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GPX1 knockout, not catalase knockout, causes accelerated abnormal optical aberrations and cataract in the aging lens

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Purpose: Glutathione peroxidase 1 (GPX1) and catalase are expressed in the lens epithelial cells and cortical fiber cells, where they detoxify H2O2 to reduce oxidative stress, which is a major cause for cataractogenesis. We sought to find out, between these two enzymes, which is critical for transparency and homeostasis in the aging lens by investigating alterations in the lens's refractive property, transparency, and gap junction coupling (GJC) resistance.
Methods: Wild-type (C57BL/6J), GPX1 knockout ($GPX1^{-/-}$) and catalase knockout ($CAT^{-/-}$) mice were used. Lens transparency was quantified using dark-field images and ImageJ software. For optical aberration evaluation, each lens was placed over a copper electron microscopy specimen grid; the grid image was captured through the lens using a digital camera attached to a dark-field binocular microscope. Optical aberrations were assessed by the quality of the magnified gridlines. Microelectrode-based intact lens intracellular impedance was measured to determine GJC resistance.
Results: In contrast to wild-type (WT) and $CAT^{-/-}$ lenses, $GPX1^{-/-}$ lenses developed accelerated age-related cataracts. While two-month-old lenses were normal, at nine months of age, $GPX1^{-/-}$ mice started to show the development of abnormal optical distortion aberrations and loss of transparency. At 12 months of age, $GPX1^{-/-}$ lenses developed significant opacity and abnormal optical distortion aberrations compared to $CAT^{-/-}$ and WT ($p<0.001$); these aberrations gradually increased with age and matured into cataracts by 24 months of age. There was also a significant increase ($p<0.001$) in GJC resistance in the differentiating and mature fiber cells of $GPX1^{-/-}$ lenses at 12 months of age compared to that in similar areas of age-matched $CAT^{-/-}$ and WT lenses.
Conclusions: Changes in the refractive and physiological properties of the lens occurred before cataract formation in $GPX1^{-/-}$ lenses but not in $CAT^{-/-}$ lenses. GPX1 is more critical than catalase for lens transparency, optical quality, and homeostasis in the aging lens under normal physiological conditions. GPX1 could be a promising therapeutic target for developing potential strategies to reduce adverse oxidative stress and delay/treat/prevent age-related cataracts.

The lens is an avascular organ. It has a layer of epithelial cells (ECs), an outer cortical zone of differentiating fiber cells (DF), and a central zone of mature fibers (MF), where a wide range of membrane proteins are cleaved and organelles are degraded. About 15% of the distance into the lens is the transition zone of DF to MF. A microcirculation involving aquaporin (AQP) water channels (AQP0, AQP1, and AQP5), gap junction (GJ) channels (Connexin [Cx] 43, 46, and 50), and ion channels helps to nourish the lens and dispose of metabolic waste [1-5]. GJ channels constituted by Cx membrane proteins form cell–cell aqueous pores that allow the cell-to-cell passage of water, ions, metabolites, and second messengers [6,7]. Cx43 and Cx50 are present in the lens epithelial cells, whereas Cx46 and Cx50 are expressed in the fiber cells [8,9]. Microcirculation is a key physiological process in lens homeostasis.

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Active oxidative metabolism occurs in the epithelial cells and the outer cortical region of the lens [10]. Since there is no intracellular protein turnover or replacement of lens cells, with age progression, the lens nucleus becomes vulnerable to oxidation by reactive oxygen species (ROS) and cataractogenesis [11-13]. Oxidative stress causes lens protein methionine oxidation, glycation, loss of sulfhydryl groups, thiolation, cross-linkage by non-disulfide bonds, and the formation of high molecular weight insoluble aggregates, all of which ultimately lead to cataract formation [14,15]. H_2O_2 is a major ROS that contributes to cataracts [16]. In the lens, it is mainly produced in the mitochondria and endoplasmic reticulum of ECs and DFs. H_2O_2 also passes from the aqueous and vitreous humors into peripheral lens cells [17-19]. It plays a paradoxical dual role. Under normal physiological conditions, low-level H2O2-induced redox signaling is needed for maintaining and modulating homeostatic functions. Supraphysiological levels cause oxidative distress in cells and evoke mechanisms of the inflammatory response, growth loss, and apoptosis [20,21]. The lipid phase of the membrane has a relatively low permeability for H_2O_2 or H_2O. Cells express AQPs to facilitate faster transmembrane diffusion of both [22-24].

Normally, the lens compensates for mild oxidant stress by increasing reduced Glutathione (GSH) and activating antioxidant enzymes such as superoxide dismutase (SOD), peroxiredoxins, catalase, and GPX1 [25-29]. Antioxidant enzyme activity decreases with age [30], leading to increased H_2O_2 levels in the lens cells. GPX1 is ubiquitously expressed in the cytosol and the mitochondrial matrix. It oxidizes GSH and reduces H_2O_2 to H_2O. Catalase is localized in the peroxisomes of mammalian cells and helps to eliminate H_2O_2produced during fatty acid oxidation; it reduces two H_2O_2 to two H_2O plus O_2. High production of ROS during aging or a significant decrease in the ROS scavenging ability of the lens cause adverse oxidative stress and lead to cataracts [31]. H_2O_2 concentration in the aqueous humor is typically about 0.03–0.07 mM; higher levels are seen in certain cataract patients [17,32].

Oxidative damage leads to reductions in lens GJ channels and compromise of the lens microcirculation [33-35], which are major factors in the homeostasis of the intracellular milieu. The purpose of the current study was to determine whether GPX1 and catalase antioxidant enzymes are equally relevant to homeostasis in the aging lens and whether there are detectable changes in the lens and gap junction coupling (GJC) due to the loss of GPX1 or catalase before cataract formation. We tested the lenses of GPX1 knockout ($GPX1^{-/-}$) and catalase knockout ($CAT^{-/-}$) mouse models at appropriate ages for optical aberrations, transparency, and GJC to evaluate the impact of the individual loss of these antioxidant enzymes on lens homeostasis and cataractogenesis.

METHODS

Animals: Wild-type (WT) C57BL/6J (C57; Jackson Laboratories, Bar Harbor, ME), GPX1−/− [36,37] and $CAT^{-/-}$ [38] mice were used. Knockouts and controls were bred back for 15–20 generations to bring them to C57BL/6J background. Animal procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" (Bethesda, MD), and protocols approved by the Stony Brook University Animal Care and Use Committee.

Evaluation of lens transparency and aberration: Eight lenses from each mouse model were used for the study. Lens transparency was assessed as previously described [39,40]. In short, lenses of WT, $GPX1^{-/-}$, and $CAT^{-/-}$ mice were dissected out and kept in prewarmed (37 °C) mammalian physiological saline and imaged under the same magnification and lighting conditions; a Zeiss dark-field binocular microscope equipped with an Olympus digital camera was used for imaging. From the lens images, the transparency was quantified using the pixel brightness intensity (SigmaScan Pro 5.0 and SigmaPlot 10 software programs). The higher the pixel brightness intensity is, the lower the transparency becomes. Pixel brightness intensity data were translated into percentage transparency and shown as bar diagrams using an arbitrary unit. Qualitative evaluation of aberrations in the lens was done using the dark-field optical grid focusing technique [40]. Each lens was placed on a copper electron microscope specimen grid with the anterior side facing the grid and imaged. The quality of the focused grid lines was used to assess light scatter and aberrations due to alterations in the refractive index gradient.

Lens impedance measurement: Lenses (12 months old) were dissected out from WT, $GPX1^{-/-}$, and $CAT^{-/-}$ mice and mounted as previously described [33,41-43]. For the study, six lenses were used for each mouse model. Impedance is a measure of GJC resistance. For impedance measurement, each lens was mounted in the perfusion chamber that was attached to a microscope stage and perfused with normal Tyrode's solution. Microelectrodes filled with 3M KCl with initial resistances of 1.5 to 2 MΩ [44] were used for GJC resistance measurements. A wide-band stochastic current was injected into a microelectrode that was inserted into a central fiber cell. At a distance r (cm) from the center of a lens of radius a (cm), another microelectrode was inserted into a peripheral fiber cell to record the induced voltage. Using a fast Fourier analyzer (Hewlett Packard, Palo Alto, CA), the frequency domain impedance (induced voltage ÷ injected current) of the lens was recorded in real time. At high frequencies, the magnitude of the lens impedance asymptotically approaches to the series resistance (Rs). Rs(KΩ) is the resistance of all GJs between the point of voltage recording and the surface of the lens. It was measured at multiple depths into the lenses of WT, $GPX1^{-/-}$, and $CAT^{-/-}$ mice.

Statistical analysis: The Student *t* test using SigmaPlot 10 software (SPSS Inc., Chicago, IL) was used to assess significance. Error bars represent standard deviations. P values <0.05 were considered significant.

RESULTS

Lens optical aberrations and transparency: Two-month-old lenses from WT, $GPX1^{-/-}$, and $CAT^{-/-}$ were tested for optical quality. Dark-field images are shown in Figure 1A (upper panel). The same lenses were photographed over a metal grid to assess the quality of the focused grid lines (Figure 1A, lower panel). There was no significant light scattering; all three lenses of different genotypes magnified the grid lines with a positive barrel distortion aberration, which is typical of a wild-type young lens. In this type of

aberration, the straight lines bend outward from the image center due to the spherical nature of the lens. Quantification representation by bar graphs (Figure 1B) shows that there was no statistically significant difference in lens transparency among the three genotypes ($p>0.05$) at two months of age. Twelve-month-old GPX1$^{-/-}$ and CAT$^{-/-}$ mouse lenses were tested for transparency and focusing ability and compared with age-matched WT lenses to determine which of these two enzymes is critical for protecting the lens from developing age-related optical aberrations, opacity, and cataract. There was increased light scattering and abnormal optical distortion aberration in GPX1$^{-/-}$ lenses (as revealed by the distorted grid lines), but not in CAT$^{-/-}$ lenses when compared to the WT (Figure 2A ; compare lower panel copper grid-focusing images). The quantification of lens transparency shown in Figure 2A revealed a statistically significant ($p<0.05$) increase in opacity in GPX1$^{-/-}$ lenses compared to the WT lenses; the values for the CAT$^{-/-}$ lenses were similar to those for the WT (Figure 2B).

A more thorough analysis of the GPX1$^{-/-}$ lens showed three zones (Figure 2C) of aberration; Zone I in the cortex showed a barrel distortion aberration (positive radial distortion) as in the WT lenses (Figure 2C). Zone II at the nuclear area showed an abnormal barrel distortion as evident from the highly distorted grid lines, compared to the matching area of the WT. The lenses exhibited a tendency toward transitioning to a pincushion distortion aberration (negative radial distortion) in the nuclear region. A new area, Zone III, was established between Zones I and II at the cortico-nuclear junction with a greater barrel distortion aberration than that of cortical Zone I.

We studied the changes in lens refractive properties and opacity from 9 to 24 months. Representative lens images at 9, 12, 16, and 24 months are shown in Figure 3A . Abnormal distortion aberration began at 9 months of age in the GPX1$^{-/-}$ lenses. At 12 months of age, the area corresponding to Zone II (see Figure 2C for the zones) of the lens nucleus showed increased abnormal aberrations compared to the corresponding zone of the 9-month-old GPX1$^{-/-}$ lenses. The abnormal aberration zones progressively increased in size as the lenses aged (compare Figure 3A, 12 - and 16-month-old GPX1$^{-/-}$ lenses) and developed into mature cataracts (Figure 3A, 24 -month-old lens), occupying almost the entire lens area in GPX1$^{-/-}$ in comparison to age-matched WT lenses. Like the 12-month-old lenses of CAT$^{-/-}$ (Figure 2A), the 24-month-old lenses were comparable only to those of the WT (data not shown). The quantification of lens transparency showed statistically significant ($p<0.001$) early onset and rapid progression in the severity of cataracts in the GPX1$^{-/-}$ lenses (Figure 3B) compared to the WT.

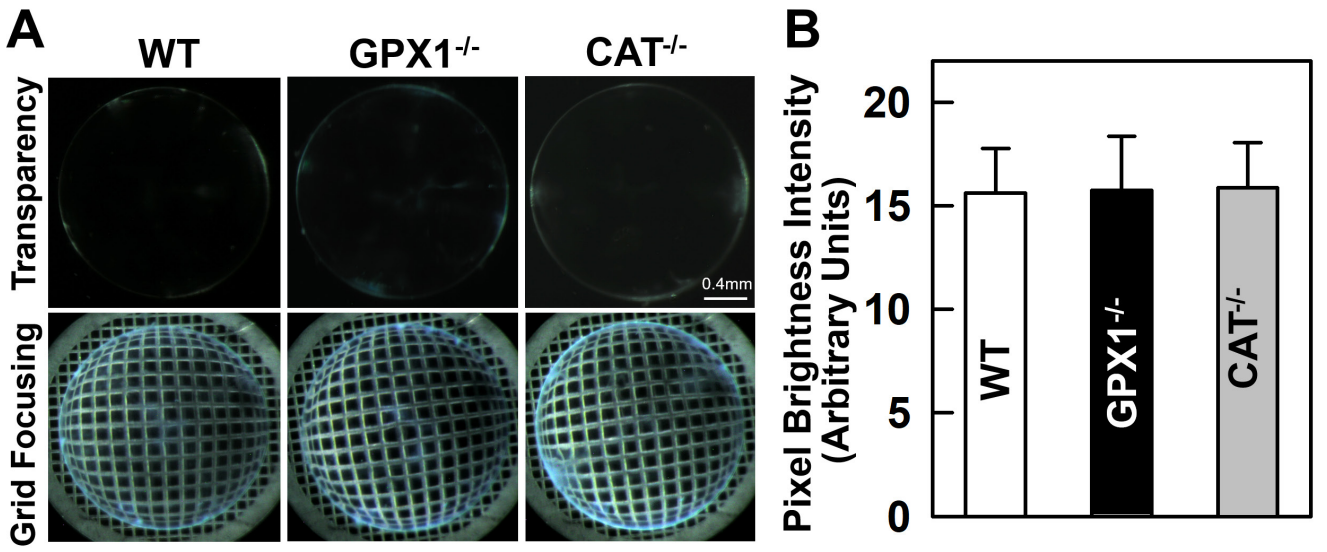

Figure 1. Lens transparency evaluation and quantification. **A**: Comparison of lens transparency in 2-month-old WT, GPX1$^{-/-}$, and CAT$^{-/-}$ mice. The lower panel shows the focusing of a metal grid by the lenses. **B**: Quantification of pixel brightness intensity to assess transparency in WT, GPX1$^{-/-}$, and CAT$^{-/-}$ mouse lenses. The higher the pixel brightness intensity is, the lower the lens transparency. There is no statistically significant difference in transparency in the lenses of the GPX1$^{-/-}$ and CAT$^{-/-}$ mice compared to the WT lenses. Eight lenses were used for each mouse model. Error bars represent standard deviations.<Fig_Large></Fig_Large>

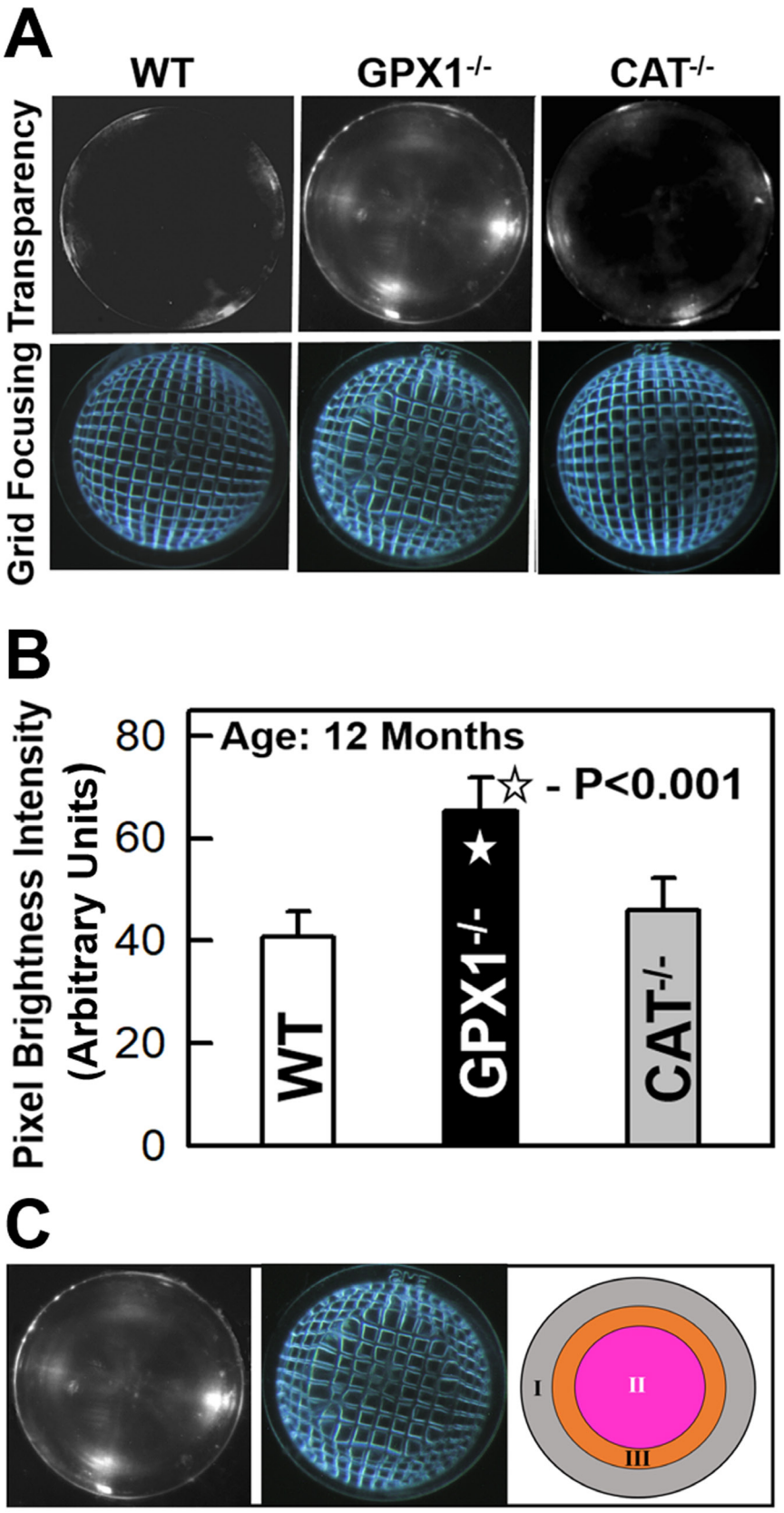

Figure 2. Knockout of GPX1, not catalase, causes alterations in lens transparency and refractive properties. **A**: Comparison of lens transparency and grid focusing ability of 12-month-old lenses of WT, $GPX1^{-/-}$ and $CAT^{-/-}$ mice. $GPX1^{-/-}$ mouse lenses showed considerable loss of transparency compared to WT and $CAT^{-/-}$ (Upper Panel). Of note is the abnormal optical distortion aberration with the formation of three zones in the $GPX1^{-/-}$ lens in contrast to the $CAT^{-/-}$ lens, which resembles the WT lens (Lower Panel). There is no significant difference in 12-month-old WT, $GPX1^{-/-}$ and $CAT^{-/-}$ lens diameters (2 mm). **B**: Quantification of pixel brightness intensity to assess lens transparency. The higher the pixel brightness intensity, the lower would be the lens transparency. Loss of lens transparency is statistically significant in ($p<0.001$) $GPX1^{-/-}$ and not in $CAT^{-/-}$ ($p>0.05$) compared to the WT. **C**: Dark-field image, grid focusing and schematics (not drawn to scale) of the three-zone formation in $GPX1^{-/-}$ (12- month-old lenses; lens diameter 2 mm). Eight lenses were used for each mouse model. Error bars represent standard deviations.<Fig_Med></Fig_Med>

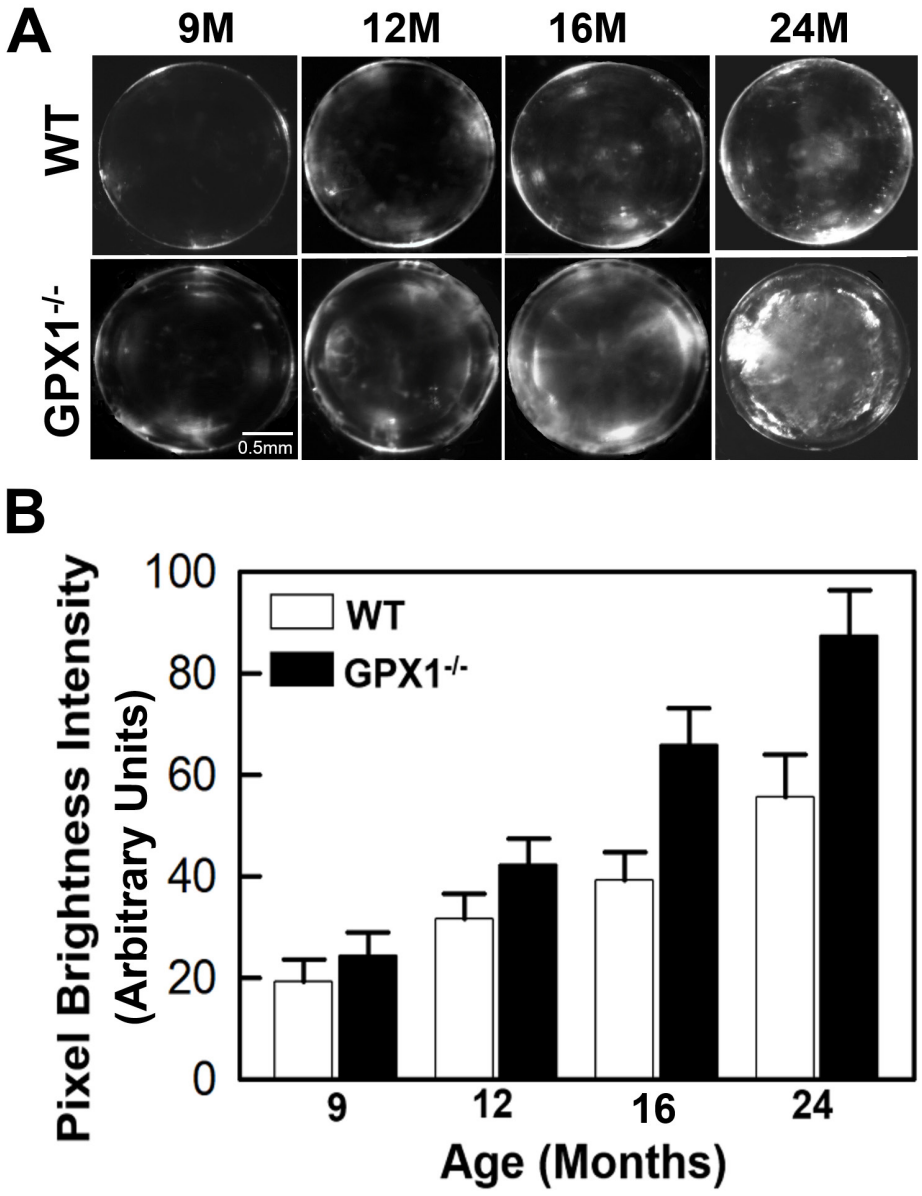

Figure 3. Lens transparency as a function of age. **A**: Dark-field images comparing lens transparency in WT and GPX1$^{-/-}$ mice at 9, 12, 16 and 24 months (M) of age. Lens refractive alterations as aberrations started around 9 months of age and continued. As age progressed, loss of lens transparency increased and manifested as mature cataracts in GPX1$^{-/-}$, signifying the role of GPX1 for lens transparency. The increase in the loss of lens transparency is statistically significant compared to the age-related loss of lens transparency in the WT type. **B**: Quantification of pixel brightness intensity to assess lens transparency. The higher the pixel brightness intensity, the lower would be the lens transparency. At each point, GPX1$^{-/-}$ mouse lenses showed a significant ($p<0.01$) increase in the severity of cataracts compared to WT lenses. Eight lenses were used for each mouse model. Error bars represent standard deviations.

Impedance (GJC resistance): We investigated the status of intracellular homeostasis by examining 12-month-old WT, GPX1$^{-/-}$, and CAT$^{-/-}$ mouse lenses for GJC resistance (Figure 4), which is a major component of the microcirculatory system. The higher the resistance is, the lower the GJC becomes. Twelve-month-old GPX1$^{-/-}$ lenses showed decreased GJC ($p<0.001$) compared to age-matched WT; however, 12-month-old CAT$^{-/-}$ lenses showed no statistically significant changes in GJC compared to WT lenses (Figure 4). Table 1 compares the coupling conductance values per unit area of cell-to-cell contact for specific regions in the lens in WT and GPX1$^{-/-}$ or in WT and CAT$^{-/-}$. DF and MF zones showed a statistically significant loss of GJC ($p<0.001$) in GPX1$^{-/-}$ but not in CAT$^{-/-}$ compared to the matching zones in WT.

DISCUSSION

Although GPX1 and catalase in mouse lenses have been studied by several investigators [37,45-48], the impact of these enzymes on lens microcirculation, and whether they play comparable roles in the prevention of age-related cataracts, is largely unknown. Several GPX antioxidant enzymes are present in mammalian cells. Ex vivo studies show that

Table 1. Twelve-month-old wild-type, GPX1−/− and CAT−/− lens fiber cell gap junction coupling conductances. Regional values of resistivity and normalized coupling conductance of WT, GPX1−/− and CAT−/−.

Genotype	Zone	Ri, KΩ-cm	Gi, S/cm²	Gi(WT)/Gi(KO)
Wild Type	DF	10.3	0.32	-
Wild Type	MF	13	0.26	-
$GPX1^{-/-}$	DF	14.3	0.23	1.4*
$GPX1^{-/-}$	MF	37.6	0.09	2.9*
$CAT^{-/-}$	DF	9.6	0.35	0.9
$CAT^{-/-}$	MF	15.4	0.22	1.2

Ri, resistivity; Gi, conductance; DF, differentiating fiber cells; MF, mature fibers; WT, wild-type. (* $p<0.05$).

GPX1 and catalase together contribute to only a small fraction of the H_2O_2 degradation; GSH contributes significantly higher levels of non-enzymatic H_2O_2 degradation [46]. In the current study, we tested the role of GPX1 and catalase in age-related cataractogenesis under normal aging conditions. $GPX1^{-/-}$ mouse lenses at two months of age did not develop cataracts; however, older $GPX1^{-/-}$ lenses, not $CAT^{-/-}$ lenses, developed age-related cataracts faster than the WT lenses.

Contradictory reports exist regarding the development of cataracts in $GPX1^{-/-}$ mice. Previous investigations on both $GPX1^{-/-}$ [47] and $CAT^{-/-}$ [38] used young mice of about eight-to-ten-weeks old and did not find any lens abnormalities; older $GPX1^{-/-}$ (age: 15 months [37]; 26 months [49]) developed mature lens cataracts. In our previous study, two-month-old $GPX1^{-/-}$ mice did not show significant differences in lens transparency, size, resting voltage, membrane conductance, or fiber cell membrane water permeability compared to those of age-matched WT; however, $GPX1^{-/-}$ lenses showed a significant reduction in GJC and the normal circulation of Ca^{2+} and Na^{+} [33]. We assume this phenomenon continues as age progresses, manifesting as cataracts at later stages, as we observed in the current study. An important finding of the present investigation was the recording of the presence of abnormal optical aberrations as a prelude to cataract

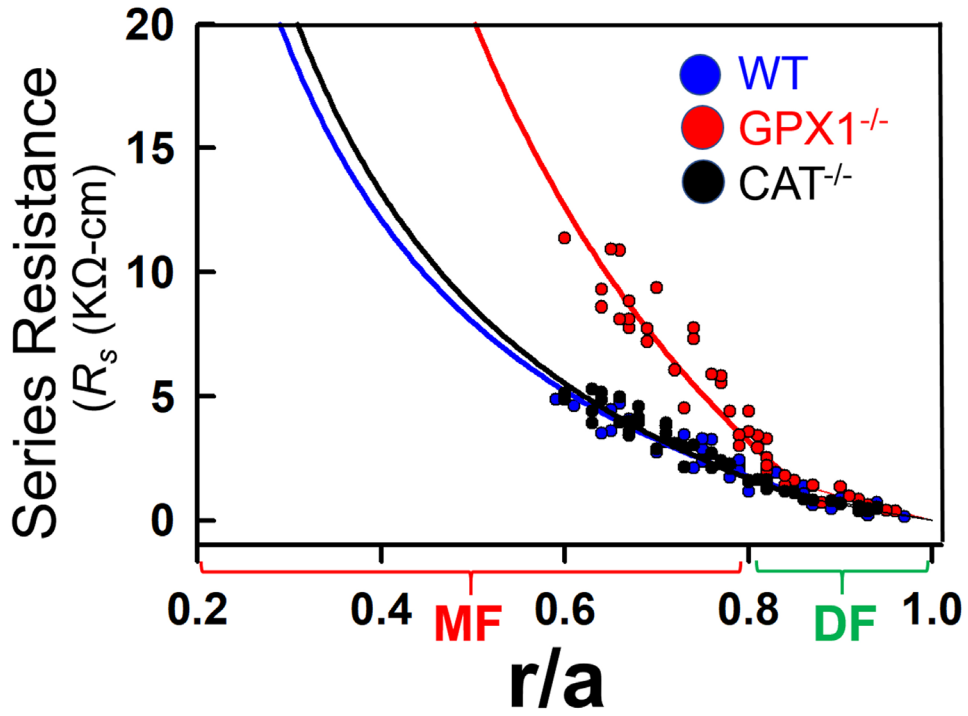

Figure 4. Impedance analyses of WT, $GPX1^{-/-}$, and $CAT^{-/-}$ lenses. Twelve-month-old $GPX1^{-/-}$ lenses, not $CAT^{-/-}$ lenses, showed a significant decrease in GJC. Series resistance (Rs) of lenses from WT (n=6) $GPX1^{-/-}$ (n=6), and $CAT^{-/-}$ (n=6) mice as a function of distance from lens center (r/a), where r (cm) is actual distance and a (cm) is lens radius. The higher the Rs is, the lower the GJC conductance. Lenses of $GPX1^{-/-}$ mice showed a significant ($p<0.001$) increase in Rs; hence, the decrease in conductance in both DF and MF was comparable to the resistance in similar areas in WT. DF: differentiating fiber cells; MF: mature fibers.

development in GPX1$^{-/-}$ lenses. Optical aberrations began around nine months of age and progressed to form different zones (Figure 2A, Figure 3A). Although the development of cataracts by GPX1$^{-/-}$ lenses has been reported by several investigators, the current study is the first to observe and record the presence of abnormal optical distortion aberrations that appear as a prelude to cataract formation.

When examined with a slit lamp, Reddy et al. [37] found that the GPX1$^{-/-}$ lens cortex was normal and only the lens nucleus showed light scattering. Ultra-structural examination of the lens nucleus using a transmission electron microscope showed an age-dependent increase in the distortion of fiber membranes and swelling of inter-fiber space at the apex of GPX1$^{-/-}$ fiber cells compared to the matching-areas in the WT lenses. Fiber cell membrane distortions were observed as early as three weeks in the lens nucleus of GPX1$^{-/-}$ [37]. The abnormal optical distortion aberration and zone formation we observed in the GPX1$^{-/-}$ lenses represent a unique phenotype, different from the global light scattering in the nucleus. The abnormal optical aberration was observed in the cortex around nine months of age in all the mice (54) examined. The current study reports a phenotypic change in GPX1$^{-/-}$ lenses with the development of abnormal distortion aberration, zone formation, and cataract development compared to the WT. GPX1$^{-/-}$ is a suitable model to study the etiology, progression, and impacts of age-related cataracts. The occurrence of abnormal optical aberrations can be used as a marker in drug-treatment studies to determine effective doses for preventing cataract formation and for early diagnosis of the onset of age-related cataracts in humans.

Antioxidant mechanisms are significantly reduced in the lens nucleus, making this area susceptible to oxidative damage [50,51]. Oxidative damage due to reactive free radicals and/or H_2O_2 may be the major factor(s) in age-related lens cataract development [17,29,52,53]. The time-dependent accumulation of such damage, particularly in central fiber cells, could cause severe impairment to lens functions. Jara et al. [54] noted that cataract development in a Cx46 mutant (Cx46fs380) may not be due to the loss of intercellular passage of GSH to the nucleus but possibly due to impaired microcirculation. It is reasonable to assume that loss of GJC, which occurs more in the MF than in the DF, is due to oxidative damage, which accrues with time. The loss of homeostasis in GPX1$^{-/-}$ could be due to reduced GJC resulting in a defective microcirculation. We did not find any adverse effects due to the loss of catalase even in older lenses under normal conditions. Unlike the loss of GPX1, the loss of other H_2O_2 degrading enzymes such as catalase (current study) and peroxiredoxins (Prdxs), especially Prdx6 [55,56], did not induce lens cataracts, suggesting GPX1 is critical to prevent cataractogenesis. Based on the ex vivo studies, catalase is more important for the decomposition of H_2O_2 at higher concentrations [46,57]. Giblin et al. [28] reported that for low concentrations of H_2O_2-induced damage, GPX1 is the primary source of protection in mammalian cells.

We noticed that the GPX1$^{-/-}$ lens phenotype and reduction in GJC were comparable to those in the lenses of a transgenic mouse model (AQP0ΔC/ΔC [39,40,58], which express only end-cleaved AQP0. Why is this so? Is there accelerated C-terminal end-cleavage of AQP0 due to the accumulation of H_2O_2 and functional alteration in GPX1$^{-/-}$ lenses? It is important to investigate the mechanistic and molecular mechanism(s) involved in causing GPX1$^{-/-}$ lenses to develop abnormal distortion aberrations and lose transparency, similar to that in AQP0ΔC/ΔC lenses. That is one of our future research goals.

Overall, our data suggest that between the two antioxidant enzymes studied, GPX1 is critical for preventing age-related lens abnormal optical distortion aberrations and cataract development under normal conditions; catalase may not play as significant a role as GPX1 protecting the lenses from oxidative damage under normal physiological conditions. The appearance of abnormal optical aberrations can be used as a morphological marker to assess treatment efficacies for early intervention. Our data suggest that GPX1 could be a potential therapeutic target to reduce adverse oxidative stress and delay/treat/prevent age-related cataracts.

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