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ORIGINAL RESEARCH ARTICLE

Small Molecule Destabilizer of β-Catenin and Ras Proteins Antagonizes Growth of K-Ras Mutation-Driven Colorectal Cancers Resistant to EGFR Inhibitors

Jung Kyu Choi[1] · **Heeyeong Cho**[2] · **Byoung-San Moon**[2,3]

Abstract

Background Oncogenic *K-Ras* mutations in colorectal cancer (CRC) combined with *APC* mutations worsen CRC prognosis and lower drug effectiveness. Thus, inhibition of both Wnt/β-catenin and Ras-MAPK signaling may be a rational strategy to improve the treatment of this cancer.

Objective To identify a novel compound inhibiting both Wnt/β-catenin and Ras-MAPK signaling in CRC.

Methods and Patients We developed a two-part screening system consisting of analysis of TOP flash reporter cells and then potential toxicity effects on primary neural stem cells (NSCs). We then screened 2000 chemical compounds and tested efficacy of candidates against isogenic colon cancer cells harboring wild-type or mutant *K-Ras*. We employed immunohistochemistry and immunocytochemistry to determine marker signatures associated with development of disease phenotypes.

Results We identified CPD0857, a compound that inactivates Wnt/β-catenin signaling and promotes ubiquitin-dependent proteasomal degradation of β-catenin and Ras proteins. CPD0857 effectively decreased proliferation and increased apoptosis of CRC cell lines, and overcame resistance of CRC harboring *APC* and *K-Ras* mutations to treatment with an EGFR monoclonal antibody (mAb). Moreover, CPD0857 attenuated invasiveness of highly migratory CRC cells in vitro. Accordingly, xenograft mice treated with CPD0857 showed slower tumor growth and significant decreases in both β-catenin and Ras protein expression.

Conclusions CPD0857 may be a potential drug for treating aggressive CRC carrying mutations that aberrantly activate Wnt/β-catenin and Ras-ERK pathways.

Key Points

CPD0857 was identified as a non-toxic inhibitor suppressing both Wnt/β-catenin and Ras-ERK signaling pathways in colorectal cancer cells (CRCs).

CPD0857 increases the level of Axin protein and reduces the level of β-catenin and Ras protein via a ubiquitin-dependent proteasomal degradation mechanism.

CPD0857 overcomes resistance to the anti-EGFR cetuximab therapy seen in CRCs harboring K-Ras gene mutations.

CPD0857 decreases cell proliferation and increases apoptosis via suppressing Wnt/β-catenin, Ras-ERK, and PI3K/AKT pathways.

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1 Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, and the second leading cause of cancer death [1]. Development of therapies, including chemotherapy and targeted approaches, has improved median overall survival time for patients with aggressive and metastatic CRC. Nonetheless, treatment remains challenging due to drug toxicity and development of tumor resistance [2].

Wnt/β-catenin and Ras/extracellular-signal-regulated kinase (ERK) pathways play a critical role in tumorigenesis and metastasis of cancers, especially CRC. CRC arises through frequent genetic abnormalities, such as loss-of-function of the tumor suppressor adenomatous polyposis coli (*APC*) in the Wnt/β-catenin pathway and gain-of-function *K-Ras* mutations in the Ras/ERK pathway, both of which function in initiation and progression of tumorigenesis. Recent studies indicate that *APC* and *K-Ras* mutations are concurrent during different stages of CRC tumorigenesis and metastasis [2, 3].

Generally, K-Ras functions as a molecular switch to regulate ERK and phosphatidyl inositol 3-kinase (PI3K)-Akt signaling [3, 4]. Oncogenic *K-Ras* mutations lead to hyperplasia crypt architecture that is not normal, but have low risk of progression to CRC [5]. Moreover, patients with metastatic CRC-bearing oncogenic *K-Ras* mutations are resistant to EGFR monoclonal antibodies, including cetuximab, a current standard of care in treating metastatic colorectal cancer [6]. Ras protein stability and signaling are also regulated by the Wnt/β-catenin pathway [3]. These findings provide a rationale for identifying drugs that target both Wnt/β-catenin and Ras/ERK pathways simultaneously.

Here, to define such compounds, we screened a small-molecule library to identify compounds that reduce levels of both β-catenin and Ras proteins by inhibiting Wnt/β-catenin signaling. Our analysis identified CPD0857, which markedly reduced proliferation and transforming capacity of various CRC cell lines, most likely by destabilizing β-catenin and Ras proteins. CPD0857 also overcame cetuximab chemoresistance in CRC cells harboring *APC* and *K-Ras* mutations and promoted tumor cell apoptosis. Moreover, CPD0857 showed anti-metastatic properties in vitro. Finally, CPD0857 significantly suppressed tumor growth and progression in mouse CRC xenograft models. Our study identifies a novel compound by chemical library screening that may be applicable to various cancer types with activated Wnt/β-catenin as well as Ras/ERK pathways.

2 Materials and Methods

2.1 Experimental Animals and Ethics Statement

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center. Xenograft experiments and tissue processing for immunostaining were previously described [3]. Briefly, BALB/c nu/nu mice were purchased from the Central Lab Animal Inc. (Seoul Korea). All animals were housed in filter-topped shoebox cages equipped with a computerized environmental control system. Room temperature was maintained at 24 °C with 40–70% relative humidity. After acclimatization for 1 week, mice were subcutaneously injected with 1×10^6 DLD-1-K-Ras mutant (D-*K-Ras* MT) cells in 200 μL phosphate-buffered saline/Matrigel (1:1) in the dorsal flank. When mean tumor volumes reached ~ 200 mm^3, mice were randomly divided into two groups (four per group) and administered either CPD0857 suspended in 0.5% methyl cellulose/0.5% Tween 80 or vehicle intraperitoneally at a drug dose of 25 mg/kg, twice a week. Tumor volume was measured every 3–4 days using Vernier calipers, applying the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. Animals were euthanized when tumor volume exceeded 1500 mm^3. Tumors were then excised, weighed, and fixed in 4% paraformaldehyde for further analysis.

2.2 Dual-Cell-Based High-Throughput Screening

Screening for chemical compounds that destabilize both β-catenin and Ras by inhibiting Wnt/β-catenin signaling was previously described [2]. Briefly, from 2000 compounds (Chemdiv chemical library), we initially identified 100 that efficiently inhibit Wnt/β-catenin signaling using the HEK293-TOP flash reporter stable cell line. For primary screening, reporter cells were seeded into 96-well plates (black polystyrene plate; Greiner Bio-One) at 2×10^4 cells per well and grown for 24 h. Each compound or control (DMSO) along with Wnt3a conditioned media (CM) was added to wells at 10 μM, and luciferase activity was measured 24 h later by FLUOstar OPTIMA. As a secondary screening, we treated neural stem cells (NSCs) with 100 compounds selected to check toxicity. NSCs had been surgically extracted from the forebrain of E14.5 rats and maintained in an undifferentiated state by culture in medium [DMEM/F12 with 10 ng/mL bFGF (Peprotech)]. Among phenotypes analyzed were cell number and morphology, as assessed by capturing phase contrast images of cells after 48 h. Nine of the original 100 compounds showed no toxicity.

2.3 Cell Culture

CRC cell lines, including HCT15, RKO, SW480, HCT116, and LoVo, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Isogenic cell lines (DLD-1 (D)-*K-Ras* WT, D-*K-Ras* MT & *PI3K* WT, and D-*K-Ras* MT & *PI3K* MT) were provided by B. Vogelstein (John Hopkins University School of Medicine, Baltimore, MD, USA) [7]. HEK293 and HEK293-TOP flash reporter cells were grown in DMEM (Gibco) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 10 µg/mL streptomycin at 37 ˚C. Human CRC lines were grown in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 10 µg/mL streptomycin at 37 °C. Cells were stored in CRYO-GOLD (Revive Organtech Inc, Irvine, CA, USA), checked based on morphology, and *Mycoplasma*-tested using MycoFluor Mycoplasma Detection kit (Invitrogen) over a period of 6 months. In analyses of protein stability, cultured cells were treated with MG132 (carbobenzoxy-Leu-Leu-leucinal) (Sigma-Aldrich, St. Louis, MO, USA).

2.4 Plasmids and Constructs

FLAG-WT-β-catenin-pcDNA3.0 and FLAG-S33Y-β-catenin-pcDNA3.0 plasmids were kindly provided by Eric R. Fearon (University of Michigan, Ann Arbor, MI, USA). Constructs were confirmed by nucleotide sequencing analysis (Cosmogenetech).

2.5 Immunoblotting Assay

Cells or tissues were gently lysed in RadioImmunoPrecipitation Assay (RIPA) buffer (Upstate Biotechnology, Lake Placid, NY, USA) for 1 h on ice and centrifuged at 12,000 rpm at 4 °C for 15 min. Lysates were boiled for 5 min at 95 °C in SDS sample buffer and separated on 10% SDS-PAGE. After blocking, membranes were incubated first with primary antibodies and then with a peroxidase-conjugated secondary antibody. Bound secondary antibody (anti-mouse or anti-rabbit 1:10,000) was detected using the enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). Images were taken using the luminescent image analyzer LAS-3000 (Fujifirm, Tokyo, Japan).

2.6 Antibodies and Reagents

Antibodies used in this study were anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-panRas (Upstate Biotechnology, Lake Placid, NY, USA; Abcam Inc., Cambridge, MA, USA), anti-phospho-β-catenin (Ser33/37/Thr41) (Cell Signaling Biotechnology, Beverly, MA, USA), anti-Axin (Santa Cruz Biotechnology), anti-β-actin (Abcam Inc.), anti-Flag (Sigma-Aldrich, St. Louis, MO, USA), anti-BrdU (Sigma-Aldrich), anti-PCNA (Santa Cruz Biotechnology), anti-pERK (Thr202/Tyr204) (Cell Signaling Biotechnology), anti-pAKT (Ser473), and HRP-conjugated anti-mouse (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescent anti-rabbit secondary antibodies (Calbiochem, La Jolla, CA, USA) were used for detection by a luminescent image analyzer, LAS-3000 (Fujifilm, Tokyo, Japan).

2.7 Immunohistochemistry and Immunocytochemistry

For immunohistochemistry, xenograft tissues were dissected and fixed in 4% paraformaldehyde (PFA) at 4 °C. Paraffin sections were incubated with primary antibody at 4 °C for 18 h. For immunocytochemistry, cells cultured on coverslips were fixed with 4% PFA/PBS for 2 h and immunostained after permeabilizing with 0.2% Triton X-100. Cells and tissues were then incubated with indicated primary antibodies overnight at 4 °C, followed by Alexa Fluor 488 (Life Technologies, Carlsbad, CA, USA) or Alexa Fluor 555 (Life Technologies) secondary antibodies at room temperature for 1 h and counterstained in 4′-6′diamidino-2-phenylindole (DAPI) (Boehringer Mannheim, Mannheim, Germany). Images were visualized using confocal microscopy (LSM5 PASCAL; Zeiss, Jena, Germany). Values obtained from at least three independent experiments were averaged and reported as mean ± SD. Student's two-tailed *t* test was used to compare two experimental groups.

2.8 Wound-Healing Assay

CRC cell lines (D-*K-Ras* WT, D-*K-Ras* MT & *PI3K* WT, D-*K-Ras* MT & *PI3K* MT, SW480, HCT15, LoVo, and RKO) were seeded at a density of 2.5×10^5 cells in six-well plates. After cells reached confluence, they were scratched with a p200 pipet tip and then the media was changed to DMEM either with or without CPD0857 (at 10, 25, or 50 µM). When required, Wnt3a CM was used to stimulate cells. Scratched cells were then grown in a tissue culture incubator at 37 °C and imaged using an ECLIPSE TE2000-U fluorescence microscope (Nikon) 12 and 24 h later.

2.9 Automated Cell Migration Assay

We used Electrical Cell-substrate Impedance Sensing (ECIS, Applied BioPhysics) to electronically measure drug effects on migration of cultured CRC cells in real time. D-*K-Ras* MT cells were grown to confluence on polyethylene eight-well (8 W) 10E + arrays (Applied BioPhysics. NY, USA). After wounding, cells were treated with CPD0857 (25 µM) or DMSO and monitored for changes in resistance at 1000 Hz using an ECIS Zθ instrument. Data were

normalized to resistance values at the point of wounding and subtracted from DMSO-treated resistance values.

2.10 Invasion Assay

D-*K-Ras* MT (3×10^4) cells were seeded on matrigel-coated chambers, and either CPD0857 or DMSO was added to lower chambers. Cells were allowed to invade for 24 h. After clearing cells on the inner surface of the chamber, cells on the outer surface were fixed using 4% paraformaldehyde (PFA) for 15 min and stained with crystal violet for 15 min. Chambers were dipped in distilled water to remove excess stain and allowed to dry. Photographs were taken using ECLIPSE TE2000-U fluorescent microscope (Nikon).

2.11 Statistical Analysis

Statistical analyses were performed using the Excel statistical tools or Prism 5 (GraphPad Software). Group differences were determined with Student's *t* test ($*P < 0.05$, $**P < 0.005$, and $***P < 0.0005$). One-way ANOVA tests (Tukey's multiple comparison test) and two-way ANOVA tests (Bonferroni post-tests) were used to analyze data from multiple groups.

3 Results

3.1 Identification of a Compound that Decreases Intracellular β-Catenin and Ras Protein Levels

High expression of both β-catenin and Ras proteins due to relevant gene mutations is common in aggressive and metastatic CRC [2, 3]. To screen for novel compounds facilitating degradation of both β-catenin and Ras proteins, we used a dual-cell-based high-throughput screening system previously described [2]. Of 2000 chemical compounds from the ChemDiv drug libraries, we selected nine of the most effective based on lack of toxicity toward neural stem cells (NSCs) (Fig. 1a). To determine whether treatment with compounds reduced levels of β-catenin and Ras proteins, we performed western blot analysis of NSCs treated with candidate compounds (Fig. 1b). We observed significantly reduced β-catenin protein expression in cells treated with

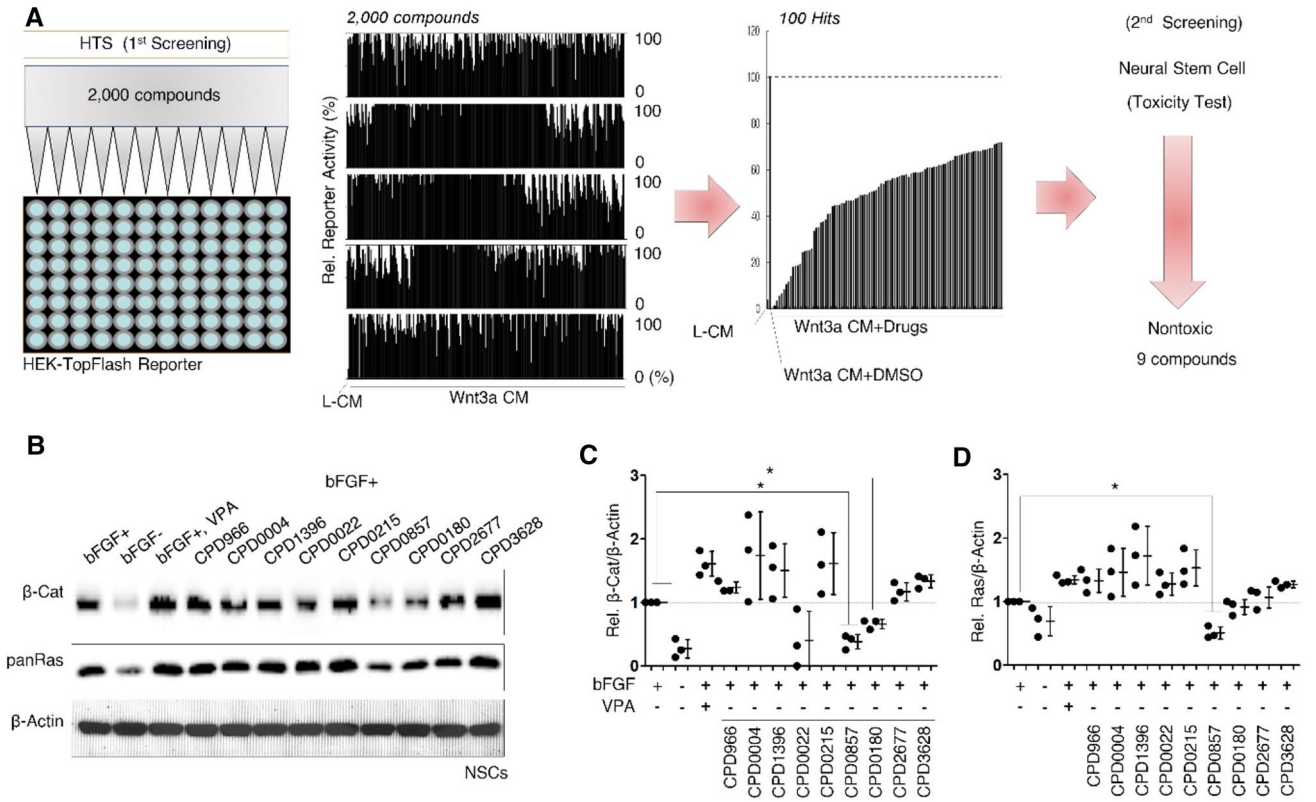

Fig. 1 Screen for drugs that promote β-catenin and Ras protein degradation. **a** A dual-cell-based high-throughput screening system using the HEK293 TOP flash line and primary neural stem cells (NSCs) was used to identify candidate chemical compounds, as described previously [2]. Among 2000 chemical compounds screened for inhibition of TOP flash reporter activity, 100 showed > 25% inhibition. Those were subjected to secondary screening using primary NSCs to assess toxicity. **b** Immunoblot (IB) analysis of NSC lysates with indicated antibodies. **c**, **d** Relative intensity of bands shown in **b**, as quantified using Image J software ($n = 3$)

CPD0022 (40 ± 45%), CPD0857 (37 ± 21%), and CPD0180 (66 ± 7.5%), but not other CPDs (Fig. 1c). Among those three, only CPD0857 (50.8 ± 9.6%) also reduced Ras protein expression (Fig. 1d). Thus, we chose CPD0857 for further analysis.

3.2 CPD0857 Promotes Ubiquitin-Dependent Proteasomal Degradation of Both β-Catenin and Ras Protein

We found that CPD0857 has a half-maximal inhibitory concentration (IC_{50}) of 1.4 μM in the HEK293 reporter cell line (Fig. 2a, right). Immunocytochemical analysis of HEK293 cells grown in Wnt3a CM confirmed depletion of both cytosolic and nuclear β-catenin following CPD0857 treatment (Fig. 2b). Moreover, CPD0857 treatment of HEK293 cells also promotes dose-dependent decreases of both β-catenin and Ras proteins expression by Wnt3a CM stimulation (Fig. 2c). Given that β-catenin and Ras are reportedly regulated at the protein level in cancer cells [8, 9], we focused on β-catenin and Ras protein stability by treating HEK293 cells with CPD0857 and the proteasome inhibitor MG132 (10 μg/mL) and assessing β-catenin and Ras protein levels. Treatment with CPD0857 alone reduced levels of β-catenin and Ras protein, an effect significantly rescued in the presence of MG132 (10 μg/mL) (Fig. 2d). To confirm these effects, we transiently overexpressed HA-Ubiquitin (HA-Ub) vector in the D-*K-Ras* MT CRC cell line and then treated cells with CPD0857 plus MG132 (10 μg/mL). Immunoprecipitation analysis of endogenous β-catenin and Ras proteins followed

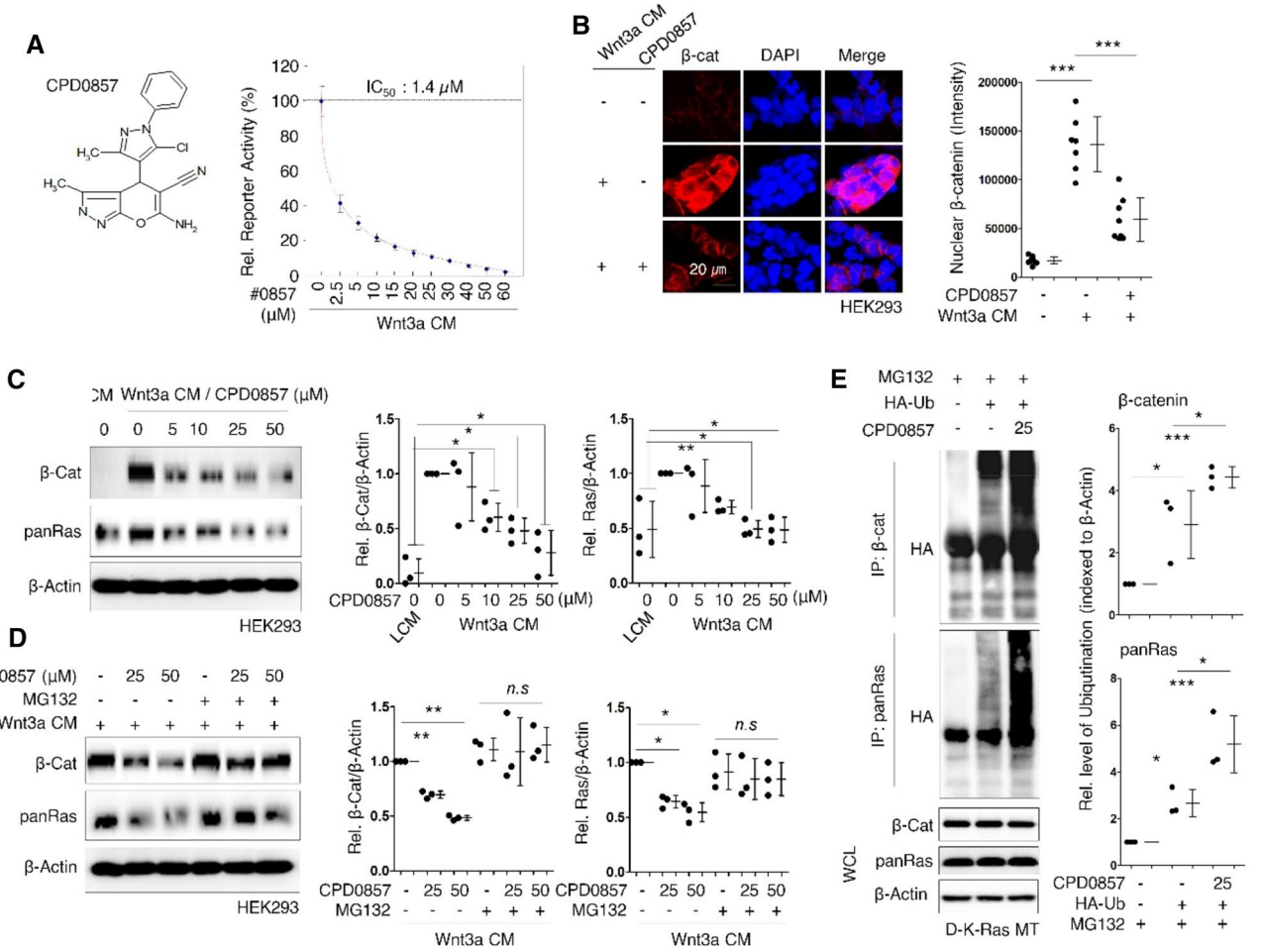

Fig. 2 CPD0857 effects on β-catenin and Ras protein destabilization in CRC cells. **a** (Left) structure of CPD0857; (right) relative reporter activity in the HEK293 TOP-flash reporter line grown in Wnt3a CM and with increasing concentrations of CPD0857. **b** Immunofluorescence analysis of 293T cells grown in the presence and/or absence of Wnt3a CM and CPD0857 using indicated antibodies; nuclei are counter-stained with DAPI. Scale bars, 20 μm ($n = 3$). **c** (Left) Immunoblot (IB) of HEK293T cells with indicated antibodies; (middle and right) quantification of respective β-catenin and Ras proteins in the presence of increasing CPD0857 doses. **d** Immunoblot (IB) analysis as in (**c**) but with some samples treated for 12 h with MG132 (10 μM) ($n = 3$). **e** Ubiquitylation assay of endogenous β-catenin and Ras proteins in lysates of D-*K-Ras* MT cells transfected with HA-Ub plasmids and treated 1 day later with MG132 (10 μM) for 6 h before harvest. IP was performed with β-catenin or Ras antibody. WCLs were analyzed by IB for indicated antibodies ($n = 3$)

by anti-HA immunoblot indicated that both endogenous proteins were polyubiquitylated, and that polyubiquitylated proteins accumulated in MG132-treated cells (Fig. 2e).

3.3 CPD0857 Promotes Proteasomal β-Catenin and Ras Degradation Independently of GSK3β

To address the role of CPD0857 in CRC cells, we cultured CRC cell lines with diverse genetic backgrounds including *APC* mutant isogenic D-*K-Ras* WT and MT cells. We observed that β-catenin, pan-Ras, and pAKT protein expressions were significantly reduced upon treatment with CPD0857 in various CRC cell lines; however, expression of both β-catenin and pan-Ras proteins except pAKT were not significant different in HCT116 cell line harboring mutant *β-catenin* (Fig. 3a). Because Ras protein degradation by controlling Wnt/β-catenin signaling does not occur in cells harboring mutant β-catenin [10], we also further assessed the effect of CPD0857 in NIH3T3 cells expressing wild-type or mutant *β-catenin* (S33Y) constructs, respectively. CPD0857 significantly reduced the expression of both proteins in cells expressing wild-type β-catenin, but this effect was not observed in cells expressing mutant *β-catenin* (S33Y) (Fig. 3b, c). To determine whether regulation of Ras and β-catenin protein stability occurs through the epidermal growth factor receptor (EGFR), which is upstream of Ras, we tested CPD0857 effects in EGFR knockout ($EGFR^{-/-}$) MEFs or in HEK293 cells stimulated with EGF. Interestingly, CPD0857 significantly reduced the expression of Ras and β-catenin proteins (Fig. 3d, e). Overall, these findings suggest that CPD0857 treatment decreases Ras and β-catenin protein levels in CRC cells and likely acts upstream of β-catenin but downstream of the EGFR.

β-Catenin destruction complex components, such as Axin, APC, glycogen synthase kinase-3 beta (GSK3β), and casein kinase-1 (CK1), bind to β-catenin, triggering its phosphorylation, ubiquitination, and subsequent degradation [2]. Among these components, GSK3β enhances β-catenin degradation via phosphorylation at S33, S37, and T41 [2], and also catalyzes Ras protein phosphorylation at T-144 and T-148 [8, 10–12]. To investigate whether GSK3β is a CPD0857 target, we used TOP Flash reporter cells in which GSK3β kinase activity was blocked by treatment with GSK3β inhibitors such as LiCl, BIO, or valproic acid (VPA). CPD0857 co-treatment significantly decreased TOP Flash activity in cells also treated with LiCl, BIO, or VPA (Fig. 3f), results confirmed by dose-dependent decreases in expression of β-catenin and Ras proteins in CPD0857-treated *GSK3β* wild-type or knock-out cells (Fig. 3g, h). These results suggest that CPD0857-mediated β-catenin and Ras destabilization in CRCs occurs independently of GSK3β.

3.4 Axin Loss Rescues CPD0857-Mediated Polyubiquitination of β-Catenin and Ras Protein in Colorectal Cancers (CRCs)

Previously, we identified a compound that destabilizes both β-catenin and Ras protein by targeting regulators of the G-protein signaling domain of Axin [2]. Therefore, we asked whether CPD0857 treatment altered Axin expression. To do so, we treated RKO, SW480, and LoVo cell lines, which harbor *APC*, *K-Ras (or B-Raf)*, and *PI3K* mutations with varying CPD0857 doses and monitored endogenous Axin protein levels using immunofluorescence analysis. Interestingly, CPD0857 treatment increased Axin protein levels, dose-dependently, and reduced β-catenin, Ras, pERK, and pAKT protein level in all CRC cell lines tested (Fig. 4a–c), results confirmed by immunoblot analysis (Fig. 4d). We asked whether such increases in Axin protein levels regulated Wnt/β-catenin signaling by knocking down Axin in 293 T cells, treating them with Axin siRNA, and then performing immunoblot analysis. CPD0857-dependent Wnt/β-catenin pathway inhibition was abolished in Axin knockdown compared to control siRNA-treated cells (Fig. 4e). To determine whether Axin regulates β-catenin and Ras protein stability, we carried out a ubiquitination assay. We found that CPD0857-mediated β-catenin and Ras protein polyubiquitination was abolished in Axin knockdown RKO cells (Fig. 4f), suggesting overall that CPD0857-dependent increases in Axin protein levels promote β-catenin and Ras protein degradation in CRC cells.

3.5 CPD0857 Treatment Inhibits Tumor Cell Growth and Overcomes Resistance to Anti-EGFR Treatment in CRC Cells Harboring Mutant K-Ras

To determine whether CPD0857 blocks CRC growth, we treated various CRC cell lines with CPD0857 and assessed proliferation and cellular transformation capacity. Proliferation of D-*K-Ras* WT, D-*K-Ras* MT, SW480, and LoVo cell lines was efficiently inhibited dose dependently following CPD0857 treatment (Fig. 5a). Moreover, CPD0857 treatment of all CRC cells tested significantly decreased the number and size of foci (Fig. 5b–d).

mAbs targeting EGFR have been used to treat CRC, but have no effect against cancers harboring *K-Ras* mutations [6]. To assess whether CPD0857 treatment could eradicate CRCs harboring mutant *K-Ras*, we compared the effect of CPD0857, the EGFR mAb cetuximab, or co-treatment with both on proliferation of D-*K-Ras* WT and D-*K-Ras* MT, and SW480 cells, which bear mutant *K-Ras* (Fig. 6a–c). Based on an MTT assay performed 24, 48, and 72 h after drug treatment, cetuximab alone reduced proliferation of D-*K-Ras* WT but not of D-*K-Ras* MT or SW480 cells (Fig. 6a). However, treatment with cetuximab plus CPD0857 efficiently reduced

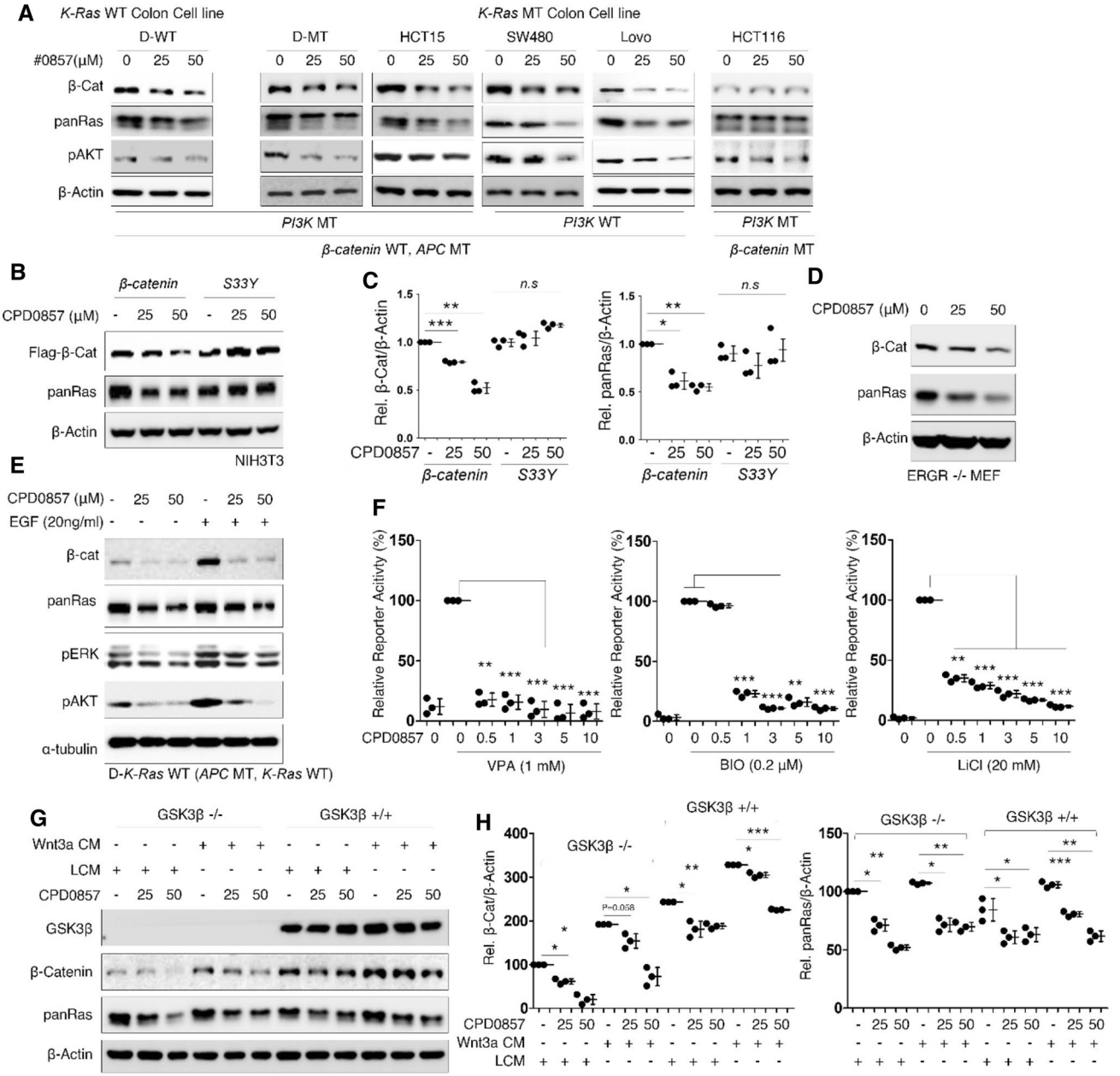

Fig. 3 CPD0857 destabilizes β-catenin and Ras proteins independent of GSK3β. **a** Immunoblot analysis of indicated CRC cell lines with indicated antibodies. **b** Immunoblot of NIH3T3 cells transfected with WT β-catenin-Flag or mutant β-catenin (S33Y)-Flag and probed with indicated antibodies. **c** Relative band intensity in blot shown in (**b**). Data are means ± SD ($n = 3$). **d** EGFR−/− MEFs were treated with indicated CPD0857 doses for 24 h. WCLs were immunoblotted using indicated antibodies. Data are means ± SD ($n = 3$). **e** D-*K-Ras* WT cells were grown in the presence or absence of EGF (20 ng/mL) and immunoblotted (IB) with indicated antibodies. **f** Relative band intensity of blot shown in (**d**). Data are means ± SD ($n = 3$). **g** GSK3β+/+ or GSK3β−/− MEF cells grown in L-cell-conditioned medium (L-CM) or Wnt3a-CM were treated with indicated amounts of CPD0857 for 24 h, and WCLs were immunoblotted using indicated antibodies. **h** Relative band intensity of blot shown in (**g**). Data are means ± SD ($n = 3$)

proliferation in all CRC cells tested (Fig. 6a). Moreover, cellular-transforming capacity decreased in D-*K-Ras* WT, D-*K-Ras* MT, and SW480 cells following CPD0857 treatment (Fig. 6b, c). We observed no synergistic effects of cetuximab and CPD0857 in terms of proliferation or cell transformation, although some synergy was seen in terms of cellular transforming capacity in D-K-Ras WT cells (Fig. 6a–c). CRC cells harboring *K-Ras* mutations exhibit more invasive or metastatic capacity than those with WT *K-Ras* [3]. To test whether CPD0857 antagonized migration of CRC cells harboring mutant *K-Ras*, we monitored wound healing capacities of cells treated with vehicle (DMSO) or varying

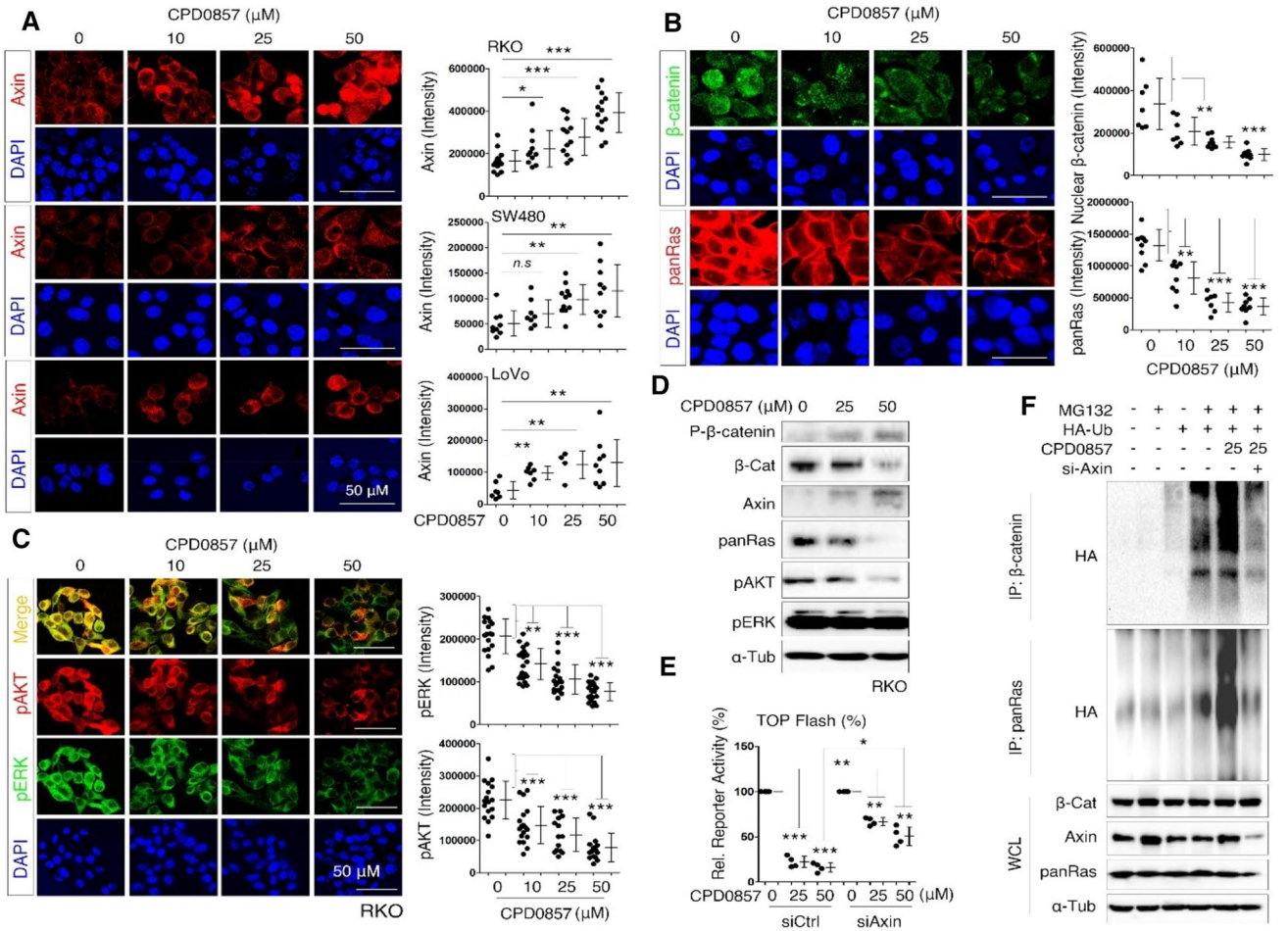

Fig. 4 CPD0857 destabilization of β-catenin and Ras proteins is Axin-dependent. **a** (Left) Immunofluorescence analysis of RKO, SW480, and LoVo CRCs treated for 24 h with increasing CPD0857 doses and incubated with Axin (red) antibodies; DAPI serves as nuclear stain; (right) quantification of Axin levels. **b** (Left) Immunofluorescence of RKO cells treated 24 h with increasing CPD0857 doses and stained with β-catenin and pan-Ras antibodies; (right) quantification of analysis at left. **c** Expression of pERK, and pAKT in the immunostained cells was quantified using Image J software. All data are shown as means ± SD ($n = 3$). Significance was determined by one-way ANOVA (*$P < 0.05$, **$P < 0.005$, ***$P < 0.005$). **d** Immunoblot analysis of RKO cells using indicated antibodies. **e** Reporter activity of HEK293-TOP flash reporter cells transduced with Axin or control siRNAs (50 nM) and treated with indicated CPD0857 doses. **f** Ubiquitylation assay of β-catenin and Ras proteins in indicated lysates of RKO cells. Cells were transduced with HA-Ub plasmids and control or Axin siRNAs and then treated a day later with or without CPD0857 for 24 h. Cells were also treated with MG132 6 h before harvest. IP was performed with β-catenin or Ras antibodies. WCLs were analyzed by IB for indicated antibodies ($n = 3$)

CPD0857 doses at the different time points. CPD0857 treatment significantly decreased migratory capacity of D-*K-Ras* MT cell line even at low (10 μM) concentrations (Fig. 6d, e). The inhibitory effect of cell migration by CPD0857 was further confirmed by the live cell wound-healing assay. These effects were confirmed using a live wound-healing assay of LoVo cells in which CPD0857 treatment significantly reduced the normalized impedance value, which indicates resistance of electrodes placed under a plate, a value that increases as cell migration increases (Fig. 6f). Next, to determine whether CPD0857 inhibited cell invasion, we performed matrigel invasion assays using D-*K-Ras* MT cells. We observed significantly reduced numbers of invading cells in samples treated with two doses of CPD0857 (Fig. 6g, h). Overall, CPD0857 exhibited an anti-proliferation effect in cells resistant to EGFR mAb therapy and antagonized growth and invasiveness of CRC cells harboring mutant *K-Ras*.

3.6 CPD0857 Inhibits Tumor Growth of CRC Harboring Mutants of both APC and K-Ras

To further investigate CPD0857 effects on cell proliferation, we undertook BrdU incorporation analysis and compared PCNA expression in D-*K-Ras* WT and MT cells following treatment with CPD0857 (at 25 or 50 μM). We observed

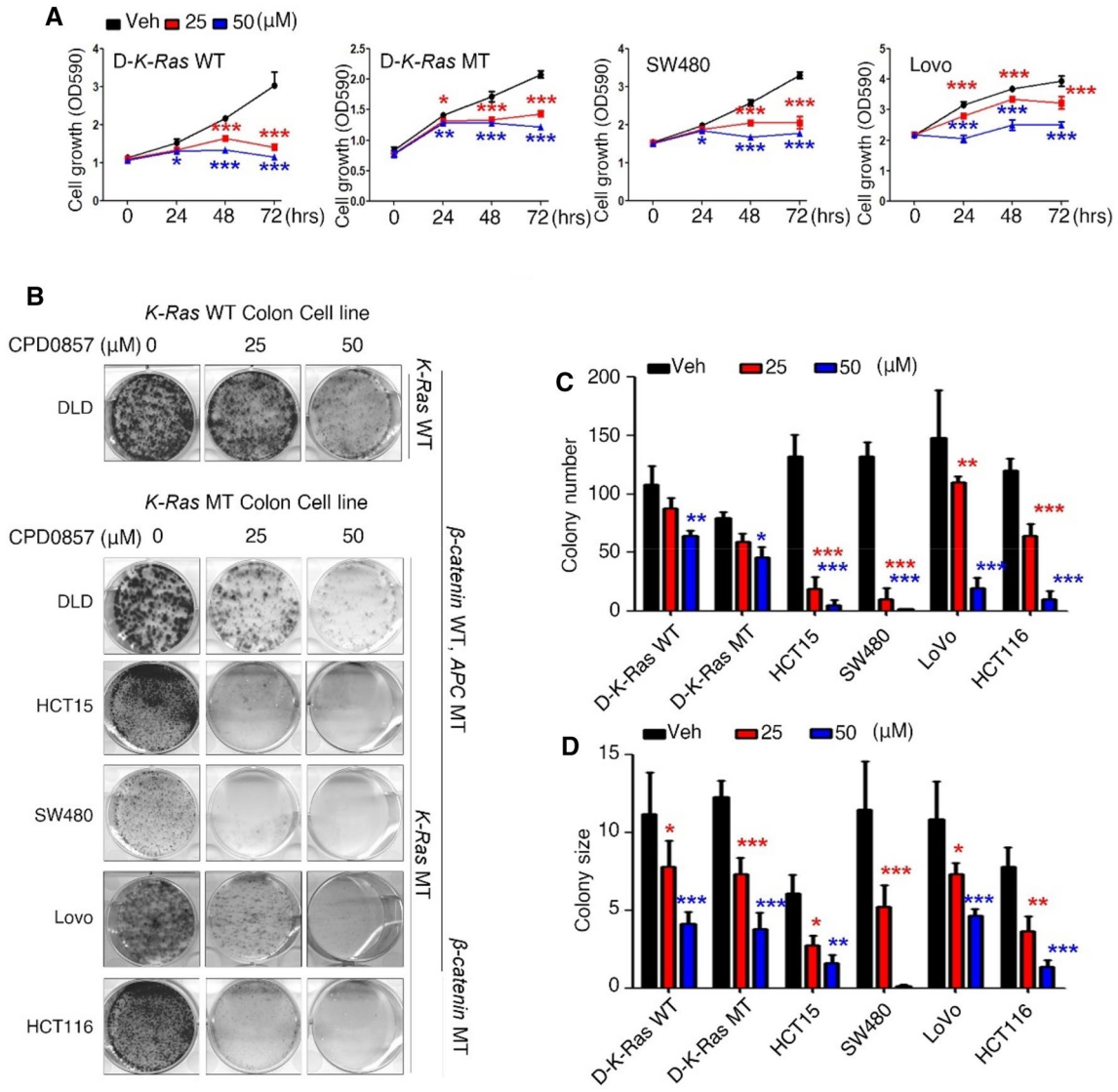

Fig. 5 Effects of CPD0857 on CRC proliferation and transforming activity. **a** Analysis of cell proliferation as measured by an MTT assay in indicated lines treated 72 h with or without CPD0857. **b** Colony-forming assay of indicated D-*R-Ras* WT and MT lines treated 2 weeks with CPD0857. **c**, **d** Colony number (**c**) and size (**d**), as quantified using Image J software. Data is presented as average ± SD ($n = 3$). Significance was determined by one-way ANOVA (*$P < 0.05$, **$P < 0.005$, ***$P < 0.005$)

a decrease in the number of BrdU-positive cells as well a significant reduction in PCNA expression (Fig. 7a, b). Next, to analyze potential effects of CPD0857 on apoptosis, we performed apoptosis assay by FACS analysis using various CRC cell lines. Interestingly, we observed the presence of a distinct sub-G1 peak (sub-diploid DNA content), suggestive of apoptotic cells, following CPD0857 treatment in D-*K-Ras* MT cells (Supplementary Fig. S1A, see Online Supplementary Material (OSM)). The height of this peak increased dose dependently with CPD0857 treatment (Supplementary Fig. S1A (OSM)). These findings were confirmed by performing a DNA fragmentation assay (Supplementary Fig. S1B (OSM)).

Next, to investigate the in vivo effects, we injected CPD0857 intraperitonially (i.p.) into mice carrying xenograft tumors from the D-*K-Ras* MT cell line. CPD0857 administration (25 mg/kg) significantly reduced the volume of tumors by 40% (Fig. 7c, d). To investigate mechanisms underlying CPD0857 tumor suppressive activity, we assessed expression of markers of Wnt/β-catenin, Ras/ERK, and PI3K/AKT signaling in tumors excised from xenograft mice. Interestingly, CPD0857 treatment significantly reduced levels of active proteins such as β-catenin, Ras, p-ERK, and p-AKT as shown by immunoblot analysis (Fig. 7e). Immunohistochemical analysis of tumors further revealed decreased levels of β-catenin

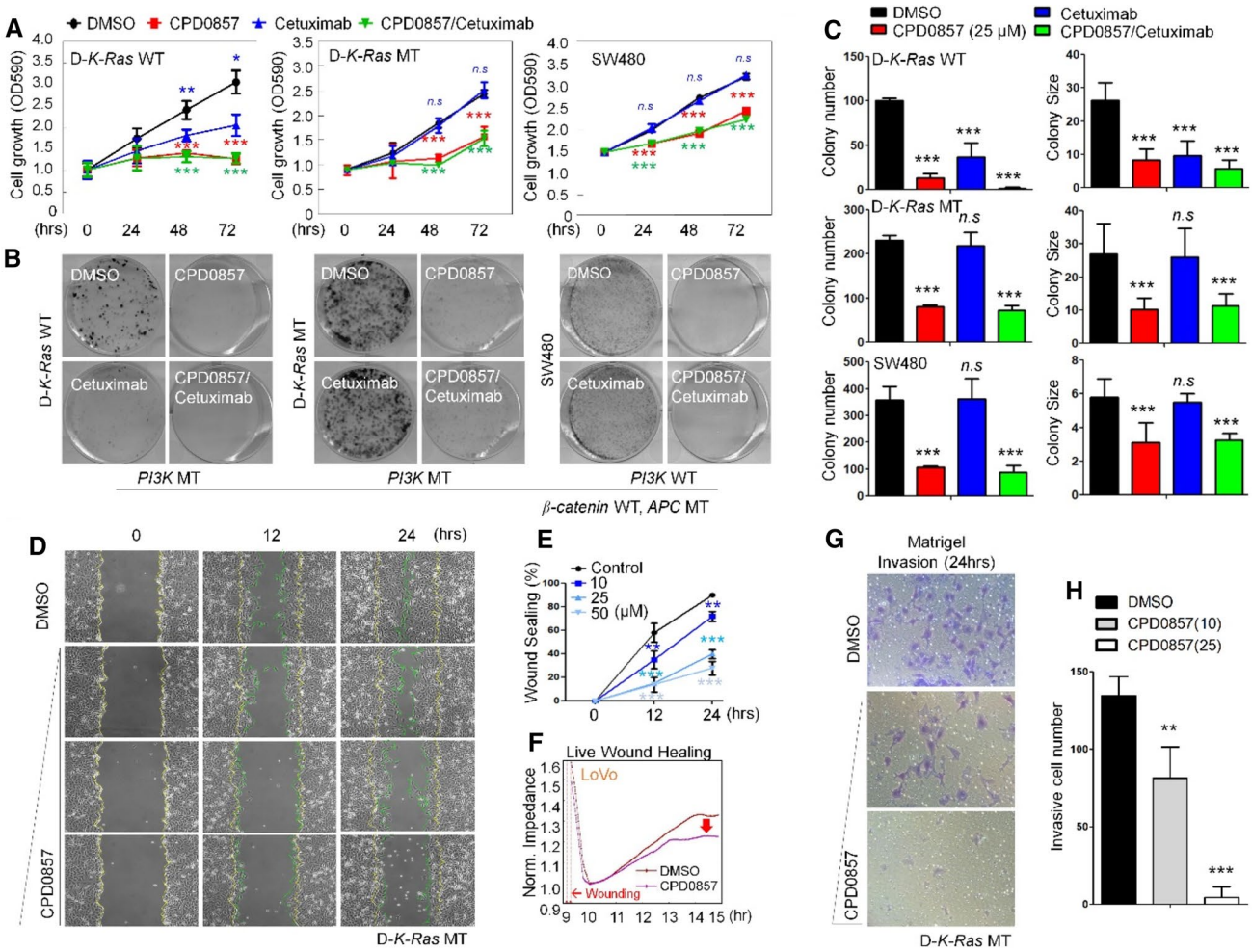

Fig. 6 CPD0857 effects on proliferation of Cetuximab-resistant CRCs harboring *K-Ras* mutations and on CRC cell migration. **a** Analysis of proliferation in indicated lines as determined by MTT assays. Cetuximab, 5 μg/mL; CPD0857, 25 μM ($n=3$). **b** Colony-formation assay of indicated lines using various drug combinations. **c** Colony number (left) and size (right), as quantified using Image J software ($n=3$). **d** Wound-healing assay of D-*K-Ras* MT cells in the presence or absence (DMSO) of CPD0857. **e** Quantification of analysis shown in (**d**). **f** Automated live wound-healing assay of LoVo cells in the presence or absence (DMSO) of CPD0857. **g**, **h** Matrigel invasion assay of D-*K-Ras* MT cells. **h** Quantification of analysis shown in (**g**). All data are presented as average ± SD ($n=3$). Significance was determined by one-way ANOVA (*$P<0.05$, **$P<0.005$, ***$P<0.005$)

(by ~ 44.3%), panRas (by ~ 48.4%), p-ERK (by ~ 64.2%), and p-AKT (by ~ 36.4%) in CPD0857-treated tumors relative to vehicle controls (Fig. 7f, g). Moreover, we observed a ~ 20% increase in levels of Axin protein expression, suggesting that CPD0857 effects may be mediated by an increase of Axin protein (Fig. 7f, g). To further examine the effect of CPD0857 on proliferation, we counted PCNA-positive cells in tumors from mice treated with or without CPD0857. Consistent with in vitro results, the number of PCNA-positive cells in CPD0857-treated tumors decreased by 41% relative to vehicle-treated mice (Fig. 7g). Taken together, these data suggest that CPD0857 inhibits tumor and that these effects are due to inhibition of Wnt/β-catenin, Ras/ERK, and AKT signaling (Fig. 7h).

4 Discussion

In this study, we assessed how a small molecule that downregulates Wnt/β-catenin and Ras/ERK signaling pathways can suppress CRC tumorigenesis. CPD0857, the novel chemical compound identified here, reduced levels of both β-catenin and Ras protein in multiple CRC cell lines via the ubiquitin-dependent proteasomal degradation pathway and subsequently suppressed tumor progression and invasive capacity.

We also found that CPD0857 significantly inhibited tumor progression without downregulating either β-catenin or Ras protein in the HCT116 colon cancer line

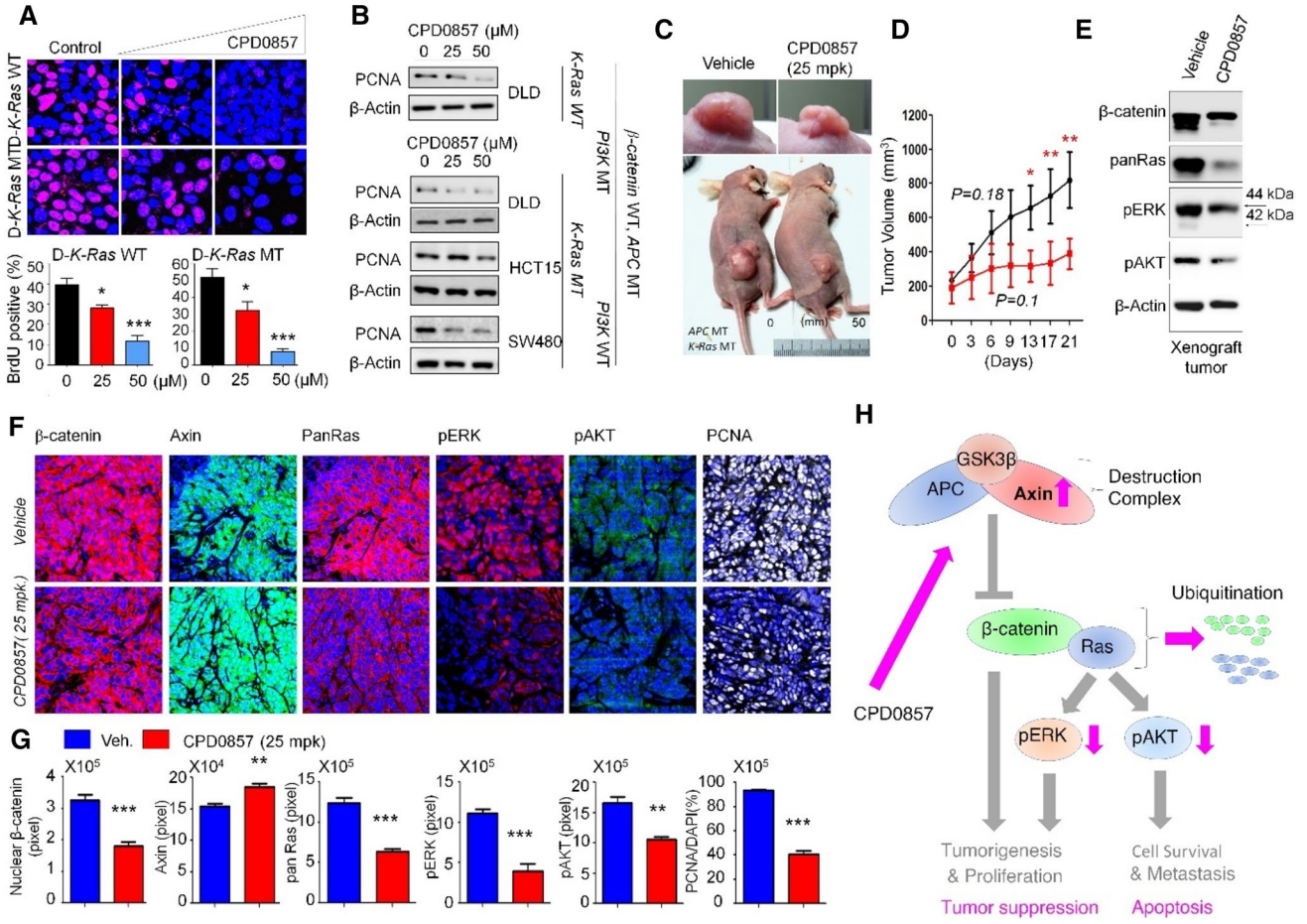

Fig. 7 Effect of CPD0857 on progression of CRC tumors with mutant *K-Ras*. **a** BrdU incorporation assay was performed in D-*K-Ras* WT and MT cell treated with varying doses of CPD0857 ($n=3$). **b** Immunoblot analysis of D-*K-Ras* WT, MT, HCT15, and SW480 cells with indicated antibodies. **c** Representative tumors in nude mice injected in the flank with D-*K-Ras* MT cells and then treated for 21 days by IP injection of vehicle or 25 mg/kg CPD0857 once every 3 days ($n=6$). **d** Tumor volumes of xenograft mice treated as in (**c**) ($n=5$). **e** Immunoblot analysis excised tumors described in (**c**) with indicated antibodies. **f** Fixed sections from excised tumors described in (**c**) were incubated with indicated antibodies. **g** Quantification of immunostaining of tumor tissues shown in (**f**), based on analysis with Image J software. Representative images were selected from at least three different fields. **h** Model of CPD0857 inhibition of Wnt/β-catenin, Ras-MAPK, and PI3K/AKT pathways. CPD0857 favors activity of the β-catenin destruction complex by increasing Axin protein levels and enhancing β-catenin and Ras degradation via the proteasome. All data are shown as means ± SD for at least three independent specimens. Significance was determined by one-way ANOVA (*$P<0.05$, **$P<0.005$, ***$P<0.005$)

expressing a non-degradable mutant β-catenin (Figs. 3a, 5b–d), suggesting that the effects of CPD0857 in this line occur by an alternative mechanism. The phosphatidylinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway is a major signaling cascade downstream of the EGFR in colon cancers [13] and is known to be important for progression of many solid cancers [14–19]. The PI3K/AKT pathway also plays an important role in cell survival by inactivating apoptogenic factors in many cell types [20]. Interestingly, CPD0857 treatment of CRC cells, including HCT116, decreased pAKT expression and increased apoptosis, based on our observation of increased populations of early and late apoptotic cells (Fig. 3a and Supplementary Fig. S2A–C (OSM)). This result strongly suggests that CPD0857 inhibits growth of HCT116 cells by inhibiting the PI3K/AKT pathway. To assess this possibility, we treated CRC cells harboring wild-type or mutant *PI3K* with CPD0857 and observed decreased pAKT levels in all CRC cells bearing wild-type or mutant *PI3K* (Figs. 3a, 4c, d). Treatment also reduced tumor growth, migration and invasion in vitro and in vivo (Fig. 6d–h). PI3K/AKT pathway activation is reported in 60–70% of CRCs, and inhibitors targeting pathway components have been suggested as therapeutic agents in several studies [21–23]. Therefore, CPD0857 could also serve as an inhibitor of PI3K/AKT signaling to promote cellular apoptosis and subsequent tumor suppression.

We found that CPD0857 significantly reduced levels of both β-catenin and Ras protein in EGFR knock-out cells (Fig. 3d), suggesting that the effects of CPD0857 in CRC cell lines do not occur via EGFR. Moreover, we previously revealed that the α-interface of Ras protein can directly interact with the *c*-term region of β-catenin, and prior degradation of β-catenin promotes initiation of Ras protein degradation by exposure of phosphorylation sites on the α-interface region of the Ras protein [2]. Based on these observations, we hypothesize that CPD0857 may first reduce β-catenin protein by inhibiting the Wnt/β-catenin pathway and subsequently decreases Ras, and these could strongly support recent stepwise model for sequential degradation of both β-catenin and Ras in CRCs [10].

TGFβ-Smad signaling is often perturbed in human cancers, including CRC [24]. Others have suggested that Axin negatively regulates basal stability of Smads by promoting their ubiquitination and thereby inhibiting the TGFβ-Smad pathway [25]. Axin negatively regulates the Wnt/β-catenin pathway and functions in β-catenin degradation [11]. Axin has also recently received attention as a small-molecule drug target for modulating assembly of the destruction complex for β-catenin degradation [2]. Importantly, we observed increased Axin protein levels in cells and xenograft tumors administered CPD0857 (Fig. 4a, d). Therefore, CPD0857 likely promotes Axin expression and induces subsequent inhibition of TGFβ-Smad signaling by destabilizing Smad3 protein, although mechanisms underlying that activity remain unclear. Nonetheless, our results suggest that Axin may be a potential target of CPD0857 in CRC, a possibility that warrants further investigation in future studies.

5 Conclusions

In summary, we used a dual-cell-based high-throughput screening system to identify CPD0857, which significantly suppresses tumorigenesis and metastatic properties by inhibiting multiple signaling pathways such as Wnt/β-catenin, Ras/ERK and PI3K/AKT pathways. Moreover, CPD0857 overcame chemoresistance to standard therapeutics such as EGFR mAbs.

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Declarations

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Conflict of interest The authors Jung Kyu Choi, Heeyeong Cho, and Byoung-San Moon declare that they have no conflicts of interest that might be relevant to the contents of this manuscript.

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Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material Not applicable.

Code availability Not applicable.

Author contributions JKC and B-SM designed and performed the all experiments. HC supported materials, edited the manuscript and performed data analysis. B-SM wrote the manuscript and organized the project.

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