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LZTR1 Loss Reduces Vimentin Expression and Motility in Hep3B Hepatocellular Carcinoma Cells

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Abstract

Hepatocellular carcinoma (HCC) remains a leading cause of cancer-related mortality, underscoring the need to elucidate molecular mechanisms that regulate tumor cell state and behavior. Leucine zipper-like post-translational regulator 1 (LZTR1) regulates RAS/mitogen-activated protein kinase (MAPK) signaling, yet LZTR1-dependent transcriptional alterations in HCC cells remain poorly defined. To address this gap and determine how LZTR1 loss reshapes signaling, transcriptional programs, and cellular phenotypes, we established a LZTR1 knockout (KO) Hep3B model and combined pathway profiling with transcriptomic and functional analyses. Immunoblotting revealed increased phosphorylation across the RAF–MEK–ERK–RSK cascade in LZTR1 KO cells. Transcriptome-wide RNA sequencing (RNA-Seq) identified differentially expressed genes, and selected findings were validated by qRT-PCR. Gene set enrichment analysis indicated that the epithelial–mesenchymal transition (EMT) gene set was enriched in control cells. At the protein level, LZTR1 loss remodeled EMT-associated markers in a hybrid epithelial–mesenchymal pattern consistent with epithelial–mesenchymal plasticity (EMP). Vimentin was suppressed at transcript and protein levels. Functionally, LZTR1 KO cells exhibited impaired wound closure and reduced transwell migration and invasion. Collectively, these findings define an EMP-related molecular and phenotypic state associated with LZTR1 deficiency in Hep3B cells, providing insight into how LZTR1 loss reshapes tumor cell behavior in HCC.

Keywords: epithelial–mesenchymal plasticity; genome editing; hepatocellular carcinoma; LZTR1; RAS/MAPK signaling; RNA sequencing; Vimentin



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1. Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 80–90% of primary liver cancers and represents a major global health burden, ranking as the sixth most frequently diagnosed malignancy and the third leading cause of cancer-related mortality worldwide [1]. The high lethality of HCC is largely attributable to late-stage diagnosis, which severely limits curative treatment options [2]. In addition, current systemic therapies show limited and often transient efficacy in advanced disease due to intrinsic and acquired resistance mechanisms [2,3]. At the molecular level, HCC arises through a multistep

process characterized by progressive dysregulation of core cellular programs governing proliferation, differentiation, survival, and cell-state plasticity [4].

At the molecular level, among the signaling pathways implicated in HCC pathogenesis, the RAS/mitogen-activated protein kinase (MAPK) cascade occupies a central position [5]. This pathway regulates diverse cellular processes, including growth, survival, differentiation, migration, and epithelial–mesenchymal transition (EMT) [6]. Importantly, RAS/MAPK signaling is frequently hyperactivated in HCC through upstream receptor activation and post-translational regulatory mechanisms, even in the absence of canonical oncogenic RAS mutations [7]. Consequently, aberrant RAS/MAPK activity constitutes a key driver of hepatocarcinogenesis and tumor progression, underscoring the need to identify regulatory factors that fine-tune pathway output in HCC.

In this context, Leucine zipper-like post-translational regulator 1 (*LZTR1*; Gene ID: 8216) is encoded at the chromosomal locus 22q11.2 and was initially identified based on sequence homology to leucine zipper-containing proteins [8]. Subsequent studies identified *LZTR1* as a member of the BTB–Kelch superfamily [9]. Localization analyses revealed predominant cytoplasmic and Golgi localization, with additional nuclear presence, consistent with multifunctional regulatory roles [9–12]. Functionally, *LZTR1* acts as a substrate-specific adaptor for the CUL3 ubiquitin ligase complex, promoting the ubiquitination and proteasomal degradation of canonical RAS proteins (KRAS, NRAS, HRAS) as well as the non-canonical RAS-family member RIT1, thereby restraining RAS/MAPK pathway activity [10,11,13].

Consistent with its role as a negative regulator of oncogenic signaling, *LZTR1* has been predominantly characterized as a tumor suppressor in multiple malignancies, including glioblastoma [14], schwannomatosis [15], and HCC [16]. However, accumulating evidence indicates that *LZTR1* function is lineage- and context-dependent, as demonstrated by focal amplification and pro-tumorigenic roles in acral melanoma [17]. Conversely, reduced *LZTR1* expression in HCC has been associated with poor prognosis and early recurrence, supporting a tumor-suppressive role in this disease context [16,18].

Despite these insights, critical gaps remain in the understanding of *LZTR1* biology in HCC. Most prior studies have relied on knockdown-based approaches, leaving the cellular consequences of complete *LZTR1* loss largely unexplored [16,19]. However, while *LZTR1* is widely recognized as a regulator of RAS/MAPK signaling, it remains unclear how *LZTR1*-dependent changes in signaling output are translated into genome-wide transcriptional programs and associated phenotypic plasticity in HCC cells. *LZTR1*-dependent transcriptomic alterations have been examined in Noonan syndrome-associated cardiomyopathy [20,21] and leukemia models [10,22]; however, such analyses have not yet been performed in HCC cells. Collectively, these gaps reveal an incomplete understanding of the transcriptional and cellular consequences of *LZTR1* loss in HCC.

In this study, we established and applied an *LZTR1* knockout (KO) model in Hep3B cells to systematically characterize the molecular and cellular consequences of complete *LZTR1* loss in HCC. Specifically, we examined how *LZTR1* deficiency affects RAS/MAPK signaling output, reshapes transcriptome-wide regulatory programs, and modulates EMT-associated protein levels and tumor cell motility and invasiveness. By integrating signaling, transcriptional, and phenotypic analyses, our work provides mechanistic insight into the context-dependent roles of *LZTR1* in shaping tumor cell states in HCC.

2. Results

2.1. Generation of *LZTR1* KO Hep3B Cells and Analysis of RAS/MAPK Signaling

To generate *LZTR1*-deficient Hep3B cells, a Clustered Regularly Interspaced Palindromic Repeat (CRISPR)/Cas9 nickase (CRISPR/Cas9n)-mediated genome-editing strategy

was designed to target exon 1 of the human *LZTR1* gene (Figure 1A). Two guide RNAs (gRNAs) flanking the target region were selected (Figure 1A).

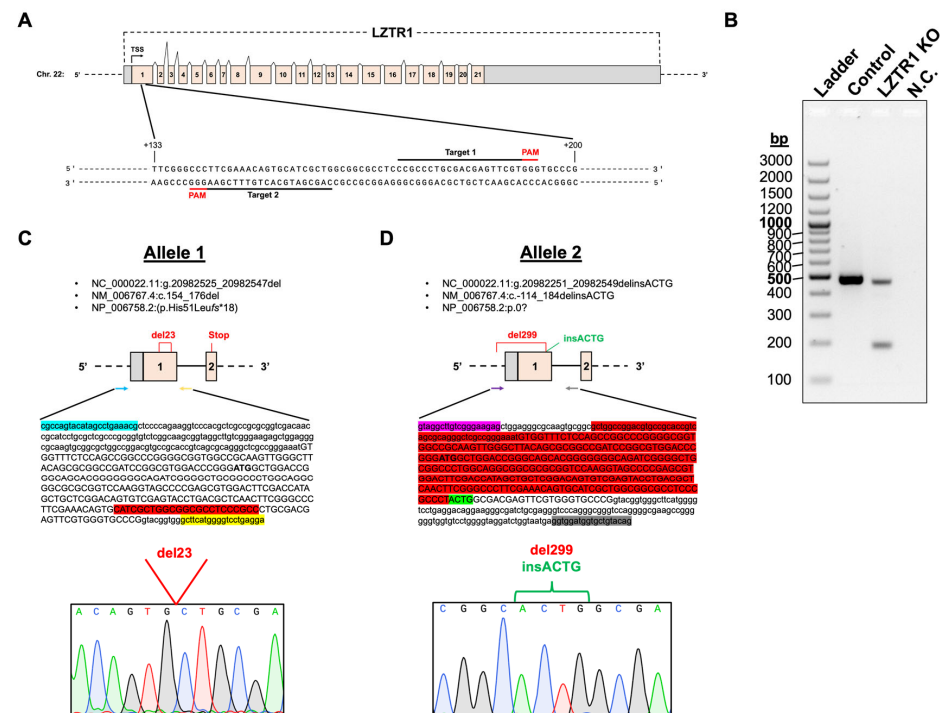


Figure 1. Generation and genetic validation of *LZTR1* KO Hep3B cells. (A) Schematic overview of the human *LZTR1* gene showing exon–intron organization and CRISPR/Cas9n target sites within exon 1, including sgRNA target sequences (Target 1 and Target 2) and corresponding protospacer adjacent motif (PAM) sites. (B) Agarose gel electrophoresis of PCR products amplified from the *LZTR1* exon 1 region. A single band at the expected size (486 bp) was detected in control cells, whereas two distinct bands corresponding to allele-1 (upper band) and allele-2 (lower band) were detected in *LZTR1* KO cells. (C) Molecular characterization of allele-1, showing a 23 bp deletion (del23) within exon 1. Primer binding sites used for PCR amplification are indicated by colored arrows (blue, forward; yellow, reverse). The deleted region is highlighted in red in the sequence alignment, and representative Sanger sequencing chromatograms are shown below. (D) Molecular characterization of allele-2, showing a 299 bp deletion (del299) accompanied by an ACTG insertion (insACTG) within exon 1. Primer binding sites are indicated by colored arrows (purple, forward; black, reverse). Deleted (red) and inserted (green) sequences are highlighted, with representative Sanger sequencing chromatograms shown below. TSS: transcription start site; bp: base pair; N.C.: non-template control.

Following clonal selection, genomic PCR analysis of edited Hep3B cells revealed two distinct amplicons absent in control cells (Figure 1B). Based on their relative sizes, the larger PCR product was designated as allele-1, and the smaller product as allele-2. Sanger sequencing of allele-1 demonstrated a 23-base pair (bp) deletion within exon 1 of *LZTR1* (Figure 1C). This variant corresponds to NC_000022.11:g.20982525_20982547del and NM_006767.4:c.154_176del, resulting in a frameshift with a predicted premature termination codon at the protein level (NP_006758.2:p.His51Leufs*18). In contrast, sequencing analysis of allele-2 revealed a larger genomic alteration consisting of a 299 bp deletion with an accompanying ACTG insertion at the target locus (Figure 1D). This sequence variation was annotated as NC_000022.11:g.20982251_20982549delinsACTG at the genomic level and as NM_006767.4:c.-114_184delinsACTG at the transcript level. Due to disruption of the 5' untranslated region (UTR) and the coding sequence, including the transcription start site, the corresponding protein-level annotation was assigned as NP_006758.2:p.0?. No sequence variation was detected at the targeted locus in control cells.

To assess how LZTR1 loss affects RAS/MAPK signaling in Hep3B cells, the levels and activation states of key pathway proteins were evaluated (Figure 2). Levels of canonical RAS proteins (pan-RAS) increased to 4.15-fold of control in LZTR1 KO cells ($p < 0.0001$; Figure 2B), while RIT1 increased to 4.86-fold of control ($p < 0.0001$; Figure 2C). In line with these changes, RAF1 phosphorylation at the activating site Ser338 increased to 1.37-fold of control ($p = 0.042$; Figure 2D), whereas phosphorylation at the inhibitory site Ser259 remained 0.98-fold of control and was not significantly different between groups ($p = 0.8176$). Further along the pathway, MEK1/2 phosphorylation at Ser217/221 increased to 1.60-fold of control ($p = 0.0005$; Figure 2E), ERK1/2 phosphorylation at Thr202/Tyr204 increased to 2.96-fold of control ($p < 0.0001$; Figure 2F), and RSK1–3 phosphorylation at Ser380 increased to 1.65-fold of control ($p < 0.0001$; Figure 2G). Together, these findings document increased RAS family protein abundance accompanied by elevated phosphorylation across the RAF-MEK-ERK-RSK cascade in LZTR1 KO Hep3B cells.

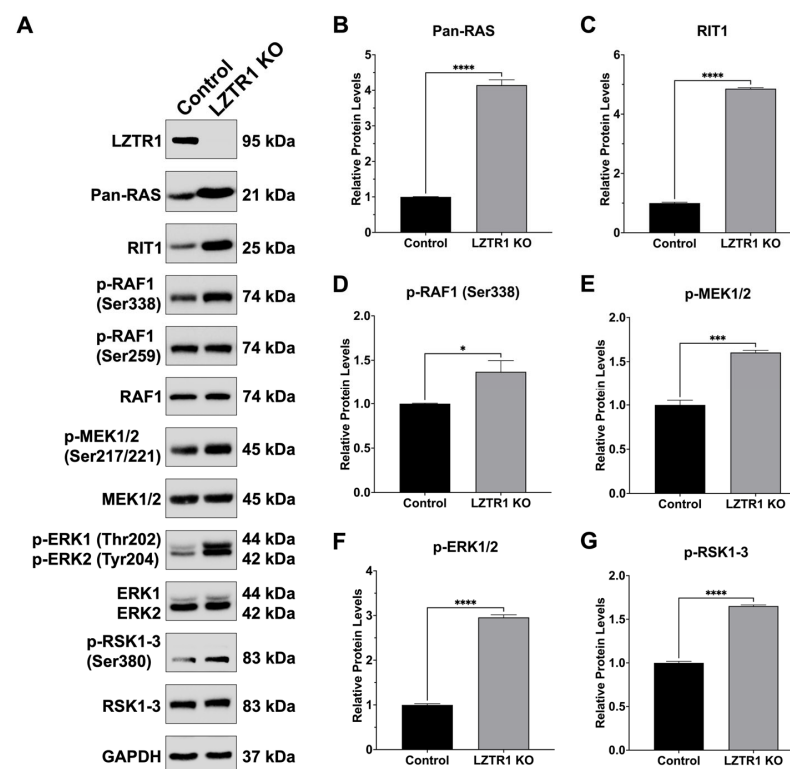


Figure 2. Analysis of RAS/MAPK pathway components in control and LZTR1 KO Hep3B cells. (A) Representative Western blot images showing LZTR1 expression, canonical RAS proteins (pan-RAS), RIT1, and key components of the RAS/MAPK signaling cascade, including total and phosphorylated (p) forms of RAF1 (Ser338 and Ser259), MEK1/2 (Ser217/221), ERK1/2 (Thr202/Tyr204), and RSK1–3 (Ser380) in control and LZTR1 KO Hep3B cells. (B–G) Densitometric analysis of Western blot band intensities, normalized to the corresponding GAPDH levels and expressed relative to control cells. Data represent mean $\pm$ SEM from three independent biological replicates. Statistical significance was determined using unpaired two-tailed *t*-tests. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$.

2.2. RNA-Sequencing (RNA-Seq) Analysis Reveals LZTR1-Dependent Transcriptional Alterations in Hep3B Cells

To define the transcriptomic consequences of LZTR1 loss in Hep3B cells, high-depth, paired-end transcriptome-wide bulk RNA-Seq analysis was performed. Inspection of LZTR1 transcript alignments revealed expression of the allele harboring the 23-nucleotide deletion previously identified by Sanger sequencing (allele-1) (Figures 1C and S1). In contrast, no transcript originating from allele-2 was detected, consistent with the predicted frameshift and absence of expression from this allele.

Comparative differential expression analysis between control and LZTR1 KO cells identified a total of 77 genes with statistically significant expression changes exceeding a $|\log_2 \text{fold change}| > 2.0$ threshold (Table S1). Among these differentially expressed genes (DEGs), 37 were upregulated and 40 were downregulated in LZTR1-deficient cells relative to controls. Hierarchical clustering of these genes revealed a clear separation between control and LZTR1 KO transcriptomes (Figure 3A).

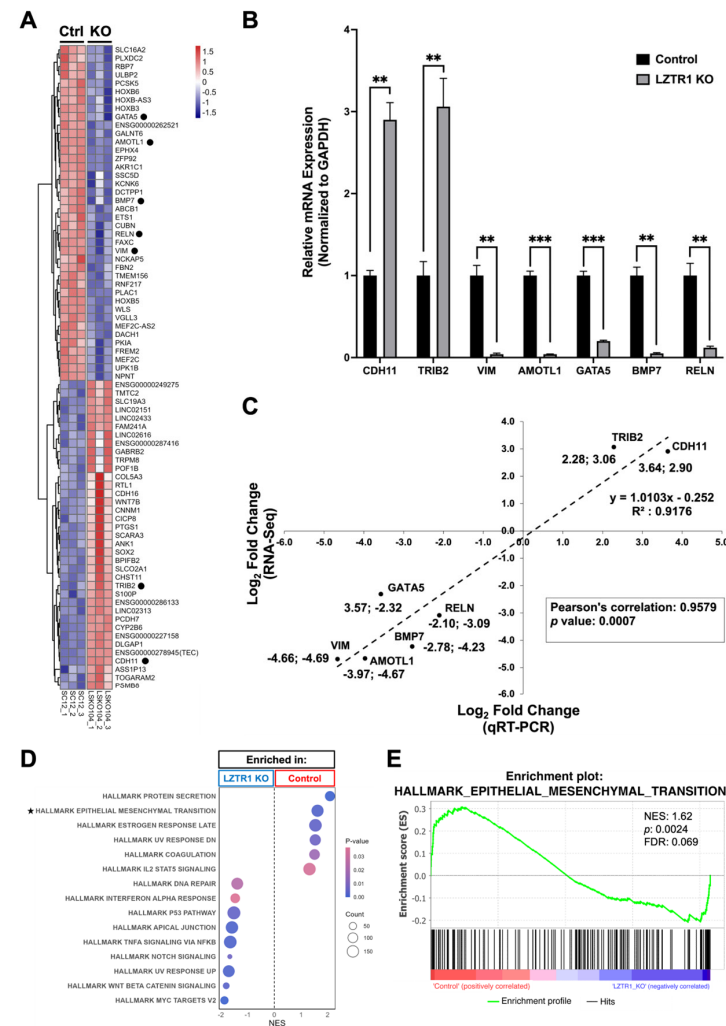


Figure 3. Transcriptomic alterations induced by LZTR1 loss in Hep3B cells. (A) Heatmap representation of DEGs identified by RNA-Seq in control and LZTR1 KO Hep3B cells. A total of 77 genes exhibiting significant expression changes ($|\log_2 \text{fold change}| > 2.0$) were identified, of which 37 were upregulated and 40 were downregulated in LZTR1 KO cells. Genes selected for qRT-PCR validation are indicated by black dots. (B) qRT-PCR validation of selected DEGs identified by RNA-Seq. Data are presented as log₂ fold changes relative to control cells. (C) Correlation analysis between RNA-Seq and qRT-PCR results showing log₂ fold change values for the validated genes. Pearson's correlation coefficient and the corresponding p value are indicated, demonstrating strong concordance between the two approaches. (D) GSEA of Hallmark gene sets of the MSigDB comparing control and LZTR1 KO Hep3B cells. The bubble plot summarizes significantly enriched pathways (nominal $p < 0.05$, FDR < 0.25), with positive normalized enrichment scores (NES) indicating enrichment in control cells and negative NES indicating enrichment in LZTR1 KO cells. The EMT gene set is indicated by a star symbol in the plot. (E) GSEA enrichment plot for the HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION gene set, which was significantly enriched in control cells relative to LZTR1 KO cells. The running enrichment score, gene set distribution, and enrichment statistics are shown. **: $p < 0.01$, ***: $p < 0.001$.

To experimentally validate the RNA-Seq findings, seven representative genes were selected based on their differential expression patterns and analyzed by quantitative real-time PCR (qRT-PCR). qRT-PCR analysis confirmed both the direction and magnitude of expression changes observed in the RNA-Seq data (Figure 3B). Correlation analysis of $\log_2$ fold change values derived from RNA-Seq and qRT-PCR demonstrated a strong positive association (Pearson's correlation coefficient = 0.9579; $p = 0.0007$), indicating high concordance between the two approaches (Figure 3C).

To further elucidate biological processes associated with LZTR1 presence or absence, Gene Set Enrichment Analysis (GSEA) was performed using the Hallmark gene sets of the Molecular Signatures Database (MSigDB). Among the 50 Hallmark gene sets analyzed, six were significantly enriched in control samples, whereas nine gene sets were significantly enriched in LZTR1 KO samples based on a nominal p value < 0.05 and a false discovery rate (FDR) < 0.25 (Figure 3D). Notably, the HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION gene set showed preferential enrichment in control cells (Figure 3D,E). Collectively, RNA-Seq analysis identified a discrete set of DEGs and associated pathway-level enrichment patterns that distinguish control and LZTR1 KO Hep3B cells.

2.3. LZTR1 Loss Alters EMT-Associated Protein Levels and Vimentin Distribution in Hep3B Cells

To assess whether LZTR1 deficiency alters EMT-related protein levels in Hep3B cells, the abundance of key EMT transcription factors, epithelial junction proteins, and mesenchymal markers was examined (Figure 4). Analysis of EMT-associated transcription factors revealed marked alterations upon LZTR1 loss. ZEB1 protein levels were significantly reduced in LZTR1 KO cells, decreasing to 0.73-fold of control ($p < 0.0001$; Figure 4B). In contrast, Snail protein levels were markedly increased following LZTR1 loss, reaching 4.69-fold of control ($p < 0.0001$; Figure 4C). Evaluation of epithelial markers further demonstrated a differential response to LZTR1 depletion. E-Cadherin protein levels showed a modest and non-significant increase to 1.17-fold of control ($p = 0.6152$; Figure 4D). In contrast, Claudin-1 protein abundance was significantly reduced to 0.41-fold of control cells ($p = 0.0032$; Figure 4E). Assessment of mesenchymal markers revealed additional pronounced alterations associated with LZTR1 loss. Vimentin protein levels were reduced to near-undetectable levels compared with control cells ($p = 0.0003$; Figure 4F). Vimentin expression was additionally examined in an independent LZTR1 KO clone (LSKO-98), which similarly exhibited loss of detectable Vimentin protein (Figure S2). Conversely, N-Cadherin abundance was markedly increased, reaching 4.34-fold of control ($p < 0.0001$; Figure 4G). Notably, this divergent regulation of mesenchymal markers occurred alongside Snail induction, indicating a non-canonical remodeling of EMT-associated markers upon LZTR1 loss. Collectively, these data demonstrate that LZTR1 loss is accompanied by substantial remodeling of EMT-associated protein profiles in Hep3B cells.

Given the pronounced reduction in Vimentin protein levels observed by immunoblotting, immunofluorescence (IF) analysis was performed to examine Vimentin subcellular distribution in control and LZTR1 KO Hep3B cells. IF analysis revealed marked differences in Vimentin protein distribution between control and LZTR1 KO Hep3B cells. In control cells, Vimentin displayed a strong and well-defined filamentous cytoplasmic staining pattern in the far-red channel (Figure 5A). In contrast, LZTR1 KO cells exhibited a pronounced loss of Vimentin signal, with the filamentous network largely absent and only weak, sparse punctate staining detectable (Figure 5A). Phalloidin staining demonstrated preserved actin cytoskeletal organization and overall cell morphology in both conditions. Secondary-only negative control stainings showed no detectable far-red signal in either control or LZTR1 KO cells, while phalloidin and DAPI signals remained clearly visible

(Figure 5B). These findings confirm the specificity of Vimentin immunostaining and further support the reduced Vimentin protein levels in LZTR1-deficient Hep3B cells.

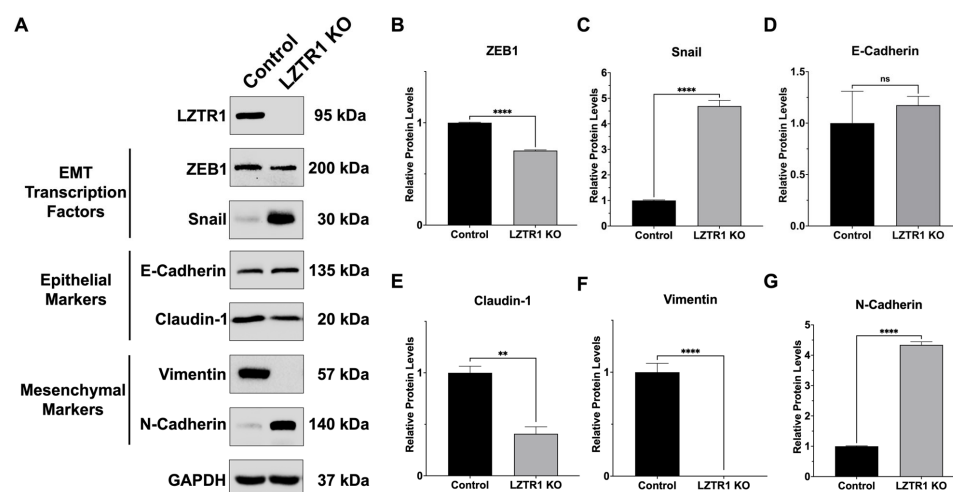


Figure 4. EMT-associated protein levels in control and LZTR1 KO Hep3B cells. (A) Representative Western blots showing EMT-associated transcription factors (ZEB1, Snail), epithelial markers (E-Cadherin, Claudin-1), and mesenchymal markers (Vimentin, N-Cadherin) in control and LZTR1 KO Hep3B cells. GAPDH was used as the loading control. Representative blots are shown; quantification includes all replicates. (B–G) Densitometric quantification of (B) ZEB1, (C) Snail, (D) E-Cadherin, (E) Claudin-1, (F) Vimentin, and (G) N-Cadherin, normalized to the corresponding GAPDH signals and expressed relative to control cells. Data represent mean $\pm$ SEM from three independent biological replicates ($n = 3$). Statistical significance was determined using unpaired two-tailed t -tests. ns: not significant, ** $p < 0.01$, **** $p < 0.0001$.

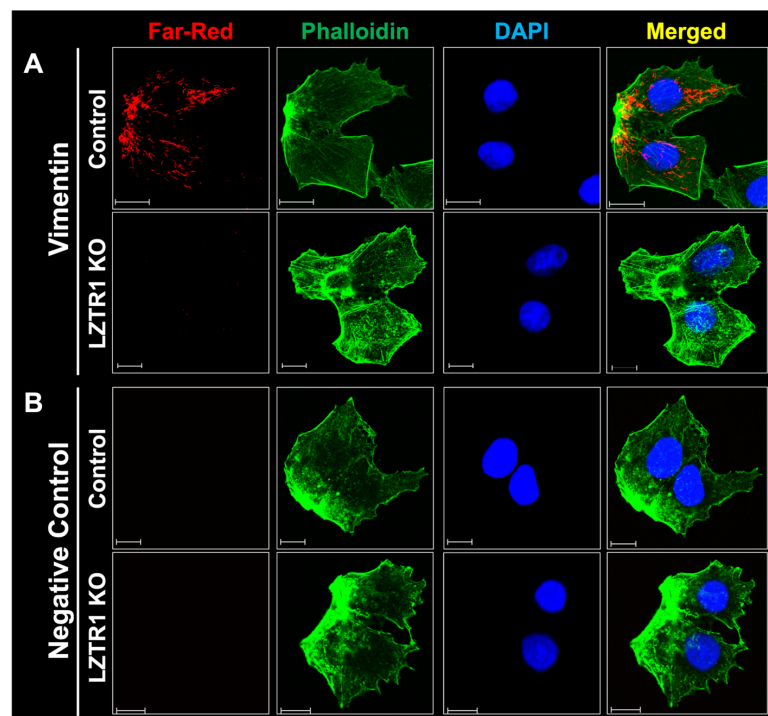


Figure 5. IF analysis of Vimentin in control and LZTR1 KO Hep3B cells. (A) Representative IF images of control and LZTR1 KO Hep3B cells showing Vimentin (far-red), Alexa Fluor 488-conjugated phalloidin (green), DAPI (blue), and merged images. Filamentous Vimentin staining is evident in control cells but markedly reduced in LZTR1 KO cells. (B) Secondary-only negative controls show no detectable far-red signal, confirming antibody specificity. Images were acquired using a 63 $\times$ objective under identical imaging conditions. Scale bars: 10 μ m.

2.4. LZTR1 Deficiency Reduces Migratory and Invasive Capacities of Hep3B Cells

To determine whether LZTR1-dependent alterations in EMT marker expression are associated with functional changes in cell motility and invasiveness, we assessed the migratory and invasive behavior of control and LZTR1 KO Hep3B cells using complementary functional assays. As an initial evaluation of collective cell migration, a wound-healing (scratch) assay was performed and wound closure was monitored over 72 h (Figure 6A). Quantification of the remaining wound area, normalized to the initial wound area at 0 h, showed consistently delayed wound closure in LZTR1 KO cells compared to control cells (Figure 6B). At 24 h, the residual wound area was $50.18 \pm 1.94\%$ in control monolayers, whereas LZTR1 KO monolayers retained a larger open area ($72.57 \pm 1.16\%$). At 48 h, the residual wound area was $21.12 \pm 2.55\%$ in control cells and $59.67 \pm 3.66\%$, in LZTR1 KO cells. At 72 h, control cells reached near-complete wound closure ($0.72 \pm 0.20\%$), whereas LZTR1 KO cells retained a substantial residual wound area ($36.73 \pm 2.75\%$).

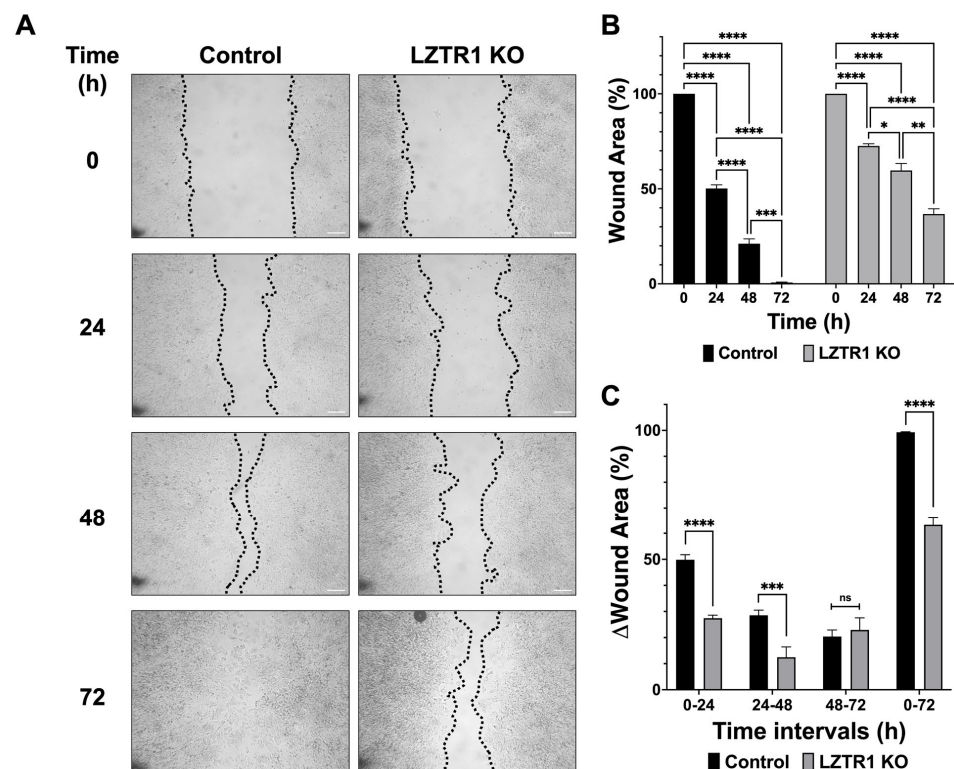


Figure 6. Wound-healing analysis in control and LZTR1 KO Hep3B cells. (A) Representative phase-contrast images of wound areas in control and LZTR1 KO Hep3B cell monolayers at 0, 24, 48, and 72 h following scratch generation. Images were acquired using a 4× objective; dashed lines indicate the wound margins. Scale bars, 500 μm. (B) Quantification of the remaining wound area over time, expressed as a percentage of the initial wound area at 0 h. Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. (C) Comparison of wound closure across consecutive time intervals (0–24 h, 24–48 h, and 48–72 h) between control and LZTR1 KO cells. Data represent mean ± SEM from three independent experiments, each performed in triplicate ($n = 9$ per condition). Statistical significance was determined using unpaired two-tailed t -tests with Welch's correction. Statistical significance is indicated in the graphs. ns: not significant. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

To directly compare closure kinetics between genotypes, wound area changes were examined across consecutive time intervals (Figure 6C). During the 0–24 h interval, control cells closed $49.82 \pm 1.94\%$ of the wound, compared with $27.43 \pm 1.16\%$ closure in LZTR1 KO cells ($p < 0.0001$). During the 24–48 h interval, wound closure reached $29.05 \pm 1.80\%$ in control cells and $12.90 \pm 3.97\%$ in LZTR1 KO cells ($p = 0.0005$). During the late 48–72 h

interval wound closure was $20.40 \pm 2.50\%$ in control cells and $22.94 \pm 4.63\%$ in LZTR1 KO cells ($p = 0.6382$). Overall, cumulative closure over 72 h reached $99.28 \pm 0.20\%$ in control cells and $63.27 \pm 2.75\%$ in LZTR1 KO cells ($p < 0.0001$).

To further evaluate whether loss of LZTR1 affects the migratory and invasive behavior of Hep3B cells, transwell migration and invasion assays were performed (Figure 7). Quantitative analysis revealed a significant reduction in the migratory capacity of LZTR1 KO cells compared to control cells. At 72 h, LZTR1-deficient cells exhibited an approximately 1.6-fold decrease in migration, with a mean of 7.5 migrated cells per field compared with 12.5 cells per field in control cells ($p < 0.0001$; Figure 7A). In invasion assays, LZTR1 loss was associated with reduced the invasive capacity of Hep3B cells. In Matrigel-based invasion assays conducted over 72 h, LZTR1 KO cells displayed an approximately 5.3-fold reduction in invasion, with 4.7 invading cells per field compared to 24.9 cells per field in control cells ($p < 0.0001$; Figure 7B). Collectively, transwell migration and invasion assays demonstrated reduced migratory and invasive capacities in LZTR1 KO Hep3B cells compared with control cells.

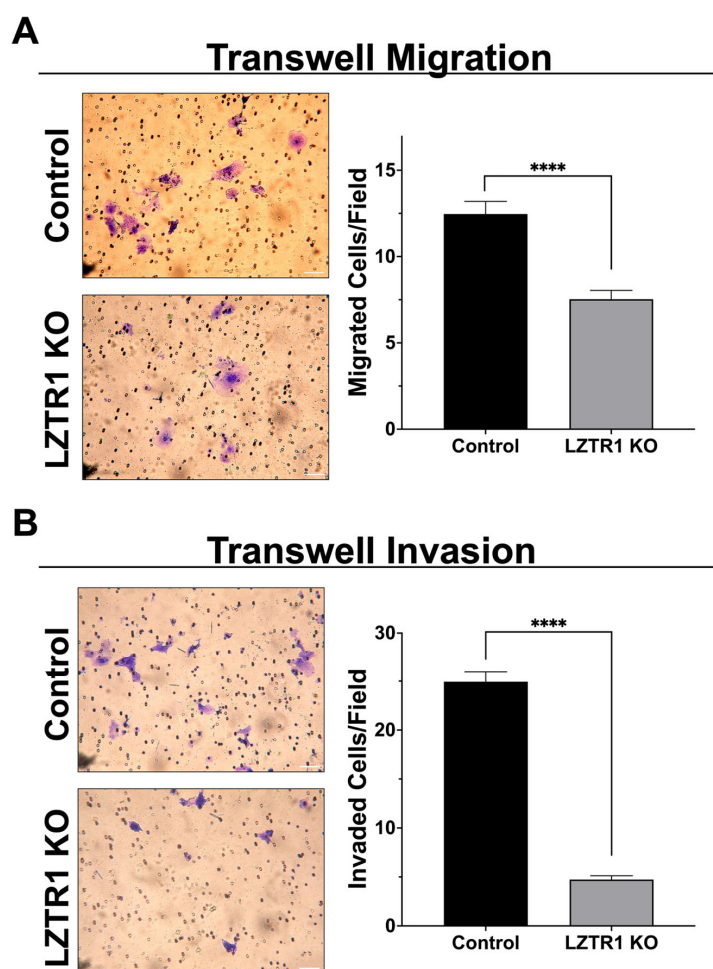


Figure 7. Transwell migration and invasion analysis of control and LZTR1 KO Hep3B cells. (A) Representative images of migrated Hep3B cells following transwell migration assays performed for 72 h in control and LZTR1 KO cells. (B) Representative images of invaded Hep3B cells obtained from Matrigel-coated transwell invasion assays conducted for 72 h in control and LZTR1 KO cells. Images were acquired using a $10\times$ objective; scale bar: $200\ \mu\text{m}$. Non-stained circular structures correspond to membrane pores. Quantitative analyses were based on three independent experiments, with cell counts obtained from five randomly selected microscopic fields per experiment. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using an unpaired two-tailed t -test with Welch's correction. ****: $p < 0.0001$.

3. Discussion

HCC, which constitutes the vast majority of primary liver cancers, continues to rank among the leading causes of cancer-related mortality worldwide, underscoring the aggressive biology and limited therapeutic durability of this disease [1]. Tumor progression and metastatic dissemination in HCC are driven by profound intratumoral heterogeneity and dynamic phenotypic plasticity, enabling malignant cells to transition between epithelial, hybrid, and mesenchymal states [23]. In this framework, epithelial–mesenchymal plasticity (EMP) has emerged as a central regulator of invasion, therapeutic resistance, and disease progression, extending beyond the constraints of a binary EMT model [24]. Here, we use EMP to denote reversible intermediate or hybrid epithelial/mesenchymal (E/M) phenotypic states, rather than a complete and unidirectional EMT. In transcriptomic studies, such intermediate states are commonly quantified along an epithelial–hybrid–mesenchymal spectrum using established EMT/EMP scoring frameworks, including the 76-gene signature (76GS), Kolmogorov–Smirnov (KS), and multinomial logistic regression (MLR) metrics. These approaches provide a principled means to distinguish partial or hybrid EMT programs from a fully mesenchymal transcriptional state [25,26]. Recent single-cell transcriptomic studies have further underscored EMP as a defining feature of aggressive malignant states in HCC [27]. Despite these advances, the upstream mechanisms governing EMP in HCC remain incompletely understood, highlighting a critical gap in current mechanistic models.

LZTR1 has been implicated as a tumor suppressor in HCC, and reduced LZTR1 expression has been associated with adverse clinical features, including poor prognosis and early recurrence, in patient-based studies. Previous studies, predominantly relying on partial LZTR1 knockdown approaches, have suggested that reduced LZTR1 enhances proliferation and migration through MAPK pathway activation [16,18,19]. However, the transcriptional programs that accompany LZTR1 loss, as well as the cellular consequences of complete genetic ablation, remain insufficiently characterized. To overcome these limitations, we established a CRISPR/Cas9-mediated LZTR1 KO Hep3B model and systematically integrated signaling, transcriptomic, and functional motility analyses.

Consistent with the established role of LZTR1 as a negative regulator of RAS-family protein stability [10,11,13], complete LZTR1 loss resulted in accumulation of canonical RAS proteins and RIT1, accompanied by robust activation of the RAF–MEK–ERK signaling cascade and its downstream effectors, including RSK1-3 (Figure 2). These findings align with seminal studies identifying LZTR1 as a key modulator of RAS/MAPK signaling output [10,11,13] and are particularly relevant in HCC, where MAPK hyperactivation frequently occurs independently of oncogenic RAS mutations [7]. Notably, the consequences of LZTR1 loss on signaling events downstream of ERK, such as RSK activation, have been explored in only a limited number of studies and not previously in an HCC context [28,29]. In this setting, our findings extend prior observations by demonstrating that complete LZTR1 loss in HCC cells is associated with enhanced ERK-to-RSK signal propagation, raising the possibility that augmented MAPK signaling may contribute to downstream transcriptional changes (Figure 2A,G).

Although MAPK activation is frequently associated with EMT and invasive behavior in HCC, ERK pathway output is highly context-dependent. Accordingly, ERK activation can be uncoupled from cell motility when key cytoskeletal effectors are transcriptionally suppressed or when parallel signaling constraints dominate. In our Hep3B model, complete LZTR1 loss robustly increased phosphorylation across the RAF–MEK–ERK–RSK cascade (Figure 2). This signaling activation was accompanied by marked suppression of VIM at the transcript level, as detected by RNA-Seq and qRT-PCR (Figure 3). At the protein level, Vimentin was almost completely lost, as demonstrated by Western blotting and IF analyses

(Figures 4 and 5). Vimentin intermediate filaments are key determinants of mesenchymal cell mechanics and migratory capacity. Accordingly, depletion of this cytoskeletal module may contribute to the reduced wound closure, migration, and invasion observed in LZTR1 KO cells (Figures 6 and 7), despite pronounced upstream MAPK hyperactivation [30]. Importantly, ERK/RSK pathway hyperactivation and Vimentin repression occur concurrently in LZTR1-deficient Hep3B cells. However, our data do not establish a causal relationship between these events. While the coexistence of enhanced epithelial features with reduced motility may appear paradoxical, our findings are consistent with an EMP-associated state and highlight an important area for further mechanistic investigation.

The key unresolved question is how sustained MAPK hyperactivation can coexist with suppression of VIM transcription in this cellular context. Our data indicate that the dominant regulatory change occurs upstream of protein stability, because VIM is suppressed at the mRNA level and the protein loss mirrors this transcriptional deficit (Figures 3–5). At present, it remains unclear whether ERK/RSK activity directly drives VIM repression or whether ERK hyperactivation instead represents a parallel consequence of RAS protein accumulation following LZTR1 loss. Importantly, ERK signaling is subjected to strong feedback regulation and adaptive transcriptional programs, including DUSP/SPRY-mediated feedback. As a result, sustained ERK hyperactivation can produce nonintuitive phenotypic outputs, depending on cellular lineage and signaling network architecture [31]. Within this framework, our data support a testable model in which LZTR1 loss is associated with a signaling-state “uncoupling”, characterized by maintained ERK/RSK activation alongside selective attenuation of the EMT cytoskeletal arm concurrent with VIM transcriptional repression and broader EMP remodeling (Figures 3–7) [32].

To establish a causal link underlying this working model, future experiments should examine whether pharmacologic inhibition of MEK/ERK signaling restores VIM mRNA and protein expression. A key question is whether normalization of ERK signaling is sufficient to rescue cell motility. In parallel, it will be important to identify ERK-responsive transcriptional and post-transcriptional regulators of VIM in HCC cells. Integration of these regulatory factors with the RNA-Seq dataset will clarify whether VIM repression represents an ERK-dependent adaptive transcriptional program or instead reflects an ERK-independent component of the LZTR1-deficient cellular state.

In addition to the observed alterations in MAPK signaling, a major contribution of this study is the identification of an LZTR1-dependent transcriptional program in HCC cells. RNA-Seq analysis identified 77 DEGs that clearly separated control and LZTR1 KO Hep3B cells by hierarchical clustering (Figure 3A; Table S1). These DEGs formed coherent functional modules, including extracellular matrix regulation, epithelial polarity, transcriptional control, and non-coding RNA-mediated adaptation. Within this transcriptional landscape, Nephronectin (*NPNT*) was markedly downregulated, consistent with its reported role in tumor–matrix interactions and metastatic competence in HCC [33]. In contrast, expression of the polarity-associated POF1B actin-binding protein (*POF1B*) was increased, in line with its involvement in epithelial polarity and junctional organization [34]. Additional changes included reduced expression of ETS proto-oncogene 1 (*ETS1*), a regulator of invasion-associated transcriptional programs, and polypeptide N-acetylgalactosaminyltransferase 6 (*GALNT6*), which has been linked to aggressive HCC phenotypes and therapy adaptation, in LZTR1 KO cells [35,36]. LZTR1 loss also affected non-coding RNA layers, as evidenced by downregulation of HOXB-AS3 and upregulation of LINC02616, both previously associated with HCC prognosis [37]. Conversely, the LZTR1-repressing lncRNA LL22NC03-N14H11.1 (ENSG00000272872) was not detected among the DEGs, arguing against reciprocal transcriptional regulation by LZTR1 in this setting [19].

To strengthen the interpretation of the RNA-Seq findings, seven representative DEGs were independently validated by qRT-PCR (Figure 3B). Among these genes, Cadherin-11 (*CDH11*) was upregulated, consistent with its established role in adhesion remodeling and cell–microenvironment interactions in liver pathology and epithelial malignancies [38–40]. In parallel, increased expression of tribbles pseudokinase 2 (*TRIB2*), a stress-responsive signaling adaptor, suggests activation of adaptive survival and stress-response pathways [41,42]. By contrast, vimentin (*VIM*) was robustly downregulated, despite its central role in mesenchymal cytoskeletal architecture and its association with aggressive HCC phenotypes [43]. Consistent with altered junctional regulation, reduced expression of angiomin-like 1 (*AMOTL1*) further indicates disrupted coupling between cell junctions and Hippo/YAP–TAZ signaling, a pathway central to epithelial growth control in HCC [44,45]. Additional coordinated changes, including downregulation of GATA binding protein 5 (*GATA5*), reelin (*RELN*), and bone morphogenetic protein 7 (*BMP7*) point to attenuation of differentiation-associated and tumor-suppressive programs, together with context-dependent rebalancing of the TGF- β /BMP axis [46–48]. Importantly, RNA-Seq and qRT-PCR measurements showed a strong positive correlation (Figure 3C), confirming the robustness of these transcriptional changes. Collectively, these findings indicate that LZTR1 loss drives coordinated reprogramming of adhesion, signaling, and differentiation pathways, thereby reshaping epithelial cell-state control in Hep3B cells.

At the pathway level, Hallmark GSEA integrated these gene-level changes into coordinated biological programs (Figure 3D,E). The HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION gene set was enriched in control cells, consistent with the observed suppression of *VIM* in LZTR1 KO cells. By contrast, enrichment of the HALLMARK_APICAL_JUNCTION and HALLMARK_DNA_REPAIR gene sets in LZTR1 KO cells indicates rewiring of epithelial polarity programs and activation of compensatory genome-maintenance responses [49,50]. Although LZTR1 loss has been shown to enhance EMT responsiveness in other cancer models [51], our data show that in Hep3B cells, these pathway-level changes occur without execution of a fully mesenchymal EMT program. Instead, they coincide with selective suppression of Vimentin-dependent cytoskeletal modules, reinforcing a non-canonical EMP-associated state.

To assess the clinical relevance of our findings, we analyzed LZTR1 and Vimentin expression in the Cancer Genome Atlas (TCGA)—Liver Hepatocellular Carcinoma (LIHC) cohort ($n = 371$). Correlation analysis revealed a positive association between LZTR1 and Vimentin expression (Spearman $\rho = 0.33$, $p = 1.31 \times 10^{-10}$; Figure S3A), supporting the association between LZTR1 and Vimentin observed in our in vitro Hep3B model. Furthermore, LZTR1 expression showed positive correlations with mesenchymal markers, including ZEB1 ($\rho = 0.33$) and CDH2 ($\rho = 0.16$). In contrast, inverse correlations were observed with epithelial markers CDH1 ($\rho = -0.11$) and CLDN1 ($\rho = -0.14$) (Figure S3B; Table S5). Stratification of tumors by median LZTR1 expression further supported this association. Low-LZTR1 tumors exhibited significantly reduced Vimentin expression ($p = 9.10 \times 10^{-8}$) and EMT scores ($p = 3.08 \times 10^{-13}$) compared to high-LZTR1 tumors (Figure S3C,D). Together, these data indicate a shift toward a less mesenchymal transcriptional state in low-LZTR1 tumors and suggest that the LZTR1–VIM relationship identified in Hep3B cells may extend to human HCC.

Reports on LZTR1 loss indicate pronounced context dependence across tumor types. In lung adenocarcinoma models, LZTR1 deficiency has been shown to increase RAS accumulation and enhance sensitivity to EMT-inducing cues, which has been linked to increased metastatic behavior [51]. In HCC models, reduced LZTR1 expression has likewise been associated with enhanced proliferation and migration, largely through activation of the RAS–RAF–MEK–ERK signaling cascade [16,18]. Our findings in the Hep3B LZTR1 KO model

diverge from these pro-migratory scenarios. Although we observe robust MAPK pathway hyperactivation (Figure 2), we do not detect a canonical Vimentin-high mesenchymal program. Instead, LZTR1 loss induces a modular pattern of EMT-associated remodeling. This pattern is characterized by increased Snail and N-Cadherin and by profound suppression of Vimentin (Figures 4 and 5). Such uncoupled execution of EMT-associated modules is a defining feature of EMP rather than classical EMT. This interpretation is consistent with contemporary EMP frameworks that emphasize partial, hybrid, and context-dependent state transitions [24,52]. Within this framework, suppression of Vimentin is consistent with the reduced migratory and invasive behavior observed in LZTR1 KO Hep3B cells (Figures 6 and 7), given the established role of Vimentin in mesenchymal cell mechanics and motility [53]. However, our data do not establish whether ERK/RSK signaling is causally responsible for Vimentin repression in this setting.

This EMP-centered interpretation is further supported by the pronounced remodeling of EMT-associated proteins following LZTR1 depletion (Figures 4 and 5). LZTR1 KO Hep3B cells showed strong induction of Snail and N-Cadherin, indicating activation of core EMT-regulatory pathways involved in junctional remodeling and acquisition of mesenchymal traits (Figure 4A,C,G) [54,55]. In contrast, ZEB1 protein levels were reduced and Vimentin expression was almost completely lost at both transcript and protein levels, reaching near-undetectable abundance by Western blotting and IF (Figures 3–5). Claudin-1 levels were also significantly decreased, whereas E-Cadherin abundance remained unchanged (Figure 4A,D,E). Together, these changes define an EMP state that deviates from the classical “Vimentin-up/E-Cadherin-down” EMT model. Instead, they reflect selective activation of distinct EMT-associated modules. Consistent with EMP concepts, our marker remodeling reflects a hybrid/discordant EMT program rather than coordinated induction of a canonical high-Vimentin, low-E-Cadherin mesenchymal state [56,57]. Importantly, loss of Vimentin was consistently validated across multiple experimental platforms, including RNA-Seq (Figure 3A), qRT-PCR (Figure 3B), Western blotting (Figure 4A,F), and IF analysis (Figure 5A). This concordance confirms that Vimentin suppression represents a robust and reproducible consequence of LZTR1 depletion. Given the central role of Vimentin in mesenchymal cytoskeletal organization and cell motility [54,55], its loss is consistent with the reduced migration and invasion observed in LZTR1 KO Hep3B cells (Figures 6 and 7), positioning this phenotype within an EMP-associated state that warrants further mechanistic dissection.

While the integrated signaling, transcriptomic, and functional analyses presented here provide a coherent framework linking LZTR1 loss to altered epithelial state control and cell motility in Hep3B cells, several limitations should be acknowledged. All experimental results in this study were using a single HCC cell line (Hep3B). This model was selected because it is well characterized and widely used in mechanistic studies of MAPK signaling and epithelial–mesenchymal state regulation in liver cancer [58,59]. At the same time, Hep3B cells represent a specific biological context (HBV-positive and TP53-deficient), which can influence oncogenic signaling dynamics and EMT/EMP-associated programs [30,60]. Accordingly, the phenotypic consequences of complete LZTR1 loss described here should be interpreted within this defined cellular background. Importantly, our TCGA-LIHC analyses show that LZTR1 expression is significantly correlated with both Vimentin expression and EMT scores at the tumor level (Figure S2). However, these correlative findings do not replace experimental validation across additional HCC cell models, and broader generalizability will require future validation in independent HCC cell lines with distinct molecular backgrounds.

Collectively, these findings demonstrate that complete LZTR1 loss is associated with MAPK pathway hyperactivation and coincides with EMP remodeling characterized by

attenuated EMT execution and impaired motility. Importantly, while these data support linked changes, they do not demonstrate a causal ERK/RSK-to-Vimentin repression mechanism. Together, our results position LZTR1 as a context-dependent regulator of signaling-state coupling in HCC. These observations emphasize the importance of considering EMP, rather than strictly binary EMT models, when interpreting LZTR1-dependent phenotypes.

4. Materials and Methods

4.1. Cell Culture

Hep3B human HCC cells were used in all experiments. This cell line was originally established from the liver tumor tissue of an 8-year-old male patient with HCC and harbors an integrated hepatitis B virus genome, as previously described [61]. The Hep3B cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (Catalog Number (Cat. No): 41966029; Thermo Fisher Scientific, Waltham, MA, USA), which is based on Earle's salt solution (EBSS), and supplemented with 10% fetal bovine serum (FBS) (Cat. No: A5256801; Thermo Fisher Scientific), 2 mM L-glutamine, 2.2 g/L sodium bicarbonate (NaHCO_3), 1% penicillin-streptomycin (Cat. No: 15140122; Thermo Fisher Scientific), and non-essential amino acids (Cat. No: 11140050; Thermo Fisher Scientific). Cells were cultured as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2 . Trypsin-EDTA solution (Cat. No: 25200056; Thermo Fisher Scientific) was used for cell detachment and passaging. For cryopreservation, cells were frozen in antibiotics-free freezing medium consisting of 20% FBS and 10% dimethyl sulfoxide (DMSO) (Cat. No: D8418; Sigma-Aldrich, St. Louis, MO, USA).

4.2. CRISPR/Cas9n Plasmid Design and Genome Engineering in Hep3B Cells

LZTR1 KO Hep3B cells were generated using a CRISPR/Cas9n-based genome-editing approach [62]. To identify target sequences for genome editing and to design sgRNAs, the human *LZTR1* gene was analyzed using the CCTop and CRISPOR online tools [63,64]. Two target sites within exon 1 of *LZTR1* were selected. Oligonucleotides were designed to include BbSI restriction enzyme recognition sites and synthesized by Macrogen (Seoul, Republic of Korea). These oligonucleotides were cloned into the PX462 expression vector (plasmid No: 62987; Addgene, Watertown, MA, USA). The oligonucleotides used to generate sgRNAs targeting the human *LZTR1* gene were as follows: sgRNA-1F (5'-caccgCCGCCCTGCGACGAGTTCGT-3'), sgRNA-1R (5'-aaacACGAACCTCGTCGAGGGCGGc-3'), sgRNA-2F (5'-caccgCAGCGATGCACTGTTTCGAA-3'), and sgRNA-2R (5'-aaacTTCGAAACAGTGCATCGCTGc-3'). Previously described scrambled control (SC) sgRNA sequences were used as negative control [65]. The oligonucleotides used to construct SC gRNAs were: SCsgRNA-F (5'-caccgTATTACTGATATTGGTGGG-3') and SCsgRNA-R (5'-aaacCCCACCAATATCAGTAATAc-3'). Double-stranded sgRNA oligonucleotides were generated by annealing complementary sgRNA pairs using T4 polynucleotide kinase (Cat. No: M2622; New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Annealed oligonucleotide pairs and the PX462 vector were digested with BbSI (Cat. No: ER1011; Thermo Fisher Scientific) and ligated to generate sgRNA expression constructs. Plasmids encoding *LZTR1*-targeting sgRNAs—were designated as PX462-*LZTR1*-1 and PX462-*LZTR1*-2, whereas SC gRNA plasmid was designated as PX462-SC.

To obtain *LZTR1* KO and control Hep3B clones, 1.5×10^6 Hep3B cells were co-transfected with 1.25 µg each of PX462-*LZTR1*-1 and PX462-*LZTR1*-2 or 2.5 µg of PX462-SC using FuGENE HD transfection reagent (Cat. No: E2311; Promega, Madison, WI, USA). Twenty-four hours after transfection, cells were trypsinized and seeded into 96-well plates at single-cell density (1 cell/well). Twelve hours later, puromycin selection was initiated by

adding 1.75 µg/mL puromycin (Cat. No: A11138; Thermo Fisher Scientific) to each well. After 72 h of selection, puromycin was removed and cells were maintained in puromycin-free medium. One week later, colonies were transferred sequentially to 24-well and then 12-well plates for expansion. After two weeks of culture, cells were trypsinized and divided into two fractions: one for continued culture and one for molecular validation. LZTR1 protein expression and genomic alterations were assessed by Western blotting and Sanger sequencing, respectively. Based on these analyses, one LZTR1 KO Hep3B clone (LSKO-104), which lacked WT LZTR1 expression due to biallelic genomic deletions, and one SC clone expressing WT LZTR1 (SC-12) were selected for all subsequent experiments.

4.3. Sanger DNA Sequencing

Genomic alterations in the *LZTR1* gene of selected LZTR1 KO and SC CRISPR/Cas9n Hep3B clones were analyzed by Sanger DNA sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genomic DNA was isolated from Hep3B clones using NucleoSpin DNA Isolation Kit (Cat. No: 740952; Macherey-Nagel, Düren, Germany). Exon 1 of *LZTR1* was amplified by PCR using gene-specific primers. All primers used for Sanger sequencing are listed in Table S2. PCR reactions were performed in a total volume of 25 µL, containing 0.1 µL Go Taq DNA polymerase (Cat. No: M3001; Promega), 5 µL 5× GoTaq Buffer, 1.3 µL genomic DNA (350 ng/µL), 1.5 µL MgCl₂ (25 mM), 0.6 µL 10 mM dNTP mixture (10 mM each), 0.6 µL of each primer (10 pmol/µL), and 15.3 µL nuclease-free water. PCR cycling conditions consisted of initial denaturation at 94 °C for 6 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 40 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 6 min and hold at 10 °C. PCR products were verified by agarose gel electrophoresis and purified using the HiPure Gel Extraction Kit (Cat. No: D211102; Magen, Guangzhou, China). To assess biallelic genomic alterations, purified PCR products were sub-cloned into the pJET1.2/blunt vector (Cat. No: K1232; Thermo Fisher Scientific) according to the manufacturer's instructions. Recombinant plasmids were transformed into competent bacteria, and plasmid DNAs from individual colonies were isolated and sequenced. Sanger sequencing reactions were performed using the Brilliant Dye Terminator Kit (Cat. No: BRD03-100, Nimagen, Nijmegen, The Netherlands) following the manufacturer's protocol. Sequencing data were analyzed using SnapGene Viewer software version (v.) 7.2 and compared with the corresponding human reference genomic sequences.

4.4. RNA Extraction, RNA-Seq, and Data Analysis

Total RNAs were extracted from three independent biological replicates of control and LZTR1 KO Hep3B cell pellets using the NucleoSpin RNA Isolation Kit (Cat. No: 740955.50; Macherey-Nagel) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and RNA integrity was assessed using 4150 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with an RNA integrity number (RIN) > 9.5 were used for downstream analyses. cDNA libraries were prepared from six samples using Stranded Total RNA Preparation with Ribo-Zero Plus Kit (Cat. No: 20040529; Illumina, San Diego, CA, USA). Libraries were subjected to 150 bp paired-end sequencing on an Illumina NovaSeq 6000 platform, generating at least 60 million uniquely mapped reads per sample. Raw RNA-Seq reads obtained from rRNA-depleted RNA-Seq libraries of control and LZTR1 KO samples were processed and analyzed using computational resources provided by the National Center for High Performance Computing (UHcM). Quality filtering and trimming were performed using Trimmomatic v. 0.39 [66] and fastp v. 0.24.0 [67]. Filtering steps included adapter removal, exclusion of reads with Phred quality scores < Q30, removal

of reads shorter than 50 bp, trimming of low-quality bases at read ends, and removal of Poly(A/T) tails. The FastQC v. 0.12.1 tool [68] was used for quality control before and after filtering, and MultiQC v. 1.27.1 [69] was used to generate consolidated quality reports. RNA-SeQC v. 2.4.2 [70] was additionally applied to evaluate mapping statistics, including the proportion of reads mapping to exonic and intergenic regions. High-quality RNA-Seq reads were aligned to the human reference genome (GRCh38.p14) obtained from GENCODE [71]. Subsequent computational analyses (including genomic alignment, transcript reconstruction, and differential expression profiling) were executed utilizing the HISAT2 v. 2.2.1 and StringTie v. 2.2.2 bioinformatic pipeline in accordance with the optimized methodological framework [72]. The alignment files (".bam" format) were analyzed using Integrative Genomics Viewer (IGV) v. 2.17.2. The alignment files underwent sorting and indexing via Samtools v. 1.21.1 [73]. Gene-level quantification was carried out using featureCounts v. 2.0.5 [74], establishing the count matrix essential for subsequent differential expression analyses. Differential gene expression analysis was conducted using DESeq2 v. 1.46.0 [75]. Genes with an adjusted p value < 0.05 , an absolute $\log_2$ fold change ($|\log_2FC| \geq 2.0$), and a $\log_2FC$ standard error ($\log_2FCSE$) < 1.0 were considered differentially expressed. Differential expression results were visualized using heatmap generated with DEBrowser v. 1.26.0 R package [76]. GSEA was performed using MSigDB v. 2024.1.Hs gene sets and GSEA software v. 4.4.0, with 1000 permutation, a Signal2Noise metric, and a weighted enrichment statistics [77,78]. Gene sets with a Benjamini–Hochberg corrected FDR value < 0.25 were considered significantly enriched, and gene sets with higher absolute NES values were prioritized. Hallmark gene set enrichment results were visualized using bubble plots generated with the ggplot2 R package v. 4.0.2 [79].

4.5. qRT-PCR Analysis

Expression levels of selected genes in the DEGs list were further examined by qRT-PCR. Primer pairs were designed using Primer3 software v. 4.1.0 [80] and synthesized by MacroGen. All primers used for qRT-PCR analyses are listed in Table S3. Total RNAs were isolated from control and LZTR1 KO Hep3B cells and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Cat. No: 1708891; Bio-Rad, Hercules, CA, USA). qRT-PCR reactions were performed on a Lightcycler 480 II system (Roche Diagnostics, Basel, Switzerland). Each reaction contained 2.5 ng of cDNA, 5 μ L BlasTaq qPCR Mastermix (G891; ABM, Richmond, BC, Canada), 0.3 μ L of each forward and reverse primers (10 pmol/ μ L), and 1.6 μ L nuclease-free water. All samples were analyzed in technical triplicates. Relative gene expression levels were calculated using the comparative $\Delta\Delta C_t$ method, with GAPDH used as the endogenous control for normalization. Fold changes were expressed as $2^{-(\Delta\Delta C_t)}$.

4.6. Protein Extraction and Western Blot Analysis

Proteins were extracted from cells using TNTE lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100 (Cat. No: X-100; Sigma-Aldrich), 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 $\times$ protease inhibitors cocktail (Cat. No: S8830-20TAB; Sigma-Aldrich). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Cat. No: 23225; Thermo Fisher Scientific). Equal amounts of protein (30 μ g per sample) were denatured in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.25 M Tris-Cl, pH 6.8, 10% SDS, 50% glycerol, 0.01% bromophenol blue) and separated on 7.5% or 10% SDS-PAGE gels, depending on target protein size. Proteins were transferred onto nitrocellulose membranes (Cat. No: 1620115; Bio-Rad). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20

(TBS-T). Primary and secondary antibodies listed in Table S4 were used according to the manufacturers' instructions. After antibody incubation, membranes were washed three times with TBS-T buffer. Protein signals were detected using enhanced chemiluminescence (ECL) (Cat. No: 1705060; Bio-Rad) and visualized with the ChemiDoc MP Imaging System (Bio-Rad). When required, membranes were incubated with acetic acid and reprobed with primary antibodies raised in a different host species to determine additional target proteins on the same membrane [81]. Band intensities were quantified using Image Lab software v. 6.1.0 (Bio-Rad). Target protein signals, including phosphorylated proteins, were normalized to the corresponding GAPDH signal to correct for variations in protein loading and transfer efficiency for each membrane. Normalized values were expressed relative to control samples, which were set to 1, and were used for all subsequent graphical and statistical analyses.

4.7. IF Staining and Confocal Microscopy

Cells were seeded on glass coverslips, fixed with 4% paraformaldehyde (PFA) (Cat. No: P6148, Sigma-Aldrich) in phosphate-buffered saline (PBS) for 15 min at room temperature (RT), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at RT, and blocked with 5% bovine serum albumin (BSA; Cat. No: BSA-1S; Capricorn Scientific, Ebsdorfergrund, Germany) for 1 h at RT. Coverslips were incubated overnight at 4 °C with anti-Vimentin primary antibody (Cat. No: 5741; Cell Signaling Technology, Danvers, MA, USA) at a 1:150 dilution. After washing three times with PBS, coverslips were incubated with far-red fluorophore-conjugated anti-rabbit Alexa Fluor-647 secondary antibody (Cat. No: ab150083; Abcam, Cambridge, UK) at a 1:750 dilution for 1 h at RT. All primary and secondary antibodies were diluted in the blocking solution. F-actin was visualized by incubation with Phalloidin-iFluor488 reagent (Cat. No: ab176753; Abcam) at a 1:1000 dilution for 30 min at RT. Following staining, coverslips were washed with PBS and mounted with ProLong Gold Anti-fade reagent containing 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No: P36935; Thermo Fisher Scientific). IF stainings were performed in three independent experiments. Images were acquired using a Leica SP8 laser-scanning confocal microscope (Leica, Wetzlar, Germany) and processed using LAS X software v. 3.5.5.

4.8. Wound-Healing Assay

Cells were seeded into 24-well plates and grown until reaching approximately 90% confluence. To inhibit cell proliferation, cells were treated with mitomycin C (10 µg/mL) (Cat. No: 51854; Cell Signaling Technology) for 2 h. Uniform linear wounds were generated in each well using a sterile pipette's tip. Wells were subsequently washed three times with PBS to remove detached cells, and 1 mL of complete culture medium was added to each well. Wound images were acquired at 0, 24, 48, and 72 h using an inverted light microscope under identical imaging conditions. Wound areas were quantified using ImageJ software v. 1.54p, and wound closure was calculated as the percentage reduction in wound area relative to the initial wound area at 0 h.

4.9. Transwell Migration and Invasion Assays

Cell migration and invasion assays were performed using permeable transwell inserts (Boyden chambers) with 8.0 µm pore size membranes in 24-well plates (Cat. No. 353097; Corning, Corning, NY, USA). For migration assays, cells were serum-starved in serum-free DMEM for 24 h, trypsinized, and resuspended in serum-free medium. Cells were seeded at a density of 2.0×10^5 cells/mL, and 0.4 mL of the cell suspension was added to the upper chamber of plates in triplicate. The lower chambers were filled with 0.6 mL DMEM supplemented with 10% FBS as a chemoattractant. For invasion assays, transwell inserts

were pre-coated with Matrigel (Cat. No: 356234; Sigma-Aldrich), and cells were seeded under the same conditions as described for migration assays. Following 72 h of incubation in a tissue culture incubator, non-migrated or non-invaded cells on the upper surface of the membrane were gently removed using a cotton swab. Cells that had migrated or invaded to the lower surface of the membrane were fixed with 4% PFA for 20 min, stained with 0.1% crystal violet for 20 min, and washed three times with distilled water. For quantification, five randomly selected microscopic fields per transwell insert per replicate were imaged using a light microscope. Migrated and invaded cells were quantified using ImageJ software, and the resulting values were used for statistical analysis.

4.10. TCGA Dataset Acquisition and Bioinformatic Analysis

Gene expression data derived from RNA-Seq for the TCGA LIHC cohort were retrieved from the UCSC Xena functional genomics explorer [82]. Expression values were obtained as log₂-transformed transcripts per million (TPM). The final dataset comprised 371 primary HCC tumor samples with complete gene expression profiles. Spearman's rank correlation coefficients (ρ) were calculated to evaluate the associations between LZTR1 transcript abundance and the expression of key EMT-related genes. For comparative analyses, samples were stratified into high-LZTR1 and low-LZTR1 subgroups using the median LZTR1 expression value as the dichotomization cutoff. An aggregate EMT score was derived for each sample by calculating the difference between the mean expression of mesenchymal markers (VIM, CDH2, SNAI1, ZEB1, FN1, TWIST1) and the mean expression of epithelial markers (CDH1, CLDN1), adapting established EMT scoring methodologies based on differential expression of epithelial and mesenchymal gene signatures [83,84]. Inter-group statistical comparisons were assessed using unpaired two-tailed Student's *t*-tests, given the large sample size of each group. All bioinformatic and statistical analyses were executed in the Python programming environment (v. 3.11.5; Python Software Foundation) utilizing the pandas v. 2.1.1 and SciPy v. 1.11.3 libraries [85].

4.11. Statistical Analyses

Statistical analyses were performed using GraphPad Prism software v. 9.5, except for RNA-Seq data analyses, which were conducted using dedicated bioinformatics pipelines as described above. Data were obtained from at least three independent biological experiments and are presented as mean $\pm$ standard error of the mean (SEM). Pearson's correlation analysis was used to assess the relationship between log₂ FC values derived from RNA-Seq and qRT-PCR analyses for the seven selected genes. Data from Western blotting, transwell migration, and invasion assays were analyzed using unpaired two-tailed *t*-tests. Changes in wound area over time were analyzed using two-way ANOVA, with experimental group and time as independent factors, followed by Tukey's post hoc test for multiple comparisons. Pairwise comparisons of wound closure between control and LZTR1 KO cells at individual time points were additionally performed using unpaired two-tailed *t*-tests with Welch's correction. A *p* value < 0.05 was considered statistically significant. Statistical significance is indicated in figures as follows: * for *p* < 0.05, ** for *p* < 0.01, *** for *p* < 0.001, and **** for *p* < 0.0001.

5. Conclusions

Although reduced LZTR1 expression has been associated with poor prognosis and early recurrence, our findings demonstrate that complete LZTR1 loss does not uniformly enhance migration or invasion in the Hep3B HCC model. Instead, LZTR1 ablation reveals a cellular state in which MAPK pathway hyperactivation coincides with suppression of Vimentin-dependent cytoskeletal programs and reduced motile capacity. From a

translational perspective, our findings provide mechanistic insight into how LZTR1 loss can reshape epithelial–mesenchymal state control in HCC cells, offering a biological framework to contextualize prior clinical observations.

By integrating signaling, transcriptomic, protein-level, and functional analyses, we propose an EMP-centered model in which LZTR1 acts as a contextual regulator of signaling–state coupling, rather than a unidirectional driver of classical EMT. In this framework, loss of LZTR1 induces coordinated but modular remodeling of epithelial-state programs, yielding phenotypic outcomes that diverge from simplified binary EMT paradigms.

Collectively, this study positions LZTR1 as a modulator of epithelial state balance in HCC cells and underscores the importance of EMP-based frameworks for interpreting heterogeneous and context-specific consequences of LZTR1 dysregulation in HCC.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041866/s1>.

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