

IMP-1 Displays Cross-Talk with K-Ras and Modulates Colon Cancer Cell Survival through the Novel Proapoptotic Protein CYFIP2

Perry S. Mongroo^{1,2}, Felicite K. Noubissi⁴, Miriam Cuatrecasas⁵, Jiri Kalabis^{1,2}, Catrina E. King^{1,2}, Cameron N. Johnstone^{1,2}, Mark J. Bowser^{1,2}, Antoni Castells⁵, Vladimir S. Spiegelman⁴, and Anil K. Rustgi^{1,2,3}

Abstract

Insulin-like growth factor 2 mRNA-binding protein-1 (IMP-1) is an oncofetal protein that binds directly to and stabilizes oncogenic c-Myc and regulates, in turn, its posttranscriptional expression and translation. In contrast to normal adult tissue, IMP-1 is reexpressed and/or overexpressed in human cancers. We show that knockdown of c-Myc in human colon cancer cell lines increases the expression of mature *let-7* miRNA family members and downregulates several of its mRNA targets: IMP-1, Cdc34, and K-Ras. We further show that loss of IMP-1 inhibits Cdc34, Lin-28B, and K-Ras, suppresses SW-480 cell proliferation and anchorage-independent growth, and promotes caspase- and lamin-mediated cell death. We also found that IMP-1 binds to the coding region and 3'UTR of *K-Ras* mRNA. RNA microarray profiling and validation by reverse transcription PCR reveals that the p53-inducible proapoptotic protein CYFIP2 is upregulated in IMP-1 knockdown SW480 cells, a novel finding. We also show that overexpression of IMP-1 increases c-Myc and K-Ras expression and LIM2405 cell proliferation. Furthermore, we show that loss of IMP-1 induces Caspase-3- and PARP-mediated apoptosis, and inhibits K-Ras expression in SW480 cells, which is rescued by CYFIP2 knockdown. Importantly, analysis of 228 patients with colon cancers reveals that IMP-1 is significantly upregulated in differentiated colon tumors ($P \leq 0.0001$) and correlates with K-Ras expression ($r = 0.35$, $P \leq 0.0001$) relative to adjacent normal mucosa. These findings indicate that IMP-1, interrelated with c-Myc, acts upstream of K-Ras to promote survival through a novel mechanism that may be important in colon cancer pathogenesis. *Cancer Res*; 71(6); 2172–82. ©2011 AACR.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the United States. Deregulated expression of the oncogenic transcription factor c-Myc occurs in a broad range of human cancers and is often associated with poor prognosis, indicating a key role for this oncogene in tumor progression. Importantly, c-Myc expression is elevated in approximately 70% of colon tumors due to defective Wnt signaling (1, 2). Recent evidence indicates that c-Myc induc-

tion causes global transcriptional repression of human microRNA (miRNA) genes, including members of the *let-7* family that are downregulated in human cancer cells and tumors (3, 4). The *let-7* family of miRNAs act as tumor suppressors and inhibit the translational expression of oncogenic mRNAs including *K-Ras*, *c-Myc*, *Hmga2*, and *Cdc34*, and suppress cancer cell growth, proliferation, and tumor formation *in vivo* (5–12). *K-Ras* is frequently mutated in human tumors and plays key roles in regulating diverse cellular pathways important for cell growth, differentiation, and survival (13). Indeed, 40% to 50% of human colon cancers harbor activating mutations in the *K-Ras* proto-oncogene and is associated with progression from an adenoma to adenocarcinoma. Thus, the K-Ras signaling pathway represents an attractive target for cancer therapy (14–18).

The human *c-Myc* mRNA coding region determinant-binding protein, also known as insulin-like growth factor 2 mRNA-binding protein-1 (IMP-1), is expressed during early embryonic mammalian development and functions in translational stability by binding and shielding several mRNAs that play critical roles in cell growth and proliferation from proteolytic degradation including *c-Myc* (19–24). Consistent with its oncofetal function, loss of *IMP-1* in mice causes perinatal lethality, dwarfism, and impaired intestinal morphogenesis (25). In striking contrast to normal adult tissues, IMP-1

Authors' Affiliation: ¹Division of Gastroenterology, Department of Medicine, ²Abramson Cancer Center, and ³Department of Genetics, University of Pennsylvania, Philadelphia, Pennsylvania; ⁴Department of Dermatology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin; and ⁵Gastroenterology Department, Hospital Clinic, CIBERehd, IDIBAPS, Barcelona, Spain

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Corresponding Author: Anil K. Rustgi, Division of Gastroenterology, Departments of Medicine and Genetics, Abramson Cancer Center, University of Pennsylvania, 600 Clinical Research Building, 415 Curie Blvd., Philadelphia, PA 19104. Phone: 215-898-0154; Fax: 215-573-2024; E-mail: anil2@mail.med.upenn.edu

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reexpression has been reported in breast, ovarian, and colorectal tumors (26). Furthermore, IMP-1 is a positive predictor of poor clinical outcome in colon cancer patients (27). Recent work has revealed that the β -catenin/Tcf complex upregulates IMP-1 mRNA and protein expression, necessary for the stabilization and induction of *c-Myc* and β -*TrCP1* mRNAs in CRCs, and maybe involved in the suppression of apoptosis (24, 28). Moreover, increased IMP-1 levels positively correlate with activation of β -catenin/Tcf signaling in primary colorectal tumors (24). Importantly, IMP-1 is a direct let-7 target and promotes cell cycle progression, growth, and migration (29). These studies suggest that IMP-1 plays a role in regulating human cancer progression.

Herein, we report a molecular mechanism by which c-Myc positively modulates IMP-1 expression in colon cancers, in part by negative regulation of let-7 miRNAs. We also show that loss of IMP-1 downmodulates K-Ras expression downstream of β -catenin, and concomitantly inhibits colon cancer cell proliferation, anchorage-independent growth, and survival in monolayer and organotypic (3D) cell culture. Furthermore, we identify a novel proapoptotic gene target, cytoplasmic FMR1-interacting protein 2 (*CYFIP2*), which is downregulated by IMP-1, and mediates the regulation of cell survival and K-Ras expression in colon cancer cells. In contrast to our knockdown studies, IMP-1 overexpression increases c-Myc and K-Ras expression and colon cancer cell proliferation. For the first time we find that IMP-1 directly interacts with *K-Ras* mRNA and is highly elevated in colon cancer cells and tumors and positively correlates with K-Ras relative to normal mucosa, thus suggesting a novel interrelationship with K-Ras *in vivo*.

Materials and Methods

Cell culture, transfection, virus production, and stable line derivation

Caco-2, SW-480, and LIM2405 cells were purchased from the American Type Culture Collection (ATCC) and maintained in accordance with the manufacturer's recommendations. Cells were transfected with human β -catenin siRNA (gift from Dr. Jeffrey Drebin, University of Pennsylvania), SignalSilence c-Myc siRNAs I and II (Cell Signaling Technology), IMP-1 siRNA, *CYFIP2* siRNA (Santa Cruz Biotechnology), or scrambled negative control siRNA (Ambion) by using Lipofectamine 2000 reagent (Invitrogen), per manufacturers' instructions. The IMP-1/pMSCV-PIG retroviral vector was purchased from Addgene (plasmid 21659) and used to stably infect LIM2405 cells as described previously (30, 31).

Total RNA isolation, reverse transcription PCR, let-7 miRNA quantitative real-time PCR analysis

Total RNA was isolated from tissues and cultured cells by using the mirVana miRNA isolation kit (Ambion) and analysis of mRNA expression by reverse transcription PCR (RT-PCR) as described previously (32). Normal human colon was obtained from the Cooperative Human Tissue Network at the Hospital of the University of Pennsylvania [with Institutional Review Board (IRB) approval]. Human primary *let-7a3-b* intron,

CYFIP2, and β -*actin* PCR products were amplified by the following oligonucleotide primer pairs:

hlet-7a3-b intron
 5'-GGGGCCGCCTACACTGAGAAG-3' (Forward)
 5'-CTGGGGCACGTGCTGGGAACCT-3' (Reverse)
 hCYFIP2
 5'-TGGCGTCATCATTCCTATCC-3' (Forward)
 5'-GTCAGGTCCTCACTCAAGC-3' (Reverse)
 h β -actin
 5'-AGAAATCTGGCACCACACC-3' (Forward)
 5'-AGAGGCGTACAGGGATAGCA-3' (Reverse)

RT-PCR products were resolved by 1% TAE agarose gel electrophoresis.

Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems 7900HT Real-Time PCR System. The reverse transcription was performed by the TaqMan miRNA Transcription kit, followed by quantification of hsa-IMP-1 and mature hsa-let-7a and -7b, using predesigned TaqMan Assays (Applied Biosystems), according to the manufacturer's recommendations. β -Actin or U47 endogenous controls (Applied Biosystems) were used as an internal standard to normalize. PCR reactions were performed in triplicate. Data were analyzed by ABI PRISMs 7000 sequence detection system software (Applied Biosystems).

Antibodies

We purchased the following antibodies: IMP-1 [for immunohistochemistry (IHC)], c-Myc, β -catenin, cleaved Caspase-3 (Asp175) (5A1E) and PARP (Asp214), Lamin A/C (Cell Signaling Technology), β -catenin (for IHC), Cdc34, Cyclin D1 (BD Transduction Laboratories), Ras clone 10 (Upstate), IMP-1, K-Ras (Santa Cruz), Caspase-8 (Enzo Life Sciences), Cyfip2 (Abcam), Lin28B (Abgent), and K-Ras (for IHC; Bioscience).

Immunoblotting

Immunoblotting was performed as described previously (33). The membranes were stripped by using Blotfresh Western Stripping Reagent (SigmaGen) and reprobed for anti- β -actin (Sigma-Aldrich) to confirm equal loading. Relative band intensities were quantified by Adobe Photoshop software, and normalized to the most intense band, for each antibody.

Tissue microarrays

Twelve tissue microarrays (TMA) were constructed with two cores of representative areas of each carcinoma and two cores of normal adjacent mucosa under IRB approval. A uniform cohort of 228 patients (133 male and 95 female) with colon cancer, 88 in stage 2, and 140 in stage 3, which were diagnosed between November 1993 and October 2006. Rectal tumors were excluded from the study. The pathology reports were available in all cases. Hematoxylin and eosin (H&E) slides and paraffin blocks were retrieved from the Surgical Pathology files of the Hospital Clinic, Barcelona. H&E slides from the original surgery and subsequent tumors were examined. All patients were treated with surgery and 5-fluorouracil. Slides

were imaged by a Nikon TE2000 Eclipse microscope with a QiCam (Q Imaging) camera and IPLab imaging software (BD Bioscience Bioimaging). H-score is the product of the intensity of the staining (0, 1, 2, or 3) by the percent of stained cells (0 to 100). The distributions of nuclear β -catenin, K-Ras, and IMP-1 intensity scores, percent stained cells, and H-scores were right skewed and did not follow a normal distribution. Thus, Wilcoxon's signed rank tests were used to compare normal versus tumor tissue, paired within each patient. Spearman's correlation coefficients, stratified by tumor type, were used to assess the strength of linear association between nuclear β -catenin, K-Ras, and IMP-1 measures. Spearman's correlation coefficients greater than 0.50 indicate a strong association and those greater than 0.30 indicate a moderate association. All analyses were performed by SAS version 9.2 (SAS Institute).

Cell proliferation and anchorage-independent growth assays

WST-1 cell proliferation and anchorage-independent growth were performed as described previously (31).

Gene expression profiling and bioinformatics analysis

Gene expression profiling and bioinformatics analysis was performed as described previously (33), using total RNA isolated from IMP-1 and scramble control siRNA SW-480 cells.

Organotypic cultures

SW480 cells were transiently transfected with either scramble control or IMP-1 siRNAs, seeded on Type I collagen embedded with human fetal colonic fibroblasts (ATCC), grown in organotypic culture (OTC), harvested, and fixed as previously described (34).

Template preparation, *in vitro* transcription of labeled RNA with α -³²P and UV cross-linking

The K-Ras coding cDNA and its 3'UTR subcloned into pGEM-T easy vector were linearized by *Nco*I and *Sal*I restriction enzymes, respectively. GLI1 expression plasmid and the fragment encompassing nucleotides 2,713 to 3,600 of GLI1 cDNA (35) were used, respectively, as positive and negative controls for the binding assay of K-Ras coding region with IMP-1. The full-length GLI1 subcloned into pOTB7 (ATCC) was linearized immediately 3' to the target DNA insert by *Bgl*II restriction enzyme. Fragments encompassing nucleotides 17 to 597 and 577 to 1,182 of β -TrCP1 cDNA (24) were subcloned into pcDNA3.1, linearized by *Xba*I restriction enzyme, and used, respectively, as negative and positive controls for the binding assay of the 3'UTR region of K-Ras with IMP-1. *In vitro* transcription, transfection, whole cell extraction, and UV cross-linking were performed as described previously (35).

Results

IMP-1 regulation by c-Myc in human colon cancer cells

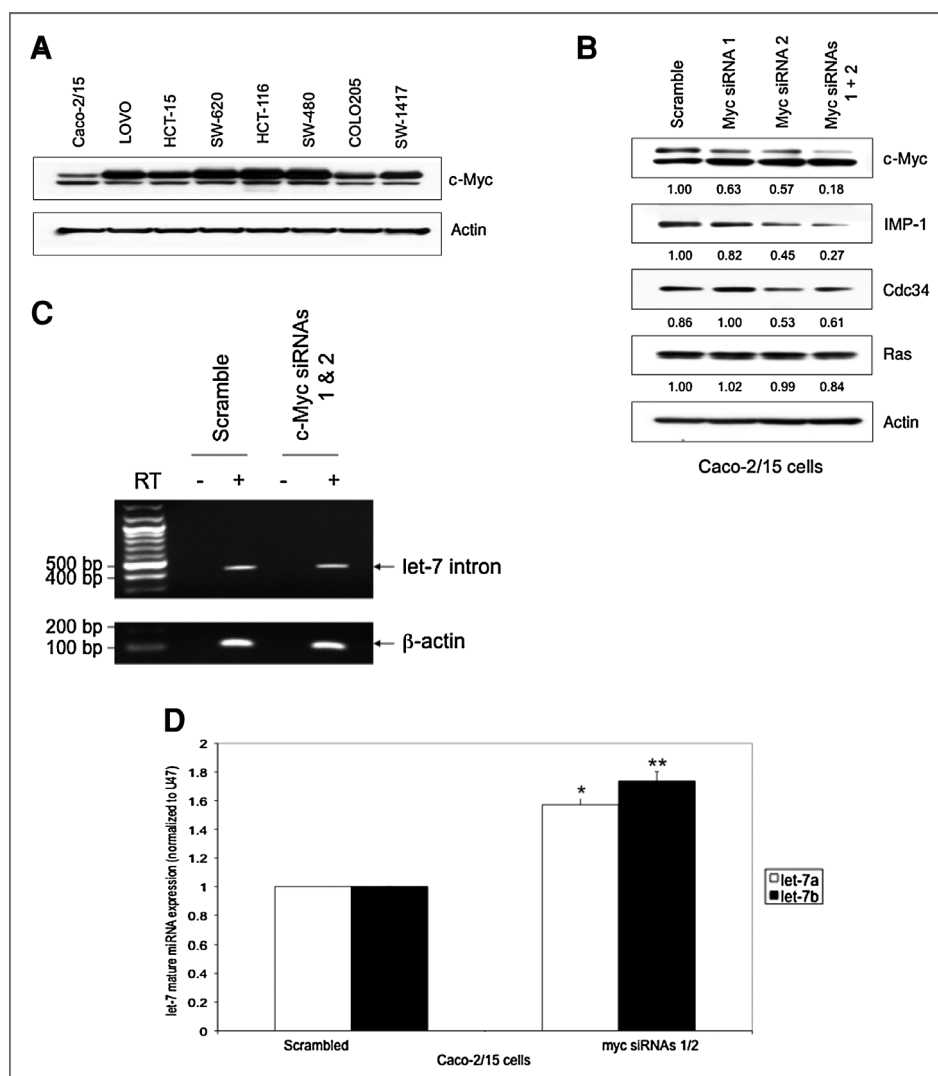
We first sought to determine the relative abundance level of endogenous c-Myc protein in 8 human colon cancer cell lines by Western blot analysis and selected Caco-2/15 cells for further c-Myc knockdown and biochemical studies based

on its lower abundance of c-Myc relative to the other cell lines surveyed (Fig. 1A). Caco-2/15 cells were transiently transfected with two different c-Myc siRNAs either individually or together. We found that a combination of the two c-Myc siRNAs (1 and 2) more effectively reduced endogenous c-Myc protein expression by 82% compared with transfection with the individual c-Myc siRNA-1 (37%) and -2 (43%), or the scramble control cells (Fig. 1B). Interestingly, we also observed a concomitant decrease in expression of the let-7 miRNA target IMP-1 by 73%, and Cdc34 and total Ras, by 30% and 16%, respectively, compared with control cells (Fig. 1B), suggesting let-7 regulation by c-Myc. To test this, total RNA was isolated from Caco-2/15 cells transfected with either a scramble control or c-Myc siRNAs. By PCR analysis using let-7 intron-specific primers, we detected no change in primary transcript levels of the *let-7a3-b* bicistronic cluster in c-Myc knockdown cells relative to scramble controls (Fig. 1C). However, qRT-PCR showed an increase in both mature let-7a and let-7b miRNA expression (Fig. 1D), suggesting that c-Myc modulates expression of known let-7 targets, IMP-1, Cdc34, and Ras, in part by negatively regulating endogenous mature let-7 miRNA levels in human colon cancer cells.

IMP-1 modulates K-Ras expression in colon cancer cells and binds to K-Ras mRNA

Our novel finding that c-Myc siRNA inhibits IMP-1 expression suggests a potential positive feedback mechanism. To explore this possibility further, we first determined the relative mRNA and protein expression level of IMP-1 in several human colon cancer cell lines by qRT-PCR and immunoblot analysis, respectively. We discovered that IMP-1 mRNA was upregulated by 3-fold or more in 8 of 14 human colon cancer cell lines surveyed, relative to normal colonic epithelium (NCE; Fig. 2A). IMP-1 mRNA levels were most notably increased in Caco-2 (511-fold) and SW-480 (38-fold) cells (Fig. 2A). Consistent with this finding, IMP-1 protein was highly expressed in Caco-2, SW-480, and SW-620 cells, and abundant levels of β -catenin, a positive regulator of IMP-1 (Fig. 2B). To examine the biochemical role of IMP-1 in c-Myc signaling, SW480 cells were transiently transfected with control, c-Myc, or IMP-1 siRNAs. By Western blot, we found that loss of IMP-1 decreased endogenous levels of Cdc34 (68%), Lin-28B (53%), a let-7 repressor and mediator of c-Myc-driven cellular proliferation, and K-Ras (60%), relative to scramble controls, but not to the same extent as c-Myc inhibition (36; Fig. 2C). Furthermore, we did not detect any significant changes in c-Myc or mature let-7a or -7b miRNA expression levels (data not shown). To assess for effects of IMP-1 in β -catenin signaling, we transfected SW-480 cells with control scramble, c-Myc, IMP-1, and β -catenin siRNAs. Western blot analysis showed that loss of β -catenin reduced endogenous protein levels of oncogenic c-Myc (89%), IMP-1 (67%), and Cyclin-D1 (98%), relative to scramble control cells (Fig. 2D). Interestingly, K-Ras was also reduced in these cells (90%) to a greater extent than by IMP-1 knockdown (42%), compared with controls (Fig. 2D). Because IMP-1 has been shown to bind and stabilize several mRNAs including oncogenic *c-Myc*, we hypothesized that it

Figure 1. IMP-1 expression is regulated by c-Myc in human colon cancer cells. **A**, immunoblot analysis of c-Myc in cell extracts from human colon cancer cell lines. β -Actin was used as a loading control. **B**, c-Myc knockdown inhibits IMP-1 and let-7 protein targets, Cdc34 and Ras. Immunoblot of lysates from Caco-2/15 cells transiently transfected with scramble control or c-Myc siRNAs were probed with the indicated antibodies. β -Actin was used as a loading control. **C**, loss of c-Myc does not affect let-7a3-b primary transcript levels but reduces let-7a and -7b mature miRNA levels. Human let-7 intronic PCR and mature qRT-PCR analyses of total RNA isolated from control and c-Myc siRNA Caco-2/15 cell transfectants, as described in Materials and Methods. **D**, RT minus control revealed no PCR product. Mature let-7a and -7b miRNA expression was normalized to U47. Error bars represent \pm SD. *, $P \leq 0.05$; **, $P \leq 0.05$.



might regulate K-Ras expression by binding to *K-Ras* mRNA (37). Indeed, protein-RNA binding assays revealed that IMP-1 interacts directly with both the coding region and 3'UTR of *K-Ras* mRNA, compared with negative controls (Fig. 2E). Taken together, these findings suggest that IMP-1 functions downstream of β -catenin and may modulate K-Ras expression in human colon cancer cells via binding to *K-Ras* mRNA.

Loss of IMP-1 impairs colon cancer cell proliferation, growth, and survival

To determine the biological role of IMP-1 in human colon cancer cell lines, we transfected SW-480 cells with scramble control or IMP-1 siRNAs and examined the effects on regulating cell proliferation and anchorage-independent growth. We found that loss of IMP-1 expression reduced colon cancer cell proliferation in WST-1 assays by approximately 2-fold ($P = 0.0001$) and significantly suppressed both colon cancer colony size formation and number in soft agar assays by 3-fold,

compared with scramble controls ($P = 0.008$; Fig. 3A and B). Intriguingly, transfection with IMP-1 siRNA also increased trypan blue uptake by 2.5-fold ($P = 0.013$) compared with scramble control cells (Fig. 3C). Loss of IMP-1 also induced cell rounding, membrane blebbing, and floating cells, suggesting the induction of cell death (Fig. 3C). To examine this further, adherent and floating SW-480 cells were collected and harvested 24 and 48 hours posttransfection with scramble or IMP-1 siRNAs. By immunoblot analysis we found that two markers of apoptosis, Caspase-3 and Lamin A/C cleavage products, were both increased in IMP-1 knockdown cells by 2-fold compared with control cells (Fig. 3D). In addition, we also observed a 3.6-fold increase in cleaved Caspase-8 in IMP-1 siRNA-transfected cells but no change in Caspase-9 cleavage (data not shown) relative to scramble controls, suggesting that IMP-1 functions via induction of an extrinsic or mitochondria-mediated cell death pathway (Fig. 3E). Taken together, these data support the notion that IMP-1 is a positive modulator of

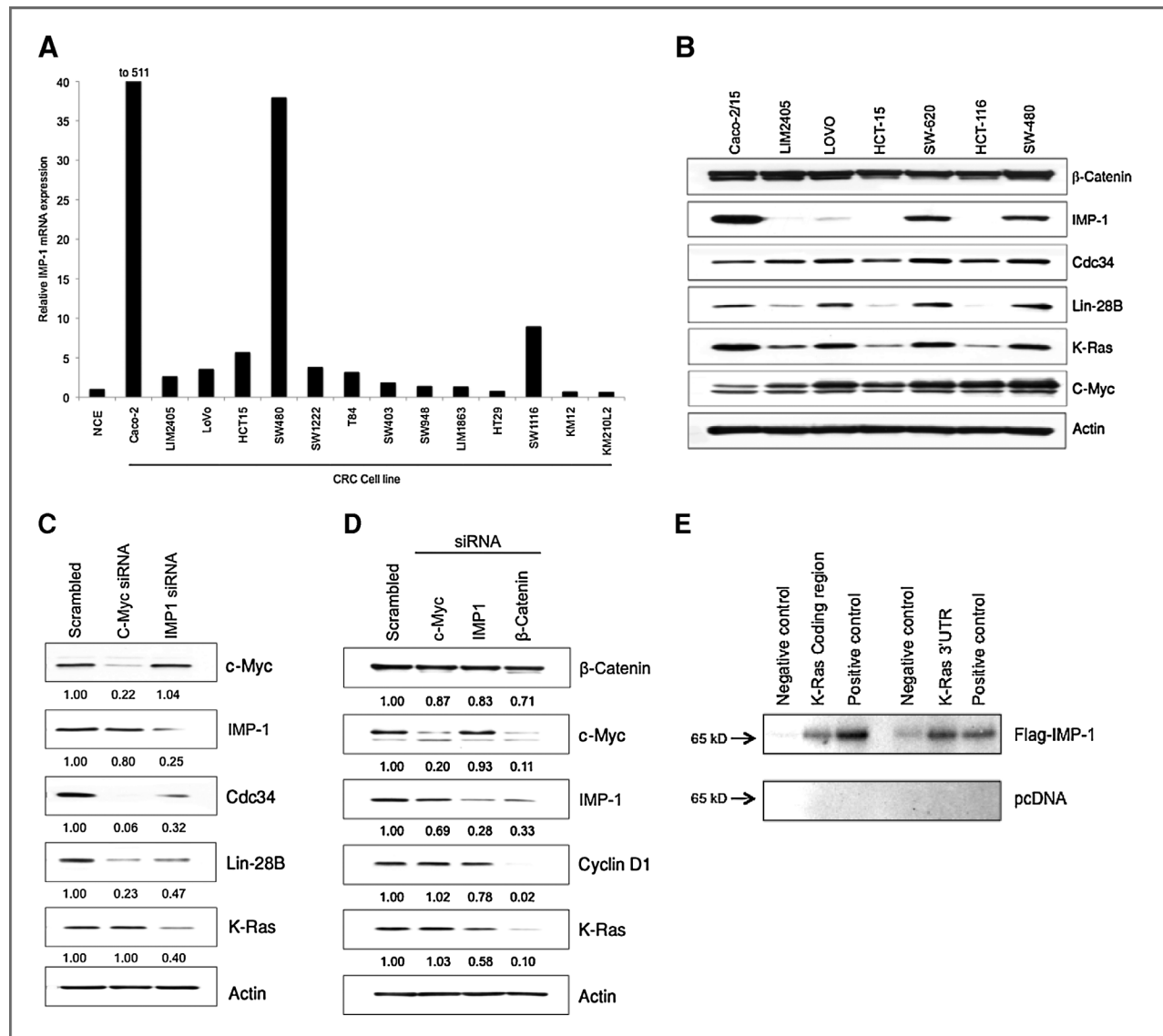


Figure 2. IMP-1 upregulates K-Ras expression in human colon cancer cells, downstream of β -catenin, and binds to K-Ras mRNA. **A**, qPCR analysis of IMP-1 mRNA levels in human colon cancer cells normalized to β -actin mRNA levels. NCE were used as a control. **B**, IMP-1 protein expression in human colon cancer cell lines. Lysates from human CRC cell lines were subjected to Western blotting by using IMP1 antibody. **C**, IMP-1 knockdown inhibits Cdc34, Lin-28, and K-Ras protein expression. Immunoblotting analysis of extracts from scramble control, c-Myc, and IMP-1 siRNA SW480 cell transfectants were probed by using the indicated antibodies. **D**, β -catenin modulates IMP-1 and K-Ras levels in colon cancer cells. Scrambled control, c-Myc, IMP-1, or β -catenin siRNAs SW480 cell lysates were assayed for levels of the proteins indicated by Western blot. β -Actin was used as a loading control. **E**, IMP-1 binds to the coding region of K-Ras mRNA and the 3'UTR. Flag immunoprecipitation of UV cross-linked complexes of the K-Ras coding region as well as the 3'UTR and proteins from 293T cells transfected with Flag-IMP-1 or pcDNA3.1 plasmid. Negative and positive controls were used as described in Materials and Methods.

colon cancer cell proliferation, anchorage-independent growth, and survival.

IMP-1 regulates survival and K-Ras expression through *CYFIP2* in colon cancer cells

To identify novel genes that are involved in mediating IMP-1 functions, expression profiling analysis was performed on total RNA isolated from scramble control and IMP-1 siRNA

SW-480 cells. Gene expression analysis confirmed that *IMP-1* was reduced by approximately 1.6-fold in knockdown cells compared with scramble controls (Supplementary Table S1). We focused on the *CYFIP2*, which was the most upregulated gene target (2.3-fold; $P = 0.00001$) by IMP-1 loss compared with control cells (Supplementary Table S1). Importantly, *CYFIP2* is a p53-inducible gene that is sufficient for inhibition of colon cancer proliferation, caspase activation, and the

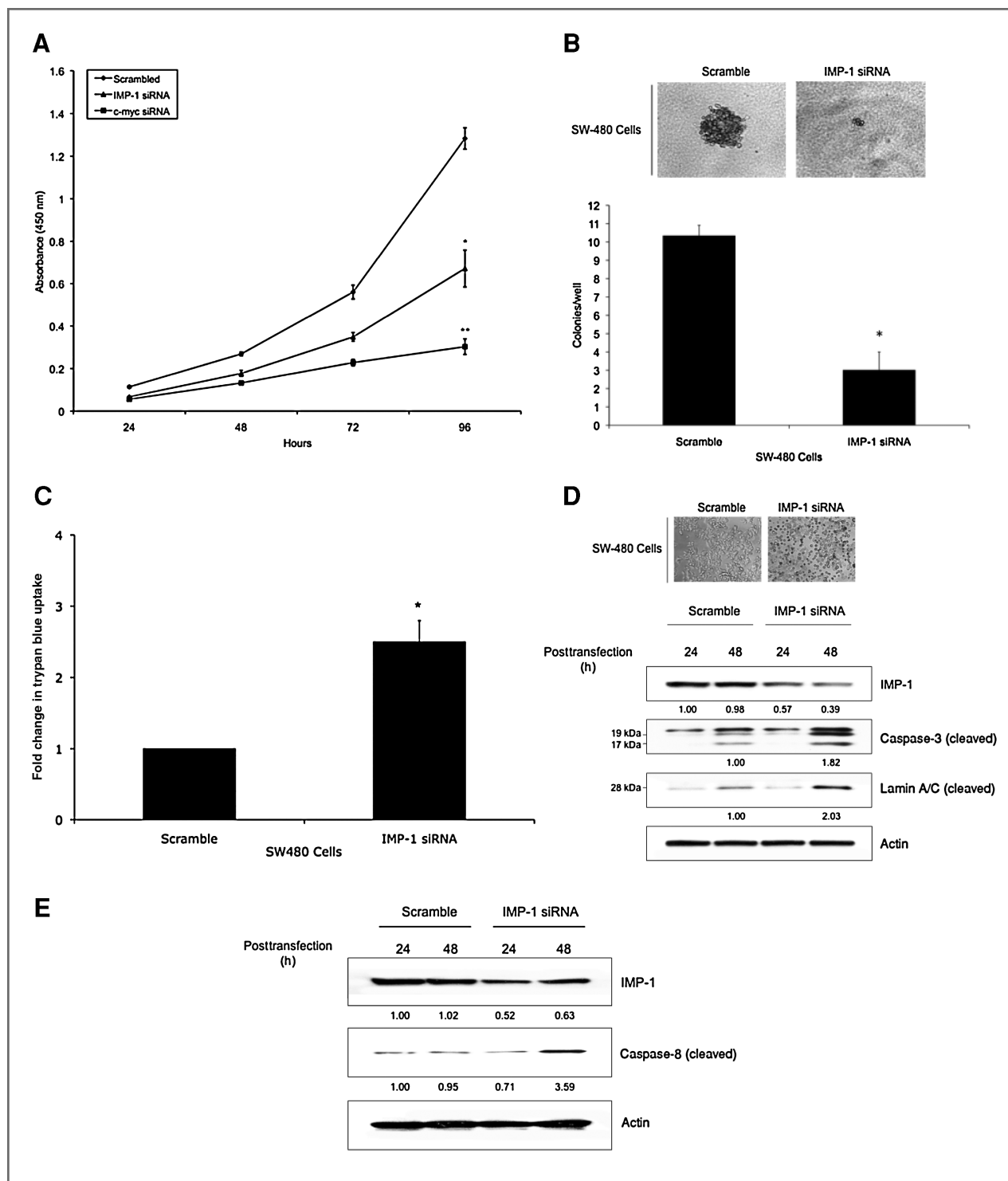


Figure 3. Loss of IMP-1 suppresses colon cancer cell proliferation, anchorage-independent growth, and induces caspase- and Lamin A/C-mediated cell death. **A**, SW-480 cells transfected with scramble control, IMP-1 siRNA, or c-Myc siRNA were assayed for differences in proliferation by using WST-1 dye assay, as described in Materials and Methods. Error bars represent \pm SD for quadruplicate determinations. *, $P \leq 0.05$. **B**, IMP-1 knockdown represses SW-480 colony size and number in soft agar assays. Scramble control or IMP-1 siRNA SW-480 cells were examined for effects on anchorage-independent growth, as described in Materials and Methods. Error bars represent \pm SD for quadruplicate determinations. *, $P \leq 0.05$. **C**, viable versus nonviable cells were assayed by trypan blue exclusion by using a Countess (Invitrogen) automated cell counter and normalized to scramble control cells ($n = 3$). Error bars represent \pm SD. *, $P \leq 0.05$. **D**, immunoblotting analysis of lysates from scramble control or IMP-1 siRNA SW-480 cells by using Caspase-3, Lamin A/C, and actin. **E**, Caspase-8 antibodies. β -Actin was used as a loading control.

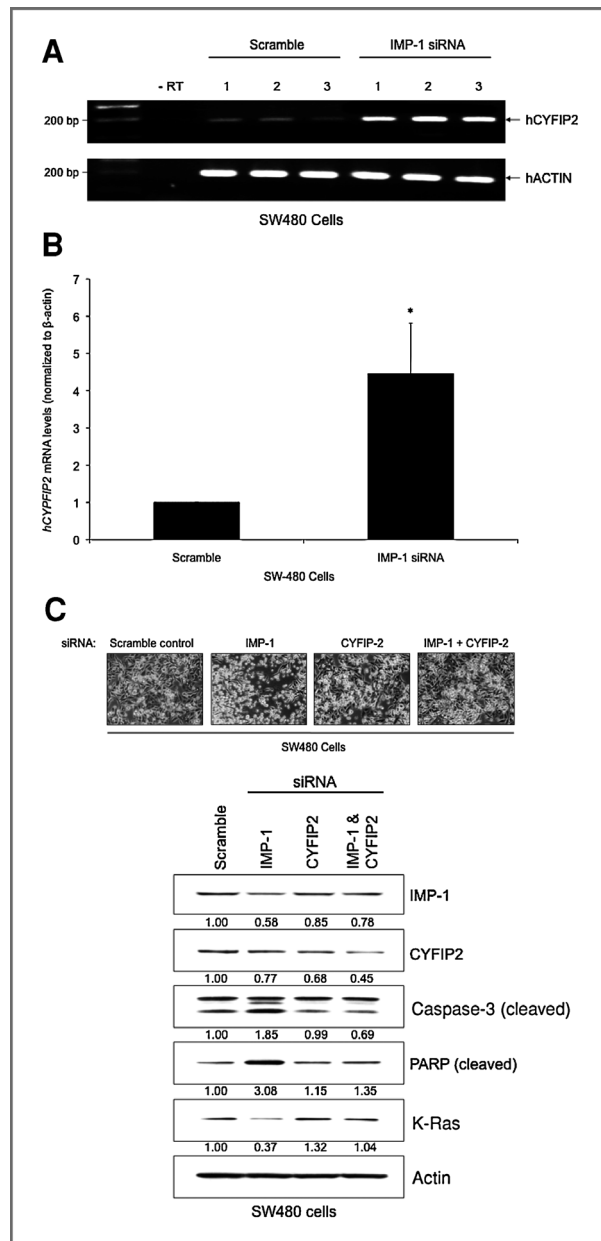


Figure 4. IMP-1 modulates colon cancer cell survival and K-Ras expression by negative regulation of CYFIP2 expression. **A**, inhibition of endogenous IMP-1 upregulates CYFIP2 mRNA in SW-480 cells. Human *CYFIP2* RT-PCR analysis. β -Actin was used as an internal control. **B**, quantification of CYFIP2 mRNA levels in IMP-1 knockdown cells normalized to β -actin by densitometry. Error bars represent \pm SD. *, $P \leq 0.05$. **C**, CYFIP2 knockdown rescues the induction of Caspase-3- and PARP-mediated apoptosis and K-Ras inhibition by IMP-1 loss in colon cancer cells. Lysates of SW-480 cells transiently transfected with scramble control, IMP-1, CYFIP2, or a combination of IMP-1 and CYFIP2 siRNAs was probed with the indicated antibodies by immunoblot. β -Actin was used as a loading control.

induction of apoptosis (38). Consistent with the gene microarray results, PCR analysis confirmed that *CYFIP2* mRNA levels were increased by 4.5-fold in cells transfected with IMP-1 siRNA but was reduced or undetectable in scramble

controls (Fig. 4A and B). We next asked whether CYFIP2 inhibition by IMP-1 mediates cell survival and K-Ras expression. To test this, SW-480 cells were transiently transfected with scramble control, IMP-1, CYFIP2, or a combination of IMP-1 and CYFIP2 siRNAs. By Western blot, we found that cotransfection of IMP-1 and CYFIP2 siRNAs reversed Caspase-3 and PARP cleavage and rescued K-Ras inhibition by IMP-1 loss, similar to levels observed in control cells (Fig. 4C). On the basis of these findings, we propose that IMP-1 promotes colon cancer cell survival and regulates K-Ras expression, in part by suppressing CYFIP2.

IMP-1 increases c-Myc and K-Ras levels and cell proliferation

As a complementary approach to our knockdown studies and to further investigate the role of IMP-1, we chose to modify LIM2405 cells, which express low endogenous IMP-1 mRNA and protein levels (Fig. 2A and B), with a retrovirus expressing IMP-1 or the vector alone as a control. We found that IMP-1 upregulated endogenous levels of c-Myc and K-Ras by 3.8-fold and 2.6-fold, respectively, relative to vector controls in LIM2405 cells (Fig. 5A). IMP-1 also increased LIM2405 cell proliferation in WST-1 assays by 1.5-fold ($P = 0.00125$; Fig. 5B and C). We next wanted to examine whether IMP-1 expression can rescue K-Ras inhibition by β -catenin knockdown. To test this, IMP-1 and vector-alone LIM2405 cells were transfected with scramble or β -catenin siRNAs. Western blot analysis showed that K-Ras levels were 1.7-fold more in IMP-1 cells transfected with β -catenin siRNA compared with vector-alone cells (Fig. 5D). These results support the notion that IMP-1 regulates K-Ras expression downstream of β -catenin signaling.

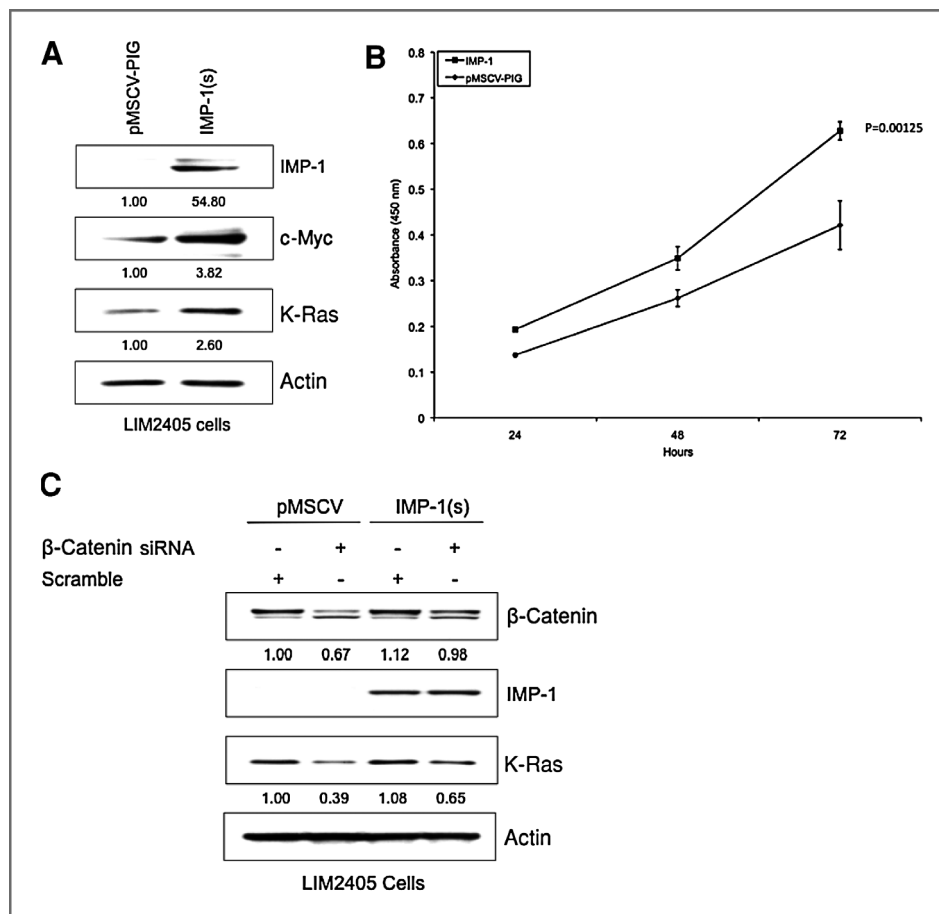
Loss of IMP-1 promotes Caspase-3 cleavage in organotypic culture

We next examined the effect of IMP-1 on colonic epithelium in OTC. SW480 cells were transiently transfected with IMP-1 or scramble control siRNAs and seeded on Type I collagen matrix embedded with colonic fetal fibroblasts. In contrast to scramble controls, IMP-1 siRNA induced the formation of apoptotic bodies indicative of cells undergoing death (Fig. 6A). Consistent with this notion and our biochemical monolayer culture results, we also detected a 2.4-fold ($P = 0.02$) increase in cleaved Caspase-3 positive cells relative to scramble control cells by IHC (Fig. 6A and B). Together these findings suggest that IMP-1 plays a key role in modulating cell survival.

IMP-1 is elevated in colon tumors and correlates with K-Ras

On the basis of our biochemical finding that IMP-1 expression is elevated in human colon cancer cell lines, we sought to determine whether there is a correlation between IMP-1, β -catenin, and K-Ras expression levels, in a cohort of 228 paired patient normal and adjacent colon cancer tissue specimens by IHC. In comparison with adjacent normal colonic mucosa, we observed a significant upregulation of nuclear β -catenin, K-Ras, and IMP-1 in paired colon cancers by 9.9-fold, 8.3-fold, and 33.8-fold ($P \leq 0.0001$), respectively (Fig. 7A).

Figure 5. IMP-1 induces c-Myc and K-Ras protein expression and colon cancer cell proliferation. **A**, expression of recombinant IMP-1 protein in LIM2405 cells. Protein lysates from LIM2405 cells stably infected with retrovirus expressing IMP-1 or empty vector control were assayed for endogenous levels of c-Myc and K-Ras by immunoblot. **B**, IMP-1 increases LIM2405 cell proliferation. IMP and vector-alone LIM2405 stable cell transfectants were assayed for differences in proliferation by using WST-1 dye assay, as described in Materials and Methods. Error bars represent \pm SD for quadruplicate determinations. *, $P \leq 0.05$. **C**, IMP-1 prevents K-Ras inhibition by β -catenin knockdown. Western blot analysis of cell extracts from empty vector and IMP-1 LIM2405 cells transfected with scramble or β -catenin siRNA probed for the indicated proteins.



Importantly, IMP-1 predominantly localized in the cytoplasmic compartment of colon tumors and positively associated with K-Ras intensity ($r = 0.35$, $P \leq 0.0001$) and H-scores ($r = 0.32$, $P \leq 0.0001$) among the tumor tissues (Fig. 7B; Supplementary Fig. S1) compared with adjacent normal colonic mucosa (Fig. 7B). Collectively, our findings suggest that IMP-1 expression is upregulated in human colon cancers and correlates with K-Ras levels.

Discussion

We report that loss of IMP-1 inhibits expression of let-7 miRNA oncogenic protein targets, Cdc34 and K-Ras, and the let-7 repressor Lin-28B, and concomitantly suppresses colon cancer cell proliferation, anchorage-independent growth, and triggers caspase-mediated cell death in both monolayer and 3D culture. By gene profiling analysis and PCR analysis we have discovered a novel proapoptotic target, CYFIP2, which is upregulated by IMP-1 knockdown. Importantly, we have identified a new function of IMP-1 to bind to *K-Ras* mRNA and regulate cell survival and K-Ras expression via inhibition of CYFIP2. We further show that a gain of IMP-1 expression occurs in human colon cancer cells and correlates with K-Ras levels in patient colon tumors.

To date, little is known about the molecular mechanisms that are involved in the complex regulation of IMP-1 in mammalian cells (26). Constitutive activation of β -catenin/Tcf has been shown to upregulate IMP-1 levels in colon cancers and is necessary for the stabilization and induction of *c-Myc* (24, 29). In addition, a recent study showed that overexpression of the antitumorigenic protein 15S-Lipoxygenase (15S-Lox-2) suppresses human prostate carcinoma cell growth and proliferation by downregulation of IMP-1 (39). Our novel finding that loss of c-Myc reduces IMP-1 expression and reexpression of IMP-1 increases c-Myc levels suggests a new potential feedback mechanism whereby c-Myc and IMP-1 may reciprocally modulate expression of each other in colon cancer cells. Oncogenic c-Myc induction has been shown to repress members of the let-7 family in human cancer cells and IMP-1 is a direct let-7 target (3, 4, 29). Consistent with this, inhibition of IMP-1 by targeted knockdown of c-Myc resulted in an increase in mature let-7 miRNA expression, suggesting that c-Myc regulation of IMP-1 is mediated in part by let-7 in colon cancer cells. Furthermore, loss of IMP-1 inhibited expression of let-7 oncogenic targets, Cdc34, K-Ras, and Lin-28B, but did not alter the endogenous levels of c-Myc, pri- or mature let-7 miRNAs (data not shown), suggesting that IMP-1 is regulated by additional genes involved in promoting the cancer cell phenotype.

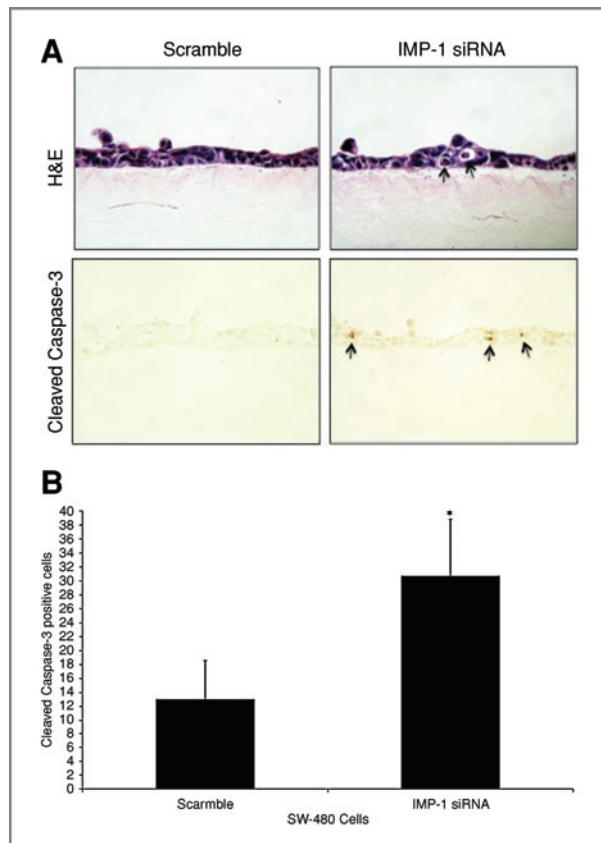


Figure 6. Loss of IMP-1 promotes induction of apoptotic cell bodies and Caspase-3 activation in organotypic cell culture. **A**, H&E-stained sections and cleaved Caspase-3 IHC of OTCs of SW480 cells transiently transfected with IMP-1 or scramble control siRNAs ($\times 40$ magnification). **B**, cleaved Caspase-3 positive cells were quantified ($n = 3$). Error bars represent \pm SD. *, $P \leq 0.05$.

We showed that loss of IMP-1 suppressed colon cancer cell proliferation and anchorage-independent growth, and increased trypan blue uptake, caspase- and lamin-mediated cell apoptosis. By gene profiling microarray analysis, we also identified a new IMP-1 target, *CYFIP2*, a direct target of the tumor suppressor p53 that is sufficient for caspase activation and colon cancer apoptosis (Supplementary Table S1; ref. 38).

K-Ras mutations are relatively frequent and appear in colonic adenomatous polyps and colon cancers of mice and humans (40). Importantly, we observed that knockdown of IMP-1 or its upstream regulator, β -catenin/Tcf, decreased levels of K-Ras protein levels in colon cancer cells. Conversely, IMP-1 expression rescues inhibition of K-Ras by β -catenin siRNA. In addition, we found that IMP-1 binds directly to the coding region and 3'UTR of *K-Ras* mRNA. Furthermore, we also showed that *CYFIP2* knockdown reverts Caspase-3- and PARP-mediated apoptosis, and inhibition of endogenous K-Ras expression due to loss of IMP-1. These findings indicate that K-Ras may be a novel downstream target of IMP-1 signaling and is regulated via direct interaction with *K-Ras*

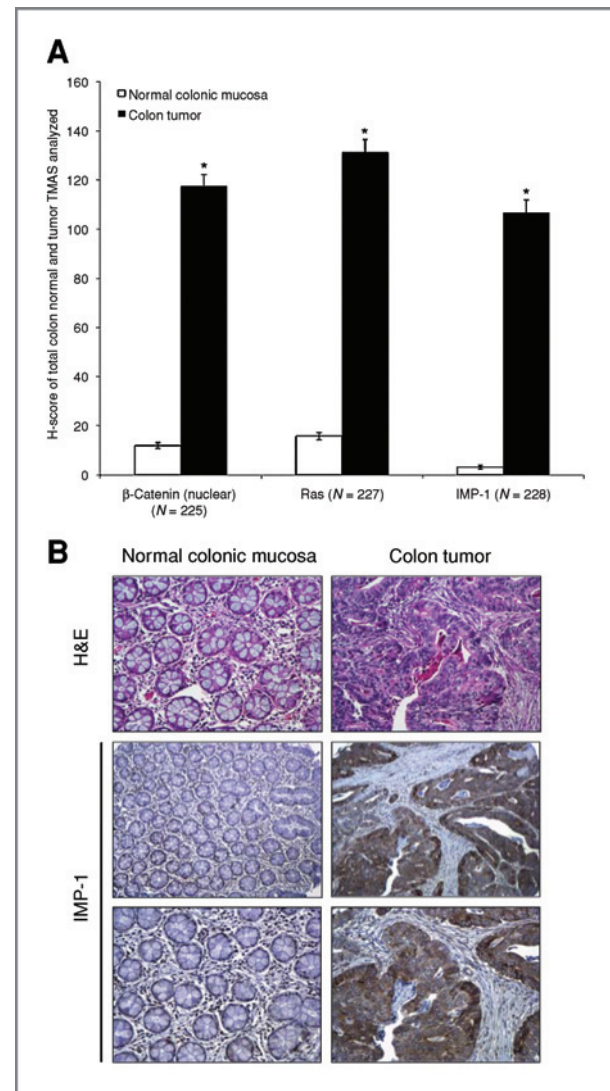


Figure 7. IMP-1 is elevated in human colon cancers. **A**, β -catenin, K-Ras, and IMP-1 IHC staining results of 228 human patient normal colon and tumor TMA samples. Error bars represent \pm SEM. *, $P \leq 0.0001$. **B**, representative image of a human colon tumor specimen with matched normal mucosa stained for IMP-1 as described in Materials and Methods, and accompanying H&E staining ($\times 10$ and $\times 20$ magnifications).

mRNA and the suppression of *CYFIP2*. Interestingly, IMP-1 was upregulated in patient colon tumors and correlated with K-Ras expression, suggesting that gain of IMP-1 maybe a novel mechanism to increase endogenous K-Ras levels.

Overexpression of IMP-1 has been reported in a variety of cancers, suggesting an important role of IMP-1 in cancer development (41–44). Constitutively active β -catenin/Tcf transcription factor upregulates IMP-1 in colon cancer cells, and elevated levels of IMP-1 correlate with β -catenin/Tcf signaling in primary colorectal tumors (24, 28). We found that nuclear β -catenin levels were significantly increased in colon cancers compared with normal colonic mucosa; however, there was no correlation between IMP-1 and β -catenin

expression in tumors, suggesting that IMP-1 dysregulation may occur independently of β -catenin *in vivo*.

In summary, we report that IMP-1 binds to *K-Ras* mRNA and plays an important role in regulating K-Ras expression potentially via repression of the proapoptotic protein, CYFIP2, downstream of β -catenin/Tcf, and promotes colon cancer cell proliferation, anchorage-independent growth, and survival (Supplementary Fig. S2). Furthermore, we show that human colon tumors express high levels of IMP-1 relative to NCE and present new evidence that IMP-1 positively correlates with K-Ras in colon cancers. On the basis of our novel findings, we propose that IMP-1 maybe a candidate for targeted therapeutic intervention in human cancers with dysregulation of K-Ras expression and signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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IMP-1 Displays Cross-Talk with K-Ras and Modulates Colon Cancer Cell Survival through the Novel Proapoptotic Protein CYFIP2

Perry S. Mongroo, Felicite K. Noubissi, Miriam Cuatrecasas, et al.

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