LncRNA *NEAT1* Recruits SFPQ to Regulate MITF Splicing and Control RPE Cell Proliferation

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Purpose. Retinal pigment epithelium (RPE) cell proliferation is precisely regulated to maintain retinal homoeostasis. Microphthalmia-associated transcription factor (MITF), a critical transcription factor in RPE cells, has two alternatively spliced isoforms: (+)MITF and (−)MITF. Previous work has shown that (−)MITF but not (+)MITF inhibits RPE cell proliferation. This study aims to investigate the role of long non-coding RNA (lncRNA) nuclear-enriched abundant transcript 1 (*NEAT1*) in regulating MITF splicing and hence proliferation of RPE cells.

Methods. Mouse RPE, primary cultured mouse RPE cells, and different proliferative human embryonic stem cell (hESC)–RPE cells were used to evaluate the expression of (+)*MITF*, (−)*MITF*, and *NEAT1* by reverse-transcription PCR (RT-PCR) or quantitative RT-PCR. *NEAT1* was knocked down using specific small interfering RNAs (siRNAs). Splicing factor proline- and glutamine-rich (SFPQ) was overexpressed with the use of lentivirus infection. Cell proliferation was analyzed by cell number counting and Ki67 immunostaining. RNA immunoprecipitation (RIP) was used to analyze the co-binding between the SFPQ and *MITF* or *NEAT1*.

Results. *NEAT1* was highly expressed in proliferative RPE cells, which had low expression of (−)*MITF*. Knockdown of *NEAT1* in RPE cells switched the MITF splicing pattern to produce higher levels of (−)*MITF* and inhibited cell proliferation. Mechanistically, *NEAT1* recruited SFPQ to bind directly with *MITF* mRNA to regulate its alternative splicing. Overexpression of SFPQ in ARPE-19 cells enhanced the binding enrichment of SFPQ to *MITF* and increased the splicing efficiency of (+)*MITF*. The binding affinity between SFPQ and *MITF* was decreased after *NEAT1* knockdown.

Conclusions. *NEAT1* acts as a scaffold to recruit SFPQ to *MITF* mRNA and promote its binding affinity, which plays an important role in regulating the alternative splicing of *MITF* and RPE cell proliferation.

Keywords: RPE, *MITF*, *NEAT1*, splicing, proliferation

The retinal pigment epithelium (RPE) cell is important for retinal homeostasis, as it supports a number of critical retina functions including secreting growth factors and antioxidants, maintaining the blood–retinal barrier, and phagocytizing detached photoreceptor outer segments, among others.[1–3] In vertebrates, RPE cells are derived from the dorsal portion of the optic vesicle and undergo a phase of rapid cell division early in development, whereas mature pigmented quiescent RPE cells reside in a monolayer between the neural retina and choroid.[4] Normally, mature RPE cells remain in G_0, out of the cell cycle. In some pathological states such as proliferative vitreoretinopathy (PVR), RPE cells will detach from the retina to enter the vitreous humor, undergo the epithelial–mesenchymal transition (EMT), and re-enter the cell cycle in a proliferative state.[5–7]

RPE cell hyperproliferative diseases are highly prevalent in patients with retinal surgery; they can affect visual function and may lead to blindness.[8,9]

Cell proliferation is an important cellular event that is complex and precisely regulated, including by non-coding RNAs and alternative splicing of pre-mRNAs. RNA splicing, in which introns are removed from eukaryotic gene transcripts, is an essential step for mRNA maturation. It is precisely regulated by coordinated interactions between *cis*-regulatory elements on the pre-mRNA and binding of splicing factors. Alternative splicing of pre-mRNA increases protein diversity from a rather limited number of genes, contributing to cell proliferation, tissue development, and organ physiology.[10–13] Dysregulation of pre-mRNA alternative splicing has been implicated in a variety of different

Investigative Ophthalmology & Visual Science

genetic diseases and cancers. Mutations in genes that affect splicing have been implicated in both retinitis pigmentosa and cataract.[14–16]

Microphthalmia-associated transcription factor (MITF) plays an irreplaceable role in controlling RPE cell proliferation, development, and function.[17] *Mitf* mutant mice display microphthalmia and retinal degeneration secondary to structural abnormalities and dysfunction of the RPE.[18–20] The dorsal retina of *Mitf* mutant mice shows hyperproliferation and formation of multiple layers of RPE cells.[21] Overexpression of dominant-negative mutant MITF increases the proliferation of chick RPE cells, although expression of wild-type MITF does not affect their proliferation.[22] Under normal conditions, MITF produces two alternative splicing isoforms in exon 6, (+)MITF and (−)MITF; (+)MITF contains exon 6a of the sequence ACIFPT upstream of the basic domain, but (−)MITF lacks this sequence.[23] Both (+)MITF and (−)MITF were reported to be expressed at similar levels in melanocytes and some melanoma cell lines,[24,25] but they have different transcriptional activities and diverse functions in cell proliferative regulation.[24,26] Mice with the mutation resulting in the production of only (−)*Mitf* (*Mitf*[mi-sp]) have hypopigmentation, retinal dystrophy and reduced electroretinography amplitude in a compound heterozygous state with *Mitf*[Mi-wb].[23,27,28] Our previous work demonstrated that (−)MITF inhibits the proliferation of RPE cells by regulating the axis of MSI2/miR-7/DAPL1, but (+)MITF did not affect the proliferation of RPE cells.[29] However, the expression states of (+)MITF and (−)MITF and their regulation in RPE cells are unclear. Nuclear-enriched abundant transcript (*NEAT1*) is a long non-coding RNA (lncRNA) that is essential for formation of nuclear body paraspeckles. It has been shown to regulate cell proliferation by sponging miRNAs, but the functional roles of *NEAT1* in RPE cells are largely unknown.[30–32] In addition, *NEAT1* also interacts with other regulatory proteins to regulate gene expression or RNA processing, including splicing factor proline- and glutamine-rich (SFPQ, or PSF) and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4 (SMARCA4, or BRG1).[33–35] SFPQ was first shown to be required for pre-mRNA splicing and was later implicated in transcriptional regulation, 3′-end processing of mRNAs, and RNA and DNA repair.[36] Although the functional role of SFPQ in RNA splicing is well documented,[37] its role in regulating *MITF* mRNA alternative splicing is currently unknown.

In this paper, we show that *NEAT1* is highly expressed in proliferative RPE cells and that these cells express only low levels of (−)MITF. Knockdown of *NEAT1* in RPE cells changes the *MITF* splicing pattern to produce higher levels of (−)*MITF* and inhibit cell proliferation. We demonstrate that *NEAT1* recruits SFPQ to bind to *MITF* mRNA and thus regulates its splicing. Overexpression of SFPQ in ARPE-19 cells enriches the binding of SFPQ to *MITF* mRNA and increases the splicing efficiency to produce (+)*MITF*. Knockdown of *NEAT1* in RPE cells decreases the binding affinity of SFPQ to *MITF* mRNA, which switches the splicing pattern to produce higher (−)*MITF* levels and conversely decreases splicing to produce (+)*MITF*. Hence, our findings suggest that *NEAT1* acts as a scaffold to recruit SFPQ to *MITF* mRNA, increasing its binding affinity and switching alternative splicing isoforms of *MITF* to reduce the levels of (−)*MITF* and promote the proliferation of RPE cells.

MATERIALS AND METHODS

Mouse RPE Isolation and Primary Culture

Mouse RPE cells were isolated from 2-month-old C57BL/6J mice immediately after euthanasia. The RPE layer was separated from the neural retina layer by digestion with 2% dispase for 30 minutes and scraped off of the choroid using an iris separator. RPE sheets were gently collected by pipetting and were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 15% fetal bovine serum (FBS). Alternatively, some RPE sheets were collected similarly for gene expression analysis. All animal experiments were carried out in accordance with the approved guidelines of the Wenzhou Medical University Institutional Animal Care and Use Committee.

Cell Culture and *NEAT1* Knockdown

ARPE-19 and D407 cells were cultured in the DMEM/F-12 and DMEM medium separately, supplemented with 10% FBS and antibiotics under 5% CO_2 at 37°C. The human embryonic stem cell (hESC) cell line (H9) was cultured using xeno-free Gibco Essential 8 Medium (A1517001; Thermo Fisher Scientific) to induce differentiation in RPE cells as described in our previous work.[38] For small interfering RNA (siRNA) studies, cells were cultured in 12-well plates to reach about 50% confluency prior to transfection, then 40 pmol of siRNA was transfected using LipoJet Reagent (SignaGen Labs, Frederick, MD, USA). The siRNA sequences were designed and synthesized by Gene Pharma Co., Ltd. (Shanghai, China) as follows:

si-NC: 5′UUCUCCGAACGUGUCACGUTT
si-NEAT1-1: 5′CCCAAGAGUACAUAAAAUAUTT
si-NEAT1-2: 5′GCCAUCAGCUUUGAAUAAATT

SFPQ Overexpression

SFPQ (NM_005066.3) overexpressing lentivirus was purchased from GeneCopoeia Co., Ltd. (Guangzhou, China). ARPE-19 cells were cultured in 12-well plates at about 50% confluency 1 day before the infection. Then, 10 μL of the SFPQ overexpression lentivirus (2×10^8 transducing units/mL) was added to the FBS-free DMEM/F-12 for about 6 hours, and the cells were cultured in the complete culture medium. Seventy-two hours after the infection, SFPQ mRNA was analyzed to test the overexpression efficiency.

MITF mRNA and Splicing Isoform Analysis

Total RNA was isolated using Invitrogen TRIzol (Thermo Fisher Scientific) and reverse-transcribed into cDNA using a reverse transcription kit and random primers (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was used for examining gene expression using reverse-transcription PCR (RT-PCR) or quantitative PCR (qRT-PCR). The primers were designed to test the total *MITF* mRNA or *MITF* splicing isoform as shown in Supplementary Figure S1, and the primer sequences used in this study are listed in Supplementary Table S1. As for the RT-PCR, primers were designed flanking exon 6a resulting in a PCR product of 206 bp for (+)*MITF* but 188 bp for (−)*MITF*. The PCR mixes include 2× Taq Master Mix (Dye Plus; Vazyme Biotech, Nanjing, China), 10-pM primer 2 + 2 μL, cDNA

sample (1 µL), and H$_2$O (up to 50 µL). PCR was performed at 94°C for 40 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. This procedure was repeated for 34 cycles, followed by a prolonged elongation step at 72°C for 5 minutes. PCR production was separated in the 4% agarose gel electrophoresis.

For qRT-PCR, primers to test the total *MITF* mRNA were designed in exons 2 and 3, which can test both (+)*MITF* and (−)*MITF*. The forward primer to test (+)*MITF* was designed in exon 6a, and the reverse primer was in exon 7, which can only test the expression of (+)*MITF* (Supplementary Fig. S1). Primers to test total mouse *Mitf* mRNA were designed in exons 5 and 7. The cDNA was processed for real-time PCR using SYBR Green (Thermo Fisher Scientific). The percentage of (+)*MITF* was normalized to total *MITF*, and the percentage of (−)*MITF* was set equal to 100% minus (+)*MITF*. Each experiment was repeated three times independently. The amplification efficiency of the primers was greater than 0.98, and single products were confirmed by melting profiles.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 25 minutes at room temperature and permeabilized with 0.4% Triton X-100 for 10 minutes. Immunostaining was carried out using rabbit Anti-Ki67 Antibody (1:200; ab9260; MilliporeSigma, Burlington, MA, USA) at 37°C for 2 hours. For bromodeoxyuridine (BrdU) analysis, cells were cultured in complete medium containing 20 µm BrdU for 2 hours. Anti-BrdU (1:200; b8434; Sigma-Aldrich, St. Louis, MO, USA) was used for immunostaining. Staining was indicated by appropriate secondary antibodies. The results were photographed with a Zeiss fluorescence microscope (Carl Zeiss Microscopy, White Plains, NY, USA), and photographs were processed digitally.

Western Blotting

Cells were washed with PBS and then lysed in radioimmunoprecipitation assay buffer. Cell debris was pelleted by centrifugation at 6000g for 5 minutes. Equal amounts of protein from the parallel samples were loaded onto the SDS-PAGE gel, separated by electrophoresis, and transferred to nitrocellulose membrane. The membrane was blocked with 5% fat-free milk and incubated with the primary specific antibody at 4°C (MET, 8198; E2F1, 3742S; P-Rb, 3590 and P-AKT, 4060; AKT, 4691; Cell Signaling Technology, Danvers, MA, USA). After washing with PBS containing 0.01% Tween 20, the membranes were incubated with fluorescein-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 2 hours, and the blots were analyzed using the Odyssey CLx system (LI-COR Biosciences). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

RNA Binding Protein Immunoprecipitation Assay

ARPE-19 or ARPE-19 + SFPQ cells were cultured for 24 hours in 10-cm dishes. RNA immunoprecipitation (RIP) experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-700; MilliporeSigma) and antibody to SFPQ (P2860; Sigma-Aldrich) following the manufacturer's protocol. Co-precipitated RNAs were used for RT-PCR or qRT-PCR analysis.

Statistical Analysis

Each experiment was repeated three times, and results are presented as mean ± SD. Statistical significance between experimental and control groups was assessed with Student's *t*-test, and $P < 0.05$ was considered to be a significant difference.

RESULTS

Switched Expression Pattern of MITF Alternative Splicing Isoforms in Different Proliferative State RPE Cells

Our previous work demonstrated that alternative splicing isoforms of MITF play different roles in regulating RPE cell proliferation, as (−)MITF but not (+)MITF inhibits the proliferation of ARPE-19 cells.[29] However, control of MITF expression and regulation of its splicing are poorly understood in RPE cells. In order to address this question, we first analyzed the expression of (−)*MITF* and (+)*MITF* in RPE cells in different proliferative states. Mature mouse RPE cells are able to reinitiate proliferation after being cultured in vitro.[38] When RPE cells were isolated from 2-month-old C57BL/6J mice and cultured in vitro for 7 days (passage 1), BrdU incorporation was detected in cultured primary RPE cells but not isolated RPE tissue (Fig. 1A), indicating that quiescent isolated RPE cells could re-enter the proliferative state after being cultured in vitro. Next, expression levels of (−)*MITF* and (+)*MITF* in quiescent and proliferative mouse RPE cells were estimated by RT-PCR. Although both (−)*MITF* and (+)*MITF* were equally expressed in quiescent RPE tissue, after the RPE cells re-entered the cell-cycle expression of (−)*MITF* decreased markedly (Fig. 1B). qRT-PCR results indicated that the percentage of (+)*Mitf* was about 50% in the quiescent mouse RPE cells, which was increased to more than 80% when the primary cultured mouse RPE cells were in the proliferative state (Fig. 1C). This pattern of expression was also seen in hESC-derived RPE cells, which can be divided into low-proliferative (highly pigmented cells about 20% positive for Ki67) and highly proliferative (low pigmented cells about 75% positive for Ki67) groups (Figs. 1D, 1E). When the expression of (−)*MITF* and (+)*MITF* in these groups was analyzed by RT-PCR and qRT-PCR, (−)*MITF* and (+)*MITF* were expressed equally in the highly proliferative hESC–RPE cells, but the low-proliferative group of hESC–RPE cells expressed higher levels of (−)*MITF* than (+)*MITF* (Fig. 1F). qRT-PCR results showed that more than 75% of the highly proliferative hESC–RPE cells expressed (+)*MITF* in contrast to only about 45% of the low-proliferative hESC–RPE cells (Fig. 1G). These results are consistent with the expression pattern of MITF splicing isoforms seen in different proliferative states of isolated RPE cells. Similar expression levels of (+)*MITF* and (−)*MITF* are associated with low-proliferative activity, and higher expression of (+)*MITF* relative to (−)*MITF* is seen in highly proliferative RPE cells, although the mechanisms controlling the alternative splicing are unclear.

LncRNA *NEAT1* is Highly Expressed in Proliferative RPE Cells

Although *NEAT1* has been demonstrated to mediate alternative splicing of pre-mRNA and regulate cell proliferation,[39,40] its role in RPE cells is currently unclear. To investigate this,
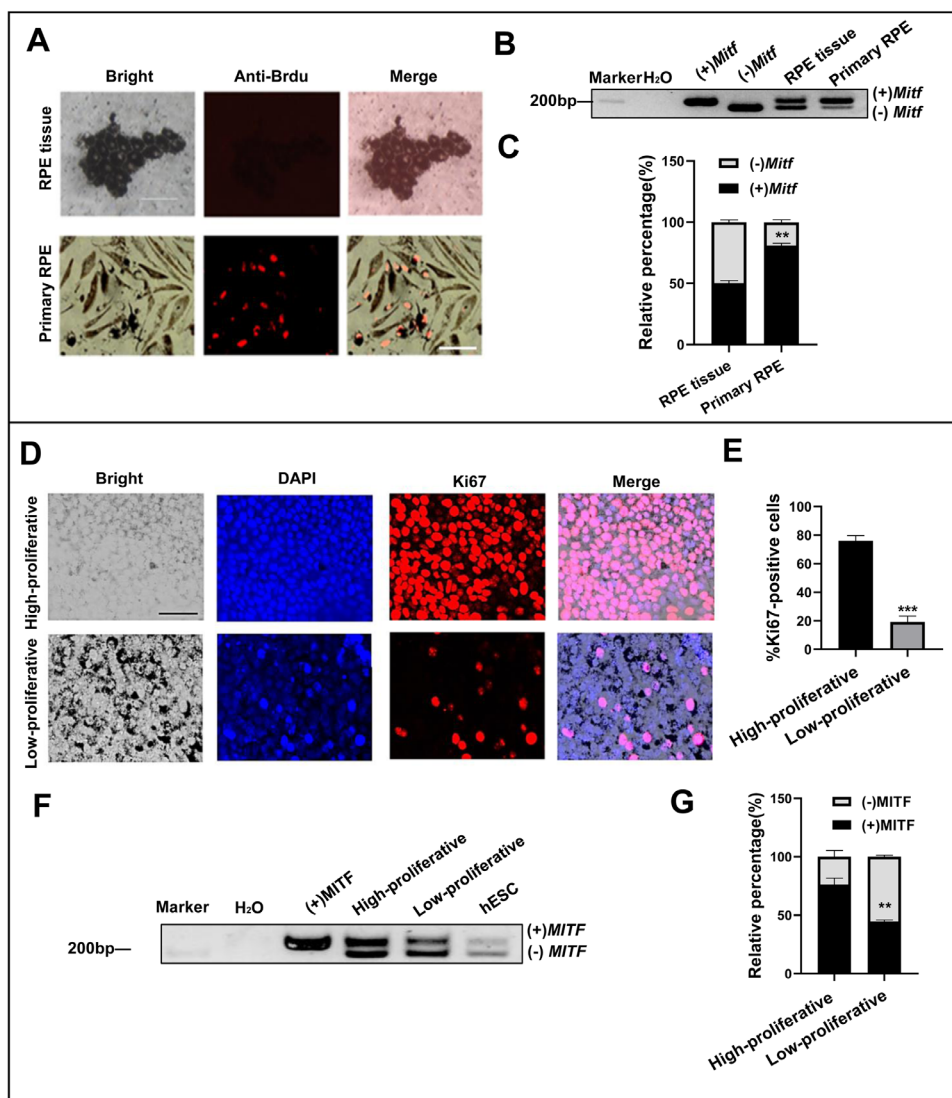
FIGURE 1. (−)*MITF* was expressed at low levels relative to (+)*MITF* in proliferative RPE cells. (**A**) RPE cells from 2-month-old mice were isolated and cultured in vitro for 7 days. BrdU-positive signals (*red*) could be detected in primary cultured RPE cells but not in isolated RPE tissue. (**B**) Expression of (−)*Mitf* and (+)*Mitf* analyzed by RT-PCR. (**C**) qRT-PCR analysis of the percentages of (−)*Mitf* and (+)*Mitf* in the isolated RPE tissue and primary cultured RPE cells. (**D**) Ki67 immunostaining in hESC–RPE cells. Highly pigmented RPE cells had lower Ki67-positive signals and lower pigmented RPE cells had higher Ki67-positive signals. (**E**) Ki67-positive percentages based on (**D**). (**F**) The expression of (−)*MITF* and (+)*MITF* analyzed by RT-PCR in the low-proliferative and highly proliferative hESC–RPE cells. (**G**) qRT-PCR analysis of the percentage of (−)*MITF* and (+)*MITF* in low-proliferative and highly proliferative hESC–RPE cells. *Scale bar*: 50 μm. **P < 0.01, ***P < 0.001; *n* = 3.

its expression was measured in RPE cells in different proliferative states. Both RT-PCR and qRT-PCR results showed that the expression of *NEAT1* was higher in proliferating primary RPE cell cultures than quiescent mouse RPE cells in isolated tissue (Figs. 2A, 2B). Similarly, expression of *NEAT1* was found to be higher in the highly proliferative than lower proliferative hESC–RPE cells (Fig. 2C). *NEAT1* was also expressed at high levels in subconfluent ARPE-19 and D407 cell lines, which are highly proliferative in vitro (Fig. 2D). In addition, when the ARPE-19 and D407 cells were maintained in cultures for 1 week after confluence, the postconfluent cells showed a decrease in proliferative activity, as indicated by a lower level Ki67 positivity when compared with the subconfluent cells (at about 30% or 70% confluence) (Supplementary Figs. S2A, S2C, S2E–S2H). The

qRT-PCR results indicate that the expression of *NEAT1* is higher in the subconfluent cells (highly proliferative) than the postconfluent cells (low-proliferative) (Supplementary Figs. S2B, S2D). These results show that *NEAT1* is highly expressed in proliferative RPE cells, suggesting that it might be involved in regulation of RPE cell proliferation.

Knockdown of *NEAT1* Inhibits RPE Cell Proliferation

NEAT1 was knocked down by siRNAs in ARPE-19 and D407 cells in order to investigate its regulation of RPE cell proliferation. As shown in Figure 3A, both si-NEAT1-1 and si-NEAT1-2 knocked down the expression of *NEAT1* efficiently in
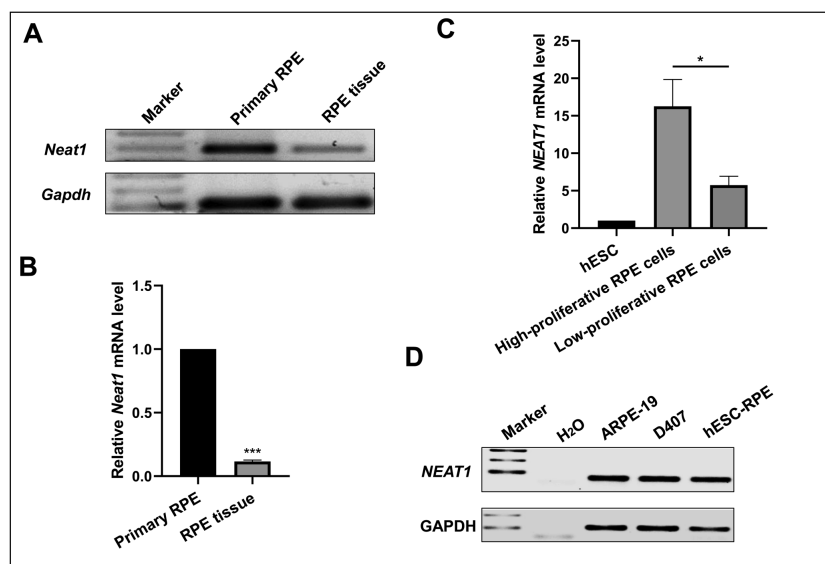
FIGURE 2. LncRNA *NEAT1* was highly expressed in proliferative RPE cells. (**A**, **B**) Expression of *Neat1* in primary cultures of mouse RPE cells and isolated RPE tissue was analyzed by RT-PCR (**A**) and qRT-PCR (**B**). (**C**) qRT-PCR estimation of *NEAT1* levels in high and low proliferation groups of hESC-derived RPE cells. (**D**) Expression of *NEAT1* was detected by RT-PCR in proliferating ARPE-19 and D407 RPE cell lines; the highly proliferative hESC–RPE cells were used as positive controls. *$*P < 0.05$, ***$P < 0.001$; $n = 3$.

ARPE-19 cells. Forty-eight hours after knockdown of *NEAT1*, the cell numbers were lower relative to the negative control (NC) group (Figs. 3B, 3C). Cell growth curves show that *NEAT1* knockdown ARPE-19 cells have lower proliferative activity compared with the control groups (Fig. 3D). In addition, the percentage of cells staining positive for Ki67, which marks proliferating cells, was found to decrease relative to the NC cells (Figs. 3E, 3F). Analysis of other markers of cell proliferation after knockdown of *NEAT1* by western blotting has shown that the expressions of MET, E2F1, and P-RB proteins decreased in the *NEAT1* knockdown APRE-19 cells, although no obvious change was seen in P-AKT and P-ERK (Figs. 3G, 3H). In order to confirm these results, *NEAT1* was also knocked down in D407 cells. Compared with the NC group, cell proliferative activity was also decreased in *NEAT1* knockdown D407 cells (Supplementary Figs. S3A–S3E). Taken together, these results indicate that knockdown of *NEAT1* inhibits RPE cell proliferation.

Knockdown of *NEAT1* Changes the Splicing Pattern of *MITF*

The above results show that (−)*MITF* is expressed at low levels in proliferative RPE cells, which express high levels of *NEAT1*, and we have previously demonstrated that (−)*MITF* inhibits RPE cell proliferation,[29] but it is unclear whether *NEAT1* regulates alternative splicing of *MITF* mRNA. In order to clarify this, we knocked down *NEAT1* in ARPE-19 cells and analyzed its effects on the expression of (+)*MITF* and (−)*MITF*. The qRT-PCR analysis showed that knockdown of *NEAT1* did not affect the expression of total *MITF* mRNA in ARPE-19 cells (Fig. 4A). However, knockdown of *NEAT1* in ARPE-19 cells decreased the percentage of (+)*MITF* relative to (−)*MITF*, which increased (Fig. 4B). The qRT-PCR results showed that the percentage of (+)*MITF* decreased from near 50% to about 32% in the *NEAT1* knockdown ARPE-19 cells, but the percentage of (−)*MITF* increased to about

68% (Fig. 4C). These results were also confirmed by knockdown of *NEAT1* in D407 cells, which did not alter expression of total *MITF* mRNA (Figs. 4D, 4E) but increased the ratios of (−)*MITF* while decreasing the ratios of (+)*MITF* mRNA (Figs. 4F, 4G). These results indicate that knockdown of *NEAT1* changes *MITF* splicing patterns by increasing the ratios of (−)*MITF* and decreasing the ratios of (+)*MITF*.

NEAT1 Recruits SFPQ to Increase Binding to *MITF* mRNA and Regulate Its Splicing

The above results show that *NEAT1* regulates *MITF* splicing and inhibits RPE cell proliferation, but how *NEAT1* regulates *MITF* splicing is unclear. mRNA splicing is regulated by specific splicing factors, and it has been reported that *NEAT1* can bind the RNA splicing factor SFPQ.[33,34] In order to verify the hypothesis that *NEAT1* might regulate *MITF* splicing by recruiting SFPQ to bind it, we used RIP to detect binding of SFPQ to *NEAT1* and *MITF* mRNA directly in ARPE-19 cells. As shown in Figures 5A and 5B, SFPQ binds directly with *NEAT1*, as well as both (+)*MITF* and (−)*MITF* mRNA, suggesting that SFPQ might potentially have a role in the regulation of *MITF* splicing. As a negative control, an amplicon of lncRNA *MIR497HG* showed no positive signal in the anti-SFPQ pull-down lane (Fig. 5C). When lentivirus-mediated SFPQ was overexpressed in AREP-19 cells by approximately fivefold, there was no effect on total *MITF* mRNA levels (Figs. 5D, 5E), but binding of SFPQ to *MITF* was approximately doubled in ARPE-19 + SFPQ cells relative to control ARPE-19 cells (Figs. 5F–5H). Finally, analysis of the percentages of (−)*MITF* and (+)*MITF* by qRT-PCR showed that the expression of (+)*MITF* increased from 51% to 72% in ARPE-19 + SFPQ cells, whereas expression of (−)*MITF* decreased to about 38% (Figs. 5I, 5J). In combination, these results demonstrate that SFPQ not only directly binds to *MITF* mRNA but also regulates its splicing to increase (+)*MITF* relative to (−)*MITF* mRNA.
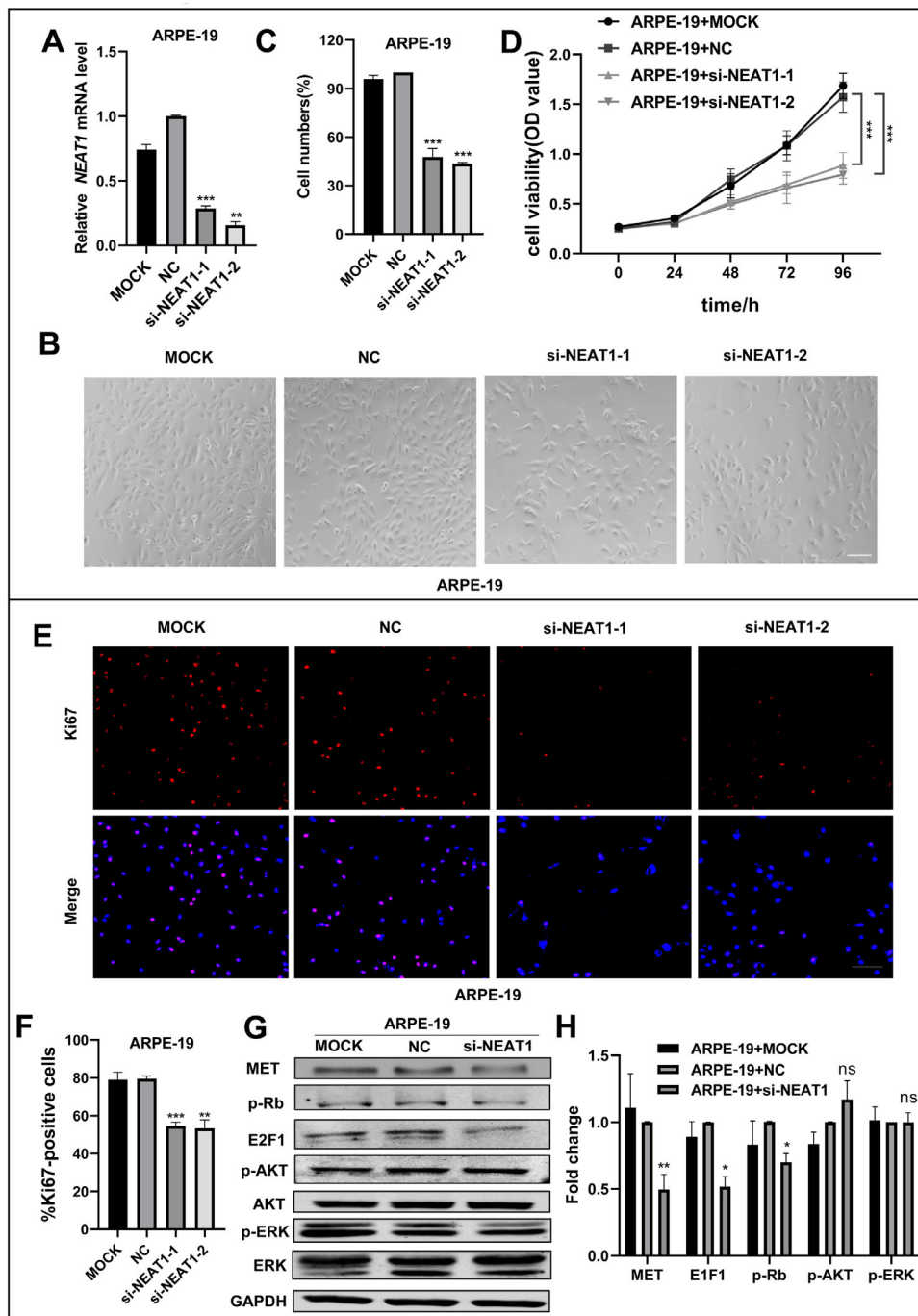
FIGURE 3. Knockdown of *NEAT1* inhibited RPE cell proliferation. (**A**) qRT-PCR showing the knockdown efficiency of *NEAT1* in ARPE-19 cells. (**B**, **C**) ARPE-19 cells were transfected with si-NEAT1 or negative control (NC); cells were counted 48 hours after transfection, and the cell number in the NC group was normalized as 100. (**D**) Cell growth curves of ARPE-19 cells after *NEAT1* knockdown. (**E**, **F**) Ki67 immunostaining of ARPE-19 cells showed a decrease in the percentage of cells staining positive after knockdown of *NEAT1*. (**G**, **H**) Western blotting showing protein levels of MET, E2F1, P-Rb, P-AKT, and P-ERK in ARPE-19 cells 48 hours after knockdown of *NEAT1*. *Scale bar*: 50 μm. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $n = 3$. ns, no significant difference.

SFPQ Regulates *MITF* Splicing in a *NEAT1*-Dependent Manner

In order to demonstrate the requirement for *NEAT1* for SFPQ-mediated *MITF* mRNA splicing, we used siRNA to knock down *NEAT1* in both ARPE-19 and ARPE-19 + SFPQ cells and analyzed the binding of SFPQ to *MITF* mRNA using

SFPQ RIP in each. *MITF* mRNA binding to SFPQ decreased after siRNA knockdown of *NEAT1* in both ARPE-19 cells (Figs. 6A, 6B) and ARPE-19 + SFPQ cells (Figs. 6C, 6D), although it was more marked in the non-overexpressing cells. In addition, the increased expression of (+)*MITF* relative to (−)*MITF* in ARPE-19 + SFPQ cells was reversed after knockdown of *NEAT1* (Figs. 6E, 6F). These results show that
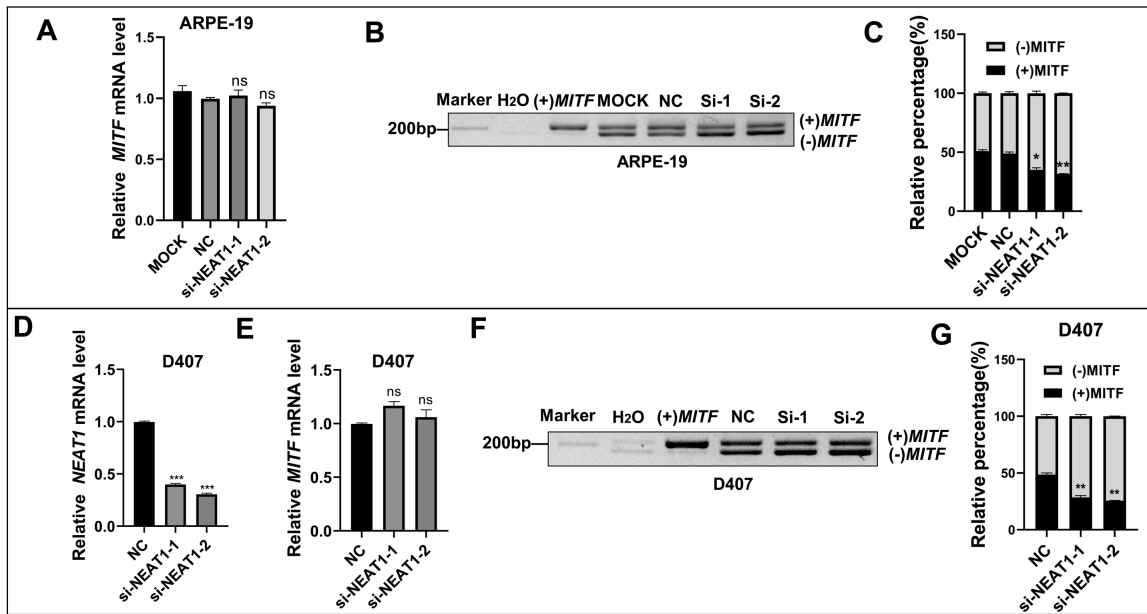
Figure 4. Knockdown of *NEAT1* changed the splicing pattern of *MITF*. (**A**) qRT-PCR was used to analyze the expression of total *MITF* mRNA in ARPE-19 cells after knockdown of *NEAT1*. (**B**) After knockdown of *NEAT1* in ARPE-19 cells, the expression of *MITF* splicing isoforms was analyzed by RT-PCR. (**C**) qRT-PCR analysis of the percentages of (−)*MITF* and (+)*MITF* in the *NEAT1* knockdown ARPE-19 cells. (**D**, **E**) qRT-PCR was used to analyze the expression levels of *NEAT1* and total *MITF* mRNA after the si-NEAT1 transfection. (**F**) After knockdown of *NEAT1* in D407 cells, the expression of *MITF* splicing isoforms was analyzed by RT-PCR. (**G**) qRT-PCR analysis of the percentages of (−)*MITF* and (+)*MITF* in the *NEAT1* knockdown D407 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 3$.
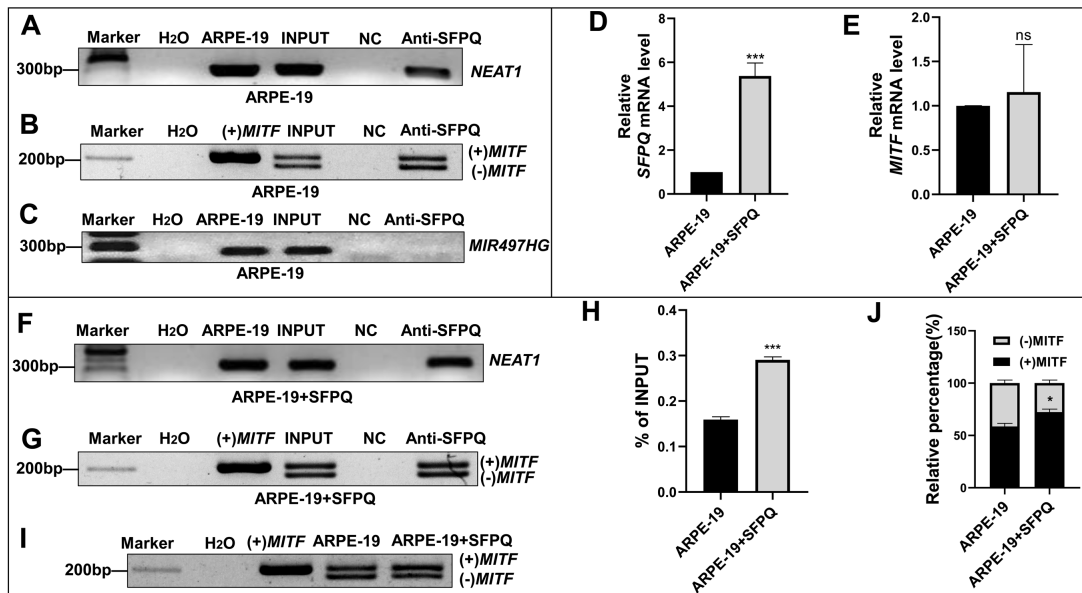

Figure 5. SFPQ bound directly to *MITF* and regulated its splicing. (**A**, **B**) RIP showing binding of SFPQ to *MITF* mRNA and *NEAT1*. (**C**) An amplicon of lncRNA *MIR497HG* was used as a negative control for RIP. (**D**) qRT-PCR quantitation of *SFPQ* in ARPE-19 cells before and after infection with lentivirus-expressing SFPQ. (**E**) qRT-PCR showing no change in total *MITF* mRNA levels in SFPQ-overexpressing ARPE-19 cells. (**F**, **G**) RIP demonstrating binding of SFPQ to *NEAT1* (**F**) and *MITF* (**G**) mRNA in ARPE-19 cells overexpressing SFPQ. (**H**) SFPQ RIP showing enrichment of *MITF* mRNA in ARPE-19 + SFPQ compared with control ARPE-19 cells. (**I**) RT-PCR showing decreased (−)*MITF* and increased (+)*MITF* expression in ARPE-19 + SFPQ relative to ARPE-19 cells. (**I**) qRT-PCR analysis of the percentages of (−)*MITF* and (+)*MITF* in ARPE-19 + SFPQ cells. *$P < 0.05$, ***$P < 0.001$; $n = 3$.

NEAT1 increases binding of SFPQ to *MITF* mRNA and regulates its splicing to increase the ratio of (+)*MITF* to (−)*MITF* mRNA.

Taken as a whole, our data suggest that the long noncoding RNA *NEAT1* works as a scaffold to recruit SFPQ binding to *MITF* mRNA, which plays an important role in
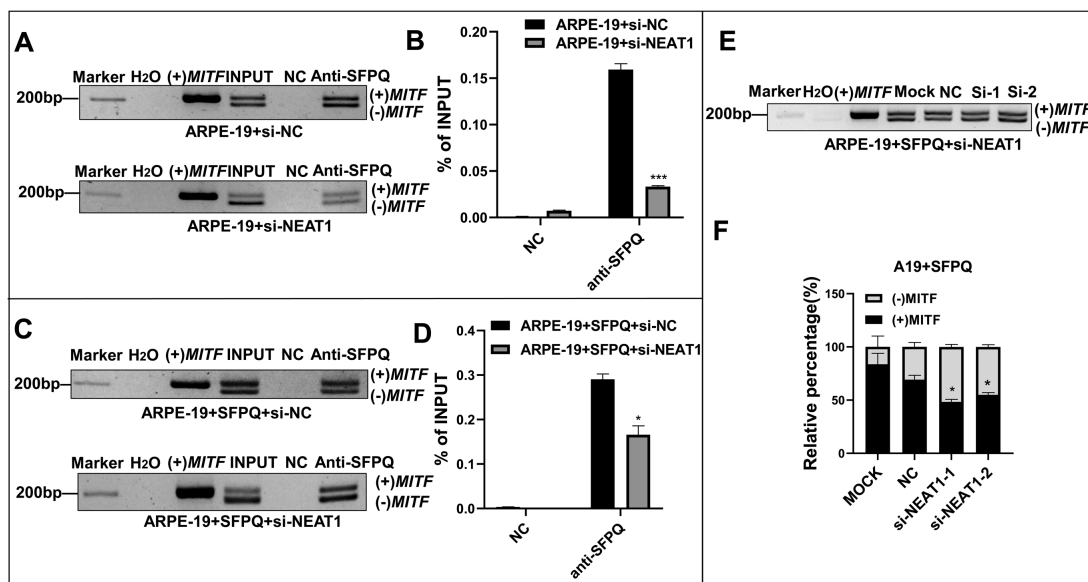
FIGURE 6. SFPQ regulated *MITF* splicing in a *NEAT1*-dependent manner. (**A–D**) RIP showed the direct binding ability of SFPQ to *MITF* after siRNA knockdown of *NEAT1* in ARPE-19 cells (**A, B**) and ARPE-19 + SFPQ cells (**C, D**), with decreased binding in *NEAT1* knockdown cells. (**E**) RT-PCR showing expression of (+)*MITF* and (−)*MITF* in ARPE-19 + SFPQ cells after siRNA knockdown of *NEAT1*. (**F**) qRT-PCR analysis of the percentages of (−)*MITF* and (+)*MITF* in the *NEAT1* knockdown ARPE-19 + SFPQ cells. *$P < 0.05$, ***$P < 0.001$; $n = 3$.
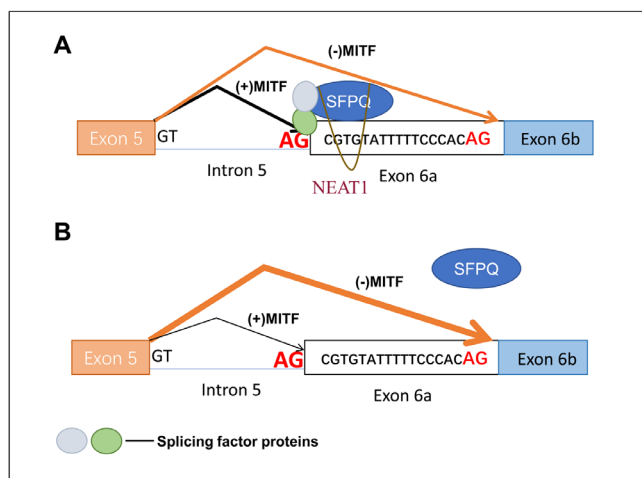

FIGURE 7. Graphical summary of *NEAT1* recruitment of SFPQ binding to *MITF* mRNA and regulation of its splicing. (**A**) *NEAT1* works as a scaffold to recruit SFPQ binding to *MITF* mRNA, which produces both (+)*MITF* and (−)*MITF* isoforms. (**B**) Knockdown of *NEAT1* decreases the binding of SFPQ to *MITF* mRNA, which increases the splicing of (−)*MITF* and decreases that of (+)*MITF*.

regulating the alternative splicing of *MITF* (Fig. 7A). Knockdown of *NEAT1* decreases the binding of SFPQ to *MITF* mRNA, which increases *MITF* splicing to produce (−)*MITF* and decreases splicing to produce (+)*MITF* (Fig. 7B).

DISCUSSION

Previously, we have shown that (−)MITF, the short MITF isoform, inhibits RPE cell proliferation.[29] Here, we show that the variations in MITF splicing isoforms with different proliferative states of RPE cells are partially regulated by the lncRNA *NEAT1* by recruiting RNA splicing factor SFPQ.

MITF plays multiple roles in regulating RPE cell development and differentiation, including its effects on antioxidant systems, growth factor expression, visual cycle activities, proliferation, and melanogenesis.[29,41–45] In RPE cells, transcriptional control of MITF expression has been shown to be regulated by signaling by bone morphogenetic protein (BMP), Wnt/β-catenin, and fibroblast growth factor (FGF), as well as transcription factors VSX2, PAX6, PAX2, OTX2, and ZEB1,[17] but the posttranscriptional regulation of MITF splicing in RPE cells is still poorly understood. Alternative splicing plays a critical role in providing protein diversity and functional activity. In this work, we showed that *NEAT1* recruits SFPQ to *MITF* mRNA to regulate its splicing in RPE cells, which provides new insight into the posttranscriptional regulation of MITF.

RPE hyperproliferation is one of the risk factors in multiple eye diseases, including PVR, malignant congenital hypertrophy of the RPE, RPE rips, and Vogt–Koyanagi–Harada disease.[46–48] The molecular mechanisms of regulating RPE cell proliferation remain incompletely understood. We previously demonstrated that (−)MITF inhibits RPE cell proliferation by regulating death-associated protein-like 1 (DAPL1).[29,38] In this work, we reveal that *NEAT1* regulates *MITF* splicing and RPE cell proliferation, suggesting that *NEAT1* might provide a new target for investigation and treatment of eye diseases related to RPE hyperproliferation. In addition, *NEAT1* was also reported to sponge various miRNAs, including miR-34a, which can inhibit RPE cell proliferation.[49,50] Hence, it is possible that *NEAT1* also regulates RPE cell proliferation through other pathways besides *MITF* splicing.

Long non-coding RNAs have a number of significant physiological functions. *NEAT1* has been reported to regulate tumor proliferation, neurodegeneration, viral infection, and immune response.[40] However, to our knowledge, the

functional roles of *NEAT1* in RPE cells are largely unknown. Our work demonstrates that *NEAT1* inhibits RPE cell proliferation, which suggests that *NEAT1* might be involved in the regulation of other physiological and pathological processes in RPE cells. Consistent with this hypothesis, *NEAT1* was reported to regulate the EMT of ARPE-19 cells when our work was under the revision.[51] *NEAT1* lncRNA is required for the formation of nuclear body paraspeckles, which contain multiple proteins, including splicing factors SFPQ and non-POU domain-containing octamer binding protein (NONO).[33] Paraspeckles are nuclear condensates that increase with changes in the state of cells, including responses to stress.[52] Mature RPE cells are believed to remain in a non-proliferative state throughout life, but in specific disease conditions, such as retinal detachment or PVR, the RPE cells undergo EMT and start to proliferate. It is still unclear whether paraspeckles regulate the cell-cycle state change in RPE cells, but our findings suggest that paraspeckle-related proteins or RNAs might be potential mechanisms through which RPE cell proliferation might be regulated.

SFPQ is a multifunctional protein that can interact with both nucleic acid and proteins to regulate gene transcription, alternative splicing, DNA damage repair, and genome stability. SFPQ has been implicated in neuronal development and various neurodegenerative diseases, including Alzheimer's disease.[37] To our knowledge, the functional role of SFPQ in RPE cells has not been investigated previously, although SFPQ was reported to inhibit TGF-1–induced VEGF upregulation in a mouse model of oxygen-induced retinopathy.[53] The NONO/SFPQ heterodimer is required for glucocorticoid induction of occludin and claudin-5 and is believed to be important for induction of the blood–retinal barrier.[54] Knockdown of SFPQ enhances visual recovery and regeneration-associated gene expression optic nerve regeneration in zebrafish.[55] RPE cell dysfunction can contribute to various retinopathies, such as age-related macular degeneration and PVR. These results suggest that SFPQ might also act as an RPE regulator, and SFPQ dysfunction could potentially contribute to retinopathies and other pathological conditions, thus offering a possible area for future studies. In addition, the questions of whether *NEAT1* recruits SFPQ to regulate *MITF* splicing in vivo and what its physiological significance is require further investigation in the future.

In the current paper we have shown that knockdown of *NEAT1* in RPE cells only partially affected the splicing of *MITF*. In addition, fivefold overexpression of SFPQ in ARPE-19 cells only increased the splicing efficiency to produce (+)*MITF* by about 20%. These results suggest that *NEAT1* and SFPQ are not the only regulators of *MITF* splicing. It is possible that multiple additional factors and/or signaling pathways might also participate in regulating *MITF* splicing. Consistent with this hypothesis, extracellular signal-regulated kinase (ERK) signaling was reported to regulate *MITF* splicing in melanoma,[25] although our data showed that knockdown of *NEAT1* did not affect ERK signaling in ARPE-19 cells. In addition, we also noticed that knockdown of *NEAT1* in ARPE-19 + SFPQ cells only partially decreased the binding affinity of SFPQ to *MITF* mRNA, suggesting that SFPQ might also bind with *MITF* mRNA in a *NEAT1*-independent manner. Hence, the *NEAT1*–SFPQ–*MITF* splicing axis that we have established is likely only one among many pathways involved in the regulation of RPE cell proliferation, and the precise mechanisms of

MITF splicing regulation still must be investigated in the future.

In summary, our work provides evidence that the lncRNA *NEAT1* plays a critical role in regulating RPE cell proliferation, acting through facilitation of the interaction between RNA splicing factor SFPQ and *MITF* mRNA to regulate its alternative splicing. The results provide molecular insights into the regulation of RPE cell proliferation, laying the groundwork for future investigations to explore and possibly modulate the underlying mechanisms of proliferative RPE pathologies and the lack of proliferative regeneration in a number of eye diseases.

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