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Targeting RICTOR sensitizes SMAD4-negative colon cancer to irinotecan

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Abstract

Deciphering molecular targets to enhance sensitivity to chemotherapy is becoming a priority for effectively treating cancers. Loss of function mutations of *SMAD4* in colon cancer is associated with metastatic progression and resistance to 5-fluorouracil (5-FU), the most extensively used drug of almost all chemotherapy combinations used in the treatment of metastatic colon cancer. Here, we report that SMAD4 deficiency also confers resistance to irinotecan, another common chemotherapeutic frequently used alone or in combination with 5-FU against colon cancer. Mechanistically, we find that SMAD4 interacts with and inhibits RICTOR, a component of the mTORC2 complex, resulting in suppression of downstream effector phosphorylation of AKT at Serine 473. *In silico* meta-analysis of publicly available gene expression datasets derived from tumors indicates that lower levels of SMAD4 or higher levels of RICTOR/AKT, irrespective of the

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SMAD4 status, correlates with poor survival, suggesting them as strong prognostic biomarkers and targets for therapeutic intervention. Moreover, we find that overexpression of SMAD4 or depletion of RICTOR suppresses AKT signaling and increases sensitivity to irinotecan in SMAD4-deficient colon cancer cells. Consistent with these observations, pharmacological inhibition of AKT sensitizes SMAD4-negative colon cancer cells to irinotecan *in vitro* and *in vivo*. Overall, our study suggests that hyperactivation of the mTORC2 pathway is a therapeutic vulnerability that could be exploited to sensitize SMAD4-negative colon cancer to irinotecan.

Keywords

Colon cancer; SMAD4; RICTOR; mTORC2; 5-FU; irinotecan; MK2206

Introduction:

Despite preventive screening, colon cancer remains as the second most lethal cancer in men and women combined in the United States with more than 50,000 deaths estimated to occur in 2019, mostly attributed to metastasis and resistance to therapy (1). The majority of colon cancer cases are of sporadic origin and surgery has limited therapeutic role in cases with metastatic colon cancer as only 10–15% of patients have resectable lesions (2). 5-fluorouracil (5-FU) is one of the most extensively used drugs in the treatment of metastatic colon cancer and remains as the clinical backbone of almost all chemotherapy combinations. It is often used along with oxaliplatin (L-OHP) or irinotecan as FOLFOX (5-fluorouracil, leucovorin [folinic acid], oxaliplatin) or FOLFIRI (5-fluorouracil, leucovorin [folinic acid], irinotecan) respectively as the standard first-line and second-line chemotherapeutic options available to combat metastatic disease in improving patient survival (3, 4). Intriguingly, screening of biomarkers to predict response to these agents is not implemented and an agent is only removed during subsequent regimens when intolerable toxicity occurs (5). Due to toxicities caused by chemotherapeutic agents, it would be more effective to combine single agents with therapies directed at biological targets to reduce the doses below toxic levels and to enhance sensitivity. Therefore, understanding the molecular basis of metastatic colon cancer will be beneficial to all affected patients in helping to design effective therapeutic strategies.

Loss of heterozygosity (LOH) at chromosome 18q has long been established as a late event during colon cancer progression (6, 7). Furthermore, several studies have suggested that LOH at 18q was an indicator of a poor prognosis in patients with tumors penetrating the bowel wall or involving regional lymph nodes (TNM stages II and III, respectively) who succumbed to disease recurrence and died within 5 years of surgical removal of their primary tumor (8, 9). To identify the target gene(s) for 18q deletions in colon cancer, we found *SMAD4* mutations or genomic deletions of this gene (10). This has been confirmed in numerous follow up studies that a high frequency of LOH at 18q was associated with an increase in the frequency of *SMAD4* mutations, which occur in about 10–30% of colon cancer and correlated to an advanced stage colon cancer (11–13). Furthermore, when tumors corresponding to different stages of colon cancer were interrogated for *SMAD4* inactivation arising from deletions or point mutations, there was a strong correlation between increasing

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frequency of *SMAD4* gene mutations and distant metastases (stage IV) relative to non-metastatic colon cancer (14, 15). A strong correlation between loss of SMAD4 expression and liver metastasis with poor prognosis in colon cancers (the most common site for colon cancer metastases) has also been established from the examination of primary tumors and the corresponding metastatic tissues (15–17). In addition to colon cancer, a tumor suppressive role corresponding to mutations, deletions and low levels of SMAD4 has been associated with poor prognosis in several other cancers (18–22).

Moreover, credence to the contribution of *SMAD4* defect in forming metastatic colon cancer was also derived from mouse models where a dramatic increase in malignant progression of intestinal polyps in *cis*-compound heterozygotes (i.e., *Apc* (+/−) *Smad4* (+/−) compared to the simple *Apc* (+/−) heterozygotes) was observed (23). Subsequently, inactivation of *SMAD4* in organoid models was crucial in showing tumor progression to the malignant and invasive stages of colon cancer (24). *In vitro* and xenotransplantation studies further supported the tumor suppressive function of SMAD4, whereupon removal it promotes malignant phenotypes including cell migration, tumorigenesis, angiogenesis, aerobic glycolysis, and metastasis (25, 26). Clinically, loss of or low SMAD4 expression correlated with the presence of metastases and has been associated with poor response to 5-FU and worse survival post-5-FU treatment (27, 28). While studies have shown that SMAD4-negative colon cancer is more resistant to 5-FU, whether and how *SMAD4* inactivation confers resistance to standard chemotherapeutic regimens, such as FOLFIRI and FOLFOX, and strategies to enhance sensitivity to these therapeutic strategies remain elusive.

Here, we report that among the three established commonly used active chemotherapeutic agents that constitute the common therapeutic regimens to treat colon cancer, SMAD4-negative colon cancer cells exhibit resistance to both 5-FU and irinotecan but not to oxaliplatin. Furthermore, we found that inactivation of *SMAD4* leads to overactivation of the mTORC2 pathway, thereby augmenting AKT signaling and resistance to irinotecan-mediated apoptosis. Consistent with these observations, targeting the mTORC2 pathway with SMAD4 overexpression, RICTOR depletion, or inhibition of the downstream effector, AKT, with MK2206 restores sensitivity of SMAD4-negative colon cancer cells to irinotecan.

Materials and methods:

Cell culture

HCT116 *SMAD4+/+* and *SMAD4−/−* isogenic cell lines are a generous gift from Dr Bert Vogelstein, while SW403, ASPC1, and CFPAC1 cells were obtained from ATCC. HCT116 and SW403 cells were cultured in McCoy's medium. ASPC1 cells were maintained in RPMI medium, while CFPAC1 cells were cultured in IMDM. All cell lines were maintained in the presence of 10% FBS and 1% penicillin/streptomycin in a 37°C incubator with 5% CO_2.

Co-immunoprecipitation

Cells were washed with PBS and incubated with 1mM dithiobis succinimidyl propionate (DSP) at room temperature for 30 min. The crosslinking reaction was quenched using

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10mM Tris for 15 min. Cells were then washed and lysed with Pierce IP buffer in the presence of protease and phosphatase inhibitors (Roche). Protein lysates were scraped from the dish using cell lifters and centrifuged for 15 min at 14 000g at 4°C. Anti-FLAG affinity gel (Sigma-Aldrich) was washed three times with Pierce IP buffer and mixed with cell lysate overnight at 4°C. The beads were then washed 3 times with Pierce IP buffer to remove unbound proteins. The beads were incubated with 3xFLAG peptide (Sigma-Aldrich) for 1 h at room temperature to elute bound proteins.

Mass spectrometry analysis

Eluate from co-immunoprecipitation was mixed with 4xLDS buffer and 10x reducing agent before being loaded onto NuPAGE 4–12% pre-cast gels and separated for 15 min at 100V. The gel area with trapped eluate was then excised and digested with trypsin. The digested samples were then analyzed with LC/MS/MS and subjected to Mascot database search for protein identification. Protein candidates detected as background in more than 10 experiments among the total 411 experiments curated by the CRAPome database were first filtered (48). Next, protein candidates that were enriched more than 5-fold based on spectral counts in the sample compared to control were then uploaded to Ingenuity Pathway Analysis for direct protein-protein interaction analysis to determine if they form any protein complexes.

Kaplan-Meier analysis

A database of colon cancer patients was established as described previously (49). Survival curves were generated based on the transcript level of a candidate gene using the Cox proportional hazards regression analysis and plotting Kaplan-Meier plots as described (30). A *p*-value below 0.05 was accepted as a significant correlation between gene expression and survival.

Tumor xenograft studies

The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all animal experiments. Six-week old female athymic nude mice (Nu/Nu) were purchased from Envigo and housed in a sterile environment with microisolator cages. The mice were subcutaneously injected with 2.5×10^6 HCT116 *SMAD4−/−* cells in 30% growth factor-reduced Matrigel (Corning). When the tumors reached around 5mm in diameter, the mice were exposed to vehicle, MK2206 (MedChemExpress) alone, irinotecan (MedChemExpress) alone, or a combination of MK2206 and irinotecan. MK2206 (360mg/kg) in 30% Captisol was administered on days 1, 8, 15, and 22 via oral gavage. Irinotecan (20mg/kg) was administered on days 1, 8, 15, and 22 via intraperitoneal injections. Tumor volume was determined using $(L \times W^2)/2$, where L represents length and W represents width.

Statistical analysis

For two group comparisons, Student's *t*-test (two-tailed, type two) was applied. Significance of multiple condition experiments was determined using one-way ANOVA. A *p*-value below

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0.05 was considered statistically significant. All data shown in in the bar graphs are the mean ± SD of at least three biological replicates. Error bars represent standard deviation.

Please refer to supplemental file for additional materials and methods.

Results:

SMAD4-negative colon cancer cells exhibit higher migratory ability and resistance to 5-fluorouracil and irinotecan

To determine whether SMAD4 suppresses cancer progression in our colon cancer model, we first compared the migratory ability of a pair of isogenic SMAD4-positive and negative HCT116 cells. While the *SMAD4* gene was knocked out using targeted homologous integration, *TGFBRII* was restored to reconstitute intact TGFβ signaling in HCT116 cells as previously described (25, 29). As expected, SMAD4-negative cells exhibited higher migratory potential than SMAD4-positive cells, which was in concurrence with previous reports that *SMAD4* inactivation promoted malignant progression of colon adenoma to carcinoma (Supp. Fig. S1) (24). To assess whether *SMAD4* expression level could serve as a prognostic biomarker in colon cancer patients, we performed *in silico* Kaplan-Meier analyses and found that higher levels of *SMAD4* associated with increased probability of overall survival (OS; HR = 0.62, $p < 0.05$), relapse-free survival (RFS; HR = 0.75, $p < 0.015$), and post-progression survival (PPS; HR = 0.42, $p = 0.05$) decreased significantly in patients with low levels of *SMAD4* expression (30). Overall, these findings provide additional credence in support of the role of SMAD4 as a tumor suppressor gene (Fig. 1A).

Next, to test whether *SMAD4* inactivation confers resistance to common chemotherapeutic agents used for treating colon cancer, we exposed the colon cancer model cells to increasing concentrations of 5-FU, irinotecan, or oxaliplatin. Interestingly, compared to SMAD4-positive, SMAD4-negative cells exhibited significant resistance to 5-FU (IC_{50} = 1.78μM versus 4.7μM) and irinotecan (IC_{50} = 0.73μM versus 6.5μM), but not to oxaliplatin (IC_{50} for both at around 0.37μM) (Fig. 1B and C). While resistance to 5-FU has been previously reported by others and us (25–28), here we also found that there was significant increase in viability of SMAD4-negative compared to SMAD4-positive cells upon exposure to irinotecan.

Mass spectrometry reveals RICTOR as a novel SMAD4 interacting protein in colon cancer cells

Several reports have indicated that overexpression of hypoxia-inducible factor 1-alpha (HIF-1α) is associated with poor prognosis in colon cancer patients (31–33). Our previous studies found that one of the mechanisms for the tumor suppressive role of SMAD4 in colon cancer is due to its interaction with and inhibition of tumor promoting transcriptional activation mediated by HIF-1α to suppress its target gene, VEGF, which promotes angiogenesis (25). These observations suggested that SMAD4 could similarly interact with other transcription factors or tumor promoting pathway factors to suppress oncogenic events including metastasis and drug resistance. To dissect the SMAD4 interactome, we elected to use FLAG-tagged SMAD4 to capture proteins that interact to form complexes in colon

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cancer cells. We constructed a FLAG-SMAD4 overexpression plasmid and confirmed that the FLAG-SMAD4 protein was functional based on the ability to induce expression of the luciferase reporter gene downstream of a SMAD-binding element (SBE4) in response to TGFβ treatment (Fig. 2A and B). Next, co-immunoprecipitation (co-IP) of the FLAG-SMAD4 with other protein factors was performed and followed by mass spectrometry (MS) to identify the protein components of the complex. These analyses revealed 1200 protein hits, which were subjected to CRAPome, spectral count enrichment, and Ingenuity Pathway analyses (Fig. 2C, Suppl. Table 1). Interestingly, three members of the mTORC2 complex, mTOR, RICTOR, and TELO2, were among the proteins bound by SMAD4 (34, 35). Therefore, we predicted that these interactions could be of functional relevance and decided to focus on the novel interaction between SMAD4 and RICTOR, where the latter is a unique component of the mTORC2 complex making it as a potential precision therapeutic target for colon cancer (Fig. 2C) (36).

Because mTORC2 phosphorylation of oncoprotein AKT at serine 473 activates the downstream events of the mTORC2 pathway to promote cell survival, we predicted that it could be a major mediator of chemoresistance and decided to undertake functional characterization of RICTOR as a potential target for therapeutic intervention in SMAD4-negative colon cancer (36, 37). First, we followed up with MS data and confirmed that SMAD4 interacted with RICTOR *in vitro* (Fig. 2D). Next, to evaluate if the interaction between SMAD4 and RICTOR is dependent upon TGFβ mediated downstream effects, we performed Western blot analysis of the SMAD4 protein complexes formed in the presence or absence of TGFβ. Interestingly, this interaction appeared to be independent of TGFβ stimulation, indicating that SMAD4 may have other non-canonical roles in suppressing colon cancer progression that are not dependent upon active TGFβ signaling (Fig. 2E).

SMAD4-negative colon cancer exhibits hyperactivation of mTORC2 pathway

To better understand whether mTORC2 is a major contributor to chemoresistance and could thereby serve as a precision therapeutic target in SMAD4-negative colon cancer, we first assessed the activation status of mTORC2 pathway by examining the level of phospho-AKT^{S473}, a downstream oncogenic target activated by mTORC2. We found that SMAD4-negative cells displayed pronounced phospho-AKT^{S473} levels compared to SMAD4-positive cells, consistent with the notion that SMAD4 may play a role in suppressing this pathway (Fig. 3A) (26). In line with these observations, SMAD4-negative cells were highly sensitive to AKT inhibition, indicated by significantly reduced viability upon treatment with MK2206, an allosteric AKT inhibitor (Fig. 3B and C).

Next, to elucidate if restoration of SMAD4 could be directly involved in inhibiting RICTOR-mediated downstream signaling, we assessed the level of phospho-AKT^{S473} in colon cancer cells overexpressing FLAG-SMAD4. We found that SMAD4 overexpression resulted in suppressed levels of phospho-AKT^{S473}, which serves as a functional readout of mTORC2 pathway activation (Fig. 3D). Importantly, SMAD4 overexpression resulted in enhanced sensitivity of the colon cancer cells to irinotecan, with a corresponding increase in the levels of the apoptotic marker, cleaved caspase 3 (Fig. 3E and F). Overall, these data

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suggested that *SMAD4* deletion in colon cancer might lead to uninhibited mTORC2/AKT signaling activity, thereby promoting resistance to irinotecan-mediated apoptosis.

Depletion of RICTOR suppresses AKT signaling activity and increases sensitivity of SMAD4-negative colon cancer cells to irinotecan

To determine whether RICTOR is critical to mTORC2 functionality in SMAD4-negative colon cancer, we knocked down *RICTOR* and assessed the expression level of phospho-AKT^{S473} in SMAD4-negative HCT116 cells using two different shRNAs (Fig. 4A and Supp. Fig. S2) (34). We noticed a dramatic decrease in phospho-AKT^{S473} level upon RICTOR depletion, confirming that RICTOR is an essential component to the kinase function of mTORC2 required for activating AKT signaling pathway in our model system (Fig. 4B and Supp. Fig. S2). In addition, we observed that knockdown of *RICTOR* increased sensitivity of SMAD4-negative colon cancer cells to irinotecan (Fig. 4C and Supp. Fig. S2). Corresponding to RICTOR and phospho-AKT^{S473} depletion, the cells also displayed higher levels of cleaved caspase 3 in response to irinotecan treatment, suggesting that mTORC2/AKT signaling drives resistance to irinotecan by blocking apoptosis (Fig. 4D and Supp. Fig. S2). Interestingly, depletion of RICTOR in SMAD4-negative HCT116 cells also impaired their migratory ability, suggesting that RICTOR could serve as a potential therapeutic target to suppress colon cancer progression (Supp. Fig S3). To further support the universality of the phenomenon that mTORC2 activation correlated with SMAD4 deficiency in colon cancer, we examined an additional SMAD4-negative SW403 cell line and observed a decrease in pAKT^{S473} levels and sensitization of the cells to irinotecan upon depletion of RICTOR using siRNA (Supp. Fig. S4).

Despite the correlation between functional activation of mTORC2 pathway and malignant progression of colon cancer in SMAD4-negative cells, we wondered if overall levels of *RICTOR* or *AKT1* could predict survival differences in all patients with colon cancer irrespective of their *SMAD4* status. Interestingly, we found that higher levels of *RICTOR* associated with decreased OS (HR = 1.72, $p < 0.01$), RFS (HR = 1.34, $p < 0.05$), and PPS (HR = 3.02, $p < 0.01$) in colon cancer patients (Supp. Fig. S5). Similarly, we found that higher levels of *AKT1* corresponded with decreased OS (HR = 1.84, $p < 0.01$), RFS (HR =1 .56, $p < 0.01$), and PPS (HR = 1.8, $p < 0.05$) in colon cancer patients (Supp. Fig. S5). Overall, these observations suggest that higher levels of *RICTOR* or *AKT1* could predict worse prognosis of colon cancer patients.

Targeting AKT with MK2206 sensitizes *SMAD4*-negative colon cancer cells to irinotecan

Since there are no drugs currently available to specifically target RICTOR and mTORC2 (38), we hypothesized that inhibiting their downstream effector target AKT could restore sensitivity of SMAD4-negative colon cancer cells to irinotecan. Unlike the mTOR inhibitor sirolimus, the addition of MK2206 was able to drastically deplete the level of phospho-AKT^{S473} in a manner similar to RICTOR knockdown (Fig. 4B and 5A) (39). These observations are highly consistent with the notion that mTORC2 pathway activation is the primary mediator of AKT^{S473} phosphorylation in these cells. Interestingly, prolonged exposure to sirolimus did not affect the levels of phospho-AKT^{S473} in these cells, suggesting that mTORC2 is rapamycin-insensitive in our model (Fig. 5A). Subsequently, we found that

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the combination of MK2206 and irinotecan was able to suppress the viability of SMAD4-negative HCT116 cells two-fold more effectively *in vitro* than irinotecan alone, on par with the sensitivity observed in SMAD4-positive cells treated with irinotecan alone (Fig. 5B). The addition of MK2206 also induced higher levels of cleaved caspase 3 in the presence of irinotecan, further supporting that AKT activation is a major driver of resistance to irinotecan-mediated apoptosis (Fig. 5C). To ensure that this phenomenon is not cell line-specific, we also examined the SMAD4-negative SW403 colon cancer cells and found that the cells exhibited enhanced suppression of viability upon combination treatment with a corresponding increase in cleaved caspase 3 levels (Supp. Fig. S6). Furthermore, the use of a different allosteric AKT inhibitor (40), AKTi-1/2 in combination with irinotecan also exhibited enhanced sensitivity of SMAD4-negative cells *in vitro*, suggesting targeted inhibition of AKT is the common phenomenon responsible for the additive therapeutic effect (Supp. Figure S7).

Having demonstrated the effects of drug treatment *in vitro*, we next examined the efficacy of the anti-tumor activities of combination therapy *in vivo* using nude mice harboring HCT116 *SMAD4–/–* xenograft tumors. The tumor bearing mice were randomized to receive vehicle, MK2206, irinotecan, or a combination of MK2206 and irinotecan. Compared to single agents, we found that the combinatorial treatment with chemotherapeutic agent irinotecan and targeted inhibition of the mTORC2 pathway was the most effective in remarkable tumor growth suppression *in vivo* (Fig. 6A–B). Interestingly, the more dramatic tumor suppression with combination treatment was not associated with increase in host toxicity (Supp. Figure S8).

Discussion:

Despite the use of irinotecan-based (FOLFIRI) and oxaliplatin-based (FOLFOX) chemotherapy consisting of the 5-FU backbone to significantly improve survival in metastatic colon cancer, 5-year OS of patients remains at 5–15% and resistance to chemotherapy is mounting. As such, it warrants the development of new strategies to combat this disease a priority. At this time, an exponential increase in our knowledge-base on the identity of genetic alterations in colon and other cancers is still unable to deliver precision medicine as it has lagged due to the difficulties in pinpointing biological targets that become functionally active in the various tumor types. *SMAD4* mutation or loss of expression, which occurs frequently in late-stage colon cancer (10–13), correlates with poor OS, RFS, and PPS (Fig. 1A). Interestingly, several studies reported that loss of SMAD4 functionality corresponded to resistance to 5-FU in the clinic, a standard first-line treatment for the disease (27, 28). Although 5-FU resistance has been associated with SMAD4-defective colon cancers, the applicability of this resistance phenomenon to other standard chemotherapeutics, such as oxaliplatin and irinotecan as well as potential biological targets for inhibition to enhance the therapeutic benefit, has remained elusive. Here, we report that while SMAD4-negative colon cancer exhibits resistance to both 5-FU and irinotecan, sensitivity to oxaliplatin is unaffected by the *SMAD4* status.

On the contrary to blinded use of inhibitors to common oncogenic signaling pathways, such as MEK-ERK, p38-MAPK, and PI3K/AKT alone or in combination with chemotherapy

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with uncertain therapeutic benefit for colon cancer patients, here we present an attempt to identify specific biological targets to sensitize chemoresistant SMAD4-negative colon cancer to reap the maximum benefit with minimal side effects (25, 26). Previously, our group reported that SMAD4 interacts with HIF-1α to suppress the expression of *VEGF*, a well-established HIF-1α target gene that promotes angiogenesis (25). Based on these observations, we hypothesized that SMAD4 may also act by inhibiting other critical protein factors involved in conferring resistance to chemotherapeutic agents such as irinotecan. Mass spectrometry analysis revealed candidate proteins bound by SMAD4, including mTOR and TELO2, both of which are common in mTORC1 and mTORC2, as well as RICTOR, which is an essential constituent of mTORC2, a protein complex that primarily phosphorylates and fully activates the oncogene AKT at Serine 473 (34). Because the activation of AKT at Serine 473 has been shown to promote colon cancer cell migration and antagonizes apoptosis (36, 37), we decided to characterize the role of RICTOR, which is unique to mTORC2, in SMAD4-negative colon cancer (36). Indeed, we found that RICTOR depletion not only impairs AKT signaling and cell migration but also sensitizes the cells to irinotecan-mediated cell death. Interestingly, Kaplan-Meier analyses also revealed that high *RICTOR/AKT1* expression, independent of the *SMAD4* status, significantly correlated with worse OS, RFS and PPS in colon cancer patients, indicating the roles of these two genes in promoting disease progression and thus could serve as potential therapeutic targets for colon cancer in general (Supp. Fig. S5).

Currently, there are no drugs that specifically and effectively target RICTOR or mTORC2 with precision (37, 38, 41, 42). Therefore, to inhibit mTORC2 signaling activity in SMAD4-negative colon cancer in our proof of principle experiments, we opted to block its downstream effector target AKT using MK2206, a commercially available, most clinically advanced and well-tolerated allosteric inhibitor of AKT, which blocks S473 phosphorylation of AKT, the primary target of mTORC2 pathway (39). We found that SMAD4-negative colon cancer cells are more sensitive to MK2206 treatment compared to SMAD4-positive cells, and that MK2206 can further suppress the growth of SMAD4-negative cells in the presence of irinotecan (Fig. 5B). Importantly, the additive effect of these two drugs resulted in increased apoptosis of the treated SMAD4-negative cells *in vitro* (Fig. 5C). The suppression of tumor growth using the combination therapy was also confirmed in xenograft models derived from SMAD4-negative colon cancer cells and it was not associated with increase in host toxicity (Supp. Figure S8).

Interestingly, we also found that the use of MK2206 with irinotecan could also significantly enhance suppression of the viability of SMAD4-negative pancreatic cancer cell lines (ASPC1, CFPAC1) displaying active AKT signaling *in vitro*, suggesting that the combination therapy could be of general applicability for cancers exhibiting loss of SMAD4 (Supp. Fig S9). Additionally, we also noted that high *RICTOR* or *AKT1* expression corresponded significantly to poor overall survival (OS) in pancreatic cancer patients, indicating these as potential therapeutic targets for the affected individuals (Supp. Fig S10).

The Cancer Genome Atlas (TCGA) data has indicated that KRAS mutations frequently occurs with SMAD4 defects in metastatic colon cancers (43). These observations are consistent with previous studies that correlated SMAD4 defects to advanced stages of colon

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cancer and as such the probability of simultaneously finding KRAS mutations and SMAD4 alterations in these tumors is very high (10, 25 *and references therein*). On the other hand, clinical benefit for targeting epidermal growth factor receptor (EGFR) with the use of humanized monoclonal antibodies, such as cetuximab or panitumumab, has been restricted to patients with wild-type KRAS metastatic colon cancers (44). Therefore, our finding of mTORC2 pathway activation (i.e., AKT activation) with SMAD4 loss of function provides a rationale for RICTOR/AKT as potential precision therapeutic targets in colon cancers with low levels of SMAD4 with activated EGFR. Further credence to this notion is also derived from the recent finding that patients carrying SMAD4 mutations had a higher possibility of a less effective response to EGFR blockade with a shorter progression-free survival (45). Thus, targeting mTORC2 pathway activation as suggested from our studies is likely to be beneficial to patients exhibiting poor response to therapy using antibody therapy targeting EGFR and clinical trials in the future are required to take advantage of these findings.

In conclusion, our observations suggest that overactivation of the mTORC2 pathway, which has been associated with poor survival in a growing number of cancers (46–47), may be the driver of metastatic cancer progression and resistance to apoptosis induced by chemotherapeutic agents. We report here for the first time that SMAD4 interacts with RICTOR to suppress mTORC2 functionality and therefore the loss of SMAD4 function results in oncogenic activation of the mTORC2 pathway, leading to enhancement in malignant colon cancer progression and resistance to chemotherapeutic agents such as irinotecan (Fig. 6C). Thus, inactivation of AKT^{S473} phosphorylation or more specifically its upstream regulator, RICTOR, emerged as legitimate strategies to enhance the sensitivity of SMAD4-negative colon cancer cells to irinotecan as shown in our studies. Interestingly, our studies also found that overexpression of *RICTOR* or *AKT1* could serve as biomarkers for poor prognosis, independently of the *SMAD4* status. Overall, we suggest that design of therapies involving established chemotherapeutic agents such as irinotecan might be highly effective when combined with targeted inhibitors for RICTOR/AKT when the colon cancer cells are either SMAD4-negative or exhibit overexpression of RICTOR/AKT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications:

Hyperactivation of the mTORC2 pathway in SMAD4-negative colon cancer provides a mechanistic rationale for targeted inhibition of mTORC2 or AKT as a distinctive combinatorial therapeutic opportunity with chemotherapy for colon cancer.

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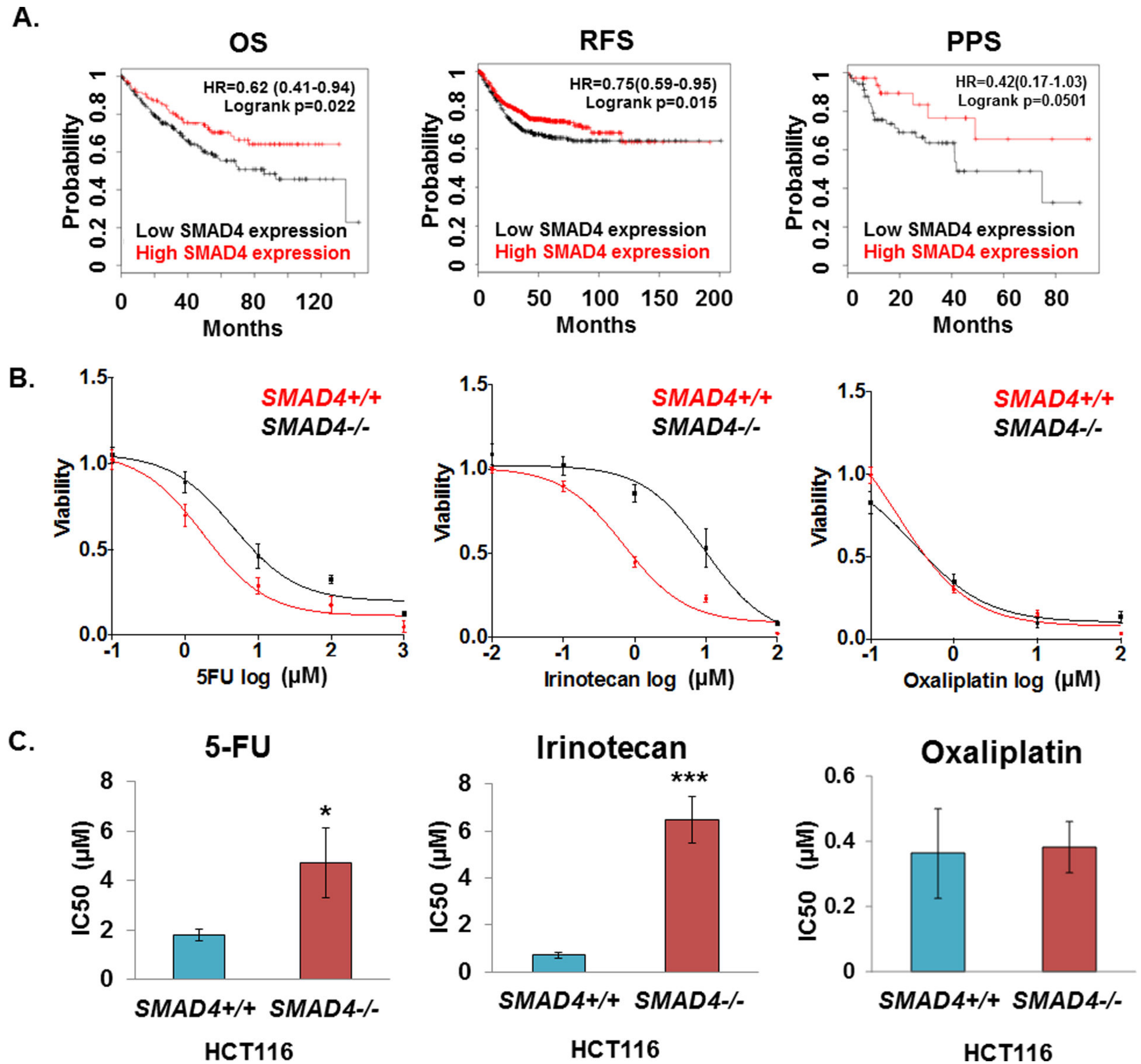

Figure 1: SMAD4-negative colon cancer cells are resistant to 5-FU and irinotecan but not to oxaliplatin.

A, *In silico* Kaplan-Meier analyses showing the correlation between *SMAD4* expression and overall survival (OS), relapse-free survival (RFS), and post-progression survival (PPS) in colon cancer patients. The analyses ran on a cohort of 304 (OS), 1045 (RFS), and 105 (PPS) patients, respectively. **B,** *SMAD4*+/+ and *SMAD4*–/– cells were treated with 5-FU, irinotecan, or oxaliplatin, and the viability of cells relative to DMSO-treated controls was determined after 72 hours (cell viability assay; mean ± SD, n = 3 biological replicates). **C,** The concentration at which 50% of growth was inhibited (IC50) was calculated using Prism for each drug (IC50 analysis; mean ± SD, n = 3; *$P < 0.05$, ***$P < 0.001$).

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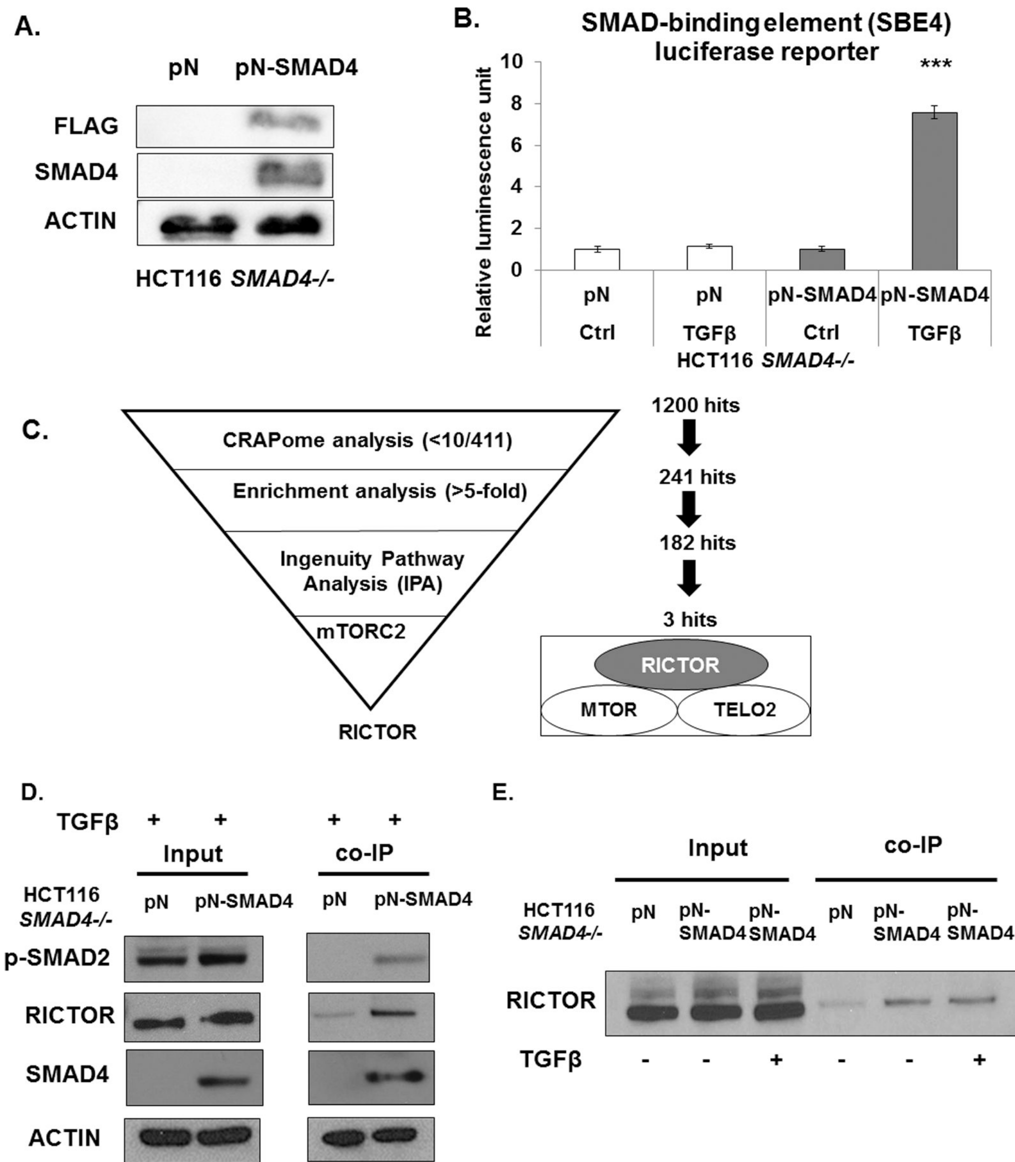

Figure 2: Mass spectrometry analysis reveals novel SMAD4-interacting partners.

A, Overexpression of FLAG-tagged SMAD4 in *SMAD4*–/– cells was monitored using western blotting. **B,** Cells were transfected with SBE4-luciferase reporter plasmid for 72 hours, serum-starved overnight, and treated with 5ng/ml of TGFβ for 4 hours prior to lysis (RLU, relative luminescence unit; mean ± SD, n = 3 biological replicates; ***$P < 0.001$). **C,** FLAG-SMAD4 protein complexes were immunoprecipitated from cell lysates and identified using mass spectrometry and Mascot database search. Schematic shows our strategy for the selection of RICTOR, which is part of mTORC2, as the top candidate targeted by SMAD4. **D,** The presence of phospho-SMAD2, a known SMAD4-interacting protein, and RICTOR, a novel SMAD4-interacting candidate, was determined in FLAG-SMAD4 complexes using western blotting. β-Actin level was used as the loading control. **E,** Colon cancer cells were serum-starved and treated with or without 5ng/ml of TGFβ prior to cell lysis and co-

immunoprecipitation. Western blotting shows the relative levels of RICTOR in FLAG-SMAD4 complexes with or without TGFβ treatment.

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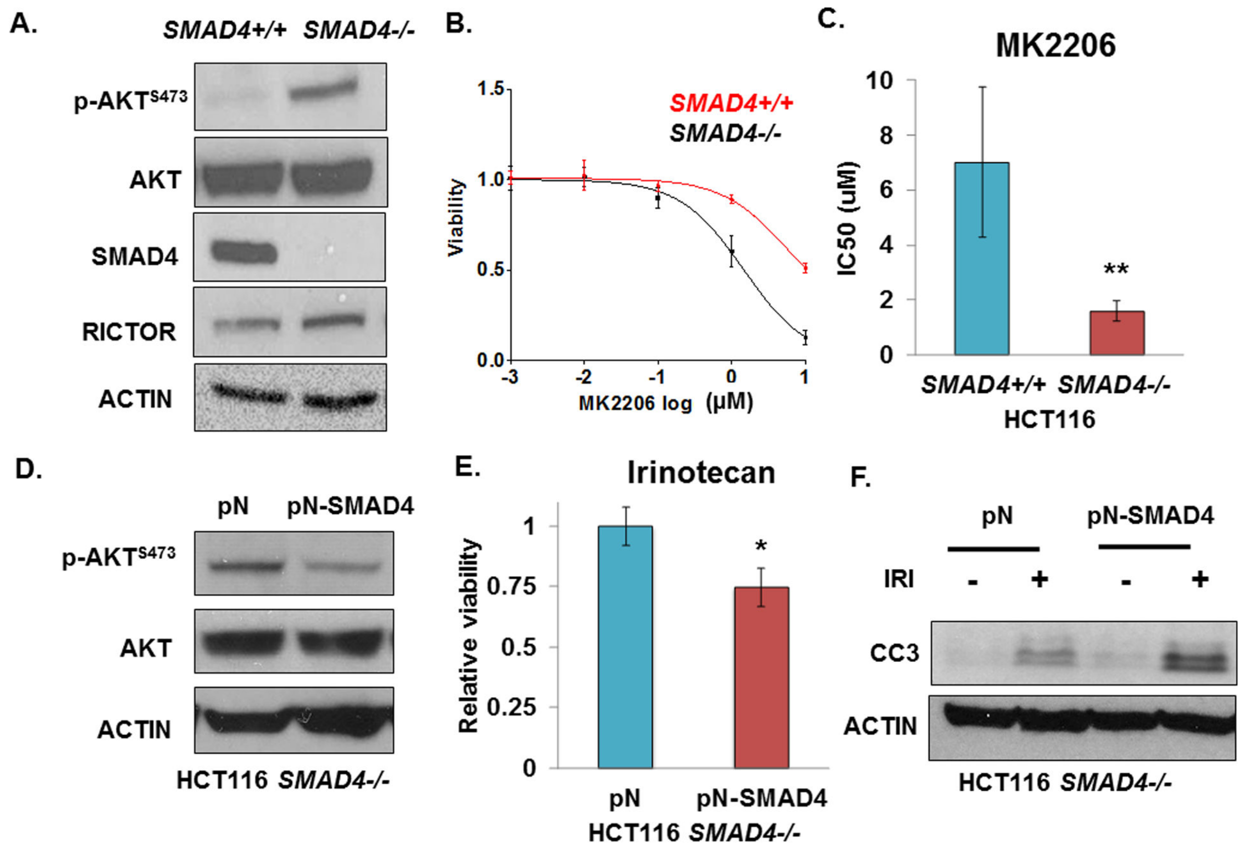

Figure 3: SMAD4-negative colon cancer cells exhibit hyper AKT signaling activity and sensitivity to MK2206.

A, Western blotting shows the relative levels of phospho-AKT^{S473} in *SMAD4*+/+ and *SMAD4*–/– cells. **B,** *SMAD4*+/+ and *SMAD4*–/– cells were treated with MK2206, an allosteric AKT inhibitor, and the viability of cells relative to DMSO-treated controls was determined after 72 hours. **C,** The IC50 of MK2206 in each cell line was calculated using Prism (IC50 analysis; mean ± SD, n = 3 biological replicates; $^{**}P < 0.01$). **D,** Western blotting shows the relative levels of p-AKT^{S473} in cells overexpressing SMAD4. **E,** The indicated cell lines were treated with irinotecan (10μM) for 72 hours. Viability of cells was normalized to DMSO-treated controls (cell viability assay; mean ± SD, n = 3 biological replicates; $^{*}P < 0.05$). **F**, Western blotting shows the relative levels of cleaved caspase 3 in the indicated cell lines after treatment with irinotecan (50μM) for 18 hours.

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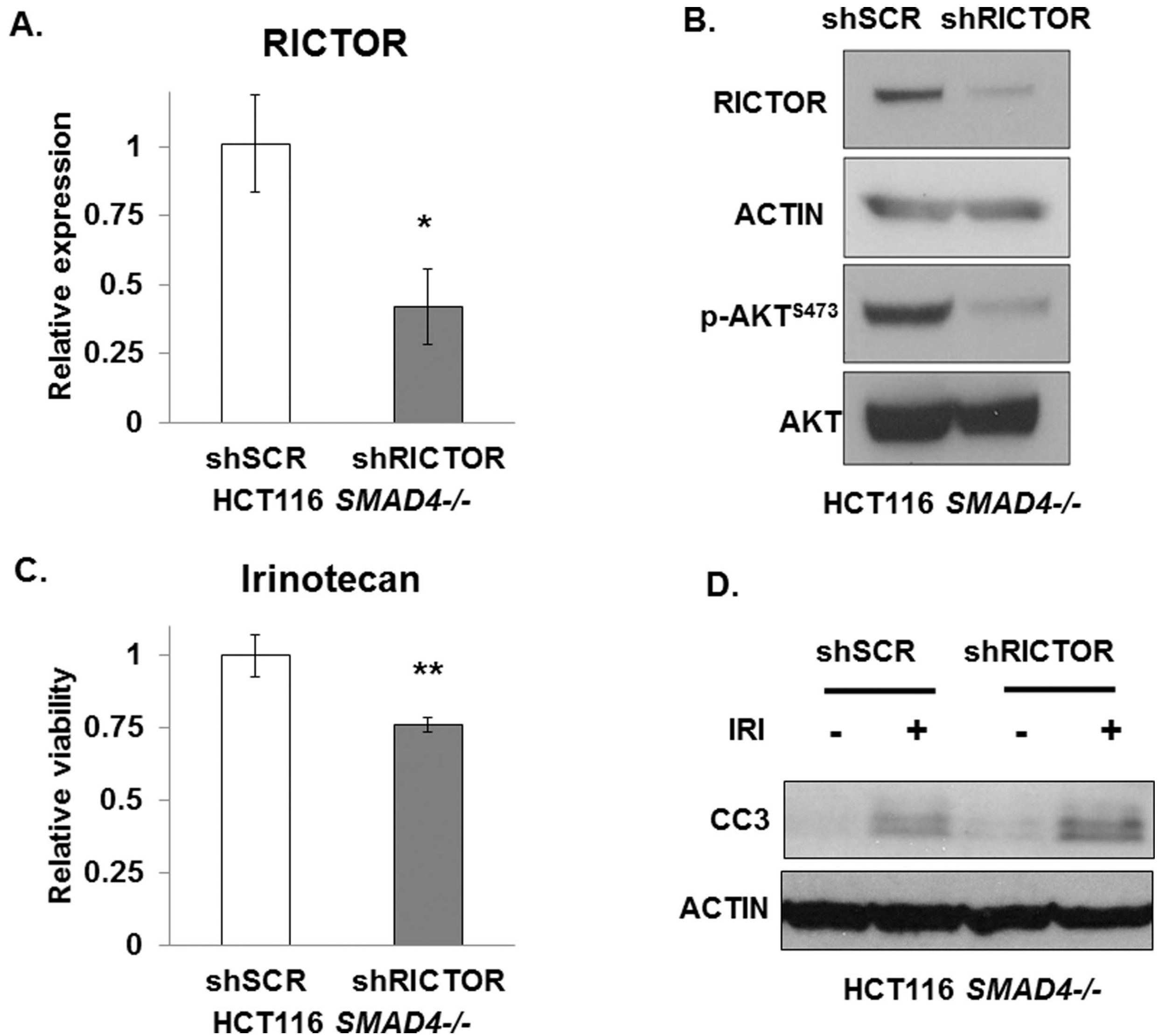

Figure 4: Knockdown of RICTOR suppresses AKT signaling and enhances sensitivity of SMAD4-negative colon cancer cells to irinotecan.

A, RICTOR depletion in SMAD4-negative colon cancer cells using shRNA was measured by RT-qPCR (RT-qPCR; mean ± SD, n = 3 technical replicates; ***$P < 0.001$). **B,** Western blotting shows the relative levels of RICTOR and p-AKT^{S473} in the indicated cell lines. **C,** The indicated cell lines were treated with irinotecan (10μM) for 72 hours. Viability of cells was normalized to DMSO-treated controls (cell viability assay; mean ± SD, n = 3; **$P < 0.01$). **D**, Western blotting shows the relative levels of cleaved caspase 3 in the indicated cell lines after treatment with irinotecan (50μM) for 18 hours.

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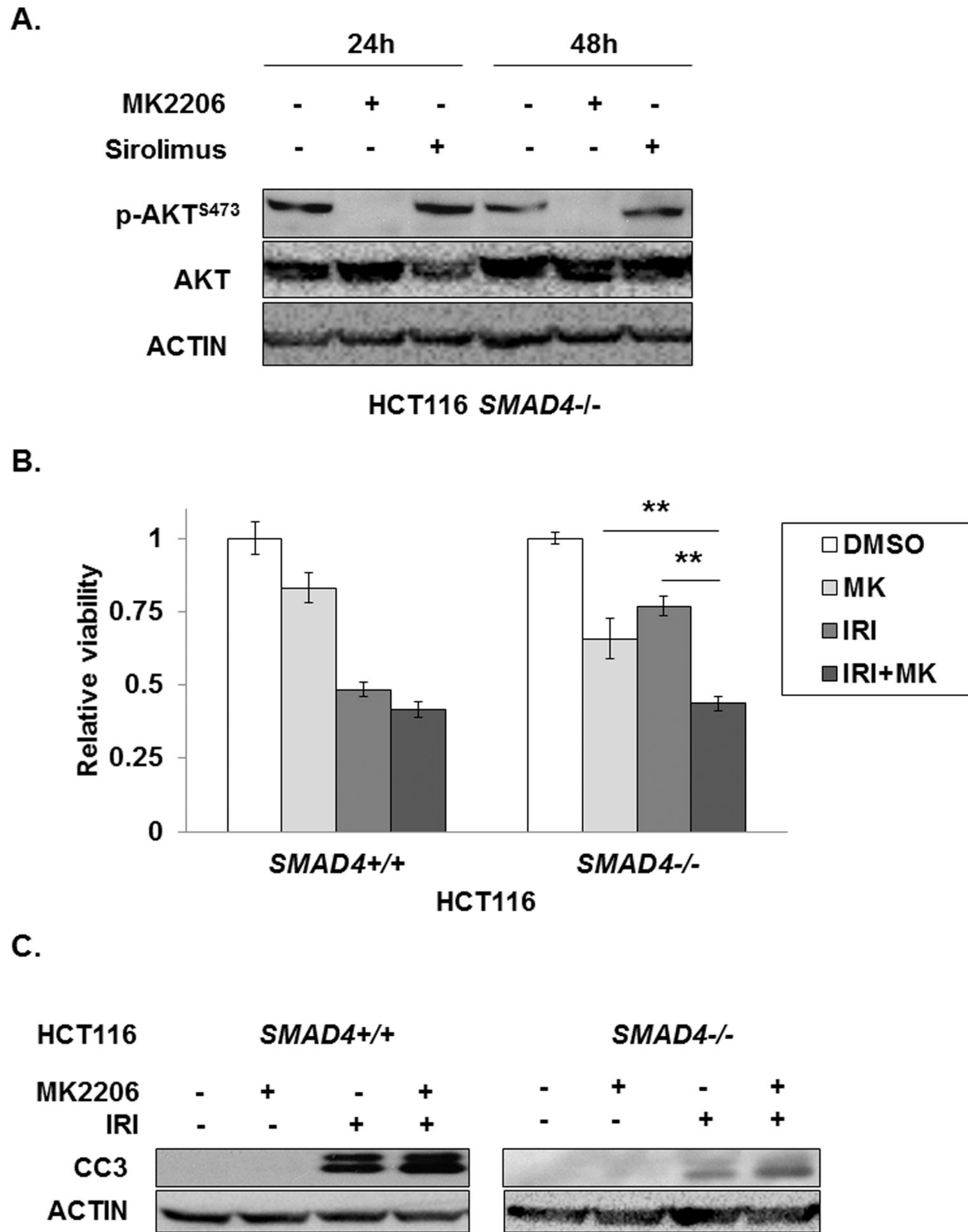

Figure 5: Targeting AKT with MK2206 suppresses p-AKT^{S473} level and sensitizes SMAD4-negative colon cancer cells to irinotecan.

A, Western blotting shows the relative levels of p-AKT^{S473} in SMAD4-negative cells after treatment with MK2206 (1μM) or of sirolimus (10μM), an mTOR inhibitor, for 24 and 48 hours. **B**, SMAD4-positive and negative cells were treated with MK2206 (1μM), irinotecan (1μM), or both for 72 hours (cell viability assay; mean ± SD, n = 3 biological replicates; ***P* < 0.01). **C,** The levels of cleaved caspase 3 were determined in SMAD4-positive and negative cells after treatment with MK2206 (1μM), irinotecan (50μM), or both for 18 hours.

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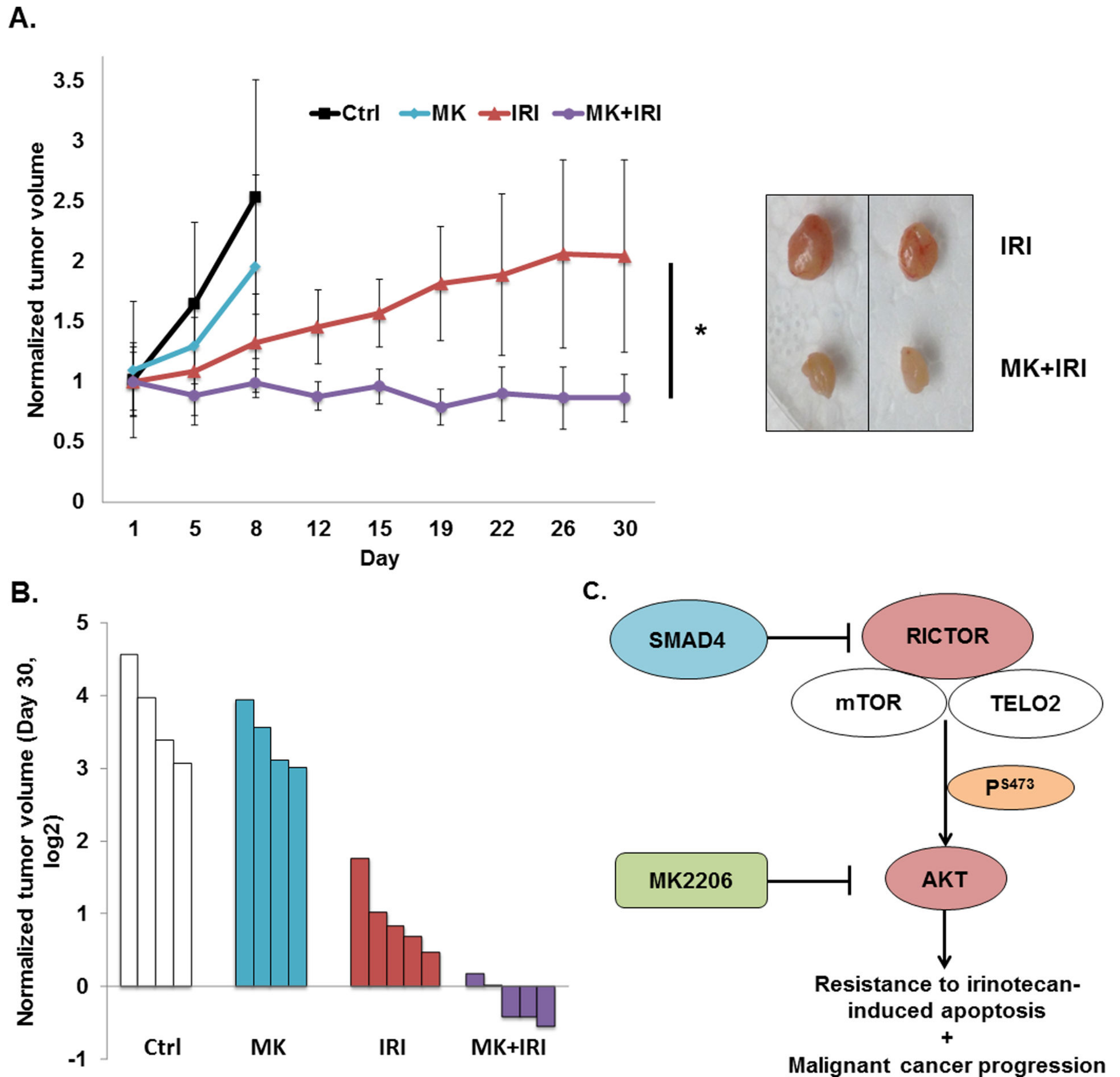

Figure 6: Combination treatment with MK2206 suppresses growth of SMAD4-negative xenografts.

A, HCT116 *SMAD4–/–* xenografts in nude mice were treated with vehicle (n = 4 biological replicates), MK2206 (360mg/kg, n = 4 biological replicates), irinotecan (20mg/kg, n = 5 biological replicates), or a combination of MK2206 and irinotecan (n = 5 biological replicates). Tumors were monitored twice a week using a caliper (relative tumor volume; mean ± SD, $*P < 0.05$ by ANOVA). Representative images of tumors at the end of the experiment are shown. **B,** Waterfall plot shows the relative volume of tumors between treatment arms on day 30. **C,** Our working model shows that *SMAD4* inactivation leads to uninhibited mTORC2/AKT signaling activity and resistance to irinotecan-mediated apoptosis.