IPO11 mediates β-catenin nuclear import in a subset of colorectal cancers

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Activation of Wnt signaling entails β-catenin protein stabilization and translocation to the nucleus to regulate context-specific transcriptional programs. The majority of colorectal cancers (CRCs) initiate following APC mutations, resulting in Wnt ligand—indepedendent stabilization and nuclear accumulation of β-catenin. The mechanisms underlying β-catenin nucleocytoplasmic shuttling remain incompletely defined. Using a novel, positive selection, functional genomic strategy, DEADPOOL, we performed a genome-wide CRISPR screen and identified IPO11 as a required factor for β-catenin-mediated transcription in APC mutant CRC cells. IPO11 (Importin-11) is a nuclear import protein that shuttles cargo from the cytoplasm to the nucleus. IPO11−/− cells exhibit reduced nuclear β-catenin protein levels and decreased β-catenin target gene activation, suggesting IPO11 facilitates β-catenin nuclear import. IPO11 knockout decreased colony formation of CRC cell lines and decreased proliferation of patient-derived CRC organoids. Our findings uncover a novel nuclear import mechanism for β-catenin in cells with high Wnt activity.

Introduction

During embryonic development and adult tissue homeostasis, the Wnt-β-catenin signaling pathway governs context-dependent transcriptional programs regulating stem cell renewal, cell proliferation, and differentiation (Wodarz and Nusse, 1998; Steinhart and Angers, 2018). In the absence of Wnt ligand, the destruction complex composed of APC, Axin, GSK3α/β, and CK1α earmarks β-catenin for proteosomal degradation (Dominguez et al., 1995; Marikawa and Elinson, 1998; Behrens et al., 1998; Peters et al., 1999). Activation of the pathway by Wnt stabilizes β-catenin, where it translocates into the nucleus to promote transcription of context-dependent target genes (Angers and Moon, 2009; Cadigan and Waterman, 2012).

Aberrant activation of Wnt-β-catenin signaling is a driver of colorectal cancer (CRC) initiation. APC loss-of-function mutations occur in 80% of CRCs (Cancer Genome Atlas Network, 2012) and inactivate the destruction complex, leading to stabilization and nuclear accumulation of β-catenin (Morin et al., 1997). Subsequent mutations in KRAS, TP53, and SMAD4 further promote tumor progression into carcinoma (Fearon, 2011; Fearon and Vogelstein, 1990); however, β-catenin signaling is still required for advanced tumor maintenance (Scholer-Dahirel et al., 2011; Dow et al., 2015). Despite this, there are no clinically approved therapeutics to target the Wnt-β-catenin pathway.

The nuclear localization of β-catenin is controlled by its rate of nuclear entry versus exit, along with retention factors anchoring β-catenin in the cytoplasm or nucleus. Pygo/Pygopus constitutively localizes to the nucleus and recruits Lgs/BCL9, which binds β-catenin to promote its nuclear localization (Townsley et al., 2004). TCF7L2/TCF4 acts similarly, binding β-catenin in the nucleus, leading to increased nuclear retention, whereas in the cytoplasm, β-catenin is bound by APC and Axin, preventing β-catenin nuclear localization (Krieghoff et al., 2006). Normally, APC continuously shuttles in and out of the nucleus. Mutations in APC in CRC remove its nuclear export signal, resulting in nuclear accumulation and promotion of β-catenin nuclear retention (Henderson, 2000; Rosin-Arbesfeld et al., 2003).

The molecular mechanisms underlying β-catenin nuclear transport remain unclear (Jameson et al., 2014). Proteins with a nuclear localization signal (NLS) are recognized by Importin-α proteins, a scaffold for Ran-binding Importin-β proteins (Rexach and Blobel, 1995). The directionality of cargo transport is regulated by the Ran-GTP gradient. It is thought that β-catenin binds the nuclear pore complex directly via its armadillo repeats,
which share homology with Importin-α and Importin-β. However, β-catenin does not associate with the same nuclear pore proteins that Importin-β1 (also known as KPNB1) utilizes for transport (Suh and Gumbiner, 2003), does not contain a classical NLS, nor does it bind Importin-β1 in vitro (Yokoya et al., 1999). The role of Ran in β-catenin nuclear import is still up for debate. Although β-catenin lacks a Ran-binding domain, its transport is inhibited by a nonhydrolyzable GTP analog or a dominant-negative Ran mutant (Fagotto et al., 1998). A conflicting study reported that β-catenin nuclear localization was independent of the Ran gradient (Yokoya et al., 1999). These inconsistencies point toward uncharacterized mechanisms of β-catenin transport, where additional factors may mediate β-catenin nuclear localization in parallel, or to different mechanisms that are context dependent. Nucleocytoplasmic trafficking mediated by Importin-β carrier proteins is necessary for a variety of cellular functions. The Importin-β family comprises 11 protein-coding genes in humans (KPNB1, IPO4, IPO5, IPO7, IPO8, IPO9, IPO11, IPO13, TNPO1, TNPO3, and TNPO3), and each has its own biological roles and transports unique sets of cargo (Kimura et al., 2014; Shalem et al., 2014; Hart et al., 2015). Given that specificity (Shalem et al., 2014; Hart et al., 2015). Given that β-catenin-mediated signaling (Fig. 2 F). This expression of TCF/LEF target genes independent of β-catenin, was resistant to induction of cell death triggered by AP20187 (Fig. 1 D). The DEADPOOL platform constitutes a robust system to conduct genetic suppressor screens for the identification of genes involved in signaling systems.

**IPO11** is required for β-catenin-dependent signaling in CRC cells

We performed a genome-wide β-catenin-DEADPOOL CRISPR screen to identify genes required for β-catenin-mediated transcriptional activity in the context of CRC harboring APC mutations (Figs. 1 C and 2 A). Strikingly, only eight genes with at least three gRNA with an enrichment Z score >3 were identified. Among these, β-catenin (CTNNB1) and its known cotranscriptional activator BCL9L (Kramps et al., 2002) were the top- and third-ranked genes in the screen, respectively (Fig. 2, A and B). The second-highest ranked gene was IPO11, a gene with no previous link to β-catenin-mediated signaling (Fig. 2, A and B). Validating the screen results, DLD-1 β-catenin-DEADPOOL cells infected with two independent gRNA targeting IPO11 were resistant to 1 nM AP20187 (Fig. 2 C). Overexpression of FLAG-IPO11 using cdNA resistant to IPO11 #1 and #2 gRNAs reactivated the reporter to 1 nM AP20187 (Fig. 2 C). Knockout (KO) of IPO11 reduced the activity of the pBAR β-catenin-dependent luciferase reporter, confirming that IPO11 is required for β-catenin-mediated transcription and not direct iCasp9 activity (Fig. 2 D). Expression of several β-catenin-dependent target genes previously found to be induced in CRC lines (Major et al., 2008) was also inhibited in IPO11/−/− cells (Fig. S1, B and C), confirming that the function of IPO11 is not limited to synthetic transcriptional reporters (Fig. 2 E). Overexpression of LEF1-VP16, which induces expression of TCF/LEF target genes independent of β-catenin, sensitized DLD-1 β-catenin-DEADPOOL cells infected with IPO11 gRNA to iCasp9-mediated cell death, suggesting that IPO11 functions upstream or at the level of β-catenin (Fig. 2 F). This indicates that IPO11 is required for expression of the β-catenin transcriptional program in DLD-1 CRC cells.

**IPO11** is required for β-catenin nuclear import in APC mutated CRC cells

We next set out to determine the mechanism underlying IPO11 action in the β-catenin signaling pathway. Compared with control, DLD-1 IPO11/−/− cells showed reduction in nuclear β-catenin protein (Fig. 3 A). In support, CRISPR-Cas9–mediated targeting of IPO11 in SW480 cells, another APC mutated CRC cell line, strongly inhibited the nuclear localization of β-catenin as detected by immunofluorescence (Fig. 3 B). Subcellular fractionation of HCT116 CRC cells containing an activating Ser45 mutation of β-catenin revealed that gRNA targeting of IPO11 had marginal effect on nuclear β-catenin in this context (Fig. 3 C). A similar observation was made in RKO CRC cells, which
do not harbor mutations within Wnt signaling components. Stimulation of RKO cells with WNT3A led to nuclear accumulation of β-catenin in both control and IPO11−/− RKO cells (Fig. 3 D). Furthermore, when we analyzed publicly available genome-wide CRISPR-Cas9 fitness screen data from CRC cell lines, we found the presence of APC mutation to be positively correlated with dependency on IPO11 for fitness (Fig. 3 E). These data suggest that IPO11 is required for β-catenin nuclear accumulation and signaling selectively in CRC cells harboring APC mutations.

IPO11 binds to β-catenin and mediates its nuclear import
IPO11 is known to directly interact with its cargos UBE2E3 and PTEN to mediate their nuclear transport (Plafker and Macara, 2000; Chen et al., 2017). Given that the observed decrease in nuclear β-catenin levels in IPO11−/− CRC cells is not attributable to changes in β-catenin mRNA levels (Fig. S1 A), we next determined whether IPO11 participated in β-catenin nucleocytoplasmic shuttling. First, using an in vitro binding assay, we observed that β-catenin interacted directly with IPO11 in a Ran-dependent manner (Fig. 4 A). Binding was confirmed in HEK293T cells

Figure 1. Development and validation of DLD-1 β-catenin-DEADPOOL cells. (A) Schematic of the β-catenin-dependent iCasp9 system. (B) AP20187 (AP) dose–response curve in DLD-1 β-catenin-DEADPOOL cells. Mean ± SD, n = 3 independent experiments. (C) Schematic of genome-wide suppressor screen to identify genes required for β-catenin signaling in CRC. (D) Crystal violet stain of DLD-1 iCasp9/Cas9 line transduced with lentiviral vectors enabling expression of LacZ, CTNNB1 #1, or CTNNB1 #2 gRNA and treated with EtOH or 1 nM AP20187. Representative of three independent experiments.
when overexpressed IPO11, but not Importin-β1, was coimmunoprecipitated with a constitutively active β-catenin S33A/S37A/T41A/S45A mutant (Fig. 4 B). Expression of a dominant-negative Ran mutant, Ran(Q69L), which constitutively binds GTP and disrupts Ran-dependent shuttling of cargo from the cytoplasm to the nucleus, inhibited the association of β-catenin with IPO11, suggesting this nuclear import mechanism is Ran dependent (Fig. 4 B). Accordingly, expression of ΔN-IPO11, which is a mutant predicted to be impaired in Ran binding, in the DLD-1 β-catenin-DEADPOOL cells rescued the AP20187-dependent killing (Fig. 4 C).

We next set out to identify β-catenin’s structural domains underlying its IPO11-dependent nuclear localization. To do so, we fused full-length β-catenin as well as three independent domains (N-terminal, armadillo repeats, and C-terminal) to eGFP-β-galactosidase to allow visualization by microscopy and to sufficiently increase the molecular weight of these fusion proteins to prevent passive diffusion through the nuclear pores (Fig. 4 D and Fig. S2 C). Each domain (N-terminal, armadillo repeats, and C-terminal) localized to the nucleus of control cells, but nuclear localization of full-length β-catenin and the isolated C-terminal domain was significantly decreased in IPO11−/− cells (Fig. 4, E and F). We conclude that determinants present on the C-terminus of β-catenin are required for IPO11-mediated nuclear translocation.

To distinguish whether IPO11 plays a role in active nuclear import or nuclear retention of β-catenin, we tagged β-catenin with a classical NLS to reroute its nuclear import through the
Importin-α/Importin-β complex. Since NLS-β catenin localized properly to the nucleus in IPO11−/− DLD-1 cells (Fig. 4 G), IPO11 does not play a role in the retention of β catenin in the nucleus.

Finally, a nuclear import assay was performed using purified FITC-conjugated β catenin and IPO11 in semi-permeabilized HeLa cells (Fig. S3, A and B). Although β catenin alone, in the absence of nuclear import factors, is able to transit into the nucleus, the addition of recombinant IPO11 increased its nuclear localization (Fig. 4 H). The addition of dominant-negative Ran(Q69L) blocked the IPO11 effect and hence confirmed the requirement for Ran GTPase activity in this process. We conclude that IPO11 interacts with β catenin and mediates its nuclear import in a Ran-dependent manner (Fig. 5 E).

**IPO11 mediates β catenin nuclear import in cancer**

Activating mutations within Wnt pathway components are required during CRC initiation and progression. Consistent with a role for IPO11 to sustain high levels of nuclear β catenin activity, its expression is upregulated in CRC and rectal tumor samples compared with normal matched tissues (Fig. S2 A; TCGA mRNA expression dataset via Oncomine, https://www.oncomine.org; Cancer Genome Atlas Network, 2012). To confirm the functional importance of IPO11 in this context, we observed a reduction of colony formation in IPO11−/− DLD-1 cells infected with gRNA targeting IPO11 (Fig. 5 A). For all CRC cell lines containing an APC mutation, their growth dependency on IPO11 positively correlated with CTNNB1 dependency (R = 0.56, P = 0.016; Avana...
Figure 4. **IPO11 binds β-catenin to mediate its nuclear transport.** (A) GST-pulldown assay using purified IPO11 and β-catenin proteins. (B) HEK293T cells were transfected with the indicated expression plasmids, and lysates were subjected to coimmunoprecipitations followed by Western blot. (C) Crystal violet stain of DLD-1 β-catenin-DEADPOOL cells expressing FLAG-ΔN-IPO11 and treated with EtOH or 1 nM AP20187. (D) Schematic of eGFP-β-galactosidase-β-catenin fusion constructs. FL, full length. (E) Confocal microscopy–based quantification of nuclear to cytoplasmic eGFP intensity ratios for the indicated constructs expressed in control or IPO11−/− clones. Each point represents a single cell, with data collected over two independent experiments. n > 25 for each group. Two-way ANOVA Sidak’s multiple comparison test. **, P < 0.01; ****, P < 0.0001. (F) Representative images of E. (G) DLD-1 cells overexpressing FLAG-β-catenin or FLAG-NLS-β-catenin and LacZ, IPO11 #1, and IPO11 #2 gRNA were fractionated into cytoplasmic and nuclear compartments and immunoblotted for indicated proteins. (H) FITC-β-catenin nuclear import assay in semipermeabilized HeLa cells, imaged by confocal microscopy. Each point represents a single cell, pooled
CRISPR library via DepMap, https://depmap.org; Fig. S2 B; Meyers et al., 2017).

To support these findings, we next studied the requirement of IPO11 for the growth of APC mutant CRC patient-derived three-dimensional organoids, which rely on cancer stem cell activity for growth (van de Wetering et al., 2015). KO of IPO11 in the organoids led to reduced expression of the β catenin target gene ASCL2, suggesting that IPO11 is required for β catenin activity in this context (Fig. S2 B). We then delivered gRNA in APC mutant CRC organoids and monitored their growth using a fluorescence-based imaging assay over multiple days (Fig. S3 C). Transduction of gRNAs targeting CTNNB1 or IPO11 reduced organoid growth only in Cas9-expressing organoids in which gene editing occurred (Fig. 5 C and D; and Fig. S3, D and E). We conclude that IPO11 is required for growth of CRC cells.

In this study using the DEADPOOL system, we identified IPO11 as a nuclear import factor for β catenin in CRC cells harboring APC mutations and high levels of constitutive ligand-independent signaling. Interestingly, IPO11 was identified as a site of insertion for the mouse mammary tumor virus in mammary tumors (Theodorou et al., 2007), the same context in which Wnt proteins were discovered (Nusse et al., 1984). In bladder cancer, IPO11 overexpression promotes migration and correlates with poor survival (Zhao et al., 2016, 2018). IPO11 also regulates PTEN nuclear localization, where IPO11 KO leads to tumor progression in the prostate (Chen et al., 2017). IPO11 may therefore play context-dependent, sometimes opposite, roles in different cancers.

Our study reveals that IPO11 is required in most CRC cell lines harboring APC mutations, whereas other tested CRC cell lines such as RKO and HCT116 show less IPO11 dependency for β catenin localization and activation. This genotype-specific requirement reveals that context-dependent mechanisms for β catenin nuclear translocation exist. It is possible that these mechanisms coexist in cells and may be differentially engaged in zones of low or high Wnt activity. Passive diffusion of β catenin to the nucleus may be sufficient for ligand-dependent β catenin signaling and in the context of direct β catenin activating mutations; however, high β catenin signaling requirements in tissue stem cells or in APC mutant tumor cells may require additional β catenin nuclear import provided by an active IPO11-mediated process. It is important to further understand the biochemical nature of β catenin–IPO11 interaction in the context of truncated APC proteins. It is clear from our study that in most APC mutant contexts, β catenin relies on IPO11 for nuclear import. This further our understanding of the molecular pathology of CRC.

The molecular mechanism underlying β catenin nuclear transport remains incompletely understood. Due to the homology of β catenin and Importin-α armadillo repeats, it has been suggested that β catenin mediates its own nuclear entry through the nuclear pore. In fact, overexpression of Importin-β1 reduces β catenin nuclear localization (Fagotto et al., 1998), which was thought to be due to competition between Importin-β1 and β catenin for nuclear pore binding. Importin-β proteins compete with one another for binding sites at the nuclear pore. However, β catenin overexpression does not block Importin-β nuclear localization (Yokoya et al., 1999), and β catenin fails to directly interact with nuclear pore proteins (Suh and Gumbiner, 2003), perhaps indicating that β catenin may be transported by an alternative Importin-β other than Importin-β1. For example, overexpression of Importin-β1 reduces nuclear transport of the IPO11 cargo UB2E3 (Pfankner and Macara, 2000). In agreement with a Ran/Importin-β mechanism, β catenin nuclear transport is temperature dependent and sensitive to depletion of ATP/GTP or the addition of nonhydrolyzable GTP analogues indicative of an active and energy-dependent process (Fagotto et al., 1998). We speculate that β catenin nuclear transport occurs in a similar manner to Importin-α, via parallel routes using both Ran/Importin-β-dependent and –independent mechanisms that could be engaged through two different nuclear targeting signals previously identified in β catenin (Suh and Gumbiner, 2003; Fig. 5 E). Interestingly, our results indicate that whereas the isolated N-terminal, armadillo repeats and C-terminal domains of β catenin all localize to the nucleus of CRC cells, only the C-terminal domain does so in an IPO11-dependent manner. The separation of β catenin molecular determinants supporting passive diffusion versus IPO11-mediated nuclear localization offers an additional level of context-dependent regulation of signaling output but also may help explain, in part, the conflicting reports describing the mechanisms of β catenin nuclear transport.

Materials and methods

Plasmids

To make the pBAR-iCas9 plasmid, the DNA fragment encoding for the FKBP-Casp9 (iCas9) fusion protein was PCR amplified from pMSCV-F-del Cas9/iRES.GFP (#15567; Addgene) and ligated downstream of 12× LEF-TCF elements (#12456) in a puromycin-resistant lentiviral plasmid. Lenti Cas9-2A-BsdR (#73310) and pLCKO (#73311) were obtained from Addgene. pH3 vector expressing (#12555; Addgene) IPO11 cDNA was a gift from Dr. Ian Macara (Vanderbilt University, Nashville, TN) that was used to clone IPO11 into a pcDNA3 backbone with Flag tag. HA-β catenin SA cDNA was cloned into pIRESpuro-CBP-2xTEV-StrepTag backbone (#17132; Addgene). β catenin, Ran, and IPO11 inserts were PCR amplified from cDNA and cloned into the respective backbones pET-28a (+), pMAL, and pGEX-GST. Flag-LeF1-VI16, Flag-IPO11, Flag-β catenin, and Flag-NLS-β catenin cDNA were PCR amplified and replaced the Cas9-BFP2 expression cassette in pAAVS1-tet-Cas9-BFP2 (#125519; Addgene) plasmid backbone. IPO11, β catenin, and Ran mutants were generated by PCR-based site-directed mutagenesis. eGFP-β gal-β catenin full-length and truncation plasmids were constructed by Gibson assembly and placed into pcDNA3 backbone.
Figure 5.  **IPO11 is required for CRC organoid growth.**  

(A) Crystal violet stain of colony formation of DLD-1 βcatenin-DEADPOOL cells transduced with LacZ, IPO11 #1, and IPO11 #2 gRNA.  

(B) RT-qPCR of ASCL2 in CSC171C organoid line transduced with LacZ, IPO11 #1, or IPO11 #2 gRNA. Mean fold change ± SD, representative of \( n = 3 \) independent experiments. Statistical analysis was performed by one-way ANOVA Dunnett’s test. *, \( P \leq 0.05 \); **, \( P \leq 0.01 \).  

(C) Fluorescence images of CSC171C organoid line expressing Cas9 and gRNA for LacZ, CTNNB1 #1, IPO11 #1, or IPO11 #2.  

(D) Points represent mean fold change ± SD of fluorescence of C representative of \( n = 3 \) independent experiments. Statistical analysis was performed by one-way ANOVA Dunnett’s test. ***, \( P \leq 0.001 \); ****, \( P \leq 0.0001 \).  

(E) Proposed model of βcatenin nuclear translocation through direct binding of βcatenin armadillo repeats and nuclear pore proteins via a Ran-independent mechanism. IPO11 directly binds βcatenin in the cytoplasm to mediate βcatenin nuclear import. In the nucleus, IPO11 binds to Ran-GTP, which leads to the dissociation of the cargo-importin-β complex. βcatenin accumulates in the nucleus.

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Development of the DLD-1 βcatenin-DEADPOOL cell line

DLD-1 cells were cotransduced with lent Cas9-2A-BsdR (#73310; Addgene) and pBAR-iCasp9 and selected with 8 µg/ml blasticidin and 100 µg/ml hygromycin B. Single cells were isolated by serial dilution. Clonal lines were tested for response to B/B homodimerizer (AP20187 #635058; Takara Bio) and for Cas9 cutting efficiency.

Cell culture

DLD-1, SW480, HeLa, and HEK293T cells were cultured in DMEM (4.5 g/liter D-glucose and L-glutamine; #11965; Thermo Fisher) and 5% FBS (Thermo Fisher) with 10% fetal bovine serum (Thermo Fisher) and 5% chloride. gDNA samples were bar coded by PCR amplification using i5 and i7 adapter primers for Illumina next-generation sequencing.

TruSeq adapter primers with i5 bar codes: 5'-AATGATACGCGACACCGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3'; 5'-AATGATACGCGACACCGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3'; 5'-AATGATACGCGACACCGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3'; 5'-AATGATACGCGACACCGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3'.

Viral production and transductions

Lentivirus was produced in HEK293T by calcium phosphate transfection of a 50% confluent 100-mm dish with 2 µg vesicular stomatitis virus glycoprotein, 5 µg psPAX2, and 5 µg lentiviral vector. 24 h after transfection, medium was discarded and fresh medium was added. 48 h after transfection, virus-containing medium was collected and centrifuged at 1,000 g for 5 min and 0.45 µm filtered. DLD-1 and SW480 cells were transduced with virus in the presence of 8 µg/ml polybrene.

Virus intended for organoid cultures was concentrated with Lenti-X Concentrator (Takara) and incubated at 4°C overnight, centrifuged at 1,500 g for 45 min, and resuspended in 200 µl of 1× PBS. To transduce organoids with lentivirus, the protocol was adapted from Koo et al. (2012). In brief, pellets were resuspended in 200 µl infection media (CRCA media plus 10 µM Y-27632, 10 µM CIHR-99021, 10 mM nicotinamide, and 8 µg/ml polyan brene) and counted. 2.5 × 10⁴ cells were transfected to a round bottom tube, and 5 µl of concentrated virus was added. Transductions were spinoculated for 1 h at 600 g at 32°C, then transferred to a 37°C 5% CO₂ incubator for 6 h. Transduced organoids were transferred to an Eppendorf tube and centrifuged for 45 min, and resuspended in 200 µl of CRC media for 3 min at RT. Virus-containing supernatant was removed, and pellets were resuspended in 125 µl of Matrigel (Corning). 25 µl of Matrigel was seeded into four wells of a 48-well plate, and 250 µl CRC media was added per well and changed every 2-3 d.

Positive selection screen

The screen was adapted from Hart et al. (2015) using the TKOv1 CRISPR/Cas9 gRNA library. Briefly, the DLD-1 βcatenin-DEADPOOL cell line was infected with the TKOv1 library, which contains 91,320 gRNA targeting 17,232 human genes, with 8 µg/ml polybrene at an MOI of 0.3 at 24 h after infection. Cells were subsequently treated with 3.5 µg/ml of puromycin for 48 h. At 72 h, this population of gene-edited cells was split and cultured in the presence of EtOH (vehicle control) or 1 nM AP20187 and cultured in biological triplicate populations. EtOH-treated cells were passaged every 3 d and harvested on day 23. AP20187-treated cells were not passaged, and medium was changed every 3 d.

Genomic DNA was extracted using the QiAamp DNA Blood Maxi Kit (Qiagen) and precipitated using ethanol and sodium chloride. gDNA samples were bar coded by PCR amplification using i5 and i7 adapter primers for Illumina next-generation sequencing.

TruSeq adapter primers with i7 bar codes: 5’-CAAGCAGAAGACGGCATACGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3’; 5’-CAAGCAGAAGACGGCATACGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3’; 5’-CAAGCAGAAGACGGCATACGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3’; 5’-CAAGCAGAAGACGGCATACGAGATGTTCAGACTCGTATGCCGTCTTATACGAATAC-3’.

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counted. 500 cells were seeded per condition and were cultured for 12 d with media changes every 2–3 d. The cells were fixed with 100% ice-cold MeOH for 20 min at −20°C. Cells were stained with crystal violet solution (0.5% crystal violet and, 25% methanol) for 45 min at RT. Crystal violet solution was removed, and cells were washed five times with distilled H2O.

Organoids were monitored by fluorescence imaging using the Cytation 5 Imaging Reader (BioTek) every 2–3 d. Multiple fluorescence images were stitched, and the intensity was quantified using GenS software.

**CRC organoid passaging**

Embedded Matrigel culture of CRC organoids (obtained from Dr. Catherine O’Brien, University Health Network, Toronto, Ontario, Canada) was adapted from Sato et al. (2011). Briefly, media were removed, and 500 µl of TrypLE Express (Thermo Fisher) was added per well. Matrigel fragments were scraped, transferred to an Eppendorf tube, and incubated at 37°C for 20 min. 2 ml of cold Advanced DMEM/F12 supplemented with Pen-Strep, 2 mM GlutaMAX, and 10 mM Heps was added. Dissociated organoids were pelleted by centrifugation at 250 g for 5 min at 4°C, and supernatant was removed. To keep organoids for passaging, 225 µl of Matrigel was added per dissociated well. 25 µl of Matrigel was seeded per well of a 48-well plate, and 250 µl of organoids media (Advanced DMEM/F12, 2 mM GlutaMAX-1, 10 mM Heps, 100 U/ml penicillin/streptomycin, B27, 1.25 mM N-acetyl-L-cysteine, 20 mM [Leu15]-Gastrin I, 50 ng/ml mouse EGF, 100 ng/ml mNoggin, and 0.5 µM A83-01) was added per well and changed every 2–3 d.

**TOPFlash reporter assay**

Lentivirus containing the TOPFlash β-catenin-dependent Firefly luciferase and a normalizing Renilla luciferase were used to generate a stable DLD-1 TOPFlash cell line with 100 µg/ml hygromycin B selection. DLD-1 TOPFlash cells were subsequently transduced with lentivirus containing gRNA. After 24 h, transduced cells were selected for gRNA integration with 2 µg/ml puromycin. 7 d after gRNA infection, DLD-1 TOPFlash cells were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (ΔΔCt method (Bookout et al., 2006). The qPCR primer sequences are as follows: ASCL2 forward: 5’-CCCTCCACAGCTCAAGTTA-3’, ASCL2 reverse: 5’-GGACCAACACCTTGAGATT-3’, AXIN2 forward: 5’-CTCCCCACCTTGAAATGAGA-3’, AXIN2 reverse: 5’-TGGCTCTGTGCAAGACATAG-3’, KIAA1999 forward: 5’-AGA GTGAGCCAGCTGATGTT-3’, KIAA1999 reverse: 5’-ACTGTCTCG GCTACAGACCC-3’, ND1 forward: 5’-TGAAGAGATGGAGAG AGTGAGCCA-3’, ND1 reverse: 5’-GGTGACCTGCGTTGTT GTGAAA-3’, TDDG1 forward: 5’-AAATGGCCCATGTCACACTC-3’, TDDG1 reverse: 5’-CGATGCTAAGCCTCTTTT-3’, TNFRSF19 forward: 5’-GGTGGTGTACTAAAGAATGGT-3’, TNFRSF19 reverse: 5’-GCTGACCAATTTTCTTCTG-3’, cyclinophilin B forward: 5’-GGATGAGCACAAGGAGAA-3’, cyclinophilin B reverse: 5’-GCCGTAGTGCTTCAGTTTT-3’, IP011 forward: 5’-TCTGCT TTCAGGATCTTCCG-3’, IP011 reverse: 5’-TTTTCAGCCTTGGCT TTGCT-3’, CTNNB1 forward: 5’-TACCTCCAAAGCTGTAATGA G-3’, CTNNB1 reverse: 5’-TGAGGAGCATCAAAGCTGTAGT-3’. 50 ng/ml mouse EGF, 100 ng/ml mNoggin, and 0.5 µM A83-01 were added per well. Matrigel fragments were scraped, transferred to the manufacturer’s protocol (NE-PER kit; Thermo Fisher). Fractions were quantified and 10 µg of protein was loaded into each well.

**Western blot antibodies**

Primary antibodies were purchased from the respective vendors: rabbit anti-BCL9 (A303-152A; Bethyl), rabbit anti-IP011 (A304-811A; Bethyl), rabbit ERK1/2 (9102; Cell Signaling), rabbit β-catenin for Western blot (#9587; Cell Signaling), rabbit Lamin B1 (#16048; Abcam), mouse anti-β-tubulin clone TUB 2.1, rabbit anti-Flag (2368; Sigma-Aldrich), mouse anti-HA (clone 16B12; Covance), mouse myc antibody (#9E10; Abcam), and GST antibody (B-326, MA4-004). Secondary antibodies were conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc).
Cell Signaling) 1:50 antibodies in 0.2% Triton X-100 in PBS with 10% normal donkey serum at 4°C for 16 h. Coverslips were washed three times with PBS, then incubated with secondary anti-rabbit antibodies conjugated to Alexa Fluor 488 (Thermo Fisher) 1:500 in 0.2% Triton X-100 in PBS containing 10% normal donkey serum for 1 h in the dark at RT. Coverslips were washed five times for 5 min in PBS and mounted onto slides with Vectashield mounting medium (Vector Laboratories) containing DAPI. Images were acquired at room temperature on a laser scanning confocal microscope (LSM700; Carl Zeiss) at 12-bit with a Plan-Apochromat 63×/1.4 NA oil immersion objective using Zen software. To quantify β-catenin localization, images were analyzed in Imagej, the nucleus and whole cell were traced to two regions of interest, and intensity values for these areas were calculated. The relative nuclear intensity of β-catenin was determined by measuring the mean fluorescence intensity of β-catenin in the cytoplasm and nucleus of 50 cells per condition. The intensity and area of the cytoplasm was calculated by subtracting the intensity and area of the nucleus from the whole cell. Statistical analysis was done by one-way ANOVA Dunnett’s test.

Various plasmids encoding fusion constructs between eGFP-β-galactosidase and either full-length β-catenin or isolated β-catenin domains were transfected along with a plasmid coding for H2B-mCherry (to label nuclei) in DLD-1 cells with lipofectamine2000 (Thermo Fisher) for 8 h followed by media change and overnight recovery. 24 h after transfection, cells were plated onto coverslips. After 24 h, cells were fixed in 2% paraformaldehyde at RT for 10 min. Coverslips were mounted onto slides with Vectashield. Images were acquired at room temperature on a laser scanning confocal microscope (LSM700; Carl Zeiss) at 12-bit with Plan-Apochromat 20×/1.4 NA oil immersion objective using Zen software. Nuclear and cytoplasmic fluorescence intensity was quantified using CellProfiler (Broad Institute; https://cellprofiler.org). Briefly, cells were identified using H2B-mCherry expression to localize the nuclei. Cell bodies were determined using the Distance-B method. Mean fluorescence intensity of the eGFP constructs was measured in each compartment: nucleus and cytoplasm (defined as nucleus minus cell body). Ratios of nuclear/cytoplasmic mean fluorescent intensity were calculated and normalized to LacZ-eGFP-β-gal condition, which was set to 1. Statistical analysis was done by two-way ANOVA Sidak’s multiple comparison test.

**GST-pulldown assay**

BL21 bacteria were transformed with protein expression vectors pGEX-4T1 and pET-28B for GST and His-tagged proteins, respectively. Induction of protein expression was initiated at OD600 of 0.8-1.0 with 0.5 mM IPTG as follows: GST, GST-Importin-β1, GST-IPO11 4 h at RT, His-β-catenin 3 h at 37°C. Bacterial pellets were lysed with GST lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.2 mg/ml lysozyme, and 1 mM T-Cep) or His lysis buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, with 1 mM T-Cep, and 1 mM PMSF) and sonicated. Lysates were cleared by centrifugation at 16,000 g for 30 min at 4°C and incubated with glutathione-sepharose 4B (GE Healthcare) or Hislink (Promega) resin. Bound proteins were washed with GST lysis buffer or His wash buffer (His lysis buffer with 60 mM imidazole), and His-β-catenin was eluted from beads with 500 mM imidazole in lysis buffer. GST beads were resuspended in 500 µl of binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 20% glycerol, and protease inhibitor cocktail [Sigma-Aldrich]), incubated with purified His-β-catenin protein for 2 h at 4°C, washed with binding buffer, and eluted with 4× Laemmli buffer.

**Nuclear import assay**

The protocol was adapted from a previous study (Cassany and Gerace, 2009). GST-purified β-catenin was conjugated to FITC using a commercial kit (Thermo Fisher). Dye ratio was estimated to be 1.2 molecules per molecule of protein. pcDNA3.1-FLAG-IPO11 was transfected in 293T cells and 48 h after transfection was purified using FLAG-M2 beads (Sigma-Aldrich). IPO11 was eluted using 100 µg/ml FLAG peptide (Sigma-Aldrich). Both β-catenin and IPO11 were concentrated using Amicon 30-kd filters (Millipore). For the import assay, HeLa cells were grown in eight-well chamber slides. The following day, cells were permeabilized in 0.004% digitonin (Thermo Fisher), the cytoplasm was washed out, and the indicated components (360 µM FITC-β-catenin, 2 µM Ran or RanQ69L, and 600 nM IPO11) in transport buffer (20 mM Hepes, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, and protease inhibitor [Sigma-Aldrich]) were added. Cells were incubated for 30 min at 37°C. Following transport washout, cells were fixed in 4% paraformaldehyde and coverslips were mounted using Vectashield containing DAPI (Vector Laboratories). Images were acquired at room temperature on a laser scanning confocal microscope (LSM700; Carl Zeiss) at 12-bit with Plan-Apochromat 20×/1.4 NA oil immersion objective using Zen software. Nuclear and cytoplasmic fluorescence intensity was quantified using CellProfiler, the cytoplasm was washed out, and the indicated components (360 µM FITC-β-catenin, 2 µM Ran or RanQ69L, and 600 nM IPO11) in transport buffer (20 mM Hepes, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, and protease inhibitor [Sigma-Aldrich]) were added. Cells were incubated for 30 min at 37°C. Following transport washout, cells were fixed in 4% paraformaldehyde and coverslips were mounted using Vectashield containing DAPI (Vector Laboratories). Images were acquired at room temperature on a laser scanning confocal microscope (LSM700; Carl Zeiss) at 12-bit with Plan-Apochromat 20×/1.4 for quantification and 40×/1.4 for representation of NA oil immersion objective using Zen software. Nuclear intensity was quantified using CellProfiler, nuclei were identified using DAPI stain, and intensity was measured in the FITC channel. Nuclear intensity of each replicate was normalized to the β-catenin-alone condition and expressed at relative FITC intensity.

**Gene essentiality analysis**

Essentiality data for IPO11 in colon/colorectal cell lines were retrieved from PICKLES (Lenoir et al., 2018) using the 18Q4 Avana library dataset. The APC mutation status of cell lines was retrieved from COSMIC using the DepMap online tool (Meyers et al., 2017; https://depmap.org/portal) and binned according to their mutation status (wild type or mutant).

**Statistical analysis**

Statistical analysis was performed using Graphpad Prism Software. Data are represented as mean ± SD. Statistical analysis of multiple samples was performed by one-way ANOVA Dunnett’s test unless stated otherwise. In cases with uneven sample sizes, the Mann-Whitney U test was used.

**Online supplemental material**

Fig. S1 shows that IPO11 KO does not affect β-catenin transcription. Fig. S2 shows IPO11 expression and gene dependency in CRCs.
IPO11 mediates β-catenin nuclear import in cancer

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