

Exploring the lncRNA localization landscape within the retinal pigment epithelium under normal and stress conditions

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Research Article

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Abstract

Background:

Long noncoding RNAs (lncRNAs) are emerging as a class of genes whose importance has yet to be fully realized. It is becoming clear that the primary function of lncRNAs is to regulate gene expression, and they do so through a variety of mechanisms that are critically tied to their subcellular localization. Although most lncRNAs are poorly understood, mapping lncRNA subcellular localization can provide a foundation for understanding these mechanisms.

Results:

Here, we present an initial step toward uncovering the localization landscape of lncRNAs in the human retinal pigment epithelium (RPE) using high throughput RNA-Sequencing (RNA-Seq). To do this, we differentiated human induced pluripotent stem cells (iPSCs) into RPE, isolated RNA from nuclear and cytoplasmic fractions, and performed RNA-Seq on both. Furthermore, we investigated lncRNA localization changes that occur in response to oxidative stress. We discovered that, under normal conditions, most lncRNAs are seen in both the nucleus and the cytoplasm to a similar degree, but of the transcripts that are highly enriched in one compartment, far more are nuclear than cytoplasmic. Interestingly, under oxidative stress conditions, we observed an increase in lncRNA localization in both nuclear and cytoplasmic fractions. In addition, we found that nuclear localization was partially attributable to the presence of previously described nuclear retention motifs, while adenosine to inosine (A-to-I) RNA editing appeared to play a very minimal role.

Conclusions:

Our findings map lncRNA localization in the RPE and provide two avenues for future research: 1) how lncRNAs function in the RPE, and 2) how one environmental factor, in isolation, may alter lncRNA localization and play a potential role in retinal disease pathogenesis.

Background

lncRNAs are a poorly understood class of molecules that have garnered increased attention in recent years. Like mRNAs, lncRNAs are capped, spliced, and usually poly-adenylated, however, they have limited-to-no coding capacity, and are usually expressed at lower levels, with more tissue specificity [1–4]. Rather than code for a protein, lncRNAs serve to modulate gene expression through transcriptional or translational control, and to do this, they must localize to either the nucleus or the cytoplasm, depending on the mechanism by which they function [5, 6].

Particularly evident in lncRNAs, RNA localization – the distribution of RNA transcripts at the subcellular level – is inextricably tied to RNA function. While our understanding of this subject is far from complete, recent publications have begun to uncover the mechanisms underlying the various aspects of RNA

localization. Nuclear retention is one such aspect, wherein an RNA species is temporarily or permanently sequestered in the nucleus. Currently, nuclear retention is thought to occur via anchoring transcripts to structural entities, and/or preventing transcripts from recruiting nuclear export factors, with motifs within the primary sequence of the RNAs facilitating such processes [7]. An interrogation of the sequence of the nuclear localized *BMP2-OP1 responsive gene (Borg)* transcript identified a sequence, the BORG motif, whose presence was sufficient to impart nuclear localization, although the mechanism by which this occurred was not determined [8]. Another sequence, the 5' splice site (5'SS) motif, identified from the backbone of an expression plasmid, promoted nuclear retention of RNAs carrying the motif through sequestration in nuclear speckles [9]. More recently, it was found that RNAs possessing a particular sequence, the SINE-derived nuclear RNA localization (SIRLOIN) motif, were bound by HNRNPK, leading to their accumulation in the nucleus [10]. Despite these discoveries, the nuclear retention of a transcript is appreciably more complex than the presence or absence of retention motifs, as demonstrated by the variable localization of such RNAs across cell types [11]. Additionally, a linear regression model based on retention motifs and other genomic and splicing features was only able to predict 15–30% of the variability in localization among lncRNAs, alluding to unknown factors contributing to RNA localization [11].

There is also a growing interest in how the phenomenon of RNA nuclear retention is used by the cell as a means of regulatory control. Indeed, recent studies have identified subsets of mRNAs which are retained in the nucleus under normal conditions and released into the cytoplasm in response to various stimuli, including: cell stress signaling [12–14], neuronal activity [15], and developmental cues [16]. The retention of these transcripts is thought to allow the cell to quickly begin synthesizing protein in response to the relevant stimulus [7]. Furthermore, research has demonstrated that nuclear retention can buffer cytoplasmic transcript levels from the noise created by bursts of transcriptional activity, thus shielding the cell from wild fluctuations in protein levels [17]. Though it is unclear whether lncRNA nuclear retention is similarly used by the cell to enact regulatory control, it seems well within the realm of possibility, given the molecular similarities between mRNAs and lncRNAs and their localization-dependent activities.

Because of its implication in regulatory processes, RNA localization is beginning to be recognized for its role in the development and progression of human disease [7]. Mutations affecting the structure and function of RNA export factors have been linked to the pathogenesis of neurodevelopmental disorders [18, 19], motoneuron diseases [20], and neurodegenerative disease [21]. Alterations in RNA primary sequence can also contribute to disease by affecting transcript localization. Repeat expansions in *HTT* and *C9ORF72* are thought to contribute to Huntington's disease and amyotrophic lateral sclerosis, respectively, by causing the transcripts to become sequestered in the nucleus [22, 23]. Additionally, the *ApoE* transcript, a major susceptibility gene for Alzheimer's disease, has been shown to be released into the cytoplasm in response to neuronal injury in mice, implicating the dysregulation of RNA localization as a possible contributor to disease progression [24].

Age-related macular degeneration (AMD) is the third most common cause of moderate-to-severe visual impairment worldwide, currently believed to be affecting 196 million people, but the role of RNA

localization in its pathogenesis is, as yet, unknown [25]. The disease results in lesions in the macula region of the eye corresponding to the death of the retinal pigment epithelium (RPE) and the overlying photoreceptor cells [26]. Development of AMD is largely tied to environmental risk factors, with epidemiological studies finding a genetic component to account for 37–71% of AMD pathogenesis [27, 28]. Oxidative stress is a strong environmental risk factor for the progression of AMD [29]. This is attributable to the constant exposure of the retina to light, high oxygen tension, high metabolic rate, exposure to fatty acids capable of autoxidation, as well as controllable factors such as diet and smoking, which collectively contribute to the generation of reactive oxygen species in the retina and RPE [29–34]. Due to the important connection between oxidative stress in the RPE and AMD pathogenesis, several transcriptome profiling studies have examined cultured RPE exposed to oxidative stressors and native RPE from human donors with AMD [35–38]. Yet these studies have not separately examined cytoplasmic and nuclear transcriptomes, leaving unknown the subcellular distribution of RNA transcripts within the RPE cells. Thus, it is also unknown whether, how, and to what functional effect oxidative stress alters lncRNA localization in the RPE.

In this study, we examined the poly-adenylated noncoding transcriptomes of both nuclear and cytoplasmic fractions of normal human induced pluripotent stem cell derived retinal pigmented epithelium (iPSC-RPE) cells, as well as iPSC-RPE cells treated with hydrogen peroxide (H_2O_2) – exposure to which is commonly used to model oxidative stress in the field of AMD research [33, 34, 39, 40]. We found that, under normal conditions, the majority of lncRNAs were evenly distributed between the nucleus and cytoplasm, while the lncRNAs that localized to one fraction were overwhelmingly nuclear. However, H_2O_2 exposure leads more lncRNAs to commit to a subcellular compartment, with a larger proportion localizing to either the nucleus or cytoplasm. Further analysis of the data suggested that previously described nuclear retention motifs, and to a much lesser extent A-to-I RNA editing, contribute to the localization patterns under normal conditions. Yet these factors cannot explain the largescale shift in localization in response to oxidative stress, and more studies will be needed in order to understand this phenomenon.

Results

lncRNA Transcript Localization Shifts in Response to Oxidative Stress

We generated iPSC-RPE cells using the BXS0114 iPSC line. One group of iPSC-RPE samples (hereafter referred to as BXS) was left untreated to act as controls, while another group of samples (hereafter referred to as BXS- H_2O_2) was treated with 500 μM H_2O_2 for 3 hours in order to induce oxidative stress. Nuclear and cytoplasmic RNA was isolated from five technical replicates of each condition, and RNA-Seq libraries were prepared. Each sample was sequenced on an Illumina NextSeq 500, and generated a minimum of 13 million reads, with at least 85% of reads uniquely aligning to the hg38 human genome

build, and, on average, 92% of these reads counted. We have previously shown these data to be of high quality and the iPSC-RPE to be sufficiently RPE-like [41, 42].

We first examined overall transcript expression to assess sample quality and differences after exposure to H₂O₂. We considered a transcript to be expressed if it had an average reads per kilobase per million (RPKM) > 0.5 across all samples in a condition. Of the 68,792 lncRNA transcripts, we found similar numbers expressed in both conditions, with 7,264 total in BXS and 7,133 BXS-H₂O₂. We also looked at each fraction individually to determine nuclear and cytoplasmic expression. We again see similar numbers, with more transcripts expressed in the nucleus in both conditions. Specifically, in BXS, we found 5,993 transcripts expressed in the nuclear fraction and 5,542 in the cytoplasmic fraction, while in BXS-H₂O₂, we found 6,294 transcripts expressed in the nucleus and 5,294 in the cytoplasm.

To examine lncRNA localization, we compared expression in the nuclear and cytoplasmic fractions. We considered a transcript to be localized if it showed at least a two-fold greater expression in one fraction, with an adjusted p-value < 0.01. A transcript with a fold-change < 1.9 between fractions, regardless of p-value, was considered to be of mixed localization - meaning that it was similarly expressed in the cytoplasmic and nuclear fractions. We found that a large portion of transcripts did not display strongly asymmetric localization, as 3,156 and 3,553 lncRNAs were of mixed localization in the control and H₂O₂-treated iPSC-RPE, respectively (Fig. 1C, Fig. 1D). In contrast, we see far fewer transcripts localized to one fraction, with only 997 transcripts localized under control conditions. Of these, 775 were localized to the nucleus, while only 222 were cytoplasmic. Interestingly, exposure to H₂O₂ increases localization, as we find 1,773 transcripts localized in BXS-H₂O₂. We found H₂O₂ exposure increased localization in both fractions, with 1,253 nuclear and 520 cytoplasmic localized transcripts in the BXS-H₂O₂ iPSC-RPE (Fig. 1C, Fig. 1D).

In order to validate our RNA-Seq analysis, we performed RNA fluorescent in situ hybridization (RNA-FISH) using probes targeting a set of lncRNA transcripts that our analysis identified as having nuclear (NEAT1, cyto:nuc expression ratio = (< 0.001)), cytoplasmic (SNHG16, cyto:nuc expression ratio = 4.9), or mixed localization (MTND1P23, cyto:nuc expression ratio = .96). We were able to confirm localization in each case (Fig. 1E, Fig S1).

Nuclear Retention Signals Contribute to lncRNA Localization

Studies by Zhang et al. [8], Lee et al. [9], and Lubelsky and Ulitsky [10] have identified several nuclear retention motifs, however, the effects of these motifs have only been examined in small subsets of transcripts, and as such, it is unknown the extent to which such elements contribute to the overall lncRNA localization landscape. With this in mind, we set out to perform a large-scale analysis of the 5'SS, SIRLOIN, and BORG motifs to determine whether and how they influence lncRNA localization within the iPSC-RPE. Because the full SIRLOIN element is quite rare, we used the 7 nucleotide pyrimidine-rich SIRLOIN sub-element described by Lubelsky and Ulitsky [10] in our study.

Analysis of the presence of one or more nuclear retention motifs in expressed lncRNA transcripts revealed a wide range of distributions for the different motifs. Whereas lncRNAs with the 5'SS motif were relatively rare (approximately 500 expressed lncRNAs), the SIRLOIN and BORG motifs were more abundant (approximately 2000 and 1700 expressed lncRNAs, respectively). In addition, we found that similar numbers of lncRNAs containing each motif were expressed in the treated and untreated samples.

We next examined the role these motifs may play in lncRNA localization. In the control samples, lncRNAs with the 5'SS, BORG, or SIRLOIN motifs displayed similar localization patterns, with approximately 19–24% being nuclear, and only 2–4% being cytoplasmic (Fig. 2). On the other hand, our analysis of lncRNAs without these motifs revealed that approximately 15–16% were localized to the nucleus, while 6–8% localized to the cytoplasm (Fig. 2).

In the treated samples, however, we see a shift in the localization patterns of lncRNAs. Under oxidative stress conditions, lncRNAs possessing 5'SS motifs were more highly enriched in the nuclear fraction, as we found 35% nuclear localized, while only 4% localized to the cytoplasm (Fig. 2A). Those lncRNAs with the BORG or SIRLOIN elements also showed enriched nuclear localization, where approximately 25–27% were nuclear localized, and only 7% localized to the cytoplasm (Fig. 2B, Fig. 2C). Overall, in the treated samples, we see a 1.66–1.87 fold increase in lncRNAs with motifs localizing to the nucleus, and a 2.26–2.79 fold increase in those localizing to the cytoplasm, as compared to control. We also examined lncRNAs without these motifs, and found 21–22% of these to localize to the nucleus, while 11–12% localized to the cytoplasm (Fig. 2). This also denotes an increase in localization of lncRNAs without motifs, as compared to control, where we see a 2.18–2.31 fold increase in cytoplasmic localization, and a 1.78–1.88 fold increase in nuclear localization. Hence, it appears the effect of oxidative stress on localization seems to apply to all transcripts, regardless of the presence or absence of these motifs.

These data indicate that transcripts with 5'SS, BORG, or SIRLOIN motifs are more likely to localize to the nucleus than transcripts without such elements, but we wanted to ensure that these localization patterns were the result of an actual phenomenon, rather than a sampling artefact from our analysis. In an attempt to decipher this, we analyzed the localization of lncRNAs possessing random sequences of the same length and structure as the 5'SS, BORG, and SIRLOIN motifs. In both the treated and untreated data sets, transcripts with random sequence variants of the SIRLOIN motifs displayed localization patterns nearly identical to transcripts with the actual SIRLOIN element (Fig. 2C). On the other hand, lncRNAs with random sequence variants of the 5'SS or BORG motifs were less nuclear localized and more likely to be localized to the cytoplasm than those with the 5'SS or BORG motifs (Fig. 2A, Fig. 2B). H₂O₂ exposure did not appear to affect the localization of lncRNAs with random sequence variants noticeably differently from the transcripts with the 5'SS, BORG, or SIRLOIN motifs (Fig. 2).

To further our analysis, we compared transcript localization to the number of nuclear retention motifs present in each transcript. In both cell lines, and for each motif examined, we found a positive, albeit weak, correlation between the number of motifs per transcript and nuclear localization (Fig. 3). In contrast, when we performed this same analysis using random motifs of 7 and 8 nucleotides, we found a

mix of weak positive, negative, and neutral correlations between motif number and nuclear localization (Figure S2).

A-to-I RNA Editing Plays a Minimal Role in lncRNA Localization

A-to-I RNA editing has also been implicated in the nuclear retention of transcripts [14, 43, 44]. In order to better understand whether A-to-I RNA editing might be contributing to the observed localization patterns in our iPSC-RPE samples, we analyzed our RNA-Seq data for the presence of editing using SPRINT [45]. Since unequivocal identification of editing sites within the transcriptome requires whole genome sequencing, and the genome of the BXS0114 cell line has not yet been sequenced, our analysis was limited to the identification of potential editing sites in our samples. To probe the veracity of the analysis, ten regions containing putative editing sites identified by SPRINT were examined through Sanger sequencing – interrogating the genomic as well as transcriptomic sequences in order to discriminate edited sites from single-nucleotide polymorphisms (SNPs). Of the ten 200 bp regions examined, nine regions were found to have been edited at one or more sites (Fig. 4A). The remaining region possessed a SNP at a potential editing site identified by SPRINT, but otherwise showed no evidence of editing. Of note, only 11 of the 71 editing sites detected through Sanger sequencing were also identified by SPRINT. These findings suggest that the analysis yields a low false-positive rate, yet a relatively high false-negative rate, offering a conservative estimate on the full extent of A-to-I editing.

Our initial analysis examined the total number of edited lncRNA transcripts within each fraction, regardless of their localization. In the untreated samples, we found 115 A-to-I edited lncRNA transcripts in the nuclear fraction and 74 edited transcripts in the cytoplasmic fraction (Fig. 4B). Interestingly, in the H₂O₂-treated samples, we observed 74 edited lncRNA transcripts in the nuclear fraction and 102 edited transcripts in the cytoplasmic fraction – a noticeable shift in distribution compared to control (Fig. 4B).

We next sought to examine the extent to which A-to-I RNA editing might contribute to nuclear retention of lncRNAs. To this end, we identified the transcripts that were both localized to a given fraction and edited in that fraction, with the hypothesis that if A-to-I editing were driving nuclear retention, nuclear localized transcripts would be more likely to be edited than cytoplasmic transcripts. Indeed, in both the treated and untreated samples, we found a greater number of nuclear localized lncRNAs also showing A-to-I editing in the nuclear fractions (BXS: 16, BXS-H₂O₂: 17) as compared to their cytoplasmic counterparts (lncRNAs localized and edited in the cytoplasm (BXS: 0, BXS-H₂O₂: 4)) (Fig. 4C).

To help contextualize the differences in editing between the H₂O₂-treated and untreated samples, we sought to uncover whether the expression or localization of a key A-to-I RNA editing enzyme, ADAR1-p110, changes in response to oxidative stress. A-to-I RNA editing is catalyzed by a family of enzymes called Adenosine Deaminases Acting on RNA (ADARs), and ADAR1-p110, an isoform of the ubiquitously expressed ADAR1, has been shown to translocate from the nucleus to the cytoplasm in response to UV irradiation and heat shock stresses [46, 47]. Western blotting, using an antibody that targets both the

p110 and p150 isoforms of ADAR1, revealed that expression levels were not affected by H₂O₂ treatment (Figure S3A). Furthermore, immunofluorescent (IF) staining of ADAR1, using the same antibody, revealed no noticeable difference in ADAR1 localization between the control samples and those that were treated with peroxide (Figure S3B). To determine whether changes in ADAR1-p110 localization might be masked by co-detection with the p150 isoform, we co-transfected ARPE-19 cells with ZsGreen (a transfection marker) and a flag-tagged variant of ADAR1-p110. No noticeable translocation of flag-tagged ADAR1-p110 was observed via IF staining in response to H₂O₂ treatment (Figure S3C).

Discussion

In this study, we have utilized a high-throughput approach to interrogate the localization patterns of the poly-adenylated lncRNAs within the context of iPSC-RPE. We have shown that, within untreated iPSC-RPE cells, the vast majority of lncRNA transcripts displayed mixed localization between the nucleus and cytoplasm, but of the localized lncRNAs, more than 75% were nuclear (Fig. 1C). These observations are largely in agreement with previously described localization patterns of lncRNAs from other human cell lines [3, 11]. It should be noted, however, that despite the tendency of lncRNAs to localize to the nucleus, the localization spectrum is wide and can vary by cell type [3]. With this in mind, and because lncRNA function is inextricably tied to subcellular localization, it is critical to understand where a transcript is localized within the relevant cell types when exploring questions of lncRNA functionality. Thus, these data will prove to be a valuable resource in understanding the functional roles played by lncRNAs in the context of the RPE.

In addition, our data corroborate the localization patterns of transcripts possessing nuclear retention motifs. Similar to the observations of Lee et al. [9], Lubelsky and Ulitsky [10], and Zhang et al. [8], we found that transcripts with such motifs were depleted from the cytoplasmic fraction of iPSC-RPE cells (Fig. 2). Furthermore, our data support the finding of Zhang et al. [8] that the extent of nuclear fraction enrichment is positively correlated with the number of motifs, although we observed a much weaker correlation (Fig. 2). This discrepancy likely stems from methodological differences (RT-qPCR versus RNA-Seq), and demonstrates the added nuance found using RNA-Seq analysis of subcellular fractions as a means of probing RNA localization.

Since the previously described nuclear retention motifs were present in only a subset of the nuclear localized transcripts in the iPSC-RPE cells, additional motifs or mechanisms likely exist to direct RNAs to be retained in the nucleus. Even for transcripts with known nuclear retention motifs, the molecular processes controlling the nuclear localization of RNA remain largely unclear. Though the SIRLOIN motif is thought to become bound by HNRNPK, and the 5'SS motif is believed to target RNAs to nuclear speckles, the mechanisms surrounding these processes are not yet known [9, 10]. The splicing state of a transcript is another point of consideration, as incomplete splicing may target an RNA for sequestration in the nucleus [48, 49]. Further complexity is added by the fact that nuclear retention signals may exist in RNA secondary structures such as double-stranded RNA (dsRNA) regions. Indeed, RNA is known to be involved

in nucleocytoplasmic transport via interactions with proteins that specifically recognize dsRNA regions [50].

Double-stranded RNA may also be targeted for adenosine-to-inosine (A-to-I) RNA editing, which is a process that has been implicated in the nuclear retention of a number of transcripts via paraspeckle anchoring [14, 43, 44]. Despite the potentially high false negative rate in the number of editing events called by SPRINT, our data support the notion that such RNA editing could contribute to nuclear retention (Fig. 4C). However, the relatively few A-to-I editing events revealed by our analysis make it unlikely that such a mechanism could play any more than a minor role in the distribution patterns of lncRNAs we observed in the iPSC-RPE (Fig. 4B, Fig. 4C).

We also found that oxidative stress, in the form of H₂O₂ exposure, produced a dramatic shift in the lncRNA localization landscape of the iPSC-RPE cells. H₂O₂ treatment resulted in a 1.6 fold increase in the number of nuclear localized transcripts and a 2.3 fold increase in the number of cytoplasmic localized transcripts (Fig. 1). In comparing the treated and untreated samples, we did not find that the increase in localized lncRNAs in the BXS-H₂O₂ samples could be attributed to a corresponding decrease in the number of lncRNAs with mixed localization. Nor did we find appreciable numbers of lncRNAs shifting from cytoplasmic to nuclear or vice versa. Indeed, we found the increased nuclear and cytoplasmic localization observed in the BXS-H₂O₂ samples to derive primarily from transcripts in the BXS samples that had fallen outside our cutoff thresholds and been left unassigned in terms of localization. Thus, the changes we observed in localization following H₂O₂ treatment likely reflect a small shift in lncRNA expression and/or an increased coherence between samples.

While our data indicate that oxidative insults can cause a substantial alteration in lncRNA localization within the human RPE, it is not yet clear what factors are responsible for this localization shift. Upon H₂O₂ treatment, lncRNAs with previously identified RNA nuclear retention motifs displayed similar shifts in localization as lncRNA transcripts that did not have those motifs (Fig. 2). Additionally, while we did observe a shift in A-to-I RNA editing in lncRNAs after H₂O₂ exposure, very few transcripts were thus affected (Fig. 4). Curiously, we saw an increase in the number of cytoplasmic A-to-I edited lncRNAs and a decrease in the number of edited nuclear lncRNAs, a phenomenon which did not appear to be caused by a translocation or change in expression of the ADAR1-p110 isoform (Fig. 4B, Fig S2). Rather than being linked to RNA localization, this increased editing may reflect a greater need for the cell to prevent the activation of the dsRNA apoptosis pathway, which is one of the known functions of ADAR1 activity [51]. Taken together, these data suggest that while the 5'SS, BORG, and SIRLOIN motifs and A-to-I RNA editing may affect RNA localization, these factors are not responsible for the major localization shift seen in the lncRNAs of the iPSC-RPE after oxidative stress.

Altered subcellular localization patterns have previously been reported in mouse cells in response to various external stimuli [12–16], but to our knowledge, this is the first examination of such changes in human iPSC-RPE cells in response to an oxidative insult, and the first such study conducted on a transcriptome-wide scale. Considering that oxidative stress is a strong environmental risk factor for AMD

progression [29] and that there is mounting evidence for the involvement of the dysregulation of RNA localization in the development of a number of neurodegenerative diseases [21–24], these findings suggest that changes in RNA localization could play a role in the pathogenesis of AMD. Indeed, numerous lncRNAs have already been implicated in the pathology of AMD [52–55].

Much remains unknown regarding the lncRNA localization landscape within the RPE, how that localization is achieved, how it is altered by external stimuli, and how it might relate to disease pathology. Here, by mapping the localization of lncRNAs, we have set a foundation for future studies investigating the function of lncRNAs in the RPE. Further, the data presented here builds upon other studies to solidify the notion that transcript localization (and thus function) can vary based on cell type and environmental stressors, which highlights the need to study lncRNAs in the proper context in order to understand their roles in visual dystrophies and other diseases. Future studies will enable a more thorough investigation into these remaining questions, and will not only offer insight into the roles lncRNAs may play in disease pathogenesis, but also, potentially, how they might be used for the treatment of disease.

Conclusions

In this study, we have presented the first full scale analysis of lncRNA localization within human RPE cells. We have shown that, within the RPE, lncRNAs are mostly mixed between the cytoplasm and the nucleus, but of the asymmetrically localized transcripts, the majority are nuclear. Interestingly, oxidative stress led to an increase in asymmetrically localized transcripts, both cytoplasmic and nuclear. Our data are consistent with nuclear retention motifs and A-to-I RNA editing contributing to lncRNA localization in the RPE, yet these factors do not appear to undergird the shift in localization caused by oxidative stress. Our analysis provides a foundation from which further research can explore the functions of lncRNAs within the RPE, and determine whether lncRNA localization dysregulation plays a role in the pathogenesis of AMD or other ocular diseases.

Methods

Culturing of cell lines and differentiation of iPSCs:

All reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. ARPE-19 cells were purchased (line APRE-19, ATCC, CRL-2302), cultured in 49% Advanced DMEM (Fisher Scientific, Cat #: 12-491-015), 49% F-12 (Fisher Scientific, Cat #: MT10080CV), and 2% FBS (ATCC, Cat#: 30-2020), and used for RNA-FISH experimentation 24-48hrs after reaching confluence. Human iPSC cells were purchased (line ATCC-BXS0114, ATCC, ACS-1028) and seeded at 500,000 cells in a 10-cm dish coated with Matrigel (Fisher Scientific, Cat #: 08-774-552). iPSCs were maintained in TeSR-E8 media (Stem Cell Technologies, Cat #: 05990) with Rock Inhibitor (Y-27632 dihydrochloride, Santa Cruz Biotechnology, Cat #: sc-281642A) at a final concentration of 1 μ M/mL. Media without Rock Inhibitor was changed daily. The procedure for differentiating human iPSCs toward RPE was performed using the BXS0114 iPSCs as previously described [41, 56] with minor adjustments. Briefly, the iPSCs were maintained until reaching

60–70% confluency, then individual colonies were lifted using Accutase (Stem Cell Technologies, Cat #: 07920). Colonies were allowed to settle in a 15 mL conical tube, old media was carefully aspirated, and fresh TeSR-E8 media was added. The colonies were then transferred to a T25 flask to initiate differentiation (day 0). Over the course of the following 4 days, the colonies were gradually transitioned to neural induction medium (NIM) (consisting of DMEM/F12, 1% N-2 supplement (Fisher Scientific, Cat #: 17-502-048), MEM non-essential amino acids (Fisher Scientific, Cat #: 11-140-050), and 2 µg/mL heparin (Sigma Aldrich, Cat #: H3149-100KU)) from TeSR-E8 media in steps of 3:1, 1:1, 1:3 and 0:1 TeSR-E8:NIM. Upon reaching day 6, the colonies were transferred to a 10-cm dish coated with laminin (Fisher Scientific, Cat #: 23-017-015) in NIM, where the media was changed every 2 days. At day 16, rosettes were removed from the culture via vigorous pipetting, and the remaining cells were switched to retinal differentiation medium (RDM) (consisting of DMEM/F12 (3:1), 2% B-27 supplement without retinoic acid (Fisher Scientific, Cat #: 12-587-010), and 1% antibiotic/antimycotic (Fisher Scientific, Cat #: 15-240-062)). This culture was maintained in RDM until day 80, when RPE was dissected and passaged as described. This process was performed 5 times to generate the 5 technical replicates used for this study.

Cell fractionation:

Subcellular fractionation was carried out as described by Rio et al. [57] with minor adjustments. Briefly, iPSC-RPE cells were incubated either in RDM media (untreated samples) or in RDM media with 500 µM hydrogen peroxide (treated samples) for 3 hours immediately prior to sample collection. The treatment conditions (i.e. H₂O₂ concentration and treatment duration) were chosen based on previous evaluations of cell death and oxidative damage analyses in ARPE-19 and human primary RPE cells in order to minimize cell death before sample collection while still sufficiently invoking oxidative stress [58–63]. Our previous analyses, indicating that iPSC-RPE and native RPE are similar from a transcriptional standpoint, support the notion that treatment outcomes would be similar in our iPSC-RPE cells [41]. The cells were then washed three times with phosphate buffered saline (PBS). Tryple Express dissociation reagent (Fisher Scientific, Cat#: 12-605-010) was applied to the cells, which were then incubated at 37°C for 5 minutes and collected via scraping. The cells were pelleted via centrifugation and resuspended in ice-cold cell disruption buffer (10 mM KCl, 1.5 mM MgCl₂, 20 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol [DTT, added just before use]). To facilitate swelling, cells were incubated on ice for 20 minutes. The cells were then transferred to an RNase-free dounce homogenizer. Homogenization was achieved using 15–20 strokes of the pestle, and the homogenate was visualized under a microscope to ensure that greater than 90% of the cell membranes were sheared while the nuclei remained intact. The homogenate was then transferred to a new 1.5 mL microcentrifuge tube. In order to strip residual cytoplasmic material from the nuclei, Triton X-100 was added to a final concentration of 0.1%, and the tubes were mixed gently by inversion. The nuclei were pelleted via centrifugation, and the supernatant (containing the cytoplasmic fraction) was transferred to a new 1.5 mL microcentrifuge tube. To wash the nuclear pellet, 1 mL of ice-cold cell disruption buffer was added, and both the nuclear and cytoplasmic fractions were centrifuged. The cytoplasmic supernatant was transferred to a new tube and the wash was removed and discarded from the nuclear pellet.

RNA isolation:

RNA was isolated from the nuclear and cytoplasmic fractions using Tri-Reagent (Molecular Research Center Inc., Cat#: TR 118) following the manufacturer's protocol with some modifications. Briefly, after addition of the Tri-Reagent, the nuclear and cytoplasmic samples were mixed well by inversion, transferred to phase-lock heavy tubes, and incubated at room temperature for 5 minutes. 200 μ L chloroform was added to each sample, which were then mixed vigorously for 15 seconds and incubated at room temperature for 15 minutes. The samples were centrifuged, and the aqueous (top) phase was transferred to a new 1.5 mL microcentrifuge tube. To remove any contaminating phenol, 400 μ L chloroform was added to each sample, which was then vigorously mixed, incubated at room temperature for 2 minutes, and centrifuged. The aqueous phase, containing the RNA, was then transferred to a new 1.5 mL microcentrifuge tube. Each volume of RNA solution was then thoroughly mixed with 1/10th volume of 3M sodium acetate, 1 volume isopropanol, and 2.5 μ L RNA-grade glycogen. Precipitation of RNA was accomplished through incubation of the samples at -80°C for 1 hour. The samples were then centrifuged to pellet the RNA, and the supernatant discarded. To wash the RNA pellets, 75% ethanol was added to each tube, which were then briefly vortexed and centrifuged. After removal of the ethanol from the pellets, this wash step was repeated. Following the second wash, the ethanol was removed and the pellets were allowed to air dry for 5–10 minutes. The RNA was then resuspended in 22 μ L DNase-free, RNase-free water and quantified via nanodrop spectrophotometer. RNA integrity and quality were assessed on a Qubit 4 Fluorometer using a Qubit RNA IQ Assay Kit. The RNA IQ values all ranged from 7.5 to 8.2, indicating high quality RNA samples.

RNA library preparation and sequencing:

RNA-sequencing libraries were prepared using the SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing kit (Agilent, Cat#: G9691A) according to the manufacturer's protocol. Libraries were prepared using 100 ng total RNA, and each sample was indexed for multiplexing. Prior to sequencing, library quality and quantity were determined using High Sensitivity Screen Tape on a TapeStation 4150 (Agilent). Sequencing was performed using a NextSeq 500 (Illumina) generating 2 x 150 bp reads.

Sequencing analysis:

Reads were aligned to the human genome (build hg38) with STAR v2.5.2b, and counts generated for all transcripts in gencode v29 using Rsubread v1.32.4 [64–66]. Counts were normalized using reads per kilobase per million (RPKM) to examine overall expression. Differential expression analysis using DESeq was performed to determine localization and effect of treatment [67]. Motif analyses were performed with gimsan. RNA editing analyses were performed using SPRINT [45].

RNA-fluorescent in situ hybridization:

iPSC-RPE cells or ARPE-19 were seeded onto 8-well chamber slides and 48-well plate coverslips, respectively, and were grown to confluence. ARPE-19 cells were chosen for their lack of pigmentation, and

as a biological replicate. Cells were prepared using the ViewRNA Cell Plus Assay Kit (Fisher Scientific, Cat#: 88-19000-99) according to the manufacturer's protocol with the minor alteration of fixation and permeabilization using 3:1 methanol:glacial acetic acid at room temperature. To stain the nuclei, the coverslips were incubated in Hoechst solution. The cells were then mounted and visualized using a Leica TCS SPE confocal microscope.

Editing verification by sanger sequencing:

Ten potential editing sites were selected from among the sites identified by the SPRINT algorithm. Primers were designed to flank the selected sites in order to produce amplicons of approximately 200 bp. PCR amplification of these sites was performed using genomic DNA isolated from BXS0114 iPSC-RPE cells and using cDNA synthesized from RNA isolated from BXS0114 iPSC-RPE cells. Following PCR amplification and gel electrophoresis, the amplicons were excised from the gel and purified. The purified amplicons were cloned into the pCR-4Blunt-TOPO vector (Fisher Scientific, Cat #: 45-003-1), the plasmids were isolated, and the inserts were Sanger sequenced using M13 Forward primers. Alignment of the genomic and transcriptomic sequences parsed bona fide editing sites from SNPs.

Immunoblotting analysis:

ARPE-19 cells were incubated either in media (untreated samples) or in media with 500 μ M hydrogen peroxide (treated samples) for 3 hours immediately prior to sample collection. Cell lysates were collected using 1x RIPA buffer (Abcam, Cat #: ab156034), and protein concentrations were determined using a BCA protein assay (Fisher Scientific, Cat #: PI23227). The samples were electrophoresed on a 4–12% Bis-Tris protein gel (Fisher Scientific, Cat #: NP0335BOX), and proteins were then transferred to a nitrocellulose membrane (Fisher Scientific, Cat #: 45-004-012). The membranes were blocked with 1x Tris buffered saline with 1% casein (Bio-Rad, Cat #: 1610782) for 1 hour at room temperature, and incubated with either anti-ADAR1 (Abcam, Cat #: ab88574) or anti-tubulin (Novus Biologicals, Cat #: NB100-690) primary antibodies overnight at 4°C. Then, the membranes were washed with 1x Tris-buffered saline with 0.1% Tween 20 (TBST), incubated with an alkaline phosphatase conjugated secondary antibody (Krackeler Scientific, 45-A4312-.25mL) for 1 hour at room temperature, and developed using ECF substrate (Fisher Scientific, Cat #: 45-000-947).

ADAR1 transfection and immunofluorescence:

ZsGreen and flag-tagged ADAR1-p110 mRNA were made using the Invitrogen MEGAscript T7 Transcription Kit (Fisher Scientific, Cat #: AM1334) according to the manufacturer's protocol. ARPE-19 cells were transfected with these mRNAs using the Invitrogen Lipofectamine MessengerMAX Transfection Reagent (Fisher Scientific, Cat #: LMRNA001) according to the manufacturer's protocol. ARPE-19 cells were incubated either in media (untreated samples) or in media with 500 μ M hydrogen peroxide (treated samples) for 3 hours immediately prior to sample collection. For transfected samples, hydrogen peroxide treatment was carried out 24 hours post-transfection. Immunofluorescent staining was carried out as follows: cells were washed with 1x PBS, fixed with ice-cold methanol for 1 minute, permeabilized with 0.2% Triton X-100 (in 1x PBS) at room temperature for 10 minutes, blocked with

blocking buffer (1% bovine serum albumin, 0.03% Triton X-100, in 1x PBS) for 1 hour at room temperature, incubated in primary antibody (either anti-ADAR1 [Abcam, Cat #: ab88574] or anti-flag [Krackeler Scientific, Cat #: 45-F3165-1MG]) for 1 hour at room temperature, incubated in secondary antibody (either Alexa Fluor 488 goat anti-mouse [Fisher Scientific, Cat #: A11029] or Alexa Fluor 555 donkey anti-mouse [Fisher Scientific, Cat #: A31570]) for 1 hour at room temperature, counterstained with Hoechst (Fisher Scientific, Cat#: H3570), and then mounted on slides for visualization using a Leica TCS SPE confocal microscope.

Abbreviations

lncRNA: Long noncoding RNA

RPE: Retinal pigment epithelium

RNA-seq: High throughput RNA sequencing

iPSC: Induced pluripotent stem cell

A-to-I: Adenosine-to-inosine

BORG: BMP2-OP1 responsive gene

5'SS: 5' splice site

SIRLOIN: SINE-derived nuclear RNA localization

AMD: Age-related macular degeneration

iPSC-RPE: iPSC derived RPE

H₂O₂: Hydrogen peroxide

BXS: Control BXS0114 iPSC-RPE samples

BXS-H₂O₂: H₂O₂-treated BXS0114 iPSC-RPE samples

RPKM: Reads per kilobase per million

RNA-FISH: RNA fluorescent in situ hybridization

SNP: Single nucleotide polymorphism

ADAR: Adenosine deaminases acting on RNA

dsRNA: double stranded RNA

NIM: Neural induction medium

RDM: Retinal differentiation medium

PBS: Phosphate buffered saline

TBST: Tris-buffered saline with 0.1% Tween 20

Cyt: Cytoplasmic

Nuc: Nuclear

Declarations

Ethics approval and consent to participate:

Not applicable. No humans or animals were used. No human or animal tissues were used.

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets generated for this study can be found in the GEO REPOSITORY, GEO accession GSE158909.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

TJK and MHF designed the study. TJK and MHF performed experiments. EDA and MHF analyzed and interpreted the data. MHF, EDA, and TJK wrote and edited the manuscript.

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Figures

Figure 1

Distribution of lncRNA transcripts in the RPE. Volcano plots of lncRNAs in BXS (A) and BXS-H₂O₂ (B). Log₂ cytoplasm:nuclear fold change and corresponding log₁₀ adjusted p-value are plotted for each transcript. Transcripts with fold change >2 are colored blue, adjusted p<0.01 are green, both fold change >2 and adjusted p<0.01 are yellow. Genes confirmed via FISH are red (A). Pie graphs of the distribution of lncRNAs within BXS (C) and BXS-H₂O₂ (D). The cytoplasmic categorization is indicated by orange with horizontal stripes, nuclear by blue with vertical stripes, and mixed by purple with crosshatched stripes. The total number of transcripts in each category and the percentage of the whole are indicated. (E) RNA-FISH images of iPSC-RPE confirming localization of NEAT1, MTND1P23, and SNHG16 (red) and counterstained with Hoechst solution (blue). Arrows indicate some of the localized RNAs. Scale bar is 5 μm.

Figure 2

lncRNAs containing retention signal motifs are more nuclear localized. Pie graphs of the distribution of lncRNAs within BXS and BXS-H₂O₂. (A) distribution of transcripts with the 5'SS motif, without the 5'SS motif, and with a random version of the 5'SS motif. (B) distribution of transcripts with the BORG motif, without the BORG motif, and with a random version of the BORG motif. (C) distribution of transcripts with the SIRLOIN motif, without the SIRLOIN motif, and with a random version of the SIRLOIN motif. The cytoplasmic categorization is indicated by orange with horizontal stripes, nuclear by blue with vertical stripes, and mixed by purple with crosshatched stripes.

Figure 3

The number of retention signal motifs is positively correlated with nuclear localization. Graphs plotting the number of 5'SS, BORG, and SIRLOIN motifs per transcript versus log₂ cytoplasm:nuclear fold change from the BXS and BXS-H₂O₂ samples. Fold change corresponding to nuclear (nuc) and cytoplasmic (cyt) localization is indicated. The orange dotted lines plot the trendlines for the data.

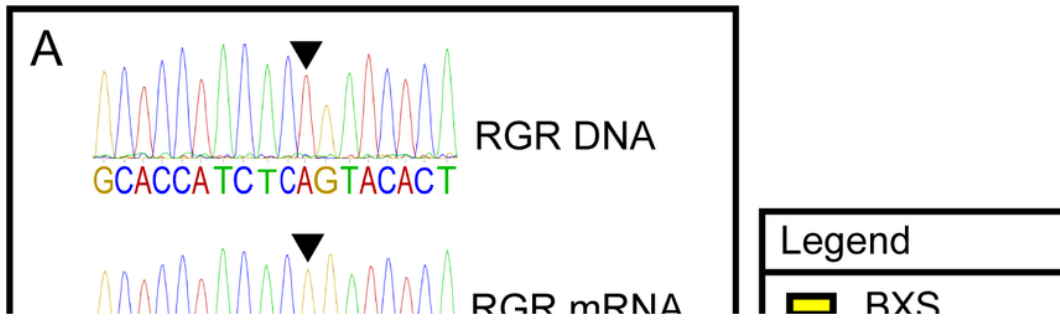


Figure 4

A-to-I RNA editing patterns change in response to oxidative stress. (A) Representative sanger sequencing trace results depicting an instance of A-to-I editing within the RGR mRNA, where an inosine is read by the sequencer as a guanine. (B) Graph depicting the number of A-to-I edited lncRNAs within the cytoplasmic (cyt) and nuclear (nuc) fractions of BXS (solid yellow) and BXS-H₂O₂ (magenta with diagonal stripes). (C) Graph depicting the number of transcripts that were both localized to a given fraction (cytoplasmic [cyt] or nuclear [nuc]) and more highly edited in that fraction for the control BXS or BXS-H₂O₂ samples.

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