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Oncogenic Role of Long Noncoding RNA DLGAP1-AS2 in Colorectal Cancer: Regulation of the Trim21/ELOA/LHPP Signaling Axis

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Abstract

Long noncoding RNAs (lncRNAs) have attracted considerable attention for their roles as either oncogenes or tumor suppressors in cancer development. Yet, the specific functions and molecular mechanisms of the majority of lncRNAs in colorectal cancer (CRC) remain poorly understood. DLGAP1-AS2 levels were quantified using RT-qPCR across several CRC patient cohorts. Its effects on CRC cell proliferation and metastatic potential were investigated through a combination of in vitro and in vivo assays. The molecular interactions and regulatory mechanisms of DLGAP1-AS2 were explored using RNA pull-down, RNA immunoprecipitation, RNA sequencing, luciferase reporter assays, chromatin immunoprecipitation, and rescue experiments. DLGAP1-AS2 was found to enhance CRC tumor growth and metastasis by binding directly to Elongin A (ELOA) and reducing its stability via Trim21-mediated ubiquitination and subsequent degradation. Furthermore, DLGAP1-AS2 suppresses LHPP expression by interfering with ELOA-driven transcriptional activation, thereby preventing LHPP-dependent inhibition of the AKT pathway. In addition, cleavage and polyadenylation factors CPSF2 and CSTF3 were shown to interact with DLGAP1-AS2, stabilizing it in CRC cells. DLGAP1-AS2 represents a novel prognostic marker and offers insight into the molecular drivers of CRC, highlighting a potential target for therapeutic intervention.

Keywords: Colorectal cancer, Long noncoding RNAs, DLGAP1-AS2, ELOA, Trim21, LHPP

Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer and ranks second among causes of cancer-related deaths worldwide. Incidence rates are rising, with around 1.9 million new cases and 935,000 deaths reported in 2020 [1]. The molecular basis of CRC is not fully resolved, and treatment outcomes remain suboptimal, emphasizing the need to uncover novel molecular targets and pathways.

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Recent studies in cancer transcriptomics have highlighted that numerous genes involved in tumorigenesis are noncoding RNAs (ncRNAs). Among them, long ncRNAs (lncRNAs) have gained particular attention due to their ability to regulate protein-protein, RNA-protein, and protein-DNA interactions, as well as to act as miRNA sponges [2]. Several lncRNAs have been implicated in CRC progression and may serve as diagnostic or therapeutic biomarkers [3-9].

Our analysis of the CRC transcriptome identified multiple differentially expressed lncRNAs, including FEZF1-AS1, LINC00152 (CYTOR), MCM3AP-AS1, SLCO4A1-AS1, SNHG6, SNHG15, SNHG17, and UCA1 [7-15]. For example, FEZF1-AS1 promotes CRC development via PKM2/STAT3 signaling and metabolic regulation [7], while CYTOR contributes to CRC progression through interactions with NCL and Sam68 [12].

In the present study, we discovered a previously uncharacterized DLGAP1-AS2 transcript that is markedly upregulated in CRC and associated with malignant characteristics and poor prognosis. Functional and mechanistic investigations revealed that DLGAP1-AS2 drives CRC progression by promoting Trim21-mediated ubiquitination and degradation of ELOA. ELOA, in turn, binds to the LHPP promoter to enhance its transcription, activating LHPP-mediated inhibition of AKT signaling. Additionally, DLGAP1-AS2 is stabilized through interactions with CPSF2 and CSTF3. Our findings reveal a novel DLGAP1-AS2/Trim21/ELOA/LHPP regulatory axis in CRC, suggesting that targeting this pathway could provide new therapeutic opportunities.

Materials and Methods

Cell lines

The colorectal cancer (CRC) cell lines Caco-2, DLD1, HCT116, HCT8, HT29, LoVo, RKO, and SW480 were obtained from ATCC and cultured according to the supplier's guidelines. Cell identity was verified through short tandem repeat profiling by Genewiz, Inc. (China), and all cultures were confirmed to be free from mycoplasma contamination.

Clinical samples

Paired samples of primary CRC tissues and adjacent noncancerous tissues (NCTs) were collected from patients at the Affiliated Hospital of Jiangnan University under informed consent. The protocol received approval from the Clinical Research Ethics Committee of the Affiliated Hospital of Jiangnan University, and all participants provided written consent.

RNA sequencing analyses

Total RNA was extracted from CRC and NCT samples using TRIzol (Invitrogen, USA). Ribosomal RNA was removed using the Ribo-off rRNA Depletion Kit (Vazyme, China). RNA-seq libraries were prepared with the VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina (Vazyme) and sequenced on an Illumina platform with 150 bp paired-end reads. Sequencing reads were aligned to the human genome GRCh38 using HISAT2. lncRNA and mRNA annotations were obtained from GENCODE (v25). Additionally, expression data and clinical information for multiple CRC cohorts were retrieved from TCGA (https://portal.gdc.cancer.gov/) and GEO (http://www.ncbi.nlm.nih.gov/geo).

Quantitative RT-PCR (qRT-PCR)

cDNA synthesis was performed with the HiFiScript cDNA Synthesis Kit (CWBIO, China). Gene expression was quantified using qRT-PCR and the Ultra SYBR Mixture (Vazyme, China). β-actin served as the internal control, and relative expression was calculated using the $2-\triangle\triangle Ct$ method.

Plasmids and siRNAs

The DLGAP1-AS2 sequence was cloned into pLenti-EF1a-F2A-Puro-CMV-MCS. CPSF2 and ELOA were inserted into pRK7-Flag, whereas CSTF3 and Trim21 were cloned into PCMV5-HA and pcDNA3.1-Myc, respectively. SiRNAs targeting DLGAP1-AS2, CPSF2, CSTF3, ELOA, and Trim21 were purchased from GenePharma (China). Verified shRNAs for DLGAP1-AS2 and ELOA were subcloned into pLKO.1 vectors. The LHPP promoter was amplified by PCR from human genomic DNA and inserted into the pGL3-Basic vector.

Cell proliferation and colony formation assays

Cell viability was measured using the CCK8 assay (Beyotime, China) according to the manufacturer's instructions. For colony formation, 800–1500 CRC cells per well were plated in 6-well plates and cultured in 10% FBS medium for 10–15 days. Colonies were fixed with methanol, stained with 0.1% crystal violet, and quantified under an inverted microscope. Each experiment was performed at least in triplicate.

Migration and invasion assays

Transwell chambers (Corning, USA) were used to evaluate cell migration and invasion, following the manufacturer's protocols.

In vivo assays

Male BALB/c nude mice were obtained from Shanghai Animal Center, Chinese Academy of Sciences, and maintained under pathogen-free conditions at Jiangnan University. For tumor growth studies, 5-week-old mice were randomly assigned to groups (n = 5 per group) and subcutaneously injected with 0.1 ml of a suspension containing 2 × 10^6 CRC cells. Tumor volumes were calculated as volume = length × width^2 × 0.5. For metastasis assays, 7-week-old mice (n = 5 per group) received tail vein injections of 2 × 10^6 CRC cells. Five weeks later, the lungs were examined for metastatic

nodules. All procedures were approved by the Jiangnan University Medical Experimental Animal Care Commission (JN.No20190615b0320925) and complied with institutional and national guidelines.

RNA pull-down and mass spectrometry

RNA-protein interactions were analyzed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher, USA) following the manufacturer's instructions. Pull-down samples were resolved by gel electrophoresis and visualized with silver staining. Distinct bands were excised for proteomic identification by mass spectrometry and cross-referenced with the Human Protein Reference Database (http://www.hprd.org/).

RNA Immunoprecipitation (RIP) Assays

RIP experiments were carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) as per the supplier's protocol. Cell lysates were incubated at 4 °C overnight with magnetic beads coated with antibodies targeting CPSF2, CSTF3, ELOA, or control IgG. RNA was then extracted from the immunoprecipitated complexes and analyzed via RT-PCR to measure DLGAP1-AS2 levels. Detailed antibody information is provided in Tab.S5.

Western blotting

Cells were lysed in Beyotime lysis buffer supplemented with protease inhibitors (Roche, USA). Proteins were separated using SDS–PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking, membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000, Thermo Fisher) at room temperature for 1 h. Signals were detected using ECL reagents (Vazyme).

Immunoprecipitation (IP) Assays

HCT116 cells transfected with the indicated plasmids were lysed in IP buffer (Beyotime) containing protease inhibitors. The lysates were incubated with antibody-coated beads overnight at 4 °C under gentle rotation. After three washes, bound protein complexes were eluted and analyzed by western blot using the appropriate antibodies.

CRISPR-Cas9–mediated trim21 knockout

Two sgRNAs targeting exon 2 of TRIM21 were designed using a CRISPR library and cloned into the pLentiCRISPR vector (Trim21-gRNA-pLentiCRISPR). HCT116 cells were transfected with these constructs to generate Trim21 knockout lines following the manufacturer's instructions. Puromycin selection was applied, and single-cell colonies were isolated. Trim21 deletion was confirmed by Sanger sequencing and western blotting.

Immunohistochemistry (IHC)

ELOA expression in CRC tissue samples was evaluated by IHC on 4-μm paraffin-embedded sections. Slides were incubated with anti-ELOA antibody (Santa, 1:200) at 4 °C overnight and processed using the GT Vision III Detection System/Mo&Rb (GeneTech, China). Two independent pathologists, blinded to the sample identities, scored the staining.

Chromatin Immunoprecipitation (ChIP)-on-chip and analysis

Protein-DNA interactions were captured using antibodies against ELOA or control IgG. Immunoprecipitated DNA was amplified, labeled, and hybridized to Nimblegen human 720 K RefSeq promoter arrays (Roche Nimblegen, USA). Peaks with FDR ≤ 0.05 were mapped to the nearest genes. Promoter sequences from differentially expressed genes were extracted, and MEME-ChIP with a Markov model was employed to identify potential ELOA-binding motifs.

Dual-Luciferase Reporter Assays

Reporter plasmids were co-transfected with ELOA-expressing constructs. Forty-eight hours post-transfection, luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Beyotime).

Statistical analysis

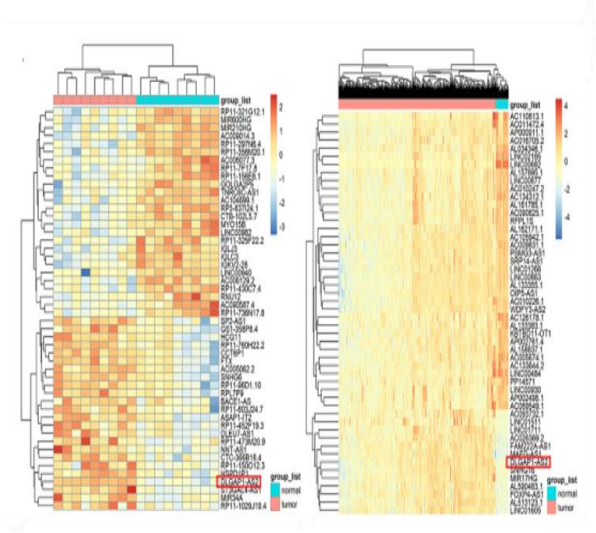
Data are presented as mean ± standard deviation. Statistical calculations were performed using GraphPad Prism 8.0 (GraphPad Software, USA) and SPSS 20.0 (SPSS Inc., USA), with p-values < 0.05 considered significant.

Results and Discussion

Upregulation of DLGAP1-AS2 predicts adverse clinical outcomes in CRC

To discover lncRNAs relevant to colorectal cancer (CRC), next-generation sequencing–based transcriptome

profiling was performed on nine matched pairs of CRC tissues and corresponding normal colorectal tissues (NCTs). The 50 most significantly altered lncRNAs were subsequently cross-validated using the TCGA CRC dataset, where DLGAP1-AS2 consistently exhibited marked overexpression in both CRC cohorts **(Figure 1a)**. This abnormal elevation was independently confirmed in four additional CRC datasets (GSE32323, GSE8671, GSE18105, and GSE22598); **(Figure 1b)**). Validation in a separate clinical cohort further demonstrated that 67% (67 of 101) of CRC specimens displayed greater than a 1.5-fold increase in DLGAP1-AS2 expression relative to matched adjacent NCTs **(Figure 1c).** Broad pancancer analyses additionally showed that DLGAP1-AS2 expression was elevated across multiple malignancies, including stomach adenocarcinoma, esophageal carcinoma, pancreatic adenocarcinoma, cholangiocarcinoma, and kidney renal papillary cell carcinoma, indicating that DLGAP1-AS2 may represent a general oncogenic lncRNA **(Figure 1d).** Based on these findings, DLGAP1-AS2 was selected for further functional investigation.



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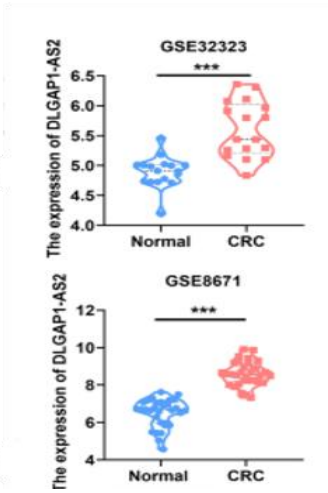

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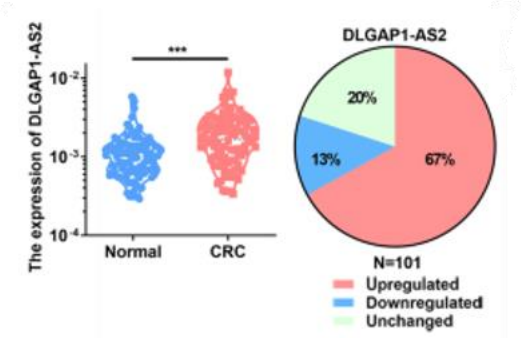

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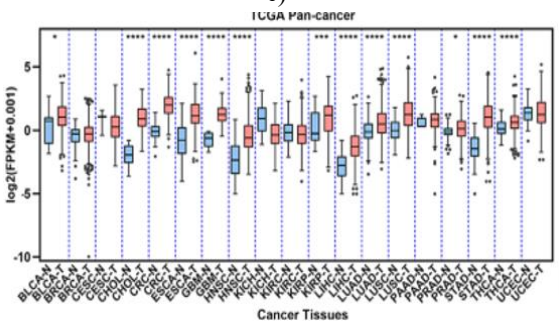

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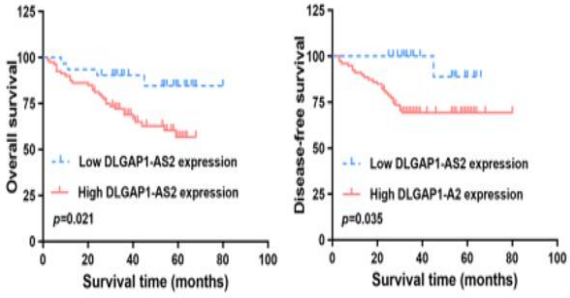

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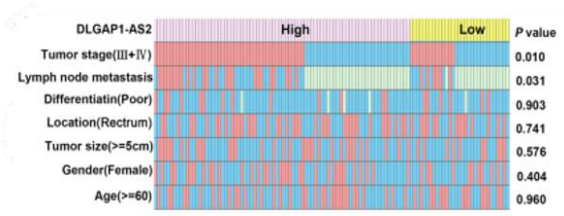

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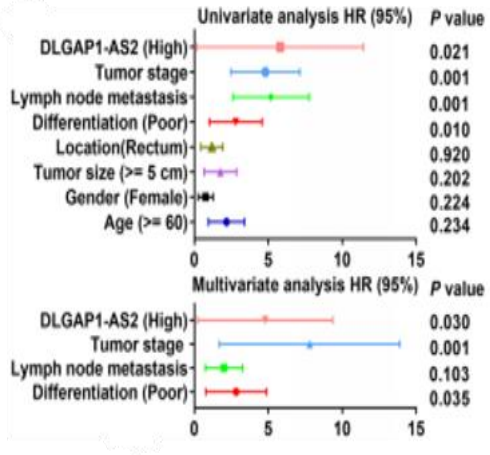

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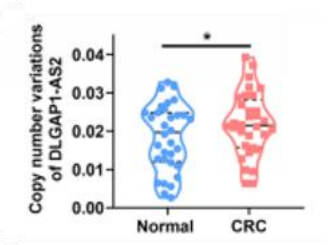

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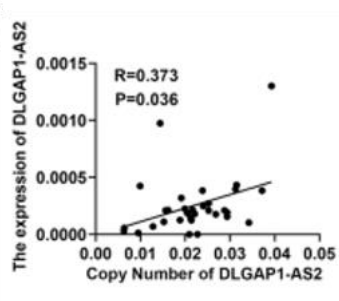

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Figure 1. Association of DLGAP1-AS2 with poor prognosis in CRC.

(a) Heatmap illustrating significantly dysregulated lncRNAs derived from RNA-seq profiling of nine CRC samples and paired normal tissues, integrated with TCGA CRC data.

(b) DLGAP1-AS2 expression levels in CRC datasets GSE32323 and GSE8671.

(c) qRT-PCR–based quantification of DLGAP1-AS2 expression in 101 paired CRC and adjacent normal tissues.

(d) Expression distribution of DLGAP1-AS2 across multiple cancer types.

(e) Kaplan–Meier curves for overall survival and disease-free survival stratified by DLGAP1-AS2 expression in CRC.

(f) Associations between DLGAP1-AS2 expression and clinicopathological characteristics in CRC.

(g) Results of univariate and multivariate regression analyses in CRC patients.

(h) Genomic copy number assessment of DLGAP1-AS2 in CRC tissues using qPCR.

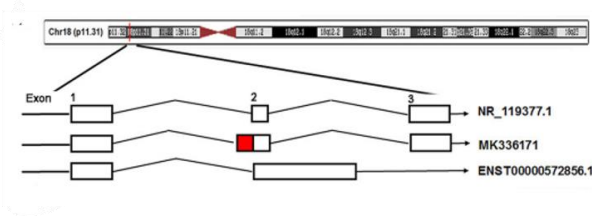
(i) Correlation analysis between DLGAP1-AS2 copy number and transcript abundance in CRC tissues.

Survival analysis using Kaplan–Meier methods demonstrated that elevated DLGAP1-AS2 expression was significantly associated with shorter overall survival and reduced disease-free survival **(Figure 1e)**. Further correlation analyses revealed significant relationships between DLGAP1-AS2 expression and tumor differentiation grade, lymph node involvement, and tumor stage **(Figure 1f)**. Importantly, both univariate and multivariate Cox regression models identified DLGAP1-AS2 expression as an independent prognostic factor in CRC patients **(Figure 1g).**

To elucidate mechanisms responsible for DLGAP1-AS2 overexpression in CRC, copy number variation (CNV) data from the TCGA CRC cohort were examined. Compared with adjacent NCTs, CRC tissues showed increased genomic copies of DLGAP1-AS2, accompanied by a weak but positive correlation between copy number and expression level. This observation was further supported by qPCR analysis of 32 paired CRC and NCT samples, confirming that CNV contributes to DLGAP1-AS2 dysregulation (**Figures 1h–1i**). Because promoter CpG island methylation commonly influences gene transcription, the promoter region of DLGAP1-AS2 was analyzed; however, no CpG islands were identified, suggesting that DNA methylation does not play a major role in regulating DLGAP1-AS2 expression. Collectively, these results indicate that increased DLGAP1-AS2 expression in CRC is partially driven by genomic copy number amplification.

Discovery of a novel DLGAP1-AS2 transcript in CRC

Integrated analysis of DLGAP1-AS2 sequence annotations from GenBank, GENECODE, and UCSC databases identified two primary transcripts with lengths of 923 bp (NR_119377.1) and 2261 bp (ENST00000572856.1), respectively **(Figure 2a)**. Notably, cloning experiments based on the GenBank-annotated transcript (NR_119377.1) revealed a previously uncharacterized isoform containing an additional 58 bp insertion within the second exon; this transcript has been deposited in GenBank under accession number MK336171 **(Figure 2b).** Subsequent qRT-PCR and semi-quantitative RT-PCR analyses demonstrated that this novel isoform represents the dominant transcript expressed in CRC tissues as well as in other cancer cell lines **(Figures 2c and 2d)**. Moreover, analyses using the Coding Potential Assessment Tool (CPAT) and PhyloCSF confirmed the absence of protein-coding capacity for DLGAP1-AS2. Accordingly, this newly identified predominant transcript was selected for subsequent CRC studies. DLGAP1-AS2 was found to be highly expressed across multiple CRC cell lines and localized to both the nuclear and cytoplasmic compartments of CRC cells.



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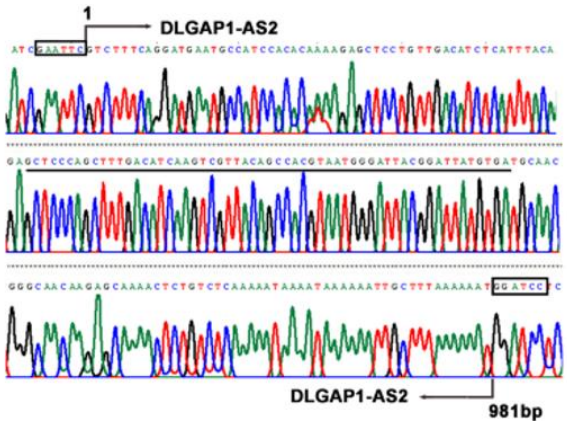

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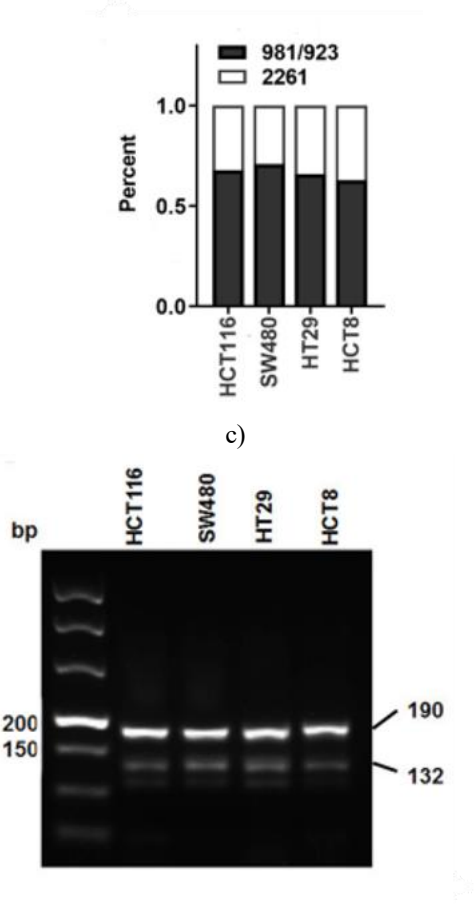
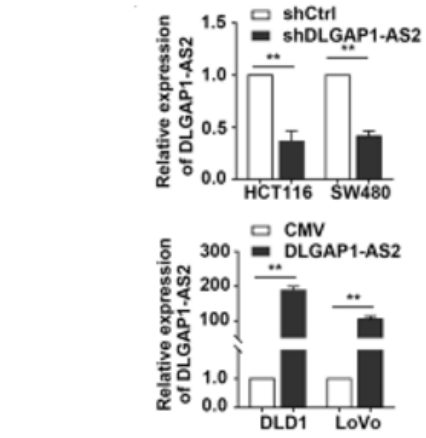


Figure 2. Discovery of a novel DLGAP1-AS2 transcript in CRC.
(a) Genomic schematic illustrating alternative DLGAP1-AS2 transcript isoforms and their chromosomal location at human chromosome 18p11.31 based on UCSC Genome Browser annotations (GRCh38/hg38).
(b) Sequencing validation of the newly identified DLGAP1-AS2 transcript, highlighting the inserted 58 bp segment (underlined).
(c) Quantitative comparison of the expression abundance of individual DLGAP1-AS2 transcript variants in CRC cells.
(d) Semi-quantitative RT-PCR detection of the two DLGAP1-AS2 isoforms in CRC cell lines. Amplified products of 190 bp and 132 bp correspond to the 981 bp and 923 bp DLGAP1-AS2 transcripts, respectively.

DLGAP1-AS2 enhances colorectal cancer cell proliferation and metastatic potential

CRC cell lines exhibiting comparatively high endogenous DLGAP1-AS2 expression (HCT116 and

SW480) or low expression (DLD1 and LoVO) were chosen for knockdown or ectopic expression experiments, respectively, followed by functional characterization. Three independent siRNAs targeting DLGAP1-AS2 were designed, and the most effective sequence (siDLGAP1-AS2-1) was subsequently used to generate shRNA constructs and establish stable DLGAP1-AS2–depleted CRC cell lines. Functional analyses using CCK-8 and colony formation assays revealed that suppression of DLGAP1-AS2 markedly reduced, whereas enforced expression significantly increased, CRC cell proliferation and clonogenic growth **(Figures 3a–3c**). Transwell-based assays further demonstrated that depletion of DLGAP1-AS2 substantially impaired migratory and invasive capacities in HCT116 and SW480 cells, while DLGAP1-AS2 overexpression enhanced these behaviors in DLD1 and LoVO cells **(Figures 3d–3e).**



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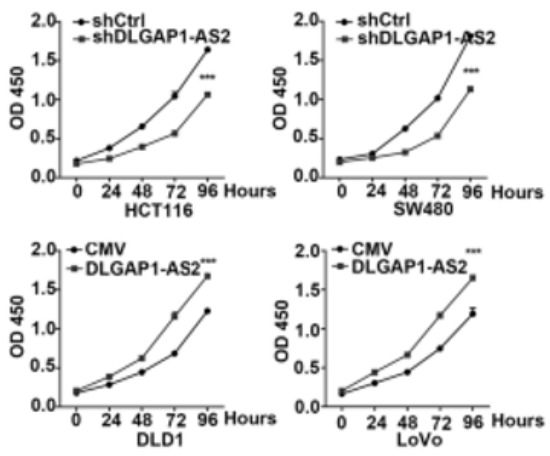

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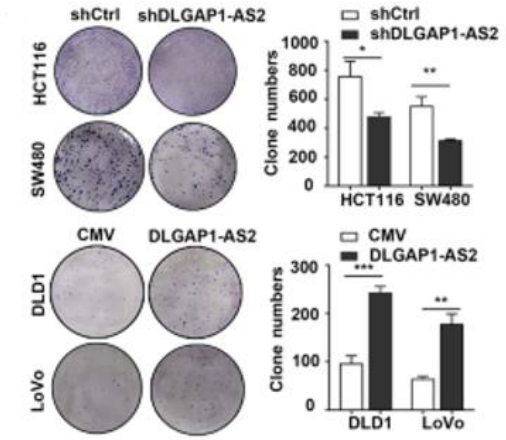

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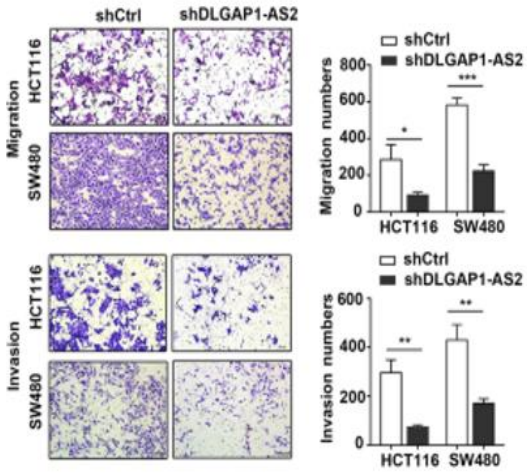

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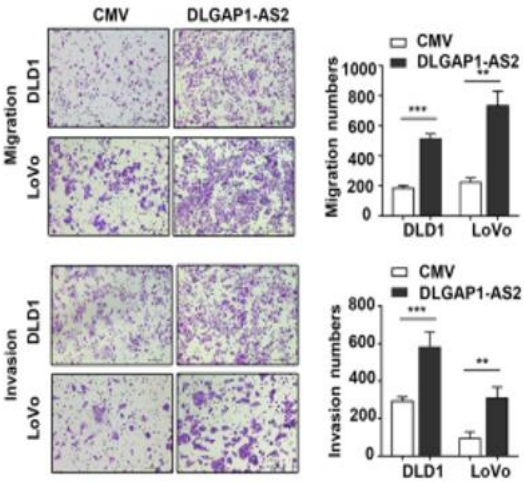

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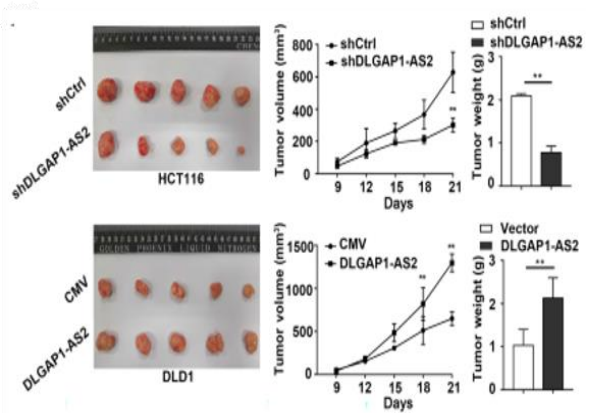

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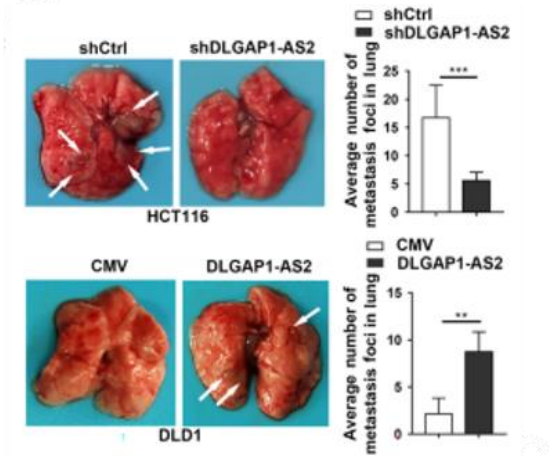

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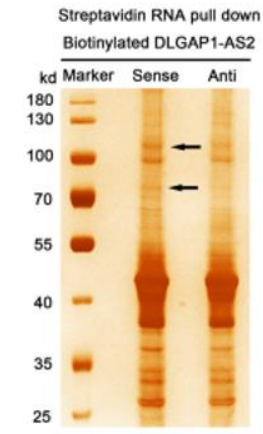
Figure 3. DLGAP1-AS2 drives CRC cell growth and metastatic behavior.
(a) qRT-PCR confirmation of DLGAP1-AS2 silencing or overexpression in CRC cells.
(b–c) Effects of DLGAP1-AS2 modulation on CRC cell proliferation (b) and colony-forming ability (c), assessed by CCK-8 and colony formation assays.
(d–e) Evaluation of CRC cell migration and invasion using Transwell assays following DLGAP1-AS2 manipulation.
(f) Assessment of DLGAP1-AS2–mediated colorectal tumor growth in a xenograft mouse model (n = 5).
(g) Analysis of DLGAP1-AS2 function in CRC metastasis using a lung colonization mouse model (n = 5).

To validate the tumor-promoting role of DLGAP1-AS2 in vivo, CRC cells with stable DLGAP1-AS2 knockdown or overexpression were subcutaneously implanted into nude mice. Tumors derived from DLGAP1-AS2–silenced cells exhibited significantly reduced volume and mass compared with control tumors, whereas forced expression of DLGAP1-AS2 markedly accelerated xenograft tumor growth **(Figure 3f).**

A lung metastasis model was further employed to determine the role of DLGAP1-AS2 in CRC dissemination. The results showed that loss of DLGAP1-AS2 dramatically suppressed metastatic burden, whereas its overexpression significantly enhanced metastatic colonization of the lungs **(Figure 3g)**. Collectively, these findings indicate that DLGAP1-AS2 functions as a potent promoter of CRC growth and metastasis.

DLGAP1-AS2 directly associates with CPSF2, CSTF3, and ELOA in CRC cells

To investigate the molecular mechanism underlying the oncogenic activity of DLGAP1-AS2, RNA pull-down assays were conducted to identify interacting proteins in CRC cells. Proteins captured by DLGAP1-AS2 were separated by SDS–PAGE and subjected to mass spectrometry and subsequent immunoblot analysis. These experiments identified CPSF2, CSTF3, and ELOA as candidate DLGAP1-AS2–binding proteins **(Figures 4a and 4b)**. RNA immunoprecipitation (RIP) assays further confirmed the physical association between DLGAP1-AS2 and these three proteins (**Figure 4c).**



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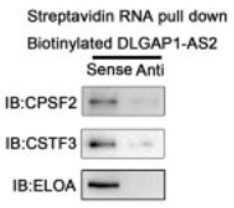

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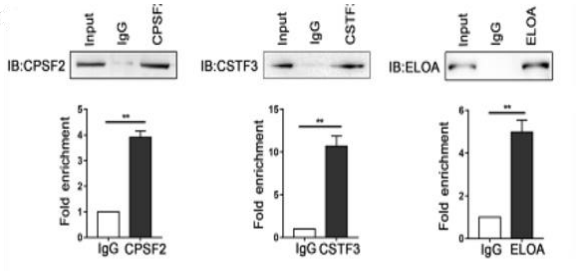

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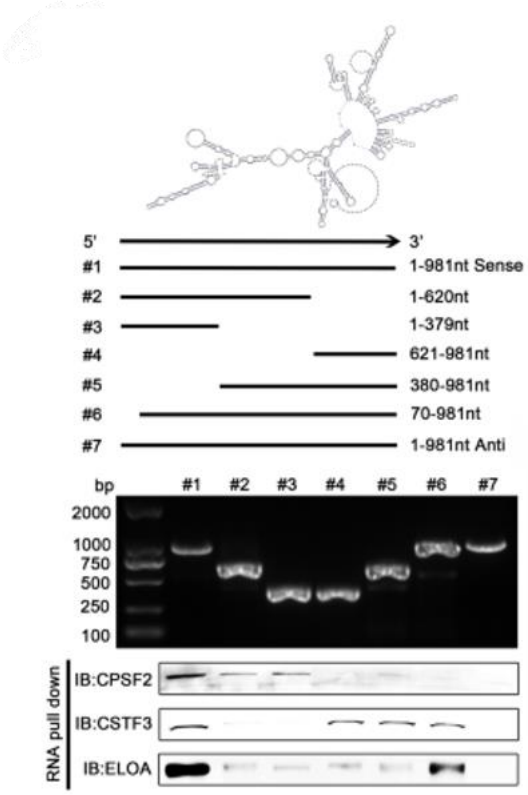

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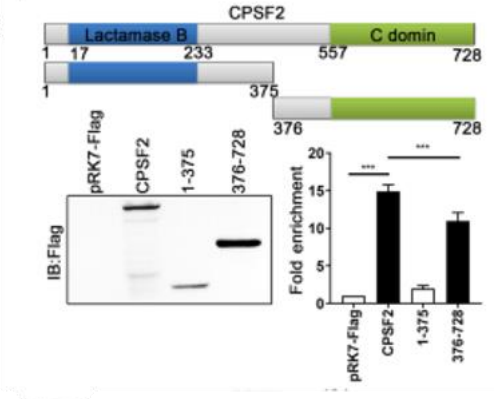

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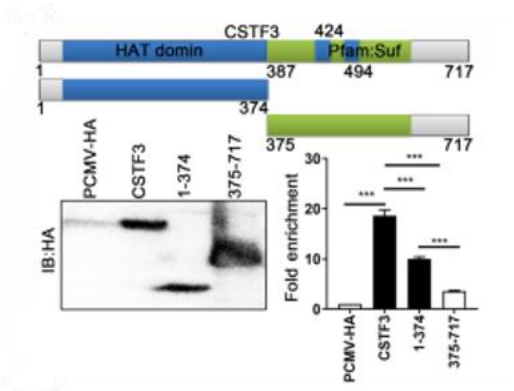

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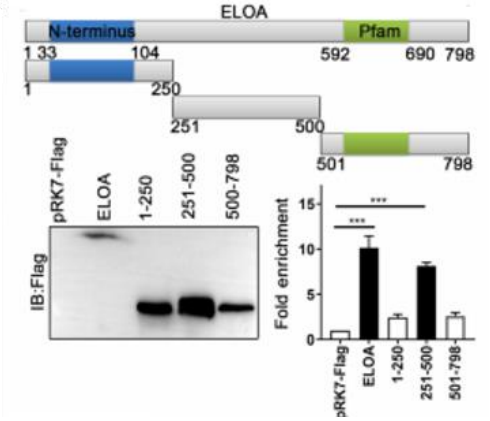

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Figure 4. Physical interaction between DLGAP1-AS2 and CPSF2, CSTF3, and ELOA in CRC cells. (a) Silver-stained SDS–PAGE analysis of proteins isolated via DLGAP1-AS2 RNA pull-down. (b) Immunoblot detection of CPSF2, CSTF3, and ELOA in RNA pull-down fractions. (c) RIP assays validating interactions between DLGAP1-AS2 and CPSF2, CSTF3, or ELOA, with enrichment quantified by qRT-PCR. (d) Immunoblot analysis following RNA pull-down using full-length or truncated DLGAP1-AS2 probes. (e–g) RIP assays assessing DLGAP1-AS2 enrichment using full-length or truncated CPSF2 (e), CSTF3 (f), or ELOA (g) proteins.

To delineate the DLGAP1-AS2 regions responsible for protein binding, a panel of truncated DLGAP1-AS2 mutants was generated based on predicted secondary structures derived from LNCipedia and catRAPID analyses. RNA pull-down experiments demonstrated that nucleotides 1–379, 621–981, and 380–981 of DLGAP1-

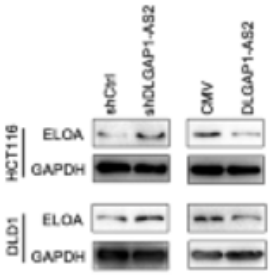
AS2 mediate interactions with CPSF2, CSTF3, and ELOA, respectively **(Figure 4d).**

Conversely, deletion constructs of CPSF2, CSTF3, and ELOA were generated for RIP analysis. Removal of amino acids 376–728 from CPSF2 largely abolished its interaction with DLGAP1-AS2 **(Figure 4e).** The association between CSTF3 and DLGAP1-AS2 was mediated by the N-terminal 1–374 amino acid region of CSTF3 **(Figure 4f),** whereas amino acids 251–500 of ELOA were required for its interaction with DLGAP1-AS2 in CRC cells **(Figure 4g)**. Together, these data demonstrate that DLGAP1-AS2 specifically and directly binds CPSF2, CSTF3, and ELOA in CRC cells.

DLGAP1-AS2 induces ELOA ubiquitination and proteasomal breakdown

Although DLGAP1-AS2 was previously shown to physically associate with CPSF2, CSTF3, and ELOA in CRC cells, the biological consequences of these interactions remained undefined. We therefore examined whether modulation of DLGAP1-AS2 affected the expression of these binding partners. Neither CPSF2 nor CSTF3 exhibited detectable alterations at the transcript or protein level following either depletion or overexpression of DLGAP1-AS2. Likewise, ELOA mRNA abundance remained unchanged under both experimental conditions. In contrast, ELOA protein accumulation was substantially elevated in CRC cells lacking DLGAP1-AS2 and markedly diminished upon forced DLGAP1-AS2 expression **(Figure 5a).**

To assess whether DLGAP1-AS2 influences ELOA protein stability, CRC cells were treated with the translation inhibitor cycloheximide (CHX). These assays revealed that loss of DLGAP1-AS2 prolonged ELOA protein persistence, whereas ectopic expression of DLGAP1-AS2 significantly shortened its half-life **(Figure 5b)**. Importantly, inhibition of proteasomal activity using MG132 completely abolished the DLGAP1-AS2–mediated reduction of ELOA protein **(Figure 5c).** Furthermore, ubiquitination assays demonstrated that both endogenous and exogenously expressed ELOA displayed enhanced ubiquitin conjugation in DLGAP1-AS2–overexpressing cells, while ubiquitin modification was markedly reduced upon DLGAP1-AS2 silencing **(Figure 5d**). Together, these observations indicate that DLGAP1-AS2 accelerates ELOA degradation through a ubiquitin-dependent proteasome pathway in CRC cells.



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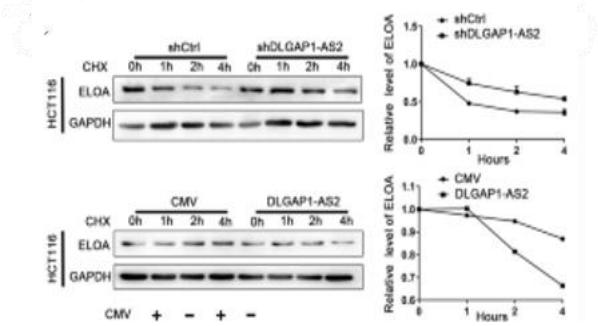

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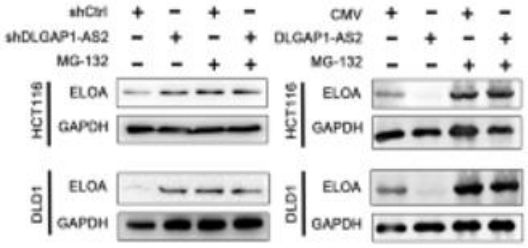

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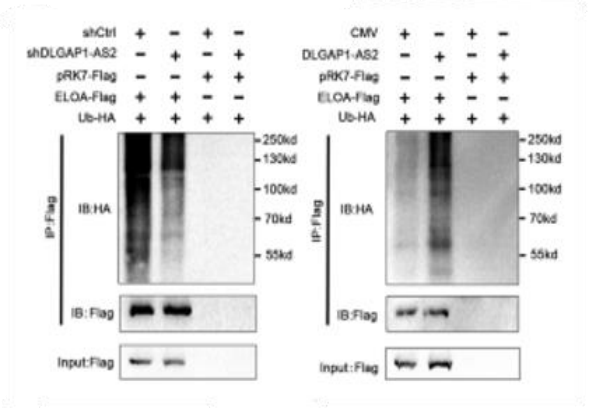

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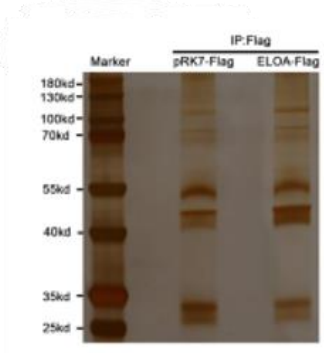

e)

Protein Name	Abundance
Trim21	1051341138
UHRF1	23837362
Stub1	1587778
Trim25	999330
RAD18	513088
UBE3C	279117

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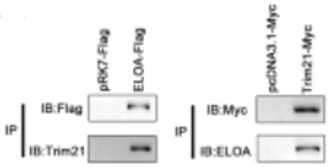

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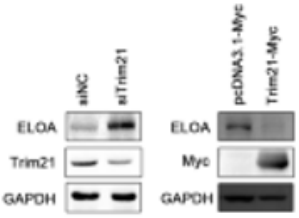

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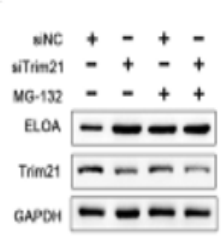

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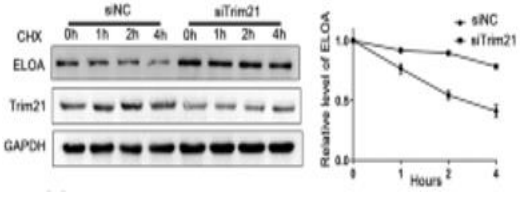

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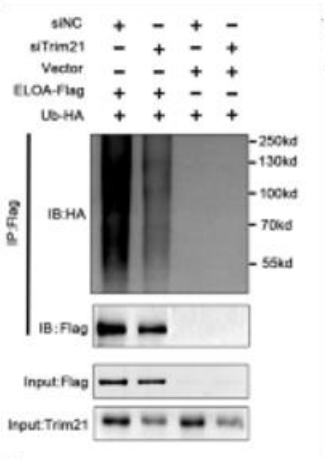

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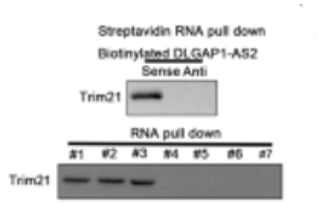

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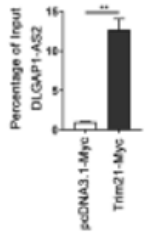

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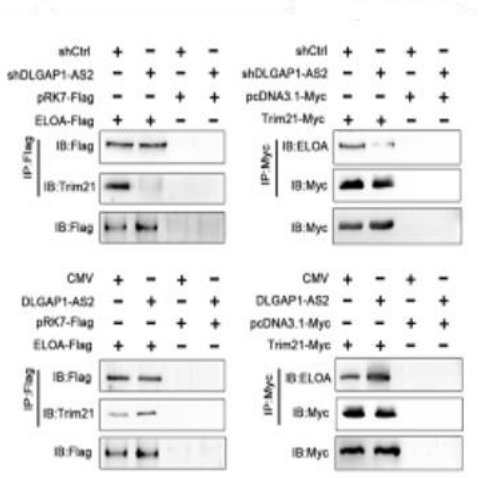

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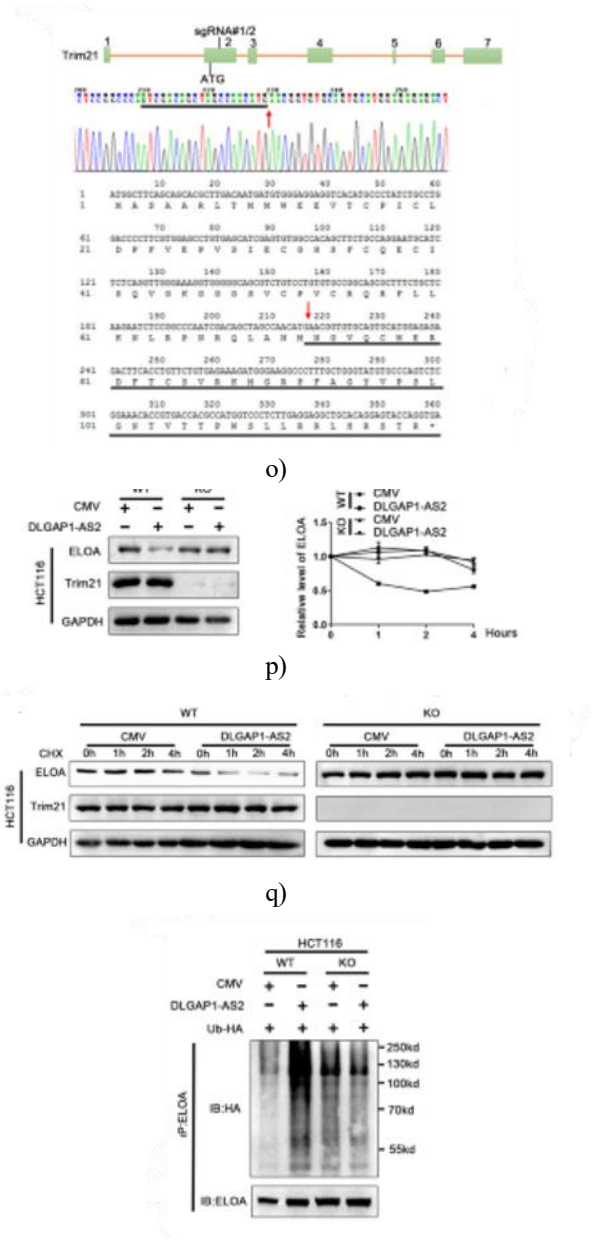


Figure 5. DLGAP1-AS2 drives Trim21-dependent ubiquitination and degradation of ELOA.
(a) Immunoblot analysis showing changes in ELOA protein levels following DLGAP1-AS2 knockdown or overexpression in CRC cells.
(b) Effects of DLGAP1-AS2 modulation on ELOA protein turnover in CRC cells treated with CHX (50 mg/L) for 0, 1, 2, or 4 h.
(c) ELOA protein abundance in DLGAP1-AS2–manipulated CRC cells after MG132 exposure (20 mmol/L, 4 h).
(d) Analysis of ELOA ubiquitination regulated by DLGAP1-AS2. Cells were co-transfected with Flag-tagged ELOA, HA-tagged ubiquitin, and/or DLGAP1-AS2 plasmids and treated with MG132 for 4 h prior to lysis. Ubiquitinated ELOA was detected by anti-HA immunoblotting following Flag immunoprecipitation.
(e) Silver-stained SDS–PAGE showing proteins co-immunoprecipitated with ELOA-Flag.
(f) Identification of candidate E3 ubiquitin ligases by mass spectrometry following ELOA immunoprecipitation.
(g) Confirmation of the ELOA–Trim21 interaction by co-immunoprecipitation in HCT116 cells.
(h) Effects of Trim21 depletion or overexpression on ELOA protein levels in HCT116 cells.
(i) Restoration of ELOA protein levels by MG132 in Trim21-overexpressing cells.
(j) CHX chase assays demonstrating increased ELOA half-life following Trim21 knockdown in HCT116 cells.
(k) Enhancement of ELOA ubiquitination upon Trim21 overexpression.
(l) Immunoblot detection of Trim21 among proteins retrieved from DLGAP1-AS2 RNA pull-down assays using probes #1–#7 (Figure 4d).
(m) RIP assays validating the interaction between Trim21 and DLGAP1-AS2.
(n) DLGAP1-AS2 enhances the physical association between ELOA and Trim21.
(o) Diagram illustrating sgRNA–CRISPR/Cas9 target sites within TRIM21 and preterminal regions in Trim21-knockout HCT116 cells.
(p) Effects of DLGAP1-AS2 on ELOA protein expression in Trim21-KO HCT116 cells.
(q) Effects of DLGAP1-AS2 on ELOA protein stability in Trim21-KO HCT116 cells.
(r) Effects of DLGAP1-AS2 on ELOA ubiquitination in Trim21-KO HCT116 cells.

DLGAP1-AS2 reinforces the association between ELOA and Trim21

To determine how DLGAP1-AS2 facilitates ubiquitin-driven destruction of ELOA, ELOA-Flag immunoprecipitation followed by mass spectrometry was performed, identifying six candidate E3 ubiquitin ligases **(Figures 5e–5f).** Among these candidates, Trim21 was the most abundantly detected protein. Subsequent co-immunoprecipitation experiments confirmed a direct interaction between Trim21 and ELOA **(Figure 5g).** Subcellular fractionation and localization analyses

revealed that ELOA and Trim21 are primarily cytoplasmic, whereas CPSF2 and CSTF3 localize predominantly to the nucleus, indicating that these proteins do not compete for DLGAP1-AS2 binding.

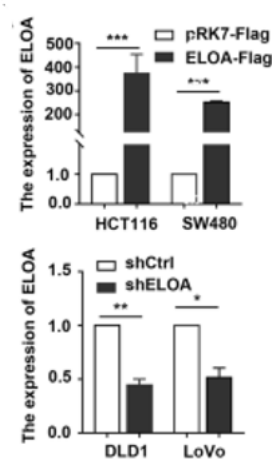
Functional studies further showed that depletion of Trim21 resulted in a pronounced accumulation of ELOA protein, whereas Trim21 overexpression caused a significant reduction in ELOA levels **(Figure 5h).** Notably, pharmacological blockade of the proteasome using MG132 reversed Trim21-induced ELOA downregulation **(Figure 5i).** Consistently, CHX chase experiments demonstrated that Trim21 knockdown markedly extended the half-life of ELOA protein **(Figure 5j)**. In addition, ubiquitination assays revealed a clear decrease in ELOA ubiquitination following Trim21 silencing, whereas enforced Trim21 expression substantially enhanced ELOA ubiquitin conjugation **(Figure 5k).**

RNA pull-down analysis demonstrated that Trim21 binding is mediated by the 1–379 nt segment of DLGAP1-AS2 **(Figures 4d and 5l).** Consistently, RNA immunoprecipitation experiments revealed a pronounced accumulation of DLGAP1-AS2 within RNA–protein complexes isolated using anti-Myc antibodies in CRC cells **(Figure 5m).** We subsequently assessed whether DLGAP1-AS2 influences the physical association between Trim21 and ELOA. Loss-of-function experiments showed a marked reduction in Trim21–ELOA interaction following DLGAP1-AS2 silencing, whereas enforced DLGAP1-AS2 expression substantially strengthened this interaction **(Figure 5n).** Collectively, these findings indicate that DLGAP1-AS2 facilitates Trim21-dependent ubiquitin-mediated proteolysis of ELOA by promoting Trim21–ELOA complex formation.

A Trim21-deficient HCT116 cell line was generated using CRISPR/Cas9 genome editing, resulting in a 52 bp deletion that introduced a frameshift mutation within the TRIM21 locus **(Figure 5o).** In Trim21-KO cells, modulation of DLGAP1-AS2 expression failed to alter ELOA protein abundance, degradation kinetics, or ubiquitination status, confirming that Trim21 is essential for DLGAP1-AS2-driven ELOA ubiquitination and turnover **(Figures 5p–5r).**

ELOA suppresses colorectal cancer growth and dissemination

ELOA, a component of the transcription factor B (SIII) complex, remains poorly characterized in the context of cancer biology, with its role in tumor progression largely unresolved. To clarify its function in CRC, a comprehensive set of in vitro and in vivo assays was performed. Forced expression of ELOA significantly restrained CRC cell proliferation and clonogenic capacity, whereas ELOA depletion produced the opposite effect, markedly enhancing these oncogenic properties **(Figures 6a–6c)**. In parallel, Transwell assays demonstrated that ELOA negatively regulates CRC cell migratory and invasive behaviors **(Figures 6e and 6e).**



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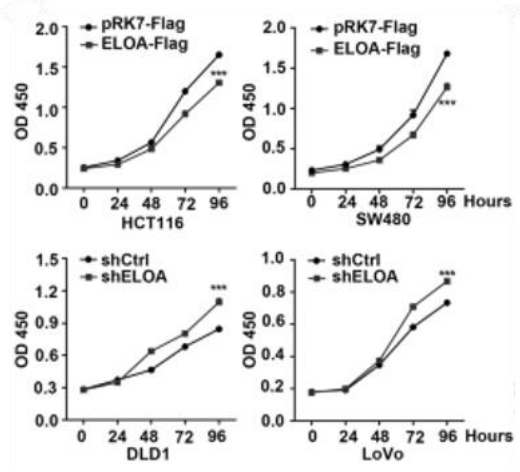

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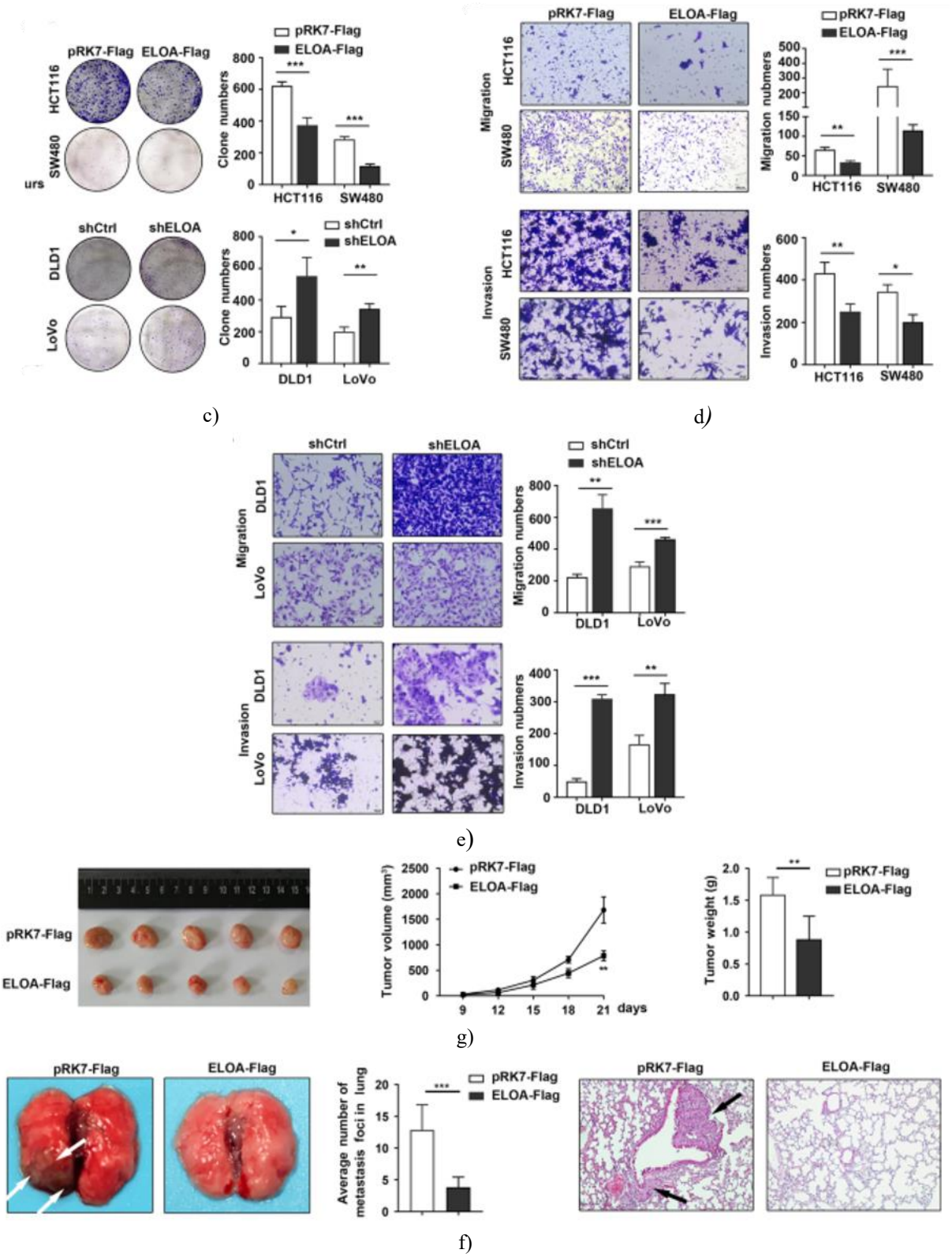


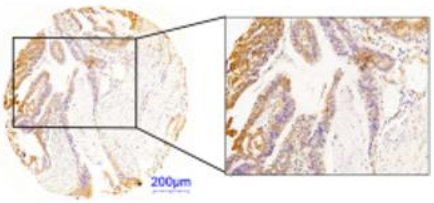
Figure 6. ELOA restrains CRC proliferation and metastatic capacity.
(a) Verification of ELOA knockdown and overexpression in CRC cells by qRT-PCR.
(b–c) Effects of ELOA modulation on CRC cell proliferation (B) and colony formation (C).

(d–e) Assessment of CRC cell migration and invasion following ELOA manipulation using Transwell assays.
(f) Evaluation of CRC tumor growth following ELOA overexpression in a xenograft mouse model (n = 5).
(g) Analysis of CRC metastatic potential using a lung metastasis mouse model (n = 5).

The tumor-suppressive role of ELOA was further validated in vivo. Xenograft studies revealed that enforced ELOA expression significantly inhibited CRC tumor expansion in nude mice **(Figure 6f).** Consistently, lung metastasis assays demonstrated a marked reduction in pulmonary metastatic nodules in mice implanted with ELOA-overexpressing cells compared with controls **(Figure 6g)**. Together, these results establish ELOA as a potent inhibitor of CRC growth and metastatic spread.

Reduced ELOA expression associates with elevated DLGAP1-AS2 and a favorable prognosis in CRC

To assess the clinical relevance of ELOA, its protein expression was examined in CRC patient specimens by immunohistochemistry **(Figure 7a).** Kaplan–Meier survival analysis revealed that diminished ELOA protein levels were significantly associated with inferior overall survival **(Figure 7b).** Correlation analyses further demonstrated a significant relationship between ELOA expression and tumor stage **(Figure 7c)**. Moreover, both univariate and multivariate Cox regression analyses identified ELOA as a prognostic variable in CRC **(Figure 7d).** Notably, ELOA protein abundance exhibited a strong inverse correlation with DLGAP1-AS2 expression in CRC tissues **(Figure 7e),** implicating ELOA in CRC pathogenesis.



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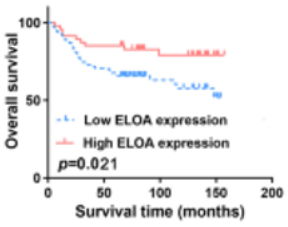

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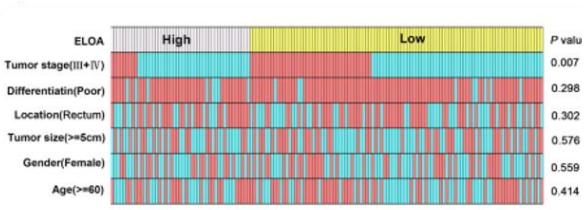

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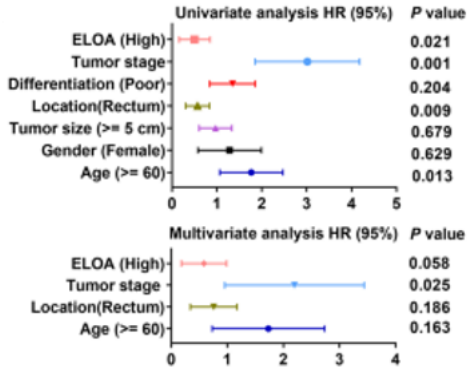

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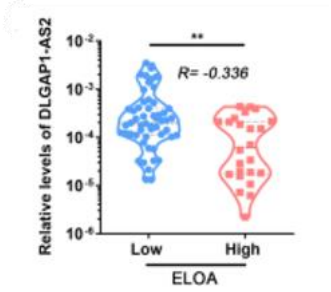

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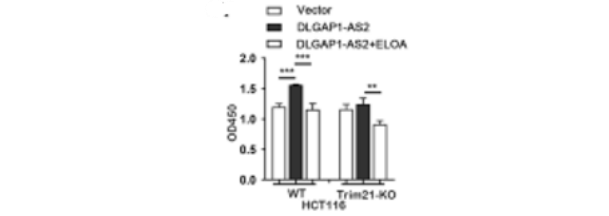
Figure 7. ELOA predicts favorable clinical outcomes in CRC**.**
(a) Immunohistochemical detection of ELOA protein in CRC tissues.
(b) Kaplan–Meier analysis of overall survival stratified by ELOA expression levels.
(c) Association between ELOA expression and clinicopathological characteristics in CRC.
(d) Univariate and multivariate regression analyses in CRC patients.
(e) Inverse correlation between DLGAP1-AS2 expression and ELOA protein abundance in CRC tissues.

ELOA functions as a downstream effector of DLGAP1-AS2 signaling

To determine whether ELOA acts downstream of DLGAP1-AS2, rescue experiments were conducted. Suppression of ELOA expression restored the impaired proliferative and clonogenic phenotypes induced by DLGAP1-AS2 knockdown, whereas enforced ELOA expression significantly attenuated the growth-promoting effects driven by DLGAP1-AS2 overexpression in HCT116 cells. Transwell assays further demonstrated that ELOA counteracted DLGAP1-AS2-mediated enhancement of CRC cell migration. Importantly, the oncogenic effects of DLGAP1-AS2 via ELOA suppression were observed in wild-type CRC cells but were completely abolished in Trim21-KO cells **(Figures 8a–8c).** These findings confirm that DLGAP1-AS2 promotes CRC progression through regulation of the Trim21–ELOA axis.



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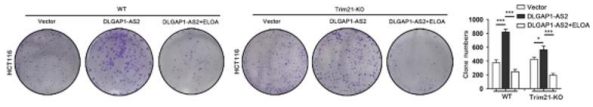

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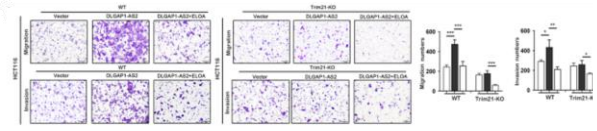

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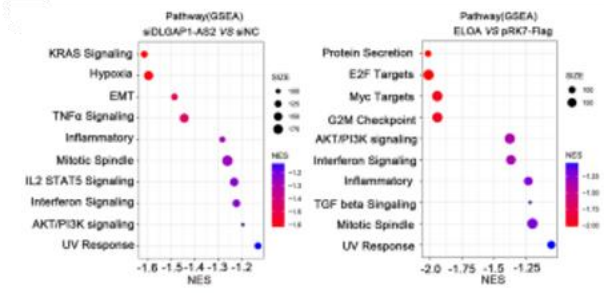

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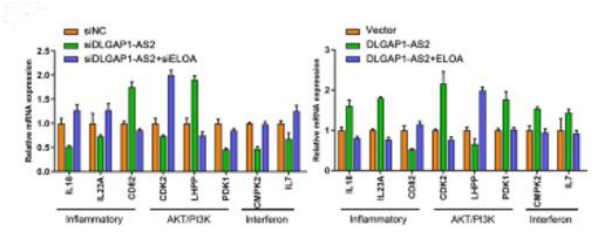

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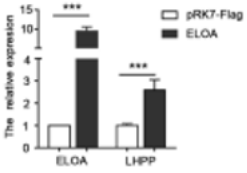

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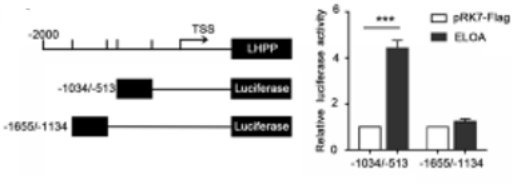

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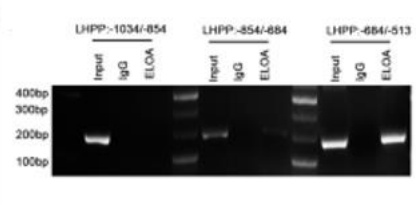

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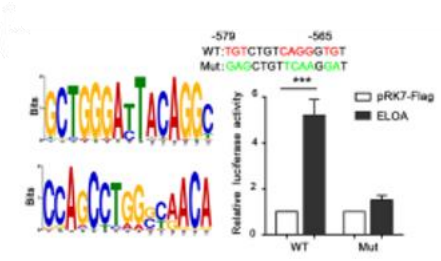

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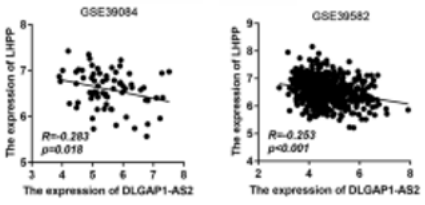

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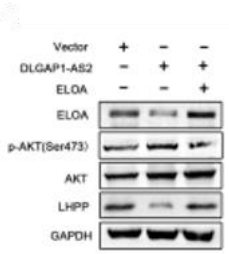


k)

Figure 8. ELOA mediates DLGAP1-AS2-driven CRC growth and metastasis.
(a–b) Cell proliferation and colony formation analyses in WT and Trim21-KO HCT116 cells following DLGAP1-AS2 and ELOA modulation.
(c) Migration and invasion assays in WT and Trim21-KO HCT116 cells transfected with DLGAP1-AS2 and ELOA plasmids.
(d) GSEA of differentially regulated genes influenced by DLGAP1-AS2 and ELOA, identified by RNA-seq in DLGAP1-AS2-silenced and ELOA-overexpressing HCT116 cells.
(e) qRT-PCR validation of selected differentially regulated genes.
(f) LHPP mRNA levels measured by qRT-PCR in ELOA-overexpressing HCT116 cells.
(g) Luciferase reporter assays assessing LHPP promoter activity following ELOA overexpression.
(h) ChIP-PCR analysis of ELOA recruitment to specific LHPP promoter regions, with IgG as a negative control.
(i) Luciferase validation of the predicted ELOA-binding motif within the LHPP promoter (Mut: binding site deleted).
(j) Correlation analysis between DLGAP1-AS2 and LHPP expression.
(k) Effects of the DLGAP1-AS2/ELOA axis on AKT signaling, assessed by pAKT (Ser473).

To further elucidate the mechanistic linkage between DLGAP1-AS2 and ELOA, transcriptomic profiling was conducted in HCT116 cells following transfection with si-DLGAP1-AS2, an ELOA overexpression plasmid, or the corresponding control vectors. In DLGAP1-AS2-depleted HCT116 cells, 682 differentially expressed genes (DEGs) were identified, consisting of 478 upregulated and 204 downregulated transcripts relative to controls. Conversely, ELOA overexpression resulted in 1420 DEGs, including 768 downregulated genes and 652 upregulated genes. Gene set enrichment analysis (GSEA) performed on these DEG datasets revealed a substantial overlap in the biological pathways influenced by DLGAP1-AS2 and ELOA, notably involving inflammatory response, mitotic spindle regulation, PI3K/AKT signaling, interferon response, and UV response pathways **(Figure 8d).** In addition, selected genes within these shared pathways were validated by qRT-PCR. Notably, enforced ELOA expression markedly reversed the transcriptional changes induced by DLGAP1-AS2 knockdown in multiple genes associated with inflammatory response, AKT signaling, and interferon-related pathways **(Figure 8e),** indicating that DLGAP1-AS2 modulates these gene programs through ELOA.

ELOA transcriptionally activates LHPP by directly targeting its promoter

To identify genes containing ELOA-specific promoter-binding regions, a ChIP-on-chip analysis was performed in HCT116 cells using NimbleGen human 720 K RefSeq promoter arrays. This assay identified 836 promoter regions significantly enriched by ELOA immunoprecipitation (false discovery rate < 0.05). Integration of these ChIP-on-chip results with transcriptomic data narrowed the list of candidate ELOA-regulated genes to three targets: LHPP, IL7, and CMPK2. Among these, only LHPP—recently characterized as a tumor suppressor [16]—was transcriptionally upregulated by ELOA and was therefore selected for further investigation. Consistent with this selection, LHPP mRNA levels were significantly elevated in CRC cells overexpressing ELOA **(Figure 8f)**.

To validate the ELOA-binding region identified by ChIP-on-chip analysis, two LHPP promoter reporter constructs were generated, encompassing either the -1034/-513 region or the -1655/-1134 region (used as a negative control). Luciferase reporter assays demonstrated that ELOA robustly enhanced transcriptional activity driven by the -1034/-513 LHPP promoter fragment, supporting a direct role for ELOA in regulating LHPP transcription **(Figure 8g).** This interaction was further confirmed by ChIP-qPCR using primers spanning the LHPP promoter (-1034/-513). A significant enrichment of ELOA binding was detected within the -684/-513 region of the LHPP promoter **(Figure 8h),** confirming that ELOA directly promotes LHPP transcription through promoter engagement.

Using the MEME-ChIP database in combination with a Markov model, conserved sequence motifs were analyzed across the promoters of the 836 ChIP-on-chip–

identified genes. Two potential ELOA-binding motifs were identified: GCTGGGATTACAGGC and CCAGCCTGGGCAACA. One motif was located within the -579/-565 region of the LHPP promoter (TGTCTGTCAGGGTGT), which partially represents a reverse complement of CCAGCCTGGGCAACA. Mutation of this region abolished ELOA-induced luciferase activity in reporter assays **(Figure 8i).** Collectively, these findings demonstrate that ELOA activates LHPP transcription by directly binding to the -579/-565 promoter region.

DLGAP1-AS2 stimulates AKT signaling via the ELOA/LHPP regulatory axis

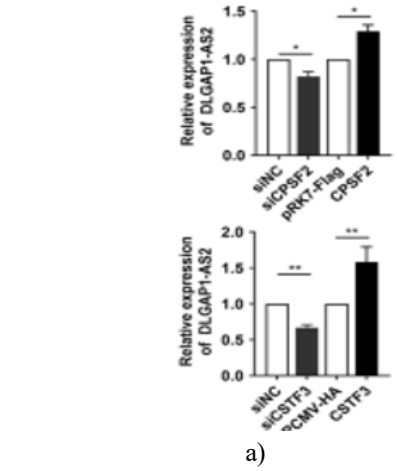
Our analyses revealed that DLGAP1-AS2 influences multiple oncogenic signaling pathways, including PI3K/AKT. Notably, previous studies have shown that LHPP functions as a negative regulator of PI3K/AKT signaling in CRC [17, 18]. Examination of several public CRC datasets revealed that LHPP expression was consistently reduced in tumor tissues relative to matched noncancerous tissues (NCTs). Correlation analyses further demonstrated an inverse relationship between LHPP and DLGAP1-AS2 expression, as well as a positive association between LHPP and ELOA levels in CRC samples **(Figure 8j)**.

To determine whether LHPP mediates ELOA-dependent modulation of PI3K/AKT signaling, AKT phosphorylation levels (pAKT at Ser473 and Thr308) were examined in ELOA-overexpressing CRC cells. These experiments confirmed that ELOA upregulated LHPP expression and concomitantly suppressed AKT pathway activation. Moreover, rescue experiments revealed that ELOA overexpression counteracted DLGAP1-AS2-induced AKT activation at Ser473, but not at Thr308 **(Figures 8k).** Together, these findings demonstrate that DLGAP1-AS2 accelerates CRC progression through modulation of the ELOA/LHPP/AKT signaling cascade.

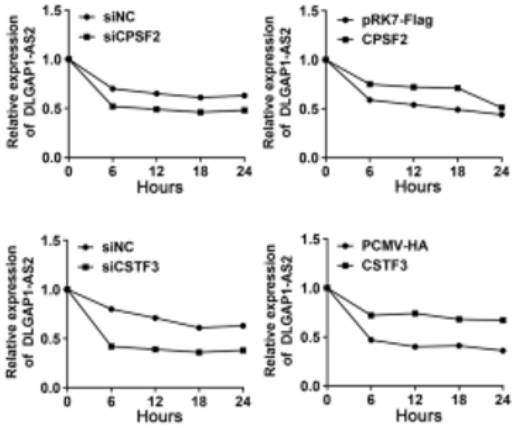
CPSF2 and CSTF3 associate with DLGAP1-AS2 and enhance its RNA stability

CPSF2 and CSTF3 are components of the cleavage and polyadenylation (C/P) machinery and function as multiprotein complexes involved in RNA maturation and stability control. Our data identified CPSF2 and CSTF3 as direct binding partners of DLGAP1-AS2. Analysis of public CRC datasets revealed that both CPSF2 and CSTF3 were significantly upregulated in tumor tissues compared with paired NCTs, and elevated expression of either gene was associated with poorer patient survival outcomes.

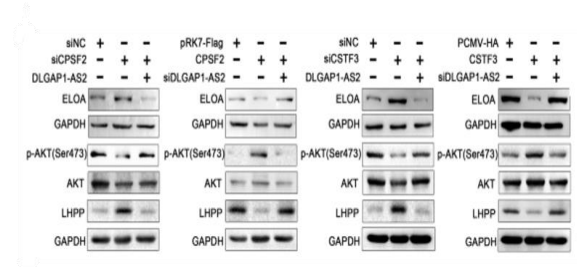
Although DLGAP1-AS2 did not influence CPSF2 or CSTF3 expression levels in CRC cells, both CPSF2 and CSTF3 positively regulated DLGAP1-AS2 abundance **(Figure 9a)**. Furthermore, actinomycin D chase assays (2 μg/ml) demonstrated that CPSF2 and CSTF3 significantly enhanced the stability of DLGAP1-AS2 transcripts **(Figure 9b**). Consistent with these findings, CPSF2 or CSTF3 overexpression led to reduced ELOA expression and activation of AKT signaling (pAKT at Ser473 but not Thr308) through a DLGAP1-AS2–dependent mechanism in CRC cells **(Figure 9c).**



a)

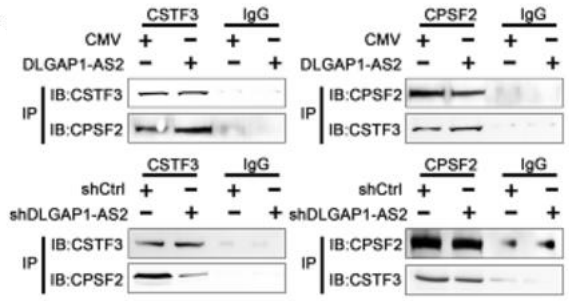


b)

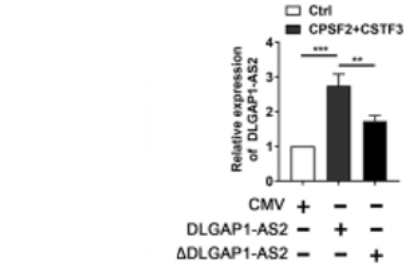


c)

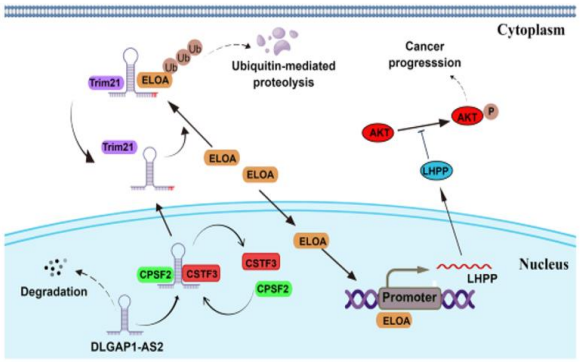
d)



e)



f)



g)

Figure 9. CPSF2 and CSTF3 enhance DLGAP1-AS2 stability in colorectal cancer cells**.**

(a) Modulation of DLGAP1-AS2 transcript levels by CPSF2 or CSTF3.
(b) Half-life of DLGAP1-AS2 measured in CRC cells treated with actinomycin D (2 μg/ml) upon CPSF2 or CSTF3 expression.
(c) Effect on ELOA protein and AKT pathway signaling (pAKT Ser473) by CPSF2 or CSTF3.
(d) Assessment of CPSF2–CSTF3 physical association via co-immunoprecipitation.
(e) DLGAP1-AS2 facilitates CPSF2–CSTF3 binding.
(f) Influence of CPSF2/CSTF3 overexpression on DLGAP1-AS2 abundance; deletion of the AAUAAA motif (ΔDLGAP1-AS2) impairs this regulation in HCT116 cells.
(g) Proposed model illustrating DLGAP1-AS2–mediated tumor-promoting mechanism in CRC.

Experimental results showed that CPSF2 can directly associate with CSTF3 **(Figure 9d),** and this interaction is strengthened in the presence of DLGAP1-AS2 (**Figure 9e).** This suggests that DLGAP1-AS2 acts as a scaffold, facilitating the assembly of CPSF2–CSTF3 complexes. Functionally, CPSF2 and CSTF3 appear to cooperate in enhancing DLGAP1-AS2 RNA levels. Analysis of DLGAP1-AS2 identified a CPSF2 recognition sequence (AAUAAA) located at nucleotides 89–94. Deletion of this motif (ΔDLGAP1-AS2) significantly reduced the ability of CPSF2 and CSTF3 to upregulate DLGAP1-AS2 **(Figure 9f)**. Together, these data indicate that CPSF2 and CSTF3 jointly contribute to the stabilization of DLGAP1-AS2 in CRC cells.

The number of annotated lncRNAs has expanded dramatically with advances in RNA sequencing. Our previous studies using microarrays identified several CRC-associated lncRNAs [7–15]. In the present study, high-throughput sequencing enabled further discovery of CRC-related lncRNAs, including DLGAP1-AS2, which is consistently upregulated in multiple cancer types.

DLGAP1-AS2 is located on chromosome 18p11.31, with three transcripts annotated in GENECODE or GenBank. We identified a previously unreported transcript that predominates in CRC and other cancer cells. The other two transcripts (NR_119377.1 and ENST00000572856.1) are expressed at lower levels but still show oncogenic potential under specific conditions (data not shown).

Analyses across multiple CRC cohorts indicate that DLGAP1-AS2 serves as a potential prognostic marker. Functional experiments confirmed that DLGAP1-AS2

promotes tumorigenicity and CRC progression. Previous reports have also demonstrated its prognostic relevance in various cancers, including rectal cancer [19–26]. For example, Zeng et al. showed that DLGAP1-AS2 contributes to radioresistance in rectal cancer stem cells via the E2F1-CD151 axis [27]. These findings support the idea that DLGAP1-AS2 is a pancancer oncogene and may represent a therapeutic target.

LncRNAs generally function via interactions with DNA, RNA, or protein partners [28]. Prior studies suggested that DLGAP1-AS2 can sequester miR-503 or miR-505 or bind transcription factors such as Six3 to regulate Wnt/β-catenin signaling in gastric cancer. Given its predominantly cytoplasmic localization and low miRNA abundance in CRC cells, alternative molecular mechanisms likely drive its tumor-promoting activity in CRC.

In this study, CPSF2, CSTF3, and ELOA were identified as bona fide DLGAP1-AS2-interacting proteins. ELOA is a subunit of the Pol II elongation factor SIII complex, enhancing transcriptional elongation through direct interactions with RNA polymerase II [29, 30]. SIII consists of ELOA and a heterodimeric Elongin B/C module, which binds the BC-box motif on ELOA and regulates transcription [31]. Additionally, ELOA serves as a substrate recognition component for a Cullin-RING E3 ubiquitin ligase that targets Pol II's RPB1 subunit for ubiquitination following DNA damage.

Although the roles of Elongins B and C in cancer have been extensively studied, ELOA's contribution to tumorigenesis and the mechanisms controlling its activity remain poorly defined. Through a series of functional experiments, we identified ELOA as a critical downstream effector of DLGAP1-AS2. Our data indicate that DLGAP1-AS2 promotes ELOA degradation by enhancing Trim21-mediated ubiquitination, unveiling a previously unrecognized posttranslational regulation of ELOA. Using a combination of in vitro and in vivo assays, we demonstrated for the first time that ELOA suppresses CRC cell proliferation and metastasis, establishing a novel tumor-suppressive role for this protein in colorectal cancer progression.

Transcriptome profiling revealed that DLGAP1-AS2 and ELOA modulate highly similar sets of signaling pathways. Rescue experiments confirmed that ELOA functions downstream of DLGAP1-AS2. ELOA, a transcription elongation factor, is known to facilitate gene activation under stress or developmental cues [32], but its downstream targets in cancer were previously unknown. We found that ELOA directly regulates the transcription of LHPP, a recently discovered tumor suppressor [16]. LHPP restricts tumor growth and metastatic potential through inhibition of the PI3K/AKT pathway, specifically by modulating phosphorylation of AKT at Ser473 [33–38]. Our study provides the first evidence that ELOA binds the LHPP promoter, activates its transcription, elevates LHPP levels, and thereby suppresses AKT signaling.

Despite reports of DLGAP1-AS2 upregulation in multiple malignancies, the mechanisms underlying its overexpression remain unclear. CPSF2 and CSTF3, components of the cytoplasmic polyadenylation factor (CPF) complex, are critical for 3′ mRNA processing [39]. CPSF2, the 100 kDa subunit of CPSF, recognizes the AAUAAA polyadenylation motif to direct cleavage and polyadenylation, influencing RNA stability, nuclear export, and translation initiation [40]. CSTF3 binds GU-rich sequences downstream of the cleavage site to add the poly(A) tail, further stabilizing transcripts [41]. Here, we demonstrate that CPSF2 and CSTF3 physically associate with DLGAP1-AS2, enhancing its stability and consequently suppressing the ELOA/LHPP axis in CRC cells. This reveals a novel posttranscriptional mechanism contributing to the elevated levels of DLGAP1-AS2 in CRC. The detailed molecular basis of CPSF2/CSTF3-mediated stabilization, and whether it operates in other cancers, remains to be investigated.

Conclusion

In summary, DLGAP1-AS2 functions as an oncogenic lncRNA in colorectal cancer by promoting tumor growth and metastasis. Mechanistically, DLGAP1-AS2 reduces ELOA protein stability via Trim21-mediated ubiquitination and proteasomal degradation. ELOA, in turn, transcriptionally activates the tumor suppressor LHPP by binding to its promoter, revealing a previously uncharacterized DLGAP1-AS2/Trim21/ELOA/LHPP regulatory pathway in CRC. Moreover, CPSF2 and CSTF3 interact with DLGAP1-AS2 to enhance its stability. Together, these findings provide mechanistic insight into colorectal carcinogenesis and suggest that DLGAP1-AS2 may serve as a prognostic biomarker and therapeutic target in CRC **(Figure 9g).**

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Conflict of Interest: None

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Ethics Statement: None

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