Am J Transl Res 2025;17(5):3842-3861
www.ajtr.org /ISSN:1943-8141/AJTR0162204

Original Article

Analysis of cullin family genes in rectal adenocarcinoma: expression, prognostic significance, and therapeutic implications

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Received November 22, 2024; Accepted May 7, 2025; Epub May 15, 2025; Published May 30, 2025

Abstract: Objectives: Cullin family genes play a critical role in ubiquitin-mediated protein degradation and have been implicated in various cancers. However, their expression patterns, prognostic significance, and functional roles in rectal adenocarcinoma (READ) remain unclear. This study aims to comprehensively analyze the expression, prognostic value, and potential biological functions of culllin genes in READ. Methods: We analyzed the transcriptional expression of CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9 in READ using publicly available databases, including UALCAN and HOA. The prognostic significance of CUL genes was evaluated using Kaplan-Meier survival analysis. Functional enrichment analysis was performed to determine the biological pathways associated with cullin gene expression. Additionally, siRNA-mediated knockdown of CUL genes was done to assess the effect on cell proliferation, migration, and colony formation. Results: The analysis revealed significant overexpression of cullin genes in READ compared to normal tissues. Survival analysis indicated that higher expression of specific CUL2 and CUL7 genes correlated with poor prognosis in READ patients. Functional enrichment analysis demonstrated that cullin genes were associated with diverse enrichment terms. In vitro experiments showed that siRNA-mediated knockdown of CUL2 and CUL47 led to a significant reduction in cell proliferation, migration, and colony formation, highlighting their potential oncogenic role in READ. Conclusion: This study provides novel insight into the role of cullin genes in READ, suggesting that CUL2 and CUL7 may be biomarkers and therapeutic targets. Further research is warranted to explore their underlying mechanisms and clinical applications in READ management.

Keywords: Cullin genes, rectal adenocarcinoma (READ), prognostic biomarkers, gene expression analysis, ubiquitin-mediated proteolysis, sirna knockdown

Introduction

Rectal adenocarcinoma (READ) originates from the epithelial cells of the rectum and is a significant subtype of colorectal cancer (CRC), a leading cause of cancer-related morbidity and mortality worldwide [1-3]. It is characterized by the abnormal growth of glandular cells in the rectum, and its clinical presentation often includes symptoms such as rectal bleeding, abdominal pain, and changes in bowel habits [4]. READ, although less prevalent than colon cancer, contributes substantially to the overall cancer burden, with an increasing incidence due to factors such as diet, age, and lifestyle changes [5, 6]. According to the World Health Organization (WHO), colorectal cancer is among the top three cancers globally, and rectal cancer, specifically, poses unique challenges in terms of treatment and prognosis [7, 8]. Despite advancements in screening, surgical techniques, and chemotherapy, the survival rates for advanced stages of READ remain relatively low [9-11]. Therefore, there is an urgent need for a deeper molecular understanding of READ to improve early detection, treatment efficacy, and patient survival [12, 13].

Cullin family genes are a group of evolutionarily conserved genes that play a crucial role in the

https://doi.org/10.62347/UNVS8140

regulation of various cellular processes, including cell cycle progression, apoptosis, DNA repair, and differentiation, through their involvement in the ubiquitin-proteasome system (UPS) [14, 15]. The cullin proteins function as scaffolding components of multi-subunit E3 ligase complexes, facilitating the ubiquitination and subsequent degradation of specific substrate proteins [16]. This process is essential for maintaining cellular homeostasis and preventing the accumulation of damaged or misfolded proteins [16]. The cullin family includes several members, such as Cullin-1 (CUL1), Cullin-2 (CUL2), Cullin-3 (CUL3), Cullin-4 (CUL4), Cullin-5 (CUL5), and Cullin-7 (CUL7), Cullin-9 (CUL9). Each forms a complex with various adaptor proteins and substrates to regulate diverse signaling pathways [17].

Recent studies have highlighted the significant role of cullin family genes in various cancers. For instance, CUL1 regulates cell cycle progression through its involvement in the SCF (Skp1-Cullin-F-box) E3 ligase complex, which targets cell cycle regulators such as cyclins and CDK inhibitors for degradation [18]. Dysregulation of CUL1 has been associated with uncontrolled proliferation in breast cancer, lung cancer, and hepatocellular carcinoma [19]. CUL3 has been shown to modulate oxidative stress and tumor suppressor pathways in colorectal cancer, where its downregulation leads to enhanced tumorigenic potential [20]. Similarly, CUL4A and CUL4B are involved in chromatin remodeling and DNA damage response, with their overexpression linked to poor prognosis in ovarian, prostate, and gastric cancers [21]. CUL5 plays a tumor-suppressive role in certain cancers, including leukemia and glioblastoma, by mediating the degradation of oncogenic proteins [22, 23]. Additionally, CUL7 and CUL9 have been implicated in tumor progression and metastasis, particularly in osteosarcoma and pancreatic cancer, where they regulate key signaling pathways involved in epithelial-to-mesenchymal transition (EMT) and cell migration [24, 25]. The widespread involvement of cullin family genes in multiple malignancies emphasize their critical role in cancer biology. These genes influence tumor development and progression by modulating key oncogenic and tumor-suppressive pathways, including PI3K/AKT, Wnt/β-catenin, and p53 signaling [26]. Given their broad effect on cellular processes essential for tumor survival and proliferation, cullin family genes are increasingly being recognized as prognostic biomarkers and therapeutic targets in cancer research. However, while the role of cullin genes has been well-characterized in several common cancers, their function in READ remains poorly understood. Investigating the expression and prognostic significance of Cullin genes in READ may show their contribution to tumor pathophysiology and help identify novel therapy.

Over the past decade, a growing body of evidence has highlighted the dysregulation of cullin family genes in multiple cancers, including breast cancer, lung cancer, hepatocellular carcinoma, melanoma, and leukemia [24, 27]. Alterations in the expression or function of cullin proteins have been linked to uncontrolled cell proliferation, enhanced survival, migration, and metastasis, making them possible targets for cancer therapy [24, 28]. For instance, CUL1 has been shown to be overexpressed in breast cancer, leading to the enhanced degradation of tumor suppressors such as p27Kip1 [29], while CUL4A is involved in the regulation of DNA repair mechanisms that are frequently disrupted in cancer cells [30]. Similarly, CUL3 has been implicated in regulating the migration and invasion of cancer cells in several malignancies [15]. Despite these insights, the role of cullin family genes in READ remains largely unexplored, so their use as biomarkers or therapeutic targets is uncertain.

In recent years, computational biology has become an essential tool for understanding the complex molecular mechanisms underlying cancer [31, 32]. The use of *in silico* approaches, such as gene expression profiling and bioinformatic analysis, allows for the identification of key genes and pathways that are altered in cancer [33-35]. These findings can be validated through *in vitro* studies using cancer cell lines, primary tumor tissues, and animal models [36, 37]. However, despite the promising potential of cullin family genes, their role in READ progression, metastasis, and resistance to treatment is uncertain [38]. In response to these gaps in knowledge, our study aimed to investigate the role of cullin family genes in rectal adenocarcinoma through a multi-level approach involving *in silico* [39, 40] and *in vitro* experiments [41, 42].

Materials and methods

Expression analysis of cullin genes in READ

The Tumor Immune Estimation Resource (TIMER2.0) database (http://timer.cistrome.org/) [43] is an interactive web platform that provides RNA-seq expression profiles for tumor and normal tissues from The Cancer Genome Atlas (TCGA). In our study, we used TIMER2.0 to analyze the transcriptional expression levels of cullin genes in READ samples compared to normal tissues.

The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/) [44, 45] is a resource that provides protein expression profiles across human tissues, including immunohistochemistry data from patient-derived samples. To validate the mRNA expression findings from TIMER2.0, we retrieved immunohistochemistry (IHC) staining images for cullin proteins in READ tissues from the HPA database. The staining intensity (weak, moderate, or strong) and protein localization were analyzed to confirm the overexpression of cullin genes at the protein level. Representative images were selected to illustrate the observed expression patterns.

Genetic alteration analysis of cullin genes in READ

To investigate the genetic alterations in cullin genes within READ samples, we utilized the cBioPortal for Cancer Genomics (https://www.cbioportal.org/) [46], a comprehensive open-access resource for exploring multidimensional cancer genomics datasets. This database provides access to large-scale cancer genomic data, including mutations, copy number variations (CNVs), and other genetic alterations.

Analysis of cullin gene expression across cancer stages and patient gender

To evaluate the differential expression of cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) in READ patients across different tumor stages and between genders, we utilized the UALCAN database (http://ualcan.path.uab.edu/) [47, 48]. UALCAN is an interactive web resource that enables transcriptomic analysis of TCGA data, allowing for exploration of gene expression patterns across various clinical features, including cancer stage and patient demographics.

Prognostic analysis of cullin genes in READ

To assess the prognostic significance of cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) in READ, we utilized the cSurvival database (https://cSurvival.org/) [49, 50], an interactive platform designed for survival analysis using TCGA transcriptomic data. This tool enables Kaplan-Meier survival curve generation and statistical comparison of gene expression levels with overall survival (OS) outcomes.

Expression analysis of cullin genes across immune subtypes in READ

The TISIDB database (http://cis.hku.hk/TISIDB/) [51] is a comprehensive resource integrating tumor-immune system interactions. It provides immune-related genomic and transcriptomic data across various cancers, including READ. This database was used in our study to evaluate the expression of cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) across distinct immune subtypes of READ.

miRNA-mRNA network, immune evasion, and drug resistance analysis of cullin genes in READ

The miRNet database (https://www.mirnet.ca/) [52], a comprehensive platform for miRNA-target interaction analysis, was used to predict miRNA-mRNA interactions and identify miRNAs that may regulate cullin gene expression in READ. To analyze the differential expression of hsa-miR-34a-5p in READ tumor and normal tissues, as well as to evaluate its effect on patient survival, we used the UALCAN database (https://ualcan.path.uab.edu/) [47], an interactive web portal for in-depth cancer transcriptome analysis based on TCGA data. Furthermore, the GSCA database (http://bioinfo.life.hust.edu.cn/GSCA/#/) [53], an integrated platform for genomic and immune correlation studies, was employed to assess the relationship between cullin gene expression and immune cell infiltration, as well as to investigate the association between cullin gene expression and drug resistance in READ.

Gene enrichment analysis of cullin genes

The DAVID (Database for Annotation, Visualization, and Integrated Discovery) tool (https://david.ncifcrf.gov/) [54] was utilized for gene

enrichment analysis to explore the functional significance of cullin family genes in READ. This tool provides comprehensive annotation and pathway enrichment analysis, allowing us to identify significantly enriched cellular components, molecular functions, biological processes, and pathways associated with cullin gene activity.

Cell culture and siRNA transfection

HT29 cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Cat# 11965092) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher, Cat# 26140079) and 1% penicillin-streptomycin (Thermo Fisher, Cat# 15140122) at 37°C with 5% CO_2. For gene silencing, HT29 cells were transfected with CUL2 and CUL7-specific siRNAs (Thermo Fisher, Silencer Select, Cat# AM16708) using Lipofectamine RNAiMAX (Thermo Fisher, Cat# 13778075), following the manufacturer's protocol. A scrambled siRNA was used as a negative control. Cells were harvested 48 hours post-transfection for further experiments.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from transfected and control HT29 cells using the PureLink RNA Mini Kit (Thermo Fisher, Cat# 12183018A) according to the manufacturer's instructions. The RNA concentration and purity were measured using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Cat# 4368814). RT-qPCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher, Cat# A25741) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher). GAPDH was used as an internal control, and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Following primer pairs were used for the amplification purpose: GAPDH-F, 5'-TGACTTCAACAGCGACACCCA-3', GAPDH-R, 5'-CACCCTGTTGCTGTAGCCAAA-3'; CUL2-F, 5'-GTCTTACTCCGTGCTGTGTCCA-3', CUL2-R, 5'-CTGACTCCACAAATAGTGTTGGC-3'; CUL7-F, 5'-CCGCAAACTCATCACCAACATCC-3', CUL7-R, 5'-GGCACAGGTATCTGAGGAACAC-3'.

Western blot assay

Protein lysates were prepared using RIPA buffer (Thermo Fisher, Cat# 89901) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Cat# 78442). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher, Cat# 23227). Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Thermo Fisher, Cat# 88518). Membranes were blocked with SuperBlock T20 (TBS) Blocking Buffer (Thermo Fisher, Cat# 37536) and incubated with primary antibodies against CUL2 (Thermo Fisher, Cat# A302-476A), CUL7 (Thermo Fisher, Cat# A300-224A), and GAPDH (loading control, Thermo Fisher, Cat# MA5-15738) overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibodies, and protein bands were visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Cat# 34580).

Cell proliferation assay

Cell proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Thermo Fisher, Cat# G3582). Transfected and control HT29 cells were seeded in 96-well plates (5,000 cells/well) and incubated at 37°C. At 24-, 48-, and 72-hours post-transfection, 20 µL of MTS reagent was added per well and incubated for 2 hours. Absorbance was measured at 490 nm using a microplate reader.

Colony formation assay

Transfected HT29 cells were seeded at low density (500 cells/well) in 6-well plates and maintained for 10-14 days. Colonies were fixed with methanol (Thermo Fisher, Cat# A452-4), stained with 0.5% crystal violet (Thermo Fisher, Cat# C581-25), and counted using ImageJ software.

Wound healing assay

Transfected and control HT29 cells were seeded in 6-well plates and grown to ~90% confluence. A uniform scratch was made using a 200 µL pipette tip (Thermo Fisher, Cat# 9406075). Cells were washed with PBS to remove debris and incubated in serum-free DMEM. Images of

wound closure were captured at 0 and 24 hours using a phase-contrast microscope. Wound healing was quantified by measuring the wound closure area using ImageJ software.

Statistical analysis

All experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Differences between two groups were evaluated using an unpaired two-tailed Student's t-test, while multiple group comparisons were analyzed using one-way ANOVA followed by Tukey's post hoc test. The Kruskal-Wallis test was applied for non-parametric comparisons in gene expression analyses across immune subtypes. Correlation analyses were performed using Pearson's or Spearman's correlation tests as appropriate. Kaplan-Meier survival analysis was used to assess the prognostic significance of gene expression, and log-rank tests were employed for statistical comparisons. *P-value < 0.05, **P-value < 0.01, and ***P-value < 0.001 were considered significant.

Results

Cullin genes were up-regulated in READ samples

To investigate the expression of cullin family genes in READ, we conducted a comprehensive analysis using TIMER2.0 and HPA databases. Initially, we analyzed the expression of cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) across READ samples from the TCGA dataset using the TIMER2.0 database. The results indicated significant (p-value < 0.05) overexpression of these cullin genes in READ tissues compared to normal controls (**Figure 1A**). To confirm the observed expression patterns, we further validated the expression of cullin genes using immunohistochemistry data from the HPA database. As shown in **Figure 1B**, tissue samples from READ patients exhibited strong staining for cullin proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9), indicating high protein expression levels in tumor tissues (**Figure 1A**). The consistent high staining intensity further supports the upregulation of cullin family genes in READ (**Figure 1B**). Taken together, our analyses using the TIMER2.0 and HPA databases provided strong evidence that cullin genes were significantly upregulated in READ.

Genetic alteration analysis of cullin genes in READ

Analysis of cullin genetic alterations in READ samples, using the cBioPortal database, revealed a diverse spectrum of alteration types, affecting gene expression and protein function. 100% (14 out of 14) of the READ samples harbored genetic alterations in at least one of the analyzed cullin genes (**Figure 2A**). Specifically, the mutation analysis indicated that CUL9 was altered in 43% of samples, CULT in 29%, CUL5 in 29%, CULI in 29%, CUL2 in 21%, CUL3 in 21%, CUL7 in 21%, CUL4B in 7%, and CUL4A in 7% of the READ cohort (**Figure 2A**), encompassing missense, nonsense, and splice site mutations (**Figure 2B**, **2C**). Furthermore, copy number variation analysis demonstrated heterozygous deletions in several cullin genes and, less frequently, heterozygous amplifications, with CUL2, CUL4A, and CUL5 showing some instances of heterozygous amplification (**Figure 2D**, **2E**). These findings collectively indicated that genomic alterations of cullin genes, including both mutations and copy number changes such as amplification, were highly prevalent in READ tissue.

Association of cullin genes with cancer stage and patient gender

The transcriptional expression analysis of individual cullin genes in READ using the UALCAN portal revealed distinct patterns across different clinical variables. When examining cancer stages, the expression of CUL1 (**Figure 3A**) showed an increase in later stages (Stage 3-4) compared to normal tissue, suggesting a possible upregulation during tumor progression. Similarly, CUL2 (**Figure 3B**), CUL3 (**Figure 3C**), CUL4A (**Figure 3D**), CUL4B (**Figure 3E**), CUL5 (**Figure 3F**), CUL7 (**Figure 3G**), and CUL9 (**Figure 3H**) exhibited higher expression levels in tumor stages compared to the normal control. Furthermore, when stratified by patient gender, subtle differences in expression were observed for several cullin genes, including CUL1 (**Figure 3A**), CUL2 (**Figure 3B**), CUL3 (**Figure 3C**), CUL4A (**Figure 3D**), CUL4B (**Figure 3E**), CUL5 (**Figure 3F**), CUL7 (**Figure 3G**), and CUL9 (**Figure 3H**),

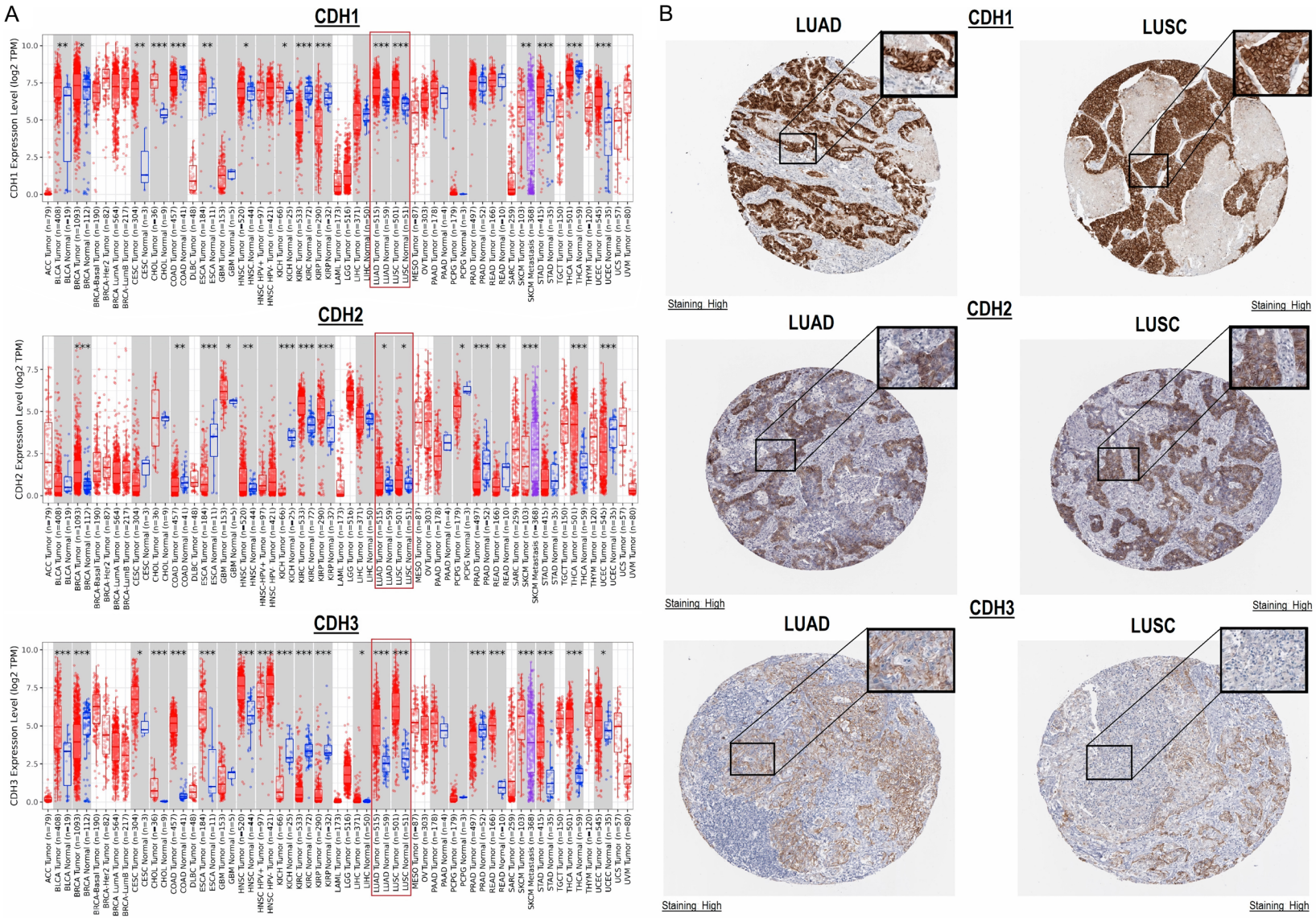


Figure 1. Differential expression of cullin genes in rectal adenocarcinoma (READ). A: Boxplot showing the transcriptional expression levels of cullin genes in READ and normal tissues from The Cancer Genome Atlas (TCGA) dataset. B: Immunohistochemistry (IHC) images from the Human Protein Atlas (HPA) database demonstrating strong staining intensity for cullin proteins in READ tumor tissues. **P*-value < 0.05, ***P*-value < 0.01, and ****P*-value < 0.001.

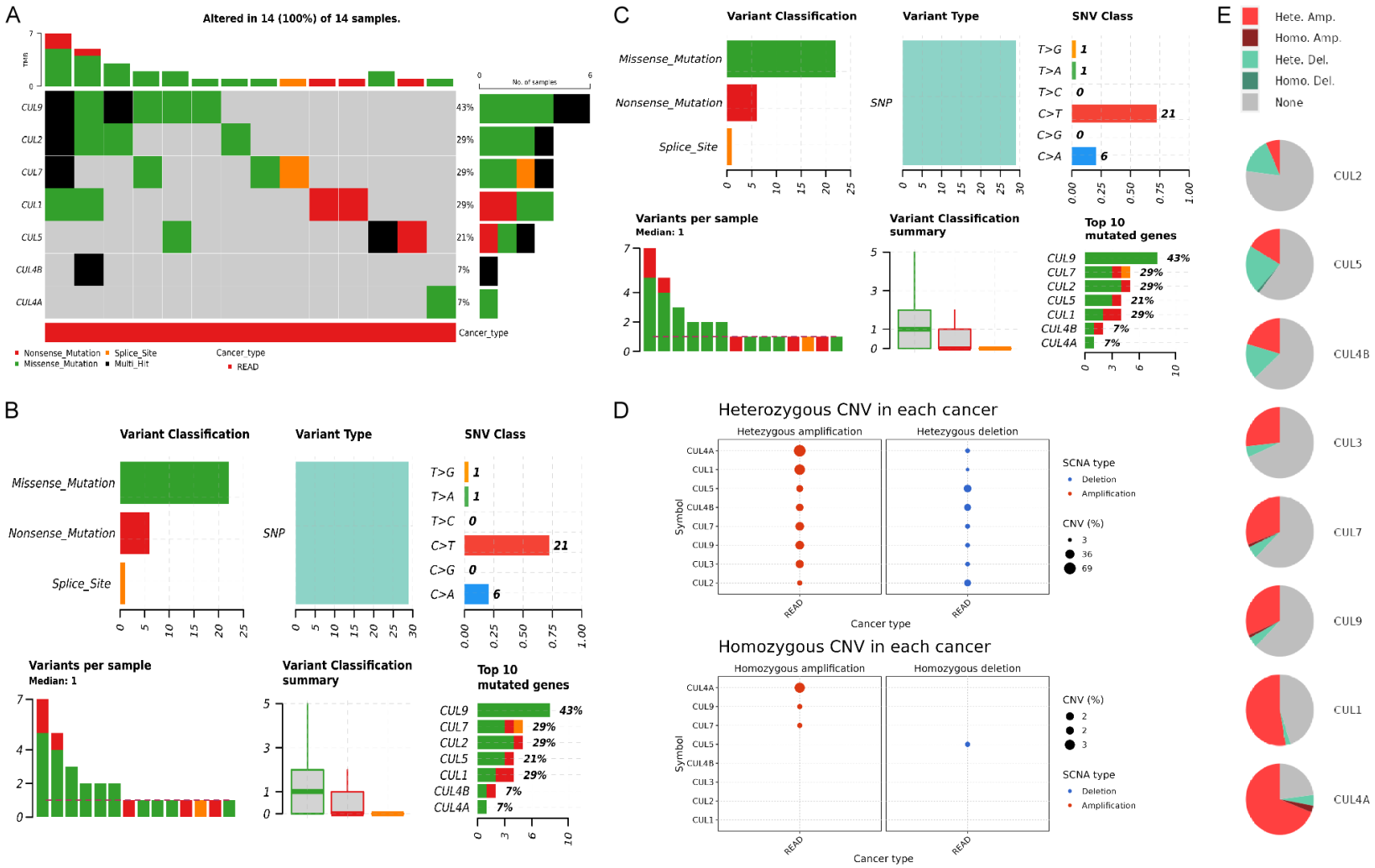


Figure 2. Genomic alterations in cullin genes across rectal adenocarcinoma (READ) samples. A: Bar graph depicting the frequency of genetic alterations in cullin genes among READ samples. B: Mutation analysis illustrating different types of mutations, including missense, nonsense, and splice site mutations. C: Lollipop plots indicating the specific mutation sites within cullin proteins. D, E: Copy number variation (CNV) analysis showing deletions and amplifications in cullin genes across READ samples.

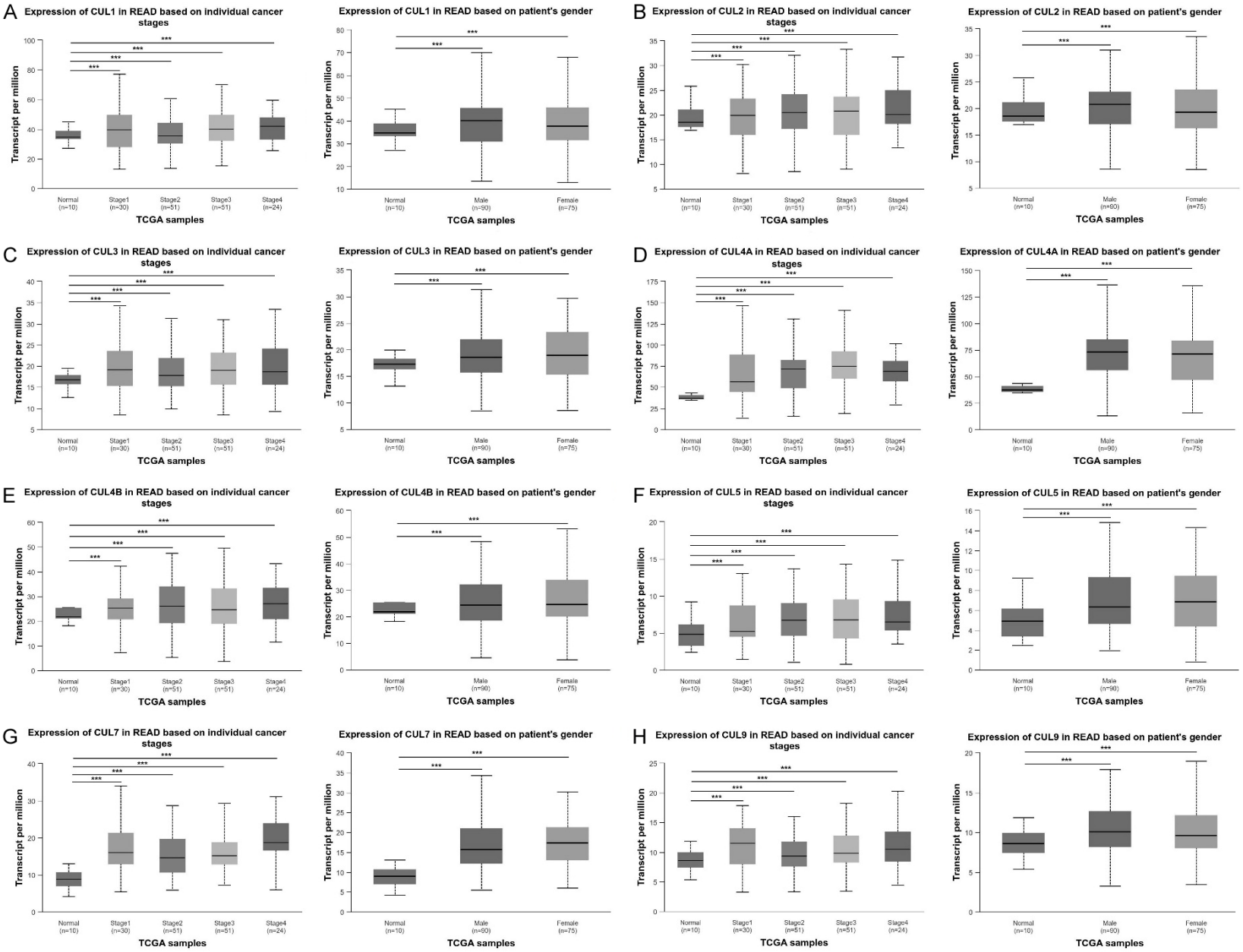

A
Expression of CUL1 in READ based on individual cancer stages
Transcript per million
Normal (n=10)
Stage1 (n=30)
Stage2 (n=51)
Stage3 (n=51)
Stage4 (n=24)
TCGA samples
Expression of CUL1 in READ based on patient's gender
Normal (n=10)
Male (n=90)
Female (n=75)
TCGA samples
B
Expression of CUL2 in READ based on individual cancer stages
Expression of CUL2 in READ based on patient's gender
C
Expression of CUL3 in READ based on individual cancer stages
Expression of CUL3 in READ based on patient's gender
D
Expression of CUL4A in READ based on individual cancer stages
Expression of CUL4A in READ based on patient's gender
E
Expression of CUL4B in READ based on individual cancer stages
Expression of CUL4B in READ based on patient's gender
F
Expression of CUL5 in READ based on individual cancer stages
Expression of CUL5 in READ based on patient's gender
G
Expression of CUL7 in READ based on individual cancer stages
Expression of CUL7 in READ based on patient's gender
H
Expression of CUL9 in READ based on individual cancer stages
Expression of CUL9 in READ based on patient's gender

Figure 3. Association of cullin gene expression with cancer stage and patient gender in rectal adenocarcinoma (READ). A-H: Boxplots showing the transcriptional expression levels of individual cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) across different tumor stages and between male and female patients, as analyzed using the UALCAN portal. *P*-value < 0.05.

suggesting sex-specific overexpression of these genes in tumor tissues.

Prognostic analysis of cullin genes in READ

Based on the survival analysis performed using the cSurvival database and visualized in **Figure 4**, we observed varying associations between the expression levels of different cullin genes and OS. Higher expression of CUL2 and CUL7 were significantly associated with poor prognosis in READ patients (**Figure 4**). However, there was no significant difference in OS for other genes, including Cul1, CUL3, CUL4A, CUL4B, CUL5, or CUL9 (**Figure 4**). These findings suggest that the higher expression levels of CUL2 and CUL7 genes have prognostic value in READ.

Expression analysis of cullin genes across immune subtypes in READ

The expression of cullin family genes was analyzed across different immune subtypes of READ using data from the TISIDB database. The results, visualized through violin plots, reveal distinct expression patterns of CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9 across the immune subtypes C1, C2, C3, C4, and C6. The statistical significance of differential expression was assessed using the Kruskal-Wallis test, with p-values provided for each gene. Notably, CUL1 (P = 3.66e-03), CUL3 (P = 1.32e-02), CUL4A (P = 4.25e-02), CUL7 (P = 1.00e-02), and CUL9 (P = 2.40e-04) exhibited significant differences in expression among the immune subtypes (**Figure 5**), indicating potential immune-related regulatory roles in READ. Conversely, CUL2 (P = 2.87e-01), CUL4B (P = 9.50e-02), and CUL5 (P = 1.16e-01) did not show a significant variation (**Figure 5**), suggesting relatively stable expression across immune subtypes.

miRNA-mRNA network, immune evasion, and drug resistance analysis of cullin genes in READ

The integrative analysis of cullin family genes in READ provided valuable insights into their regulatory networks, immune associations, and potential therapeutic implications. The miRNA-mRNA interaction network predicted using the miRNET database (**Figure 6A**) revealed several miRNAs that may regulate CUL gene expression, highlighting their possible role in post-transcriptional gene regulation. In this network, hsa-miR-34a-5p was noted to target all hub cullin genes simultaneously (**Figure 6A**). Expression analysis conducted by the UALCAN database demonstrated a significant upregulation of hsa-miR-34a in READ tumor samples compared to normal tissues (**Figure 6B**), suggesting its involvement in tumorigenesis. However, survival analysis indicated that hsa-miR-34a expression was not significantly associated with OS in READ patients (**Figure 6C**), implying that while dysregulated, its prognostic significance remained unclear. Furthermore, correlation analysis between CUL gene expression and immune infiltrates using the GSCA database revealed strong negative associations, particularly for CUL4A, with key immune cell populations such as Tfh cells, TH2 cells, NK, and MAIT cells (**Figure 6D**). This suggests that cullin genes may contribute to immune evasion in the tumor microenvironment by suppressing immune cell infiltration. Additionally, drug sensitivity analysis identified significant positive correlations between the expression of CUL4B and CUL7 with resistance to multiple chemotherapeutic agents, including PAC-1 and topoisomerase inhibitors (**Figure 6E**), indicating that elevated expression of these genes may contribute to drug resistance in READ.

Gene enrichment analysis of cullin genes

The gene enrichment analysis of cullin family genes was performed using the DAVID tool to explore their functional significance. The cellular component analysis revealed a strong enrichment in ubiquitin ligase complexes, including the SCF ubiquitin ligase complex, Cullin-RING ubiquitin ligase complex, and various RING E3 ubiquitin ligase complexes, highlighting the critical role of cullin proteins in ubiquitin-mediated protein degradation (**Figure 7A**). Molecular function analysis demonstrated significant enrichment in ubiquitin protein ligase

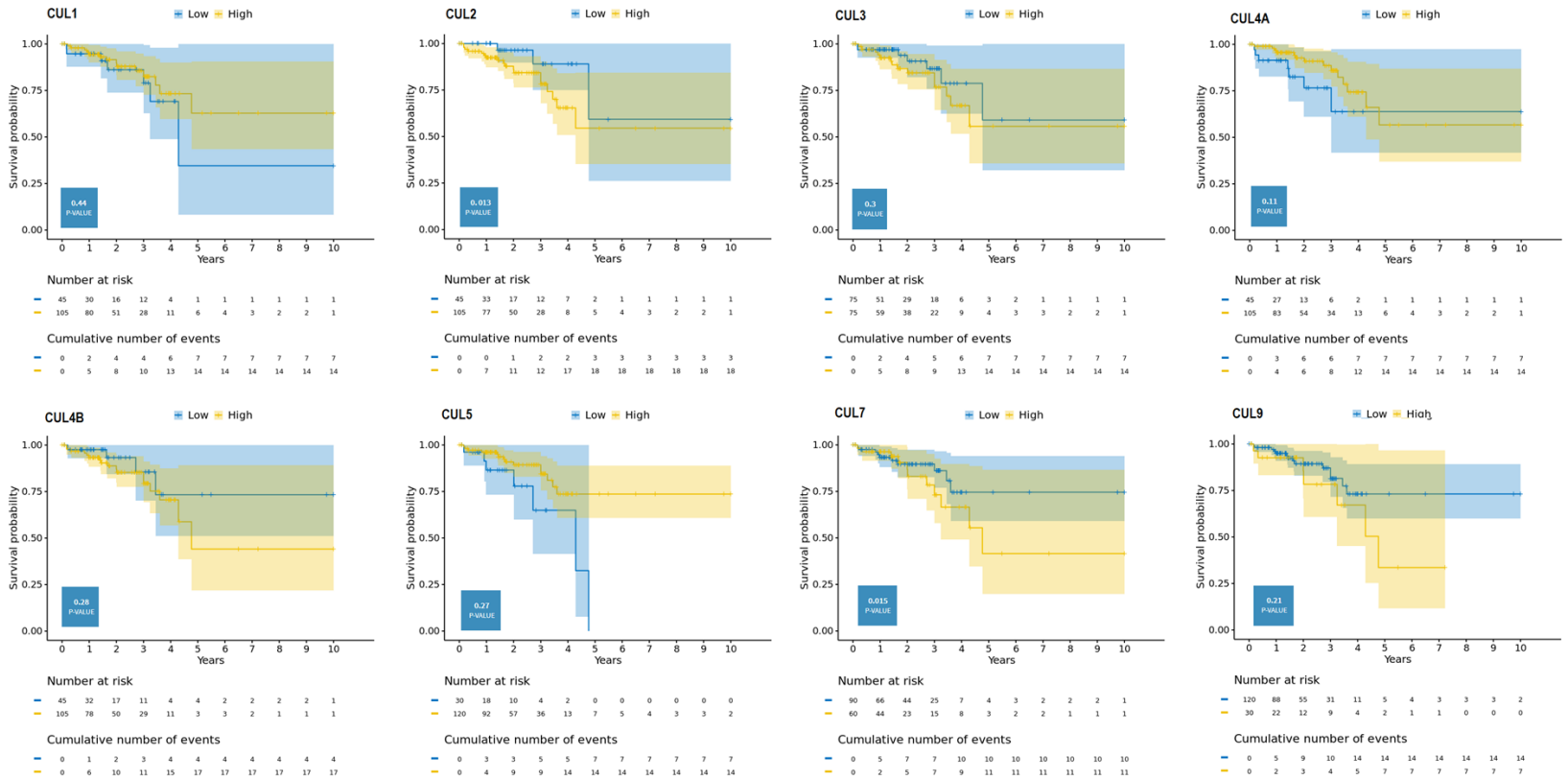


Figure 4. Prognostic significance of cullin genes in rectal adenocarcinoma (READ). Kaplan-Meier survival curves generated using the cSurvival database showing overall survival (OS) differences based on cullin gene expression levels in READ patients. Higher expression of CUL2 and CUL7 was significantly associated with worse prognosis ($P < 0.05$), while no significant OS differences were observed for CUL1, CUL3, CUL4A, CUL4B, CUL5, or CUL9. *P*-value < 0.05.

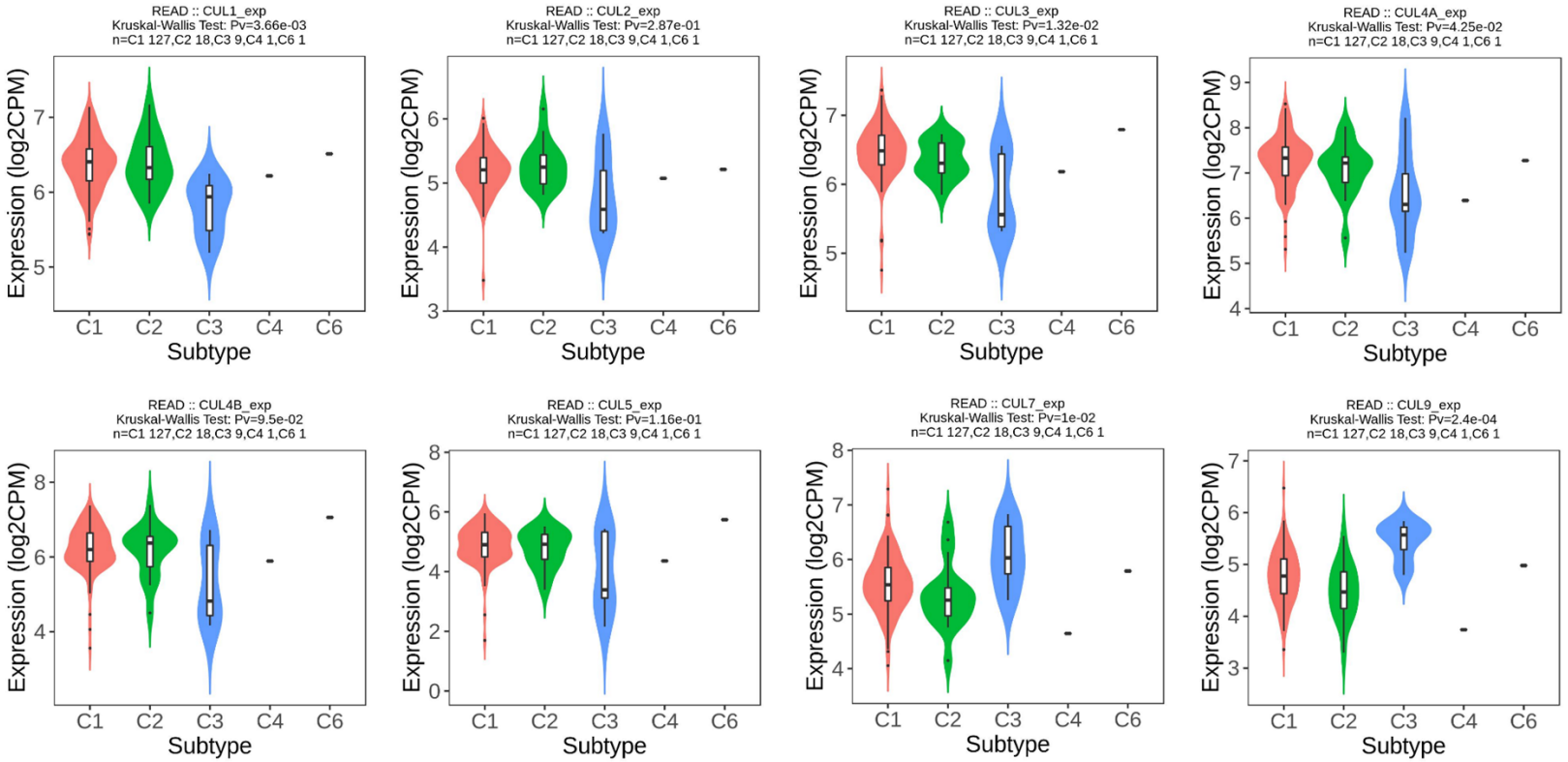
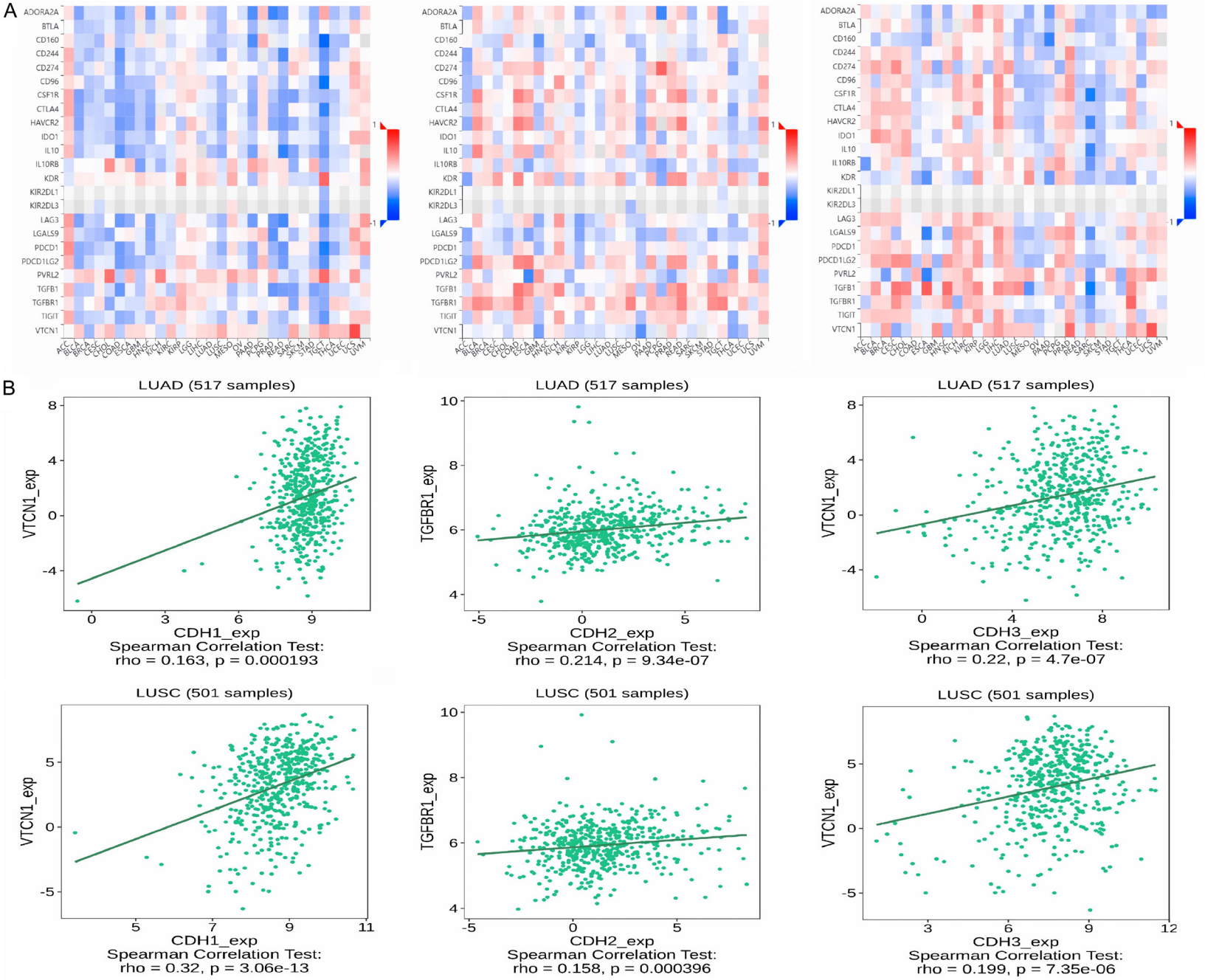


Figure 5. Expression of cullin genes across immune subtypes in rectal adenocarcinoma (READ). Violin plots illustrating the expression levels of CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9 across five immune subtypes (C1, C2, C3, C4, and C6) in READ. Significant differences were observed for CUL1, CUL3, CUL4A, CUL7, and CUL9, suggesting immune-related regulatory roles. *P*-value < 0.05.


A
ADORA2A
BTLA
CD160
CD244
CD274
CD96
CSF1R
CTLA4
HAVCR2
IDO1
IL10
IL10RB
KDR
KIR2DL1
KIR2DL3
LAG3
LGALS9
PDCD1
PDCD1LG2
PVRL2
TGFB1
TGFBR1
TIGIT
VTCN1
B
LUAD (517 samples)
VTCN1_exp
CDH1_exp
Spearman Correlation Test:
rho = 0.163, p = 0.000193
TGFBR1_exp
CDH2_exp
Spearman Correlation Test:
rho = 0.214, p = 9.34e-07
VTCN1_exp
CDH3_exp
Spearman Correlation Test:
rho = 0.22, p = 4.7e-07
LUSC (501 samples)
VTCN1_exp
CDH1_exp
Spearman Correlation Test:
rho = 0.32, p = 3.06e-13
TGFBR1_exp
CDH2_exp
Spearman Correlation Test:
rho = 0.158, p = 0.000396
VTCN1_exp
CDH3_exp
Spearman Correlation Test:
rho = 0.199, p = 7.35e-06

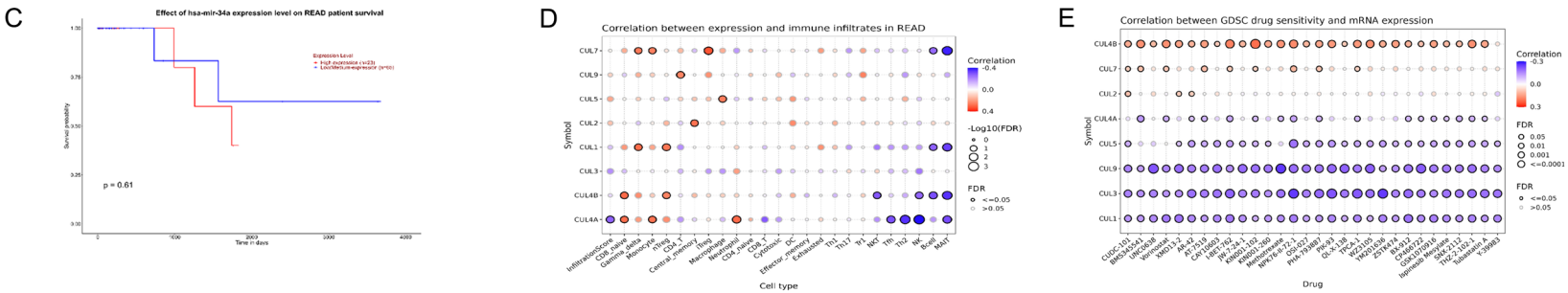


Figure 6. miRNA-mRNA network, immune evasion, and drug resistance analysis of cullin genes in rectal adenocarcinoma (READ). A: miRNA-mRNA interaction network showing predicted miRNA regulators of cullin genes, with hsa-miR-34a-5p targeting multiple cullin genes. B: Boxplot showing significant upregulation of hsa-miR-34a in READ tumor tissues. C: Kaplan-Meier survival plot indicating no significant association between hsa-miR-34a expression and overall survival in READ patients. D: Correlation heatmap depicting strong negative associations between CUL4A expression and immune cell infiltration, suggesting a role in immune evasion. E: Drug sensitivity analysis demonstrating that high expression of cullin genes correlates with resistance to multiple drugs. *P*-value < 0.05.

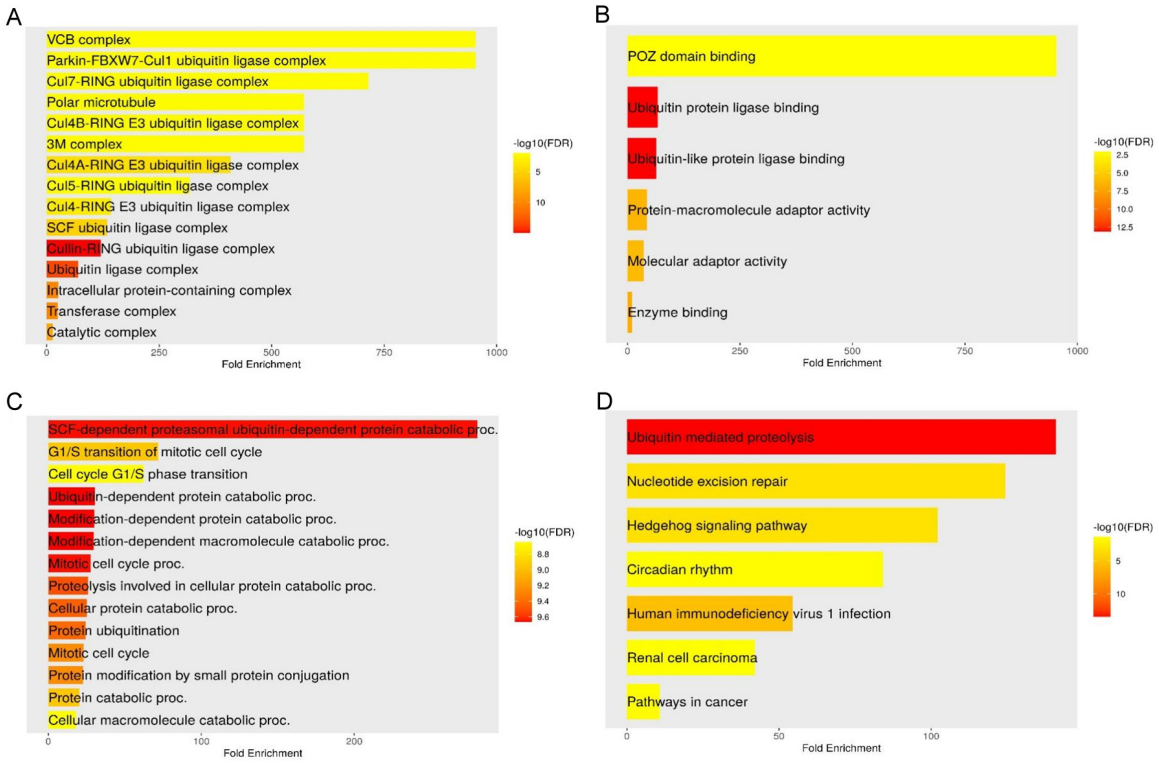


Figure 7. Gene enrichment analysis of cullin genes in rectal adenocarcinoma (READ). A: Cellular component enrichment analysis. B: Molecular function enrichment analysis. C: Biological process enrichment analysis. D: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. *P*-value < 0.05.

binding and protein-macromolecule adaptor activity, indicating the involvement of cullin genes in protein ubiquitination and molecular interactions necessary for proteasomal degradation (**Figure 7B**). The biological process analysis emphasized the contribution of cullin genes to SCF-dependent proteasomal ubiquitin-dependent protein catabolic processes, cell cycle regulation, and protein modification, particularly in the context of the G1/S transition of the mitotic cell cycle (**Figure 7C**). Additionally, pathway enrichment analysis identified ubiquitin-mediated proteolysis as the most significantly enriched pathway, along with pathways related to nucleotide excision repair, the Hedgehog signaling pathway, circadian rhythm regulation, and renal cell carcinoma, suggesting that cullin genes play a role in various oncogenic and regulatory pathways (**Figure 7D**).

CUL2 and CUL7 knockdown suppresses proliferation, colony formation, and migration in READ cells

The functional roles of CUL2 and CUL7 in READ cells were investigated by performing siRNA-mediated knockdown, followed by a series of functional assays. RT-qPCR and western blot analysis confirmed a significant reduction in the expression levels of CUL2 and CUL7 upon siRNA transfection, indicating efficient gene silencing (**Figure 8A**, **8B** and Supplementary Figure 1). Knockdown of CUL2 and CUL7 led to a significant decrease in cell proliferation, as evidenced by reduced proliferation rates compared to control cells (**Figure 8C**). Colony formation assays further demonstrated a marked reduction in the number of colonies formed in CUL2- and CUL7-silenced cells, suggesting impaired clonogenic potential (**Figure 8D**, **8E**). Additionally, wound healing assays revealed a substantial decrease in wound closure percentages in si-CUL2 and si-CUL7 cells relative to control cells, indicating a diminished migratory capacity (**Figure 8F**, **8G**). Collectively, these results suggest that CUL2 and CUL7 play crucial roles in promoting cell proliferation, colony formation, and migration in READ cells, suggesting significance in tumor progression.

Discussion

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide, with rectal ade-

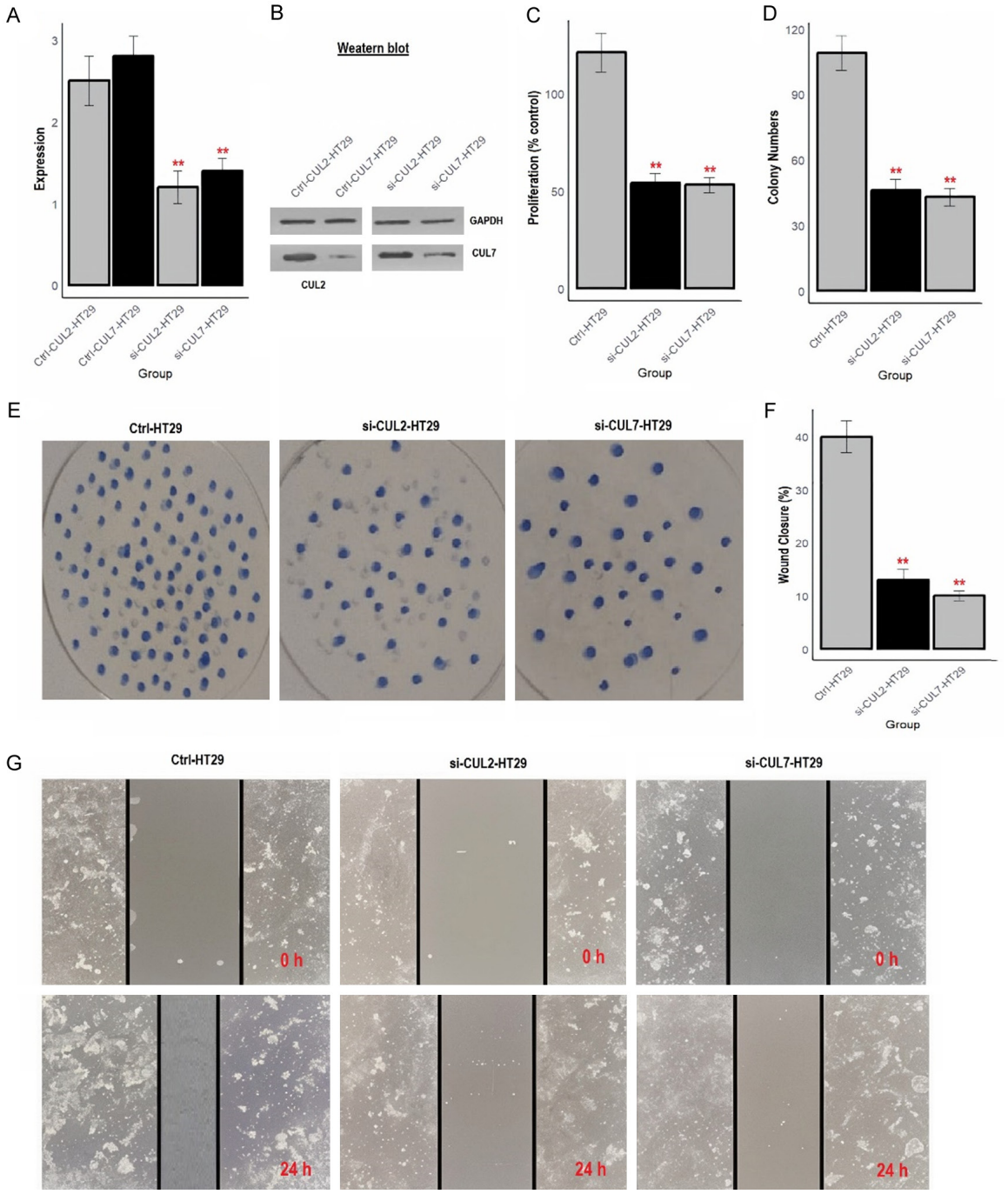


Figure 8. Functional impact of CUL2 and CUL7 knockdown in rectal adenocarcinoma (READ) cells. A, B: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis confirming efficient knockdown of Cullin 2 and Cullin 7 following small interfering RNA (siRNA) transfection. C: Cell proliferation assay showing a significant reduction in proliferation rates upon CUL2 and CUL7 silencing. D, E: Colony formation assay demonstrating decreased clonogenic potential in si-CUL2 and si-CUL7 transfected cells. F, G: Wound healing assay illustrating a significant reduction in wound closure percentage, indicating impaired migratory capacity upon CUL2 and CUL7 knockdown. ***P*-value < 0.01.

nocarcinoma (READ) being a major subtype [55-58]. Despite advances in treatment, the prognosis of READ remains poor due to late-stage diagnosis, tumor heterogeneity, and therapy resistance [56]. The cullin protein family, which plays a pivotal role in ubiquitin-mediated

proteolysis and cell cycle regulation, has been increasingly implicated in tumorigenesis [14, 59]. However, their specific involvement in READ remains largely unexplored. Our study systematically analyzed the expression, genetic alterations, prognostic significance, immune associations, and functional roles of Cullin genes in READ.

Previous studies have reported the overexpression of cullin genes in various cancers. For instance, CUL1 and CUL4A have been shown to be upregulated in hepatocellular carcinoma (HCC) and breast cancer, respectively, contributing to enhanced tumor growth and metastasis [60-62]. Similarly, CUL3 and CUL5 overexpression has been implicated in non-small cell lung cancer (NSCLC) and gastric cancer, respectively [60, 63]. Our study extends these findings by demonstrating significant overexpression of all eight cullin genes (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9) in READ tissues compared to normal controls. This strong upregulation, validated using both RNA expression data and protein expression, suggests an oncogenic role of cullin genes in READ progression. Moreover, recent reports show that dysregulation of cullin-mediated ubiquitination contributes to oncogenesis by promoting the degradation of tumor suppressors, such as p53 and RB1, while stabilizing oncogenic factors like MYC and β-catenin [64, 65]. Specifically, CUL1 and CUL4 have been implicated in the regulation of Wnt/β-catenin and PI3K/AKT signaling, both of which are frequently altered in READ and drive tumor growth, invasion, and therapeutic resistance [66, 67]. Additionally, CUL3 dysfunction has been associated with enhanced epithelial-mesenchymal transition (EMT), leading to increased metastatic potential in colorectal cancers [68]. The aberrant expression of cullin genes in READ underscores their significance as biomarkers and therapeutic targets, offering novel insight into their mechanistic role for tumorigenesis and treatment response.

Genetic alteration analysis revealed that all READ samples harbored mutations in at least one cullin gene. Notably, CUL9 exhibited the highest mutation frequency (43%), followed by CUL1, CUL5, and CUL7 (each 29%). These findings contrast with previous reports in glioblastoma and renal cell carcinoma, where CUL3 and CUL4B were the most frequently mutated cullins [27, 69]. Additionally, our CNV analysis showed amplification across multiple cullin genes, which is in contrast to pancreatic cancer, where CUL4A and CUL5 deletion were more prevalent [14, 15]. These differences suggest that cullin gene alterations may exhibit cancer-type specificity, further necessitating tissue-specific investigation.

Our survival analysis demonstrated that high expression of CUL2 and CUL7 was significantly associated with poor OS in READ patients. This aligns with prior findings in ovarian cancer and melanoma, where CUL2 overexpression was linked to unfavorable prognosis [23], whereas CUL7 upregulation correlated with poor survival outcomes in lung adenocarcinoma [70]. However, our study found no significant prognostic effect, of CUL1, CUL3, CUL4A, CUL4B, CUL5, or CUL9. This differs from findings in triple-negative breast cancer [71, 72], where CUL4B was a strong predictor of poor OS. These discrepancies emphasize the need for cancer-specific survival analyses of cullin genes.

Our immune subtype analysis revealed significant differential expression of CUL1, CUL3, CUL4A, CUL7, and CUL9 across immune subtypes, suggesting an immune-regulatory role in READ. Notably, CUL4A expression negatively correlated with Tfh, TH2, NK, and MAIT cell infiltration, supporting its potential involvement in immune evasion. This is consistent with findings in hepatocellular carcinoma, where CUL4A was implicated in suppressing immune responses by modulating T-cell infiltration [73]. Our miRNA-mRNA network analysis identified hsa-miR-34a-5p as a possible regulator of multiple cullin genes in READ, suggesting a post-transcriptional regulatory mechanism. While hsa-miR-34a-5p has been previously reported to target CUL4B in head and neck cancer [74], our study provides new evidence that it may simultaneously regulate multiple cullin genes in READ. Additionally, our drug sensitivity analysis demonstrated that CUL4B and CUL7 overexpression correlated with resistance to PAC-1 and topoisomerase inhibitors, a finding not been previously reported in READ, and this may have therapeutic implications.

Functional validation experiments demonstrated that knockdown of CUL2 and CUL7 significantly reduced proliferation, colony formation,

and migration in READ cells. While previous studies have linked CUL2 silencing to suppressed growth in renal cancer [69, 75], our study was the first to report the tumor-promoting roles of CUL2 and CUL7 in READ. These findings suggest that targeting CUL2 and CUL7 could be a promising therapeutic approach for READ treatment.

Despite the comprehensive nature of our study, several limitations must be acknowledged. First, our analyses were primarily based on publicly available datasets and *in vitro* experiments, necessitating further validation using patient-derived xenograft (PDX) models or clinical tissue samples. Second, while we identified miRNA interactions and drug resistance patterns, mechanistic studies are required to elucidate the exact pathways through which cullin genes contribute to immune evasion and chemoresistance in READ. Third, our study focused on transcriptional and genetic alterations; future investigations should integrate epigenetic modifications and post-translational modifications to gain a more holistic understanding of cullin gene regulation in READ. Finally, the effect of CUL2 and CUL7 knockdown on tumorigenesis needs further validation by *in vivo* models to establish their therapeutic potential.

Conclusion

Our study provided the first comprehensive analysis of Cullin genes in READ, revealing their significant upregulation, genetic alterations, prognostic implications, immune associations, and functional roles. Our findings suggest that CUL2 and CUL7 may be biomarkers and therapeutic targets in READ, warranting further exploration.

Acknowledgements

This work was supported by the Tianjin Health Research Project (TJWJ2023XK014).

Disclosure of conflict of interest

None.

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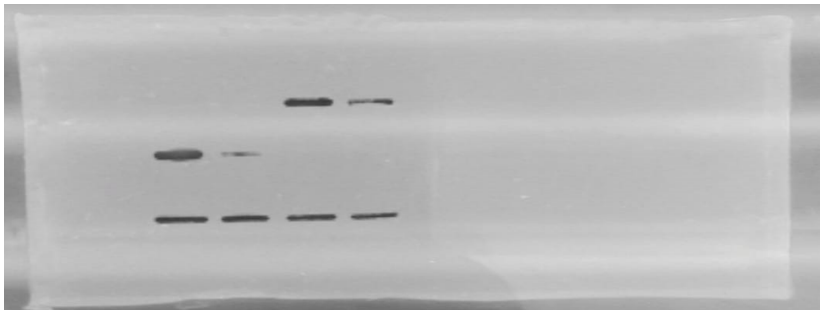

Supplementary Figure 1. Uncut western blot bands of CUL, CUL7, and GAPDH.