microRNA-4331 Promotes Transmissible Gastroenteritis Virus (TGEV)-induced Mitochondrial Damage Via Targeting RB1, Upregulating Interleukin-1 Receptor Accessory Protein (IL1RAP), and Activating p38 MAPK Pathway In Vitro∗§

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Transmissible gastroenteritis virus (TGEV), a member of the coronaviridae family, could cause fatal diarrhea of piglets and result in numerous economic losses. Previous studies demonstrated that TGEV infection could lead to mitochondrial damage and upregulate miR-4331 level. So miR-4331 may play an important regulatory role in the control of mitochondrial function. To explore the potential role of miR-4331 in mitochondrial damage, we adopted a strategy consisting of quantitative proteomic analysis of porcine kidney (PK-15) cells in response to miR-4331 and TGEV infection. Eventually, 69 differentially expressed proteins were gained. The target of miR-4331 was identified. The effects of miR-4331 and its target RB1 on mitochondrial Ca2+ level, mitochondrial membrane potential (MMP), interleukin-1 receptor accessory protein (IL1RAP), p38 MAPK signaling pathway were investigated. The results showed that miR-4331 elevated mitochondrial Ca2+ level, mitochondrial membrane potential (MMP), interleukin-1 receptor accessory protein (IL1RAP), p38 MAPK signaling pathway during TGEV infection. RB1 was identified as the direct targets of miR-4331 and downregulated IL1RAP, suppressed the activation of p38 MAPK, and attenuated TGEV-induced mitochondrial damage. In addition, IL1RAP played a positive role in activating p38 MAPK signaling and negative role in TGEV-induced mitochondrial damage. The data indicate that miR-4331 aggravates TGEV-induced mitochondrial damage by repressing expression of RB1, promoting IL1RAP, and activating p38 MAPK pathway. Molecular & Cellular Proteomics 17: 10.1074/mcp.RA117.000432, 190–204, 2018.

Transmissible gastroenteritis virus (TGEV)† is an enveloped enteropathogenic coronavirus with positive-sense single-stranded RNA genome (1). TGEV infects pigs and especially causes piglets up to 14 days of age high mortality, which can reach to 100% (2, 3) and result in numerous economic losses. Our previous work demonstrated that TGEV infection can reduce MMP, leading to mitochondrial damage (4, 5). Mitochondrion, an organelle of eukaryocyte, not only acts as the energy metabolism factory, but also is involved in many key biological processes, such as apoptosis, pathogenic infection (6, 7). Mitochondrion is a very sensitive organelle to microenvironmental changes in cells and much easier becomes dysfunction than other organelles (8). Mitochondria, the major source of energy, in the form of ATP, are closely interconnected with cell death. Ca2+ is essential for maintain mitochondrial function. However, Ca2+ accumulation in mitochondria can impair mitochondrial function, resulting in a transient depolarization of MMP and reducing ATP production (9–11). Both Ca2+ accumulation and MMP depolarization can cause mitochondrial damage, so the alterations of Ca2+ level and MMP are used to assess mitochondrial function (12). miRNAs are small noncoding RNA species containing about 22 nucleotides and contribute to regulating many cellular processes including apoptosis, development, differentiation, and cell cycle (13). We previously reported that miR-4331 level was...
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**EXPERIMENTAL PROCEDURES**

**Antibodies and Plasmids**—Anti-RB1 was purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Pierce (US). Anti-p-p38 and anti-β-actin antibody were purchased from Cell Signaling Technology (US). Anti-IL1RAP and anti-GAPDH primary antibody was purchased from Genetex (US). The psiCHECK-2 plasmid and dual-luciferase reporter assay system were purchased from Promega (US). The primers and siRNAs were synthesized by Ribo Biotech (RiboBio, China).

**Virus and Cells**—The TGEV Shaanxi strain was isolated from TGEV-infected piglets (15). PK-15 cells were obtained from ATCC (CCL-33) and grown in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, US), 100 IU of penicillin, and 100 mg/ml streptomycin, at 37 °C in 5% CO2 atmosphere incubator.

**Experimental Design and Statistical Rationale for Proteomics**—In order to investigating the effect of mir-4331 on mitochondrial damage in response to TGEV infection, PK-15 cells were transfected with 100 nM mir-4331 mimics or negative control using Lipofectamine 3000 and infected with TGEV at an MOI of 1.0 at 24 h post-transfection (hpt). The cells were collected for quantitative proteomic analysis at 24 h post-infection (hpi). Two biological replicates preparations labeled with TMT were analyzed. To evaluate the transfection efficiency, the mir-4331 level was measured by real-time PCR. Reverse transcription reactions and real-time PCR were performed as described previously (14). The relative quantification of miRNAs was normalized to U6 using the two-ddCt method (16).

**Protein Isolation, Digestion, and Labeling with TMT**—The PK-15 cells were sonicated on ice in lysis buffer (8 M urea, 1% Triton-100, 65 mM DTT and 0.1% protease inhibitor mixture) and centrifuged at 12,000 × g for 20 min at 4 °C. The clarified supernatant was collected. Finally, the protein was precipitated with cold 15% TCA for 2 h at −20 °C and centrifuged at 4 °C for 10 min. The precipitate was redissolved in buffer (8 M urea, 100 mM TEAB, pH 8.0). One hundred micrograms of each sample was digested with trypsin. The two control samples and two treatment samples were respectively labeled with TMT (126, 129, 127, and 130).

**LC-MS/MS**—The samples were fractionated by high pH reverse-phase High Performance Liquid Chromatography (HPLC). Briefly, peptides were dried by vacuum centrifugation. Then the Peptides were dissolved in 0.1% formic acid and directly loaded onto a reversed-phase precolumn (Acclaim PepMap 100, Thermo Scientific). Peptides were separated using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific) and analyzed by Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM (Thermo Fisher Scientific) coupled online to the HPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 32. Ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 2E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap. SE4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800. Fixed first mass was set as 100 m/z.

**Analysis of LC-MS/MS Data**—MS/MS data were analyzed by Mascot (version 2.3.0) according to a previously described protocol (17). Briefly, raw MS data files were processed using the LC/MS software Proteome Discoverer (version 1.3.0.339) (Thermo scientific, US) and converted into the Mascot generic format (mgf) files. Mascot software performed peak generation, precursor mass recalibration, extraction.
of TMT 6-plex reporter ions intensity, and calculation of TMT 6plex reporter ions intensity ratios. For each MS/MS spectra, top 10 most intense peaks in every 100 Da window were extracted for database search. Then Mascot search was performed by searching tandem mass spectra against Uniprot Sus scrofa database (UniProt release 2015_04, 26054 entries) (http://www.uniprot.org) concatenated with reversed decoy database and protein sequences of common contaminants. Cleavage enzyme was specified as trypsin/P, maximum number of missing cleavages was set to 2. Mass tolerance for precursor ion was set to 10 ppm and mass tolerance for MS/MS ions was set to 0.02 Da. Carbamyiodomethylation on Cys and TMT6plex (N-term) and TMT6plex (K) were specified as fixed modification and oxidation on Met, acetylation on protein N-terminal were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 1%. Mascot software assembled the peptide/protein groups, calculated false discovery rates, filter the identifications. Protein quantification was set to use only unique peptides bearing any modification. The median ratio of TMT 6-plex reporter intensity of unique peptides was set as protein relative abundance changes. In this study, we prepared two duplicate
samples. For each protein, we set the average ratio calculated from two duplicate samples as the final quantitation of protein. Student’s test was explored to calculate differential significance degree of protein relative abundance changes. \( t \) test \( p \) value less than 0.05 was considered significantly differentially. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD008174 (http://www.ebi.ac.uk/pride/archive/) (18).

Bioinformatic Analysis of Differentially Expressed Proteins—Gene Ontology (GO) annotation of proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate proteins pathways. The Wolf Psort database (https://wolfpsort.hgc.jp/) was used to predict subcellular localization.

Analysis of Protein-Protein Interactions—The differentially expressed protein name identifiers (Uniprot accession) were searched against the STRING database (version 10.0) for protein-protein interactions (19).

Vector Construction—To overexpress RB1 and IL1RAP, the full-length sequences of RB1 and IL1RAP were obtained by PCR from PK-15 cells and cloned into plasmid pCI-neo (Promega, USA). The constructions were respectively named pCI-neo-RB1 and pCI-neo-IL1RAP. The primer sequences are shown in supplemental Information S1.

Western Blot Analysis—PK-15 cells were treated with RIPA lysis buffer containing phenylmethyl sulfonylfluoride (PMSF). The proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvi-
nylidene difluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 2 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The HRP-conjugated secondary antibodies were used to incubate the membrane for 1 h at room temperature.

Reverse Transcription PCR and Real-time PCR—Total RNA was extracted with TRIzol (Invitrogen, US). 2 μg of total RNA was treated with DNase I (Fermentas, Germany) for 30 min at 37 °C and reversely transcribed using the First-strand cDNA synthesis kit (Invitrogen). The cDNA was used as the template for real-time PCR. The real-time PCR was performed on Bio-Rad iQ5 Real-Time PCR System (Bio-Rad). The reverse transcription primers and real-time PCR primers are shown in supplemental Information S1.

Dual-luciferase Reporter Assay—3’ UTRs of candidate targets containing the miR-4331 binding sites were respectively amplified by PCR and were cloned into the vector psiCHECK-2 (Promega). The binding sites of miR-4331 in RB1-wt 3’ UTR were mutated following the mutagenesis protocol (20) to generate RB1-mut. The PCR primers are shown in supplemental Information S1. PK-15 cells were co-transfected with plasmid RB1-wt (or RB1-mut) and miR-4331 mimics (or miR-4331 inhibitors) using Lipofectamine 3000 (Invitrogen, US). The luciferase activities were detected at 48 hpt using a Dual-Glo Luciferase Assay System (Promega).

RNA Interference—The siRNAs of RB1 and IL1RAP were synthesized by GenePharma (GenePharma, China) (supplemental Information S1). PK-15 cells were transfected with 100 nM siRNA or irrelevant siRNA using Lipofectamine 3000 (Invitrogen, US).

Evaluation of MMP—MMP of PK-15 cells was detected using JC-1 as an indicator (21). PK-15 cells were transfected with miR-4331 mimics or miR-4331 inhibitors and were subsequently infected with TGEV at 1 MOI. PK-15 cells were treated with PBS containing JC-1 and incubated at 37 °C for 20 min. The absorbance was measured at 550 ex/590 em.

Measurement of Mitochondrial Ca2+ Level—The mitochondrial calcium level was detected using Rhod-2 kit (GENMED, China) following the manufacturer’s protocol. The absorbance was measured at 490 ex/590 em. The relative fluorescence unit (RFU) was calculated.

RESULTS

TGEV Infection Increases Mitochondrial Ca2+ Level and Decreases MMP—PK-15 cells were infected with TGEV at 1 MOI for 24 h. The mitochondrial Ca2+ level and MMP of PK-15 cells were evaluated. The results showed that TGEV infection

| No. | Protein       | Fold change | p value  |
|-----|---------------|-------------|----------|
| 1   | GBP1          | 1.636       | 2.86082E-08 |
| 2   | SDC4          | 1.6075      | 0.010935485 |
| 3   | Uncharacterized protein | 1.4665       | 0.003411649 |
| 4   | MRPL11        | 1.4465      | 0.023362276 |
| 5   | HK2           | 1.417       | 0.138772E-07 |
| 6   | LOC100522887  | 1.411       | 0.004014107 |
| 7   | LAMA3         | 1.382       | 0.014635659 |
| 8   | HELLs         | 1.322       | 0.009530397 |
| 9   | LOC100512420  | 1.3185      | 0.029473603 |
| 10  | LOC100516293  | 1.3175      | 0.01026956 |
| 11  | SLCTA2        | 1.312       | 0.009473936 |
| 12  | HMGS31        | 1.311       | 0.00560657 |
| 13  | PIHA1         | 1.303       | 0.002036451 |
| 14  | NDUF5A1       | 1.294       | 0.018110837 |
| 15  | IL1RAP        | 1.28        | 0.001803519 |
| 16  | TACSTD2       | 1.275       | 0.004011772 |
| 17  | E-cadherin    | 1.272       | 0.004320702 |
| 18  | PLO1D1        | 1.272       | 0.022204118 |
| 19  | ITM2B         | 1.2665      | 0.011878292 |
| 20  | COX5A         | 1.262       | 0.017952372 |
| 21  | HSPG2         | 1.256       | 0.001915952 |
| 22  | F11R          | 1.2465      | 0.018738586 |
| 23  | TOP1          | 1.243       | 6.08874E-08 |
| 24  | NDUF5B        | 1.238       | 0.048158995 |
| 25  | ANX1A         | 1.227       | 0.033945569 |
| 26  | NDUF5S1       | 1.224       | 3.01379E-06 |
| 27  | MRPL47        | 1.222       | 0.006827477 |
| 28  | RELA          | 1.219       | 0.002508153 |
| 29  | CD47          | 1.2185      | 0.042185225 |
| 30  | LOC100513756  | 1.214       | 0.2904697 |
| 31  | MRPL24        | 1.214       | 0.023142371 |
| 32  | CYP51A1       | 1.213       | 0.002272823 |
| 33  | FN1           | 1.204       | 0.021222E-06 |
| 34  | STAT5a        | 0.8315      | 0.005207431 |
| 35  | TAP2          | 0.83        | 0.005291123 |
increased mitochondrial Ca\textsuperscript{2+} level (Fig. 1A) and decreased mitochondrial MMP (Fig. 1B).

**Gene Ontology Enrichment Analysis of miR-4331 Targets**—miR-4331 targets were predicted using TargetScan and miRanda, by which 618 targets were obtained (supplemental Information S2). The 618 targets of miR4331 were searched against Gene Ontology (GO) database to provide enrichment information on biological processes, molecular functions, and cellular components. Among total 618 predicted targets, 26 targets were related to mitochondria, 4.2\% of total targets. GO enrichment of the 618 targets of miR-4331 showed that 89.6\% of targets were enriched in metabolic process and 31.6\% of targets were linked to mitochondria in cellular component (Fig. 2).
miR-4331 Increases Mitochondrial Ca\(^{2+}\) Level and Decreases MMP—We previously reported that TGEV infection caused the reduction of MMP through inducing ROS accumulation and increased miR-4331 level (5, 14). To investigate the effects of miR-4331 on mitochondria damage during TGEV infection, the PK-15 cells were transfected with miR-4331 mimics, or miRNA mimics control, miR-4331 inhibitors, inhibitors, and subsequently infected with TGEV at 1 MOI for 24 h. The miR-4331 level remarkably increased by miR-4331 mimics (Fig. 3A) and suppressed by miR-4331 inhibitors (Fig. 3B). miR-4331 mimics led to a decrease of mitochondrial Ca\(^{2+}\) level (Fig. 3C) and an increase of MMP (Fig. 3E). miR-4331 inhibitors reduced mitochondrial Ca\(^{2+}\) level (Fig. 3D) and increased MMP (Fig. 3F).

Quantitative Proteomic Analysis—A total of 4209 proteins were identified by high-resolution LC-MS/MS analysis, among which 4165 proteins were quantified (supplemental Information S3). When setting ≥1.2-fold as the upregulated threshold and ≤0.83-fold as the downregulated threshold, 69 differentially expressed proteins were obtained, including 33 upregulated proteins and 36 downregulated proteins (Table I). Raw data of MS are available via ProteomeXchange with identifier PXD008174.

GO Enrichment Analysis of the 69 Differentially Expressed Proteins—The 69 differentially expressed proteins were searched against Wolf Psort database (https://wolfpsort.hgc.jp/) for prediction of subcellular localization. The subcellular localization analysis revealed that the distribution of differentially expressed proteins was distributed in cytosol (43.48%), nuclear (15.49%), mitochondria (13.04%), extracellular (11.59%), plasma membrane (7.25%), cytosol and nuclear (2.9%), endoplasmic reticulum (2.9%), and peroxisome (2.9%) (Fig. 4A). The 69 differentially expressed proteins were annotated by UniProt-GOA database or InterProScan. The proteins were enriched by GO annotation based on three categories: biological process, cellular component, and molecular function. The differentially expressed proteins are primarily enriched in cellular process (66.67%), metabolic process (55.07%), and single-organism process (53.62%). The molecular functions of the differentially expressed proteins
are mainly involved in binding (52.17%) and catalytic activity (42.03%). In addition, the differentially expressed proteins are the component of cell part (86.96%), cell (86.96%), and organelle (65.22%) (Fig. 4B).

KEGG Pathway Enrichment Analysis of Differentially Expressed Proteins—Kyoto Encyclopedia of Genes and Genomes (KEGG) online service tools KAAS were used to annotate protein’s KEGG database description. Then the annotations were mapped on KEGG pathway database using KEGG mapper. KEGG enrichment analysis revealed the differentially expressed proteins were enriched in Glucagon signaling pathway, Inflammatory mediator regulation of TRP channels, Aldosterone synthesis and secretion, ECM-receptor interaction, cAMP signaling pathway, Oxidative...
phosphorylation, Ca\textsuperscript{2+} signaling pathway, MAPK signaling, and NF-κB signaling pathway (Fig. 5). The differentially expressed proteins were introduced into the web-tool STRING to generate protein-protein interaction networks (Fig. 6).

**Verification of Differentially Expressed Proteins by Western Blot and Real-Time PCR**—The transcription and expression levels of some differentially expressed proteins were verified using Western blot and real-time PCR. The Western blot analysis revealed that the protein levels of IL1RAP and RELA in PK-15 cells were dramatically increased by miR-4331 overexpression (Fig. 7A). The mRNA levels of 9 mitochondria-related differentially expressed proteins, including IL1RAP, COX5A, NDUFB5, MARPL24, MARPL1, RELA, CAMKA2D, PCK2, and BCAT1, were confirmed by real-time PCR (Fig. 7B). The Western blot and real-time PCR results were consistent with the proteomic data.

**IL1RAP Promotes TGEV-induced Mitochondrial Damage and Activates p38 MAPK Signaling**—According proteomic analysis, overexpression of miR-4331 may lead to an increase of IL1RAP protein level. It is reported that IL1RAP is a component of IL-1 protein complex (22) and IL-1 is a activator of p38 MAPK (23). We previously found that TGEV infection reduced MMP and activated p38 MAPK pathway (5). Therefore, we presume that IL1RAP might play a role in TGEV-induced decrease of MMP and activation of p38 MAPK. To explore the effects of IL1RAP on mitochondria and p38 MAPK pathway, we constructed pCI-neo-IL1RAP to overexpress IL1RAP and synthesized siRNA of IL1RAP, siIL1RAP, to silence IL1RAP. The results showed that pCI-neo-IL1RAP led to an increase IL1RAP at mRNA level (Fig. 8A) and protein level (Fig. 8B) and siIL1RAP reduced mRNA level (Fig. 8A) and protein level (Fig. 8D). Mitochondrial Ca\textsuperscript{2+} level was upregulated by pCI-neo-IL1RAP and downregulated by siIL1RAP (Fig. 8C). In addition, MMP level was suppressed by pCI-neo-IL1RAP and increased by siIL1RAP (Fig. 8D). As expected, phosphorylation level of p38 was enhanced by pCI-neo-IL1RAP and attenuated by siIL1RAP (Fig. 8E).

**Activation of p38 MAPK Pathway Facilitates TGEV-induced Mitochondrial Damage**—To investigate whether the activation of p38 MAPK promotes TGEV-induced mitochondrial damage, PK-15 cells were treated with SB203580, the specific inhibitor of p38 MAPK, and infected with TGEV. As predicted, TGEV-induced phosphorylation of p38 was attenuated by SB203580 (Fig. 9A). Inhibition of p38 phosphorylation led to a decrease of mitochondrial Ca\textsuperscript{2+} level (Fig. 9B) and an increase of MMP during TGEV infection (Fig. 9C), indicating that p38 promotes TGEV-induced mitochondrial damage.

**miR-4331 Promotes TGEV-induced Activation of p38 MAPK Pathway**—We demonstrated that miR-4331 could upregulated IL1RAP expression via quantitative proteomic analysis and IL1RAP contributed to activation of p38 MAPK pathway via phosphorylating p38. However, whether miR-4331 can activate p38 pathway is unknown. To investigate the
Effect of miR-4331 on p38 MAPK. miR-4331 mimics or inhibitors was introduced into PK-15 cells. The phosphorylation level of p38 was detected. The result showed that TGEV-induced phosphorylation of p38 was enhanced by miR-4331 mimics and attenuated by miR-4331 inhibitors (Fig. 10), suggesting miR-4331 caused activation of p38 MAPK pathway.

RB1 is the Direct Target of miR-4331—To identify whether miR-4331 directly binds to the 3′ UTRs of miR-4331 targets, the miR-4331 binding sites of target mRNA were mutated with a 4-bp substitution (Fig. 11A). The wild type sequences of 3′ UTRs containing miR-4331 binding sites and mutated 3′ UTRs sequences of miR-4331 targets, in which miR-4331 seed sequence was mutated, were cloned into the 3′ UTR of Renilla luciferase in dual-luciferase reporter plasmid psiCHECK-2 to generate constructions, RB1-wt and RB1-mut (Fig. 11B). The constructs were co-transfected into PK-15 cells with either miR-4331 mimics/mimics control or miR-4331 inhibitors/inhibitors control. The Renilla luciferase activities of RB1-wt and RB1-mut were respectively reduced and

Fig. 8. Effects of IL1RAP on mitochondrial damage and p38 MAPK pathway. A, The relative mRNA level of IL1RAP in PK-15 cells transfected with pCI-neo-IL1RAP and siIL1RAP; B, The effects of pCI-neo-IL1RAP and siIL1RAP on expression of IL1RAP; C, The effect of IL1RAP on mitochondrial Ca\[^{2+}\] level during TGEV infection; D, The effect of IL1RAP on MMP during TGEV infection; E, The effect of IL1RAP on p38 phosphorylation. Data are representative three independent experiments. *p < 0.05; **p < 0.01.
improved by miR-4331 mimics (normalized to Firefly luciferase activity) (Fig. 11C). In contrast, the Renilla luciferase activities of RB1-wt and RB1-mut were respectively raised and decreased by miR-4331 inhibitors (normalized to Firefly luciferase activity) (Fig. 11D). The results revealed that miR-4331 directly binds to 3′ UTR of RB1 mRNA. To determine whether miR-4331 inhibits RB1 expression, either miR-4331 mimics or miRNA inhibitors control were transfected into PK-15 cells. RB1 protein level was tested using Western blot, showing that expression of RB1 was suppressed by miR-4331 mimics and improved by miR-4331 inhibitors (Fig. 11E).

RB1 Attenuates TGEV-induced Mitochondrial Ca$^{2+}$ Level and Improves MMP During TGEV Infection—We demonstrated that miR-4331 facilitated mitochondrial damage and directly targeted RB1. However, whether miR-4331 affects mitochondria through targeting RB1 is unclear. To investigate the effect of RB1 on TGEV-induced mitochondrial damage, three siRNAs of RB1, siRB1–1, siRB1–2, and siRB1–3, were synthesized and respectively transfected into PK-15 cells to silence RB1. The silencing efficiency of RB1 siRNAs were evaluated, showing siRB1–3 was the most effective siRNA for RB1 silencing (Fig. 12A and 12B). Moreover, RB1 gene was cloned into eukaryotic expression plasmid pCl-neo, named pCl-neo-RB1, to overexpress RB1. pCl-neo-RB1 and pCl-neo were respectively transfected into PK-15 cells, followed by evaluation of mRNA and protein level. Expectedly, both the mRNA and protein level were upregulated (Fig. 12C and 12D). Then, PK-15 cells were transfected with either siRB1–3 or pCl-neo-RB1 and subsequently infected with TGEV for 24 h. The mitochondrial Ca$^{2+}$ level and MMP were measured. The results showed that mitochondrial Ca$^{2+}$ level was decreased by pCl-neo-RB1 and increased by siRB1–3 (Fig. 12E). In addition, MMP was upregulated by pCl-neo-RB1 and decreased by siRB1–3 (Fig. 12F). The results reveal that miR-4331 promotes TGEV-induced mitochondrial damage via targeting RB1.

RB1 Suppresses IL1RAP Expression and p38 MAPK Signaling—We showed that miR-4331 could aggravate TGEV-induced mitochondrial damage via targeting RB1 and activating p38 MAPK pathway. Therefore, we presumed that miR-4331 is likely to regulate IL1RAP and p38 MAPK signaling.
through its target RB1. We silenced RB1 using siRB1–3 and overexpressed RB1 using pCI-neo-RB1. IL1RAP, p38, and p-p38 were analyzed by Western blotting. It was found that RB1 was suppressed by siRB1–3 (Fig 13). Silencing RB1 caused an upregulation of IL1RAP and p-p38 (Fig. 13). Conversely, overexpression of RB1 led to a suppress of IL1RAP and p-p38 (Fig. 13).

**DISCUSSION**

In this study, we investigated the effects of miR-4331 on mitochondrial damage induced by TGEV infection via targeting RB1. The results showed that miR-4331 promoted TGEV-induced mitochondrial damage via targeting RB1, upregulating IL1RAP, and enhancing TGEV-induced activation of p38 pathway. Our findings reveal a novel regulatory effect of miR-4331 on TGEV-induced mitochondrial damage.

It is reported that TGEV infection caused the alterations of proteomes in PK-15 cells, including upregulated STAT1 and many ISGs (ISG15, IFIT1, IFIT2, IFIT3, IFIT5, OAS1, OAS2, and Mx1) (24). In this study, Mx1 was differentially decreased by miR-4331 in contrast to upregulation in TGEV-infected PK-15 cells. It indicates that miR-4331 plays a negative role in
TGEV-induced Mx1 expression. In addition, although STAT1 and many ISGs, including ISG15, IFIT1, IFIT2, IFIT3, OAS1, OAS2, Mx1, were detected using TMT approach, only Mx1 was altered by miR-4331 in TGEV-infected PK-15 cells. The likely reason is that miR-4331 downregulated TGEV-induced STAT1 and these ISGs, including ISG15, IFIT1, IFIT2, IFIT3, OAS1, OAS2, to normal level. In contrast to upregulation in TGEV-infected PK-15 cells, Mx1 was differentially decreased by miR-4331 in TGEV-infected PK-15 cells. It indicates that miR-4331 plays a negative role in TGEV-induced Mx1 expression.

In this study, RB1 is identified as the target of miR-4331 and affects mitochondrial function. RB1 is a suppressor of cell death via binding and repressing transcription factor E2Fs. The hypophosphorylated RB1, the active form of RB1, binds to and sequesters transcription factor E2F1 to operate as an inhibitor of E2Fs (25). C-terminal region of RB1 has a E2F1-specific binding site that is sufficient to repress E2F1-induced apoptosis, so the C-terminal interaction site on RB1 acts as a potent inhibitor of E2F1 (26). A pool of evidence shows RB1 localizes in mitochondria (27). Therefore, a set of reports highlights a series of links between RB1 and mitochondrial function. RB1 promotes mitochondrial biogenesis for erythropoiesis (28). Loss of RB1 caused a decrease of mitochondrial mass, downregulated mitochondrial function, oxidative phosphorylation, MMP, and accumulation of hypopolarized mitochondria (29), implying that suppression of RB1 leads to mitochondrial damage. These results further support our conclusion that RB1 weakens TGEV-induced mitochondrial damage. Here, RB1 was identified as the target of miR-4331 and inhibited by miR-4331. Whereas, RB1 was not identified as the differentially expressed protein using TMT, it may be because of low expression level of RB1 and the insufficient sensitivity of this technology.

**Fig. 12. Effect of RB1 on TGEV-induced mitochondrial damage.** A and B, Effect of RB1 siRNAs on RB1 transcription and expression; C and D, Effect of pCI-neo-RB1 on RB1 transcription and expression; E, Effect of RB1 on mitochondrial Ca2+ level during TGEV infection; F, Effect of RB1 on mitochondrial membrane potential level during TGEV infection. Data are representative three independent experiments. **p < 0.01.
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Cytokines of the IL-1 family are considered critical regulators of intestinal mucositis (30). IL-1 is a key proinflammatory cytokine that can initiate a series of signaling resulting in activation of NF-κB signaling pathway. IL-1 interacts with L1RAP and IL-1 receptor type I (IL-1R) to form IL-1 complex (31), which mediates the activation of p38 MAPK signaling in presence of IL-1β treatment (32). It is consistent with our finding that IL1RAP can lead to the activation of p38 MAPK pathway. Moreover, we found miR-4331 caused an upregulation of RELA, which is the p65 subunit of NF-κB. NF-κB is a key activator of inflammation and is activated during TGEV infection (33, 34). Therefore, we speculate miR-4331 may function as a regulator of TGEV-induced NF-κB pathway through regulating IL1RAP and RELA.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD008174 (http://www.ebi.ac.uk/pride/archive/) (18).

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