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LRRFIP1 enhances the Wnt/β-catenin pathway by binding to DVLs in myelodysplastic syndrome

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Abstract

Background Due to high heterogeneity, diagnosing MDS can be challenging. Consequently, investigating its pathogenesis and progression mechanisms, and seeking novel targets for diagnosis and treatment, are critical issues that require urgent attention. This study aimed to investigate whether *LRRFIP1* might contribute to MDS pathogenesis by modulating the Wnt/β-catenin signaling pathway.

Methods In MDS cell lines, mRNA transcriptome sequencing and Dual luciferase reporter gene assays were employed to assess Wnt/β-catenin signaling pathway activity. To explore the biological characteristics of MDS cell lines, CCK8, Annexin-V APC/7-AAD and Annexin V-FITC/PI double staining and fluorescence TUNEL assay, and PI single staining were used.

Results In MDS cell lines, proliferation was notably higher in *LRRFIP1*-overexpressing cells compared to silenced ones, while cell apoptosis was lower in the former. mRNA transcriptome sequencing revealed *LRRFIP1*'s involvement in modulating the Wnt/β-catenin signaling pathway. *LRRFIP1* was found to positively regulate Wnt/β-catenin pathway activity, and synergy between *LRRFIP1* and *Dvl* was observed in enhancing canonical Wnt signaling. *LRRFIP1* overexpression significantly upregulated key genes in Wnt signaling pathway (such as *β-catenin*, *Dvl2*, *Dvl3* and *Wnt*) at the mRNA level, and notably upregulated non-phosphorylated β-catenin at the protein level. Moreover, BCL-2 and CyclinD1 protein expression was significantly higher in *LRRFIP1*-overexpressing cells compared to silenced ones, with even greater expression observed in *LRRFIP1/Dvl3* co-overexpressing cells.

Conclusions *LRRFIP1* can promote the proliferation of MDS cells and inhibit apoptosis, and *LRRFIP1* and *Dvl* can synergistically enhance the activity of the Wnt/β-catenin signaling pathway in MDS, thus providing evidence for *LRRFIP1*'s involvement in the pathogenesis of MDS.

Keywords Myelodysplastic syndromes, *LRRFIP1*, *Dvl*, Pathogenesis, Wnt/β-catenin pathway

Background

Myelodysplastic syndrome (MDS) is a clonal stem-cell-derived myeloid neoplasm characterized by variable cytopenia, as well as morphological and functional abnormalities in hematopoietic cells. It is also associated with an increased risk of developing acute myeloid leukemia (AML) [1–6]. Due to its high heterogeneity and complexity, diagnosing MDS can be challenging and prone to missed or incorrect diagnoses [7, 8]. Consequently, investigating its pathogenesis and progression mechanisms,

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and seeking novel targets for diagnosis and treatment, are critical issues that require urgent attention.

Previous studies conducted by our research team have identified the MDS CpG island methylation phenotype (CIMP), which includes leucine-rich repeat in Flightless-1 interaction protein 1 (*LRRFIP1*), as an independent prognostic factor for MDS. This phenotype holds promise as a molecular marker for early diagnosis and prognosis prediction in MDS [9]. Subsequent functional investigations have elucidated that LRRFIP1 exerts multiple regulatory roles across various biological systems and processes. These include involvement in immune responses against microorganisms and autoimmunity, cytoskeletal system remodeling, signal transduction pathways, and transcriptional regulation of genes [10–24]. Furthermore, *LRRFIP1* has been identified as one of the cancer-associated genes promoting cell invasion and metastasis [25, 26]. Within several signal transduction pathways, the LRRFIP1 protein interacts with related proteins such as disheveled proteins (Dvls), contributing to signal transduction, cell proliferation, apoptosis, and cell-cycle regulation [27].

The pathogenesis of MDS remains incompletely understood, involving genetic abnormalities, immune dysregulation, epigenetic regulation, environmental factors, and others. Prior research, including our own and multiple studies, has highlighted the pivotal role of the Wnt/β-catenin pathway in the onset and progression of MDS [1, 2, 4, 5, 28–30]. The preliminary results of our research team's study indicated epigenetic regulation of the Wnt pathway in MDS cell lines. Deregulation of the genes involved in the Wnt signaling pathways may contribute to the phenotypical abnormalities of MDS bone marrow-derived mesenchymal stem cells [3, 4]. Growing evidence suggested that aberrant Wnt signaling in leukemia stem cells and/or the bone marrow niche can lead to MDS/AML [1]. LRRFIP1 interacted with Dvls, positively modulating both canonical and noncanonical Wnt signaling pathways in cancer cells [31]. Dvls mediated canonical and noncanonical Wnt signaling through specific protein domains [32]. Increased *Dvl* expression has been shown to enhance Wnt signaling activation, and its upregulation is implicated in various cancers [33, 34]. Therefore, we hypothesized that *LRRFIP1* interacted with *Dvls* in the Wnt signaling pathway in MDS cells, influencing cell proliferation and apoptosis. Our study aimed to provide further insights into MDS pathogenesis.

Methods

Cell culture

The MDS cell lines SKM-1 and MUTZ-1 were obtained from the China Center for Type Culture Collection (Wuhan, China). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO2.

Construction of recombinant lentiviral vectors and transfection

Recombinant lentiviruses carrying *LRRFIP1*, *Dvl3*, and their controls (LRRFIP1-NC, Dvl3-NC) were constructed and transfected into MDS cell lines. Co-transfection of *LRRFIP1* and *Dvl3* was also done. Fluorescence microscopy, qRT-PCR, and Western blot analysis assessed lentivirus expression and signal transduction as per the manufacturer's instructions.

Knockdown of *LRRFIP1* and *Dvl3* using siRNA

Small interfering RNA (siRNA) targeting *LRRFIP1* and *Dvl3* were utilized to inhibit *LRRFIP1* and *Dvl3* expression. A scrambled siRNA served as a negative control (NC). Cells were transfected with siRNA using the transfection reagent designed for recombinant lentivirus-*LRRFIP1* and lentivirus-*Dvl3* (Keygen Biological Co. Ltd. Nanjing, China), following the manufacturer's instructions. Knockdown of *LRRFIP1* and *Dvl3* expression was confirmed using qRT-PCR and Western blot analysis.

qRT-PCR

Total RNA was extracted from both treated and untreated MDS cells using the Total RNA extraction kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. qRT-PCR was conducted. Primer sequences are provided in Supplementary Table 1. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as the internal control, and the $2^{-\Delta\Delta CT}$ method was employed to analyze the relative changes in gene expression.

mRNA transcriptome sequencing, data processing and analysis

To purify messenger RNA (mRNA) from total RNA using the unique polyA structure of mRNA, mRNA was fragmented into 200–300 bp through ion disruption. Random primers and reverse transcriptase were then used to synthesize the first strand of cDNA. The second strand of cDNA was synthesized using the first strand cDNA as a template. The double-stranded cDNA underwent end repair and poly-A tail addition, followed by ligation of sequencing adapters under the action of ligase. The product with adapters added was subjected to fragment selection using magnetic beads. PCR amplification enriched the library fragments, followed by size selection. The mixed library was denatured to single-stranded form and sequenced on the Novaseq 6000 PE150 platform. Raw sequencing data was generated. In raw data, there was generally a small portion of reads that contained artificial sequences such as sequencing primers and adaptors.

Removing low-quality regions that could impact data quality and subsequent analysis, raw data was filtered for clean data, and the quality assessment reports were generated by FastQC. Then clean data was aligned to the reference genome, and assembled for transcriptome analysis. Gene expression profiles were constructed, and GO/KEGG/Reactome analyses were performed on differential genes to elucidate functions and regulatory networks.

Immunoblotting and immunoprecipitation

Whole-cell lysates were pretreated with rabbit immunoglobulin G (IgG) and protein G sepharose (Amersham). Using rabbit anti-LRRFIP1 polyclonal antibodies and protein G sepharose, endogenous LRRFIP1 was immunoprecipitated from supernatants. Endogenous Dvl2, Dvl3 was immunoprecipitated using rabbit anti-LRRFIP1 monoclonal antibodies (Santa Cruz). Conversely, endogenous DVL2 and DVL3 were used to immunoprecipitate endogenous LRRFIP1.

Luciferase assay

TOPFlash or FOPFlash reporter plasmids were utilized. Cells were harvested 24 h post-transfection, and luciferase activities were quantified using SpectraMax M3 (Molecular Devices, USA). All luciferase activities were normalized for protein concentration and transfection efficiency using β-galactosidase. All experiments were conducted in triplicate and expressed as the mean ± standard deviation.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer (Boster, Wuhan) to isolate proteins, quantified by bicinchoninic acid protein assay. The proteins were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Keygen Biological Co., Ltd., Nanjing, China) and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, United States). Membranes were blocked, incubated with primary antibodies overnight at 4 °C, then with secondary antibodies for 1 h at 20 °C. The following antibodies were used: anti-LRRFIP1, anti-Dvl2, anti-Dvl3, anti-BCL-2, anti-Caspase-3, anti-CyclinD1, anti-p21, and anti-GAPDH from Abcam (Britain), as well as anti-β-catenin, anti-GSK3β, anti-Wnt, and goat anti-rabbit IgG-HRP from Keygen Biological Co. (China).

Cell proliferation and viability assays

Cells were seeded in 96-well plates and incubated for 2 to 10 days. Cell Counting Kit-8 (DOJINDO CP736, Japan) was added (10μL/well), incubated for 1–4 h, and absorbance was measured at 450 nm using ELx800 (Biotech, USA).

Analysis of the cell cycle and apoptosis

For cell cycle analysis, MDS cell lines were stained with propidium iodide (PI, Beyotime Biotechnology, China). Apoptotic cells were double-stained with Annexin V-APC/7-AAD or Annexin V-FITC/PI (Keygen Biological Co. Ltd., Nanjing, China) and quantified using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (BD Biosciences). Before analyzing DNA content using PI fluorescence, debris and doublets were excluded through FSC/SSC gating. The relative distribution of cells in the sub-G1, S, and G2 phases was then determined based on the PI fluorescence histogram. Cell apoptosis was also evaluated via the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The formula used to determine the cell apoptotic index was as follows: apoptotic index = apoptotic cells/total cells 100%. All procedures followed manufacturers' instructions.

Statistical analyses

Statistical analysis was performed using Stata 11. Data are presented as means ± standard deviations. The significance of differences was assessed using Student's t-test, with $p < 0.05$ considered statistically significant.

Results

Effect of *LRRFIP1* expression on the biological characteristics of MDS cell lines

In this study, cell proliferation was compared between *LRRFIP1* overexpression and knockdown cell lines with untreated MDS cell lines. Figure 1A illustrated that the proliferation of *LRRFIP1* overexpression cell lines was significantly higher than that of *LRRFIP1* knockdown cells. Additionally, cell apoptosis (Fig. 1B–D) was evaluated in *LRRFIP1* overexpression and knockdown cell lines, as well as untreated MDS cell lines. The degree of apoptosis observed in *LRRFIP1* overexpression cell lines was lower than that in *LRRFIP1* knockdown cell lines.

The impact of *LRRFIP1* on cell-cycle progression was investigated. Figure 1E depicted the distribution of MDS cell lines in each cell-cycle phase between *LRRFIP1* overexpression and knockdown cell lines. In *LRRFIP1* overexpression SKM-1 cell lines, the proportions of cells in the G1, S, and G2 phases were 50.71%, 31.44%, and 17.85%, respectively. Conversely, the proportion of S-phase cells was significantly increased (47.22%), while those of G1 and G2 phase cells were significantly decreased (40.22% and 12.56%, respectively) in *LRRFIP1* underexpression SKM-1 cell lines. Notably, *LRRFIP1* knockdown led to S-phase cell-cycle arrest. In the MUTZ-1 cell line, the proportions of cells in S phase and G2 phase were 44.54% and 21.71%, respectively, in the *LRRFIP1* overexpression

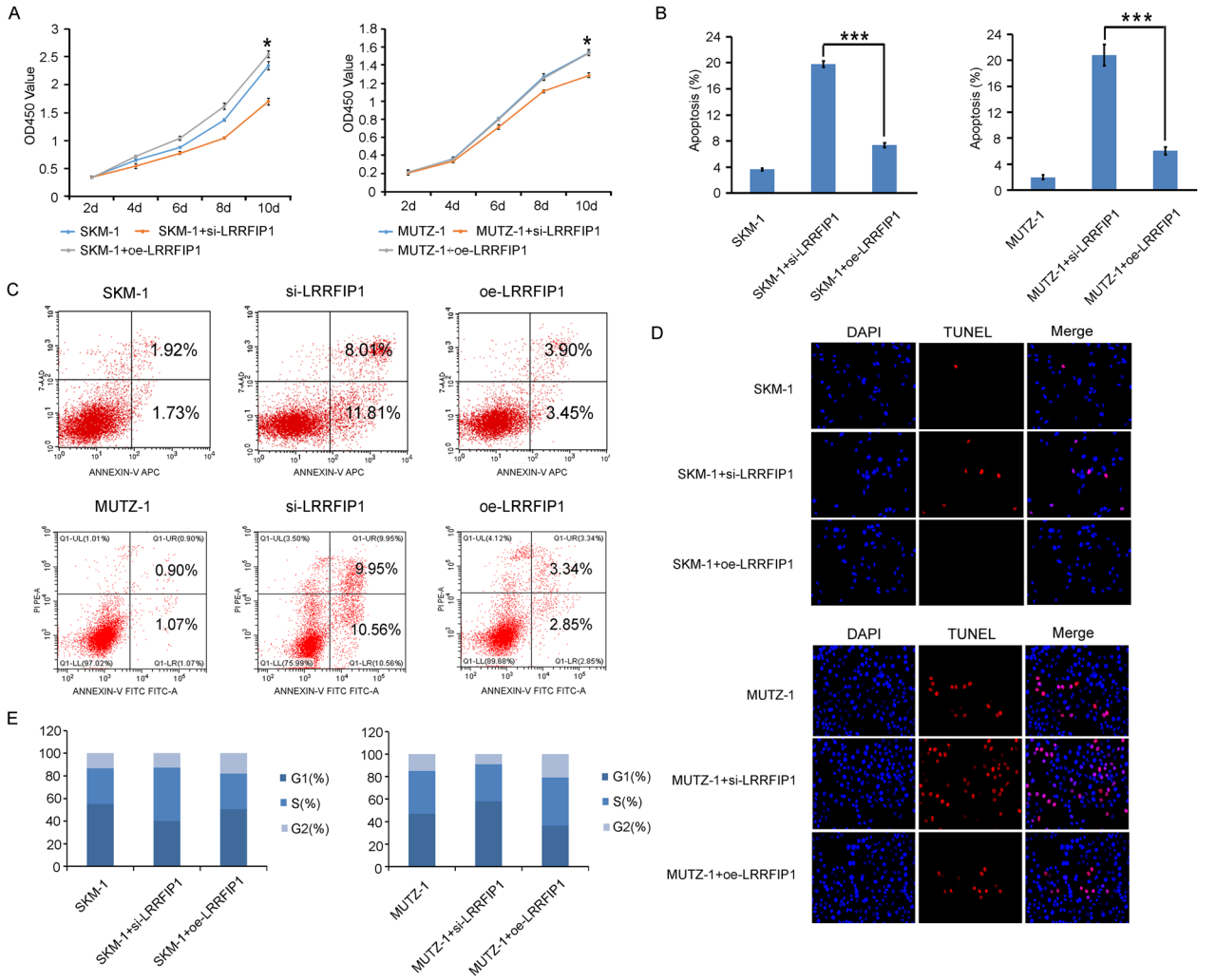

Fig. 1 Effect of *LRRFIP1* expression on the biological characteristics of MDS cell lines. **A** The CCK8 assay revealed that overexpression of *LRRFIP1* promoted cell proliferation. **B** and **C** Annexin-V APC/7-AAD or Annexin V-FITC/PI double staining coupled with flow cytometry analysis demonstrated that overexpression of *LRRFIP1* inhibits cell apoptosis. **D** TUNEL assay confirmed that overexpression of *LRRFIP1* inhibits apoptosis in MDS cell lines. **E** Cell cycle analysis using the PI single staining method revealed that silencing the expression of *LRRFIP1* led to cell cycle arrest in the G1/S phase, thereby inducing apoptosis. Data shown were representative of three replicates of each experiment. *, $p < 0.05$. ***, $p < 0.001$

cell line, whereas in the *LRRFIP1* silenced cell line, these proportions were 32.74% and 9.14%, respectively. Overall, these findings demonstrated that *LRRFIP1* knockdown induced cell-cycle arrest in the G1/S phase, thereby promoting cell apoptosis.

Transcriptomic analysis of the impact of different expression of *LRRFIP1* on the Wnt/β-catenin signaling pathway

To explore the impact of varying levels of *LRRFIP1* expression on the Wnt/β-catenin signaling pathway in the SKM-1 cell line, we employed mRNA transcriptome sequencing technology to analyze SKM-1 cells with *LRRFIP1* overexpression, *LRRFIP1* silenced expression, and untreated SKM-1 cells. Genes exhibiting significant differences in expression levels across the groups were identified as differentially expressed genes (DEGs). Figure 2A–C illustrated that gene expression across the nine samples was comparable, with good reproducibility within each group, weak correlation between samples in different groups, and notable differences in mRNA expression between different groups.

Gene Ontology (GO) enrichment analysis was conducted on the DEGs from the three groups, focusing on biological processes (BP), cellular components (CC), and molecular functions (MF). The top 20 most significantly enriched biological processes among the three groups of DEGs with varying *LRRFIP1* expression levels were

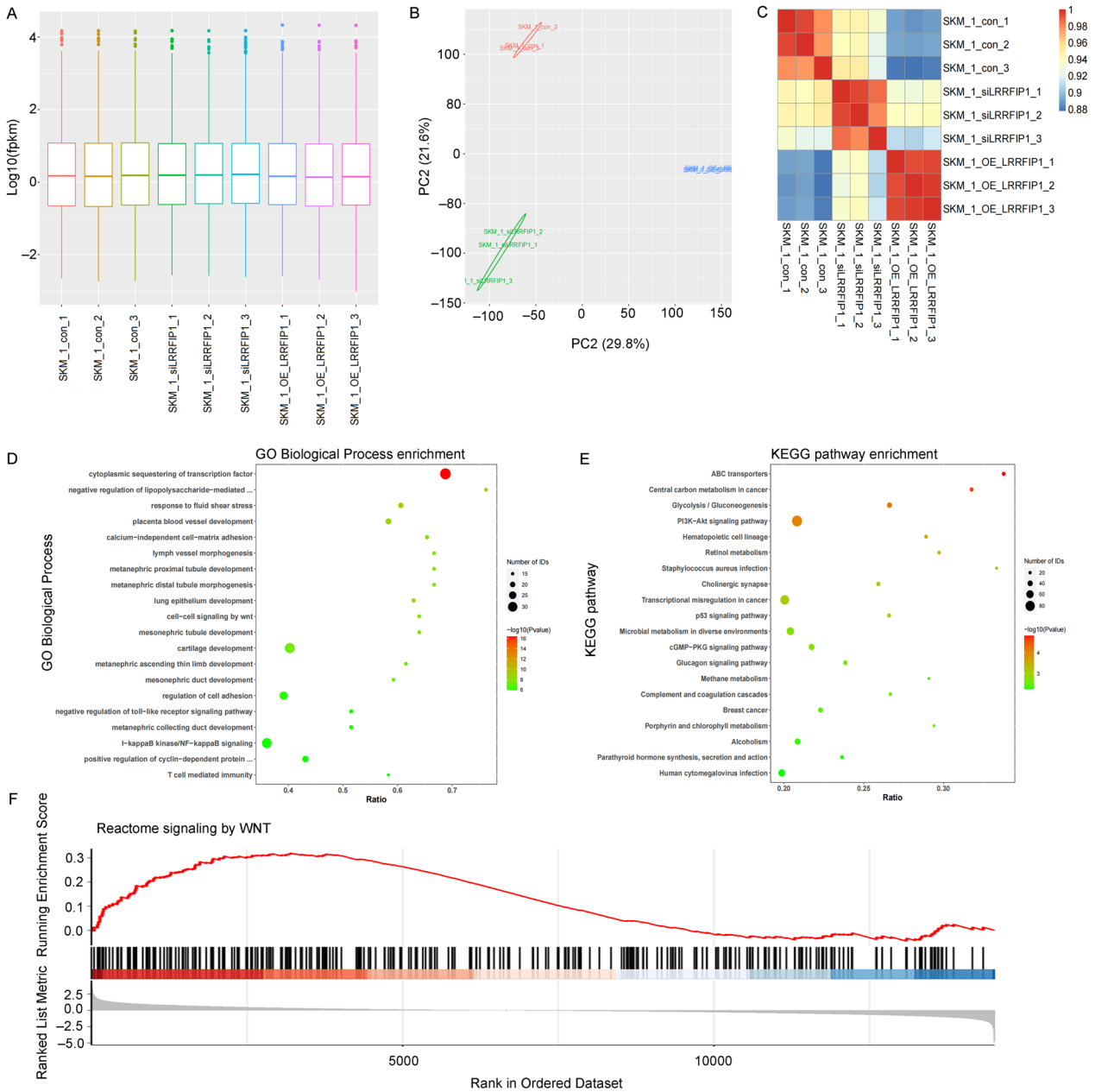

Fig. 2 Transcriptomic analysis of the activation of the Wnt/β-catenin signaling pathway by *LRRFIP1* overexpression. **A** The box plot of FPKM distribution in gene expression illustrated the log10(FPKM) values across different samples, providing insights into the distribution and variability of gene expressions. The gene expression distribution of the nine samples was similar and comparable. **B** Principal component analysis (PCA) was performed on the mRNA of these 9 samples. The PCA plot showed that the centroids of the three groups did not overlap, suggesting clear differentiation between the groups. Additionally, the samples within each group clustered together with good repetition, indicating significant differences in mRNA expression between the three groups. **C** The heatmap of pairwise correlations among samples displayed the squared value of the correlation coefficient, showing the degree of correlation between each pair of samples. The correlation between samples within each group was the strongest, indicating good repetition within the group. The correlation between samples between groups was weaker, suggesting gene expression differences between the groups. **D** The bubble plot for Gene Ontology (GO) analysis of differentially expressed genes highlighted the top 20 most significantly enriched biological processes, comparing *LRRFIP1* overexpressed SKM-1 cell line with untreated SKM-1 cell line. **E** The bubble plot for Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes depicted the top 20 most enriched signaling pathways, comparing *LRRFIP1* overexpressed SKM-1 cell line with untreated SKM-1 cell line. **F** Reactome enrichment analysis confirmed that *LRRFIP1* overexpression positively regulated the canonical Wnt signaling pathway

presented in a bubble chart. Overexpression of *LRRFIP1* resulted in enrichment in biological processes such as cytoplasmic sequestration of transcription factors, regulation of Toll-like receptor signaling pathway, NF-kB signaling pathway, and Wnt-mediated intercellular signaling (Fig. 2D).

Furthermore, KEGG pathway enrichment analysis was performed. Overexpression of *LRRFIP1* led to enrichment in the PI3K-Akt signaling pathway, hematopoietic cell lineage, transcriptional dysregulation in cancer, p53 signaling pathway, and Wnt signaling pathway which didn't list in the top 20 most enriched signaling pathways (Fig. 2E). Additionally, enrichment analysis through Reactome confirmed the involvement of *LRRFIP1* in the positive regulation of the Wnt/β-catenin signaling pathway (Fig. 2F).

Interactions between LRRFIP1 and DVLs in MDS cell line

mRNA transcriptome sequencing data analysis suggested that the overexpression of *LRRFIP1* positively regulated the Wnt/β-catenin signaling pathway. Previous studies have shown that Dvl enhances Wnt signaling pathway activity by stabilizing the β-catenin/TCF transcription complex, which is essential for Wnt/β-catenin signal transduction. We hypothesized that LRRFIP1 participated in the regulation of the Wnt/β-catenin signaling pathway in MDS through its interaction with Dvl. Immunoprecipitation was performed to examine the interaction between LRRFIP1 and Dvls. Western blot analysis revealed the presence of both Dvl2 and Dvl3 proteins in the immunoprecipitates of the experimental group. Similarly, immunoprecipitation was performed on whole-cell lysates of SKM-1 and MUTZ-1 cell lines using Dvl2 antibody, Dvl3 antibody, and control IgG antibody, respectively. LRRFIP1 protein was detected in the immunoprecipitates of the experimental group. These findings indicated interactions between LRRFIP1 protein and Dvl2 protein, as well as between LRRFIP1 protein and Dvl3 protein in SKM-1 and MUTZ-1 cell lines (Fig. 3).

LRRFIP1 positively regulated Wnt/β-catenin signaling by interacting with *DVLs*

To validate the involvement of *LRRFIP1* in the canonical Wnt signaling pathway, a *LRRFIP1* overexpression vector was constructed and transfected into MDS cell lines to generate stable cell lines with elevated *LRRFIP1* expression (Fig. S1A). Additionally, three types of siRNA were utilized to generate MDS cell lines with reduced *LRRFIP1* expression, and cell lines with the most stable low expression of *LRRFIP1* were chosen (Fig. S1B). Subsequently, luciferase reporter assays were employed to evaluate the impact of *LRRFIP1* on the activities of the Wnt signaling pathways in these cell lines. In SKM-1 (Fig. 4A) and MUTZ-1 (Fig. 4B) cell lines, *LRRFIP1* overexpression significantly increased the TOPFlash activity of Wnt signaling, while minimal activation was observed in the *LRRFIP1* knockdown cells. These findings provided evidence supporting the positive roles of *LRRFIP1* in the regulation of Wnt/β-catenin signaling.

A *Dvl3* overexpression vector was also constructed and transfected (Fig. S1C). *Dvl3* expression was verified in the three siRNA-transfected cell lines, and the cell line transfected with siRNA-3 was ultimately selected for subsequent experiments (Fig. S1D). Similarly, the *Dvl3* overexpression lentiviral vector and siRNA-3 were transfected into the MUTZ-1 cell line too. Additionally, MDS cell lines co-transfected with the *LRRFIP1* and *Dvl3* overexpression vectors were constructed. Concurrently, MDS

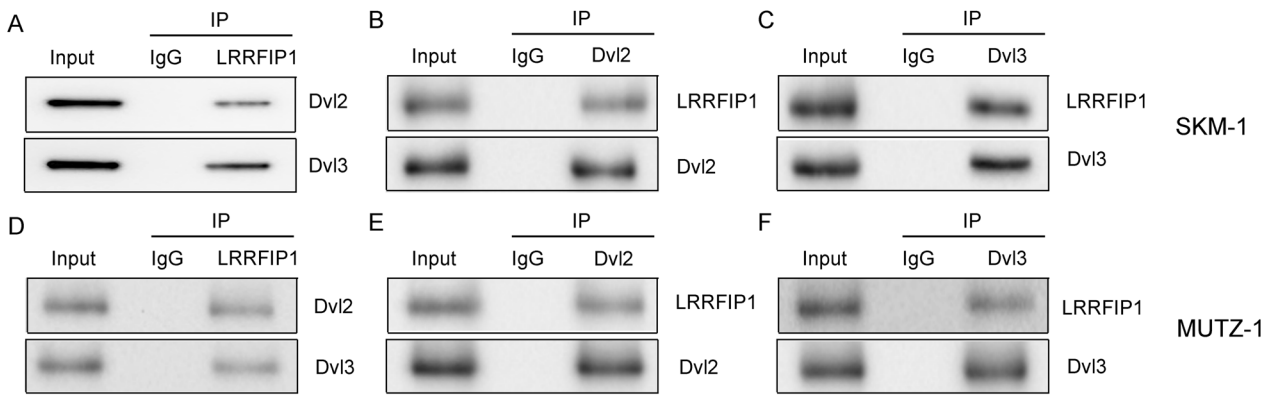

Fig. 3 An interaction between LRRFIP1 protein and Dvl protein in MDS cell lines. The interaction between LRRFIP1 protein and Dvl2 protein, as well as Dvl3 protein, was detected through immunoprecipitation in SKM-1 and MUTZ-1 cell lines. Input represents the positive control; IgG represents the negative control; IP stands for immunoprecipitation

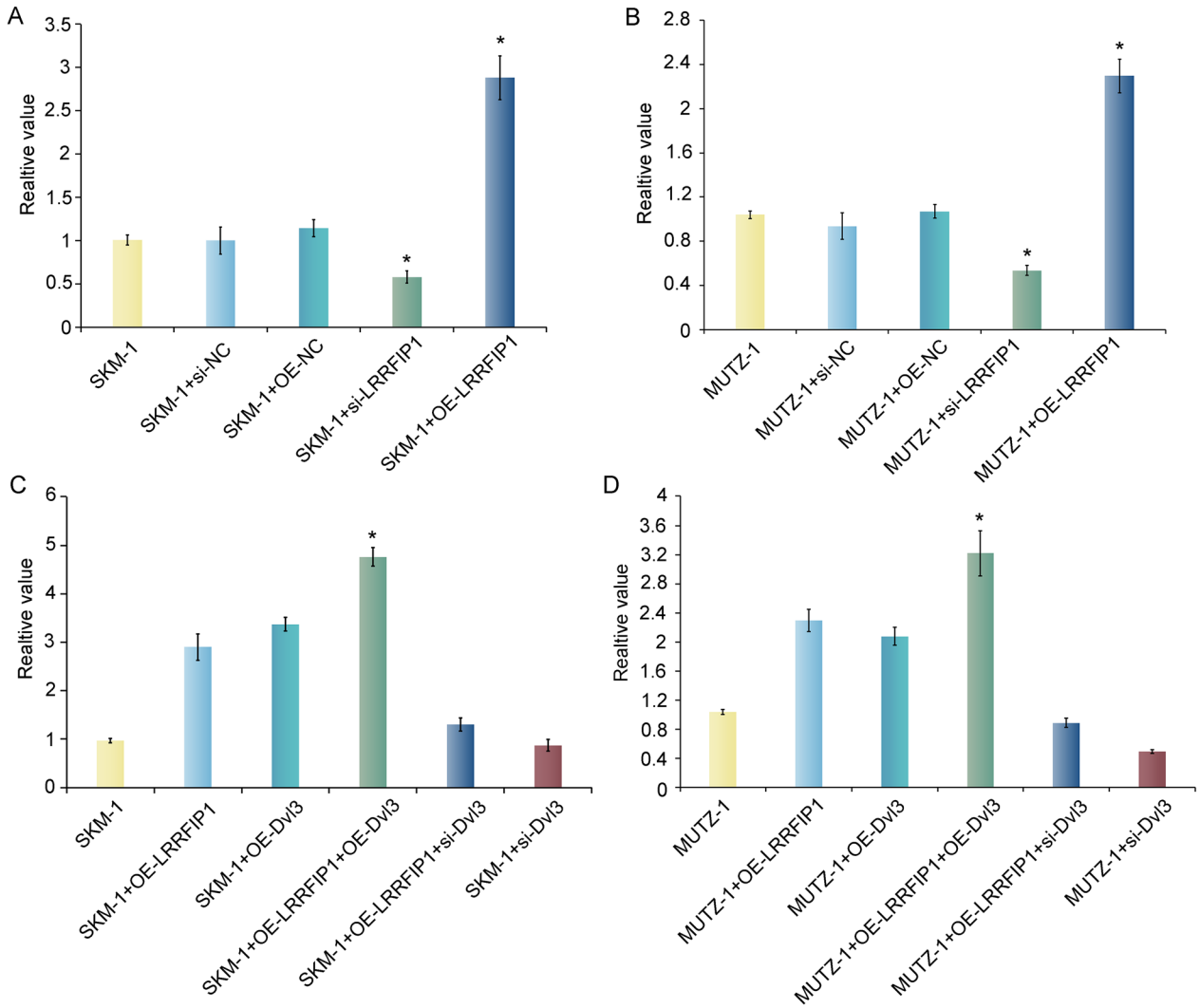

Fig. 4 Overexpression of *LRRFIP1* activated the Wnt/β-catenin signaling pathway. In SKM-1 (**A**) and MUTZ-1 (**B**) cell lines, *LRRFIP1* overexpression significantly increased the TOPFlash activity of Wnt signaling, while minimal activation was observed in the *LRRFIP1* knockdown cells through dual-luciferase reporter gene experiments. Co-transfection of both overexpression vectors resulted in a significant increase in TOPFlash activity compared to *LRRFIP1* overexpression alone. When *LRRFIP1* overexpression and *Dvl3* silencing were co-transfected, TOPFlash activity decreased significantly in SKM-1 (**C**) and MUTZ-1 (**D**) cell lines. *, $p < 0.05$

cell lines with overexpression of *LRRFIP1* and silencing of *Dvl3* were obtained.

The expressions of *LRRFIP1* and *Dvl3* in the *LRRFIP1/Dvl3* co-overexpression cell line were significantly higher than that in the *LRRFIP1* overexpression/*Dvl3* silencing cell line ($p < 0.05$) (Fig. S1E, S1F). Western blot was used to validate the protein expression levels of LRRFIP1 (Fig. S2A) and Dvl3 (Fig. S2B) in the corresponding overexpressing and silenced cell lines. The results were consistent with those obtained from qRT-PCR.

The interaction between *LRRFIP1* and *Dvl3* in the canonical Wnt signaling pathway were elucidated. When *LRRFIP1* was overexpressed alone, it increased TOP flash activity. Co-transfection of both overexpression vectors resulted in a significant increase in TOPFlash activity compared to *LRRFIP1* overexpression alone. However, when *LRRFIP1* overexpression and *Dvl3* silencing were co-transfected, TOPFlash activity decreased significantly ($p < 0.05$), indicating that *LRRFIP1* and *Dvl* can synergistically enhance the activity of the canonical Wnt signaling pathway (Fig. 4C, D).

The expression of key factors in the Wnt signaling pathway and their target genes was assessed. In the SKM-1 cell line, *LRRFIP1* overexpression notably upregulated the expression of non-phosphorylated *β-catenin*, *Dvl2*, *Dvl3*, and *Wnt*. LRRFIP1 overexpression significantly

increased β-catenin expression, while no significant difference was observed in Dvl2, Dvl3, and Wnt protein levels. These results all indicated that *LRRFIP1* could positively regulate Wnt signaling (Fig. 5A, B). Comparatively, co-overexpression of *LRRFIP1* and *Dvl3* significantly upregulated the expression of non-phosphorylated *β-catenin*, *GSK-3β*, and *Wnt*, along with elevated expression of β-catenin protein, GSK-3β protein, and Wnt protein. These results all indicated that *LRRFIP1* and *Dvl* could synergistically enhance the transduction of the Wnt signaling pathway (Fig. 5C, D).

In the MUTZ-1 cell line, *LRRFIP1* overexpression significantly increased the expression of non-phosphorylated *β-catenin*, *Dvl2*, *Dvl3*, and *Wnt*. Moreover, *LRRFIP1* overexpression notably elevated the expression of non-phosphorylated β-catenin protein, Dvl2 protein, Dvl3 protein, and Wnt protein, consistent with the mRNA expression of these genes. Comparatively, co-overexpression of *LRRFIP1* and *Dvl3* significantly upregulated *Dvl2*, *β-catenin* and *Wnt* at the mRNA level when compared to *LRRFIP1* overexpression/*Dvl3* silencing. Additionally, Dvl2 protein, β-catenin protein, GSK-3β protein, and Wnt protein were markedly upregulated. These findings collectively indicated that *LRRFIP1* and *Dvl* could synergistically enhance the transduction of the Wnt signaling pathway (Fig. 6).

Effect of *LRRFIP1* and *Dvl3* co-expression on the biological characteristics of MDS cell lines

The differences in cell proliferation between *LRRFIP1*/*Dvl3* co-overexpression, *LRRFIP1* overexpression/*Dvl3* silencing cell lines, and untreated MDS cell lines were detected. As depicted in Fig. 7A, the proliferation of *LRRFIP1* and *Dvl3* co-overexpression

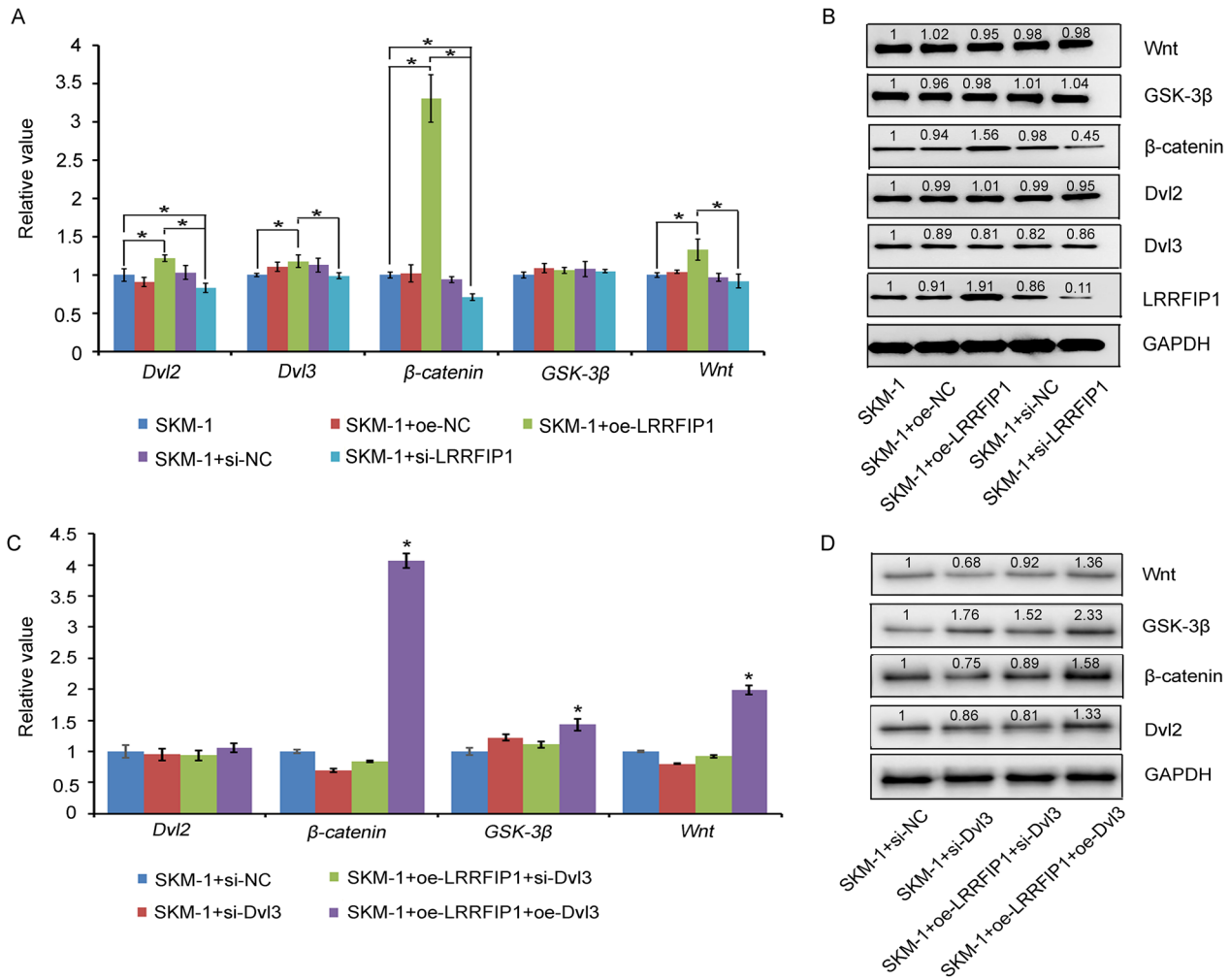

Fig. 5 The gene and protein expression of the Wnt signaling pathway components (Dvl2, Dvl3, β-catenin, GSK-3β, and Wnt) in SKM-1 cell lines with *LRRFIP1* overexpression and silencing, *LRRFIP1*/*Dvls* co-overexpression, and *LRRFIP1* overexpression/*Dvl3* silencing. **A**, **C**. qRT-PCR detection of gene expressions. *, $p < 0.05$. **B**, **D** Western blot detection of protein expression

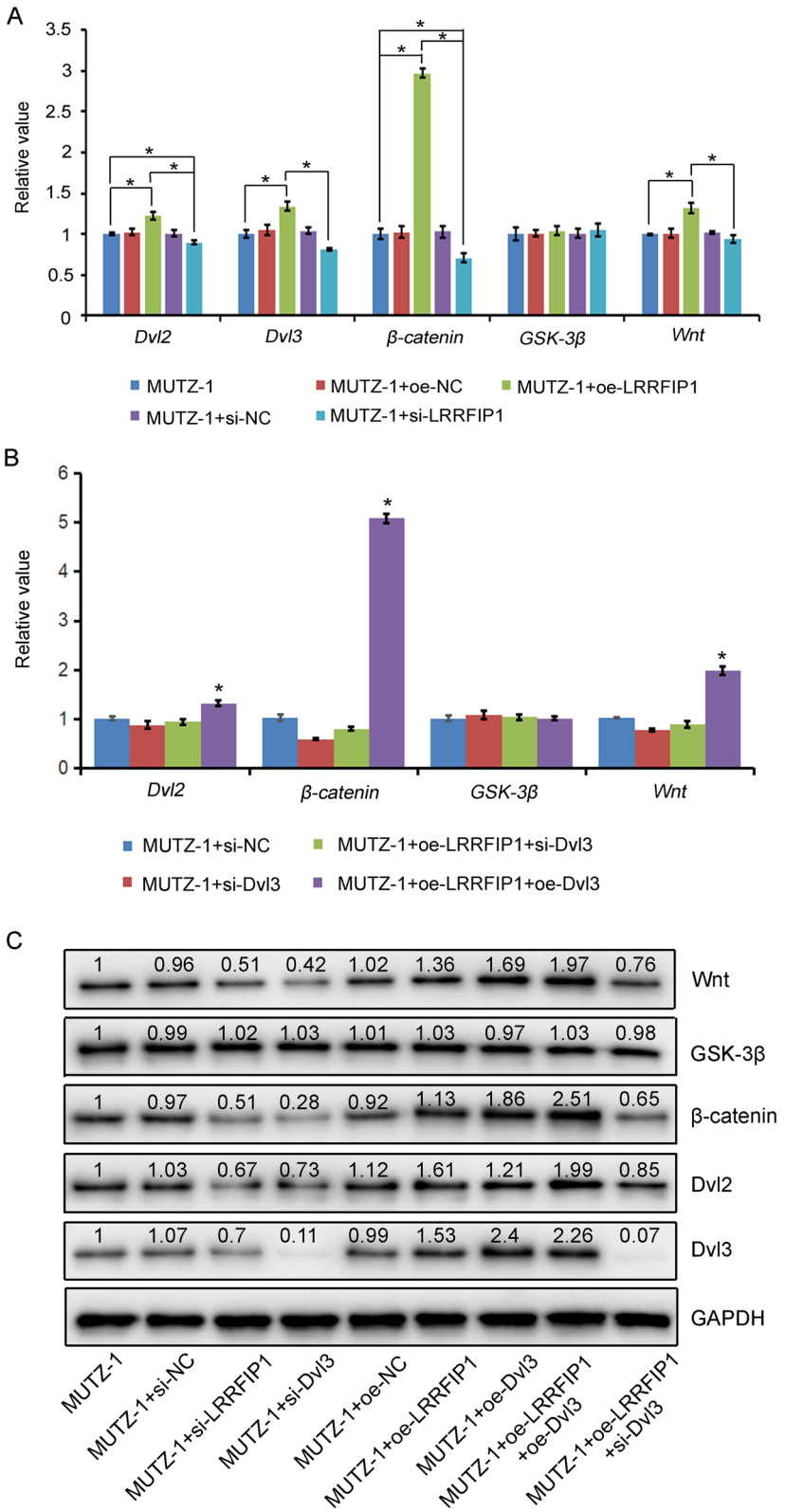

Fig. 6 The gene and protein expression levels of the Wnt signaling pathway components (Dvl2, Dvl3, β-catenin, GSK-3β, and Wnt) in MUTZ-1 cell lines with *LRRFIP1* overexpression and silencing, *LRRFIP1/Dvls* co-overexpression, and *LRRFIP1* overexpression/*Dvl3* silencing. **A**, **B** qRT-PCR detection of gene expressions *, $p < 0.05$. **C** Western blot detection of protein expression

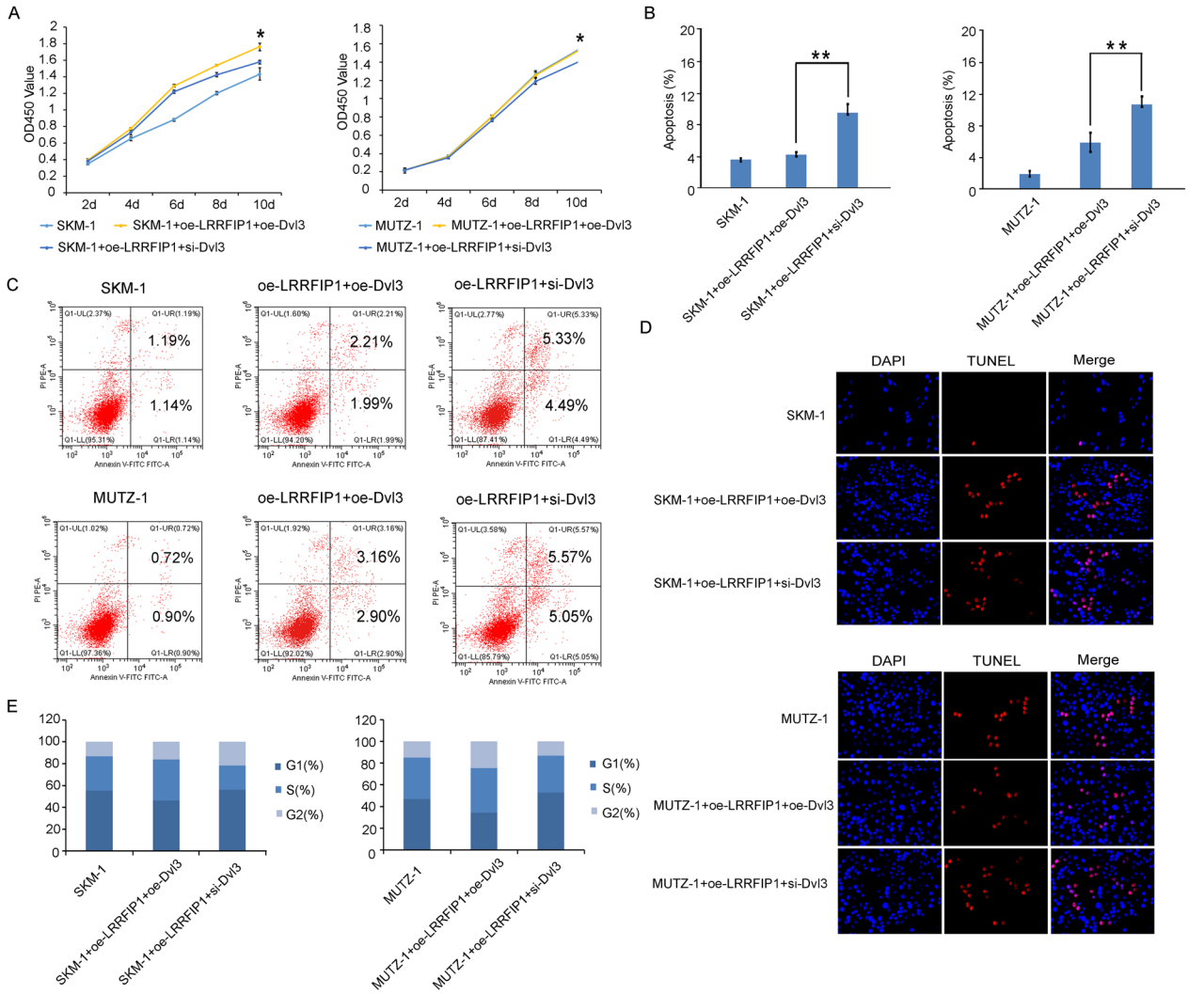

Fig. 7 The effect of co-expression of *LRRFIP1* and *Dvl3* on cell biological characteristics. **A** The cell proliferation assay using the CCK8 method revealed that co-overexpression of *LRRFIP1* and *Dvl3* promoted cell proliferation. **B**, **C** Analysis of cell apoptosis through Annexin V-FITC/PI double staining and flow cytometry showed that co-expression of *LRRFIP1* and *Dvl3* inhibits cell apoptosis compared to the co-transfection of *LRRFIP1* overexpression and *Dvl3* silencing. **D** TUNEL assay was conducted to detect apoptosis in MDS cell lines with co-overexpression of *LRRFIP1* and *Dvl3*, as well as in those with *LRRFIP1* overexpression and *Dvl3* silencing. The results indicated that co-expression of *LRRFIP1* and *Dvl3* inhibited apoptosis in MDS cell lines. **E** Cell cycle analysis using the PI single staining demonstrated that overexpression of *LRRFIP1* combined with silencing of *Dvl3* led to cell cycle arrest in the G1 phase, inducing apoptosis in cells. Data shown were representative of three replicates of each experiment. *, $p < 0.05$. **, $p < 0.01$

cell lines was notably higher than that in the cell line co-transfected with *LRRFIP1* overexpression/*Dvl3* silencing. Furthermore, the apoptosis rate was significantly lower (Fig. 7B–D).

Cell cycle progression was investigated by examining the effects of LRRFIP1 and Dvl3 co-expression. Figure 7E illustrated the distribution of MDS cell lines in each cell-cycle phase. In the SKM-1 cell line, compared with the co-transfection of *LRRFIP1* overexpression/*Dvl3* silencing (where cell proportions in S phase and G2 phase were 22.12% and 21.76% respectively), the cell proportions in S phase and G2 phase in the *LRRFIP1/Dvl3* co-overexpression cell line were 37.60% and 16.25% respectively, indicating that *LRRFIP1/Dvl3* co-overexpression could promote cell proliferation. In the MUTZ-1 cell line, the cell proportions in S phase and G2 phase in the *LRRFIP1/Dvl3* co-overexpression cell line were 42.17% and 24.39% respectively, while those in the cell line co-transfected with *LRRFIP1* overexpression and *Dvl3* silencing were 35.30% and 13.48% respectively. This finding also confirmed that the co-overexpression of *LRRFIP1* and *Dvl3* could promote cell proliferation.

Apoptosis-related marker proteins (BCL-2, Caspase-3) and cell cycle arrest proteins (CyclinD1, p21) were further examined. The expression levels of BCL-2 protein and CyclinD1 protein were significantly higher in the *LRRFIP1*-overexpressing cell line compared to the *LRRFIP1*-silenced cell line. Similarly, the expression of these two proteins was also elevated in the *LRRFIP1/Dvl3* co-overexpression cell lines than in the cell lines co-transfected with *LRRFIP1* overexpression and *Dvl3* silencing. The expression of Caspase-3 protein and p21 protein were contrary (Fig. 8). *LRRFIP1* overexpression and co-overexpression of *LRRFIP1* and *Dvl3* could promote cell proliferation and inhibit cell apoptosis. These findings provided further evidence for the involvement of *LRRFIP1* and its interaction with *Dvl3* in the regulation of cell proliferation and apoptosis in MDS.

Discussion

This study provided comprehensive insights into the role of *LRRFIP1* in the pathogenesis of MDS by regulating the Wnt/β-catenin signaling pathway. Initially, we demonstrated that overexpression of *LRRFIP1* promoted cell proliferation and suppressed apoptosis in MDS cell lines. Subsequent mRNA transcriptome sequencing unveiled the involvement of *LRRFIP1* in regulating the Wnt/β-catenin signaling pathway. The positive regulation of *LRRFIP1* on Wnt/β-catenin pathway activity was further confirmed by a dual luciferase reporter gene assay. Given that Dvl played a crucial role in enhancing Wnt signaling by stabilizing the β-catenin/TCF transcriptional complex, we further investigated whether *LRRFIP1* regulated this pathway via interaction with Dvl. Immunoprecipitation assays confirmed the interaction between LRRFIP1 and Dvl proteins. Subsequent dual luciferase reporter gene assays provided further confirmation that *LRRFIP1* and *Dvl* synergistically enhanced Wnt/β-catenin pathway activity. This comprehensive analysis shed light on the intricate mechanisms underlying MDS pathogenesis, emphasizing the significance of the LRRFIP1-Dvl interaction in modulating Wnt/β-catenin signaling.

The significance of the Wnt/β-catenin signaling pathway in human health and disease, including cancer and developmental disorders, cannot be overstated [35, 36]. Studies have highlighted the importance of Wnt/β-catenin signaling in the survival, proliferation, and differentiation of hematopoietic stem cells, underscoring its role in leukemia pathogenesis [2, 37]. Many cases of human birth defects and other diseases such as cancer have been attributed to the dysfunction of the Wnt signaling pathway. Alterations of this pathway have been implicated in the pathogenesis of MDS [30]. Down-regulation of Wnt signaling pathway-related gene expression may be one of the reasons for abnormal phenotype of

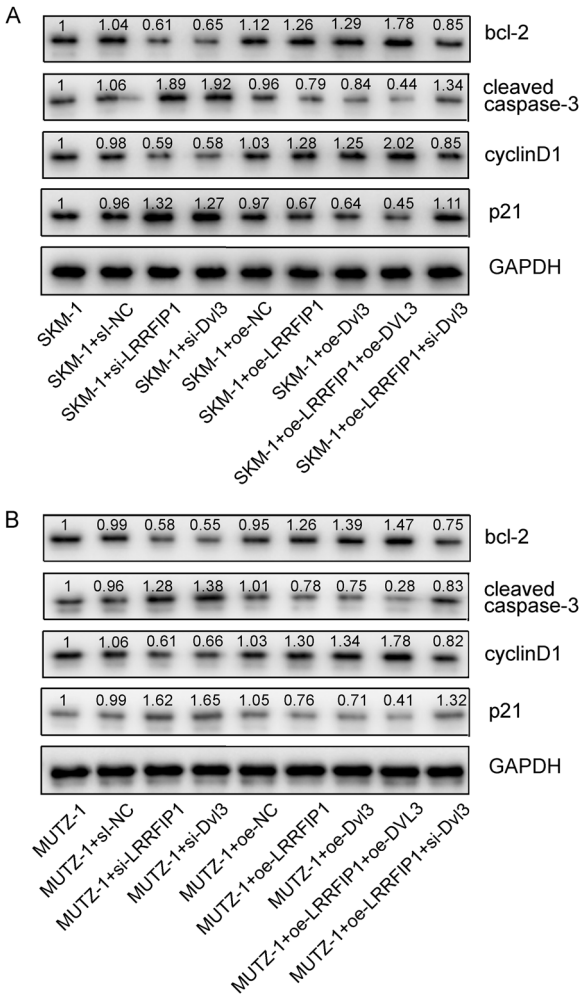

Fig. 8 The impact of *LRRFIP1* expression on apoptotic proteins and cell cycle proteins. Overexpression of *LRRFIP1* in SKM-1 cell lines. **A** and MUTZ-1 cell lines. **B** could upregulate the expression of BCL-2 and CyclinD1 proteins.The same trend was also observed in the *LRRFIP1/Dvl3* co-overexpression cell line compared to the cell line co-transfected with *LRRFIP1* overexpression and *Dvl3* silencing. The expression of Caspase-3 protein and p21 protein were contrary

MDS bone marrow mesenchymal stem cells. Increasing evidence suggested that abnormal Wnt signaling in leukemic stem cells and the bone marrow niche can lead to the occurrence of MDS/AML.The gene expression profiles of hematopoietic cells further supported the involvement of Wnt signaling activation in the pathogenesis of MDS, AML, and related myeloid neoplasms [1]. Notably, MDS mesenchymal stem cells exhibited abnormal methylation patterns, which could affect Wnt/β-catenin signaling [3]. Experimental evidence, including studies on mouse models, has shown that abnormal activation of β-catenin in bone marrow mesenchymal stem cells was associated with MDS progression [2]. Previous evidence

has reported the mechanisms underlying Dvl-mediated activation of the Wnt signaling pathway and the causes of developmental disorders [38, 39]. In this study, we elucidated the role of *LRRFIP1*, a cancer-associated gene, in promoting Wnt/β-catenin signaling by interacting with Dvls in MDS. This finding contributed to our understanding of the molecular mechanisms underlying MDS pathogenesis and highlights potential therapeutic targets for intervention.

There have been several studies indicating that *LRRFIP1* was involved in the regulation of the Wnt/β-catenin signaling pathway [40]. *LRRFIP1* targeted the canonical Wnt/β-catenin signaling pathway in cervical cancer and colon cancer cell lines [31]. *LRRFIP1* was also shown to promote epithelial-mesenchymal transition in pancreatic cancer through the Wnt/β-catenin pathway [41]. However, few studies have reported the effect of *LRRFIP1* on MDS mediated through the Wnt signaling pathway. TOPFlash reporter assays were used in this study to investigate the role of *LRRFIP1* in the canonical Wnt signaling pathway because TOPFlash/ FOPFlash assay functions as an important approach in studying the activation of the Wnt signaling pathway [39]. The overexpression of *LRRFIP1* in MDS cells resulted in the activation of TOPFlash reporter activity. In addition, when *LRRFIP1* was knocked down, it did not induce TOPFlash activity, which confirmed that *LRRFIP1* played an active role in the regulation of the Wnt/β-catenin signaling pathway. Furthermore, the study investigated the effect of *LRRFIP1* on key genes involved in the Wnt pathway. This discrepancy between SKM-1 and MUTZ-1 cell lines may suggest divergent pathogenic mechanisms in different MDS cell lines.

Dvls mediated canonical and noncanonical Wnt signaling pathways [32, 42]. The overexpression of *Dvl* potentiated the activation of Wnt signaling, and its upregulation was involved in several cancers [33]. The Dvl family comprises three isoforms: Dvl1, Dvl2, and Dvl3 [43–45]. Typically, all the three proteins located in the cytoplasm are implicated in phosphorylation and mediate the downstream signal transduction of various Wnt proteins [42]. Some studies have identified LRRFIP1 as a binding partner of Dvl2 and Dvl3 in tumor cells [31]. Through coimmunoprecipitation assays, it was demonstrated that LRRFIP1 interacted with Dvl2 and Dvl3 in MDS cells. Furthermore, the synergistic activation of TOPFlash reporter activity by *LRRFIP1* and *Dvl3* indicated their collaborative role in activating the canonical Wnt signaling pathway.

In several signal transduction pathways, LRRFIP1 bounded to related proteins such as Dvls and was involved in cell cycle regulation [27]. The findings regarding *LRRFIP1*'s involvement in cell cycle regulation and its impact on cell proliferation and apoptosis aligned with previous research in other cancer types, such as hepatocellular carcinoma. In hepatocellular carcinoma tissues and cell lines, *LRRFIP1* has been found to be highly expressed, suggesting its potential role in influencing cell proliferation and apoptosis [46]. Similarly, in this study, the researchers observed that *LRRFIP1* promoted cell proliferation and suppressed apoptosis in MDS cell lines. Silencing *LRRFIP1* expression led to cell cycle arrest in the S phase and induced apoptosis, highlighting the regulatory role of *LRRFIP1* in cell cycle progression and apoptosis.

The differential expression of key proteins associated with cell proliferation and apoptosis further supported these findings. Overexpression of *LRRFIP1* resulted in higher levels of BCL-2 and CyclinD1 proteins, which were known to promote cell survival and proliferation. Conversely, the expression levels of Caspase-3 and p21 proteins, which were associated with apoptosis induction and cell cycle arrest, respectively, were lower in cells overexpressing *LRRFIP1*. Overall, these results reinforced the notion that *LRRFIP1* played a critical role in modulating cell proliferation and apoptosis, potentially through its influence on cell cycle progression and the expression of key regulatory proteins.

Currently, high-risk MDS patients who are refractory to hypomethylating agents (HMA) or experience disease progression after HMA treatment have a poor prognosis, with a median survival of only 4–6 months [47, 48]. There is currently no standard treatment for this patient group, making the need for effective treatment options urgent [49]. The application of Venetoclax in high-risk MDS patients is currently being explored. In this study, it was observed that the expression of BCL-2 protein was significantly higher in *LRRFIP1* overexpression cell lines compared to *LRRFIP1* silenced cell lines. Venetoclax, an oral BCL-2 inhibitor, may offer a potential therapeutic approach. Moving forward, we will continue to explore the biological characteristics of cell lines with different expression levels of *LRRFIP1* treated with various concentrations of Venetoclax, providing further evidence for the treatment of MDS with Venetoclax. Certainly, there were also limitations in this study. We have only explored the regulation of the Wnt/β-catenin signaling pathway by *LRRFIP1* in the MDS cell lines, and further validation was needed in mouse models, which would provide more robust evidence for identifying new diagnostic and therapeutic targets for MDS.

Conclusions

The identification of the CIMP of MDS, which included *LRRFIP1*, was an independent prognostic factor of MDS [9]. Building upon this finding, our study further

explored the role of *LRRFIP1* in MDS and shed light on its potential mechanisms. We demonstrated that *LRRFIP1* acted as an oncogene in MDS, promoting cell proliferation and inhibiting apoptosis. Importantly, our transcriptome analysis revealed that *LRRFIP1* was involved in positively regulating the Wnt/β-catenin signaling pathway. The confirmation of *LRRFIP1*'s role in Wnt/β-catenin signaling was further validated through dual-luciferase reporter gene experiments, highlighting its ability to enhance the activity of this pathway, particularly when in synergy with Dvl proteins. These findings contributed to a deeper understanding of the pathogenesis of MDS and provide a foundation for the exploration of novel diagnostic and therapeutic targets. By unraveling the molecular mechanisms underlying *LRRFIP1*'s involvement in MDS, we pave the way for the development of targeted interventions aimed at modulating Wnt/β-catenin signaling for the treatment of this disease.

Abbreviations

MDS	Myelodysplastic syndrome
AML	Acute myeloid leukemia
CIMP	CpG island methylation phenotype
LRRFIP1	Leucine-rich repeat in Flightless-1 interaction protein 1
Dvls	Disheveled proteins
siRNA	Small interfering RNA
NC	Negative control
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IgG	Immunoglobulin G
PI	Propidium iodide
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
DEG	Differentially expressed gene
GO	Gene Ontology
BP	Biological processes
CC	Cellular components
MF	Molecular functions

Supplementary Information

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Supplementary material 1.

Supplementary material 2.

Supplementary material 3.

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Author contributions
JY L and Y Z designed the experiments and revised the manuscript; XL Z,YT L and H Z performed the experiments, and contributed to the data analysis. XL Z wrote the manuscript. WY S and SX Q participated in the revision of the manuscript. All authors discussed the results and reviewed the manuscript.

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Availability of data and materials
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human:HRA007918) that are publicly accessible at https://ngdc.cncb.ac. cn/gsa-human. Other data will be made available upon reasonable request. Data requestors will need to sign a data access agreement and submit a proposal to Prof. Yu Zhu and Prof. Jianyong Li.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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