Oxidative Stress Modulates Complement Factor H Expression in Retinal Pigmented Epithelial Cells by Acetylation of FOXO3

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Age-related macular degeneration (AMD), the leading cause of severe vision loss in the elderly, is a complex disease that results from genetic modifications that increase susceptibility to environmental exposures. Smoking, a major source of oxidative stress, increases the incidence and severity of AMD, and antioxidants slow progression, suggesting that oxidative stress plays a major role. Polymorphisms in the complement factor H (CFH) gene that reduce activity of CFH increase the risk of AMD. In this study we demonstrate an interaction between these two risk factors, because oxidative stress reduces the ability of an inflammatory cytokine, interferon-γ, to increase CFH expression in retinal pigmented epithelial cells. The interferon-γ-induced increase in CFH is mediated by transcriptional activation by STAT1, and its suppression by oxidative stress is mediated by acetylation of FOXO3, which enhances FOXO3 binding to the CFH promoter, reduces its binding to STAT1, inhibits STAT1 interaction with the CFH promoter, and reduces expression of CFH. Expression of SIRT1, a mammalian homolog of NAD-dependent protein deacetylase sir2, attenuated FOXO3 recruitment to the CFH regulatory region and reversed the H2O2-induced repression of CFH gene expression. These data suggest an important interaction between environmental exposure and genetic susceptibility in the pathogenesis of AMD and, by elucidating molecular signaling involved in the interaction, provide potential targets for therapeutic intervention.

The strongest environmental risk factor for AMD is cigarette smoking, which is associated with substantially higher incidences of geographic atrophy and choroidal neovascularization (11, 12). The mechanism by which smoking increases the incidence and severity of AMD is uncertain, but cigarette smoke is known to contain numerous oxidants (13). Exposure of mice to cigarette smoke or hydroquinone, an oxidant known to be present in cigarette smoke, resulted in sub-RPE deposits and diffuse thickening of Bruch’s membrane (14), suggesting that oxidative stress is capable of promoting these phenotypic characteristics of AMD. Oxidative stress has also been implicated in AMD by the Age-Related Eye Disease Study, which showed that antioxidants and zinc reduce the risk of individuals with large drusen progressing to advanced AMD, defined as choroidal neovascularization or severe geographic atrophy involving the center of the fovea (15). Also, proteomic analysis has demonstrated that drusen contain numerous proteins with adducts that are commonly caused by oxidative damage (16).

Thus, genetic studies suggest that the level of expression of (drusen) along Bruch’s membrane, atrophy of photoreceptors and retinal pigmented epithelial (RPE) cells (geographic atrophy), and enhanced risk of choroidal neovascularization.

Several genetic susceptibility loci for AMD have been identified, but some variants in and around the complement factor H (CFH) gene are associated with particularly high risk, and others are associated with reduced risk (3–7). A polymorphism encoding the sequence variation Y402H is associated with increased risk (3–6), and in vitro studies have shown that CFH His-402 has reduced binding to C-reactive protein, heparin, and RPE cells, which reduces its complement control activity (8, 9). In addition, detailed analysis of single nucleotide polymorphisms in and around the CFH gene has identified variants, some in noncoding regions, with high risk (7). No single polymorphism could account for the contribution of CFH, and instead, haplotypes associated with increased or decreased risk were identified. The authors hypothesized that these haplotypes might modulate risk of AMD not by disrupting protein function but, rather, by altering expression. Therefore, either reducing the amount of CFH or reducing its complement-modulating activity may increase the risk of AMD.

High risk and protective variants have also been identified in other genes that code for proteins involved in the regulation of complement, complement factor B, and complement component 2 (10). This supports the hypothesis that dysregulation of complement activity contributes to the development of AMD.

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2 The abbreviations used are: AMD, age-related macular degeneration; CFH, complement factor H; sir2, silent information regulator 2; IFN-γ, interferon-γ; RPE cells, retinal pigmented epithelial cells; STAT, signal transducers and activators of transcription; ChIP, chromatin immunoprecipitation; ANOVA, analysis of variance; JNK, Jun-N-terminal kinase.
CFH may play a role in the pathogenesis of AMD and several other lines of evidence implicate oxidative stress. In this study we sought to test the hypothesis that oxidative stress influences the expression of CFH.

MATERIALS AND METHODS

Reagents, Cells, and Transfections—Interferon-γ (IFN-γ) was obtained from Abcam (Cambridge, MA). The inhibitors PD98059, LY294002, p38 inhibitor, and PP2 were purchased from Calbiochem and used at concentrations of 50, 20, 10, and 5 μM, respectively. These concentrations were found to be effective in previous studies (17–20). Goat polyclonal anti-human CFH antibody was obtained from Calbiochem. Anti-phospho-Akt, anti-phospho-STAT1, anti-FOXO3, anti-STAT1, anti-Akt, and anti-β-actin antibodies were obtained from Cell Signaling (Beverly, MA). Anti-acetyl-lysine (clone 4G12) was obtained from Upstate Biotechnologies (Lake Placid, NY). Human RPE cells (ARPE-19 cells) (21) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection, ARPE-19 cells were grown in six-well plates and transfected with Lipofectamine™ LTX (Invitrogen) according to the manufacturer’s instructions.

DNA Constructs—The CFH promoter-luciferase reporter constructs were generated by PCR amplification using purified genomic DNA as template and the primers listed in Table 1. The PCR products were inserted into the HindIII and XhoI sites of pGL3 basic vector (Promega, Madison, WI). The plasmids FOXO3.WT, FOXO3.TM, FOXO3.TMΔDB, SIRT1.WT, and SIRT1.H363Y were provided by Dr. Michael Greenberg (Department of Neurobiology, Center for Blood Research Institute for Biomedical Research, Harvard Medical School, Boston, MA). The FOXO1-GEF construct was provided by Dr. William Sellers (Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA). STAT1α.Y701F and STAT1α.WT plasmids were provided by Dr. Jim Darnell (The Rockefeller University, New York, NY).

Immunoblots—ARPE-19 cells were seeded in 6-well plates (10⁵ cells/well), and after various treatments they were lysed in pre-warmed Laemmli buffer (Bio-Rad). For each sample the same amount of total protein was added to a well of a 10% acrylamide gel and resolved by SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). Nonspecific binding was blocked by incubation with 5% nonfat milk at room temperature for 2 h before overnight incubation with primary antibody at 4°C. A 1:50 dilution of anti-CFH and a 1:1000 dilution of all other primary antibodies was used. The proteins were detected by SuperSignal West Pico substrate solution using horseradish peroxidase-linked anti-rabbit IgG (Pierce). Blots were quantified by densitometry using a Bio-Rad Molecular Imager FX and Quantity One software.

Quantitative Real Time Reverse Transcription-PCR—The cells were harvested after various 24-h treatments, and total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and then incubated with rDNase I (DNA-free™ kit, Ambion, Austin, TX) to remove any traces of contaminating DNA. Reverse transcription was performed at 50°C for 1 h using 2 μg of total RNA, 1 μl of Superscript™ III reverse transcriptase, 1 μl of 50 μM oligo(dT)₂₀, 1 μl of 10 mM dNTP, 4 μl of 5X first-strand buffer, 1 μl of 0.1 M dithiothreitol, and 1 μl of RNase OUT (Invitrogen). Each 20-μl PCR reaction mixture was prepared using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics). The +1118 to +1329 CFH primers (Table 1) were designed to hybridize in two different exons or at exon/intron boundaries to prevent amplification of any remaining genomic DNA. The amplification conditions were denaturation at 95°C for 15 s, annealing at 55°C for 5 s, and extension at 72°C for 12 s for a total of 40 cycles. Under optimized conditions there was a single melting curve and no primer-dimer formation. The copy number for each mRNA was determined using a standard curve generated with external standards of known copy number. A fragment of the large P0 subunit of human ribosomal protein (primers, Table 1) was amplified to provide an internal control.

Luciferase Assay—ARPE-19 cells were plated in 6-well plates, and transfections were done at 60–70% confluence. CFH promoter-luciferase reporter constructs in combination with Renilla luciferase control vector (pRL-SV40 vector, Promega) were transfected along with relevant FOXO3 constructs into ARPE-19 cells using Lipofectamine™ LTX (Invitrogen). Twenty-four hours after transfection, cells maintained in the medium containing serum were treated with IFN-γ and H₂O₂ for 5 h and lysed with passive lysis buffer (Promega). For inhibitor studies, cells were pretreated with LY294002 for 30 min before the addition of IFN-γ and H₂O₂. Luciferase activity was measured using the dual-luciferase reporter assay system with a luminometer according to the manufacturer’s instructions.

### Table 1

| Primers used for PCR amplifications | Gene | Region | Primers |
|------------------------------------|------|--------|---------|
| CFH                               | −127 | −553 to +107 | 5'-AAACTCGAGTGCACTTTTCAATTGTTGTAGTTTGAGTT-3' |
| CFH                               | −129 | −420 to +107 | 5'-AAAAGCTCTGAGCTTCTTTTAAAGGAGAGCTTCACTGTA-3' |
| CFH                               | −132 | −553 to +62 | 5'-AAACTCGAGTGCACTTTTCAATTGTTGTAGTTTGAGTT-3' |
| CFH                               | −134 | −33 to +107 | 5'-AAACTCGAGTGCTTTTAAAGGAGAGCTTCACTGTA-3' |
| CFH                               | +1118 | +1118 to +1329 | 5'-TTGTCACGACTTCAACTTCACTTAC-3' |
| Ribosomal protein (large P0)      | +19 | +19 to +127 | 5'-ATGCTGCAGCTTCACTTCGTTG-3' |
Firefly luciferase activities were normalized to Renilla luciferase activities for transfection efficiency.

**Immunoprecipitation**—ARPE-19 cells were plated (10^7/10-cm dish), and the following day at 80–90% confluence they were treated with IFN-γ and H_2O_2 for 1 h. Cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris, pH 8.0, 50 mM KCl, 10 mM EDTA, 1% Nonidet P-40) containing deacetylase inhibitors (10 mM nicotinamide and 1 μM Trichostatin A), protease inhibitors (Roche Diagnostics mixture tablet), and phosphatase inhibitors (20 mM NaF and 1 mM orthovanadate). The lysates were immunoprecipitated with indicated antibodies for 5 h followed by the addition of protein G-Sepharose (Sigma) for overnight incubation. The beads were washed three times with lysis buffer and once with phosphate-buffered saline. The immunoprecipitated proteins were released from the beads by boiling in Laemmli buffer (Bio-Rad) for 5 min and subsequently analyzed by immunoblotting with appropriate antibodies.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assays were conducted as described by Nelson et al. (22) with minor modifications. ARPE-19 cells upon reaching 80–90% confluence were treated with IFN-γ and H_2O_2 for 1 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and then cross-linked with 1.42% formaldehyde at room temperature for 15 min followed by the addition of glycine to a final concentration of 125 mM and incubated for 5 min. After 2 washes with cold phosphate-buffered saline (PBS), cells were scraped in 1 ml of PBS and collected by centrifugation. The cell pellet was lysed in 1 ml of IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% Nonidet P-40, and 1% Triton X-100) containing deacetylase inhibitors (10 mM nicotinamide and 1 μM Trichostatin A), protease inhibitors (Roche Diagnostics mixture tablet), and phosphatase inhibitors (20 mM NaF and 1 mM orthovanadate). The resulting nuclear pellet was washed once, resuspended in IP buffer, and sonicated for three rounds of 15 pulses each at 50% power output and 90% duty cycle (Branson Sonifier 250, VWR, West Chester, PA). DNA shearing was controlled by extracting the DNA from sheared chromatin and running on an agarose gel to ensure that that DNA fragment size was 300–1000 bp. The sonicated lysate was cleared by centrifugation, and 20 μl was retained as an input control. Two hundred μl of cleared lysate was used for immunoprecipitation with or without the addition of antibodies (5 μg of each antibody per immunoprecipitation reaction) in an ultrasonic water bath for 15 min at 4 °C. The immune complexes were recovered with protein G-Sepharose (Sigma) and washed five times with cold IP buffer without any inhibitors. DNA was precipitated from input control samples by the addition of 100% ethanol. The immunoprecipitated DNA and input DNA were extracted by incubation with 100 μl of 10% Chelex (Bio-Rad) directly with precipitated beads or input DNA pellet and boiled for 10 min to reverse the cross-link. The DNA was purified by removing the Chelex slurry by centrifugation. PCR was performed with the purified DNA, according to instructions, with a High Fidelity PCR kit (Roche Diagnostics) using the following primers: forward, 5′-AAA CTC GAG CCA AAT TCA TCA AGC ACT GCA TTC TTG GCA-3′; reverse, 5′-AAA AAG CTT GGA TCT TTG AAG AGG ACA TTG ACC AGC TAA-3′. Analysis of PCR products was performed on a standard 1% agarose gel.

**Site-specific Mutagenesis**—Point mutations in the FOXO3 binding site were generated by site-specific mutagenesis by overlap PCR extension. The two smaller PCR products were generated using primer A forward, 5′-AAA CTC GAG CCA AAT TCA TCA AGC ACT GCA TTC TTG GCA-3′, and reverse, 5′-TAT CAG AAA CTT TTG CAA AAG CAA TAA AAA ATC AAC CAC A-3′, and primer B forward, 5′-TGT GGT TGA TTT TT TAT ATT GCT TTG GCA AAA GTT TCT GAT A-3′, and reverse, 5′-AAA AAG CTT GGA TCT TTG CAA AAG ACA TTG ACC AGC TAA-3′.

**Statistical Analyses**—Data are expressed as the mean (±S.D.), and statistical comparisons were done by analysis of variance (ANOVA) with Dunnett’s correction for multiple comparisons using SAS version 9 (SAS Institute Inc., Cary, NC) with p < 0.05 considered statistically significant.

**RESULTS**

**Oxidative Stress Reduces IFN-γ-induced Up-regulation of CFH**—The function of CFH is to down-regulate the alternative complement pathway to minimize bystander damage (for review, see Ref. 23). The expression of CFH is increased by the inflammatory cytokine, IFN-γ (24). The liver is the major source of CFH, but the RPE is a local source of production in the eye (25). Incubation of cultured human RPE cells with IFN-γ increased expression of CFH with a maximal effect achieved with 10 ng/ml (Fig. 1A). Exposure of RPE cells to 0.5 or 0.75 mM H_2O_2 reduced the IFN-γ-induced stimulation of CFH production, with 0.75 mM causing a greater reduction. Serum promotes cell survival and provides some degree of protection from noxious stimuli, but H_2O_2 had the same suppressive effect on IFN-γ-induced stimulation of CFH production in serum-containing media (Fig. 1B) as seen in serum-free media (Fig. 1A). This effect of H_2O_2 is due to oxidative stress because it is abrogated in a dose-dependent manner by preincubation with the antioxidant N-acetylcysteine (Fig. 1, c and d). Consistent with this conclusion, H_2O_2 and two other causes of oxidative stress, paraquat and FeSO_4, at concentrations previously shown not to reduce viability over this time frame (26), significantly reduced CFH mRNA in RPE cells (Fig. 1C). This could be due to destabilization of the mRNA or reduction in its production. To distinguish between these possibilities we examined the effect of oxidative stress on CFH promoter activity using a dual luciferase reporter assay. The IFN-γ-induced stimulation of CFH promoter activity was significantly reduced by H_2O_2-induced oxidative stress (Fig. 1F).

**STAT1 Mediates IFN-γ-induced Stimulation of CFH Promoter Activity**—STAT1 is a transcription factor that mediates many effects of IFN-γ (27). To determine if STAT1 mediates the IFN-γ-induced effects in RPE cells, transfections were done to generate cells with increased levels of STAT1 or dominant-negative STAT1. Cells overexpressing STAT1 showed enhanced IFN-γ-induced stimulation of p3xSTAT1-luc artificial promoter activity, and this was sig-
significantly decreased in cells expressing dominant-negative STAT1 (Fig. 2a). Western blots confirmed that, in the presence of IFN-γ, RPE cells expressing dominant-negative STAT1 had substantially less CFH than cells expressing wild type STAT1 (Fig. 2b). This suggests that STAT1 plays an important role in IFN-γ-induced up-regulation of CFH in RPE cells.

Oxidative Stress Stimulates Phosphorylation of 46-kDa Jun-N-terminal Kinase (JNK) and Translocation of FOXO Proteins into the Nucleus of RPE Cells—IFN-γ stimulates phosphorylation of STAT1, which allows it to enter the nucleus and stimulate transcription. We postulated that oxidative stress reduces phosphorylation of STAT1 and thereby prevents IFN-γ from increasing levels of CFH in RPE cells. To test this hypothesis, we...
mammals, four highly conserved FOXO family members have been identified and implicated in many cellular processes such as cell cycle arrest, apoptosis, DNA repair, and detoxification of reactive oxygen species (29). The effect of LY294002 on CFH expression caused us to investigate for involvement of FOXO proteins.

Regulation of FOXO proteins occurs in part through subcellular localization; enhanced activity of the phosphatidylinositol 3-kinase-Akt pathway results in phosphorylation of FOXO proteins causing nuclear exclusion and altered gene expression (30). We tested whether oxidative stress could override the sequestration of FOXO by Akt. RPE cells transfected with a FOXO1-GFP fusion protein showed diffuse fluorescence throughout their cytoplasm in the presence of 10% serum (Fig. 3d). After treatment with IFN-γ, a fraction of the fluorescently labeled FOXO1 entered the nucleus. However, in response to oxidative stress, nuclear translocation was essentially complete. Because we observed that IFN-γ did not inhibit activation of Akt (Fig. 3a), we postulated that the partial translocation of FOXO1 to the nucleus by IFN-γ was not mediated by changes in the phosphorylation state of Akt.

In some tissues oxidative stress has been shown to increase nuclear localization and FOXO transcriptional activity through activation of JNK (31). Both Akt and JNK phosphorylate FOXO proteins but do so at different sites. Akt, which is activated by growth factors, phosphorylates FOXO proteins at sites that promote their export from the nucleus, whereas JNK, which is activated by oxidative stress or other causes of stress, phosphorylates FOXO proteins at sites that promote translocation to the nucleus, and when both Akt and JNK are activated, the nuclear localization effect of JNK predominates, which explains why oxidative stress overrides the effect of Akt (31, 32). This appears to be the case in RPE cells because incubation with H2O2 caused activation of JNK in the presence and absence of IFN-γ or serum (Fig. 3e), and as we noted previously, expression of CFH was reduced by H2O2 in the presence or absence of serum (Fig. 1b), consistent with the notion that oxidative stress overrides the effect of Akt on FOXO proteins.

**FIGURE 2.** STAT1 mediates IFN-γ-induced stimulation of CFH promoter activity. a, 48 h after transfection with a plasmid containing an expression construct for STAT1 or dominant-negative STAT1 (dn-STAT1) and plasmids containing STAT1-responsive or control luciferase reporter constructs, RPE cells were treated with medium alone (open bar) or 10 ng/ml IFN-γ (solid bar) for 5 h. Luciferase activity was measured and normalized using the dual luciferase reporter system. Compared with wild type cells treated with medium alone, the normalized firefly luciferase activity indicating STAT1 artificial promoter activity was markedly increased in wild type cells or cells overexpressing STAT1 treated with IFN-γ. Cells expressing dn-STAT1 treated with IFN-γ had significantly less luciferase activity than IFN-γ-treated wild type cells (**, p < 0.01 for difference from wild type cells; **, p < 0.01 for difference from IFN-γ-treated cells by ANOVA with Dunnett’s correction for multiple comparisons). b, 48 h after transfection with 1 or 2 μg of plasmid expressing dn-STAT1 or empty control plasmid pcDNA3, RPE cells were treated with medium alone or 10 ng/ml IFN-γ. After 24 h Western blots showed that CFH levels were reduced in cells expressing dominant-negative STAT1, indicating that STAT1 is important in the IFN-γ-induced increase in CFH.

We performed Western blots with an antibody that specifically recognizes phosphorylated STAT1. IFN-γ increased pSTAT1 in RPE cells, and this was not reduced by co-incubation with H2O2; instead the IFN-γ-induced increase was further increased by H2O2 (Fig. 3a).

Phosphorylation is an important component of several signaling pathways. Small molecule kinase inhibitors were used to determine if activation of Src, phosphatidylinositol 3-kinase, or mitogen-activated protein kinase was involved in oxidative stress-mediated down-regulation of CFH expression. In contrast to the mitogen-activated protein kinase inhibitors PD98059 (17) and p38 inhibitor (19) and the Src inhibitor PP2 (20), which had minimal effect, the phosphatidylinositol 3-kinase inhibitor LY294002 (18) caused substantial reduction of IFN-γ-induced expression of CFH (Fig. 3b). Treatment with LY294002 also caused significant decrease in CFH promoter activity, and the addition of H2O2 caused a further reduction suggesting an additive effect (Fig. 3c).

LY294002 is a specific inhibitor for the phosphatidylinositol 3-kinase-Akt signaling pathway. Members of the FOXO family of forkhead transcription factors are key downstream targets of the phosphatidylinositol 3-kinase-Akt pathway (28, 29). In treatment of forkhead transcription factors are key downstream targets of the FOXO family of forkhead transcription factors are key downstream targets of the phosphatidylinositol 3-kinase-Akt signaling pathway. Members of the FOXO family of forkhead transcription factors are key downstream targets of the phosphatidylinositol 3-kinase-Akt pathway (28, 29). In

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FIGURE 3. Oxidative stress stimulates phosphorylation of 46 kDa JNK and translocation of FOXO proteins into the nucleus of RPE cells. a, confluent RPE cells were incubated in 10 ng/ml IFN-γ in the presence of 0, 0.5, or 0.75 mM H2O2 for 15 min, and cell lysates were run in Western blots for phosphorylated STAT1 (pSTAT1), phosphorylated Akt (pAkt), and total Akt. IFN-γ increased pSTAT1, and in contrast to CFH, there was a further increase by co-incubation with IFN-γ and H2O2. b, RPE cells were pretreated with the kinase inhibitors PD98059 (50 μM), P38 inhibitor (10 μM), PP2 (5 μM), or LY294002 (20 μM) for 30 min before treatment with 10 ng/ml IFN-γ in the presence or absence of 0.75 mM H2O2. Western blots showed that IFN-γ-induced stimulation of CFH was decreased by LY294002. LY294002 had an additive effect with H2O2 to further diminish CFH expression. c, RPE cells co-transfected with the luciferase reporter constructs containing the CFH proximal promoter described in the legend to Fig. 1 were incubated with medium alone (open bars) or IFN-γ (solid bars) in the presence or absence of 0.75 mM H2O2 and 20 μM LY294002. CFH promoter activity was strongly compromised upon exposure of cells to LY294002 and H2O2 (**, p < 0.01 for difference from IFN-γ alone; *, p < 0.05 for difference from IFN-γ and LY294002 by ANOVA with Dunnett’s correction for multiple comparisons). d, 48 h after transfection with FOXO1-GFP reporter constructs, RPE cells were left untreated (control) in serum-containing medium, treated for 5 h with 0.75 mM H2O2 in the presence or absence of 10 ng/ml IFN-γ, or treated with LY294002 in the presence or absence of H2O2. Cells treated with IFN-γ showed some translocation of FOXO1-GFP to the nucleus, whereas those treated with both H2O2 and IFN-γ showed essentially complete nuclear translocation. LY294002 also promoted FOXO protein to enter the nucleus. e, after treatment of RPE cells with 0.75 mM H2O2 in the presence or absence of 10% serum for 20 min, Western blots showed an increase in the phosphorylated 46-kDa JNK (p-46 kDa-JNK) with no change in total 46- or 54-kDa JNK.
Oxidative Stress and Complement Factor H

FIGURE 4. FOXO3 mediates the H2O2-induced reduction in complement factor H (CFH) expression. a, a schematic representation of the CFH promoter region showing the FOXO3 and STAT1 binding regions. UTR, untranslated region, b, 48 h after transfection with 1, 2, or 3 μg of FOXO3 expression plasmid, RPE cells were treated with IFN-γ and/or H2O2 for 24 h in serum-free medium. Western blots for CFH showed that overexpression of FOXO3 reduced the levels of CFH and increased the reduction caused by H2O2. The blot was stripped and reprobed with anti-p27Kip1 antibody, which showed that p27Kip1 was also reduced in cells overexpressing FOXO3. c, 48 h after transfection with empty plasmid or plasmids containing a constitutively active T32A/S315A triple mutant (FOXO3.TM) or a dominant-negative mutant (FOXO3.TMΔDB), RPE cells were treated with IFN-γ and/or H2O2 for 24 h in serum-containing medium. Western blots showed that cells transfected with FOXO3.TM had lower levels of CFH when incubated with IFN-γ alone or when co-incubated with IFN-γ and H2O2 compared with control cells under each of these conditions. In contrast, cells expressing dominant-negative mutant FOXO3.TMΔDB did not show a decrease in the level of CFH, and the reduction by H2O2 was moderated. The level of p27Kip1 was also reduced in cells expressing FOXO3.TM. d, 24 h after transfection with 2 μg of plasmid containing a FOXO3 expression construct and the luciferase reporter constructs described in the legend to Fig. 1f, RPE cells were incubated with medium alone (open bars) or 10 ng/ml IFN-γ (solid bars) in the presence or absence of 0.75 mM H2O2, and after 5 h luciferase activity was measured. The bars represent the mean (± S.D.) calculated from three independent experiments done in triplicate. Compared with cells incubated in IFN-γ alone, cells co-incubated in IFN-γ and H2O2 showed a significant reduction in the luciferase activity (**, p < 0.01 for difference from IFN-γ alone; *, p < 0.05 for difference from IFN-γ-treated cells overexpressing FOXO3 by ANOVA with Dunnett’s correction for multiple comparisons). e, recruitment of FOXO3 to the CFH promoter by IFN-γ and H2O2. RPE cells were incubated with IFN-γ and H2O2 for 1 h and subjected to ChIP assays with indicated antibodies.

FOXO3 to DNA is required for its modulation of CFH expression. Expression of the dominant-negative mutant compromised the ability of H2O2 to reduce CFH, but did not eliminate it, suggesting that FOXO3 is unlikely to be the only factor responsible for reduction of CFH by oxidative stress. In accordance with these data, CFH promoter activity was significantly reduced in IFN-γ-stimulated RPE cells overexpressing FOXO3 (Fig. 4d).

To determine whether endogenous FOXO3 binds to the regulatory region containing the predicted FOXO3 binding site on the CFH promoter, ChIP assays were carried out in RPE cells (Fig. 4e). An antibody to FOXO3 specifically immunoprecipitated the region covering the FOXO3 binding site, and cells treated with IFN-γ and H2O2 showed increased recruitment of FOXO3 to the CFH promoter. As noted above, translocation of FOXO3 to the nucleus is maximal in the presence of both IFN-γ and H2O2, and this may be part of the explanation for why both are needed for binding of FOXO3 to the CFH promoter. To explore the role of the FOXO3 binding site in modulation of CFH promoter activity, deletion constructs were tested in the dual luciferase reporter assay (Fig. 5a). Constructs spanning −553 to +107 or −420 to +107 relative to the translation start site, which each contained the FOXO3 and STAT1 binding sites, both showed significant reduction of IFN-γ up-regulation of CFH promoter activity by H2O2 (Fig. 5b). A −553 to −62 construct lacking both the FOXO3 and STAT1 binding sites had essentially no promoter activity. A −33 to +107 construct containing the STAT1, but not the FOXO3 site, showed strong stimulation of activity by IFN-γ that was not significantly reduced by H2O2. A construct with point mutations in the FOXO3 binding site (TAC to GCT) also showed a marked increase in activity in the presence of IFN-γ that was not reduced by co-incubation with H2O2 (Fig. 5c), indicating that FOXO3 mediated the H2O2-induced repression of CFH promoter activity.

Oxidative Stress Increases Binding of FOXO3 and Reduces Binding of STAT1 to the CFH Promoter—To explore the binding of FOXO3 and STAT1 to the CFH promoter, ChIP with primers that specifically amplify a 521-bp fragment of the CFH promoter showed a striking increase in CFH promoter immunoprecipitated by anti-FOXO3 from chromatin of RPE cells treated with both IFN-γ and H2O2 compared with either alone, indicating that the association of FOXO3 with CFH promoter is increased in IFN-γ-stimulated RPE cells exposed to oxidative stress (Fig. 6a). In contrast, ChIP with anti-STAT1 showed enhanced binding of STAT1 to CFH promoter in RPE
cells treated with IFN-γ alone that was reduced by co-incubation with IFN-γ and H₂O₂, indicating that the association of FOXO3 with CFH promoter under conditions of oxidative stress reduces binding of STAT1 to its site in the CFH promoter. Immunoprecipitation of RPE cell homogenates with anti-STAT1 followed by Western blotting with anti-FOXO3 showed increased association of FOXO3 with STAT1 in IFN-γ-treated cells that was reduced in cells co-incubated with IFN-γ and H₂O₂ (Fig. 6b). Compared with wild-type cells, those overexpressing FOXO3 showed a significant increase in STAT1 artificial promoter activity in the presence of IFN-γ, and this was significantly reduced by H₂O₂ (Fig. 6c). These data suggest that in response to IFN-γ stimulation, FOXO3 preferentially binds to STAT1 rather than its binding site on the CFH promoter and may actually enhance CFH production, but in the presence of oxidative stress, FOXO3 preferentially binds to the CFH promoter, reduces STAT1 binding to the promoter, and reduces expression of CFH. This implies that oxidative stress causes a chemical modification of FOXO3 that changes its configuration in such a way that its affinity for DNA binding is enhanced and its affinity for binding STAT1 is reduced.

Acetylation Is a FOXO3 Modification Induced by Oxidative Stress That Reduces IFN-γ-stimulated Expression of CFH—Acetylation and deacetylation of FOXO proteins provides another mechanism for regulation of their transcriptional activity. Immunoprecipitation of RPE cell homogenates with anti-FOXO3 followed by Western blotting with an antibody that specifically recognizes acetylated lysine residues showed that oxidative stress stimulated the acetylation of FOXO3 (Fig. 7, a and b). SIRT1 is a mammalian homolog of the NAD-dependent protein deacetylase sir2, which has been reported to control FOXO3 function by deacetylation (34–36). Oxidative stress failed to reduce the IFN-γ-induced increase in CFH in RPE cells overexpressing SIRT1, but cells overexpressing a SIRT1 mutant lacking deacetylase activity had the usual response (Fig. 7c). Conversely, deacetylase inhibitors, which would
favor the acetylated form of FOXO3 even in the absence of oxidative stress, attenuated IFN-γ-stimulated expression of CFH (Fig. 7d).

To explore the mechanism of the SIRT1 rescue of CFH expression, we investigated whether SIRT1 affected the ability of STAT1 and FOXO3 to bind to the CFH promoter. ChIP assays in SIRT1-transfected RPE cells showed that treatment with H2O2 led to increased recruitment of STAT1 to the CFH promoter and reduced FOXO3 binding (Fig. 7e). These data suggest that the acetylation of FOXO3 is critical in controlling CFH expression, and the rescue effect of SIRT1 is attributable to increased recruitment of the activator STAT1 and decreased occupancy of the repressor FOXO3 in the CFH promoter.

**DISCUSSION**

Epidemiologic studies provided a clue indicating that oxidative stress contributes to the pathogenesis of AMD and this led to Age-Related Eye Disease Study, which provided unequivocal confirmation (15). The demonstration that a substantial amount of genetic susceptibility is conferred by variants in the CFH gene, which encodes a protein that suppresses the alternative complement pathway and has no known role in protection from oxidative stress, seemed to point in a different direction. The mechanism by which CFH variants alter risk of AMD is unknown, but it seems likely that it is related to the ability of CFH to suppress complement activity and not some alternative uncharacterized function, because variants in factor B or complement component 2, other modulators of complement activity, also alter the risk for development of AMD (10). It is possible that Bruch's membrane is highly susceptible to damage from inflammation and is seriously compromised by reduction in activity and/or levels of CFH. This is analogous to the situation in the kidney, because uncontrolled activation of the alternative complement pathway due to CFH deficiency results in membranoproliferative glomerulonephritis (37–39). The vulnerability of both Bruch's membrane and the kidney to excessive complement activation is suggested by the presence of drusen, like those seen in AMD, in young patients with type II membranoproliferative glomerulonephritis (40). If CFH plays such a critical protective role for Bruch's membrane, a local source of production would be valuable.

A previous study has shown mRNA and protein for CFH in the RPE, which populates the inner surface of Bruch's membrane, and several other tissues in the eye (25). In this study, we have confirmed that RPE cells produce CFH, and as is the case in human proximal tubular epithelial cells (41), expression of CFH in RPE cells is strongly stimulated by the inflammatory cytokine, IFN-γ. We have identified a relationship between the two major risk factors for AMD, because oxidative stress reduces IFN-γ-induced expression of CFH in RPE cells. The mechanism of this suppression involves the activator STAT1 and the repressor FOXO3.

There are four mammalian FOXO (forkhead box, type O) transcription factors, FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX), and FOXO6. All are negatively regulated by insulin signaling through Akt, and they are further regulated in the nucleus by the acetylase p300, which serves as a transcriptional co-activator (28, 42). The FOXOs are normally located in the cytoplasm when phosphorylated in certain positions by Akt and translocate to the nucleus when Akt is down-regulated and/or oxidative stress is present, in which case FOXOs are phosphorylated in a different position by

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**FIGURE 6. Oxidative stress increases binding of FOXO3 and reduces binding of STAT1 to the CFH promoter.** a, RPE cells were incubated with IFN-γ and/or H2O2, and then ChIP was done using anti-FOXO3, anti-STAT1, or control IgG. PCR using primers specific for the CFH promoter showed the predicted 521-bp band for each sample. There was a striking increase in CFH promoter immunoprecipitated by anti-FOXO3 from chromatin of RPE cells treated with both IFN-γ and H2O2, as previously seen. In contrast, there was substantial CFH promoter immunoprecipitated from chromatin of RPE cells treated with IFN-γ alone but not those co-incubated with IFN-γ and H2O2, indicating that the association of FOXO3 with CFH promoter under conditions of oxidative stress reduces binding of STAT1 to its site in the CFH promoter. b, confluent RPE cells were treated with 10 ng/ml IFN-γ and 0.75 mM H2O2 for 1 h, then cell homogenates were immunoprecipitated (IP) with anti-STAT1 or control IgG, the immunoprecipitates were run in Western blots (WB) for FOXO3 (upper panel), and then the membranes were stripped and blotted with anti-STAT1 (lower panel). There is a strong association between STAT1 and FOXO3 in IFN-γ-treated cells that is reduced in cells co-incubated with IFN-γ and H2O2. c, RPE cells were transfected with p3xSTAT1 promoter-firefly luciferase (p3xSTAT1-luc) and a plasmid containing an SV40 promoter-\( \text{Renilla luciferase} \) reporter for normalization. Some cells were also transfected with a plasmid containing a FOXO3 expression construct; and 24 h after the transfections, the cells were treated for 5 h with media alone (open bars) or 10 ng/ml IFN-γ (shaded bars) in the presence or absence of 0.75 mM H2O2. Artificial STAT1 promoter activity was significantly increased in IFN-γ-stimulated cells (third bar) and was increased further in IFN-γ-stimulated cells that also overexpressed FOXO3 (fifth bar; *, p < 0.01 for difference from media alone; **, p < 0.05 for difference from IFN-γ-treated cells by ANOVA with Dunnett's correction).
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JNK, which overrides the Akt effect (31, 32). In the nucleus, FOXOs can bind their DNA binding site and nuclear proteins such as SIRT1 (43). SIRT1 is a member of a family of deacetylases that are homologues of sir2 (silent information regulator 2), which increases lifespan in yeast and worms but requires daf-16, the homologue of FOXOs, to do so (44). SIRT1 is the mammalian family member most similar to sir2. SIRT1 deacetylates a number of proteins including FOXOs and the tumor suppressor p53 (34–36, 45). FOXOs have the ability to induce transcription of several genes including genes involved in protection from oxidative stress (SOD2), DNA repair (GADD45), cell cycle arrest (p27KIP1), and apoptosis (BIM and Fas ligand), and these activities can be either stimulated or repressed by SIRT1 depending upon the tissue and conditions (46–48).

In this study we found that under normal conditions in RPE cells, FOXO3 is localized predominantly in the cytoplasm. Treatment with IFN-γ/H9253 causes some FOXO3 to enter the nucleus, where it preferentially binds STAT1 rather than its DNA binding site on the CFH promoter and does not

**FIGURE 7.** Acetylation is a FOXO3 modification induced by oxidative stress that reduces IFN-γ-stimulated expression of CFH. **a,** confluent RPE cells were treated with 10 ng/ml IFN-γ and/or 0.75 mM H2O2, and then cell homogenates were immunoprecipitated with anti-FOXO3. Immunoprecipitates (IP) were run in Western blots (WB) with an antibody directed against acetyl-lysine, and then the blots were stripped and re-probed with anti-FOXO3. Oxidative stress from exposure to H2O2 stimulated acetylation of FOXO3. **b,** densitometry confirmed that RPE cells treated with H2O2 showed substantially higher levels of acetylated FOXO3. The bars represent the mean (±S.D.) calculated from densitometry readings done on three separate Western blots using an antibody directed against acetyl-lysine on immunoprecipitates with anti-FOXO3. **c,** 24 h after transfection with 1 or 2 µg of empty plasmid or plasmid containing SIRT1 or acetats-dead SIRT1 (dnSIRT1), RPE cells were exposed to 10 ng/ml IFN-γ and/or 0.75 mM H2O2. Western blots 24 h later showed that the reduction in IFN-γ-stimulated expression of CFH by H2O2 was blocked by expression of SIRT1 but not by expression of dnSIRT1. **d,** RPE cells were pretreated with deacetylase inhibitors Trichostatin A (30 nM) and/or nicotinamide (5 mM) for 20 min and then treated with 10 ng/ml IFN-γ and 0.75 mM H2O2 for 24 h. Western blots showed that deacetylase inhibitors attenuated IFN-γ-stimulated expression of CFH. **e,** RPE cells transfected either with pcDNA3 or human sirT1 cDNA for 72 h were incubated with 10 ng/ml IFN-γ and 0.75 mM H2O2 for 1 h. ChIP assay was immediately conducted with indicated antibodies. The indicated band is the expected 521 bp.

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impede activation of the promoter by STAT1. However, in the presence of oxidative stress, there is almost complete nuclear localization of FOXO3, and it is acetylated, which reduces its interaction with STAT1, increases its binding to the CFH promoter, decreases STAT1 occupancy of its binding site, and reduces expression of CFH (Fig. 8). The current findings extend previous observations suggesting that FOXO3 proteins may function as either transcriptional activators or repressors (47, 48).

The elucidation of the molecular mechanism of the suppression of IFN-γ-induced expression of CFH by oxidative stress provides targets for therapeutic intervention. Local administration of acetylase inhibitors could help to blunt the oxidative stress-induced suppression of CFH expression in RPE cells. Sustained increased expression of SIRT1 by gene transfer would accomplish the same goal. Ultimately, one could envision combination therapy in patients at risk for or who show early signs of AMD aimed at direct reduction of oxidative stress with antioxidants, suppression of acetylation of FOXO3, and use of other inhibitors of complement activation.

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