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Supplementary Information for

mTORC1 signaling suppresses Wnt/β-catenin signaling through DVL-dependent regulation of Wnt receptor FZD level

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This PDF file includes:

SI Materials and Methods

Cell culture

All cell lines were maintained at 37 °C with 5% CO_2. HEK293T and immortalized mouse embryonic fibroblast (MEF; kind gifts from Dr. David J. Kwiatkowski, Brigham and Women's Hospital, USA) cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone). SNU-398 cells were obtained from ATCC and cultured in RPMI1640 (Life Technologies) supplemented with 10% FBS. All cell lines were banked at Novartis Cell Bank, authenticated by SNP fingerprinting and routinely tested as mycoplasma-free. None of these cell lines was found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI BioSample.

Lentivirus production

To generate lentiviruses, 1 μg of guide RNA (gRNA) plasmid was co-transfected into HEK293T cells with approximately 80% confluence in a well of the 6-well plate (Corning) along with 1 μg packaging (Δ8.9) and 0.25 μg envelope (VSV-G) expression plasmids using FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Cell culture media was changed the following day and lentivirus-containing supernatant was harvested 48 h later and filtered through a 0.45 μm filter (Millipore). Lentiviruses were aliquoted and stored at -80 °C for later use.

Generation of cell lines

Immortalized MEF cells were infected by lentiviruses expressing Cas9 and STF-GFP reporter, and clonal cells were selected and experimentally validated. To delete *Tsc2* or *Fzd2*, MEF-Cas9-STF-GFP cells were infected by lentivirus expressing *Tsc2* or *Fzd2* targeting gRNA in the presence of a serum-containing medium supplemented with 8 μg/ml polybrene (Sigma). Following infection for 24 h, cell culture media was refreshed with media containing 10 μg/ml puromycin (Sigma) and cells that stably expressed the gRNA were selected. Knockout efficiency of *Tsc2* was examined by immunoblotting assay.

To generate *Znrf3/Rnf43* double knockout cells, MEF-Cas9-STF-GFP cells were co-transfected with plasmids expressing gRNAs targeting *Znrf3* and *Rnf43* using FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's instructions. At 24 h post transfection, cells were selected with 10 μg/ml puromycin for 2 days. Following removal of puromycin, cells were allowed to recover in regular growth media and single cell clones were expanded and screened by FACS analysis of the STF-GFP reporter activity in response to Wnt3a and RSPO-2 (R&D Systems) treatment. MEF ZR DKO cells were further confirmed by genomic DNA sequencing and used in this study. To generate *Dvl1/2/3* triple knockout cells, MEF-Cas9-STF-GFP cells were co-transfected with plasmids expressing gRNAs targeting *Dvl1/2/3*. At 24 h post transfection, cells were selected with 10 μg/ml puromycin for 2 days. Following removal of puromycin, cells were allowed to recover in regular growth media and single cell clones were expanded and screened by FACS analysis of the STF-GFP reporter activity in response to Wnt3a and CHIR99021 (Sigma) treatment. MEF DVL TKO cells were further confirmed by genomic DNA sequencing and used in this study. To generate *Ctnnb1* knockout cells, MEF-Cas9-STF-GFP cells were transfected with plasmid expressing gRNA targeting *Ctnnb1*. At 24 h post transfection, cells were selected with 10 μg/ml puromycin for 2 days. Following removal of puromycin, cells were allowed to recover in regular growth media and single cell clones were expanded and screened by FACS analysis of the STF-GFP reporter activity in response to Wnt3a and CHIR99021 (Sigma) treatment.

Guide sequences used in this study

*Tsc2*_g2: 5'-3' CAGTTGATGCGTAACCTTCT;
*Tsc2*_g4: 5'-3' AACAATCGCATCCGAATGAT;
*Fzd2*_g1: 5'-3' TTGGCGCGCGCGCTCGCAGA;
*Fzd2*_g2: 5'-3' GGACGGCACCTTGAGGACGC;
Znrf3: 5'-3' ATGATGTTGTGCCGACAGTG;
Rnf43: 5'-3' CAGGGGCGAGGAGCTCGTCG;
Dvl1: 5'-3' CCGACGTCGAAACCGCGATG;
Dvl2: 5'-3' CCGAATCTGTCGTATCACTG;

Dvl3: 5'-3' GATGAACTGTCATAGCCGCC;
Ctnnb1: 5'-3' ATGAGCAGCGTCAAACTGCG;
Apc: 5'-3' GTACACCTGCTGAATACGAG

Pooled CRISPR screening and data analysis

A mouse genome-wide sgRNA library targeting 18,360 genes was adapted from published sequences (1). For genes lacking published sgRNA sequence information, new sgRNAs were designed for these targets that contained an NGG PAM motif, filtering for GC content >40% and <80%, eliminating homopolymer stretches >4, and removing any guides with off-target locations having <4 mismatches across the genome. The sgRNA library was constructed using chip-based oligonucleotide synthesis to generate spacer-tracrRNA-encoding fragments that were PCR-amplified and cloned as a pool into the BpiI site of the pRSI16 lentiviral plasmid (Cellecta). Sequencing of the plasmid pool showed robust normalization, with >90% clones present at a representation of ±5-fold from the median counts in the pool.

The sgRNA libraries were packaged into lentiviral particles using HEK293T cells. Packaging was scaled up by growing cells in cell stacks (Corning). For each cell stack, 210 million cells were transfected 24 h after plating using 510.3 µl TransIT reagent (Mirus) diluted in 18.4 ml OPTI-MEM (Life Technologies) that was combined with 75.6 µg of the sgRNA library and 94.5 µg of lentiviral packaging mix (psPAX2 and pMD2 plasmids that encode Gag/Pol and VSV-G, respectively; Cellecta). 72 h post-transfection, lentivirus was collected, aliquoted, and frozen at -80 °C. Viral titer was determined using the Lenti-X qRT-PCR Titration Kit (Clontech) and was typically in the range of 5 x 10^6 transforming units/ml.

For the genome-wide screen, sgRNA libraries were transduced at a multiplicity of infection (MOI) of 0.5, aiming for coverage of, on average, 1,000 cells per sgRNA reagent. MOI was determined by using a 12-point dose-response ranging from 0 to 400 µl of viral supernatants in the presence of 5 µg/ml polybrene and measuring infection rate by FACS as a percentage of red fluorescent protein (RFP)-positive cells. Selection was optimized by determining the puromycin dose required to achieve >95% cell killing in 72 h. Cell viability was measured for a 6-point dose-response ranging from 0 to 20 µg/ml puromycin using the Cell Titer Glo assay (Promega). For the FACS-based screen, MEF-Cas9-STF-GFP cells were seeded into cell stacks (Corning). 24 h after plating, the culture media was replaced with fresh media containing 5 µg/ml polybrene and lentivirus at an MOI of 0.5. 24 h after infection, the culture media was replaced with fresh media containing 10 µg/ml puromycin. 72 h after puromycin selection, cells were trypsinized and plated into new cell stacks. An aliquot of cells was analyzed by FACS to confirm infection and selection efficiency, and the percentage of RFP-positive cells was typically >95%. 7 d after puromycin selection, cells were treated with Wnt3a conditioned media to stimulate the STF-GFP activity. For FACS, cells were harvested, resuspended at 30 million cells/ml, and live, single, RFP-positive cells were sorted using the BD FACSAria III cell sorter (BD Biosciences) from the lower GFP quartile (low GFP) or from the upper GFP quartile (high GFP). 100 million cells from each population were collected, and genomic DNA was isolated using the QIAamp DNA Blood Maxi Kit (Qiagen) and quantified using PicoGreen (Invitrogen). Illumina sequencing libraries were generated using PCR amplification with primers specific to the genome integrated lentiviral vector backbone sequence. The resulting Illumina libraries were purified using 1.8x SPRI AMPure XL beads (Beckman Coulter) following the manufacturer's instructions and qPCR quantified using primers specific to the Illumina sequences using standard methods. Illumina sequencing libraries were pooled and sequenced with a HiSeq 2500 instrument (Illumina). The number of reads was adjusted to cover each sgRNA with approximately 1,000 reads.

Raw sequencing reads were aligned to the appropriate library using Bowtie (2), allowing for no mismatches, and counts were generated. The R software package DESeq2 (3) was used to evaluate differential sgRNA representation in the form of log2 fold change and p-value between the GFP-high and the GFP-low samples. A robust z-score for each sgRNA was calculated using the median and mean-absolute deviation across the log2 fold changes. To summarize the results at the gene level, the sgRNAs are ranked by the robust z-score, and the statistical significances for each gene enriched toward higher rank (RSA up) and the lower rank (RSA down) were evaluated using the Redundant siRNA Activity (RSA) algorithm (4).

Flow cytometry

For frizzled staining, cells were collected using enzyme-free PBS-based cell dissociation buffer (Gibco) and resuspended in FACS buffer (PBS, pH 7.2, with 1% BSA). After blocking, cells were incubated with anti-pan-

frizzled (18R5) antibody or anti-Myc-Alexa fluor 488 (Cell Signaling Technology) antibody for 1 h on ice, followed by incubation with conjugated secondary antibodies for 1 h on ice where applicable. After extensive washes using FACS buffer, cells were stained with SYTOX Blue dead cell stain (Thermo Fisher Scientific, S34857) and subjected to multi-channel analysis using a CytoFLEX S flow cytometer (Beckman Coulter). Fluorescence signals from SYTOX Blue-negative cells were displayed in histogram plots. For STF-GFP reporter assay, cells were trypsinized, collected in cell culture media and subjected to flow cytometry analysis. Data were analyzed with FlowJo software (Treestar).

Immunoblotting and immunoprecipitation

Cells or tissues were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Thermo Fisher Scientific), followed by centrifugation at 13,000 r.p.m. for 20 min. Protein concentration was determined using the DC Protein Assay (Bio-Rad). Equal amount of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% Blotting-Grade Blocker (Bio-Rad) and then incubated overnight at 4 °C with the following primary antibodies at the indicated dilution in 5% BSA: anti-Myc-Tag (71D10) (#2278, 1:1,000), anti-IR-β (4B8) (#3025, 1:1,000), anti-TSC1 (#4906, 1:1,000), anti-TSC2 (#4308, 1:1,000), anti-LRP6 (#3395, 1:1,000), anti-phospho-LRP6 Ser1490 (#2568, 1:1,000), anti-DVL2 (#3216, 1:1,000), anti-Active β-Catenin (#8814, 1:10,000), anti-S6 (#2217, 1:10,000), anti-phospho-S6 Ser140/244 (#5364, 1:10,000), anti-GAPDH (#2118, 1:5,000), anti-GSK3β (#9315, 1:5,000), anti-phospho-GSK3β Ser9 (#9336, 1:1,000), from Cell Signaling Technology; anti-AP2M1 (ab75995, 1:1,000) from Abcam; anti-α-Tubulin (T6074, 1:20,000) and anti-β-Actin (A1978, 1:20,000) from Sigma; anti-β-Catenin (#610154, 1:10,000) from BD Biosciences. This was followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibody and visualization with the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Quantification of immunoblotting bands was performed by densitometry analysis with Fiji software (NIH).

For immunoprecipitations, cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and protease and phosphatase inhibitors. Cleared cell lysates were incubated with the indicated primary antibody with rotation overnight at 4 °C, followed by incubation with Pierce Protein A/G Magnetic Beads (Thermo Fisher Scientific) for an additional 2 h. Beads were washed four times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of SDS sample buffer and boiling for 10 min, resolved by SDS-PAGE, and analyzed via western blot analysis.

Biotinylation-based pulse-chase experiment

For assessing the half-life of membrane Myc-FZD2 protein, MEF-Myc-FZD2 cells were cultured in 10 cm tissue culture dish until 90% confluence and then treated with DMSO or 50 nM RAD001 prior to cell surface biotinylation using the Pierce Cell Surface Protein Isolation Kit (Pierce 89881). Briefly, cells were washed twice with 1x PBS (pH 7.2, Gibco 20012-027) and then incubated with 0.25 mg/ml Sulfo-NHS-SS-Biotin dissolved in PBS for 25 min at 37 °C. After terminating the cross-linking reaction using quenching solution, cells were washed twice with PBS, fed with fresh growth medium and returned to the 37 °C incubator. At the indicated time points, cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Thermo Fisher Scientific), and biotinylated proteins were isolated using NeutrAvidin agarose (100 µl slurry for each pulldown of 1 mg whole cell lysate) at 4 °C for overnight. The following day, agarose beads were washed three times with RIPA buffer and bound proteins were eluted in 1x LDS Sample Buffer (NuPAGE NP0007) supplemented with 50 mM DTT with gentle shaking on a vortexer for 40 min at room temperature. Cleared eluates were then subjected to immunoblotting analysis by running the 4-12% Criterion XT Bis-Tris Protein Gel (Bio-Rad #3450124).

RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA products were diluted and subjected to quantitative real-time PCR (qPCR) reactions using TaqMan Gene Expression Assays (Applied Biosystems). Specifically, qPCR was performed in 10 µl reactions consisting of 0.5 µl TaqMan

reagent, 5 µl TaqMan Fast Advanced Master Mix (Applied Biosystems) and 4.5 µl diluted cDNA template. Experiments were run on a ViiA 7 Real-Time PCR System (Applied Biosystems). The thermocycling conditions used were 20 s at 95 °C, followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. The threshold crossing value (Ct) was determined for each transcript and normalized to the housekeeping gene transcript (*Gusb* or *Actb*). The relative quantification of each mRNA species was assessed using the comparative ΔΔCt method.

TaqMan reagents used in this study are as follows: *Gusb* (Mm01197698_m1), *Actb* (Mm02619580_m1), *Fzd1* (Mm00445405_s1), *Fzd2* (Mm02524776_s1), *Fzd3* (Mm00445423_m1), *Fzd4* (Mm00433382_m1), *Fzd5* (Mm00445623_s1), *Fzd6* (Mm00433387_m1), *Fzd7* (Mm00433409_s1), *Fzd8* (Mm01234717_s1), *Fzd9* (Mm01206511_s1), *Fzd10* (Mm00558396_s1), *Axin2* (Mm00443610_m1), *Ascl2* (Mm01268891_g1), *Lgr5* (Mm00438890_m1), *Nkd1* (Mm00471902_m1), *Olfm4* (Mm01320260_m1), *Tsc1* (Mm01336938_m1), *Mki67* (Mm01278617_m1), *Cyp1a2* (Mm00487224_m1), *Cyp2e1* (Mm00491127_m1), *Glul* (Mm00725701_s1), and *Lect2* (Mm00521920_m1).

RNA sequencing

RNA sequencing libraries were prepared using the Illumina TruSeq RNA Library Prep Kit v2 following the manufacturer's instructions. Each library was sequenced in paired-end mode, 2 x 76 bp, using the HiSeq2500 platform. Reads were mapped to the *Mus musculus* genome (mm10) by using an in-house gene and exon quantification pipeline (5). The genome and the transcript alignments were used to derive gene counts based on the mouse Ensembl gene IDs (v76). Gene counts, which represent the total number of reads aligned to each gene, were then transformed into counts per million (CPM; normalization by total number of mapped reads per sample) and fragments per kilobase of exon per million of fragments mapped (FPKM; further normalization by effective gene length). After assessment by principal component analysis and multidimensional scaling, all samples were retained for further analysis, and genes not present with at least 1 CPM in at least half the samples of a group were excluded. Differential gene expression was calculated using edgeR and limma/voom in R (6). Results are reported in terms of log2 fold-changes and negative log10-adjusted *P* values (Benjamini-Hochberg false discovery rate).

Gene set enrichment analysis (GSEA)

The ISC signature and intestinal β-catenin targets were obtained from previous publications. For GSEA analysis, we used the GSEAPreranked tool, with the gene list ranked according to the log2 fold-change retrieved from the RNA-seq analysis, using the 'classic' method for calculating enrichment scores (7).

Mice

All animal work was performed in accordance with protocols approved by the Novartis Institutes for Biomedical Research Institutional Animal Care and Use Committee and the study was compliant with ethical regulations regarding animal research. *Rosa26-creERT2* mice were obtained from Taconic. *Tsc1*$^{fl/fl}$ mice were provided by Dr. David J. Kwiatkowski and backcrossed for at least six generations onto a C57BL/6 background in the animal facility at Novartis Institutes for Biomedical Research, Cambridge, Massachusetts. For *in vivo Tsc1* knockout, 8-week-old *Tsc1*$^{fl/fl}$*; Rosa26-creERT2* mice and control animals consisted of littermate *Tsc1*$^{fl/fl}$ mice negative for *Rosa26-creERT2* were treated with tamoxifen (Sigma T5648) suspended in corn oil (MP Biomedicals no. 901414) at a concentration of 20 mg/ml, and 10 ml per 1 kg of body weight was injected intraperitoneally into mice once daily for five successive days. Mice were euthanized for tissue collection 5 days after the last tamoxifen injection. Adult mice were used in all experiments, age-matched and not selected for gender, and both genders were included in each analysis. All mice had unrestricted access to water and food.

Immunohistochemistry (IHC) staining

Tissues were fixed in 10% (vol/vol) neutral-buffered formalin, paraffin embedded and sectioned. IHC staining was performed on formalin-fixed paraffin-embedded (FFPE) gut sections (4 µm thickness) using a Discovery Ultra platform (Ventana Medical Systems) according to the manufacturer's standard protocol. Slides were incubated for 1 h at room temperature with a rabbit anti-Ki67 polyclonal antibody (1:750, 0.3 µg/ml; Bethyl Laboratories, IHC-00375) followed by incubation with the OmniMap anti-Rb HRP secondary antibody (Ventana Medical Systems, Catalog Number 760-4311). The detection was performed with a DAB detection kit (Ventana Medical Systems) according to the manufacturer's instructions. Slides were counterstained with haematoxylin (Ventana Medical

Systems), and coverslips were added with Permount (Thermo Fisher Scientific). Image analysis was performed with Aperio ImageScope software (Leica Biosystems).

In situ hybridization (ISH)

Using the RNAscope VS Reagent Kit-BROWN (ACD) automated on the Discovery Ultra platform (Ventana Medical Systems), FFPE gut sections were baked and deparaffinized on the instrument, followed by cell conditioning for 16 min at 97 °C and protease treatment for 16 min at 37 °C. Sections were then hybridized with the *Olfm4* probe (ACD, Catalog Number 311839) for 2 h at 43 °C, followed by amplification for 32 min. DAB chromogenic detection was then performed followed by haematoxylin counterstain and dehydration. Image analysis was performed with Aperio ImageScope software (Leica Biosystems).

Crypt isolation and cell dissociation

Isolation of intestinal crypts and the dissociation of cells were largely performed as previously described (8). Small intestines were removed and the fat/mesentery was dissected away. The intestinal lumen was washed with ice-cold PBS (Mg^-/Ca^-) using a 10 ml syringe and 25G feeding needle until the intestines appeared white/pink. They were then opened longitudinally. Mucus and villi were gently scraped away using an angled forceps. The intestines were cut into 3-5 mm fragments, placed into 50 ml conical tubes that were filled with ice-cold PBS, and then washed twice. The intestine fragments were then incubated with 30 ml of 5 mM EDTA diluted in ice-cold PBS with gentle agitation on a nutator for 20 min at 4 °C, followed by manual shaking for 15 s and additional 20 min incubation on a nutator at 4 °C. Crypts were liberated and triturated with a 10 ml pipette 1 to 2 times, and the contents were filtered twice through a 70 µm cell strainer (BD Falcon) into a 50 ml conical tube to remove villous material and tissue fragments. At this point the suspension was mainly composed of crypts. Crypts were collected by centrifugation for 5 min at 200g at room temperature and used for crypt culture experiments and embedded in matrigel with crypt culture media.

Organoid culture

Isolated crypts or organoids were suspended in Corning Matrigel Basement Membrane Matrix (phenol red-free; Corning no. 356237), and 40-50 µl drops were plated onto a flat bottom 24-well plate (Corning) and allowed to solidify for 10-20 min in a 37 °C incubator. Five hundred microliters of crypt culture medium was then overlaid onto the matrigel, changed every 2-3 days, and maintained at 37 °C in fully humidified chambers containing 5% CO_2. The complete crypt culture medium consisted of Advanced DMEM/F-12 (Life Technologies no. 12634028) medium supplemented with 10 mM HEPES (Life Technologies no. 15630080), 2 mM Glutamax (Life Technologies no. 35050061), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies no. 15140122), 50 ng/ml EGF (PeproTech no. 315-09), 100 ng/ml Noggin (PeproTech 250-38), and 250 ng/ml R-Spondin1 (made in-house). For passaging of organoids, after 5-7 days of culture, organoids were passaged by mechanically disrupting with a seropipet and cold media to depolymerize the matrigel and generate organoid fragments. After washing away the old matrigel and cell debris by spinning down at 900g for 5 min followed by another spin at 200g for 5 min, organoid fragments were re-plated in liquid Matrigel as described above. Organoid images were taken using the Invitrogen EVOS FL Auto Cell Imaging System (Life Technologies).

Organoid knockout

For *Tsc1* knockout in organoids, crypts from $Tsc1^{fl/fl}$ (Cre-) littermates or $Tsc1^{fl/fl}$*; Rosa26-creERT2* (Cre+) mice were isolated and allowed for organoid formation as described above. 4-Hydroxytamoxifen (1 µM; Sigma H7904) was added to the crypt culture medium to induce *Tsc1* deletion. Media was replaced every other day. CRISPR-Cas9 mediated gene knockout was conducted by lentiviral infection as described previously (9). In brief, lentivirus was packaged as described above, concentrated using the Lenti-X Concentrator (Clontech) according to the manufacturer's instructions, and re-suspended in organoid infection medium consisted of complete crypt culture medium supplemented with 5 µM CHIR99021 (Sigma), 10 mM nicotinamide (Sigma no. N0636), 10 µM Y-27632 (Sigma) and 8 µg/ml polybrene (Sigma). Prior to infection, organoids were pre-treated with 5 µM CHIR99021 (Sigma) in crypt culture medium for 4 d. Organoids were then collected and subjected to two rounds of spin as needed for regular passaging (900g, 5 min; 200g, 5 min). Pellets were re-suspended in TrypLE Express Enzyme (Life Technologies no. 12605010) and incubated for 5 min at 37 °C in a water bath. Afterwards, crypt culture media was added and samples were centrifuged at 900g for 5 min. The prepared organoid cells were then combined with

250 µl of viral suspension in one well of a plat bottom 48-well plate (Corning). The plate was sealed with Parafilm and spinoculation was conducted by centrifugation of the plate at 900g for 40 min, followed by incubation in a tissue culture incubator (37 °C) for 5 h. Infected organoid cells were collected and spun down at 900g for 5 min, followed by re-suspension in Matrigel and cultured in infection medium without polybrene. After 2-3 days, infected organoids were selected using 1.5 µg/ml of puromycin. When puromycin-resistant organoids expanded, they were maintained in normal crypt culture medium and passaged when necessary.

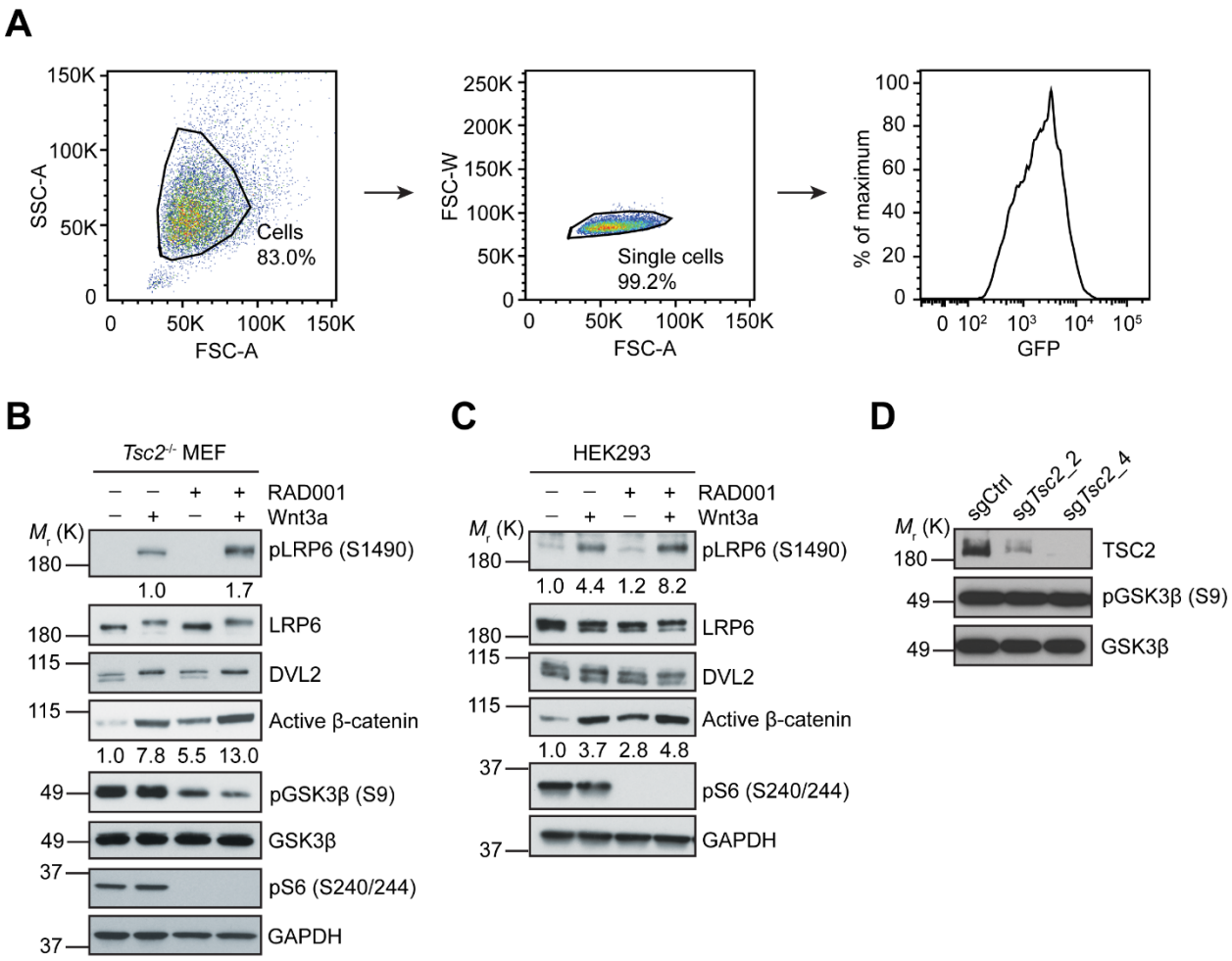

Fig. S1. mTORC1 signaling negatively modulates Wnt/β-catenin signaling. (A) Representative flow cytometry gating strategy to analyze STF-GFP reporter activity. Cells were gated using FSC-A/SSC-A characteristics, singlets were gated using FSC-A/FSC-W characteristics, and GFP signal was plotted as a histogram. (B) Immunoblots of indicated proteins in *Tsc2*$^{-/-}$ MEF cells treated with or without 50 nM RAD001 and Wnt3a conditioned media. (C) Immunoblots of indicated proteins in HEK293 cells treated with or without 50 nM RAD001 and Wnt3a conditioned media. (D) Deletion of TSC2 exerts no effect on GSK3β phosphorylation at serine 9 site in MEF cells.

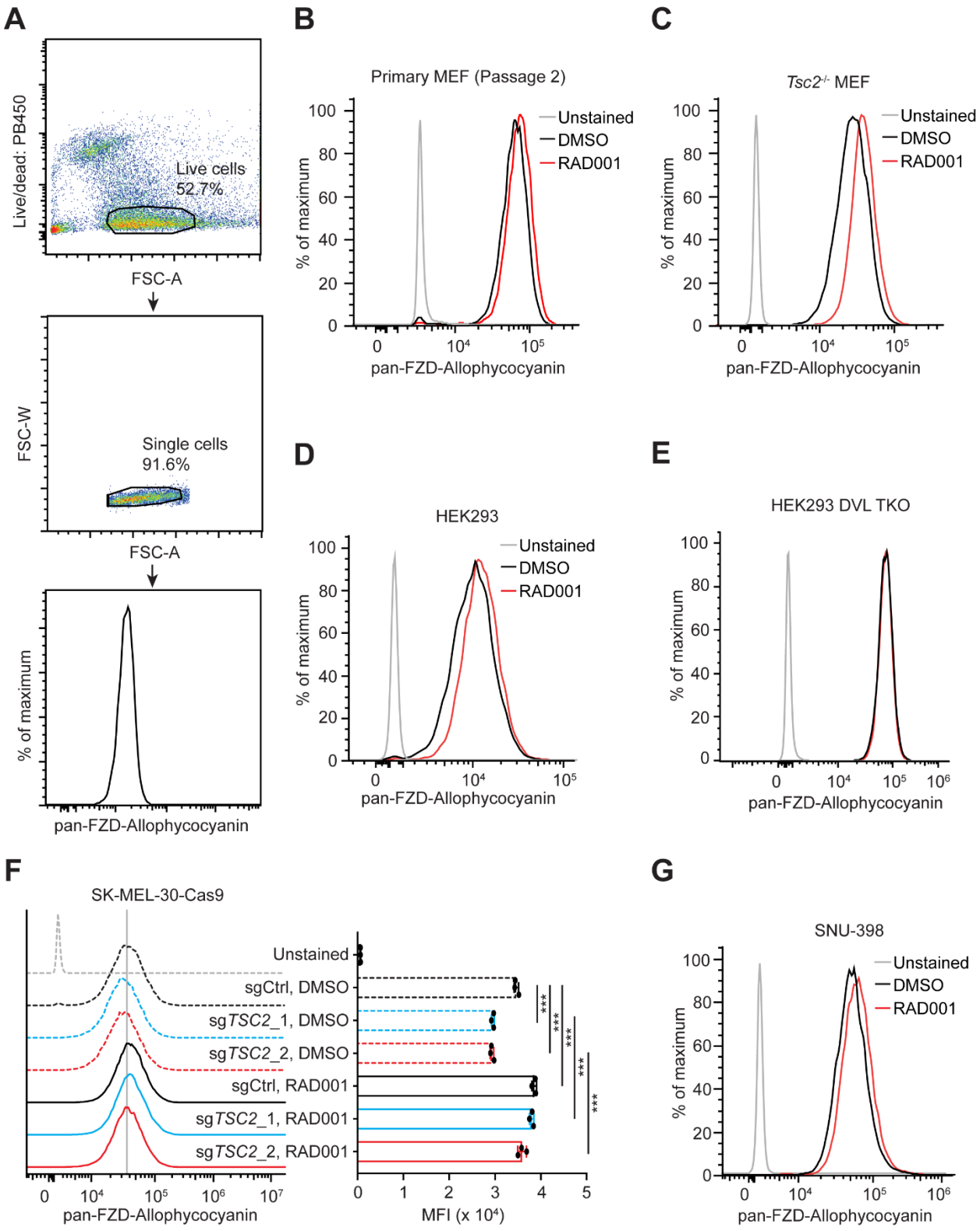

Fig. S2. Inhibition of mTORC1 activity by RAD001 treatment increases cell surface level of Wnt receptor frizzled. (A) Representative flow cytometry gating strategy to analyze cell surface level of endogenous Wnt receptor frizzled. (B-G) Flow cytometry analysis of membrane frizzled levels in primary MEF (B), *Tsc2*$^{-/-}$ MEF (C), HEK293 (D), HEK293 DVL TKO (E), SK-MEL-30 (F), and SNU-398 (G) cells treated with DMSO or 50 nM RAD001.

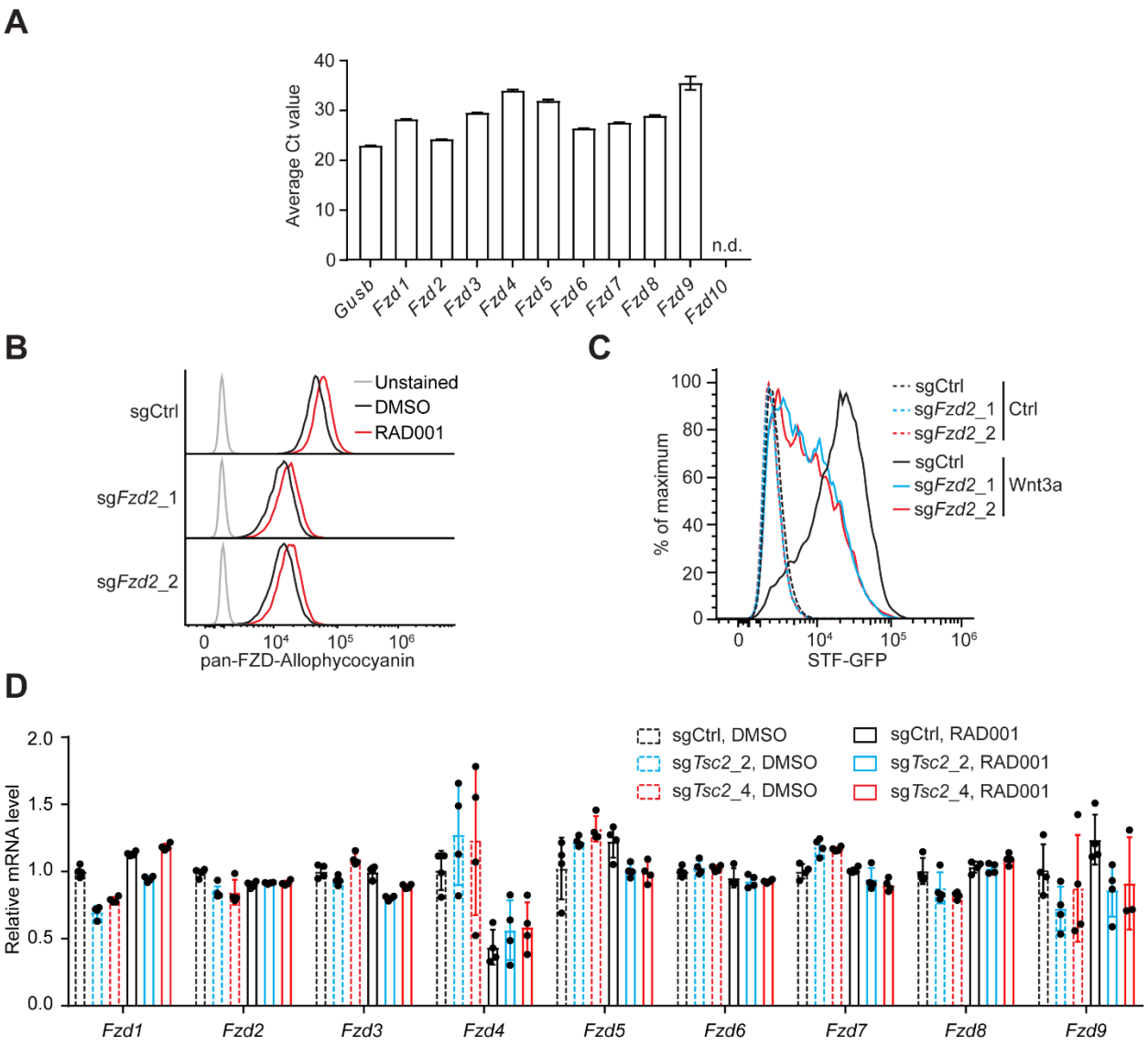

Fig. S3. FZD2 is the dominant FZD in MEF cells. (A) Quantitative RT-PCR analysis of all ten *Fzd* mRNA levels in MEF cells. Ct value of *Fzd2* is much lower than that of any other *Fzd*, suggesting that FZD2 is the dominant homolog in MEF cells. Housekeeping gene *Gusb* (glucuronidase beta) is used as an internal control. (B) Flow cytometry analysis of membrane frizzled levels in control or FZD2-deleted MEF cells treated with DMSO or 50 nM RAD001. (C) Deletion of FZD2 decreases Wnt3a-induced STF reporter activity, assessed by flow cytometry analysis of GFP. (D) Quantitative RT-PCR analysis of relative *Fzd* mRNA levels upon TSC2 deletion and/or RAD001 treatment in MEF cells.

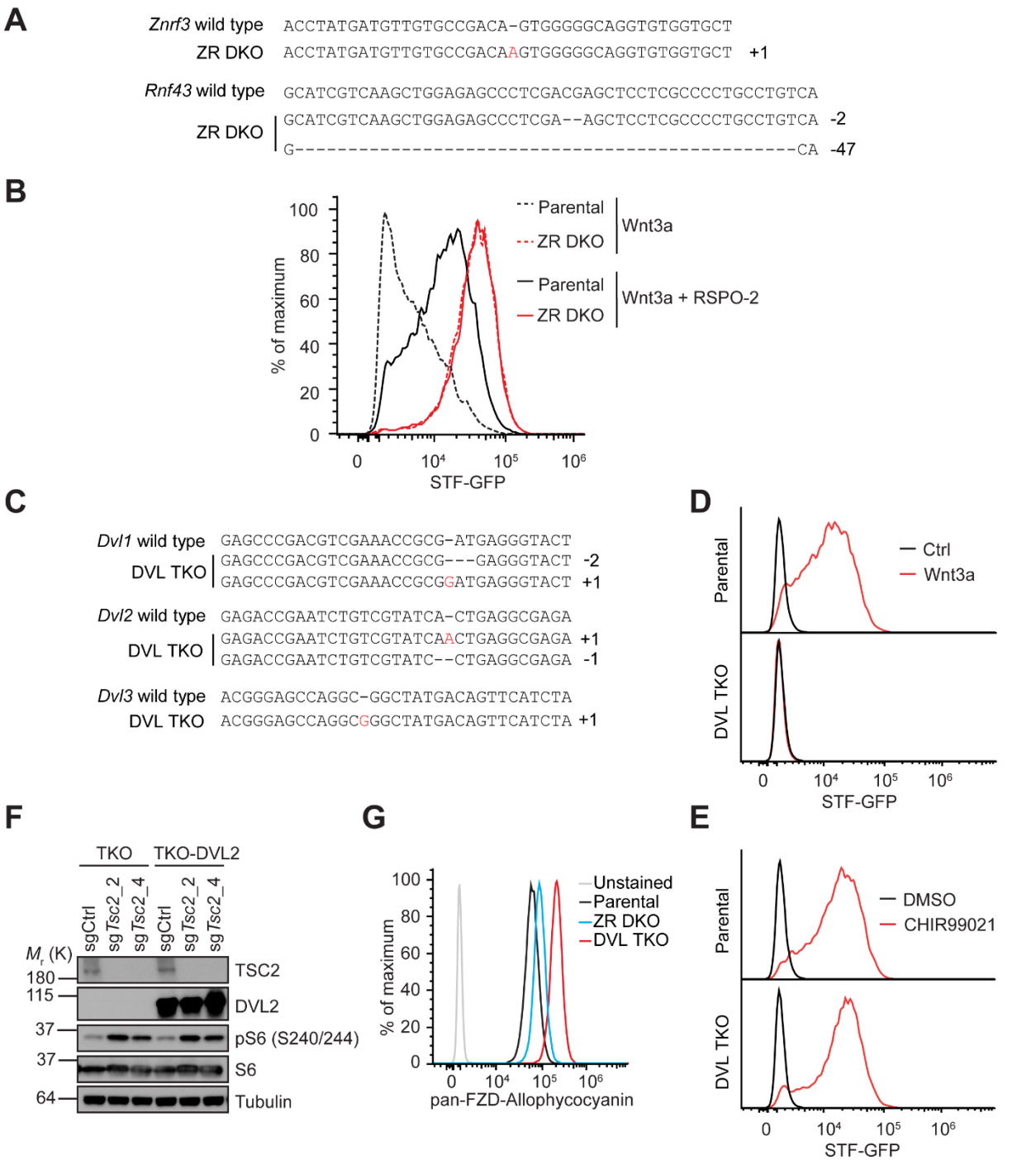

Fig. S4. Characterization of MEF ZNRF3/RNF43 double knockout (ZR DKO) cells and MEF DVL1/2/3 triple knockout (DVL TKO) cells. (A) Genomic DNA sequencing results depicting mutations in *Znrf3* and *Rnf43* genes in MEF ZR DKO cells. (B) Flow cytometry analysis showing that R-Spondin no longer increases STF reporter activity in MEF ZR DKO cells. (C) Genomic DNA sequencing results depicting mutations in *Dvl1*, *Dvl2*, and *Dvl3* genes in MEF DVL TKO cells. (D, E) Deletion of DVL1/2/3 abolishes Wnt3a (D)-, but not GSK3 inhibitor (CHIR99021) (E)-induced STF reporter activity, assessed by flow cytometry analysis of GFP. (F) Immunoblots of indicated proteins in control or TSC2-deleted MEF DVL TKO cells or MEF DVL TKO cells rescued with DVL2. (G) DVL1/2/3 knockout results in stronger increase of membrane frizzled level than ZNRF3/RNF43 knockout compared to parental MEF cells.

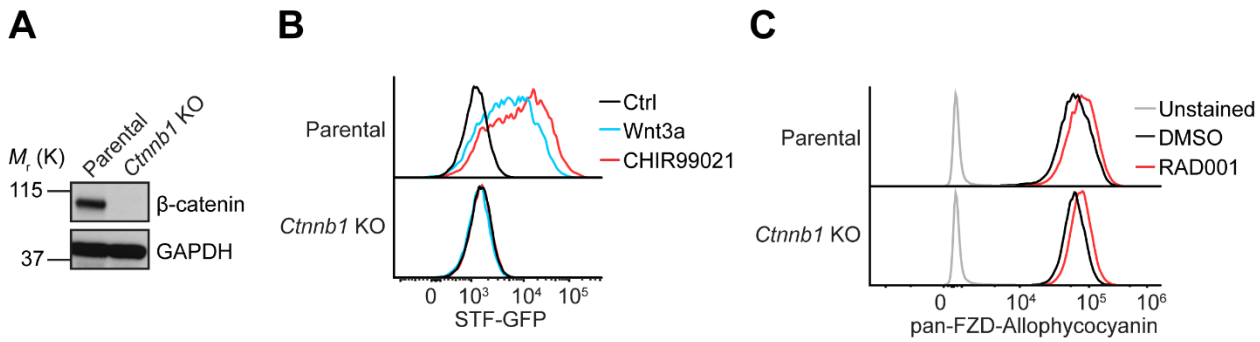

Fig. S5. Inhibition of mTORC1 activity by RAD001 treatment increases cell surface level of Wnt receptor frizzled in *Ctnnb1* KO MEF cells. (A) Immunoblots of indicated proteins in parental and *Ctnnb1* KO MEF cells. (B) Deletion of β-catenin abolishes Wnt3a and GSK3 inhibitor (CHIR99021)-induced STF reporter activity, assessed by flow cytometry analysis of GFP. (C) Flow cytometry analysis of membrane frizzled levels in parental or β-catenin-deleted MEF cells treated with DMSO or 50 nM RAD001.

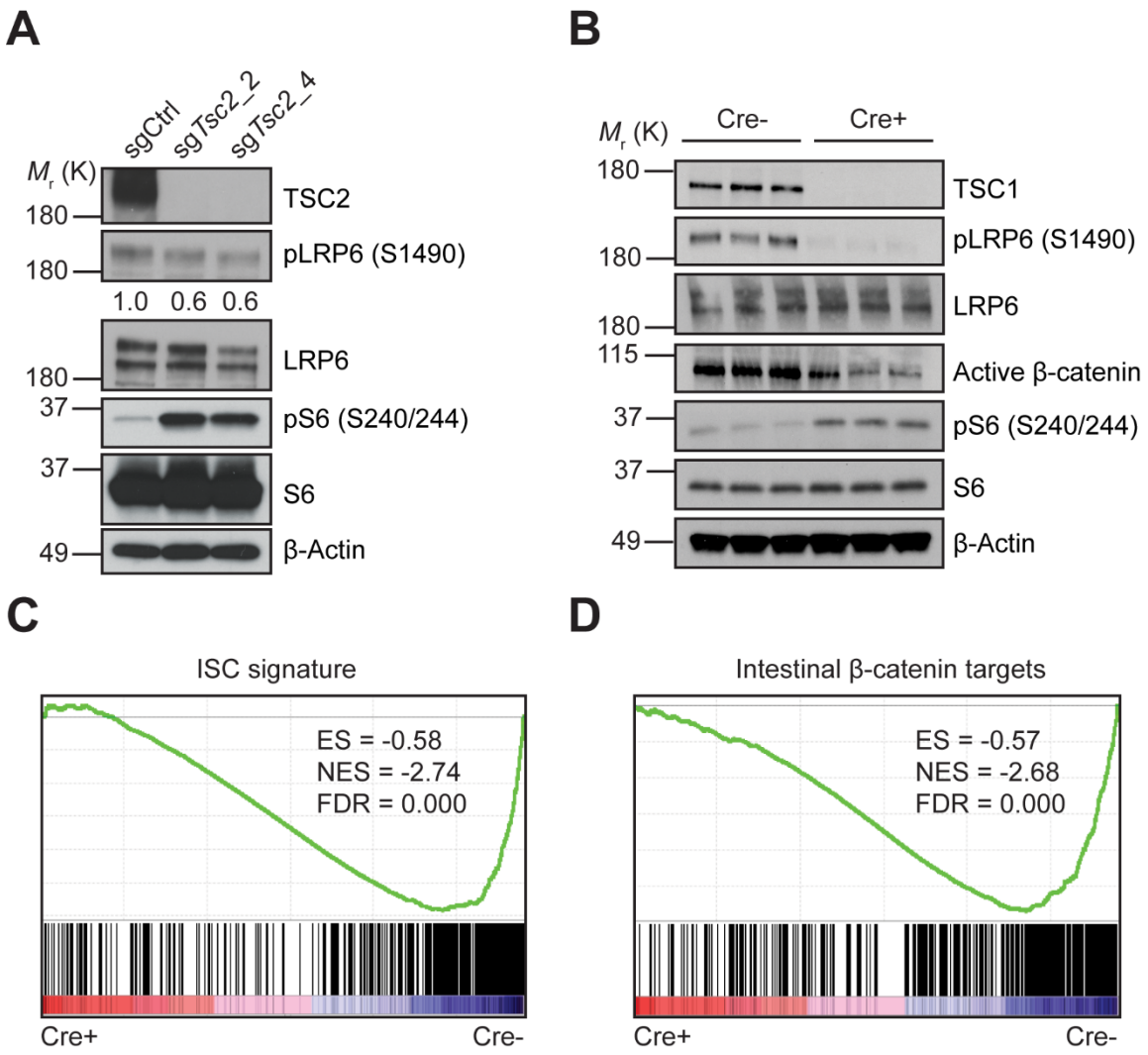

Fig. S6. Activation of mTORC1 signaling impairs Wnt/β-catenin signaling in intestinal organoids. (A) Immunoblots of indicated proteins in control or TSC2-deleted organoids cultured for 4 days. (B) Immunoblots of indicated proteins in *Tsc1*$^{fl/fl}$ (Cre-) or *Tsc1*$^{fl/fl}$; Rosa26-CreER (Cre+) organoids cultured in the presence of 1 μM 4-OHT for 4 days. (C, D) GSEA of the ISC gene signature (C) and intestine-specific β-catenin target gene sets (D) in TSC1-deleted (Cre+) versus control (Cre-) organoids. ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate.

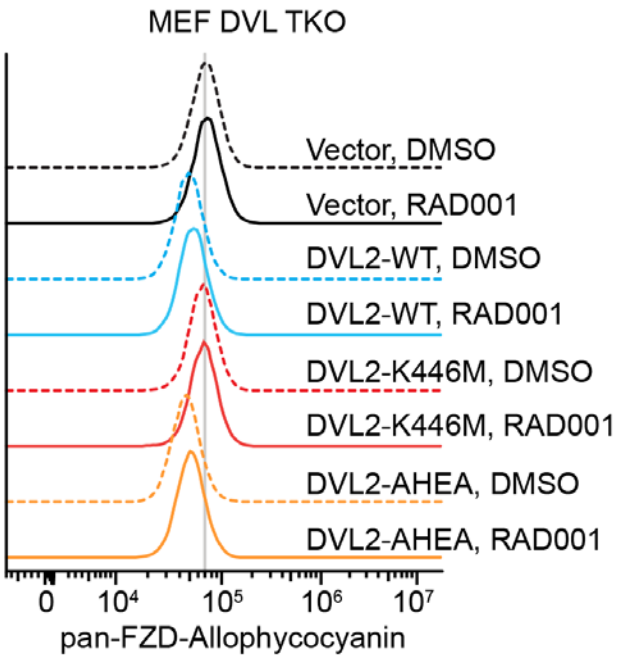

Fig. S7. K446 residue of DVL is required for DVL-mediated FZD downregulation. MEF DVL TKO cells stably expressing vector, DVL2-WT, DVL2-K446M mutant, or DVL2-AHEA mutant were treated with DMSO or 50 nM RAD001, followed by flow cytometry analysis of membrane frizzled levels.

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