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SH-SY5Y human neuronal cells with mutations of the *CDKN2B-AS1* gene are vulnerable under cultured conditions

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A B S T R A C T

Glaucoma is a common cause of blindness worldwide. Genetic effects are believed to contribute to the onset and progress of glaucoma, but the underlying pathological mechanisms are not fully understood. Here, we set out to introduce mutations into the *CDKN2B-AS1* gene, which is known as being the closely associated with glaucoma, in a human neuronal cell line *in vitro*. We introduced gene mutations with CRISPR/Cas9 into exons and introns into the *CDKN2B-AS1* gene. Both mutations strongly promoted neuronal cell death in normal culture conditions. RNA sequencing and pathway analysis revealed that the transcriptional factor Fos is a target molecule regulating *CDKN2B-AS1* overexpression. We demonstrated that gene mutation of *CDKN2B-AS1* is directly associated with neuronal cell vulnerability *in vitro*. Additionally, Fos, which is a downstream signaling molecule of *CDKN2B-AS1*, may be a potential source of new therapeutic targets for neuronal degeneration in diseases such as glaucoma.

1. Introduction

Glaucoma, the leading worldwide cause of blindness, is a type of neurodegenerative optic neuropathy that results in irreversible visual field defects [1]. It shows familial aggregation with varying prevalence in different ethnic groups [2]. This has prompted many institutions to make sustained efforts to conduct genome-wide association studies (GWASs) to discover single-nucleotide variants associated with glaucoma [3]. Primary open-angle glaucoma (POAG), the most common form of glaucoma, has a complex etiology that includes genetic predisposition [4]. Recently, progress has been made using GWASs to identify POAG risk loci across European, Asian, and African ancestries [5,6]. Previous GWASs in Japanese subjects also identified several POAG-associated loci, some of which overlapped with those in subjects of European ancestry [7–9].

One of these overlapping genetic regions includes the *cyclin-dependent kinase inhibitor 2B antisense 1* (*CDKN2B-AS1*) gene. This gene, located in a genomic region on chromosome 9p21.3, has also been shown to be implicated in diseases such as ovarian cancer, glioma, hypertension, coronary artery disease, and chronic lymphocytic leukemia [10–14]. Specific to the development of POAG, some research has suggested that *CDKN2B-AS1* is involved in mediating senescence, inflammation, and extracellular matrix accumulation [15]. Another study, which used a transgenic mouse model, indicated that the deletion of this genomic region may predispose retinal ganglion cells to increased vulnerability to intraocular pressure (IOP), acting via strengthened activation of microglia in the retina and the optic nerve [16]. However, the molecular mechanisms underlying the association between *CDKN2B-AS1* and POAG are still poorly understood, and the effects described in previous studies could occur secondarily to other mechanisms related to the gene, as it has a variety of functions throughout the body.

Here, we investigated the direct effect of *CDKN2B-AS1* mutations on vulnerability in the immortalized human neuroblastoma cell line SH-SY5Y. We show that introducing gene mutation into *CDKN2B-AS1* promoted apoptotic cell death. Moreover, we found that c-Fos was activated downstream of *CDKN2B-AS1* in an RNA-seq and pathway analysis, suggesting that *CDKN2B-AS1* mutation may contribute to the activation of c-Fos to promote apoptotic cell death in neuronal cells.

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2. Materials and methods

2.1. Cell culture

The human neuroblastoma cell line SH-SY5Y (ECACC94030304) was purchased from ECACC. The cells were cultured in DMEM (Wako 044–29765) and Ham's F-12 (Wako, 087–08335) at a 1:1 ratio, supplemented with 10 % FBS, penicillin-streptomycin, and sodium pyruvate, at 37 °C in a humidified 5 % CO_2 incubator. Stable SH-SY5Y cell lines for SpCas9 gene overexpression with lentivirus (pLV[Exp]-EF1A > hCas9(ns):T2A:Puro) were obtained from VectorBuilder (Kanagawa, Japan). The cells were cultured in EMEM (Wako, 055–08975) and Ham's F-12 (Wako, 087–08335) at a 1:1 ratio, supplemented with 10 % FBS and penicillin-streptomycin, at 37 °C in a humidified 5 % CO_2 incubator. For genome editing targeting *CDKN2B-AS1*, a SpCas9 stable cell line was infected with dual gRNA-overexpressing lentivirus (multiplicity of infection 20) in the presence of 2 μg/ml polybrene. The medium was replaced 4 h after infection.

2.2. Lentivirus vector

The lentiviral vectors used to express gRNA and TagBFP2 in our study, pLV[2gRNA]-TagBFP2:T2A:Puro-U6>Scramble[gRNA#1]-U6>Scramble[gRNA#2], pLV[2gRNA]-TagBFP2:T2A:Puro-U6>{CDKN2B-AS1_gRNA#1}-U6>{CDKN2B-AS1_gRNA#2}, and pLV[2gRNA]-TagBFP2:T2A:Puro-U6>{CDKN2B-AS1_gRNA#3}-U6>{CDKN2B-AS1_gRNA#4}, were constructed and packaged by VectorBuilder. The vector IDs were VB210519-1003myp, VB210519-1014cjr, and VB210519-1015edw. To evaluate the downstream signaling of CDKN2B-AS1, pLV[ncRNA]-EGFP:T2A:Puro-CMV>{hCDKN2B-AS1[NR_003529.3]} was used for overexpression of CDKN2B-AS1. As a control vector, we used pLV[Exp]-EGFP:T2A:Puro-CMV > ORF_Stuffer. The vector IDs were VB211112-1184dvq and VB900122-4425pgj. These IDs can be used to retrieve detailed information about the vectors on vectorbuilder.com.

2.3. RT-PCR

Total cellular RNA was extracted from stable SpCas9-expressing SH-SY5Y cells and cDNA was synthesized using the SuperPrep II Cell Lysis and RT Kit for qPCR (Toyobo, SCQ-401). For quantitative RT-PCR, primer sets were synthesized with Eurofin genomics. The specific primers used to amplify SpCas9 were 5′-GAAGAGAACCGCCAGAAGAAG-3′ and 5′-CCACGATGTTGCCGAAGAT-3'. The primers used to amplify hGAPDH were 5′-GACTCATGACCACAGTCCATG-3′ and 5′-TCAGCTCAGGGATGACCTTG. Quantitative RT-PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo, QPS201), and results were analyzed on a real-time PCR system (7500 Fast Real-Time PCR System; Life Technologies, Inc.). In addition, to confirm the specificity of the amplification, we performed a quantitative PCR program followed by a melting curve analysis and agarose gel electrophoresis of the PCR products.

2.4. Western blotting

Protein samples were extracted with PTTS buffer that included a protease inhibitor, and concentrations were measured as previously described [17]. An anti-SpCas9 antibody was incubated at 4 °C overnight, and a secondary antibody labeled with HRP was incubated for 1 h at room temperature. Immunoreactive bands were detected and captured with chemiluminescence, as previously described [18].

2.5. Immunostaining of SpCas9

To determine if the obtained viral vectors caused the expression of SpCas9, immunocytochemistry was performed. The SpCas9 stable cell line was seeded at a density of 1.5×10^5 cells/well 24 h prior to fixing in 4 % paraformaldehyde. SpCas9 was stained using mouse monoclonal anti-SpCas9 for 60 min at room temperature (1:200, anti-SpCas9 monoclonal [7A9-3A3], Cell Signaling, #14697) in 10 % donkey serum as blocking buffer. Goat anti-mouse IgG in blocking buffer was used for second staining (1:500, Alexa Flour 488). Mounting was performed with a mounting medium including DAPI for nuclear staining (Vector Laboratories Inc., H-1200). Finally, fluorescence imaging was performed using the BZ-X810 device (Keyence).

2.6. Fluorescence-activated cell sorting (FACS)

TagBFP2-positive SH-SY5Y cells and EGFP-positive SH-SY5Y cells were sorted using a BD FACSAria SORP device (BD Bioscience), and the data were analyzed using FlowJo software (Tree Star).

2.7. In-fusion cloning and sequencing

To test the efficacy of gene editing, the SH-SY5Y cells were infected with a lentivirus-based vector expressing dual-guide RNA, genomic DNA was extracted, and amplification was performed of the DNA fragment containing the target site. PCR amplicons generated with the primers (Table S1) were cloned into the pAAV-MCS vector. Cloning plasmids were constructed for identifying CRISPR/Cas-induced target mutations using the In-Fusion HD Cloning Kit (639633, Takara Bio, Shiga, Japan). The cloning vector was transformed into *E. coli* (DNA-913F, Toyobo, Japan), and colonies were isolated for sequencing. Plasmids were propagated and were purified using FastGene PlasmidMini (FG-90502, Nippon Genetics, Japan). The plasmids were sent for Sanger sequencing (Eurofins Genomics K.K.) and BLAST-searched against the human genome to identify DNA mutations.

2.8. T7 Endonuclease 1 (T7E1) assay

We prepared plasmids expressing SpCas9 and gRNA using the In-Fusion HD Cloning Kit (Clontech,639648). In brief, SpCas9 was expressed downstream of the hPGK promoter, and each gRNA sequence was placed downstream of the U6 promoter. These plasmids were transfected into HEK293T cells using PolyJet In Vitro DNA Transfection Reagent (SignaGen Labpratories, SL100688), and genomic DNA was purified from the harvested cells after 24 h using the QIAamp DNA Mini Kit (Qiagen, 56304). Genomic DNA was tested with PCR using primers designed to contain the gRNA sequence. For gRNA1 and gRNA3, PCR was performed using the following primers: 5′-tgtacttaaccactggactacctgcc-3′ and 5′-gtctcctttatgcattttgctgcaac-3'. For gRNA2 and gRNA4, PCR was performed using the following primers: 5′-gagatatccagtcaccaatcc-3′ and 5′-gtgtaagagcagatgtactggag-3'. The PCR product was analyzed using a T7 endonuclease 1 (T7E1) mismatch detection assay according to the manufacturer's protocol with the EnGen Mutation Detection Kit (New England Biolabs, E3321S). The density of the amplicon and digested band was determined with software (Image Lab 3.0; Bio-Rad).

2.9. Dead cell detection

The vulnerability of cultured SH-SY5Y cells expressing CDKN2BAS-1 mutations was measured using the Incucyte live-cell imaging system (Sartorius). One day prior to lentiviral transduction, SH-SY5Y cells stably expressing SpCas9 were seeded in a 96-well plate (Incucyte Imagelock 96-well plate; BA-04856). The cells were infected with dual gRNA-overexpressing lentivirus (multiplicity of infection 20) in the presence of 2 μg/ml polybrene. Four hours after infection, the medium was changed to a fresh medium containing Annexin V red dye (4641, Sartorius) and Caspase3/7 green dye (4440, Sartorius). Live-cell images were captured every 6 h until the end point at 72 h.

2.10. Quantitative reverse transcription PCR

EGFP-positive SH-SY5Y cells were purified after lentivirus vector infection to drive CDKN2B-AS1 expression. The purified SH-SY5Y cells were used to extract total RNA using the RNeasy Micro kit (74004, Qiagen) and cDNA was synthesized using SuperScript III (18080051, Invitrogen). Quantitative PCR (qPCR) was performed with the 7500 Fast Real-Time PCR System (Life Technologies, Inc.) using TaqMan Fast Universal PCR Master Mix (Life Technologies, Inc.). Predesigned primers were purchased from Invitrogen and used for RT-PCR, including CDKN2B-AS1 (Hs01390879) and GAPDH (Hs02786624). Quantitative PCR (qPCR) was performed under the following cycling conditions: 95 °C for 20 s and 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Relative mRNA levels of CDKN2B-AS1 were determined from the ΔCt values and normalized to GAPDH expression.

2.11. RNA-sequencing and pathway analysis

A quality check of the total RNA was performed with Bioanalyzer (Agilent Technologies, California, USA). RNA-seq libraries were synthesized with the SMART-seq HT Kit (634455, Clontech). RNA-seq libraries were sequenced using paired end reads (150 nt for read 1 and 2) on a DNBSEQ-G400RS (MGI Tech, Shenzhen, China). The obtained reads were mapped to the mouse GRCh38.p13 (hg38) genome with STAR software (version 2.7.9). Reads of annotated genes were counted using featureCounts software (version 2.0.1). FPKM values were calculated from mapped reads by normalizing to total counts and the transcript. Differentially expressed genes were detected with the DESeq2 software package (version 1.20.0). A list of differentially expressed genes detected by DESeq2 (basemean >5 and fold change <0.25, or basemean >5 and fold change >4) was used for GO enrichment analysis with the clusterProfiler software package (Yu et al., OMICS 2012, 16:5, version 3.16.0). Pathway analysis was performed using Ingenuity Pathway Analysis software (QIAGEN) and a biological triplicate RNA-seq dataset.

2.12. Statistics

Statistical significance was determined with a one-way ANOVA followed by the Tukey-Kramer test to compare the mean in multiple groups. Statistical significance was calculated with the un-paired Student's t-test to compare pairs of groups. The significance level was set at $p < 0.05$.

3. Results

3.1. Gene mutations in CDKN2B-AS1 with CRISPR/Cas9

To confirm the insertion of SpCas9 in SH-SY5Y cells, we used RT-PCR, immunoblotting, and immunostaining. For RT-PCR and immunoblots, gapdh and beta-actin, respectively, were used as internal controls. While normal SH-SY5Y cells do not express SpCas9 at all, we confirmed, at both the mRNA and protein levels, that the stable cell line we prepared expressed SpCas9, as the bands show (Fig. 1A and B). In addition, immunocytochemistry revealed the expression of SpCas9 in each cell (Fig. 1C). With all these results suggesting the successful insertion of SpCas9, we moved on to the next procedure: genome editing mutation of *CDKN2B-AS1* using a dual gRNA lentivirus vector (Fig. 1D).

We designed four gRNAs targeting exons 2 and 3 of the human *CDKN2B-AS1* gene (Supplemental Table 1) and constructed lentivirus vectors expressing the dual gRNAs and TagBFP2. Each dual gRNA lentivirus vector was introduced into human neuroblastoma SH-SY5Y cells stably expressing SpCas9, and genome editing efficiency was examined 72 h after introduction. Cell sorting was performed to purify the TagBFP2-positive cells (Fig. 2A and B). DNA was extracted from the sorted cells, and the target site sequences were sequenced according to the method described above. One insertion-deletion mutation (2 base deletions and 521 base insertions) and 7 base deletions were identified in exons from 10 sequenced clonal amplicons targeting #1 gRNA. One base substitution and 4 base deletions were identified in introns from 10 sequenced clonal amplicons targeting #3 gRNA (Fig. 2C and D). In other

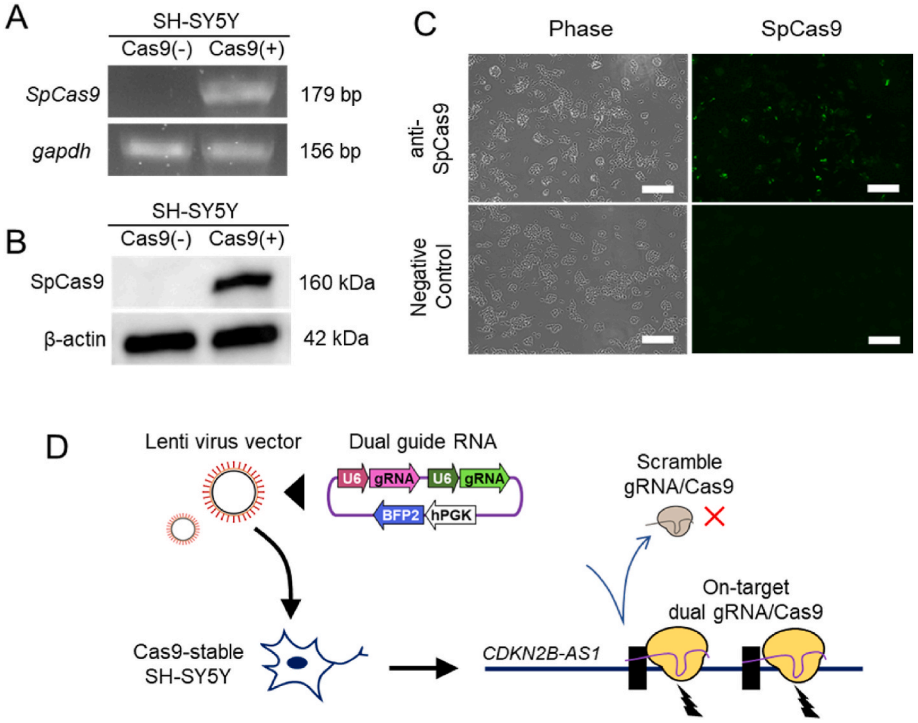
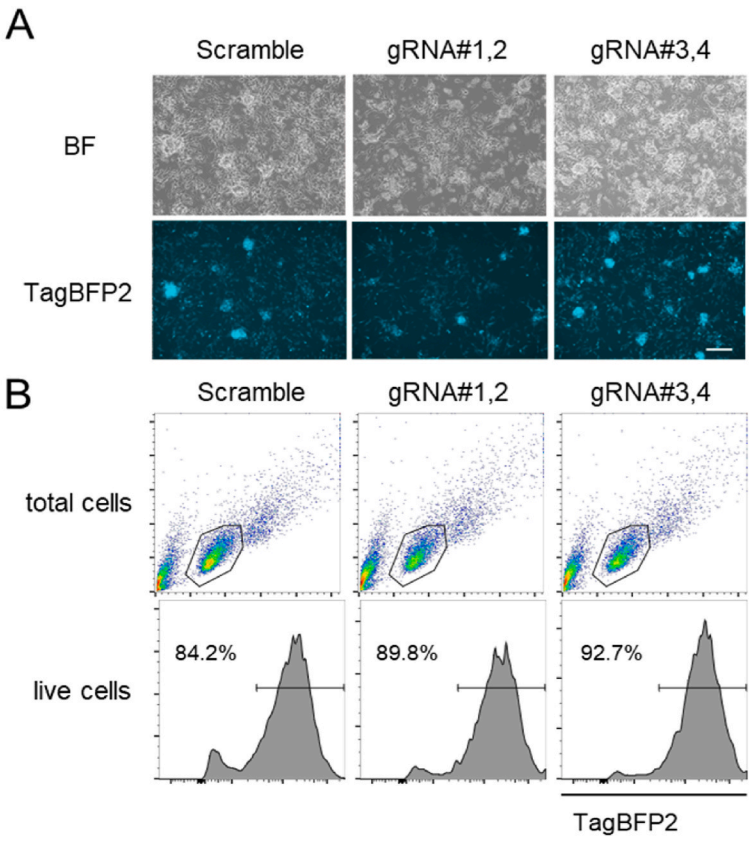
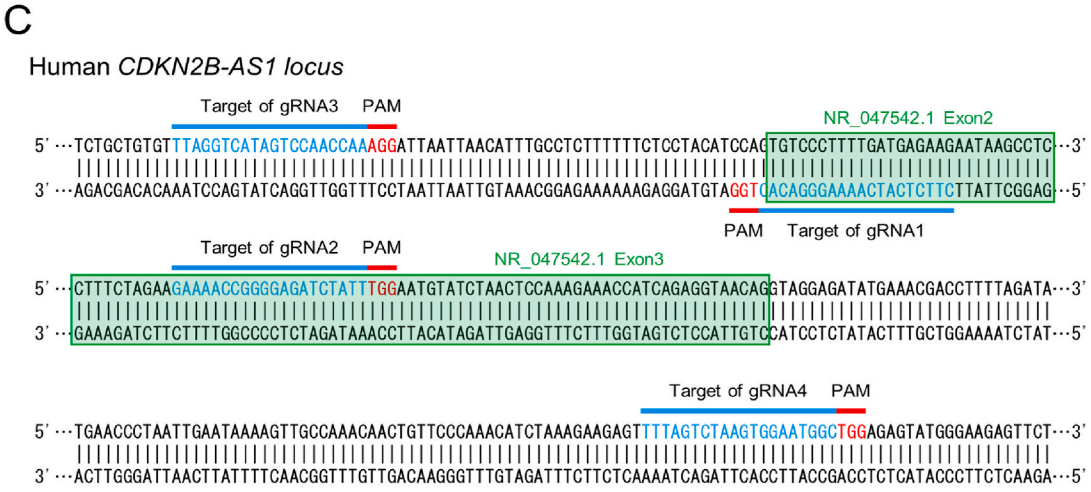


Fig. 1. Materials for CRISPR/Cas9 editing of the *CDKN2B-AS1* gene.
RT-PCR (A), immunoblotting (B), and immunostaining (C) for SpCas9 in SH-SY5Y cells. (D) Scheme of gene mutation targeting human *CDKN2B-AS1* using lentivirus vector-expressing gRNA. Scale bar = 200 μm.





D

gRNA	Allele	Target sequence
#1	WT	GGCTTATTCTTCTCATCAAAAGGGACACTGGATGTAGGAA
	-2+521bp	GGCTTATTCTTCTCATCAAAAGGGAC--TGGATGTAGGAA
	-7	GGCTTATTCTTCTCATCAAAAGGGA-------TGTAGGAA
#3	WT	TGCTGTGTTTAGGTCATAGTCCAACCAAAGGATTAATTAA
	-4bp	TGCTGTGTTTAGGTCATAGT----CCAAAGGATTAATTAA

Fig. 2. Genome editing of *CDKN2B-AS1* with lentivirus vector infection.
(A) TagBFP2-expressing SH-SY5Y cells were shown to label lentivirus-infected cells. BF; bright field. (B) TagBFP2-positive cells were purified to confirm editing of the *CDKB2B-AS1* gene with FACS. (C) The human *CDKN2B-AS1* locus and gRNA. (D) Mutation pattern of *CDKN2B-AS1*. Scale bar = 200 μm.

words, we found two mutant clones from 10 sequenced clones with #1 gRNA, and one mutant clone from 10 sequenced clones with #3 gRNA. However, there was no mutation targeting scramble gRNA as a control or #2 or #4 gRNA targeted with *CDKN2B-AS1*.

To further confirm the genome editing efficiency, we performed a T7E1 assay using HEK293T cells. Consistent with the results from Sanger sequencing, gRNA #1 and gRNA #3 were similarly effective at introducing genomic mutations (7.7 % indel at gRNA #1, and 6.8 % indel at gRNA #3). On the other hand, there was no cleaved band from gRNA #2, gRNA #4, or scramble gRNA in the T7E1 assay, i.e., there was no introduction of gene mutation (Supp Fig. 1). There results demonstrate that lentivirus vectors packaging either gRNA#1/#2 or gRNA #3/#4 can successfully introduce gene mutation in *CDKN2B-AS1* exons and introns, respectively.

3.2. Neuronal vulnerability caused by CDKN2B-AS1 mutation in vitro

To investigate whether mutation of *CDKN2B-AS1* affects cell proliferation and cell death, we monitored dead cells using a time-lapse system (Incucyte). After 4 h of infection with the lentivirus vector, cell proliferation, Annexin V red, and caspase3/7 green were monitored every 6 h (3 fields of view per well). Cell proliferation was analyzed as the confluence of cells in phase contrast images, normalizing the confluence per image to 0 h. The cell proliferation rate of stable SpCas9-expressing SH-SY5Y cells transfected with gRNA1,2 was slightly, but significantly, decreased by 72 h compared to control and #3 or #4 (Fig. 3A). However, the caspase 3/7–positive cell count (per image) also significantly increased in the *CDKN2B-AS1* genome-edited cells compared to the scramble gRNA-induced cells (gRNA #1,2; $p < 0.05$ from 6 h and gRNA #3,4; $p < 0.05$ from 36 h) (Fig. 3B). Similarly to the caspase assay, the Annexin V–positive cell area (per image) significantly increased in the *CDKN2B-AS1* genome-edited cells compared to the scramble gRNA-induced cells (gRNA #1,2; $p < 0.05$ from 24 h and gRNA #3,4; $p < 0.05$ from 54 h) (Fig. 3C). These results suggest that *CDKN2B-AS1* mutations involved neuronal cell vulnerability that promoted cell death.

To evaluate molecular mechanisms promoting apoptotic cell death related to *CDKN2B-AS1* mutation, we analyzed the downstream signaling of *CDKN2B-AS1* using a lentivirus overexpression vector. The transcriptional level of *CDKN2B-AS1* in SH-SY5Y cells infected with lentivirus overexpressing *CDKN2B-AS1* was ~15.6-fold higher than in cells infected with a control vector (Fig. 4A). Our pathway analysis revealed that the CDKN2B-AS1 molecule suppressed the Fos signaling pathway (Fig. 4B), suggesting that *CDKN2B-AS1* mutation–induced apoptotic cell death may be due to the activation of the Fos signaling pathway.

4. Discussion

Previously, GWASs have identified glaucoma-related genes and found that *CDKN2B-AS1* was one of the most important genes in Asian and European population. However, the underlying pathological mechanisms are not fully understood. Here, we introduced *CDKN2B-AS1* gene mutation with neuronal cells stably expressing Cas9 and used a lentivirus vector to express gRNA. We demonstrated that mutation in *CDKN2B-AS1* promotes neuronal cell death *in vitro*. This suggests that variants of the *CDKN2B-AS1* gene may directly explain optic nerve vulnerability. A past study showed that alleles of CDKN2B-AS1 SNPs were associated with POAG [19], and another study found that risk alleles of *CDKN2B-AS1* in glaucoma patients were also associated with normal-tension glaucoma [20]. Moreover, several haplotypes of the *CDKN2B-AS1* gene are associated with not only POAG, but also pseudoexfoliation glaucoma [21]. In addition, loci of *CDKN2B-AS1* have been found to be associated with various endophenotypes of glaucoma, such as vertical cup-disc ratio (VCDR) and optic cup area [22–24]. Interestingly, SNPs of *CDKN2B-AS1* are not associated with IOP [23], suggesting that risk alleles of *CDKN2B-AS1* are associated with glaucomatous pathology in an IOP-independent manner. These studies support our current finding that *CDKN2B-AS1* may be involved in optic nerve susceptibility.

This study is the first to introduce the use of genome editing with CRISPR/Cas9 in exon and intron regions of the *CDKN2B-AS1* gene. Interestingly, both mutations induced apoptotic cell death *in vitro*. This suggests that intronic mutations, like exonic mutations, may give rise to abnormal mature non-coding RNA in *CDKN2B-AS1*. In our experiments, we designed gRNA #3 to be upstream by 40–50 bp from the 3' splice acceptor site on exon 2 in the *CDKN2B-AS1* gene. This region is named the polypyrimidine tract; it binds with RNA processing molecules such as polypyrimidine tract-binding protein to regulate RNA splicing. Mutation of the polypyrimidine tract leads to aberrant transcripts due to the insertion of intron nucleotides and mutant proteins by frameshifting [25]. Our genome editing, which targeted introns with gRNA #3, may also have led to polypyrimidine tract mutation and produced abnormal transcripts, resulting in a pathogenic effect.

We evaluated the gRNA sequence with the computational tool CRISPOR (http://crispor.tefor.net/) to determine the score for genome editing efficiency [26]. All four gRNAs (#1-#4) had similar scores for targeting CDKN2B-AS1; thus, we do not consider that there was any problem with gRNA #2 or #4 themselves. We think that one of the reasons for the difference in genome editing efficiency was the locus of the gRNA target site. The target site of gRNA #1 and #3 (which were successful in genome editing) is around *CDKN2B-AS1* exon 2. On the other side, the target site of gRNA #2 and #4 (which failed in genome editing) is around *CDKN2B-AS1* exon 3. This is a distance of a few kbp, and there may be differences in gene structure and complexity, such as chromatin condensation. It may be hard for the gRNA complex to access

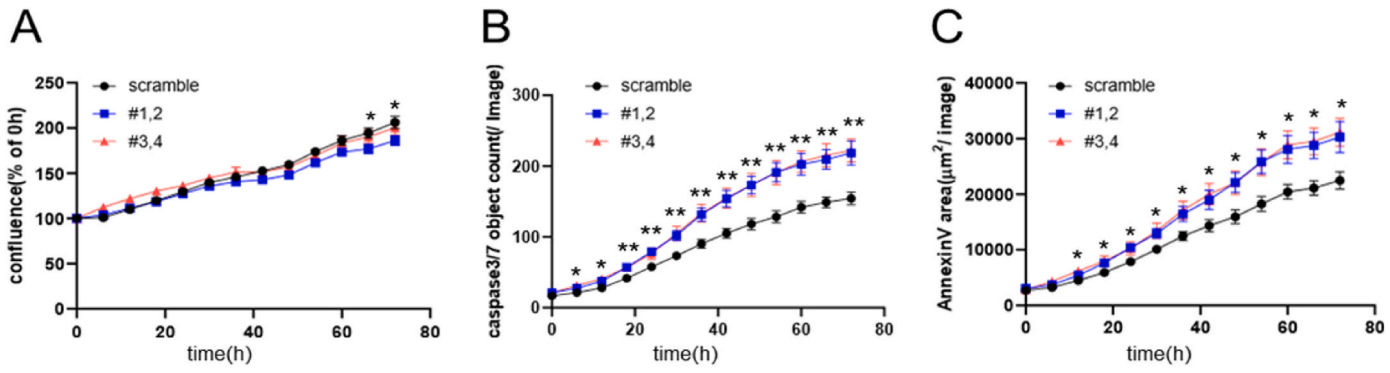


Fig. 3. Induction of cell death with *CDKN2B-AS1* genome editing *in vitro*.
Time-lapse data showing cell confluence (A), the Annexin V cell area (B), and the caspase3/7 cell area (C). Error bars denote SD (n = 8).

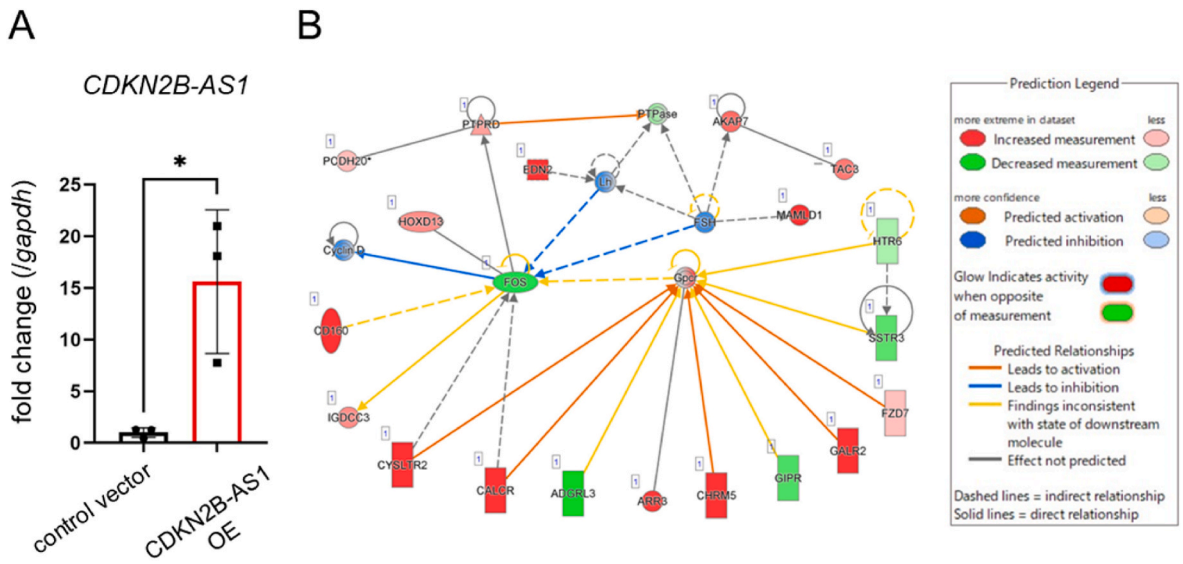


Fig. 4. Downstream analysis of *CDKN2B-AS1* in SH-SY5Y cells.
(A) Transcriptional level of *CDKN2B-AS1* in SH-SY5Y cells infected with a lentivirus vector to overexpress *CDKN2B-AS1*. (B) Gene network analysis with Ingenuity Pathway Analysis software showing downstream signaling of *CDKN2B-AS1* in an RNA-seq analysis. Error bars denote SD (n = 3).

the *CDKN2B-AS1* exon 3 region to induce genome editing.

The biological function of *CDKN2B-AS1* in neuronal cells is not fully understood. In the current study, we attempted to clarify molecular signaling downstream of *CDKN2B-AS1*. A part of the molecular network, the Fos signaling pathway, was identified; we found that *CDKN2B-AS1* may suppress Fos activation. Previously, it has been well known that the cFos molecule promotes apoptotic cell death. Our previous work has also shown that cFos is activated in RGCs by optic nerve injury in rats [27]. These findings suggest that *CDKN2B-AS1* mutation may impair cFos regulation and that activated cFos may trigger neuronal vulnerability. Selective cFos inhibitors, such as T-5224, can suppress inflammatory cytokine expression and ameliorate damage in animal models, such as models of kidney injury and skin disorders. In addition, T-5224 treatment *in vitro* has been found to have no significant cytotoxicity. These findings suggest that eye drops that include cFos inhibitors may have potential as therapeutic agents for glaucoma. Recently, we developed new drug delivery systems for sustained release of high-dose drugs into the retina. These systems would also be useful for glaucoma treatment using cFos inhibitors.

Long non-coding RNA sequences have roles in modulating gene expression via a variety of mechanisms, such as histone modification [28–30]. *CDKN2B-AS1* transcripts have also has been shown to regulate and suppress the expression of *CDKN2A/B* by epigenetic mechanisms to bind polycomb protein complexes [31]. Transcribed *CDKN2B-AS1* binds with polycomb proteins CBX7 and Suz12. These complexes interact with DNA methyltransferase DNMT3B and promote histone modification and chromatin remodeling [32]. In fact, the interaction of *CDKN2B-AS1* transcripts and methylation of histone H3 at lysine 27 (H3K27me) mediates the silencing of CDKN2A [33]. In an animal model of ocular hypertension and ischemia reperfusion, SIX6-and TBK-mediated CDKN2A elevation was associated with RGC death [34,35]. Our previous study also showed that CDKN2B promotes RGC death in the retinas of mice that undergo *N*-methyl-D-aspartic acid induced excitotoxicity [36]. Other experimental data also show that the silencing of *CDKN2B-AS1* upregulates *CDKN2B in vitro* [37]. Importantly, a GWAS on morphologic characteristics of the optic nerve head found that the *CDKN2B* locus was associated with VCDR [38]. These studies suggest that mutations in *CDKN2B-AS1* may deleteriously promote the expression of downstream genes such as CDKN2A/B, resulting in RGC death.

Interestingly, *CDKN2B-AS1* is highly expressed in atherosclerosis and regulates the expression of the cholesterol transporter ABCA1 in macrophage-derived foam cells. *ABCA1* is also known as a glaucoma-associated gene in GWASs, and the deletion of *ABCA1* in mice promotes RGC death [39]. Interestingly, *ABCA1*-deficient astrocytes upregulated chemokines such as CXCL12 and CCL5 with cholesterol efflux, resulting in low NR3A expression and RGC degeneration, causing Ca^{2+} overflow. Thus, dysregulation of the astrocyte-RGC interaction may also be involved in the pathogenesis of glaucoma. Our induced gene mutation of *CDKN2B-AS1* promoted neuronal cell death, possibly due to cholesterol dysregulation. Further investigation will be needed to clarify this point in the future.

This study had three major limitations. First, it was hard to analyze the mechanism of cell death due to the low efficiency of genome editing. We detected cells that had gene mutations induced with our CRISPR/Cas9 system, but the ratio of cells with these mutations was not high (10–20 %), even after the lentivirus-infected cells were purified by FACS. One reason for this might be that the cells infected with the lentiviral vector had already died before FACS, which was applied 72 h after virus infection. The collection of dying cells may be necessary to clarify the details of the mutation pattern of the *CDKN2B-AS1* gene with our CRISPR/Cas9 system. The cell-death mechanisms of *CDKN2B-AS1* were not clear in the current study, and it will thus be necessary to clarify these points in the future. Second, it is also hard to discuss SNPs in *CDKN2B-AS1* that were reported in previous glaucoma GWASs, because our gene mutation point in *CDKN2B-AS1* did not match the risk allele of *CDKN2B-AS1* in previous work. The setting of gRNA against SpCas9 is a necessary PAM sequence; thus, the mutation point will be limited. Finally, we used a human SH-SY5Y neuroblastoma cell line, rather than an RGC cell line, because there is no human RGC cell line. To solve this problem, the use of induced pluripotent stem (iPS) cells derived from RGCs might be considered an appropriate method. Methods for RGC differentiation and purification from three-dimensional retinal organoids were established at our laboratory [40]; further investigation will be required to clarify the pathological mechanisms of the *CDKN2B-AS1* risk allele using iPS cells from human glaucoma patients.

In conclusion, we found that *CDKN2B-AS1* gene mutations were a direct cause of neuronal cell vulnerability. This finding may have the potential to help the development of new therapeutic targets for glaucomatous optic neuropathy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author. The RNA-seq data are available at the NCBI GEO (accession number GSE236621).

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CRediT authorship contribution statement

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Declaration of competing interest

The authors report no competing interests.

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Appendix A. Supplementary data

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References

[1] J.B. Jonas, T. Aung, R.R. Bourne, A.M. Bron, R. Ritch, S. Panda-jonas, Seminar Glaucoma, vol. 390, 2017, pp. 83–93, https://doi.org/10.1016/S0140-6736(17)31469-1.

[2] A.W. Hewitt, J.E. Craig, D.A. Mackey, Complex genetics of complex traits: the case of primary open-angle glaucoma, Clin. Exp. Ophthalmol. 34 (2006) 472–484, https://doi.org/10.1111/j.1442-9071.2006.01268.x.

[3] I.F. Aboobakar, J.L. Wiggs, The genetics of glaucoma: disease associations, personalised risk assessment and therapeutic opportunities-A review, Clin. Exp. Ophthalmol. 50 (2022) 143–162, https://doi.org/10.1111/ceo.14035.

[4] H. Quigley, A.T. Broman, The number of people with glaucoma worldwide in 2010 and 2020, Br. J. Ophthalmol. 90 (2006) 262–267, https://doi.org/10.1136/bjo.2005.081224.

[5] K.P. Burdon, S. MacGregor, A.W. Hewitt, S. Sharma, G. Chidlow, R.A. Mills, P. Danoy, R. Casson, A.C. Viswanathan, J.Z. Liu, J. Landers, A.K. Henders, J. Wood, E. Souzeau, A. Crawford, P. Leo, J.J. Wang, E. Rochtchina, D.R. Nyholt, N. G. Martin, G.W. Montgomery, P. Mitchell, M.A. Brown, D.A. MacKey, J.E. Craig, Genome-wide association study identifies susceptibility loci for open angle glaucoma at TMCO1 and CDKN2B-AS1, Nat. Genet. 43 (2011) 574–578, https://doi.org/10.1038/ng.824.

[6] P. Gharahkhani, E. Jorgenson, P. Hysi, A.P. Khawaja, S. Pendergrass, X. Han, J. S. Ong, A.W. Hewitt, A. V Segrè, J.M. Rouhana, A.R. Hamel, R.P. Igo, H. Choquet, A. Qassim, N.S. Josyula, J.N. Cooke Bailey, P.W.M. Bonnemaijer, A. Iglesias, O. M. Siggs, T.L. Young, V. Vitart, A.A.H.J. Thiadens, J. Karjalainen, S. Uebe, R. B. Melles, K.S. Nair, R. Luben, M. Simcoe, N. Amersinghe, A.J. Cree, R. Hohn, A. Poplawski, L.J. Chen, S.-S. Rong, T. Aung, E.N. Vithana, , NEIGHBORHOOD consortium, ANZRAG consortium, Biobank Japan project, FinnGen study, UK Biobank Eye and Vision Consortium, GIGA study group, 23 and Me Research Team, G. Tamiya, Y. Shiga, M. Yamamoto, T. Nakazawa, H. Currant, E. Birney, X. Wang, A. Auton, M.K. Lupton, N.G. Martin, A. Ashaye, O. Olawoye, S.E. Williams, S. Akafo, M. Ramsay, K. Hashimoto, Y. Kamatani, M. Akiyama, Y. Momozawa, P. J. Foster, P.T. Khaw, J.E. Morgan, N.G. Strouthidis, P. Kraft, J.H. Kang, C.P. Pang, F. Pasutto, P. Mitchell, A.J. Lotery, A. Palotie, C. van Duijn, J.L. Haines, C. Hammond, L.R. Pasquale, C.C.W. Klaver, M. Hauser, C.C. Khor, D.A. Mackey, M. Kubo, C.-Y. Cheng, J.E. Craig, S. MacGregor, J.L. Wiggs, Genome-wide meta-analysis identifies 127 open-angle glaucoma loci with consistent effect across ancestries, Nat. Commun. 12 (2021) 1258, https://doi.org/10.1038/s41467-020-20851-4.

[7] W. Osman, S.K. Low, A. Takahashi, M. Kubo, Y. Nakamura, A genome-wide association study in the Japanese population confirms 9p21 and 14q23 as susceptibility loci for primary open angle glaucoma, Hum. Mol. Genet. 21 (2012) 2836–2842, https://doi.org/10.1093/hmg/dds103.

[8] Y. Shiga, M. Akiyama, K.M. Nishiguchi, K. Sato, N. Shimozawa, A. Takahashi, Y. Momozawa, M. Hirata, K. Matsuda, T. Yamaji, M. Iwasaki, S. Tsugane, I. Oze, H. Mikami, M. Naito, K. Wakai, M. Yoshikawa, M. Miyake, K. Yamashiro, K. Kashiwagi, T. Iwata, F. Mabuchi, M. Takamoto, M. Ozaki, K. Kawase, M. Aihara, M. Araie, T. Yamamoto, Y. Kiuchi, M. Nakamura, Y. Ikeda, K.H. Sonoda, T. Ishibashi, K. Nitta, A. Iwase, S. Shirato, Y. Oka, M. Satoh, M. Sasaki, N. Fuse, Y. Suzuki, C.Y. Cheng, C.C. Khor, M. Baskaran, S. Perera, T. Aung, E.N. Vithana, J. N. Cooke Bailey, J.H. Kang, L.R. Pasquale, J.L. Haines, J.L. Wiggs, K.P. Burdon, P. Gharahkhani, A.W. Hewitt, D.A. Mackey, S. MacGregor, J.E. Craig, R. Rand Allingham, M. Hauser, A. Ashaye, D.L. Budenz, S. Akafo, S.E.I. Williams, Y. Kamatani, T. Nakazawa, M. Kubo, Genome-wide association study identifies seven novel susceptibility loci for primary open-angle glaucoma, Hum. Mol. Genet. 27 (2018) 1486–1496, https://doi.org/10.1093/hmg/ddy053.

[9] Y. Shiga, K.M. Nishiguchi, Y. Kawai, K. Kojima, K. Sato, K. Fujita, M. Takahashi, K. Omodaka, M. Araie, K. Kashiwagi, M. Aihara, T. Iwata, F. Mabuchi, M. Takamoto, M. Ozaki, K. Kawase, N. Fuse, M. Yamamoto, J. Yasuda, M. Nagasaki, T. Nakazawa, Genetic analysis of Japanese primary open-angle glaucoma patients and clinical characterization of risk alleles near CDKN2B-AS1, SIX6 and GAS7, PLoS One 12 (2017) 1–15, https://doi.org/10.1371/journal.pone.0186678.

[10] Y. Wang, Y. Huang, H. Liu, D. Su, F. Luo, F. Zhou, Long noncoding RNA CDKN2B-AS1 interacts with miR-411–3p to regulate ovarian cancer in vitro and in vivo through HIF-1a/VEGF/P38 pathway, Biochem. Biophys. Res. Commun. 514 (2019) 44–50, https://doi.org/10.1016/j.bbrc.2019.03.141.

[11] J. Lu, Y. Chen, L. Wen, Q. Zhou, S. Yan, LncRNA CDKN2B-AS1 contributes to glioma development by regulating the miR-199a-5p/DDR1 axis, J. Gene Med. 24 (2022) 1–9, https://doi.org/10.1002/jgm.3389.

[12] S.I. Berndt, C.F. Skibola, V. Joseph, N.J. Camp, A. Nieters, Z. Wang, W. Cozen, A. Monnereau, S.S. Wang, R.S. Kelly, Q. Lan, L.R. Teras, N. Chatterjee, C.C. Chung, M. Yeager, A.R. Brooks-Wilson, P. Hartge, M.P. Purdue, B.M. Birmann, B. K. Armstrong, P. Cocco, Y. Zhang, G. Severi, A. Zeleniuch-Jacquotte, C. Lawrence, L. Burdette, J. Yuenger, A. Hutchinson, K.B. Jacobs, T.G. Call, T.D. Shanafelt, A. J. Novak, N.E. Kay, M. Liebow, A.H. Wang, K.E. Smedby, H.O. Adami, M. Melbye, B. Glimelius, E.T. Chang, M. Glenn, K. Curtin, L.A. Cannon-Albright, B. Jones, W. R. Diver, B.K. Link, G.J. Weiner, L. Conde, P.M. Bracci, J. Riby, E.A. Holly, M. T. Smith, R.D. Jackson, L.F. Tinker, Y. Benavente, N. Becker, P. Boffetta, P. Brennan, L. Foretova, M. Maynadie, J. McKay, A. Staines, K.G. Rabe, S. J. Achenbach, C.M. Vachon, L.R. Goldin, S.S. Strom, M.C. Lanasa, L.G. Spector, J. F. Leis, J.M. Cunningham, J.B. Weinberg, V.A. Morrison, N.E. Caporaso, A. D. Norman, M.S. Linet, A.J. De Roos, L.M. Morton, R.K. Severson, E. Riboli, P. Vineis, R. Kaaks, D. Trichopoulos, G. Masala, E. Weiderpass, M.D. Chirlaque, R. C.H. Vermeulen, R.C. Travis, G.G. Giles, D. Albanes, J. Virtamo, S. Weinstein, J. Clavel, T. Zheng, T.R. Holford, K. Offit, A. Zelenetz, R.J. Klein, J.J. Spinelli, K. A. Bertrand, F. Laden, E. Giovannucci, P. Kraft, A. Kricker, J. Turner, C.M. Vajdic, M.G. Ennas, G.M. Ferri, L. Miligi, L. Liang, J. Sampson, S. Crouch, J.H. Park, K. E. North, A. Cox, J.A. Snowden, J. Wright, A. Carracedo, C. Lopez-Otin, S. Bea, I. Salaverria, D. Martin-Garcia, E. Campo, J.F. Fraumeni, S. De Sanjose, H. Hjalgrim, J.R. Cerhan, S.J. Chanock, N. Rothman, S.L. Slager, Genome-wide association study identifies multiple risk loci for chronic lymphocytic leukemia, Nat. Genet. 45 (2013) 868–876, https://doi.org/10.1038/ng.2652.

[13] P. Dasgupta, P. Kulkarni, S. Majid, Y. Hashimoto, M. Shiina, V. Shahryari, N. S. Bhat, L. Tabatabai, S. Yamamura, S. Saini, Y. Tanaka, R. Dahiya, LncRNA CDKN2B-AS1/miR-141/cyclin D network regulates tumor progression and metastasis of renal cell carcinoma, Cell Death Dis. 11 (2020), https://doi.org/10.1038/s41419-020-02877-0.

[14] L.R. Pasquale, S.J. Loomis, J.A.E.H. Kang, B.L. Yaspan, W. Abdrabou, D.L. Budenz, T.C. Chen, E. Delbono, D.S. Friedman, D. Gaasterland, T. Gaasterland, C. L. Grosskreutz, R.K. Lee, P.R. Lichter, Y. Liu, C.A. Mccarty, S.E. Moroi, L.M. Olson, T. Realini, D.J. Rhee, J.S. Schuman, K. Singh, D. Vollrath, G. Wollstein, D.J. Zack, R.R. Allingham, M.A. Pericak-vance, R.N. Weinreb, K. Zhang, M.A. Hauser, J. E. Richards, J.L. Haines, J.L. Wiggs, CDKN2B-AS1 genotype-glaucoma feature correlations in primary open-angle glaucoma patients from the United States, Am. J. Ophthalmol. 155 (2013) 342–353.e5, https://doi.org/10.1016/j.ajo.2012.07.023.

[15] S. Rathi, I. Danford, H. V Gudiseva, L. Verkuil, M. Pistilli, S. Vishwakarma, I. Kaur, T.V. Dave, J.M.O. Brien, V.R.M. Chavali, in POAG Pathogenesis, (n.d.).

[16] S. Gao, T.C. Jakobs, Mice homozygous for a deletion in the glaucoma susceptibility locus INK4 show increased vulnerability of retinal ganglion cells to elevated intraocular pressure, Am. J. Pathol. 186 (2016) 985–1005, https://doi.org/10.1016/j.ajpath.2015.11.026.

[17] K. Sato, S. Li, W.C. Gordon, J. He, G.I. Liou, J.M. Hill, G.H. Travis, N.G. Bazan, M. Jin, Receptor interacting protein kinase-mediated necrosis contributes to cone and rod photoreceptor degeneration in the retina lacking interphotoreceptor retinoid-binding protein, J. Neurosci. 33 (2013) 17458–17468, https://doi.org/10.1523/JNEUROSCI.1380-13.2013.

[18] K. Sato, T. Sato, M. Ohno-Oishi, M. Ozawa, S. Maekawa, Y. Shiga, T. Yabana, M. Yasuda, N. Himori, K. Omodaka, K. Fujita, K.M. Nishiguchi, T. Nakazawa, CHOP deletion and anti-neuroinflammation treatment with hesperidin synergistically attenuate NMDA retinal injury in mice, Exp. Eye Res. 213 (2021) 108826, https://doi.org/10.1016/j.exer.2021.108826.

[19] B. Bayoglu, H. Yuksel, H.A. Cakmak, A. Dirican, M. Cengiz, Polymorphisms in the long non-coding RNA CDKN2B-AS1 may contribute to higher systolic blood pressure levels in hypertensive patients, Clin. Biochem. 49 (2016) 821–827, https://doi.org/10.1016/j.clinbiochem.2016.02.012.

[20] K.P. Burdon, A. Crawford, R.J. Casson, A.W. Hewitt, J. Landers, P. Danoy, D. A. MacKey, P. Mitchell, P.R. Healey, J.E. Craig, Glaucoma risk alleles at CDKN2B-AS1 are associated with lower intraocular pressure, normal-tension glaucoma, and advanced glaucoma, Ophthalmology 119 (2012) 1539–1545, https://doi.org/10.1016/j.ophtha.2012.02.004.

[21] N. Eliseeva, I. Ponomarenko, E. Reshetnikov, V. Dvornyk, M. Churnosov, The haplotype of the CDKN2B-AS1 gene is associated with primary open-angle glaucoma and pseudoexfoliation glaucoma in the Caucasian population of Central Russia, Ophthalmic Genet. 42 (2021) 698–705, https://doi.org/10.1080/13816810.2021.1955275.

[22] H. Springelkamp, R. Höhn, A. Mishra, P.G. Hysi, C.C. Khor, S.J. Loomis, J.N. C. Bailey, J. Gibson, G. Thorleifsson, S.F. Janssen, X. Luo, W.D. Ramdas, E. Vithana, M.E. Nongpiur, G.W. Montgomery, L. Xu, J.E. Mountain, P. Gharahkhani, Y. Lu, N. Amin, L.C. Karssen, K.S. Sim, E.M. Van Leeuwen, A.I. Iglesias, V.J. M. Verhoeven, M.A. Hauser, S.C. Loon, D.D.G. Despriet, A. Nag, C. Venturini, P. G. Sanfilippo, A. Schillert, J.H. Kang, J. Landers, F. Jonasson, A.J. Cree, L.M.E. Van Koolwijk, F. Rivadeneira, E. Souzeau, V. Jonsson, G. Menon, P. Mitchell, J.J. Wang, E. Rochtchina, J. Attia, R. Scott, E.G. Holliday, P.N. Baird, J. Xie, M. Inouye, A. Viswanathan, X. Sim, R.N. Weinreb, P.T.V.M. De Jong, B.A. Oostra, A. G. Uitterlinden, A. Hofman, S. Ennis, U. Thorsteinsdottir, K.P. Burdon, R. R. Allingham, M.H. Brilliant, D.L. Budenz, W.G. Christen, J. Fingert, D.S. Friedman, D. Gaasterland, T. Gaasterland, J.L. Haines, J.H. Kang, P. Kraft, R.K. Lee, P. R. Lichter, Y. Liu, S.E. Moroi, M.A. Pericak-Vance, A. Realini, J.E. Richards, J. S. Schuman, W.K. Scott, K. Singh, A.J. Sit, D. Vollrath, G. Wollstein, D.J. Zack, K. Zhang, I. Barroso, J.M. Blackwell, E. Bramon, M.A. Brown, J.P. Casas, A. Corvin, P. Deloukas, A. Duncanson, J. Jankowski, H.S. Markus, C.G. Mathew, C.N. A. Palmer, R. Plomin, A. Rautanen, S.J. Sawcer, R.C. Trembath, A.C. Viswanathan, N.W. Wood, C.C.A. Spencer, G. Band, C. Bellenguez, C. Freeman, G. Hellenthal, E. Giannoulatou, M. Pirinen, R. Pearson, A. Strange, Z. Su, D. Vukcevic, P. Donnelly, C. Langford, S.E. Hunt, S. Edkins, R. Gwilliam, H. Blackburn, S. J. Bumpstead, S. Dronov, M. Gillman, E. Gray, N. Hammond, A. Jayakumar, O. T. McCann, J. Liddle, S.C. Potter, R. Ravindrarajah, M. Ricketts, M. Waller, P. Weston, S. Widaa, P. Whittaker, T.D. Spector, A. Mirshahi, S.M. Saw, J. R. Vingerling, Y.Y. Teo, R.C.W. Wolfs, H.G. Lemij, E.S. Tai, N.M. Jansonius, J. B. Jonas, C.Y. Cheng, T. Aung, C.C.W. Klaver, J.E. Craig, S. MacGregor, D. A. MacKey, A.J. Lotery, K. Stefansson, A.A.B. Bergen, T.L. Young, J.L. Wiggs, N. Pfeiffer, T.Y. Wong, L.R. Pasquale, A.W. Hewitt, C.M. Van Duijn, C.J. Hammond, Meta-analysis of genome-wide association studies identifies novel loci that influence cupping and the glaucomatous process, Nat. Commun. 5 (2014), https://doi.org/10.1038/ncomms5883.

[23] H. Springelkamp, A.I. Iglesias, A. Mishra, R. Höhn, R. Wojciechowski, A. P. Khawaja, A. Nag, Y.X. Wang, J.J. Wang, G. Cuellar-Partida, J. Gibson, J. N. Cooke Bailey, E.N. Vithana, P. Gharahkhani, T. Boutin, W.D. Ramdas, T. Zeller, R.N. Luben, E. Yonova-Doing, A.C. Viswanathan, S. Yazar, A.J. Cree, J.L. Haines, J. Y. Koh, E. Souzeau, J.F. Wilson, N. Amin, C. Müller, C. Venturini, L.S. Kearns, J. H. Kang, Y.C. Tham, T. Zhou, E.M. van Leeuwen, S. Nickels, P. Sanfilippo, J. Liao, H. van der Linde, W. Zhao, L.M.E. van Koolwijk, L. Zheng, F. Rivadeneira, M. Baskaran, S.J. van der Lee, S. Perera, P.T.V.M. de Jong, B.A. Oostra, A. G. Uitterlinden, Q. Fan, A. Hofman, E.S. Tai, J.R. Vingerling, X. Sim, R.C.W. Wolfs, Y.Y. Teo, H.G. Lemij, C.C. Khor, R. Willemsen, K.J. Lackner, T. Aung, N. M. Jansonius, G. Montgomery, P.S. Wild, T.L. Young, K.P. Burdon, P.G. Hysi, L. R. Pasquale, T.Y. Wong, C.C.W. Klaver, A.W. Hewitt, J.B. Jonas, P. Mitchell, A. J. Lotery, P.J. Foster, V. Vitart, N. Pfeiffer, J.E. Craig, D.A. Mackey, C.J. Hammond, J.L. Wiggs, C.Y. Cheng, C.M. van Duijn, S. MacGregor, New insights into the genetics of primary open-angle glaucoma based on meta-analyses of intraocular pressure and optic disc characteristics, Hum. Mol. Genet. 26 (2017) 438–453, https://doi.org/10.1093/hmg/ddw399.

[24] H. Springelkamp, A. Mishra, P.G. Hysi, P. Gharahkhani, R. Höhn, C.C. Khor, J. N. Cooke Bailey, X. Luo, W.D. Ramdas, E. Vithana, V. Koh, S. Yazar, L. Xu, H. Forward, L.S. Kearns, N. Amin, A.I. Iglesias, K.S. Sim, E.M. van Leeuwen, A. Demirkan, S. van der Lee, S.C. Loon, F. Rivadeneira, A. Nag, P.G. Sanfilippo, A. Schillert, P.T.V.M. de Jong, B.A. Oostra, A.G. Uitterlinden, A. Hofman, T. Zhou, K.P. Burdon, T.D. Spector, K.J. Lackner, S.M. Saw, J.R. Vingerling, Y.Y. Teo, L. R. Pasquale, R.C.W. Wolfs, H.G. Lemij, E.S. Tai, J.B. Jonas, C.Y. Cheng, T. Aung, N. M. Jansonius, C.C.W. Klaver, J.E. Craig, T.L. Young, J.L. Haines, S. Macgregor, D. A. Mackey, N. Pfeiffer, T.Y. Wong, J.L. Wiggs, A.W. Hewitt, C.M. van Duijn, C. J. Hammond, R.R. Allingham, M.H. Brilliant, D.L. Budenz, J.N.C. Bailey, W. G. Christen, J. Fingert, D. Gaasterland, T. Gaasterland, M.A. Hauser, J.H. Kang, P. Kraft, R.K. Lee, P.A. Lichter, Y. Liu, S.J. Loomis, S.E. Moroi, M.A. Pericak-Vance, A. Realini, J.E. Richards, J.S. Schuman, W.K. Scott, K. Singh, A.J. Sit, D. Vollrath, R.N. Weinreb, G. Wollstein, D.J. Zack, K. Zhang, Meta-analysis of genome-wide association studies identifies novel loci associated with optic disc morphology, Genet. Epidemiol. 39 (2015) 207–216, https://doi.org/10.1002/gepi.21886.

[25] N. Carboni, M. Floris, A. Mateddu, M. Porcu, G. Marrosu, E. Solla, E. Cocco, M. Mura, S. Marini, M.A. Maioli, R. Piras, R. Aste, M.G. Marrosu, Aberrant splicing in the LMNA gene caused by a novel mutation on the polypyrimidine tract of intron 5, Muscle Nerve 43 (2011) 688–693, https://doi.org/10.1002/mus.21937.

[26] M. Haeussler, K. Schönig, H. Eckert, A. Eschstruth, J. Mianné, J.B. Renaud, S. Schneider-Maunoury, A. Shkumatava, L. Teboul, J. Kent, J.S. Joly, J. P. Concordet, Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR, Genome Biol. 17 (2016) 1–12, https://doi.org/10.1186/s13059-016-1012-2.

[27] K. Sato, Y. Nakagawa, K. Omodaka, H. Asada, S. Fujii, K. Masaki, T. Nakazawa, The sustained release of tafluprost with a drug delivery system prevents the axonal injury-induced loss of retinal ganglion cells in rats, Curr. Eye Res. 45 (2020) 1114–1123, https://doi.org/10.1080/02713683.2020.1715446.

[28] J.S. Mattick, I.V. Makunin, Non-coding RNA, Hum. Mol. Genet. 15 (2006) 17–29, https://doi.org/10.1093/hmg/ddl046. Spec No.

[29] W. Yu, D. Gius, P. Onyango, K. Muldoon-Jacobs, J. Karp, A.P. Feinberg, H. Cui, Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA, Nature 451 (2008) 202–206, https://doi.org/10.1038/nature06468.

[30] S. Guil, M. Esteller, Cis-acting noncoding RNAs: friends and foes, Nat. Struct. Mol. Biol. 19 (2012) 1068–1075, https://doi.org/10.1038/nsmb.2428.

[31] A. Congrains, K. Kamide, M. Ohishi, H. Rakugi, ANRIL: molecular mechanisms and implications in human health, Int. J. Mol. Sci. 14 (2013) 1278–1292, https://doi.org/10.3390/ijms14011278.

[32] N. Popov, J. Gil, Epigenetic regulation of the INK4B-ARF-INK4a locus: in sickness and in health, Epigenetics 5 (2010) 685–690, https://doi.org/10.4161/epi.5.8.12996.

[33] K.L. Yap, S. Li, A.M. Muñoz-Cabello, S. Raguz, L. Zeng, S. Mujtaba, J. Gil, M. J. Walsh, M.M. Zhou, Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a, Mol. Cell. 38 (2010) 662–674, https://doi.org/10.1016/j.molcel.2010.03.021.

[34] D. Skowronska-Krawczyk, L. Zhao, J. Zhu, R.N. Weinreb, G. Cao, J. Luo, K. Flagg, S. Patel, C. Wen, M. Krupa, H. Luo, H. Ouyang, D. Lin, W. Wang, G. Li, Y. Xu, O. Li, C. Chung, E. Yeh, M. Jafari, M. Ai, Z. Zhong, W. Shi, L. Zheng, M. Krawczyk, D. Chen, C. Shi, C. Zin, J. Zhu, P.L. Mellon, W. Gao, R. Abagyan, L. Zhang, X. Sun, S. Zhong, Y. Zhuo, M.G. Rosenfeld, Y. Liu, K. Zh, P16INK4a upregulation mediated by SIX6 defines retinal ganglion cell pathogenesis in glaucoma, Mol. Cell. 59 (2015) 931–940, https://doi.org/10.1016/j.molcel.2015.07.027.

[35] L.U. Li, Y. Zhao, H. Zhang, P16INK4a upregulation mediated by TBK1 induces retinal ganglion cell senescence in ischemic injury, Cell Death Dis. 8 (2017) 1–12, https://doi.org/10.1038/cddis.2017.169.

[36] H. Tawarayama, Q. Feng, N. Murayama, N. Suzuki, T. Nakazawa, Cyclin-dependent kinase inhibitor 2B mediates excitotoxicity-induced death of retinal ganglion cells, Investig. Ophthalmol. Vis. Sci. 60 (2019) 4479–4488, https://doi.org/10.1167/iovs.19-27396.

[37] Y. Kotake, T. Nakagawa, K. Kitagawa, S. Suzuki, N. Liu, M. Kitagawa, Y. Xiong, Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15 INK4B tumor suppressor gene, Oncogene 30 (2011) 1956–1962, https://doi.org/10.1038/onc.2010.568.

[38] W.D. Ramdas, L.M.E. van Koolwijk, M.K. Ikram, N.M. Jansonius, P.T.V.M. de Jong, A.A.B. Bergen, A. Isaacs, N. Amin, Y.S. Aulchenko, R.C.W. Wolfs, A. Hofman, F. Rivadeneira, B.A. Oostra, A.G. Uitterlinden, P. Hysi, C.J. Hammond, H.G. Lemij, J.R. Vingerling, C.C.W. Klaver, C.M. van Duijn, A genome-wide association study of optic disc parameters, PLoS Genet. 6 (2010) 1–12, https://doi.org/10.1371/journal.pgen.1000978.

[39] Y. Shinozaki, A. Leung, K. Namekata, S. Saitoh, H.B. Nguyen, A. Takeda, Y. Danjo, Y.M. Morizawa, E. Shigetomi, F. Sano, N. Yoshioka, H. Takebayashi, N. Ohno, T. Segawa, K. Miyake, K. Kashiwagi, T. Harada, S.-I. Ohnuma, S. Koizumi, Astrocytic dysfunction induced by ABCA1 deficiency causes optic neuropathy, Sci. Adv. 8 (2022) eabq1081, https://doi.org/10.1126/sciadv.abq1081.

[40] W. Kobayashi, A. Onishi, H.Y. Tu, Y. Takihara, M. Matsumura, K. Tsujimoto, M. Inatani, T. Nakazawa, M. Takahashi, Culture systems of dissociated mouse and human pluripotent stem cell–derived retinal ganglion cells purified by two-step immunopanning, Investig. Ophthalmol. Vis. Sci. 59 (2018) 776–787, https://doi.org/10.1167/iovs.17-22406.