithelium (RPE) or Bruch's membrane (Br) of either the chelated or nonchelated sections, which appear only slightly darker than the unstained section. B, Nonexudative AMD-affected macula with signs of early AMD including drusen (not shown), slightly thickened Br, and mild photoreceptor (PR) loss without RPE atrophy. The RPE and Br in the nonchelated section are darker than in the unstained section, owing to increased Perls-DAB stain corresponding to total iron accumulation. Some of the increased iron is chelatable, demonstrated by decreased Perls-DAB stain in the deferoxamine-chelated compared with the nonchelated section. C, Exudative AMD-affected macula with marked PR loss, fibrovascular

scarring, sub-RPE deposits, and thickened Br. Choroidal neovascularization is present, with the same vessel in all sections, and few RPE cells (Rs) remain. Iron is increased in the RPE cells and Br and consists of both chelatable and nonchelatable forms. Scale bars indicate 50 μm .