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KSR1 and EPHB4 Regulate Myc and PGC1β To Promote Survival of Human Colon Tumors

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Identification and characterization of survival pathways active in tumor cells but absent in normal tissues provide opportunities to develop effective anticancer therapies with reduced toxicity to the patient. We show here that, like kinase suppressor of Ras 1 (KSR1), EPH (erythropoietin-producing hepatocellular carcinoma) receptor B4 (EPHB4) is aberrantly overexpressed in human colon tumor cell lines and selectively required for their survival. KSR1 and EPHB4 support tumor cell survival by promoting the expression of downstream targets, Myc and the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1β (PGC1β). While KSR1 promotes the aberrant expression of Myc and the PGC1β protein via a posttranscriptional mechanism, EPHB4 has a greater effect on Myc and PGC1β expression via its ability to elevate mRNA levels. Subsequent analysis of the posttranscriptional regulation demonstrated that KSR1 promotes the translation of Myc protein. These findings reveal novel KSR1- and EPHB4-dependent signaling pathways supporting the survival of colorectal cancer cells through regulation of Myc and PGC1β, suggesting that inhibition of KSR1 or EPHB4 effectors may lead to selective toxicity in colorectal tumors.

Colorectal cancer (CRC) is the third most common cancer in the United States and worldwide (1). It is sporadic in nature, with only 15 to 30% having a major hereditary component (2, 3). CRC is a heterogeneous disease, with distinct molecular features of the tumor contributing to the prognosis and response to targeted therapies (4). Several critical genes and pathways are important in the initiation and progression of CRC, most notably the Wnt, RAS/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), transforming growth factor β (TGF-β), p53, and DNA mismatch repair pathways (5). Oncogenic Ras mutations commonly occur in human CRC, with approximately 43% of patients harboring activating KRAS mutations (6). Patients carrying an oncogenic form of Ras have poorer prognoses than patients harboring wild-type Ras (7–9). Their poor response to therapy can be attributed to the observed attenuation in benefit from anti-epidermal growth factor receptor (anti-EGFR) therapies (10) or resistance to RAF inhibitor therapies (11). Ras proteins are a family of small GTPases that regulate a number of cellular signaling pathways associated with the promotion of an oncogenic phenotype, particularly through the MAPK and PI3K pathways (12). The MAPK signaling pathway is composed of the downstream signaling molecules RAF, MEK, and extracellular signal-regulated kinase (ERK), whose subcellular locations are modulated by kinase suppressor of Ras 1 (KSR1) (13). KSR1 is a scaffold of the RAF/MEK/ERK kinase cascade and is required for maximal MAPK-dependent signaling (14, 15). While KSR1 is required for the survival of CRC cells, it is dispensable in normal colon epithelial cells (16). KSR1$^{-/-}$ mice develop normally with attenuated ERK signaling and display a reduced tumor burden in a polyomavirus middle-T-antigen-driven mouse tumor model (17, 18). Given that KSR1 is dispensable for normal cells but indispensable for colorectal cancer cells, we sought to detect and exploit further vulnerabilities in human colon tumor cells. To do this, we developed a gene expression-based high-throughput screen and used functional signature ontology (FUSION) (16, 19) to identify functional analogs of KSR1. From this screen, we identified EPH (erythropoietin-producing hepatocellular carcinoma) receptor B4 (EPHB4) as a KSR1-like, cancer-specific vulnerability that may be exploited by targeted therapies.

EPH receptors are the largest family of receptor tyrosine kinases (RTKs), with important roles in tissue organization and growth during development, as well as in tissue homeostasis in adults (20–22). Humans have nine EPHA and five EPHB receptors that are classified by their ability to bind their respective ligands, ephrin (EPH-receptor-interacting protein) A and ephrin B, on an adjacent cell. There are five type A and three type B ephrin ligands. Ephrin B ligands are transmembrane, and the receptor-ligand binding is capable of transmitting both forward (through the RTK) and reverse (via the ligand) signaling (reviewed in references 23 and 24). This bidirectional signaling results in repulsion between the two cells and is responsible for establishing boundaries between distinct cell types (25, 26). For example, EPHB4 binding to its ligand, ephrin B2, contributes to the establishment of capillaries in the vasculature, with EPHB4 expressed primarily in the venous endothelium and ephrin B2 in the arterial endothe-

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lium (27, 28). EPHB4 or ephrin B2 knockout mice are embryonic lethal due to their inability to develop proper vasculature systems (29–31). In the intestine and colon, EPHB-expressing cells are present in the progenitor cells of the crypts, whereas the ephrin B ligand is present in the more differentiated cells (28, 32). The repulsion of EPH-ephrin binding leads to opposing gradients and contributes to the morphology of the intestine and colonic crypts (28, 32).

We have recently shown that tumor-specific expression of peroxisome proliferator-activated receptor gamma coactivator 1β (PGC1β) is required for colon cancer survival (16). Previous work has shown that PGC1β is a direct downstream target of Myc (33, 34). Myc-dependent PGC1β transcription is inhibited by hypoxia in renal clear-cell carcinoma due to induction of MXI1 (a repressor of Myc activity) (34), a mechanism that may be operative in the hypoxic regions of many tumor types. In breast cancer cells, HER2 and insulin-like growth factor 1 (IGF1) signaling regulate PGC1β via induction of Myc mRNA and/or regulation of Myc protein stability. Tight regulation of Myc expression is essential for normal cell function (35, 36). However, in cancer cells, Myc activation can be induced through constitutive activation of a pathway (i.e., Wnt activation in tumors with APC mutations) (37) or through alterations of the Myc gene (i.e., amplification and translocation) (38, 39). Defects in the APC pathway occur in many human colon carcinomas and result in enhanced TCF-dependent transcriptional activation of Myc (37). In fact, Myc is essential for the for the "crypt progenitor cell-like" phenotype of APC-deficient cells *in vivo* (32).

Here, we examined EPHB4 and its relationship to Myc and downstream effectors of KSR1 signaling to identify pathways on which colorectal cancer cells are uniquely dependent. We show that EPHB4 has phenotypic and molecular effects in colorectal cancer cells similar to those of KSR1 and that both KSR1 and EPHB4 are essential for the survival of colorectal cancer cells but dispensable for survival of nontransformed immortalized human colonic epithelial cells (HCECs). Additionally, we demonstrate that both molecules support the expression of PGC1β, which is required for maintaining tumor cell viability. Finally, we show that EPHB4 supports Myc expression by elevating Myc mRNA, while KSR1 promotes the expression of PGC1β by enhancing the translation of Myc mRNA into protein.

MATERIALS AND METHODS

Gene expression-based high-throughput screen and functional signature ontology analysis. The gene expression-based high-throughput screen has been described previously (16, 19). The gene expression-based signature measured in the screen is based on six genes (ACSL5, BNIP3L, ALDOC, LOXL2, BNIP3, and NDRG1 genes) that are consistently affected by KSR1 depletion, as well as two housekeeping genes (PPIB and hypoxanthine phosphoribosyltransferase [HPRT] genes) that were included for normalization. To identify targets that are KSR1 functional analogs based on their gene expression-based signatures, two similarity metrics were employed, Euclidean distance (ED) and Pearson correlation (PC). KSR1-depleted positive controls cluster with a low ED and a high PC. Linear regression analysis was used to establish a cutoff ($PC > 0.25 \times ED + 0.5$) for KSR1 similarity based on the ED and PC values of KSR1-positive controls. Targets that clustered with the positive-control KSR1-depleted wells and exceeded the established cutoff based on these two metrics were designated possible KSR1 functional analogs and candidates for further evaluation.

Cell culture. The colorectal cancer cell lines HCT116, SW480, DLD1, SK-CO-1, Caco2, and HCT15 were purchased from the American Type Culture Collection (ATCC). The CBS and GEO colorectal cancer cell lines were gifts from Michael Brattain (University of Nebraska Medical Center). The cells were grown in either Dulbecco's modified Eagle's medium (DMEM) or Eagle's minimum essential medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1 mM nonessential amino acids (NEAA). All colorectal cancer cells were grown at 37°C with ambient O_2 and 5% CO_2. Immortalized nontransformed human colonic epithelial cell (HCEC) lines were a gift from J. Shay (University of Texas [UT] Southwestern) (40). HCECs were grown in medium composed of 4 parts DMEM to 1 part medium 199 (Sigma-Aldrich) with 2% cosmic calf serum (GE Healthcare), 25 ng/ml EGF, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin, 5 nM sodium selenite, and 50 μg/ml gentamicin sulfate. HCECs were grown in a hypoxia chamber with 2% O_2 and 5% CO_2 at 37°C.

TABLE 1 Sequences of individual siRNA duplexes

Target	Item no.	siRNA no.[a]	Target sequence
hKSR1	J-003570	6	CAUCAUAGACAGCAGUUUA
		7	GAGCAAGUCCCAUGAGUCU
		8	GGAAUGAAGCGUGUCCUGA
		9	AGAAAGAGGUGAUGAACUA
hEPHB4	D-003124	5	GGACAAACACGGACAGUAU
		6	GUACUAAGGUCUACAUCGA
		7	GGAGAGAAGCAGAAUAUUC
		8	GCCAAUAGCCACUCUAACA
hMyc	J-003282	23	ACGGAACUCUUGUGCGUAA
		24	GAACACACAACGUCUUGGA
		25	AACGUUAGCUUCACCAACA
		26	CGAUGUUGUUUCUGUGGAA

[a] Manufacturer's number for siRNA sequence.

siRNA transfections. Pooled or individual (Table 1) small interfering RNAs (siRNAs) targeting EPHB4 (M-003124-02), KSR1 (LU-003570-00-0002), or Myc (L-003282), as well as a nontargeting siRNA control (D-001810-01) (DharmaconGE), were introduced into the HCT116 or Caco2 cells using the Lipofectamine RNAiMax (Invitrogen) reverse-transfection protocol. Briefly, 125 pmol of siRNA and 7 μl of RNAiMax were combined in Opti-MEM for 5 min. DNA-Lipofectamine complexes were overlaid with 2 ml of cells (150,000 cells/ml) in 6-well plates. Final RNA interference (RNAi) concentrations were 50 nM. HCECs were transfected following the RNAiMax reverse-transfection protocol using 2.5 μl RNAiMax transfection reagent per 6 ml of antibiotic-free medium and 150,000 cells/ml, with a final RNAi concentration of 10 nM in 6-cm dishes (Corning; Primaria). After a 72-hour transfection, the cells were lysed in RIPA lysis buffer with protease and phosphatase inhibitors (described below).

Reagents. The EPHB4 receptor tyrosine kinase inhibitor (AZ12672857) was a gift from J. Kettle (AstraZeneca). The EPHB4 inhibitor was dissolved in dimethyl sulfoxide (DMSO) to achieve a stock concentration of 10 mM. Z-Leu-Leu-Leu-al (MG132) (C2211), cycloheximide (CHX) (C7698), bafilomycin A1 (BafA1) (B1793), poly-2-hydroxyethyl methacrylate (polyHEMA) (P3932), and propidium iodide (PI) (P4170) were purchased from Sigma-Aldrich and were used at the concentrations specified in the figures and legends.

Anchorage-independent growth on polyHEMA-coated plates. polyHEMA stock solution (10 mg/ml) was made by dissolving polyHEMA in 95% ethanol and shaking at 37°C until it was fully dissolved (6 h to overnight). Black-sided, clear-bottom 96-well plates were coated with polyHEMA by evaporating 200 μl of the 10-mg/ml stock polyHEMA solution in each well. Cells were plated in complete medium on the polyHEMA-coated wells at a concentration of 2×10^4 cells/100 μl 48 h posttransfection (as described above). Cell viability was measured according to the manufacturer's protocol using the CellTiter-Glo luminescent cell viability assay (Promega). Specifically, this was done by adding 90 μl of CellTiter-Glo reagent, shaking for 2 min to lyse the cells, incubating at room temperature for 10 min, and measuring the luminescence (Polarstar Optima).

TABLE 2 Sequences of qPCR primers

Target	Accession no.	Amplicon size (bp)	Exon(s)	T_m (°C)	Primer (5′→3′)	
					Forward	Reverse
hPPARGC1B	NM_001172699	102	12, 13	62	GAATATTTCAGTAAGCTGTCA	GCCCAGATACACTGACTACG
hMyc	NM_002467.4	119	3	58	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
hKSR1	NM_014238.1	127	20, 21	60	AGTTTCTCCAGCATGTCCATC	GAATGAAGCGTGTCCTGACT
hEPHB4	NM_004444	90	12, 13	62	AGCTGGATGACTGTGAACTG	GCCCGTCATGATTCTCACA
hGAPDH	NM_002046.1	111	2, 3	58	GGTGAAGGTCGGAGTCAACGG	GAGGTCAATGAAGGGGTCATTG
hACTB	NM_001101.1	59	4	60	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC
hHPRT1	NM_000194	128	6–8	60	GTATTCATTATAGTCAAGGGCATATCC	AGATGGTCAAGGTCGCAAG

Cell viability assay. Cells (5,000/well) were transfected on white 96-well plates. Transfections were done as described above but at a ratio of 1:25 for all of the reagents. At 0 and 72 h posttransfection, 10 μl of alamarBlue (ThermoFisher Scientific) was robotically added to each well. The plates were incubated at 37°C for 3 h, and fluorescence was measured (Polarstar Optima).

Propidium iodide. Cells were assayed for apoptosis using the sub-G_1 peak measured following PI staining. Prior to staining, all the medium in each sample well was collected and placed in a 12- by 75-mm round-bottom polystyrene tube (BD Falcon; 352054). The cells were washed once with phosphate-buffered saline (PBS), the PBS was saved, and the cells were subsequently treated with 0.25% trypsin for 5 to 10 min. The saved medium was then used to resuspend the trypsin-treated cells from the corresponding wells, which were collected and placed in the polystyrene tubes. The cells were pelleted by centrifugation for 5 min at 2,800 rpm using an Immunofuge II (Baxter/Dade). The supernatant was aspirated, and the cell pellets were resuspended in 2 ml of PBS and then pelleted again by centrifugation for 3 min at 2,800 rpm. The PBS was aspirated, and the cells were fixed in 2 ml of ice-cold 70% ethanol for at least 1 h at −20°C. The cells were then warmed to room temperature (~15 min), pelleted by centrifugation for 3 min, and then rehydrated in 2 ml of room temperature PBS and incubated at 37°C for 15 min. The cells were then pelleted, the PBS was aspirated, and the cells were resuspended in PI stain overnight. Data were acquired using a Becton-Dickinson FACSCalibur flow cytometer and analyzed using ModFit analysis software to detect a sub-G_1 peak of fluorescence.

Western blot analysis. Whole-cell lysate extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer composed of 50 mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cytoplasmic and nuclear fractionations were performed using NE-PER nuclear/cytoplasmic extraction reagents (Thermo Scientific; 78835) The protein concentration was determined using the Promega bicinchoninic acid (BCA) protein assay. Samples were diluted in 1× sample buffer (5× stock; 313 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue) with 100 mM dithiothreitol (DTT) (20× stock; 2 M). SDS-PAGE was performed, and the membranes were blocked in Odyssey PBS blocking buffer (LI-COR Biosciences; 927-40000) and incubated in primary antibody (see below) overnight at 4°C. LI-COR secondary antibodies (IRDye 800CW, 680LT, and 680RD) were diluted 1:5,000 to 1:10,000 in 0.1% Tris-buffered saline (TBS)–Tween 20 (TBST) (for nitrocellulose) or 0.1% TBST plus 0.01% SDS (for polyvinylidene difluoride [PVDF]). The membranes were imaged using the LI-COR Odyssey.

Antibodies. Primary antibodies were diluted as follows: EPHB4 (monoclonal antibody [MAb] 265; a gift from Vasgene), 1:500, and D1C7N (14960; Cell Signaling), 1:1,000; KSR1 (H-70; Santa Cruz), 1:1,000; α-tubulin (B-5-1-2; Santa Cruz), 1:2,500; β-actin (C-4; Santa Cruz), 1:2,000; PGC1β (provided by A. Kralli, Scripps Research Institute), 1:5,000; c-Myc (5605; Cell Signaling), 1:1,000; poly(ADP-ribose) polymerase (PARP) (9542; Cell Signaling), 1:1,000; pERK (9106; Cell Signaling), 1:1,000; ERK (9102; Cell Signaling), 1:1,000; pMEK (4694; Cell Signaling), 1:1,000; MEK (9122; Cell Signaling), 1:1,000; LC3B (2775; Cell Signaling), 1:1,000; p62/SQSTM1 (5114; Cell Signaling), 1:1,000; programmed cell death 4 (PDCD4) (D29C6) (9535; Cell Signaling), 1:1,000; p4E-BP1 T70 (9455; Cell Signaling), 1:1,000; eukaryotic initiation factor 4A (eIF4A) (C32B4) (2013; Cell Signaling), 1:1,000; eIF4E (9742; Cell Signaling), 1:1,000; peIF4E (S209) (9741; Cell Signaling), 1:1,000; eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (53H11) (9644; Cell Signaling), 1:1,000.

RT-qPCR. RNA was harvested using 1 ml TriReagent (MRC; TR118) and stored at −80°C until extraction. The RNA was extracted according to the manufacturer's protocol, and the final RNA pellets were resuspended in nuclease-free water. DNase digestion was performed (Qiagen; 79254), and RNA cleanup was completed (Qiagen; 74106). The RNA was quantified using a NanoDrop 2000 (Thermo Scientific). Reverse transcription (RT) was performed using iScript Reverse Transcription Supermix for RT-quantitative PCR (qPCR) (Bio-Rad; 170-8840) with 1 μg of total RNA per 20-μl reaction mixture. RT-qPCR was performed using the primers and conditions listed in Table 2. All the targets were amplified using SsoAdvanced Universal SYBR green Supermix (Bio-Rad), with 40 cycles of a 2-step program (95°C for 5 s; melting temperature [T_m] for 45 s) on an MX3000P (Stratagene). The data were normalized using two of the following normalization genes: HPRT, β-actin, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Analysis was performed according to the q-base protocol, as previously published (41).

TCGA. mRNA expression was analyzed based on the transcriptome sequencing (RNA-Seq) by expectation maximization (RSEM)-normalized RNA-Seq values of primary tumor ($n = 285$) and normal solid tissue ($n = 41$) samples, as well as patient-matched samples ($n = 26$), within The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) data set. The results were analyzed for statistical significance using unpaired and paired Student's t tests for the unpaired and patient-matched samples, respectively.

Myc translation. Myc translation reporter constructs (pGML, phpL, and phpmL) for luciferase assays were a gift from Anne Willis (Medical Research Council, Leicester, United Kingdom) (42, 43). RNAi depletions were performed in 6-well plates as described above. The following day, the cells were transfected with 3 μg of the translation vector and 1 μg of the pSV-β-galactosidase vector (Promega; E1081) using 10 μl of Lipofectamine 2000 (Invitrogen) per well. After 24 h, luciferase and β-galactosidase (β-Gal) expression was assessed using the Dual-Light system (Applied Biosciences; T1003) according to the manufacturer's instructions. Briefly, cells were rinsed twice with PBS and lysed with lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 0.5 mM DTT), and 10 μl of each lysate was added (in triplicate) to a 96-well plate. Luminescence was measured (Polarstar Optima) for 1 s per well beginning 1.5 s following injection. When quantifying basal translation from each vector, luciferase expression was normalized to β-galactosidase expression. When comparing the effect of KSR1 depletion on Myc translation, the luciferase signal was normalized to the protein input.

Statistical analyses. P values and 50% effective concentrations (EC_{50}s) were calculated using Prism software (GraphPad, La Jolla, CA). A

P value of less than 0.05 was considered statistically significant. The values presented here are shown as means ± standard deviations (SD) unless otherwise noted. EC_{50}s were calculated in Prism using an algorithm for fitting nonlinear curves with variable slopes.

RESULTS

EPHB4 is required for human colon tumor cell survival. KSR1 regulates the oncogenic potential of activated Ras (14). Our laboratory has recently shown that KSR1 also promotes anchorage-independent growth and tumor maintenance in human colon tumor cell lines (16). Importantly, we demonstrated that depletion of KSR1 is selectively toxic to CRC cells compared to immortalized, nontransformed HCECs. Using a gene expression signature representing depletion of KSR1, we developed a high-throughput screen called FUSION (19) to identify functional analogs of KSR1. Details regarding the screen, gene signature, and FUSION have been provided previously (16, 19). Based on unsupervised hierarchical clustering of reporter gene expression following RNAi-mediated depletions of individual genes, we found that knockdown of EPHB4 clustered with the RNAi-mediated KSR1 depletion (siKSR1) positive controls. We further visualized this relationship by plotting Pearson correlation versus Euclidean distance similarity metrics (Fig. 1A). Depletion of EPHB4 has a Euclidean distance of 1.44 and a Pearson correlation of 0.88, and siEPHB4 (blue) clusters with the siKSR1 (red) reference standards. Based on previous work demonstrating that gene expression-based signatures can be used to represent the functional state of a cell (16, 19, 44, 45), the similarity of siKSR1- and siEPHB4-dependent gene expression signatures suggests that EPHB4 is likely to support colon tumor cell survival similarly to KSR1.

EPHB4 expression is elevated in a variety of human cancers, including cancers of the head and neck, prostate, bladder, ovaries, large intestine, lung, brain, pancreas, and esophagus (46–54). We analyzed the expression of EPHB4 in a panel of colon tumor cells compared to its expression in HCECs. Western blotting revealed that the EPHB4 protein is overexpressed in all colon tumor cell lines tested (Fig. 1B). RT-qPCR analyses demonstrated that the abundance of protein could not be entirely attributed to an overabundance of mRNA (Fig. 1C). While there was a trend toward increased mRNA levels in all colon tumor cell lines compared to HCECs, only SK-CO-1 cells showed a statistically significant difference. To evaluate the relevance of these findings in human tumors, we examined EPHB4 gene expression in the colon adenocarcinoma data set within TCGA and demonstrated that EPHB4 was significantly increased at the mRNA level in human colon tumor samples compared to normal solid-tissue samples (Fig. 1D). These findings were consistent both using all available data (top) or using only the patient-matched tumor and normal samples (bottom). In fact, every patient-matched tumor demonstrated an increase in EPHB4 expression relative to the normal sample.

Depletion of EPHB4 is selectively toxic to colon tumor cells. Depletion of KSR1 is selectively toxic to colon tumor cells compared to HCECs (16). To determine whether EPHB4, like KSR1, is required for tumor cell survival, we measured viability, anchorage-independent proliferation, and apoptosis in two colon tumor cell lines and HCECs following knockdown of KSR1 or EPHB4 by RNAi. Cell proliferation was measured by alamarBlue cell viability assay after 72 h of KSR1 or EPHB4 depletion. In HCT116 cells, KSR1 and EPHB4 RNAi reduced cell proliferation compared to controls by 81% and 71%, respectively (Fig. 2A), whereas in the Caco2 cells, cell viability was decreased by 95% with KSR1 depletion and 69% with depletion of EPHB4. Cell proliferation was unaffected in the HCEC line. To measure anchorage-independent growth, cell proliferation was measured on a polyHEMA-coated plate (55, 56) using a CellTiter-Glo luminescent cell viability assay, as previously described (16). Following depletion of KSR1 or EPHB4, growth under anchorage-independent conditions was reduced by 57% and 53% in HCT116 cells and 74% and 51% in Caco2 cells, respectively (Fig. 2B). HCECs are unable to proliferate in an anchorage-independent environment and were not used in this experiment. Validation of target knockdown was by Western blotting (Fig. 2A and B). To determine if the reduced cell viability under normal and anchorage-independent conditions was due to increased apoptosis, PARP cleavage was assessed by Western blotting following depletion of KSR1 and EPHB4 in HCECs and HCT116 and Caco2 cells. HCECs showed no PARP cleavage following target knockdown, whereas HCT116 and Caco2 cells demonstrated PARP cleavage upon KSR1 or EPHB4 depletion (Fig. 2C). These observations show that KSR1 or EPHB4 is selectively required for colon tumor cell survival and growth and suggest that without KSR1 or EPHB4, cells undergo apoptosis.

Downstream effectors of KSR1-dependent signaling in colon tumor cell lines include the RAF/MEK/ERK kinase cascade and the PGC1 family of transcriptional regulators (14–16, 57, 58). We recently identified PGC1β as a key downstream effector of KSR1 in human colon tumor cells and showed that its expression is required for colon cancer survival both *in vitro* and *in vivo* (16). To determine whether EPHB4 disrupts either of these pathways, we assessed MEK1/2 and ERK1/2 activation and total PGC1β protein levels by Western blotting after 72 h of EPHB4 depletion in HCT116 and Caco2 cells. We observed that depletion of EPHB4 did not affect MEK1/2 or ERK1/2 phosphorylation. However, EPHB4 RNAi did suppress PGC1β levels (Fig. 2D). These data indicate that, like KSR1, EPHB4 regulates PGC1β.

Inhibition of EPHB4 kinase activity is selectively toxic to colon tumor cells. Pharmacological targeting of EPHB4 is of clinical interest (23, 24). One strategy for targeting EPHB4 is inhibition of its kinase activity. To determine the extent to which inhibition of the EPHB4 kinase, like RNAi-mediated EPHB4 depletion, is selectively toxic to colon tumor cells, we measured viability in HCECs and a panel of colon tumor cell lines treated for 72 h with increasing doses of an EPHB4 kinase inhibitor, AZ12672857 (AZ2857) (59). The EC_{50} for each cell line was determined from four independent experiments using a nonlinear curve fitted with a variable slope (Fig. 3A). HCECs (EC_{50} = 14 μM) were less sensitive to EPHB4 inhibition than HCT116 or Caco2 cells (EC_{50} = 3.2 μM and 2.6 μM, respectively). The EC_{50}s of three additional colon tumor cell lines (SW480, DLD1, and SK-CO-1) are shown in the table in Fig. 3A. HCECs tolerated doses up to 20 μM (the highest dose tested) without increasing the percentage of PI-stained cells in the sub-G_1 peak measured by flow cytometry (Fig. 3B). However, at the same dose, HCT116 and Caco2 cells had >50% sub-G_1 cells after 72 h of treatment with AZ2857. Taken together, these data indicate that inhibition of EPHB4 decreases total ATP levels as measured by the CellTiter-Glo cell viability assay in HCECs, possibly through reduced growth or induction of senescence, but that treatment with AZ2857 does not induce apoptosis. However, in the tumor cell lines HCT116 and Caco2, treatment with AZ2857 clearly reduces cell viability via induction of cell death.

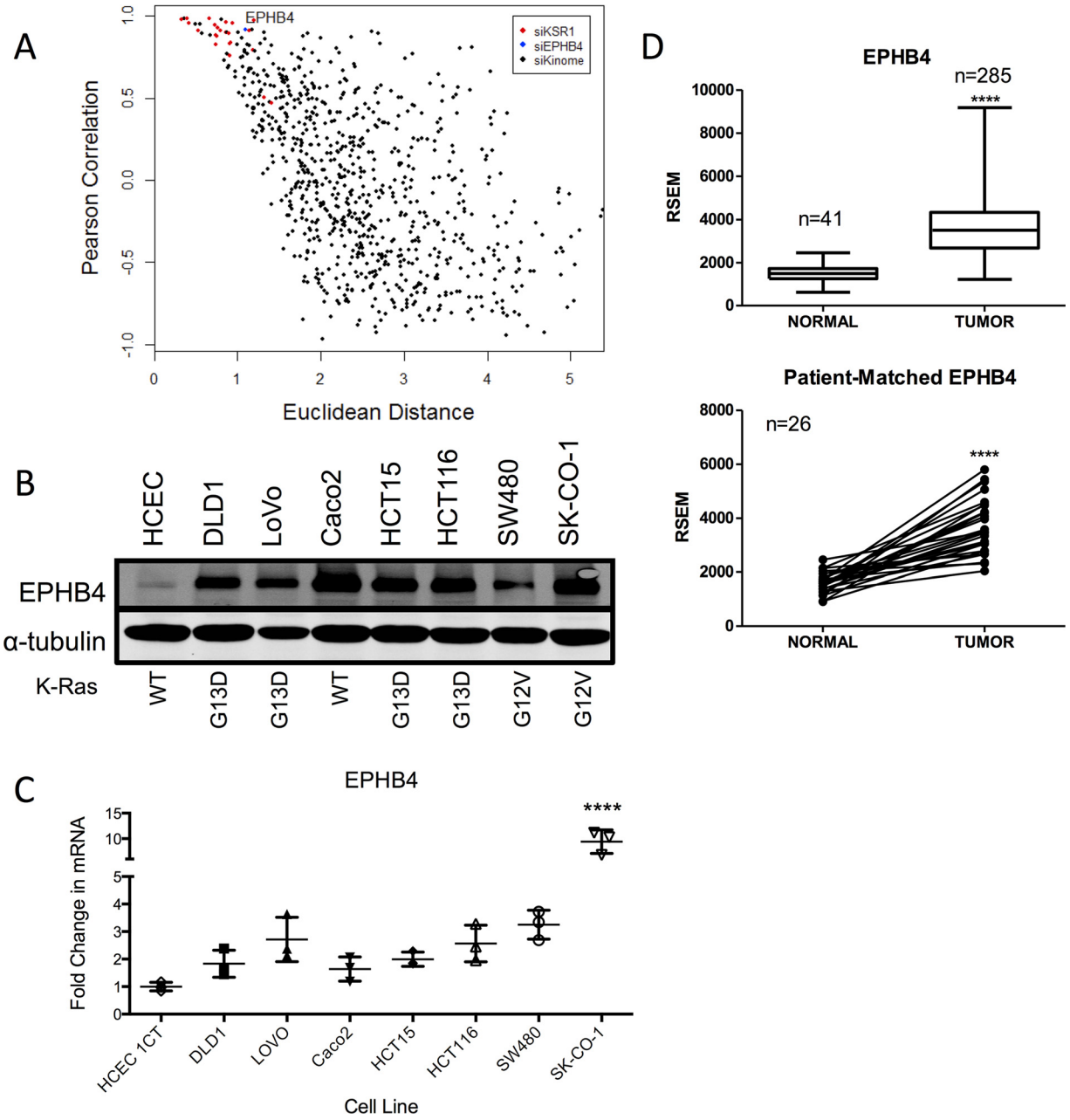

FIG 1 Genome-scale RNAi screen identifies EPHB4 as a KSR1-like effector. (A) Identification of EPHB4 as a KSR1-like effector using Pearson correlation and Euclidean distance similarity metrics. (B and C) Western blot (B) and RT-qPCR (C) of EPHB4 levels in a panel of colon tumor cell lines and immortalized, nontransformed HCECs. The RT-qPCR data are shown as means ± SD. ****, $P < 0.0001$ (matched one-way analysis of variance [ANOVA] and Dunnett's posttest). (D) EPHB4 gene expression (RNA-Seq) data from TCGA for unpaired primary colon tumors and normal solid-tissue samples (top) and primary tumors and patient-matched normal solid-tissue samples (bottom). The median is indicated with a horizontal line in the interior of the box, the first and third quartiles are represented by the ends of the box, and the ends of the whiskers mark the minimum and maximum values. The numbers (n) of samples analyzed are shown. Unpaired results were analyzed for statistical significance using Student's unpaired *t* test. ****, $P < 0.0001$. Matched results were analyzed for statistical significance using Student's paired *t* test. ****, $P < 0.0001$. The results shown here are, in whole or in part, based upon data generated by TCGA Research Network (http://cancergenome.nih.gov/).

KSR1 protects EPHB4 from lysosome-dependent degradation. To assess the possible relationships between KSR1 and EPHB4, KSR1 and EPHB4 were depleted by siRNA for 72 h in HCT116 and Caco2 cells. The levels of KSR1 and EPHB4 proteins (Fig. 4A) and mRNA (Fig. 4B) were measured by Western blotting and RT-qPCR, respectively. Depletion of KSR1 resulted in a consistent reduction in EPHB4 protein expression but not mRNA levels. EPHB4 knockdown did not affect KSR1 protein or mRNA expression in either cell line. These observations suggest that KSR1 regulates EPHB4 protein levels via a posttranscriptional mechanism.

KSR1 regulates proteins such as Myc and PGC1β, which are degraded by the proteasome (16, 60). Therefore, we tested

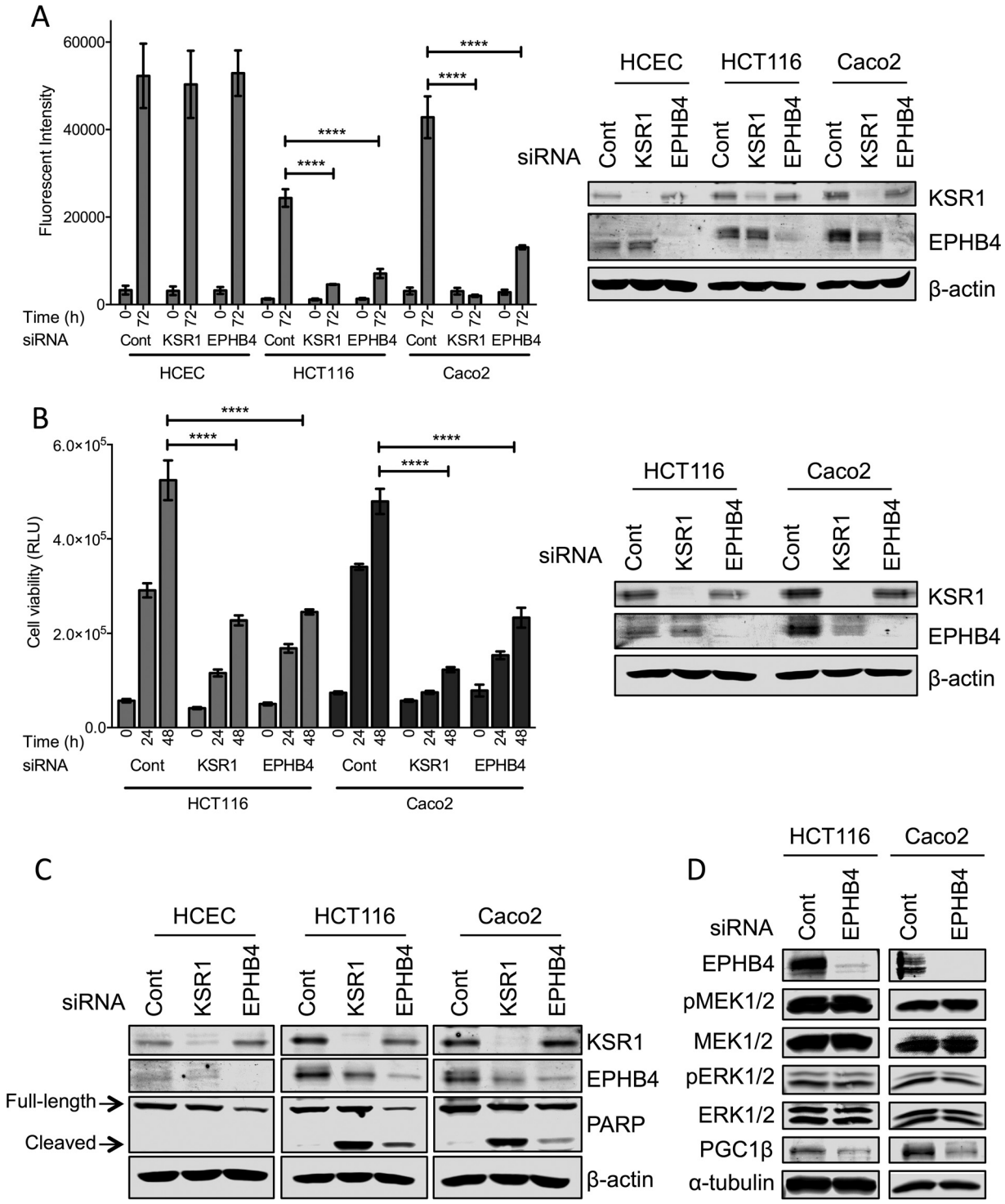

FIG 2 EPHB4 promotes CRC survival by regulating PGC1β. (A and B) The viability of HCECs and HCT116 and Caco2 cells was measured following RNAi of KSR1 or EPHB4 under normal (A) and anchorage-independent (B) conditions by alamarBlue and CellTiter-Glo assays, respectively. (Left) The data are shown as mean fluorescence intensity or relative light units (RLU) ± SD. ****, $P < 0.0001$ (matched two-way ANOVA and Dunnett's posttest for multiple comparisons). (Right) Target knockdown for the assays was assessed by Western blotting. Cont, control. (C) PARP cleavage following depletion of KSR1 or EPHB4. (D) Western blot of the indicated proteins in HCT116 and Caco2 cells following RNAi of EPHB4.

whether KSR1 depletion induces proteasome-mediated degradation of EPHB4. HCT116 and Caco2 cells were depleted of KSR1 for 72 h and incubated with and without 10 μM MG132 for the final 6 h of knockdown. Treatment with MG132 was unable to rescue the EPHB4 levels when KSR1 was depleted (Fig. 4C). Inhibition of proteasomal degradation of Myc was used as a positive control for MG132 treatment. In HCT116 cells without KSR1 depletion, MG132 treatment increased EPHB4 expression, suggest-

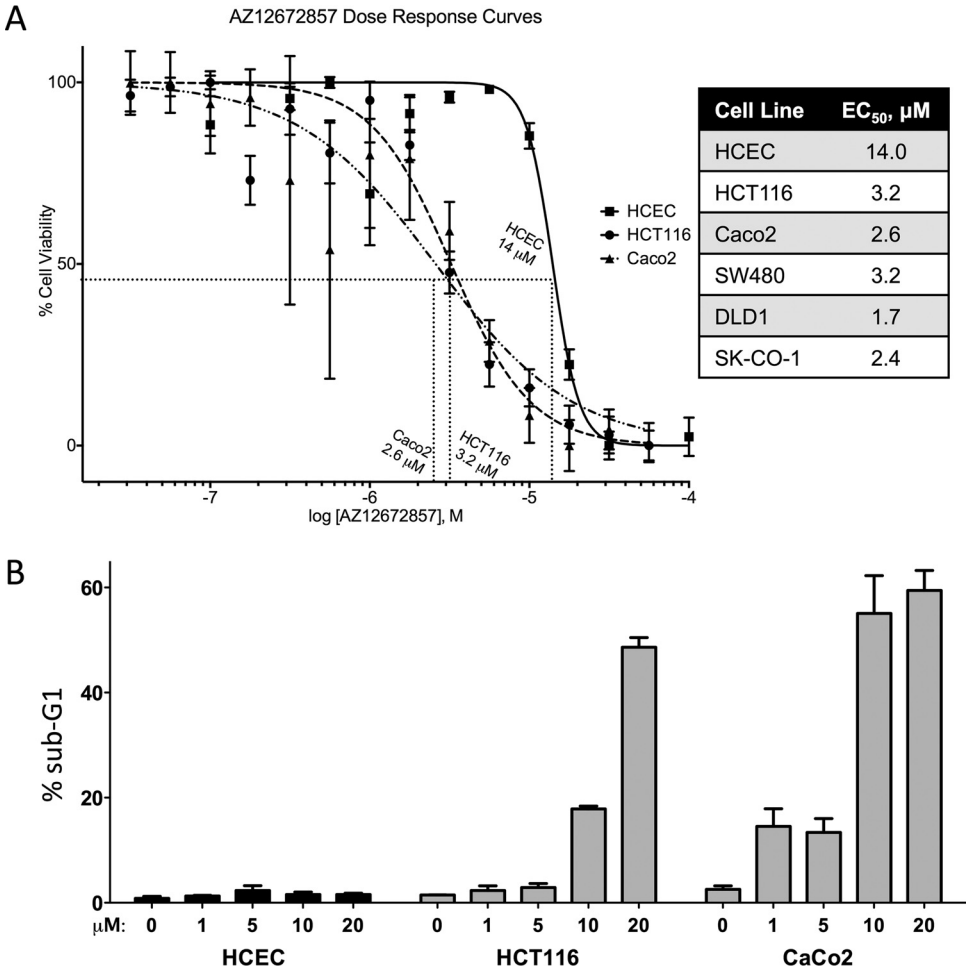

FIG 3 EPHB4 inhibitors are selectively toxic to colon tumor cell lines. (A) HCECs and Caco2, HCT116, SW480, DLD1, and SK-CO-1 cells were treated with increasing doses of AZ12672857, and cell viability was measured by CellTiter-Glo at 72 h. Each data point represents the results of four independent experiments. The data are presented as means ± standard errors of the mean (SEM). The data were normalized, and the EC_{50} for each cell line was calculated using an algorithm for fitting a nonlinear curve with variable slope in GraphPad Prism. (B) HCECs and HCT116 and Caco2 cells were treated with increasing doses of AZ12672857. The sub-G_1 peak was quantified following PI staining and analysis by flow cytometry. The error bars indicate SD.

ing that EPHB4 degradation is partially mediated by the proteasome in these cells but that the effect of KSR1 on EPHB4 stability is independent of proteasome-mediated degradation.

A canonical method of RTK signal termination is downregulation after ligand binding (61–63). Lysosome-mediated degradation of RTKs, including EPHB1, has been well documented (64–66). We assessed whether EPHB4 degradation is mediated by the lysosome and whether KSR1 stabilizes EPHB4 expression by suppressing this degradation. KSR1 was depleted in HCT116 and Caco2 cells for 72 h with and without treatment with 100 nM BafA1, an inhibitor of autophagosome-lysosome fusion, for the final 8 h of knockdown. Treatment with BafA1 alone increased the expression of EPHB4 in both HCT116 and Caco2 cells (Fig. 4D). Additionally, when cells were depleted of KSR1, treatment with BafA1 rescued EPHB4 expression. Increased LC3BII and p62 expression were used as positive controls for BafA1 treatment. Taken together, these data indicate that KSR1 stabilizes EPHB4 by suppressing lysosome-mediated degradation.

KSR1 and EPHB4 regulate Myc and PGC1β. Previous research had demonstrated that Myc regulates PGC1β transcription in renal cell carcinoma (34) and breast cancer (33) cells. Therefore, we examined whether Myc regulates PGC1β in colon tumor cell lines. In HCT116 and Caco2 cells, Myc was depleted with a pool of siRNA; PGC1β protein levels were decreased with Myc knockdown, as assessed by Western blotting (Fig. 5A). To determine if this effect was due to a single siRNA and was potentially an off-target effect, the four siRNA duplexes that compose the pool were assessed independently. Expression of PGC1β protein was correlated with the degree of Myc knockdown (Fig. 5B). The pool of all four siRNA duplexes was used in the remaining experiments. Next, PGC1β mRNA levels were measured by RT-qPCR following depletion of Myc. In both HCT116 and Caco2 cells, Myc depletion significantly decreased expression of PGC1β mRNA (Fig. 5C). Expression of Myc is required for the formation of intestinal crypts but is dispensable for homeostasis of the adult epithelium (67). To evaluate the importance of Myc expression to CRC cell viability, the tumor cell lines HCT116 and Caco2 and a nontransformed colon epithelial cell line (HCEC) were transfected with siRNA targeting Myc or a nontargeting siRNA. Cell viability was assessed 72 h posttransfection. Depletion of Myc reduced cell via-

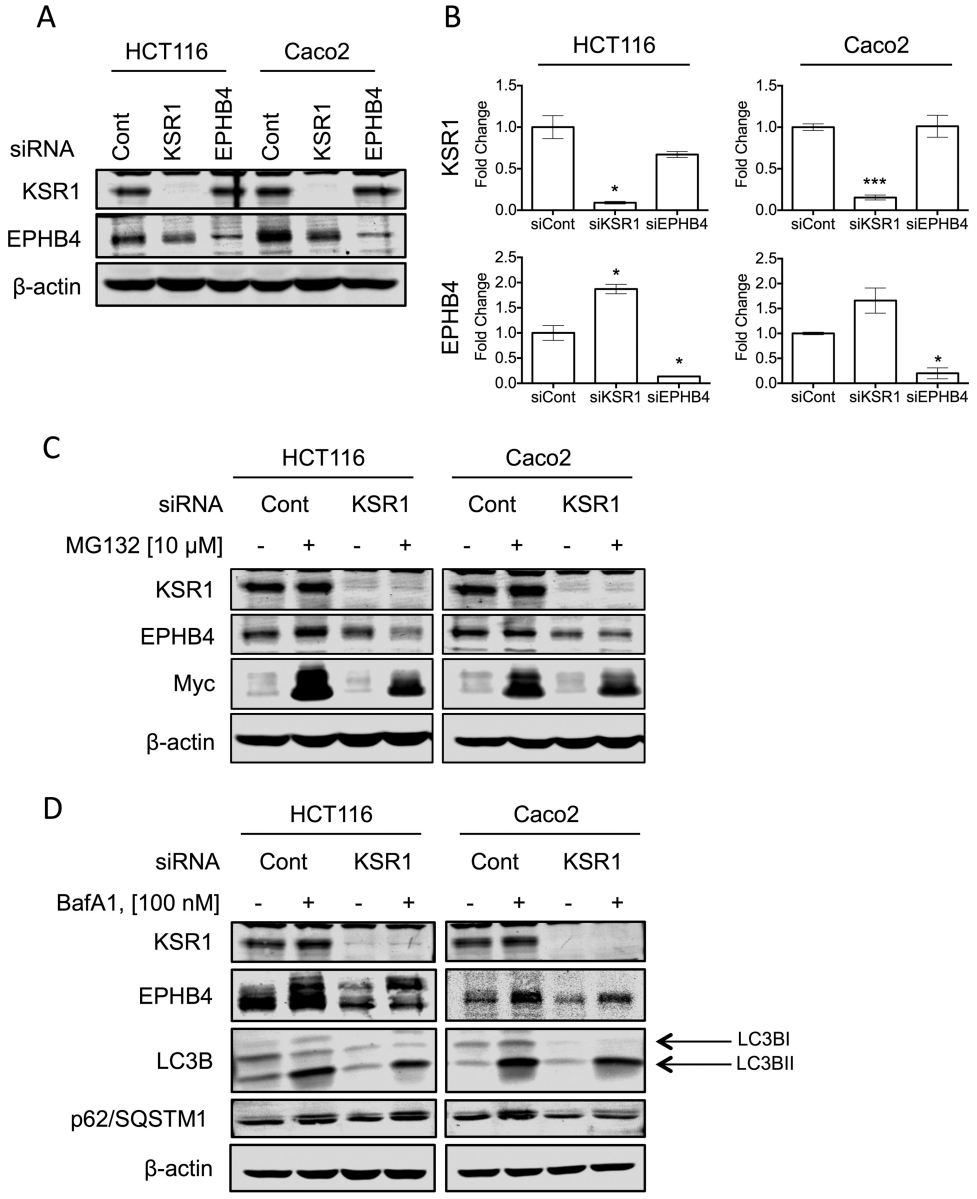

FIG 4 KSR1 protects EPHB4 from lysosome-dependent degradation. KSR1 or EPHB4 was depleted in HCT116 and Caco2 cells for 72 h. (A) KSR1 and EPHB4 protein levels were assessed by Western blotting. (B) KSR1 and EPHB4 mRNA levels were measured by RT-qPCR. The data are shown as means ± SD. *, $P < 0.05$; ***, $P < 0.001$ (repeated-measures, one-way ANOVA with Dunnett's posttest for multiple comparisons). (C) KSR1 was depleted in HCT116 and Caco2 cells for 72 h. The cells were treated with and without 10 μM MG132 for the last 8 h of knockdown. The proteins were analyzed by Western blotting. Myc was used as a positive control for MG132 treatment. (D) KSR1 was depleted in HCT116 and Caco2 cells for 72 h. The cells were treated with and without 100 nM BafA1 for the last 8 h of knockdown. The proteins were analyzed by Western blotting. LC3B and p62/SQSTRM were used as positive controls for BafA1 treatment.

bility in HCT116 (60%) and Caco2 (64%) cells but did not affect proliferation in the HCECs (Fig. 5D), indicating that the tumor cells are more reliant on the expression of Myc for cell growth. Validation of target knockdown by Western blotting is also shown.

To determine whether KSR1 and EPHB4 regulate PGC1β through a Myc-dependent pathway, we assessed Myc and PGC1β protein levels following KSR1 or EPHB4 depletion in HCT116 and Caco2 cells. In both cell lines, depletion of KSR1 or EPHB4 resulted in diminished expression of Myc and PGC1β (Fig. 6A), with EPHB4 depletion having the greatest effect on Myc levels. To confirm that these data are not the result of a single siRNA, we transfected the four individual siRNA duplexes for KSR1 (Fig. 6B, top) and EPHB4 (bottom) into HCT116 cells and measured target, Myc, and PGC1β protein expression by Western blotting 72 h posttransfection. With the exception of KSR1 siRNA 6, all the individual duplexes sufficiently depleted the expression of their targets, as well as Myc and PGC1β. Due to its lack of target knockdown, KSR1 siRNA duplex 6 was not used in the siKSR1 pool in any experiment. Additionally, HCT116 and Caco2 cells were treated with increasing doses of the EPHB4 kinase inhibitor, AZ2857, for 72 h. Western blotting indicated that pharmacological inhibition of EPHB4 decreases Myc and PGC1β protein levels, similar to that seen with depletion using siRNA (Fig. 6C).

To assess whether EPHB4 also regulates PGC1β mRNA levels,

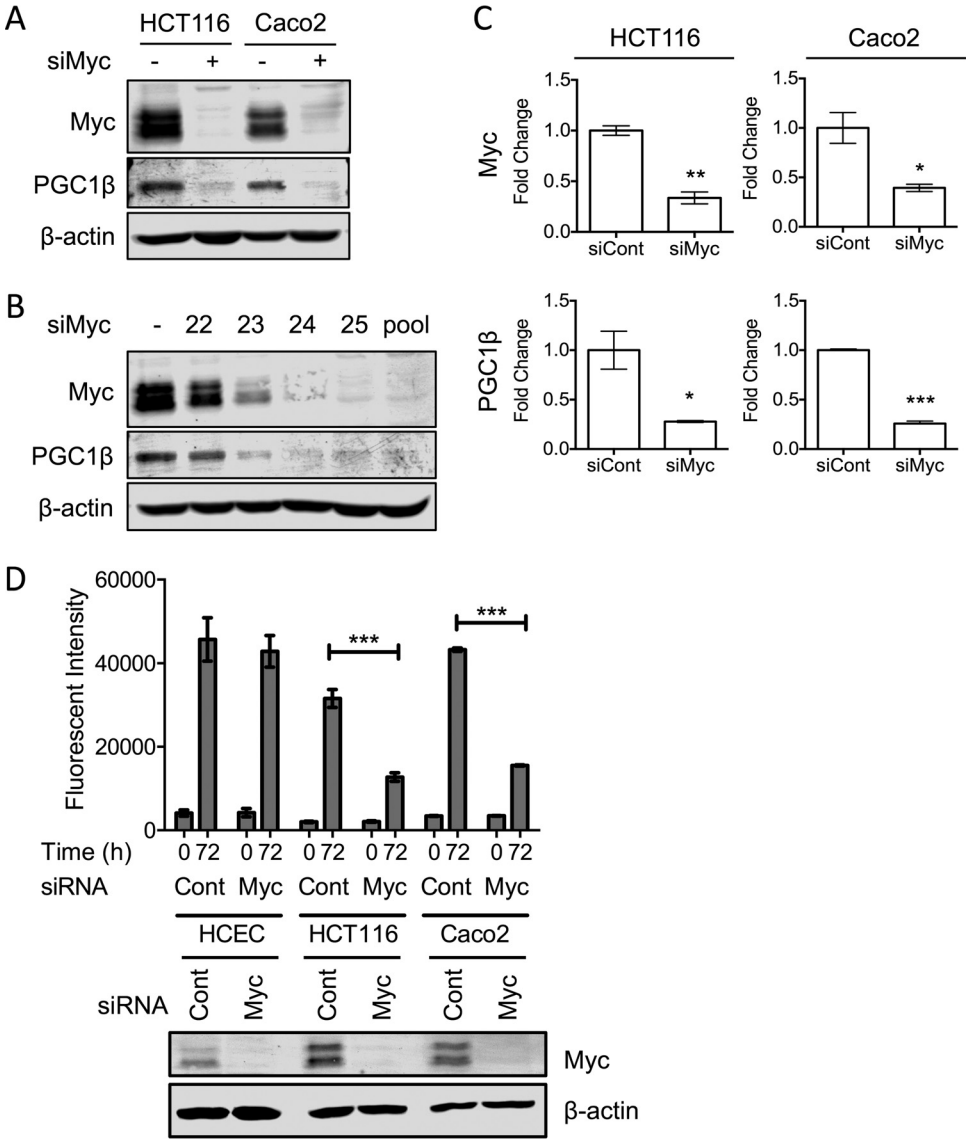

FIG 5 Myc regulates PGC1β in colon tumor cells. (A) Western blot following RNAi of Myc in HCT116 and Caco2 cells. (B) Myc and PGC1β protein expression in HCT116 cells transfected with individual or pooled (all 4) Myc siRNA duplexes. (C) RNA levels of Myc and PGC1β were measured by RT-qPCR in HCT116 and Caco2 cells following RNAi of Myc. (D) Cell viability was measured by alamarBlue following depletion of Myc. The data are shown as means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (paired, two-tailed *t* test[C] and matched, two-way ANOVA [D]).

HCT116 and Caco2 cells were transfected with siRNA targeting EPHB4 (or a nontargeting siRNA) or treated with 10 μM AZ2857 for 72 h. Myc and PGC1β mRNA levels were measured by RT-qPCR. Data from three biological replicates (each measured in triplicate) are shown in Fig. 6D (siRNA) and E (EPHB4 inhibitor). Depletion of EPHB4 by siRNA decreased Myc and PGC1β mRNA expression by 46% and 49% in HCT116 cells and 70% and 26% (not significant) in Caco2 cells, respectively. Inhibition of EPHB4 with the kinase inhibitor AZ2857 consistently decreased the levels of both Myc and PGC1β mRNAs 70% and 45% in HCT116 cells and 67% and 56% in Caco2 cells, respectively. Depletion of KSR1 did not affect Myc or PGC1β mRNA levels (Fig. 6D). These observations suggest that EPHB4 may regulate PGC1β transcription in a Myc-dependent manner. Although EPHB4 was identified by FUSION using KSR1 as a reference standard and the two proteins share common downstream effectors (Myc and PGC1β), these data reveal that the mechanisms by which EPHB4 and KSR1 regulate Myc and PGC1β are not identical.

KSR1 and EPHB4 do not promote Myc stability. To test the hypothesis that depletion of KSR1 or EPHB4 affects Myc levels by regulating protein stability, we examined the turnover of Myc in HCT116 and Caco2 cells following treatment with CHX with and without RNAi of KSR1 or EPHB4. Representative immunoblots of Myc in HCT116 cells after knockdown are shown in Fig. 7A. Data from three independent experiments in each cell line were quantified, and the half-life of Myc under each condition was calculated using GraphPad Prism software. Depletion of KSR1 or EPHB4 did not change the rate of Myc turnover (Fig. 7B).

KSR1 promotes the translation of Myc. Regulation of protein synthesis is mediated by key inhibitors of translation, 4E-BP1 and

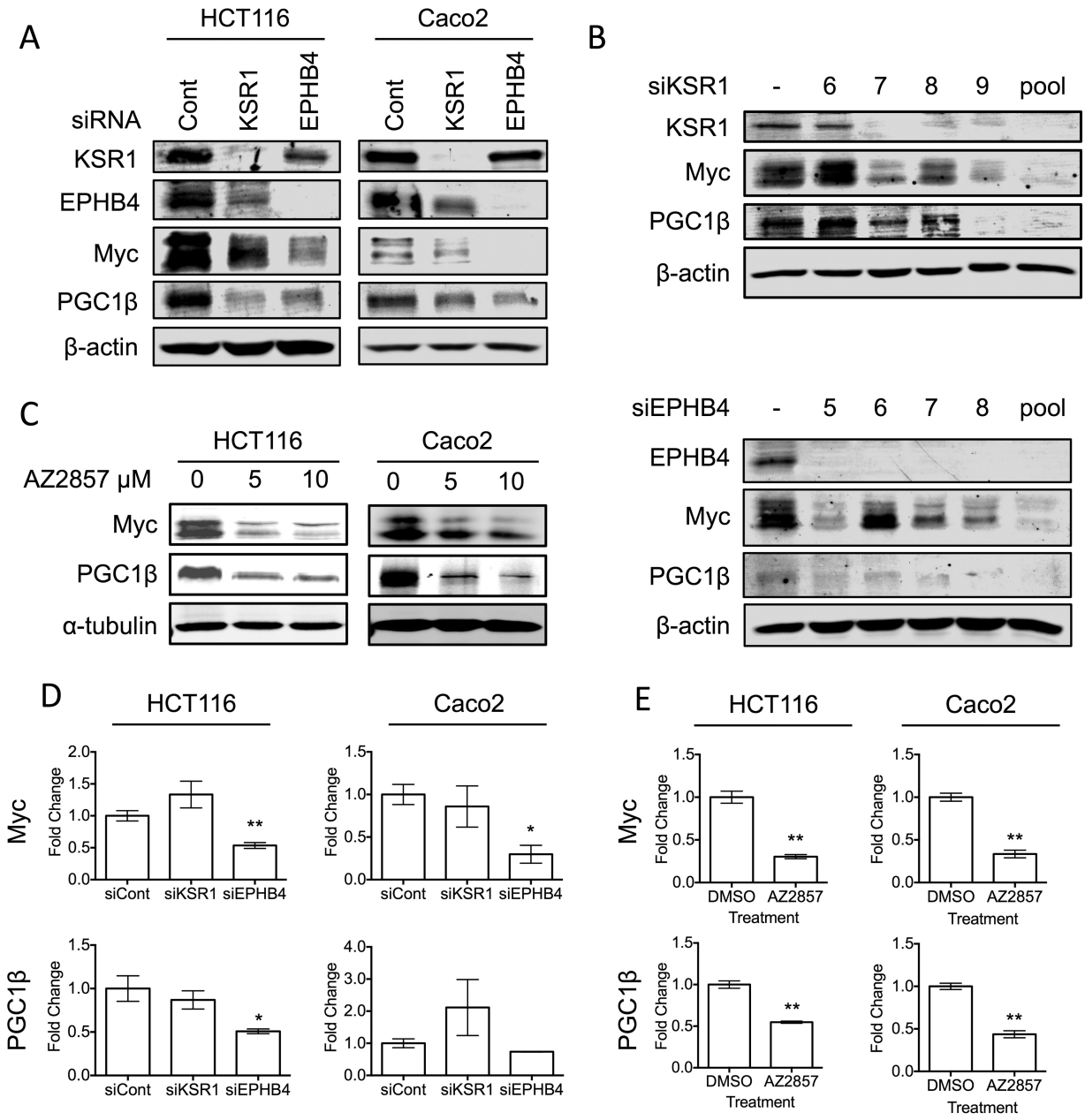

FIG 6 Inhibition of EPHB4 decreases Myc RNA and protein levels. (A and C) Myc and PGC1β protein levels were assessed by Western blotting following RNAi of KSR1 or EPHB4 (A) or treatment with AZ2857, an EPHB4 inhibitor (C), in HCT116 and Caco2 cells. (B) Individual siRNA duplexes for KSR1 (top) and EPHB4 (bottom) were transfected into HCT116 cells, and Myc and PGC1β protein expression was assessed by Western blotting. (D and E) RNA levels of Myc and PGC1β were measured by RT-qPCR in HCT116 and Caco2 cells following RNAi of EPHB4 (D) or treatment with AZ2857 (E). The data are shown as means ± SD. *, $P < 0.05$; **, $P < 0.01$ (paired, two-tailed *t* test).

programmed cell death 4 (PDCD4) (reviewed in reference 68). 4E-BP1 sequesters eukaryotic initiation factor 4E (eIF4E) to inhibit translation. Phosphorylation of 4E-BP1 releases eIF4E and derepresses protein synthesis (reviewed in reference 68). Similarly, PDCD4 sequesters eukaryotic initiation factor 4A (eIF4A). Phosphorylation of PDCD4 leads to its nuclear localization or proteasome-mediated degradation (69). PDCD4 can be phosphorylated by p70 S6 kinase (S6K) or p90 ribosomal protein S6K (RSK) (69, 70). 4E-BP1 and eIF4E regulate cap-dependent translation, while PDCD4 and eIF4A regulate both cap-dependent and -independent translation (reviewed in reference 68).

To determine if KSR1 or EPHB4 affects the expression of these key regulators of protein synthesis, KSR1 and EPHB4 were depleted by siRNA for 48 h in HCT116 and Caco2 cells. In both cell lines, depletion of KSR1 but not EPHB4 decreased 4E-BP1 and eIF4E phosphorylation (Fig. 8A). This observation suggests that depletion of KSR1 inhibits cap-dependent translation. Depletion of KSR1 and not EPHB4 also increased the total levels of PDCD4 (Fig. 8A), suggesting that KSR1 may promote both cap-dependent and cap-independent translation.

The potent role played by KSR1 in regard to key effectors of translation led us to assess the role of KSR1 in the translation of

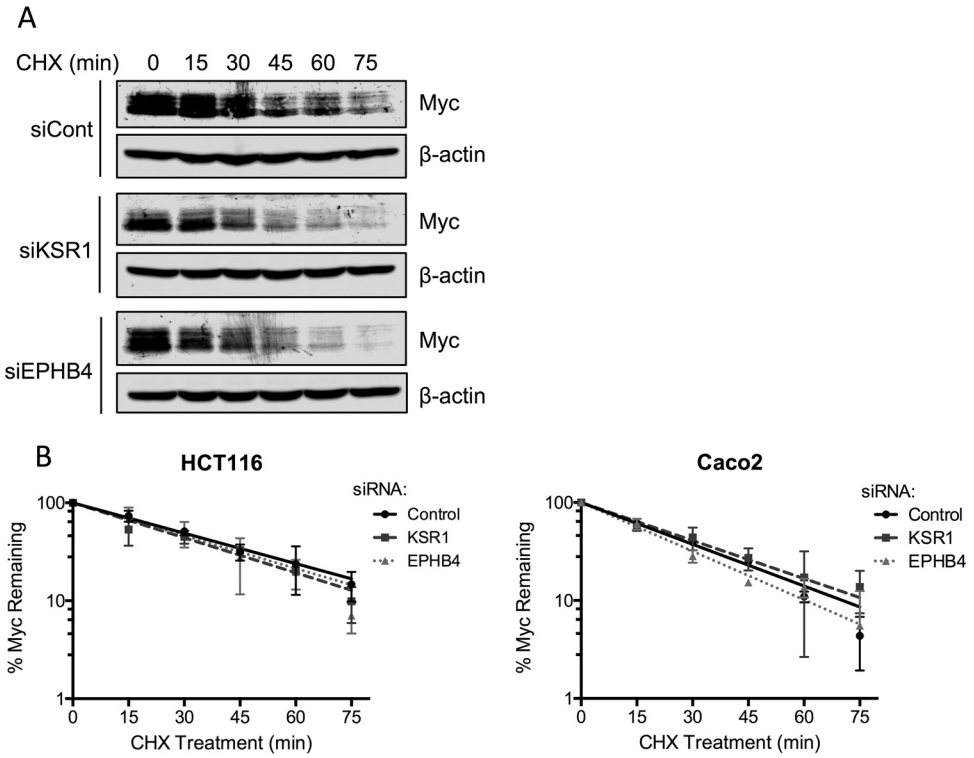

FIG 7 KSR1 or EPHB4 depletion does not affect Myc stability in HCT116 or Caco2 cells. (A) Depletion of KSR1 or EPHB4 was performed for 72 h prior to treatment with 100 μg/ml CHX or vehicle for 0, 15, 30, 45, 60, or 75 min. Myc levels were assessed by Western blotting. (B) An assay was performed three times in each cell line. Myc expression was quantified and normalized to β-actin. The Myc half-life was calculated using nonlinear, one-phase decay (Y0 = 100; plateau = 0) with automatic outlier elimination in GraphPad Prism. The error bars indicate SD.

Myc proteins using luciferase reporter constructs containing the Myc 5′ untranslated region (UTR). The 5′ UTR of Myc mRNA contains an internal ribosome entry site (IRES) (42), and therefore, Myc is translated in a cap- and IRES-dependent manner. Translation from the IRES element depends on eIF4A (helicase) but is independent of eIF4E (cap binding protein) (71). To determine the relative contribution of IRES-dependent translation of Myc to total Myc synthesis, HCT116 and Caco2 cells were transfected with pGML (Myc 5′ UTR), phpL (hairpin only), or phpmL (hairpin-Myc 5′ UTR) luciferase reporter constructs with a pSV-β-galactosidase vector for 24 h. Luciferase expression was normalized to the β-galactosidase expression in each well. IRES-dependent translation comprises 32% and 14% of total Myc translation in HCT116 and Caco2 cells, respectively (Fig. 8B).

To assess whether depletion of KSR1 affects translation via the Myc 5′ UTR, we first measured total translation of luciferase (cap and IRES dependent) using the pGML reporter construct (43, 72). The luciferase signal was normalized to total protein in each sample. Depletion of KSR1 decreased translation of Myc in HCT116 and Caco2 cells by 49% and 33%, respectively (Fig. 8C). Since KSR1 also affected the expression of PDCD4, we also measured the effect of KSR1 depletion on IRES-dependent translation of Myc. Depletion of KSR1 decreased IRES-dependent Myc translation in HCT116 and Caco2 cells by 63% and 68%, respectively (Fig. 8D).

DISCUSSION

We have identified a new pathway critical for colon tumor cell survival impacted by effectors of Ras and Wnt signaling. Two proteins, KSR1 and EPHB4, are required for increased Myc protein expression in human colon tumor cells, which then promotes the expression of its downstream effector, PGC1β. We recently showed that Ras-induced and KSR1-dependent PGC1β upregulation promotes colon cancer survival *in vitro* and *in vivo* (16). This effect is likely due, at least in part, to the ability of this transcriptional coregulator to promote the expression of genes critical to the expansion of glycolytic and oxidative metabolism (16, 33, 73). Here, we show that KSR1- and EPHB4-dependent mechanisms increase Myc expression, which drives PGC1β expression in colon tumor cell lines to promote their survival.

These data highlight the utility of an unbiased RNAi screen and FUSION analysis to identify vulnerabilities present in CRC cells but not in nontransformed colon epithelial cells. The identification of EPHB4 as a novel effector of Myc signaling prompted us to evaluate the relationship between KSR1 and Myc and revealed that EPHB4 and KSR1 promote PGC1β expression via increased Myc mRNA expression and protein translation, respectively. While the regulation of PGC1β by Myc has been detected previously in renal cell carcinoma (34) and breast cancer cells (33), we show that this pathway is critical for colon cancer cell survival. Taken together, these data reveal that tumor cells in various cancers have a unique dependence on Myc-dependent expression of PGC1β to promote cell survival, which may be exploited in the development of new cancer therapeutics.

Using KSR1 as a reference standard, we used FUSION (16, 19) to identify the EPHB4 gene as a gene that is required for colon

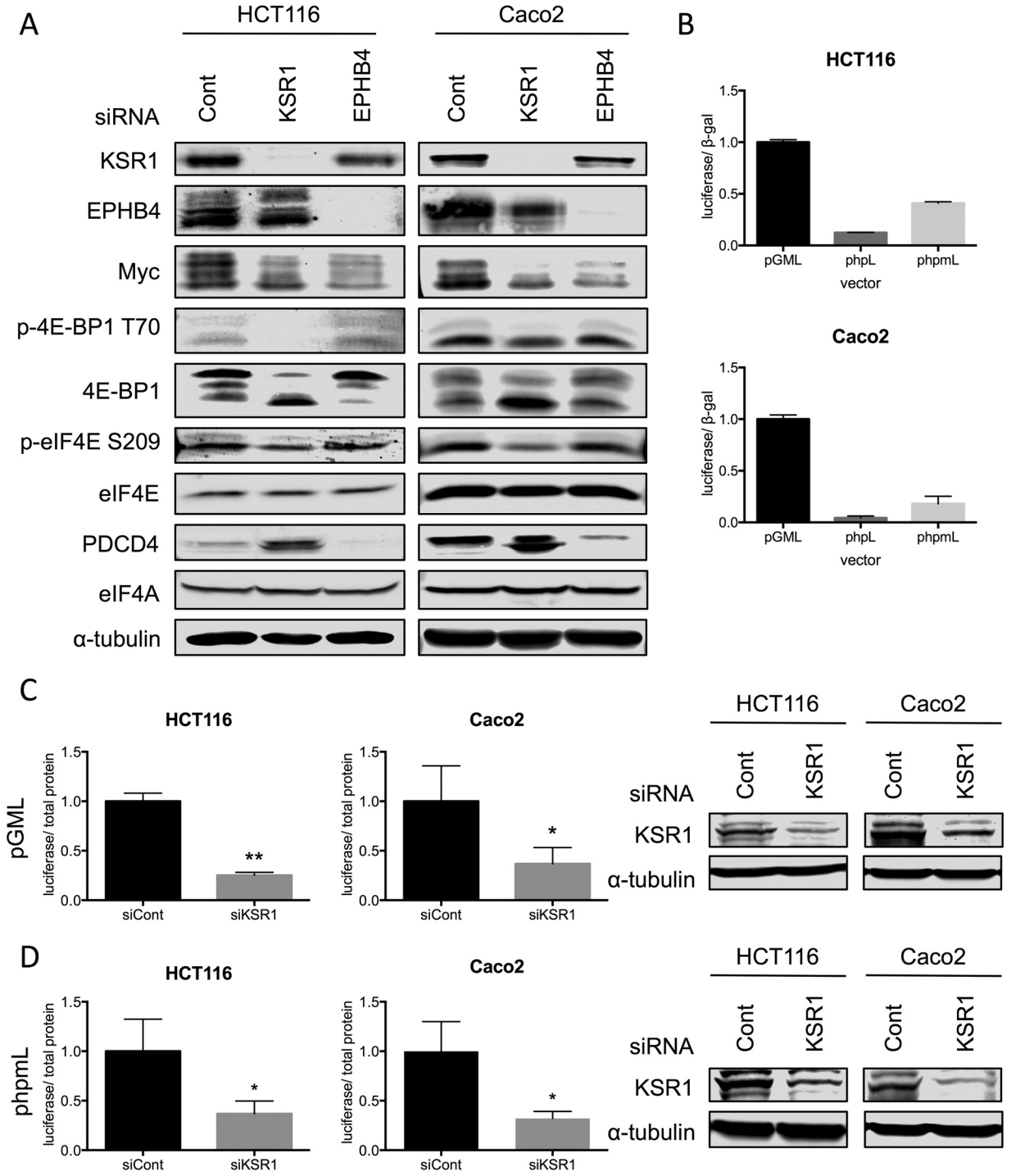

FIG 8 KSR1 promotes the translation of Myc in HCT116 and Caco2 cells. (A) HCT116 and Caco2 cells were transfected with siRNA targeting KSR1 or EPHB4 or a nontargeting siRNA (Cont) for 48 h. Protein expression levels were assessed by Western blotting. (B) HCT116 and Caco2 cells were transfected with pGML (Myc 5′ UTR), phpL (hairpin only), or phpmL (hairpin-Myc 5′ UTR) luciferase reporter constructs with a pSV-β-galactosidase vector for 24 h. Luciferase expression was normalized to β-galactosidase expression in each well. The data are shown as technical replicates of a single biological replicate. (C) HCT116 and Caco2 cells were depleted of KSR1 for 24 h and then transfected with pGML for an additional 24 h. Luciferase expression was normalized to total protein in each well. The data are shown as means and SD. *, $P < 0.05$; **, $P < 0.01$ (paired, two-tailed *t* test). (D) HCT116 and Caco2 cells were depleted of KSR1 for 24 h and then transfected with phpmL for an additional 24 h. Luciferase expression was normalized to total protein in each well. The data are shown as means and SD. *, $P < 0.05$ (paired, two-tailed *t* test).

tumor cell survival. The mechanistic role that EPHB4 plays in cancer remains controversial. However, a preponderance of data indicate that EPHB4 is overexpressed broadly in human cancers, including cancers of the head and neck, prostate, bladder, ovaries, large intestine, lung, brain, pancreas, and esophagus (46–54). Further research has shown that the ablation or inhibition of EPHB4 in a number of cancer cell types reduces tumor cell viability, including prostate (47), bladder (48), ovarian (49), colon (50), lung

(51), head and neck squamous cell carcinoma (74), and esophageal (54) cancer. Additionally, patient data have shown that EPHB4 levels negatively correlate with overall patient survival in ovarian cancer and glioblastoma (49, 52).

Expression of EPHB2 and EPHB4 is regulated by Wnt/β-catenin signaling in human CRC (50, 75). β-Catenin's binding partners, p300 and CBP, determine which gene is transcribed, with p300 promoting EPHB2 expression and CBP promoting EPHB4 (50). EPHB2 is present in the normal colon and EPHB4 is expressed only when tumors arise (50). These data contrast with studies showing that EPHB4 is expressed in human colonic crypts and early CRC lesions (76), followed by promoter hypermethylation and epigenetic silencing in more advanced stages (77). Further evidence indicated that EPHB4 expression is highest at the bases of crypts, suggesting that it plays an important role in maintaining the population of stem and progenitor cells located in that region of the crypt (76). The repulsive interaction that occurs when the ephrin-B2 ligand-expressing cells of the upper region of the crypt come in contact with the EPHB4 receptor-expressing cells located at the base of the crypt suggests that this expression pattern may aid in compartmentalizing tumor cells and reducing the dissemination of such cells, serving as a tumor suppressor (76, 78–80). While it is controversial, the majority of studies support the idea that increased EPH forward signaling promotes cell segregation and is primarily tumor suppressive, whereas reverse signaling through the ephrin ligand is tumor promoting, driving neoangiogenesis and invasion (79, 81). However, EPHB forward signaling can also be cross-activated by fibroblast growth factor and ERBB receptors, leading to noncanonical forward signaling that promotes cell proliferation (24), thereby contributing to cancer growth.

We show that EPHB4 is overexpressed in a panel of colon tumor cell lines and that, like KSR1, EPHB4 depletion via siRNA or small-molecule inhibition is selectively toxic to colon tumor cells compared to immortalized but nontransformed HCECs. The mechanism of HCEC resistance to EPHB4 inhibition may result from the fact that EPHB4 expression is minimal in the cell line and the cells do not rely on its overexpression for survival. These data are consistent with previous studies showing that EPHB4 is absent in normal colon but was expressed in 102 human colorectal cancer sections analyzed by both immunohistochemistry and RT-qPCR (50).

Lysosomal degradation of RTKs is well documented (82). Here, we show that EPHB4 is primarily degraded via the lysosome and that KSR1 forestalls that degradation. Canonical lysosomal RTK degradation occurs following activation of the receptor by its respective ligand. However, ligand-independent receptor degradation has been reported (83, 84). KSR1-dependent effects on Myc and PGC1β are not simply due to KSR1 stabilizing EPHB4. This prediction is based on the differential effects (transcriptional versus posttranscriptional regulation of Myc expression) observed with KSR1 and EPHB4 depletion. However, the observation that KSR1 promotes the stability of an RTK may allude to a broader mechanism by which KSR1 supports tumorigenesis.

Recent work from TCGA network discovered that, in a comprehensive examination of human colon and rectal cancers with diverse anatomical origins and mutation statuses, changes in Myc transcriptional targets were found in nearly 100% of the tumors (6), suggesting an important role for Myc in CRC. While a promising target for CRC, Myc is a transcription factor and traditionally considered "undruggable" (35, 85). Although there are new strategies emerging to inhibit Myc, including interrupting key dimerization events or DNA binding (35), finding additional or alternative ways to target Myc protein expression or its downstream effectors may provide therapeutic benefits to many cancer patients.

Our studies show that EPHB4 regulates Myc expression through the promotion of mRNA levels. KSR1 does not share this mechanism of action, which led us to examine alternative explanations for its ability to increase Myc levels in human colon tumor cells. Further analyses of posttranscriptional mechanisms revealed that KSR1 promotes the translation of Myc protein. Myc translation can be initiated via cap-dependent and -independent (IRES-dependent) mechanisms (42, 71). Our data suggest that KSR1 can promote both mechanisms of Myc protein synthesis (Fig. 7C and D). However, depletion of KSR1 also increases PDCD4 and suppresses the phosphorylation of 4E-BP1 (Fig. 7A), key inhibitors of global translation. This observation raises the intriguing possibility that KSR1-dependent tumors preferentially upregulate global protein synthesis in support of the transformed phenotype. Tumors can develop an enhanced ability to promote cap-dependent translation by overexpressing eIF4E or loss of 4E-BP (reviewed in reference 86). However, during apoptosis, growth arrest, mitosis, hypoxia, or amino acid starvation, cap-dependent translation is suppressed and IRES-mediated translation is induced (72, 87). In addition to Myc, IRES-dependent translation of mRNAs encoding hypoxia-inducible factor 1α (HIF1α), vascular endothelial growth factor A (VEGFA), Bcl2, X-linked inhibitor of apoptosis (XIAP), and p120 catenin, has been reported (reviewed in reference 86). It is important to note that we have tested only the ability of KSR1 to promote IRES-mediated translation of Myc. However, if KSR1 promotes IRES-mediated translation, future studies may reveal transcripts that are selectively translated in a KSR1-dependent manner to promote cancer cell survival during times of stress.

Following hypoxia, PGC1β mRNA is decreased in renal clear-cell carcinoma cells by induction of MXI1, a repressor of Myc activity (34). Subsequent work demonstrated that Myc regulates Her2- and IGF1-dependent induction of PGC1β in breast cancer cells (33). Our work indicates that Myc also mediates EPHB4- and KSR1-dependent regulation of PGC1β expression. Combined with these reports, our data suggest that tumor cells with diverse origins find multiple ways to regulate Myc-driven PGC1β expression. The observation that KSR1 promotes 4EBP1 and decreases PDCD4 expression suggests that increased Myc translation is a consequence of a KSR1-dependent increase in global protein translation. Translation is the most energy intensive activity of a proliferating cell (88). This raises the intriguing possibility that the increased energy demand in tumor cells demonstrating elevated protein translation (68) is met by a coordinate increase in Myc-dependent expression of PGC1β to expand the metabolic capacity of the cells (73).

The identification of these relationships highlights an important aspect of FUSION. Although the screen was intended to identify genes whose knockdown mimicked that of KSR1 depletion in cancer cells whose transformation is driven by oncogenic Ras, it is designed to select hits based on phenotype, which is not necessarily limited to KSR1-specific pathways. Therefore, it may identify critical effectors, such as EPHB4, whose inhibition has the same effect as depletion of KSR1 but whose mechanism of action is

different. Although the HCT116 cell line used in our screen expresses RasG13D, our results demonstrate that the screen may also identify vulnerabilities in tumor cells (e.g., Caco2 cells) that do not express constitutively active Ras. This may lead to the identification of potential targets that are applicable in a wide variety of cancer cell types.

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We declare that we have no conflicts of interest.

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