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Author manuscript
Mamm Genome. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:
Mamm Genome. 2015 June ; 26(0): 210–221. doi:10.1007/s00335-015-9568-9.

Genomic regulation of senescence and innate immunity signaling in the retinal pigment epithelium

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Abstract

The tumor suppressor p53 is a major regulator of genes important for cell cycle arrest, senescence, apoptosis and innate immunity, and has recently been implicated in retinal aging. In this study we sought to identify the genetic networks that regulate p53 function in the retina using quantitative trait locus (QTL) analysis. First we examined age-associated changes in the activation and expression levels of p53, known p53 target proteins and markers of innate immune system activation in primary retinal pigment epithelial (RPE) cells that were harvested from young and aged human donors. We observed increased expression of p53, activated caspase-1, CDKN1A, CDKN2A (p16INK4a), TLR4, and IFNα in aged primary RPE cell lines. We used the Hamilton Eye Institute (HEI) retinal dataset (www.genenetwork.org) to identify genomic loci that modulate expression of genes in the p53 pathway in recombinant inbred BXD mouse strains using a QTL systems biology based approach. We identified a significant trans-QTL on chromosome 1 (region 172–177Mb) that regulates the expression of *Cdkn1a*. Many of the genes in this QTL locus are involved in innate immune responses, including Fc-receptors, interferon-inducible family genes and formin 2. Importantly, we found an age-related increase in FCGR3A and FMN2 and a decrease in IFI16 levels in RPE cultures. There is a complex multigenic innate immunity locus that controls expression of genes in the p53 pathway in the RPE, which may play an important role in modulating age-related changes in the retina.

Keywords

p53; CDKN1A; QTL; Genetic networks; Innate immunity; Retina; Aging; Age-related macular degeneration

Introduction

Organismal aging is a complex multi-step-process that is influenced by genetic and environmental factors and results in the progressive decline of cellular and tissue function. Because aging is a risk factor for many diseases including neurodegenerative diseases of the

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eye like age-related macular degeneration (AMD), considerable research has focused on identifying the cellular and molecular mechanisms inherent in aging. Increased susceptibility to apoptosis and cellular senescence are considered hallmarks of cellular aging along with increased genomic instability, nuclear DNA damage, shortened telomeres, and oxidative stress-induced damage (Campisi 2003). Senescence and apoptosis are thought to contribute to aging and age-related disorders by decreasing the proliferative potential of progenitor stem cells, altering tissue regenerative capacity, decreasing tissue function and by altered tissue architecture and microenvironment caused by altered gene expression and secretion of inflammatory cytokines, growth factors, and proteases (Campisi 2003; Coppe et al. 2008; Garfinkel et al. 1994; Krtolica and Campisi 2002; Kuilman et al. 2008; Novakova et al. 2010; Ohtani and Hara 2013). Increased secretion of cytokines, like interleukin (IL)-1a, IL-6, IFNβ and IFNγ, can also trigger an innate immune response via activation of toll-like receptors (TLRs) (Novakova et al. 2010; Ohtani and Hara 2013). Thus, increased apoptosis, cellular senescence, and inflammation may contribute to neurodegeneration and lead to an aging phenotype.

In the retina, the retina pigment epithelium (RPE) plays an important role in the immune defense of the retina, formation of the blood retina barrier and is also known to express several innate immune receptors, including TLRs and nod-like receptors. Age-related changes in RPE functions, inflammation and apoptosis-driven RPE dysfunction may alter retinal homeostasis and contribute to degenerative retinal diseases, like AMD (Dunaief et al. 2002; Parmeggiani et al. 2012). Aging has been shown to increase expression of genes involved in local inflammation and regulation of the immune system, including the retina (Chen et al. 2010). Altered expression of genes involved in the innate immune system, including genes involved in the alternative complement pathway like the complement factor H gene, are known to play a key role in AMD disease susceptibility and pathogenesis and in advanced stages of neovascular AMD (Whitcup et al. 2013; Zipfel et al. 2010). Secretion of inflammatory cytokines and activation of immunocompetent cells in the retina may play an important role in the loss of blood-retinal barrier function and ultimately to development of neovascular AMD (Parmeggiani et al. 2012). Inflammasome-mediated activation of caspase-1 and cleavage and secretion of IL-1β and IL-18 also appear to be important in AMD and drusen formation (Whitcup et al. 2013). A better understanding of the molecular mechanisms that activate the p53 network (senescence, apoptosis) and the innate immune system (inflammation, para-inflammation) during aging may lead to a better understanding of aging and to better treatment approaches to complex age-related diseases like AMD.

The p53 transcription factor is a master regulator of cell cycle arrest and an initiator of apoptosis and thus plays a major role in the regulation of cellular lifespan. The p53 protein is a sequence-specific transcription factor that regulates the expression of many genes, including its own function (through *MDM2, MDM4*), cell cycle arrest and DNA repair (*CDKN1A* [p21CIP1], *CDK2, GADD45, TRIM22*), senescence (*PML, PAI*-1), apoptosis (*BAX, PUMA, NOXA*, survivin), growth factors (*IGFBP*-3, *PTEN*), translation (Sestrin), autophagy (Sirtuin1), and innate immune receptors (TLRs) (Barsotti et al. 2012; Hasty and Christy 2013; Kitagawa et al. 2013; Klettner 2012; Purvis et al. 2012; Suzuki et al. 2009; Valente et al. 2013; Valente and Strasser 2013; Vuong et al. 2012). Activation of p53 can

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also directly initiate apoptosis via down regulation of anti-apoptotic BCL2 family proteins, including BCL2, BCLXL, and induction of mitochondrial outer membrane permeabilization. Importantly, p53 regulates innate immune mediated processes, including increased expression of TLRs, cytokines and chemokines, enhancement and stimulation of IFN signaling, IFN regulatory factor-9, and regulates expression of IFN stimulated genes and IFI family genes, (Gugliesi et al. 2005; Kwak et al. 2003; Menendez et al. 2013, 2011; Munoz-Fontela et al. 2008; Shatz et al. 2012). Aging in the RPE is associated with increased p53 levels and increased p53-mediated apoptosis (Bhattacharya et al. 2012). Thus p53 may play an important role in regulating aging in the RPE and in development of AMD.

Recombinant inbred (RI) strains of mice are a useful tool to study the genetic and molecular networks that contribute to a phenotype, disease or process, like aging in the eye (Geisert et al. 2009). The BXD family of RI mice are the largest panel of RI mice that were generated from a cross between C57BL/6J and DBA/2J mice. The tissue transcriptome profiles from the BXD family were used to generate the Hamilton eye institute (HEI) database and the data can be easily interrogated using the interactive GeneNetwork website (www.genenetwork.org) to identify the genetic networks that regulate phenotypes or diseases (Freeman et al. 2011; Jablonski et al. 2011; Templeton et al. 2013a, 2013b; Whitney et al. 2011). The purpose of this study was to use this systems biology approach to identify genomic loci that regulate the expression of the p53 pathway genes in the retina, and thus may play an important role in the aging retina and in susceptibility to AMD.

Materials and Methods

Interrogation of the HEI Retinal Dataset

In this study we used the HEI retina database to define the genetic loci that regulates expression of the p53 pathway (*Trp53, Tlr4, Cdkn2b, Cdkn1A, Ifna2* and *Casp1*) in the retina. This dataset has been described previously and a description is also available in the GeneNetwork website (Freeman et al. 2011; Geisert et al. 2009). This database contains the eye and retina transcriptome profiles from young (7–16 wks old) mice from 75 BXD strains and 5 control strains (C57BL/6, DBA/2J, both reciprocal F1s and BALB/cByJ). The HEI Retina dataset is comprised primarily of retinal tissue but robust signals for RPE-specific transcripts, including RPE65 and Bestrophin, indicate the presence of RPE in the samples.

When possible we selected ProbeSets within the coding region for each gene (ProbeSets used: 2585183, 2684234, 1227240, 2634083, 1233138 and 1247592). QTL mapping was performed using the WebQTL module on GeneNetwork using simple whole genome analysis without bootstraps or permutations to define QTLs that modulate gene expression levels related to senescence and inflammation. The cis-acting QTLs are located on the same genetic locus as the gene, while the trans-acting QTLs are genetic loci that modulate gene expression in a different location in the genome. This analysis produces a likelihood ratio statistic (LRS) score that indicated the confidence of linkage between the genomic loci and the p53 pathway of interest. A LRS score of over 15 is considered significant.

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Primary RPE cell culture

Primary human RPE cells were isolated from postmortem de-identified donor eyes provided by the Midsouth Eye Bank. The Institutional Review Board at the University of Tennessee Health Science Center approved the use of human donor eyes. We used RPE cell lines that were derived from two young donors (age 29 or 40) and from two aged donors (ages 84 and 86) to study the effects of aging on RPE physiology. Inclusion criteria for aged donor eyes included the absence of diagnostic criteria for AMD from the postmortem metadata profile. Exclusion criteria involved known retina dystrophies, diabetic retinopathy, uveitis, trauma, or other proliferative retinal diseases. RPE cells were isolated using procedures described previously (Chaum 2001). Briefly, globes were excised, anterior segment removed, vitreous extracted, and the retina was dissected free. The eyecup was washed with Dulbecco's Modified Eagles Medium (DMEM) followed by 0.25% trypsin/EDTA for four 15-min digestion cycles. Cells were loosened, transferred to DMEM with FBS, centrifuged at 2,000g for 5mins and the pellet was re-suspended in 1media with 5% Fetal Bovine Serum (FBS) and placed on poly-L-lysine coated 12-well culture ware. The fastest growing cells with cobblestone morphology were used for our studies. Primary cultures were maintained in DMEM and Ham's F12 medium (1:1 ratio) containing L-glutamine and 10% FBS.

Western blot analysis

The protocol for western blots has been described earlier (Bhattacharya et al. 2009, 2007, 2003). Cell lysates were prepared using mammalian protein extraction buffer (Pierce, Rockford, IL) with 150mM NaCl, 1mM Na_2EDTA and a protease inhibitor cocktail followed by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore Bedford, MA, USA) and probed with primary antibodies against p53, CDKN1A, CDKN2A, TLR4, Caspase1, Actin, FMN2, FCGR3A (Novus Biologicals, San Diego, CA), IFI16 and IFNA2 (Cell Signaling, Beverly, MA) overnight at 4° C in TBS buffer containing 0.1% Tween-20 and 5% nonfat dry milk (Bio-Rad, Hercules, CA), as per manufacturers recommendations. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1h and the immunocomplexes were visualized by the ECL detection system (Perkin Elmer, Waltham, MA). Representative Western blots from three experiments are shown. Densitometric analysis of all Western blots was performed using Image J software (National Institutes of Health). All data are expressed as mean ± SE. ANOVA and appropriate post hoc test determined the significance of the differences between means from each group. Values of $P<0.05$ were considered significant.

RNA isolation and qRT-PCR

Human RPE cell lines from young and aged donor eyes were grown to confluence, RNA was isolated using TRIreagent (Sigma Aldrich) and 1μg of total RNA was reverse transcribed to cDNA (Promega) using random hexamer primers as recommended by the manufacturer. Real-time qPCR was performed using an ABI Prism® 7700 sequence detection system (Applied Biosystems) and 120nM each primer. Each reaction was performed in triplicate from a minimum of 12 samples. Cycle threshold values for each gene are normalized to GAPDH expression in each sample and to expression in young HPE samples using the delta-C_t method. All data are expressed as the mean ± SE. Statistically

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significant differences were determined using a two-tailed students T-test. Values of $P<0.05$ were considered significant. Primers used were specific for *FCER1*γ forward 5`-TGA AGA TCC AAG TGC GAA AG-3`, reverse 5`-GCA TCT ATT CTA AAG CTA CTG TGG-3`; *IFI16* forward 5`-GCC AGC GTA ACT CCT AAA ATC-3`, reverse 5`-CCA CTT CCA TCT TCC CTG TA-3`; *FMN2* forward 5`-CAG AGA AGT TTT GCT CCC G-3`, reverse 5`-GCA GCC CAG GTA TAA AGT TG-3`; and *GAPDH* forward 5`-TTC GAC AGT CAG CCG CAT CTT CTT-3`, reverse 5`-ACC AAA TCC GTT GAC TCC GAC CTT-3`.

Results

Aging Activates p53 Signaling and Inflammatory Pathways in Human RPE Cells

We have previously shown that aging in human RPE cells activates p53-mediated apoptosis through increased level and post-translational modification of p53, increased levels of the pro-apoptotic marker PUMA, activation of caspase-3, increased levels of CDKN1A, a known transcriptional target of p53, and reduced levels of antiapoptotic BCL2, all of which lead to an overall increase in apoptosis (Bhattacharya et al. 2012, 2011). To investigate age-related changes in p53-mediated senescence and inflammation pathways, we measured the levels of p53 and its target proteins in primary RPE cultures from young and aged donors. Consistent with our previous observations, we found that basal levels of p53 were low in RPE cultures from young donors but were significantly increased in RPE from aged donors (Figure 1). To determine if p53 target proteins were also modulated in aged RPE cells, we measured the protein levels of CDKN1A. We observed increased levels of CDKN1A in the aged RPE compared to young RPE (Figure 1). We also examined expression of CDKN2A, which is a biomarker of senescence that is complementary to but independent of p53 activity. Consistent with activation of senescent pathways, aging in the RPE increases expression of CDKN2A (Figure 1).

Activation p53 can regulate tissue inflammation including modulation of cellular behavior in response to stressors (Vousden and Prives 2009). Since aging robustly increased p53 levels, we asked if components of the innate immune system that are known to be regulated by p53 (Gupta et al. 2001) were also upregulated in the aged RPE. We found that aging of RPE increases expression of the pro-inflammatory caspase-1 and of TLR4 and its downstream target IFNA2 (IFNα) but not IFNG (Figure 1), suggesting an activation of type I interferon responses. Thus, aging in the RPE is associated with both increased activation of p53 and increased expression of downstream targets that regulate innate immunity and senescence.

Identification of a QTL on Chr1 that Modulates the p53 Pathway in the Retina

Because increased activation of the p53 pathway appears to play a role in aging in the RPE, we sought to identify genomic loci that regulate p53-induced innate immunity in the retina. The HEI retinal database contains retinal and RPE samples, the latter of which is confirmed by the presence of robust signals for RPE specific transcripts (RPE65 and Bestrophin). We used the HEI retina database and the interactive website GeneNetwork to determine the genetic sources of variation in the expression of the p53-mediated innate immunity genes (*Trp53, Tlr4, IFNA2, Cdkn2b, Cdkn2a, Cdkn1a*, and *Casp1*) and to identify genomic regions that control expression of these p53 pathway genes. Of these transcripts, there is sufficient

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variability in expression of *Cdkn2b, Cdkn1a*, and *Casp1* (fold change 2–2.7) in the BXD mice (Figure S1). We used the QTL mapping tool to identify genomic regions that control expression of the genes in the p53 pathway using a likelihood ratio statistic (LRS), which indicates the confidence of linkage between the QTL and the gene of interest. The regulatory loci can either be a trans-QTL (located at a different genomic locus from the gene), or a cis-QTL (located at the same locus as the gene of interest). We identified a significant trans-QTL for *Cdkn1a* on Chr 1 (172–177MB, LRS of 21), a cis-QTL for *Cdkn2b* on Chr 4 (75–100MB, at the *Cdkn2b* locus, LRS 124), and trans-QTLs for *Casp1* on Chr 4 (125–150MB) and on Chr 15 (50–75MB) (Figure 2 A, B). The trans-QTL on Chr 1 has been previously identified as a QTL "hotspot" that is referred to as *Qrr1 and exhibits functional and genetic similarities with the orthologous region of human Chr 1q21-q23.* (Vogel et al., 2013, 2012). No significant QTLs were identified for the other genes in the p53 pathway. Combined QTL analysis of *Cdkn1a, Cdkn2a*, and *Cdkn2b* identify the cis-QTL on Chr 4 and the *Qrr1* "hotspot" (Figure 3). Thus we conclude that genes in the *Qrr1* locus control expression of *Cdkn1a* in the mouse retina. Candidate genes in this *Qrr1* region may regulate expression and activation of p53, apoptosis and innate immunity, include; FC receptor IgG low affinity (*Fcgr*) 2b, 3 and 4, FC receptor IgE high affinity γ (*Fcer1*γ), the interferon activated (Ifi) gene family (*Ifi202b, Ifi203-205, Aim2*) and Formin 2 (*Fmn2*) (Table 1). Several candidate genes in the Qrr1 region have variable expression in the retina of BXD mice, including *Fcer1*γ, *Fcgr3, FMN2*, and *Ifi205* (Figure S2), thus making them strong candidate genes in this region.

To determine if the levels of candidate genes in the *Qrr1* locus were altered in aging, we analyzed mRNA expression using qRT-PCR and performed Western blot analysis of lysates from young and aged human RPE cultures. We found that aging significantly increases *FCER1*γ mRNA expression, increases FCGR3A and FMN2 levels, and decreases *IFI16* (mouse IIF202b, IFI203-205) mRNA and protein levels (Figure 4 A–C). Interestingly, increased FMN2 protein levels did not correlate with increased mRNA expression and may indicate that aging alters FMN2 protein stability or degradation. Importantly, these genes regulate both innate immune system function and expression of p53 (Figure 5). This suggests that the *Qrr1* locus may play an important role in regulating age-related expression of *Cdkn1a* in the RPE, possibly through activation of p53 and local inflammatory pathways.

Discussion

Here we demonstrate that aging in the RPE is associated with a significant age-related increase in expression of CDKN1A, CDKN2A, TLR4, IFNA2, activated caspase-1, FMN2 FCGR3A and FCER1γ and a decrease in IFI16 levels (Figure 1, 4). We identified a genomic region on Chr 1 (*Qrr1*) that controls expression of *Cdkn1a* in the mouse retina. This Qrr1 hotspot on mouse Chr 1 exhibits functional and genetic similarities with the orthologous region of human Chr1q21-q23. The candidate genes in *Qrr1* (*Fcgr*2b, *Fcgr*3-4, *Fcer1*γ, *Ifi202b, Ifi203-205, Aim2* and *Fmn2*) play an important role in innate immune responses and may play a role in aging in the retina. We hypothesize that during aging, increased exposure to harmful environmental factors activate p53 and genes in the *Qrr1* locus, which in turn stimulate cell cycle arrest, senescence, innate immunity, and activate the complement cascade, TLR-4, and caspase-1-dependent pro-inflammatory pathways, resulting in

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abnormal secretion of inflammatory factors including interferons and cytokines (Figure 5). *Thus, the Qrr1 locus strongly links multiple genetic loci implicated in senescence, innate immunity, and acquired immunity responses with p53 signaling*.

We have previously shown that age is associated with increased expression and post-translational modifications of p53 that leads to disruption of Mdm2 interactions and initiate activation of p53-dependent apoptosis and cell cycle arrest in human RPE cells (Bhattacharya et al., 2012). In this study we identified a genetic network (*Qrr1*) that regulates CDKN1A expression in the mouse retina and human RPE, thus indicating that *Qrr1* may be an important regulator of p53-dependent senescence in the mouse retina and in the aged human RPE. In addition we have demonstrated that aging up-regulates expression of several proteins involved in senescence and inflammation in the human RPE. Together our data indicates the involvement of a genetic mechanism between p53 and several components of the innate immune signaling. The precise molecular mechanisms directing p53, senescence, and pro-inflammatory signaling in response to aging in the RPE are currently unknown and warrant further investigation.

Activation of inflammation and the innate immune system play a key role in aging and age-related disorders, like AMD. As a result of normal retinal physiology and light exposure, the retina is subjected to high levels of oxidative stress, which can activate local innate immunity response (“para-inflammation”) in an effort to restore normal retinal homeostasis (Xu et al. 2009). In the aging retina, RPE cells have increased senescence, increased secretion of chemokines and cytokines, and are exposed to low levels of chronic para-inflammation, all of which may contribute to breakdown of the blood-retinal barrier (Xu et al. 2009). Immune dysfunction is thought to play a key role in the pathogenesis of AMD due to the association of polymorphisms in the complement factor H gene with AMD and due to the presence of elevated retinal- and astrocyte-specific autoantibodies in the serum and retina of AMD patients, which increase with advanced stages of AMD (Morohoshi et al. 2012a, 2012b; Mullins et al. 2001; Patel et al. 2005; Penfold et al. 1990). Activation of the pro-inflammatory complement component 4b, complement factor H and caspase-1, are also found in the aged RPE/choroidal tissue (Cao et al. 2013; Chen et al. 2008). Our observations of increased expression p53, TLR4, IFNA2 and activated caspase-1 in aged RPE cells suggest that this innate immune signaling pathway may also be important during aging in the RPE.

Appropriate regulation of cell cycle progression and senescence is important for the immune system function in aging. *CDKN1A* is a transcriptional target of p53 but also has p53 independent functions. CDKN1A and p53 are regulators of cell cycle progression, apoptosis, cell survival, differentiation and senescence. IFNγ signaling has been shown to induce cell cycle arrest in part through activation of *CDKN1A* expression (Chen et al. 2000; Hobeika et al. 1999; Napione et al. 2012; Shen et al. 2008; Xaus et al. 1999). TLR2 can inhibit cancer progression by activating innate immunity signaling cascades that trigger CDKN1A and CDKN2A mediated senescence (Lin et al. 2013). Thus, CDKN1A is an important regulator of innate immune responses.

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Our data show that *Cdkn1a* expression is controlled by the *Qrr1* locus in the mouse retina. The *Qrr1* locus contains several immunomodulatory genes and has been shown to contain QTLs that regulate obesity, behavior, metabolism, among others, and is syntenic to human chromosome 1q21-23 (Allen et al. 1999; Choubey 2012; Haywood et al. 2000; Hogarth et al. 1998; Kikuchi et al. 2005; Moser et al. 1998; Mozhui et al. 2008; Rozzo et al. 2001; Santiago-Raber et al. 2009; Vogel et al. 2013, 2012; Vyse et al. 1997; Wakeland et al. 2001). Candidate genes in *Qrr1* that may modulate p53 activity and *Cdkn1a* expression include the FC-IgG receptor, low affinity (*FCGR*) family (*Fcgr2b, Fcgr4, Fcgr3*), Fc receptor IgE, high affinity receptor 1 γ (*Fcer1g*), interferon activated gene family (*Ifi204, Ifi203, Ifi202b, Ifi205, Aim2*), and Formin 2 (*Fmn2*). These genes are important regulators of the immune system, and we have found that aging in the RPE up-regulates expression of FCRG3A *FCER1*γ, and FMN2 and down-regulates IFI16 expression (Figure 4).

FCGRs play a role in the regulation of activation of the complement pathway and the immune system in aging. Activation of FCGRs can trigger phagocytosis, expression and secretion of inflammatory cytokines and chemokines, initiation of cell cycle arrest and apoptosis (Gessner et al. 1998; Karsten and Kohl 2012; Murinello et al. 2014; Nimmerjahn and Ravetch 2008; Rittirsch et al. 2009; Schmidt and Gessner 2005; Syed et al. 2009). A linkage between FCGRs and TLR4 has been reported both *in vitro* and *in vivo* (Rittirsch et al. 2009), and cross-talk between complement C5a and FCGR signaling pathways generates cytokines and chemokines and modulates FCGR expression (Karsten and Kohl 2012; Kumar et al. 2006; Schmidt and Gessner 2005; Skokowa et al. 2005; Syed et al. 2009). A possible role for FCGRs in AMD was indicated by the observations that their expression is increased in the aged mouse retina and they are present in immune complexes found in the retinas from wet AMD patients (Chen et al. 2010; Murinello et al. 2014). The Fc high affinity IgE gamma (Fcerγ) receptor, also a candidate gene in *Qrr1*, is a regulator of immune response to allergens and may inhibit activation of immune receptors, like TLRs (Novak et al. 2010). Based on our observations that FCGR3A and IFNA2 levels are increased during aging and because cross-talk between FCGR3 and TLR4 is known, it is possible that increased FCGR3 levels in aged RPE cells increase innate immune responses through activation of TLR4 signaling.

The family of IFN-inducible (IFI) -200 gene family encode structurally related proteins, whose expression is activated by interferons and includes mouse *Ifi202b, Ifi203, Ifi204* and absent in melanoma 2 (*Aim2*) and human *IFI16* and *AIM2*. Expression of IFI16 increases with age and can be activated by p53 (Song et al. 2008; Xin et al. 2004). Depending on the cellular context and its intracellular localization, IFI16 can activate or repress p53 transcriptional activity, activate or repress senescence, increase expression of *Cdkn1a*, activate cell cycle arrest, regulate autophagy, activate IFNβ, increase expression of interferon stimulated genes, and regulate activation of caspase 1 by AIM2 and NLRP3 inflammasoms and secretion of pro-inflammatory cytokines, chemokines and adhesion molecules (Baggetta et al. 2010; Costa et al. 2011; Datta et al. 1996; Duan et al. 2011; Fujiuchi et al. 2004; Gugliesi et al. 2005; Johnstone et al. 1998; Kwak et al. 2003; Ouchi and Ouchi 2008; Shi et al. 2014; Veeranki et al., 2011; Xin et al. 2004). IFI16 has anti-inflammatory properties through its negative regulation of AIM2-mediated inflammasome

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formation, and subsequent inhibition of caspase1 and IL1β activation (Veeranki et al. 2011). Our observation of decreased IFI16 levels in aged RPE cells may lead to increased AIM2 levels, AIM2-mediated inflammasome activity, increased activation of caspase-1 and increased levels of activated inflammatory cytokines, like IL-1β. Thus, *Aim2* and IFI16 are also candidate genes in the *Qrr1* locus.

Fmn2 expression can be activated by conditions that activate p53 including, DNA damage, oncogenic stress and hypoxia, but can also be activated by p53-independent mechanisms involving CDKN2A [p14Arf] and NFκB (Faix and Grosse 2006; Yamada et al. 2013a, 2013b). Intriguingly, FMN2 is required to stabilize CDKN1A levels, by inhibiting its degradation. Given that p53 activation correlates with CDKN1A and FMN2 levels in our aged RPE cells, it is tempting to speculate that FMN2 prevents CDKN1A degradation during aging in the RPE and thereby permits CDKN1A to accumulate to a level where it can promote retinal senescence and activate innate immune responses. Here we show that aging is associated with increased FMN2 protein levels, but not with increased *FMN2* mRNA expression. Thus aging may increase FMN2 levels through increased stability of FMN2, possibly through its association with CDKN1A, or through post-translational modifications. Recent analyses indicate that protein levels correlate with the corresponding mRNA by only 20–40% of the time (Tian et al., 2004; Nei et al., 2006). For example, we have shown previously that age-related post-translational modifications of p53 lead to increased stability and accumulation of the protein (Bhattacharya et al., 2012). Therefore, increased protein levels may not always reflect corresponding changes in mRNA expression, and suggest that alterations in protein stability can significantly modulate protein levels.

There are other potential candidate genes in *Qrr1* that are involved in adaptive immunity, including signaling lymphocytic activation molecule (SLAM) family genes, ubiquitin-fold modifier conjugating enzyme 1, transformation related protein 53 binding protein 2, toll-like receptor 5, and C-reactive protein. These proteins regulate cytokine production (Th2/ IL4), inflammatory signaling through the NFκB pathway, activate innate immune responses against bacterial infection, activate apoptosis, activate T cells, play an important role in unfolded protein response pathway/ ER stress response pathway and activate the complement cascade (Cannons et al. 2011; Hayashi et al. 2001; Hertel et al. 2013; Komatsu et al. 2004; Ma and Deenick 2011; Means et al. 2003; Schwartzberg et al. 2009; Tordella et al. 2013; Wilson et al. 2013). All of these transcripts may play a role in p53-mediated inflammation during aging and AMD pathogenesis.

Additional studies are needed to demonstrate that *Qrr1* regulation of *Cdkn1a* requires activation of p53, to identify which specific genes in *Qrr1* regulate *Cdkn1a* expression, and to show that *Qrr1* modulates aging in the retina. We were not able to identify QTLs for some of the genes in the p53 pathway (*Trp53, Cdkn2b* and *Tlr4*) due to the absence of sufficient variation in the expression of these genes across the BXD strains. While the BXD mice used to generate the HEI retinal database were not aged, the expression of the p53 pathway and *Qrr1* candidate genes (*FCGR3, IFI16*, AIM2 and *FMN2*) suggest a role in the aging RPE and warrant further investigation.

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Summary

We have identified a unique genetic locus on mouse Chr 1 (*Qrr1*) that modulates expression of *Cdkn1a*, the p53 pathway, and the innate immune system in the RPE. P53 and CDKN1A are important regulators of the cell cycle, apoptosis, and innate immunity, and our data show that the p53 pathway and candidate genes in *Qrr1* are modulated in the aged RPE. Several of the genes that map to this region are involved in innate immune responses and, due to the role of p53 in regulation of the innate immune system, are considered among the candidate genes involved in the aging retinal phenotype. We hypothesize that the *Qrr1* hotspot plays a role in modulating aging in the RPE through p53-mediated regulation of innate, and possibly acquired, immune responses. Together these genes may act to promote innate immunity, inflammation, and senescence in the aging RPE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

These studies were supported in part by an unrestricted UTHSC departmental grant from Research to Prevent Blindness, New York, NY, the Plough Foundation, Memphis, TN, The Lions of Arkansas Foundation Inc., and the UTHSC Hamilton Eye Institute NEI Core Grant for Vision Research (P30 EY013080). The authors would also like to thank Dr. Rob Williams for his comments and review of the manuscript.

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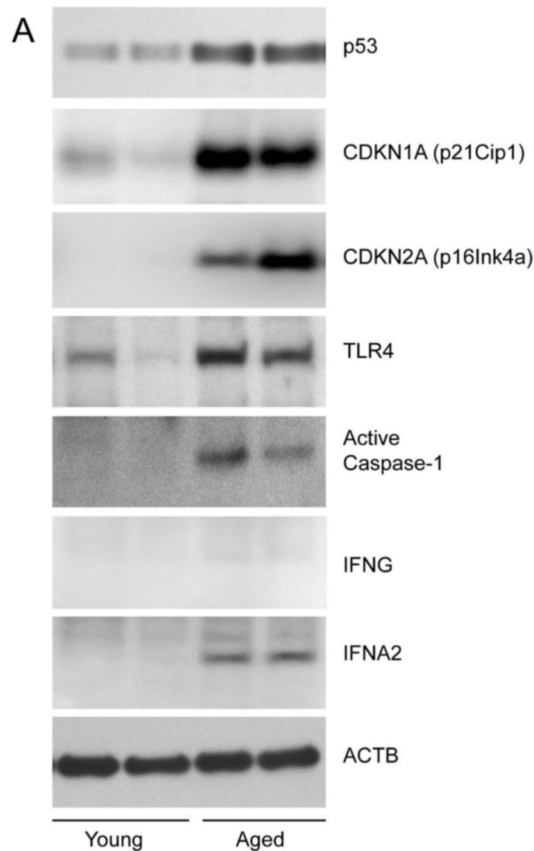

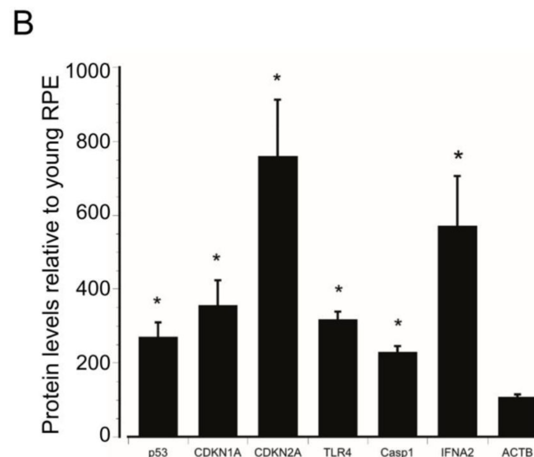

Figure 1.

Aging activates the p53 pathway in RPE cells. A) Primary cultures of RPE cells obtained from two young and two aged (29, 40, and 84, 86 years, respectively) human donor eyes and were grown to confluence. RPE cell lysates were analyzed by western blot for total-p53, CDKN1A, CDKN2A, TLR4, active caspase-1, IFNG, and IFNA2 using specific antibodies. Beta actin was used as an internal loading control. B) Densitometric values from young RPE cells were set at 100%. Data shows the mean +/− SE. *, significantly different compared with young RPE cells ($P<0.05$). N=3.

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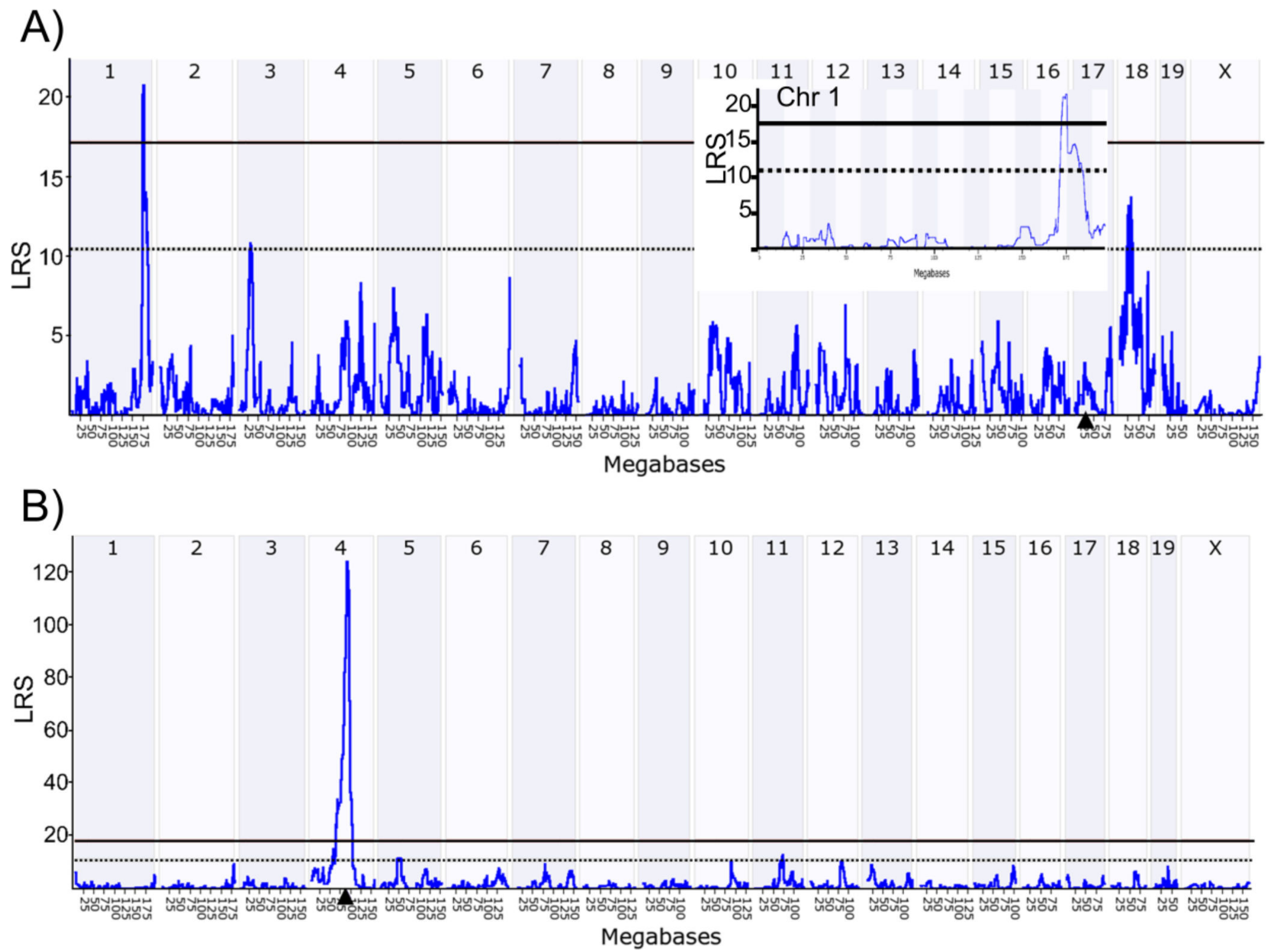

Figure 2.

Identification of genomic networks that regulate expression of *Cdkn1a*, Cdkn2a or *Cdkn2b*. The data illustrates the likelihood ratio statistic (LRS) scores for expression of (A) *Cdkn1a* (B) *Cdkn2b* in the retina of BXD RI mice. QTLs with significant LRS scores are indicated by a horizontal solid black line and regions with suggestive LRS scores are indicated by a dashed black line (~17.2, and ~10.5, respectively). Arrowhead indicates the query gene locus. Insert (A) shows the trans-QTL on Chr 1 (*Qrr1*).

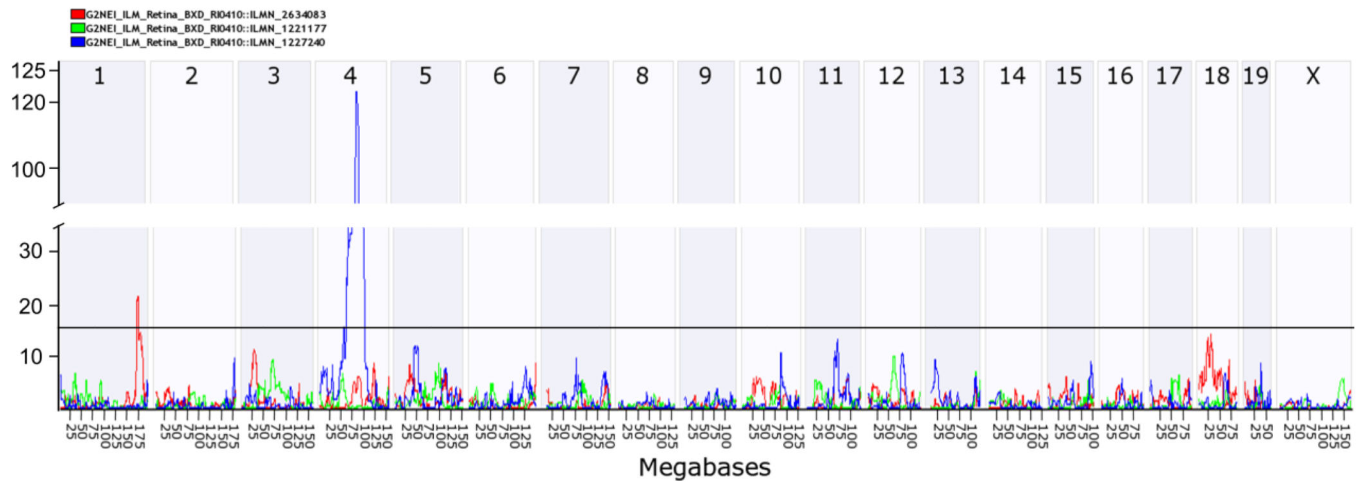

Figure 3.

Graphic representation of genomic networks that regulate the expression of p53 pathway in the retina. The data illustrates the likelihood ratio statistic (LRS) scores for the combined expression of the *Cdkn1a, Cdkn2a* and *Cdkn2b* in the retina of BXD RI mice. A significant cis-QTL peak on mouse chromosome 4 (LRS=124) at the location of the *Cdkn2b* gene and a trans-QTL on chromosome 1 (LRS= 21) is seen. Horizontal line marks the threshold for significance (LRS=15).

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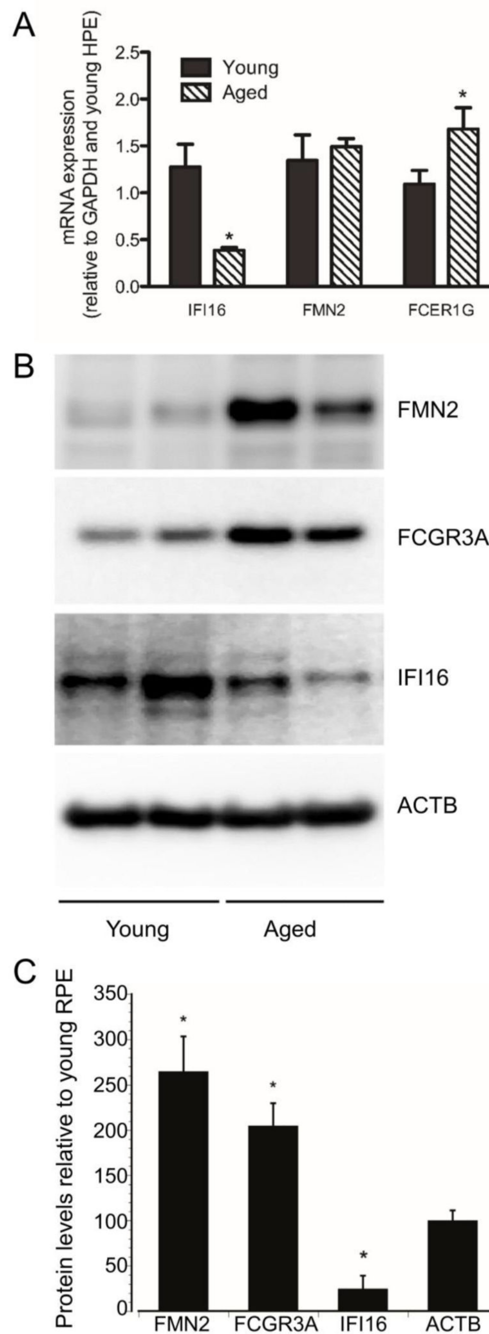

Figure 4.

Aging alters expression of genes in the *Qrr1* locus in RPE cells. A) mRNA expression from primary cultures of young or aged RPE cells were analyzed by qRT-PCR for expression *IFI16, FMN2, FCER1*γ and *GAPDH*. Cycle threshold values for each transcript were normalized to *GAPDH* and young RPE samples using the delta-Ct method. N=12. *, denotes significantly different values (P<0.05). B). Cell lysates from primary cultures of young or aged RPE cells were analyzed by Western blot for expression of IFI16, FMN2, and FCGR3A. Beta actin was used as an internal loading control. C) Densitometry analysis of

Western blot bands. Values from young RPE cells were set at 100%. Data shows the mean +/– SE. *, significantly different compared with young RPE cells ($P<0.05$). N=3

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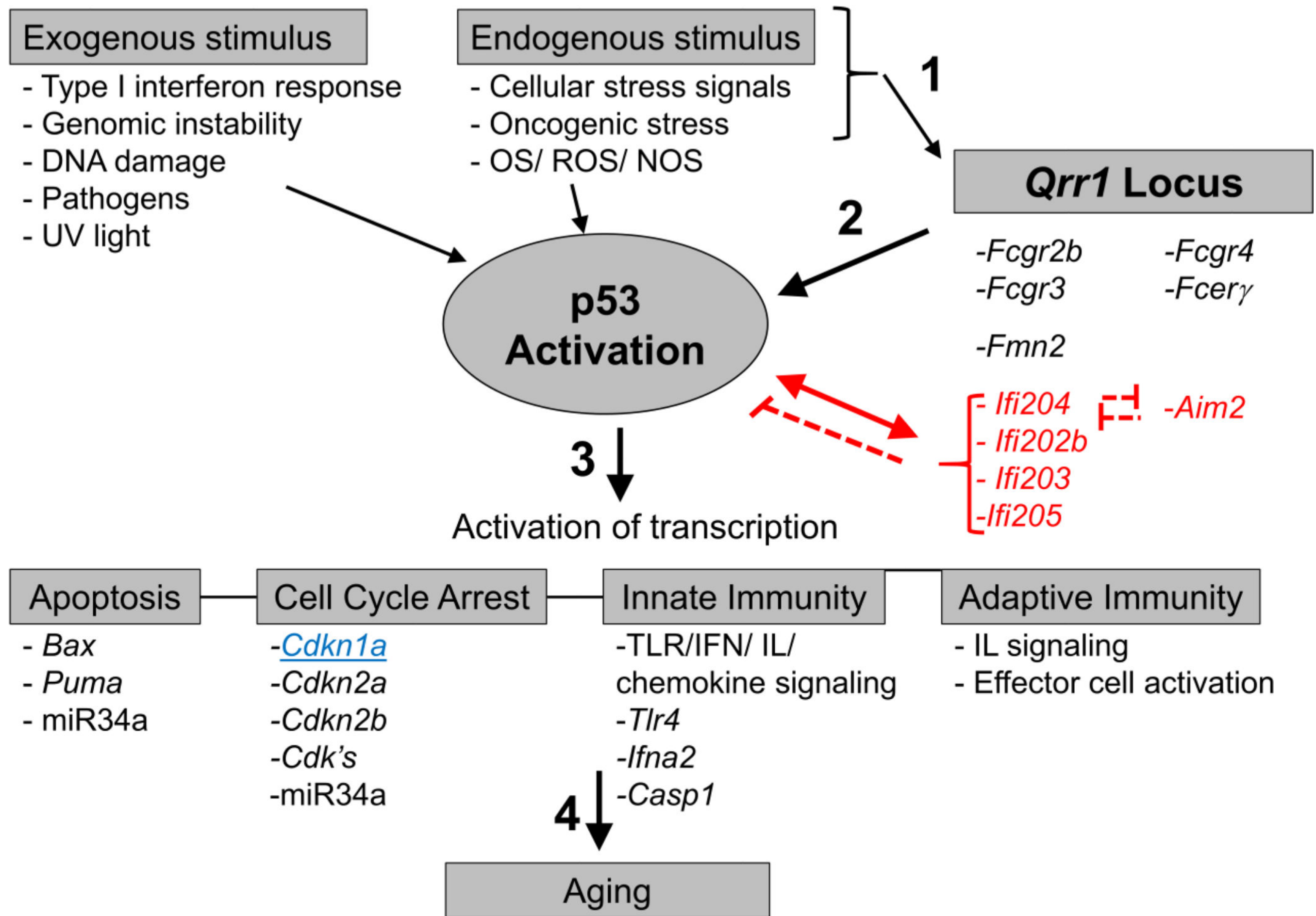

Figure 5.

Schematic showing *Qrr1* activation of *Cdkn1a* and p53 pathways that are important for immunity and aging. 1) Various exogenous and endogenous stimuli, like TLR4 and IFN signaling, DNA damage, oxidative stress and foreign dsDNA, activates proteins in *Qrr1* locus and p53. 2) *Qrr1* proteins (IFI family) can also increase or decrease p53 transcriptional activity. Importantly, p53 triggers expression of some of innate immunity genes in the *Qrr1* locus on chromosome 1. 3) Once activated, p53 initiates expression of genes that play an important role in apoptosis, cell cycle arrest, and innate and adaptive immunity. 4) Misregulation of these pathways are thought to be important for aging and AMD. *Cdkn1a*, which was used for the QTL analysis, is in blue and underlined. Genes in red indicate a possible negative regulator of p53 activity.

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Table1

Gene-interval analysis of the QTL on chromosome 1 reveals candidate genes involved in innate immunity

Gene	Description	Mouse Chr	Mouse Gene ID	SNP	LRS	Exp. level	Range (fold)	Human name	Human Chr	Human Gene ID
Fcgr2b	Fc receptor, IgG, low affinity IIb	1, 78.02	14130	43	20.5	6.84	1.43	*FCGR2B*	1q23	2213
Fcgr4	Fc receptor, IgG, low affinity IV	1, 78.53	246256	24	20.0	7.15	2.54	*FCGR3A* *FCGR3B*	1q23	2214 2215
Fcgr3	Fc receptor, IgG, low affinity III	1, 78.80	14131	59	20.0	7.31	2.44	*FCGR2A*	1q23	2212
Fce1γ	Fc receptor, IgE, High affinity gamma	1, 79.23	14127	13	20.5	7.64	6.28	*FCER1G*	1q23	2207
Aim2	Absent in melanoma 2	1, 80.33	383619	103	19.0	6.85	1.5	*AIM2*	1q22	9447
Ifi204	Interferon activated gene 204	1, 80.63	15951	15	13.0	6.58	1.51	*IFI16*	1q22	3428
Ifi203	Interferon activated gene 203	1, 80.76	15950	31	13.0	6.67	1.3	*IFI16*	1q22	3428
Ifi202b	Interferon activated gene 202B	1, 80.79	26388	76	13.0	6.83	1.91	*IFI16*	1q22	3428
Ifi205	Interferon activated gene 205	1, 80.83	226695	114	13.0	6.68	2.19	*IFI16*	1q22	3428
Fmn2	Formin 2	1, 81.04	54418	1290	13.0	9.18	2.91	*FMN2*	1q43	56776

Exp level, average retinal expression across all BXD mouse strains. LRS, likelihood ratio statistic score