

# Knockdown of the Long Noncoding RNA TUG1 Suppresses Retinoblastoma Progression by Disrupting the Epithelial–Mesenchymal Transition

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## Abstract

Taurine-upregulated gene 1 (TUG1) is a long noncoding RNA (lncRNA) that has previously been linked to the development and progression of several cancer types. Its expression and mechanistic role in retinoblastoma (RB), however, remains to be established. Herein, we found that RB tissue samples exhibited TUG1 upregulation. RB cell lines similarly exhibited marked TUG1 upregulation. Real-time cellular analysis (RTCA) and colony formation assays were then used to gauge RB cell proliferation, while transwell assays were conducted to assess the metastatic and invasive potential of these cells. In these assays, TUG1 upregulation was found to promote RB cell proliferative, migratory, and invasive activity while inducing the epithelial–mesenchymal transition (EMT). Subsequent quantitative real-time polymerase chain reaction (qPCR) and Western blotting indicated that this lncRNA functions at least in part by influencing the expression of Notch signaling pathway genes, which were downregulated following TUG1 knockdown in RB cells. Together, these data suggested that TUG1 can promote RB cell malignancy via the Notch signaling and EMT pathways, contributing to negative patient outcomes.

## Keywords

long noncoding RNA, Taurine-upregulated gene 1, retinoblastoma

## Introduction

Retinoblastoma (RB) is a cancer of the retina that most commonly develops in children<sup>1</sup>. Recent advances in tumor detection have improved the survival outcomes of RB patients<sup>2</sup>, but vision loss is nonetheless common among survivors due to advanced disease and complications of treatment<sup>2,3</sup>. As RB and associated vision loss can be debilitating, further research is urgently needed to understand the mechanisms governing the initiation and metastatic progression of RB in an effort to develop targeted drugs capable of combatting the morbidity associated with this disease.

Long noncoding RNAs (lncRNAs) are RNAs >200 nucleotides long that lack coding potential<sup>4</sup>. Key roles for specific lncRNAs, as promoters or suppressors of RB tumor development, have been identified to date<sup>5,6</sup>. Taurine-upregulated gene 1 (TUG1) is a 7.1-kb lncRNA encoded on chromosome 11A1 that was first identified as an important regulator of retinal and normal photoreceptor development that is expressed in retinal tissues<sup>7</sup>. More recent work has highlighted roles for TUG1 as a modulator of key oncogenic processes such as invasion, metastasis, cell cycle progression, proliferation, and apoptosis. TUG1 has also been linked to radioresistance and angiogenesis in the context of

hepatoblastoma<sup>8</sup>. TUG1 and Lin28a also positively regulate one another, and are associated with osteogenic differentiation<sup>9</sup>. Indeed, TUG1 has been identified as a possible regulator of pathological processes in a range of diseases including cancer and diseases of the respiratory<sup>10</sup>, digestive<sup>11</sup>, and nervous systems<sup>12</sup>.

While the biological role of TUG1 has been reported in multiple oncogenic contexts, its specific functional importance in RB remains to be established. In this study, we therefore sought to establish the clinical importance of TUG1 in RB patient samples and to characterize its mechanistic role in RB cells.

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## Materials and Methods

### Human Samples

In total, 60 RB tumor tissue samples and 60 samples of healthy retinal tissue were collected via surgery at our hospital from January 2012 to January 2015. Tumor samples were pathologically confirmed by two independent pathologists as per the American Joint Committee on Cancer guidelines (7th edition)<sup>13</sup>. Healthy retinal samples were obtained from the ruptured globe tissues harvested from children. After collection, samples were snap frozen and stored at  $-80^{\circ}\text{C}$ . RB patients were followed up for 26.4 months on average (range: 6–60 months) after surgery, with rates of overall survival (OS) being recorded. This study was conducted according to the principles of the Declaration of Helsinki. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (2016-ECFAHXJU-18092). All tissues were obtained with informed consent.

### Cell Culture

SO-RB-50, Weri-Rb1, Y79, and RBL-13 RB cell lines and control ARPE-19 retinal epithelial cells were acquired from the American Type Culture Collection (Shanghai, China). All cells were cultured in RPMI-1640 (Gibco, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) in a 5%  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$ .

### Lentiviral Production and Transduction

Lentiviral particles encoding TUG1 overexpression (LV-TUG1) or shRNA sequences (LV-shTUG1), together with corresponding Mock negative control lentiviruses, were purchased from the GenePharma Company (Shanghai). Cells were plated in six-well plates until 40% confluent, at which time they were infected with these lentiviral constructs together with polybrene (8  $\mu\text{g}/\text{mL}$ ; GenePharma, China). After 48 h, cells were collected for quantitative real-time polymerase chain reaction (qPCR) analyses and other downstream experiments.

### Quantitative Real-Time PCR

RNAiso (TaKaRa, Japan) was used based on provided directions to isolate total RNA from tissue and cell samples, after which moloney-murine leukemia virus (M-MLV) reverse transcriptase (Promega) was used to prepare cDNA. Appropriate primers (Supplemental Table 1) were then used for qPCR reactions conducted with an ABI 7900 cyclo instrument (Applied Biosystems, CA, USA). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a normalization control, and the  $2^{-\Delta\Delta\text{Ct}}$  method was used to assess relative gene expression, with samples being analyzed in triplicate.

### Cell Proliferation Assay

A real-time cellular analysis (RTCA)-based cellular proliferation assay was conducted using an xCELLigence DP instrument (ACEA Biosciences, USA). The RTCA system can monitor cell growth in real time using a label-free cell-based assay which can measure impedance variations in the culture media. In the RTCA system, the cultured cells were attached to the cell culture wells in special cell culture plates. Then, any changes in the electronic impedance were recorded through E-plate sensors and expressed as the cell index, which corresponds to cell viability. Cells were seeded in 16-well E-plates ( $5 \times 10^3/\text{well}$ ), with cell index data being recorded every 15 min over a 15-h period.

### Colony Formation Assay

Cells were harvested and plated in 6-well plates (500/well), followed by a 14-day incubation. Visible colonies were then fixed for 10 min with methanol (1 mL/well), followed by staining for 5 min with 0.1% crystal violet at room temperature. Colonies were then washed and counted to assess cell proliferation.

### Transwell and Wound Healing Assays

Wound healing assays were employed to assess cellular migratory capacity. Briefly, the cells were grown to 90%–95% confluence in a six-well plate, after which a pipette tip was used to generate a scratch wound in the monolayer surface. After rinsing cells with phosphate buffered saline (PBS), the wounded site was imaged at 24 h after wounding.

To additionally evaluate RB cell migration, 24-well transwell inserts (8- $\mu\text{m}$  pores; Millipore, MA, USA) were used based on provided directions. Briefly, appropriate cells were collected at 48 h post-transfection and added to the upper portion of each chamber ( $2 \times 10^5/\text{well}$ ) in media containing 2% FBS, while media containing 10% FBS was added to the lower chamber (500  $\mu\text{l}$ ). Cells were then incubated for 24 h, after which cells on the upper surface were removed, and those on the lower surface were fixed for 30 min with 4% paraformaldehyde, stained for 10 min with 0.1% crystal violet, and then cells in five random fields of view were counted with ImageJ software (Media Cybernetics, MD, USA). Invasion assays were conducted in an identical manner, except that the transwell insert was first coated with Matrigel (BD Biosciences, CA, USA), and half as many cells were added to the upper chamber.

### Western Blotting

Proteins were extracted from samples with a Beyotime protein extraction kit, after which a BCA kit (Qiagen, CA, USA) was used to quantify protein concentrations. Next, 30  $\mu\text{g}$  of protein per sample was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

transferred to PVDF membranes (Millipore, Temecula, CA, USA). Blots were subsequently blocked for 2 h with 5% non-fat milk at 37°C followed by staining overnight with antibodies specific for E-cadherin (1:500, Abcam, Cambridge, MA, USA), Notch1 (1:500, Abcam), or GAPDH (1:1000, CST) at 4°C. Blots were then treated for 1 h with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) at 37°C, after which enhanced chemiluminescence (Amersham Biosciences, IL, USA) was used to visualize protein bands with Image Lab 2.0 (Bio-Rad Laboratories, CA, USA).

### Immunofluorescent Staining

Cells were cultured on glass coverslips, rinsed with PBS, fixed for 10 min with 4% polyfluoroalkoxy (PFA), and then treated for 5 min with cold (−20°C) methanol. Next, 50-mM  $\text{NH}_4\text{Cl}$  in PBS was added for 5 min for aldehyde quenching. Following an additional PBS wash, cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS/3% bovine serum albumin (BSA), followed by a 30-min incubation with anti-E-cadherin. After rinsing again, cells were then stained with secondary anti-rabbit IgG antibodies conjugated to Alexa 488 (LV-TUG1 group) or Alexa 555 (LV-shTUG1 group) (Invitrogen) for 30 min. After two rinses with PBS and one rinse with water, cells were then mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), and a confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to image cells. Fields of view were selected at random for quantification, and experiments were repeated thrice.

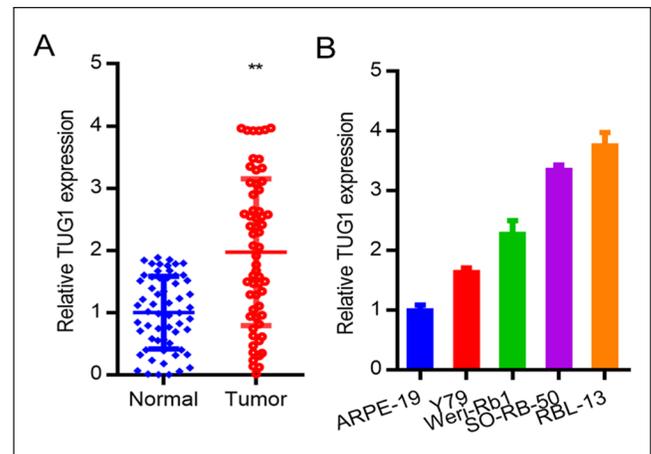
### Statistical Analysis

SPSS 19.0 and GraphPad Prism 6 (GraphPad, CA, USA) were used for all statistical analyses. Data were compared via chi-squared tests, Student's *t* tests, or one-way analyses of variance (ANOVAs) as appropriate, with  $P < 0.05$  as the significance threshold.

## Results

### RB Tumor Samples and Cell Lines Exhibit TUG1 Upregulation

To examine the possible association between TUG1 and RB, TUG1 expression was assessed via qPCR in 60 RB tumor samples and 60 samples of healthy retinal tissue, revealing significant increases in the expression of this lncRNA in RB patient tumors (Fig. 1A). Consistent with this, TUG1 upregulation was observed in the Y79 and RBL-13 cell lines relative to levels in a healthy retinal cell line (Fig. 1B). These findings indicate that TUG1 upregulation is a hallmark of RB that may be associated with disease development or progression.

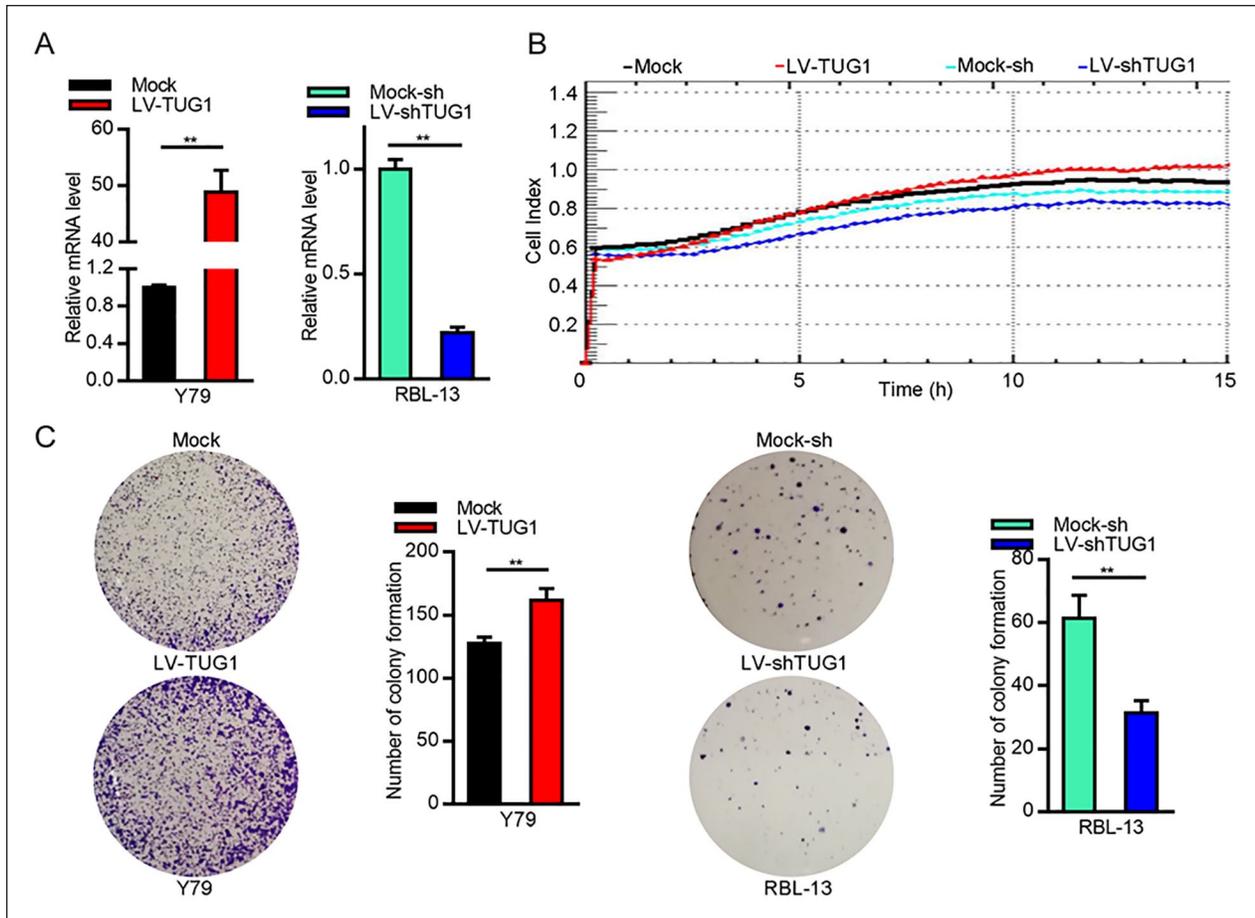


**Figure 1.** Retinoblastoma tissues and cells exhibit TUG1 upregulation. (A) qPCR was used to evaluate TUG1 expression levels in 60 RB tissue samples and healthy retinal tissue samples. Continuous data are means  $\pm$  standard deviations and were compared via *t* tests. (B) qPCR was used to assess TUG1 levels in four RB cell lines and a control epithelial cell line. Continuous data are means  $\pm$  standard deviations and were compared via one-way ANOVA. \*\* vs normal tissues, \*\* $P < 0.01$ . TUG1: Taurine-upregulated gene 1; qPCR: quantitative real-time polymerase chain reaction; RB: retinoblastoma; ANOVA: analysis of variance.

### TUG1 Drives the In Vitro Proliferative and Metastatic Activity of RB Cells

While all four analyzed RB cell lines exhibited increased TUG1 expression relative to control ARPE-19 cells, we found that the RBL-13 cells expressed the highest levels of this lncRNA. To examine the functional implications of this finding, we knocked down TUG1 in RBL-13 cells and upregulated it in Y79 cells using appropriate lentiviral constructs (Figure S1). As expected, TUG1 was successfully upregulated following LV-TUG1 transduction, whereas transduction with a lentivirus encoding a TUG1-specific shRNA reduced its expression (Fig. 2A). Subsequent RTCA analyses of RB cell proliferation indicated that TUG1 overexpression was linked to enhanced proliferative activity (Fig. 2B), with similar enhancement in the colony-forming activity of LV-TUG1-transduced Y79 cells being observed relative to Mock controls (Fig. 2C).

Subsequent wound healing and transwell assays were employed to gauge the impact of TUG1 on the metastatic potential of RB cells. Wound healing assays indicated that TUG1 overexpression enhanced Y79 cell migration relative to Mock control cells (Fig. 3A), while knocking down this lncRNA in RBL-13 cells impaired their migration (Fig. 3B). Transwell assays similarly confirmed that higher levels of TUG1 expression were associated with more robust migratory and invasive activity for these two tested RB cell lines (Fig. 3C, D), suggesting that TUG1 can promote the in vitro metastatic progression of RB cells.



**Figure 2.** TUG1 expression is associated with enhanced RB cell proliferation. (A) TUG1 overexpression or knockdown in Y79 and RBL-13 cells was confirmed via qPCR. (B) RTCA assays were used to assess the proliferation of Y79 and RBL-13 cells following TUG1 overexpression or knockdown. (C) The impact of TUG1 expression on RB cell proliferation was assessed via colony formation assay. Continuous data are means  $\pm$  standard deviations and were compared via *t* tests. \*\* vs the control group,  $P < 0.01$ . TUG1: Taurine-upregulated gene 1; qPCR: quantitative real-time polymerase chain reaction; RB: retinoblastoma; RTCA: real-time cellular analysis.

Y79 cells overexpressing TUG1 exhibited reduced E-cadherin expression, whereas the levels of this epithelial marker were increased following TUG1 knockdown in RBL-13 cells (Fig. 4A), consistent with changes in EMT-related processes. Immunofluorescent staining similarly confirmed that higher TUG1 levels in these two cell lines were associated with decreased E-cadherin levels (Fig. 4B). Taken together, these *in vitro* findings indicate that the lncRNA TUG1 promotes tumor cell migration and invasion and induces EMT.

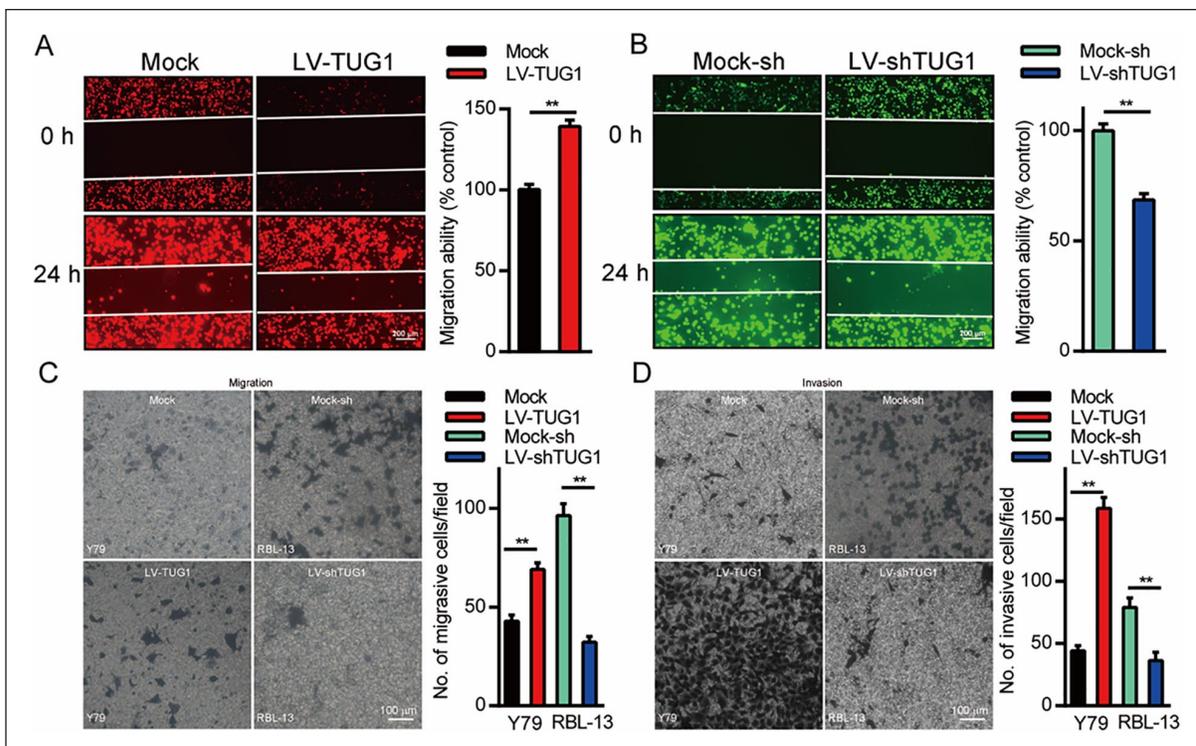
### Knocking Down TUG1 Inhibits Notch Signaling in RBL-13 Cells

The Notch signaling pathway helps balance important processes including differentiation, EMT progression, proliferation, and apoptotic cell death in a context-dependent manner<sup>14</sup>. In RB tumors, Notch signaling is closely associated with EMT progression<sup>15</sup>. We thus assessed Notch

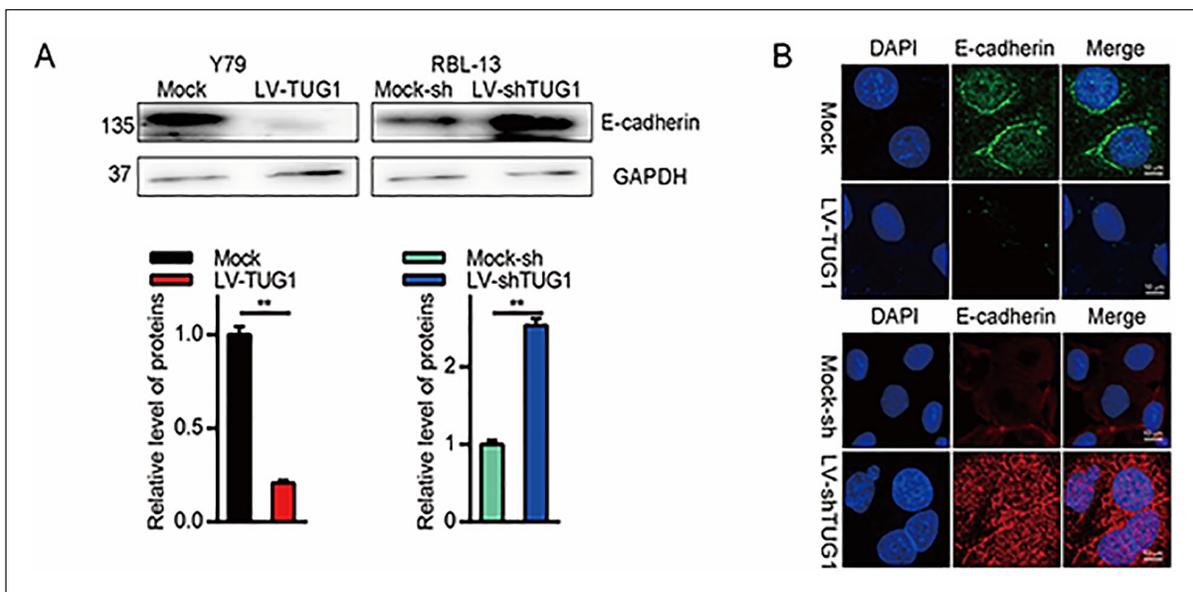
pathway component expression at the mRNA (Fig. 5A) and protein (Fig. 5B) levels, revealing that TUG1 knockdown impaired Notch1 upregulation at both these levels, while other pathway components were unaffected. These data indicated that the Notch signaling pathway may represent an integral mechanism whereby TUG1 modulates RB cell malignancy.

### Discussion

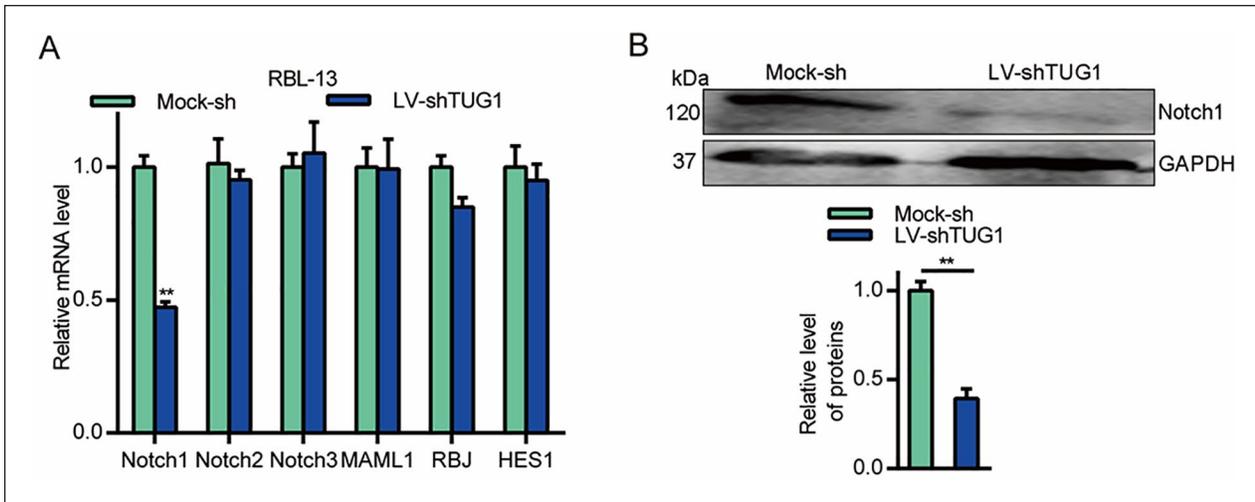
RB is the most common form of ocular malignancy in the world<sup>2</sup>. RB management is complex and relies upon a series of treatment options including radiotherapy, chemotherapy, enucleation, laser photocoagulation, thermotherapy, and cryotherapy. Chemotherapy, which can be conducted through intravenous, periocular, intra-arterial, or intravitreal approaches, is the most common treatment strategy that seeks to preserve eye integrity<sup>16</sup>. Despite these myriad treatment options, this disease remains tricky, emphasizing the



**Figure 3.** TUG1 promotes RB cell migration and invasion. (A) Wound healing assays were used to assess the migration of Y79 cells overexpressing TUG1. Scale bar: 200  $\mu$ m. (B) Wound healing assays were used to assess the migration of RBL-13 cells in which TUG1 was knocked down. Scale bar: 200  $\mu$ m. (C) Transwell assays were used to assess the migration of Y79 and RBL-13 cells in which TUG1 was overexpressed and knocked down, respectively. Scale bar: 100  $\mu$ m. (D) Transwell assays were used to assess the invasive activity of Y79 and RBL-13 cells in which TUG1 was overexpressed and knocked down, respectively. Scale bar: 100  $\mu$ m. Continuous data are means  $\pm$  standard deviations and were compared via *t* tests. \*\* vs the control group,  $P < 0.01$ . TUG1: Taurine-upregulated gene 1; RB: retinoblastoma.



**Figure 4.** TUG1 expression impacts the EMT process in RB cells in vitro. (A) E-cadherin levels were assessed via Western blotting in Y79 and RBL-13 cells. Continuous data are means  $\pm$  standard deviations and were compared via *t* tests. \*\* vs the control group,  $P < 0.01$ . (B) Representative E-cadherin immunofluorescent staining images for Y79 cells overexpressing TUG1 (LV-TUG1) or RBL-13 cells in which TUG1 had been knocked down (LV-shTUG1). Blue: 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 10  $\mu$ m. TUG1: Taurine-upregulated gene 1; EMT: epithelial-mesenchymal transition; RB: retinoblastoma.



**Figure 5.** TUG1 knockdown suppresses Notch signaling pathway activity. (A) Notch1, Notch2, Notch3, MAML1, RBJ, and Hes1 mRNA levels were assessed via qPCR in RBL-13 cells. (B) Notch1 protein levels were assessed via Western blotting, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control. Data are representative of three independent experiments. Continuous data are means  $\pm$  standard deviations and were compared via *t* tests. \*\* vs the control group,  $P < 0.01$ . TUG1: Taurine-upregulated gene 1; qPCR: quantitative real-time polymerase chain reaction.

importance of elucidating the mechanistic basis for its onset and progression. The dysregulation of many lncRNAs has recently been identified as a hallmark of RB, with several of these noncoding RNAs having been directly associated with RB malignancy and oncogenesis. Liu et al, for example, observed MALAT1 overexpression in RB cells, and found that knocking it down markedly suppressed the proliferative, migratory, and invasive activity of these cells while driving their apoptotic death<sup>17</sup>. Hao et al determined that AFAP1-AS1 was an oncogenic lncRNA commonly upregulated in RB tissues and cell lines<sup>18</sup>. Herein, we sought to expand upon these prior studies by exploring the functional importance of TUG1 in this cancer type, providing novel evidence that it is upregulated in RB tissues and cells wherein it can promote malignant tumor progression.

TUG1 has been found to be dysregulated in cancer types such as non-small cell lung cancer, colorectal cancer (CRC), hepatocellular carcinoma (HCC), and gastric cancer (GC)<sup>19</sup>. Liu et al detected increased TUG1 expression in cervical cancer tissues and cell lines from humans relative to corresponding controls, and determined that the knockdown of this lncRNA impaired the survival and proliferation of these cells<sup>20</sup>. Shen et al further determined that knocking down TUG1 in CRC cells was sufficient to suppress their migratory and invasive activity in vitro owing to the disruption of the EMT, with a corresponding impairment of lung metastasis in vivo. They found that this mechanism was linked to a disruption of transforming growth factor- $\beta$  (TGF- $\beta$ )-induced metastasis, which could not occur when this lncRNA was knocked down, leading to the characterization of a TUG1/TWIST1/EMT signaling axis in CRC<sup>21</sup>. Herein, we similarly determined that TUG1 knockdown was sufficient to

compromise RB cell migration and invasion, in line with these past findings<sup>21</sup>. We also observed TUG1 upregulation in RB tumor tissues as compared with normal tissue controls, suggesting that TUG1 may regulate the pathogenesis of RB.

The EMT plays an essential role in processes including fibrosis, cancer progression, the healing of wound tissue, and embryonic development<sup>22,23</sup>. In RB cells, the EMT process is particularly important as a promoter of metastatic progression<sup>24</sup>. The EMT is characterized by reductions in epithelial marker (E-cadherin) expression with concomitant increases in levels of mesenchymal markers (N-cadherin, fibronectin, and vimentin)<sup>25</sup>. As such, we explored E-cadherin levels in our RB cells via immunofluorescent staining and Western blotting, revealing that TUG1 overexpression resulted in E-cadherin downregulation, whereas the opposite phenotype was observed upon TUG1 knockdown. These data thus indicated that TUG1 regulates EMT progression in RB cells.

Binding of the Notch receptor to Jagged (Jag1-2) and Delta-like (DLL1,3,4) ligands on adjacent cells can initiate the Notch signaling pathway by driving the proteolytic cleavage of this receptor such that the  $\gamma$ -secretase complex mediates Notch intracellular domain (NICD) release. Upon translocation to the nucleus, NICD is able to complex with CBF1 and MAML<sup>26,27</sup>, leading to the activation of Notch target genes in the Hes and Hey families<sup>28</sup>. Upon upregulation, these proteins in turn suppress the expression of genes related to cell differentiation. Notch pathway dysregulation has been linked to the growth and development of many types of tumors, but has not been studied in detail in the context of RB. Notch signaling plays an integral role in the regulation of normal retinal cellular processes, leading us to further explore its association with RB pathogenesis.

Given that Notch1 can inhibit photoreceptor differentiation to maintain cells in a progenitor state<sup>29</sup>, it is critical to normal retinal development. Postnatal Notch1 deletion in mice has been shown to enhance the differentiation of rod photoreceptors, whereas Notch receptor knockdown during embryonic development is linked to enhanced production of cone photoreceptors<sup>29</sup>. Such Notch1 signaling has also been linked to the development of retinal ganglion cells, such that Notch1-positive progenitor cells can differentiate to give rise to photoreceptors or ganglion cells<sup>30</sup>. We observed pronounced downregulation of Notch1 in RB cells in which TUG1 had been knocked down, whereas no impact on the expression of Notch2, Notch3, MAML1, RBJ, or HES1 was observed. Notch1 was an important driver of RBL-13 cell activity, and was expressed at significantly higher mRNA and protein levels in these cells relative to corresponding control cell lines. However, the control line used in this study is subject to the caveat that there are differences in retinal tissues from infants and adults.

Together, these data suggest that TUG1 is a novel prognostic biomarker in RB and may be a viable therapeutic target in patients affected by this disease. By providing a robust foundation for future research, our findings offer multiple opportunities for the validation of TUG1 as a lncRNA worthy of clinical investigation.

## Conclusion

In summary, we herein identified TUG1 as a lncRNA that is upregulated in RB and that represents a promising prognostic biomarker that may be useful in guiding RB patient diagnosis and/or management. We additionally determined that knocking down this lncRNA was sufficient to impair the proliferative, migratory, and invasive activity of RB cells owing to its ability to function in an oncogenic manner via the Notch and EMT pathways. Together, these results suggest that TUG1 may represent a promising target for RB treatment.

## Author Contributions

L.L. designed and conducted experiments, and performed data analysis. Z.Z. and Y.Z. conducted experiments and collected data. H.W. conducted experiments, performed data analysis, wrote the article, data interpretation, and article revision.

## Ethical Approval

This study was approved by the ethics committee at the first affiliated hospital of Xian Jiaotong University, Shaanxi, China.

## Statement of Human and Animal Rights

All the experimental procedures involving patients were conducted in accordance with the Helsinki Declaration for human studies, and approved by the ethics committee at the first affiliated hospital of Xian Jiaotong University (2016-ECFAHXJU-18092).

## Statement of Informed Consent

Written informed consents were obtained from the patients for the collection and publication of clinical data.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Supplemental Material

Supplemental material for this article is available online.

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