



# mTORC1 signaling suppresses Wnt/ $\beta$ -catenin signaling through DVL-dependent regulation of Wnt receptor FZD level

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**Wnt/ $\beta$ -catenin signaling plays pivotal roles in cell proliferation and tissue homeostasis by maintaining somatic stem cell functions. The mammalian target of rapamycin (mTOR) signaling functions as an integrative rheostat that orchestrates various cellular and metabolic activities that shape tissue homeostasis. Whether these two fundamental signaling pathways couple to exert physiological functions still remains mysterious. Using a genome-wide CRISPR-Cas9 screening, we discover that mTOR complex 1 (mTORC1) signaling suppresses canonical Wnt/ $\beta$ -catenin signaling. Deficiency in tuberous sclerosis complex 1/2 (TSC1/2), core negative regulators of mTORC1 activity, represses Wnt/ $\beta$ -catenin target gene expression, which can be rescued by RAD001. Mechanistically, mTORC1 signaling regulates the cell surface level of Wnt receptor Frizzled (FZD) in a Dishevelled (DVL)-dependent manner by influencing the association of DVL and clathrin AP-2 adaptor. Sustained mTORC1 activation impairs Wnt/ $\beta$ -catenin signaling and causes loss of stemness in intestinal organoids *ex vivo* and primitive intestinal progenitors *in vivo*. Wnt/ $\beta$ -catenin-dependent liver metabolic zonation gene expression program is also down-regulated by mTORC1 activation. Our study provides a paradigm that mTORC1 signaling cell autonomously regulates Wnt/ $\beta$ -catenin pathway to influence stem cell maintenance.**

CRISPR screen | mTORC1 signaling | intestinal stem cells | Frizzled | organoids

**W**nt signaling is critical for development and tissue homeostasis, and its deregulation has been implicated in a variety of diseases (1–4). The canonical Wnt/ $\beta$ -catenin signaling pathway is initiated upon binding of Wnt ligand to the seven-pass transmembrane receptor FZD and the single-pass low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) on the cell surface. This event further triggers phosphorylation of LRP5/6, inactivation of  $\beta$ -catenin destruction complex, and accumulation of active  $\beta$ -catenin and partnering with TCF/LEF transcription factors, leading to the activation of Wnt/ $\beta$ -catenin target gene expression (2, 5). Although the core Wnt/ $\beta$ -catenin pathway components have been extensively investigated over the past decades, additional contributors and the crosstalk with other signaling pathways await further exploration.

As the core receptor of Wnt proteins, the strength of the Wnt/ $\beta$ -catenin signaling pathway is exquisitely sensitive to the level of FZD on the plasma membrane. Regulation of FZD turnover has emerged as a key regulatory node of the Wnt signaling pathway. For instance, FZD is degraded by membrane E3 ligase ZNRF3 and RNF43, and this activity of ZNRF3 and RNF43 is blocked by secreted R-spondin proteins (6, 7). Mutations of RNF43 and ZNRF3 and amplification of R-spondin genes are found in various tumors (8, 9). Later, DVL was identified to act as a negative regulator of FZD turnover, largely through recruitment of ZNRF3/RNF43 to FZD receptors (10). Considering the critical role of FZD in Wnt signaling, it would be

expected that other signaling pathways might impact Wnt signaling through regulation of FZD turnover. Nevertheless, whether and how other pathways control FZD turnover is unknown.

The mammalian target of rapamycin complex 1 (mTORC1) signaling coordinates anabolic and catabolic metabolism to support cell growth and tissue homeostasis (11–13). The mTORC1 activity is controlled by various upstream signals, including growth factors, nutrients, and stress. Numerous growth factor and mitogen-dependent pathways converge on Tuberous Sclerosis Complex (TSC) to impact mTORC1 signaling transduction (12). TSC is composed of TSC1, TSC2, and TBC1D7, among which TSC2 is the catalytic subunit with GTPase-activating protein (GAP) activity toward the small GTPase Rheb which binds and directly activates mTORC1 activity through unclear mechanisms (14). The physical interaction of TSC1 and TSC2 is also essential for maintaining the protein stability of TSC2 (15, 16). Activated mTORC1 phosphorylates a variety of downstream effectors, such as S6K, 4EBP1, ULK1, TFEB, and Grb10, and consequently controls a number of cellular processes, including protein synthesis, autophagy, and

## Significance

**The Wnt/ $\beta$ -catenin signaling pathway plays prominent roles during embryonic development and adult tissue homeostasis by maintaining somatic stem cell functions. The mammalian target of rapamycin complex 1 (mTORC1) signaling pathway has also been implicated in regulating stem cell functions in multiple tissue types. However, the crosstalk between these two pathways remains largely unclear. Herein, using *in vitro* cell lines, *ex vivo* organoids, and an *in vivo* mouse model, we made striking findings in support of a paradigm that mTORC1 signaling cell autonomously suppresses Wnt/ $\beta$ -catenin signaling through down-regulating the Wnt receptor FZD level to influence stem cell functions, with implications in the aging process.**

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Conflict of interest statement: All authors were employees of Novartis Pharma AG when the research was conducted.

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Data deposition: The raw RNA-seq reads are available in the NCBI Short Read Archive under the accession no. [SRP140499](https://www.ncbi.nlm.nih.gov/submit/SLRP140499) (for liver RNA-seq samples) and no. [SRP155719](https://www.ncbi.nlm.nih.gov/submit/SLRP155719) (for intestinal organoid samples).

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growth factor signaling, among others (12). Recent studies have highlighted the roles of mTORC1 signaling in stem cell (SC) functions and aging (11–13). For example, *Tsc1* deletion in hematopoietic stem cells (HSCs) led to elevated mTORC1 activity and concomitant loss of stemness in HSCs (17). Despite some similarities in regulating diverse fundamental cellular processes, whether Wnt and mTORC1 signaling pathways couple to exert physiological functions is still unclear.

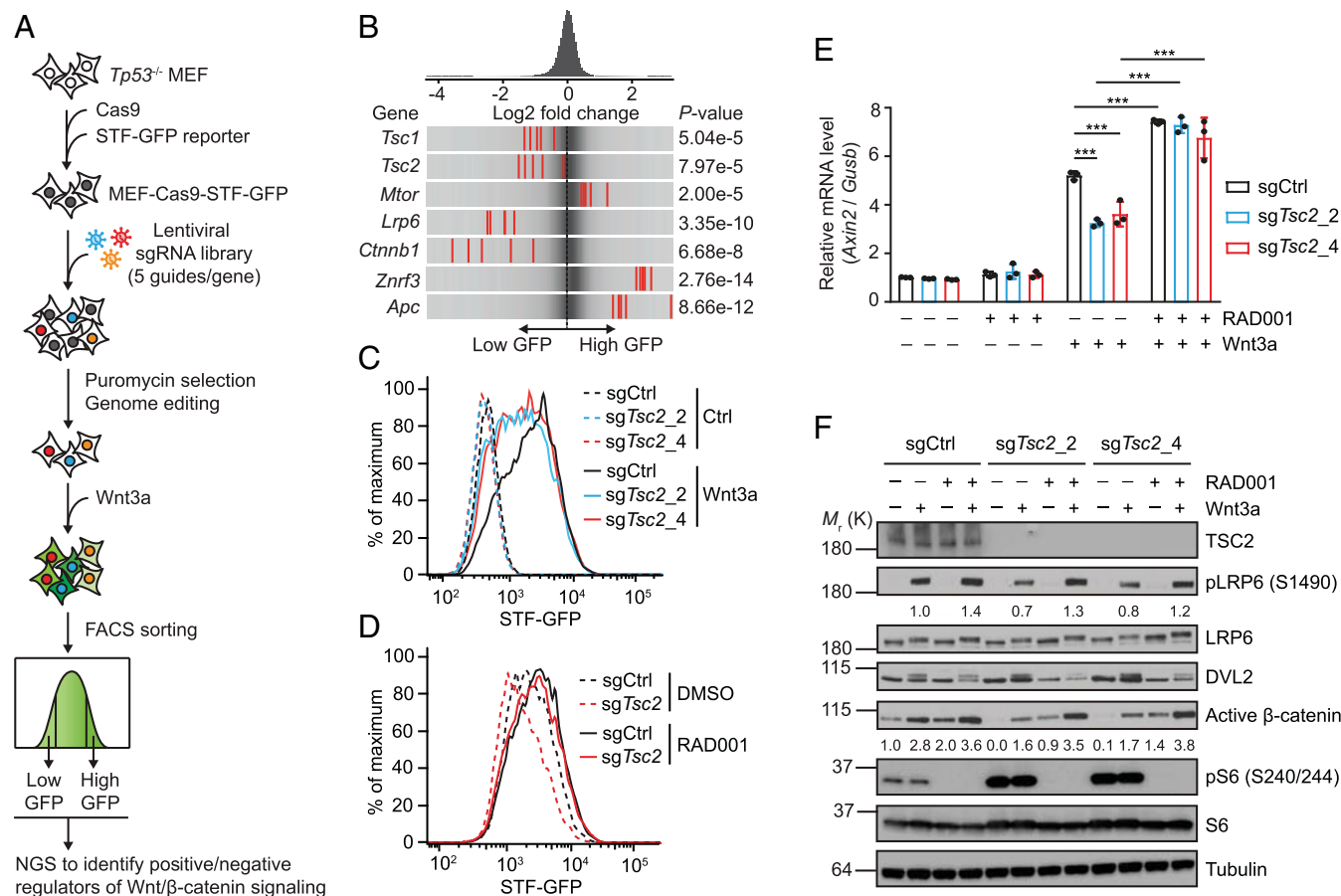
In this study, we discovered a surprising inhibitory effect of mTORC1 signaling on Wnt/ $\beta$ -catenin signaling through an unbiased CRISPR screen. Mechanistic studies revealed that mTORC1 signaling decreases the expression of membrane FZD through enhancing DVL-mediated FZD turnover. Activation of mTORC1 signaling in vivo inhibits Wnt signaling in the intestine and liver. Our findings provide a mechanistic connection of these two fundamental signaling pathways and enhance our understanding of these pathways in stem cell maintenance and tissue homeostasis.

## Results

**A Genome-Wide CRISPR-Cas9 Screening Reveals that mTORC1 Signaling Negatively Modulates Wnt/ $\beta$ -Catenin Signaling.** We set out to identify regulators of Wnt/ $\beta$ -catenin signaling by carrying out a genome-

wide CRISPR-Cas9 screening (Fig. 1A). First, we engineered *Tp53*<sup>-/-</sup> mouse embryonic fibroblast (MEF) cells to express Cas9 and Super-Topflash green fluorescent protein (STF-GFP) reporter (MEF-Cas9-STF-GFP). The MEF-Cas9-STF-GFP cells were then infected with a pooled lentiviral sgRNA library covering 18,360 genes (5 sgRNAs per gene) at a sufficiently low multiplicity of infection to bias for integration of a single lentiviral sgRNA cassette per cell. After puromycin selection and multiple passages to allow gene editing to take place, STF-GFP reporter activity was induced by Wnt3a. Cells were then sorted for populations with the lowest 25% (low GFP) and the highest 25% (high GFP) of the GFP signal and subjected to next-generation sequencing (NGS) and informatics analysis to identify positive and negative regulators of Wnt/ $\beta$ -catenin signaling, respectively (Fig. 1A).

Inspection of the list of genes targeted by sgRNAs that are recovered from low GFP and high GFP populations revealed several genes encoding known core positive (*Lrp6* and *Ctnnb1*) and negative regulators (*Znrf3* and *Apc*) of Wnt/ $\beta$ -catenin pathway (Fig. 1B). Interestingly, sgRNAs targeting *Tsc1/2* which encode core negative regulators of the mTORC1 signaling and *Mtor* itself were identified in the low GFP and high GFP populations (Fig. 1B), respectively, suggesting a suppressive role of



**Fig. 1.** A genome-wide CRISPR-Cas9 screening reveals that mTORC1 signaling negatively modulates Wnt/ $\beta$ -catenin signaling. (A) Workflow of the CRISPR-Cas9 screening process. STF-GFP reporter activity was induced by Wnt3a stimulation overnight, allowing an ideal window for identifying both positive and negative regulators of Wnt/ $\beta$ -catenin signaling. (B) Frequency histograms of sgRNAs identified in the low GFP or high GFP population. sgRNAs targeting indicated genes are shown by the red lines. (C) Deletion of TSC2 decreases the STF reporter activity, assessed by flow cytometry analysis of GFP. (D) RAD001 (50 nM) increases the STF reporter activity and rescues TSC2-deletion-induced reduction of STF reporter activity in the presence of Wnt3a, assessed by flow cytometry analysis of GFP. (E) Quantitative RT-PCR analysis of *Axin2* mRNA level (relative to *Gusb*). Data shown as mean  $\pm$  SD for  $n = 3$  independent experiments; significance tested using ANOVA; \*\*\* $P < 0.001$ . (F) Immunoblots of indicated proteins for the same cells and treatments as described in E. Densitometry quantification of pLRP6 and active  $\beta$ -catenin are shown.

mTORC1 signaling in the Wnt/ $\beta$ -catenin pathway. To validate the screening results, we deleted *Tsc2* in MEF–Cas9–STF–GFP cells using two independent sgRNAs and confirmed that loss of *Tsc2* decreased Wnt3a-induced STF–GFP reporter activity (Fig. 1C and *SI Appendix*, Fig. S1A). Conversely, inhibition of mTORC1 activity by RAD001 treatment increased Wnt3a-induced STF–GFP reporter activity and rescued *Tsc2* knockout-induced STF–GFP reduction (Fig. 1D). Consistent with the alteration in reporter activity, expression of *Axin2*, a classical Wnt/ $\beta$ -catenin target gene, was suppressed by *Tsc2* knockout and increased by RAD001 treatment (Fig. 1E). Western blot analysis further demonstrated that Wnt3a-induced accumulation of phosphorylated LRP6 (pLRP6) and active  $\beta$ -catenin were hampered by *Tsc2* knockout while enhanced by RAD001 treatment (Fig. 1F). Additionally, RAD001 treatment also increased Wnt3a-induced accumulation of pLRP6 and active  $\beta$ -catenin in *Tsc2*<sup>-/-</sup> MEF (*SI Appendix*, Fig. S1B) and HEK293 (*SI Appendix*, Fig. S1C) cells. A recent report showed that mTORC1 activation resulted in feedback inactivation of Akt, leading to reduced GSK3 $\beta$  phosphorylation (pGSK3 $\beta$ ) at serine 9 and concomitant loss of  $\beta$ -catenin activation in melanoma cell lines (18). However, mTORC1 activation in MEF cells exerted no effect on pGSK3 $\beta$  (*SI Appendix*, Fig. S1D). Moreover, inhibition of mTORC1 activity by RAD001 in *Tsc2*<sup>-/-</sup> MEF cells increased active  $\beta$ -catenin while decreasing the level of pGSK3 $\beta$  (*SI Appendix*, Fig. S1B), which is in agreement with the previous finding that S6K1 is the predominant GSK3 regulatory kinase under mTORC1-activated condition (19). These results are not consistent with the model that mTORC1 signaling inhibits Wnt signaling through decreasing GSK3 phosphorylation. Importantly, *Tsc2* knockout decreased, while RAD001 treatment increased phosphorylation of LRP6 (Fig. 1F). These results together suggest that mTORC1 signaling negatively modulates Wnt/ $\beta$ -catenin signaling, probably through regulation of Wnt receptor activation on the cell surface.

**mTORC1 Signaling Regulates the Cell Surface Protein Level of Wnt Receptor Frizzled.** Next we explored the molecular mechanisms through which mTORC1 regulates Wnt signaling. Frizzled (FZD) is the Wnt receptor responsible for Wnt/ $\beta$ -catenin signaling initiation and transduction (5). Since mTORC1 inhibits LRP6 phosphorylation, which is downstream of FZD activation, we examined whether mTORC1 signaling regulates cell surface level of FZD by flow cytometry using pan-FZD antibody 18R5 (20). Indeed, mTORC1 activation by *Tsc2* deletion or mTORC1 inhibition by RAD001 treatment resulted in decrease or increase of cell surface FZD level, respectively, in MEF cells (Fig. 2A and *SI Appendix*, Fig. S2A). We also isolated primary MEF cells ( *Tp53* wild type, nonimmortalized) from a wild-type mouse and showed that RAD001 treatment increased the cell surface FZD level (*SI Appendix*, Fig. S2B), ruling out the possibility that FZD regulation by mTORC1 activity is p53 dependent. In addition, RAD001 was also able to increase the cell surface FZD levels in *Tsc2*<sup>-/-</sup> MEF (*SI Appendix*, Fig. S2C), HEK293 (*SI Appendix*, Fig. S2D), SK-MEL-30 (*SI Appendix*, Fig. S2F), and TSC2-null SNU-398 (21) (*SI Appendix*, Fig. S2G) cells. Taken together, these results demonstrate that mTORC1 signaling regulates the membrane levels of Wnt receptor FZD in different cell types.

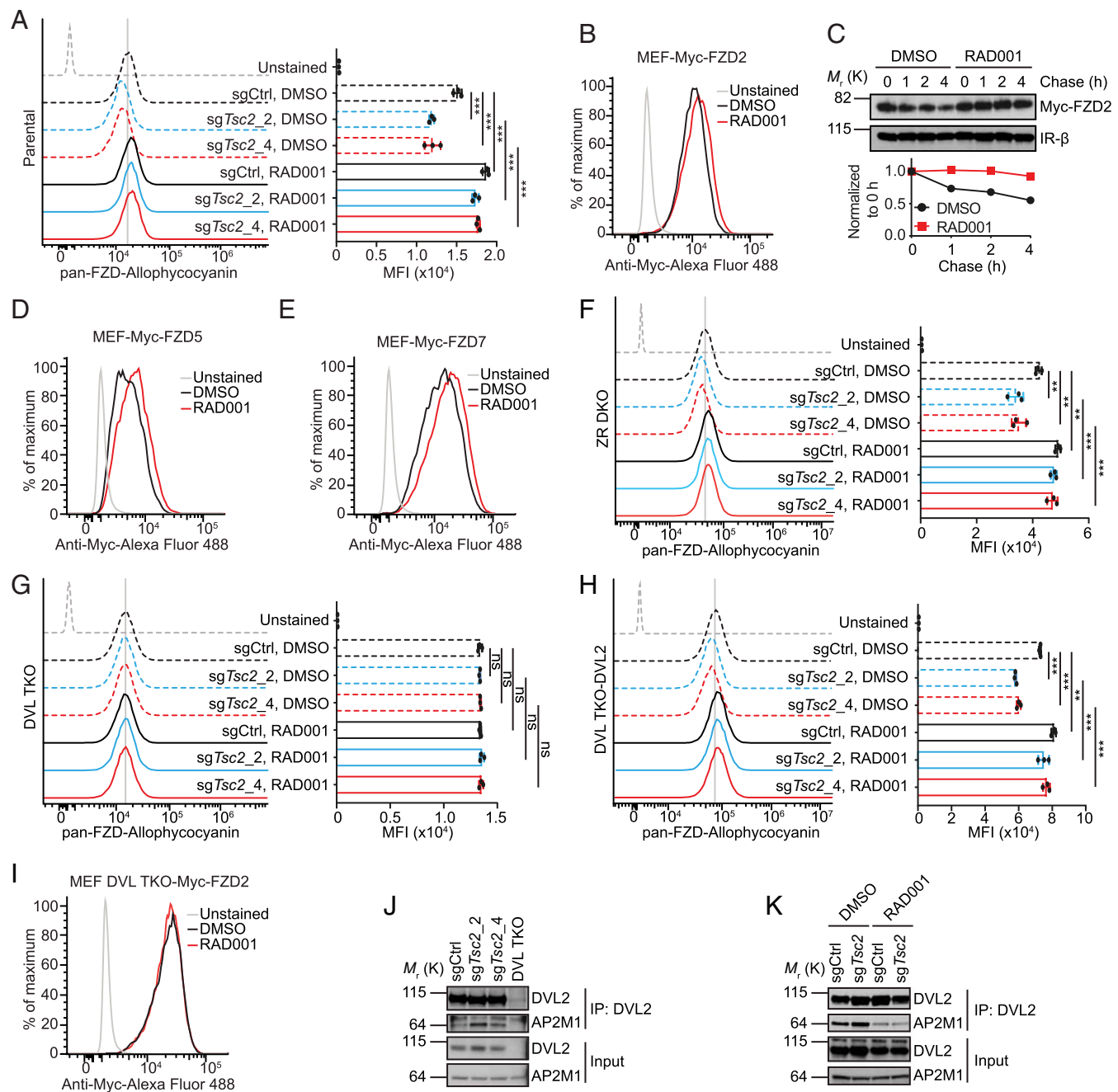
We then asked whether FZD regulation by mTORC1 signaling is on the transcript or protein level. Assessment of the mRNA levels of all 10 *Fzd* genes in MEF cells revealed that *Fzd2* is the dominant *Fzd* in MEF cells (*SI Appendix*, Fig. S3A). In line with this finding, knockout of FZD2 drastically reduced the cell surface FZD level (*SI Appendix*, Fig. S3B) and significantly reduced Wnt3a-induced STF reporter activity in MEF cells (*SI Appendix*, Fig. S3C). Importantly, perturbation of mTORC1 activity exhibited little effect on the mRNA levels of *Fzd2* and other *Fzds* (*SI Appendix*, Fig. S3D), suggesting that mTORC1 signaling regulates the cell surface FZD at the protein level. Consistently, RAD001

treatment increased cell surface Myc–FZD2 level (Fig. 2B) and extended its half-life in a cell surface protein biotinylation-based pulse-chase experiment (Fig. 2C) in MEF cells stably expressing ectopic Myc–FZD2. Interestingly, RAD001 was still able to increase the residual cell surface FZD level in FZD2-deleted cells (*SI Appendix*, Fig. S3B), implying that mTORC1 signaling regulates several, if not all, FZDs. Indeed, RAD001 treatment increased the cell surface levels of ectopic Myc–FZD5 (Fig. 2D) and Myc–FZD7 (Fig. 2E) in MEF cells. Collectively, these results indicate that mTORC1 signaling regulates cell surface protein levels of multiple FZDs.

**Regulation of FZD Protein Level by mTORC1 Signaling Is DVL Dependent.** ZNRF3 and RNF43 E3 ubiquitin ligases have previously been identified as responsible for FZD degradation (6, 7). To investigate whether the regulation of cell surface FZD level by mTORC1 signaling is through ZNRF3/RNF43, we knocked out *Znrf3* and *Rnf43* in MEF cells (*SI Appendix*, Fig. S4A and B). Interestingly, manipulation of the mTORC1 signaling could still cause the alteration of cell surface FZD level in *Znrf3/Rnf43* double knockout MEF (MEF ZR DKO) cells (Fig. 2F), in a similar manner to the observations in parental MEF cells (Fig. 2A), implying a ZNRF3/RNF43-independent mechanism by which mTORC1 signaling regulates cell surface FZD level. More recently, Dishevelled (DVL) was shown to be a negative regulator of cell surface FZD level (10). To test whether DVL is required for mTORC1-mediated FZD regulation, we knocked out *Dvl1/2/3* in MEF cells (*SI Appendix*, Fig. S4C). Consistent with *Dvl1/2/3* knockout in HEK293 cells (10), Wnt3a-induced STF reporter activity was abrogated in *Dvl1/2/3* triple knockout MEF (MEF DVL TKO) cells (*SI Appendix*, Fig. S4D), whereas the response to GSK3 inhibitor CHIR99021 was retained (*SI Appendix*, Fig. S4E). Importantly, neither *Tsc2* deletion nor RAD001 supplementation affected the cell surface FZD level in MEF DVL TKO cells (Fig. 2G), whereas the regulation of cell surface FZD level by mTORC1 signaling was restored when DVL2 was reintroduced into MEF DVL TKO cells (Fig. 2H and *SI Appendix*, Fig. S4F). Moreover, RAD001 treatment had no effect on the cell surface level of ectopic Myc–FZD2 in MEF DVL TKO cells (Fig. 2I) or endogenous FZD in HEK293 DVL TKO cells (*SI Appendix*, Fig. S2E). These findings suggest that mTORC1 signaling regulates cell surface FZD level in a DVL-dependent manner. Given that *Dvl1/2/3* knockout abolished Wnt signaling transduction from the cell membrane receptors, it is possible that manipulation of mTORC1 activity failed to alter FZD levels simply due to the loss of Wnt signaling resulting from *Dvl1/2/3* knockout. To rule out this possibility, we knocked out *Ctnnb1*, which encodes  $\beta$ -catenin protein, in MEF cells (*SI Appendix*, Fig. S5A). As expected, knockout of  $\beta$ -catenin completely abolished Wnt signaling in MEF cells (*SI Appendix*, Fig. S5B). However, RAD001 treatment still increased the cell surface FZD level in  $\beta$ -catenin–deleted cells (*SI Appendix*, Fig. S5C). These data further strengthen the idea that regulation of FZD protein level by mTORC1 signaling is DVL dependent, but ZNRF3/RNF43 or  $\beta$ -catenin independent.

Next we sought to gain further insight into how DVL is involved in mTORC1-mediated FZD regulation. Although DVL acts as an adaptor for ZNRF3/RNF43 to recognize and down-regulate FZD (10), DVL TKO cells manifested a higher cell surface FZD level than ZR DKO cells and parental cells in both MEF (*SI Appendix*, Fig. S4G) and HEK293 (10) contexts, suggesting the existence of an alternative route for DVL to regulate the cell surface FZD level. A previous report has demonstrated that association of DVL with the clathrin AP-2 adaptor is essential for FZD internalization (22). Therefore, DVL likely promotes FZD degradation through promoting ZNRF3/RNF43-mediated FZD ubiquitination and through enhancing clathrin-mediated FZD internalization. Since mTORC1-mediated FZD





**Fig. 2.** mTORC1 signaling regulates the cell surface protein level of Wnt receptor Frizzled in a DVL-dependent manner. (A) Flow cytometry analysis of membrane frizzled levels in control or TSC2-deleted MEF cells treated with DMSO or 50 nM RAD001. MFI, median fluorescence intensity. (B) Flow cytometry analysis of membrane Myc-FZD2 in MEF cells stably expressing ectopic Myc-FZD2 treated with DMSO or 50 nM RAD001. (C) mTORC1 inhibition by RAD001 treatment extends the half-life of Myc-FZD2 in a cell surface protein biotinylation-based pulse-chase assay in MEF-Myc-FZD2 cells. Insulin receptor- $\beta$  (IR- $\beta$ ) acts as a control. *Bottom*, densitometry quantification of Myc-FZD2. (D and E) Flow cytometry analysis of membrane Myc-FZD5 (D) or Myc-FZD7 (E) in MEF cells stably expressing ectopic Myc-FZD5 or Myc-FZD7 treated with DMSO or 50 nM RAD001. (F) Flow cytometry analysis of membrane frizzled levels in control or TSC2-deleted MEF ZNR/F3/RNF43 double knockout (ZR DKO) cells treated with DMSO or 50 nM RAD001. (G) Flow cytometry analysis of membrane frizzled levels in control or TSC2-deleted MEF DVL1/2/3 triple knockout (DVL TKO) cells treated with DMSO or 50 nM RAD001. (H) Flow cytometry analysis of membrane frizzled levels in control or TSC2-deleted MEF DVL TKO-DVL2 rescued cells treated with DMSO or 50 nM RAD001. (I) Flow cytometry analysis of membrane Myc-FZD2 in MEF DVL TKO cells stably expressing Myc-FZD2 treated with DMSO or 50 nM RAD001. (J) Deletion of TSC2 enhances the interaction between DVL2 and AP2M1, assessed by coimmunoprecipitation assay. (K) RAD001 decreases the interaction between DVL2 and AP2M1, assessed by coimmunoprecipitation assay. Error bars denote SD for  $n = 3$  independent experiments (A, F, G, and H); significance tested using ANOVA;  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ; ns, not significant.

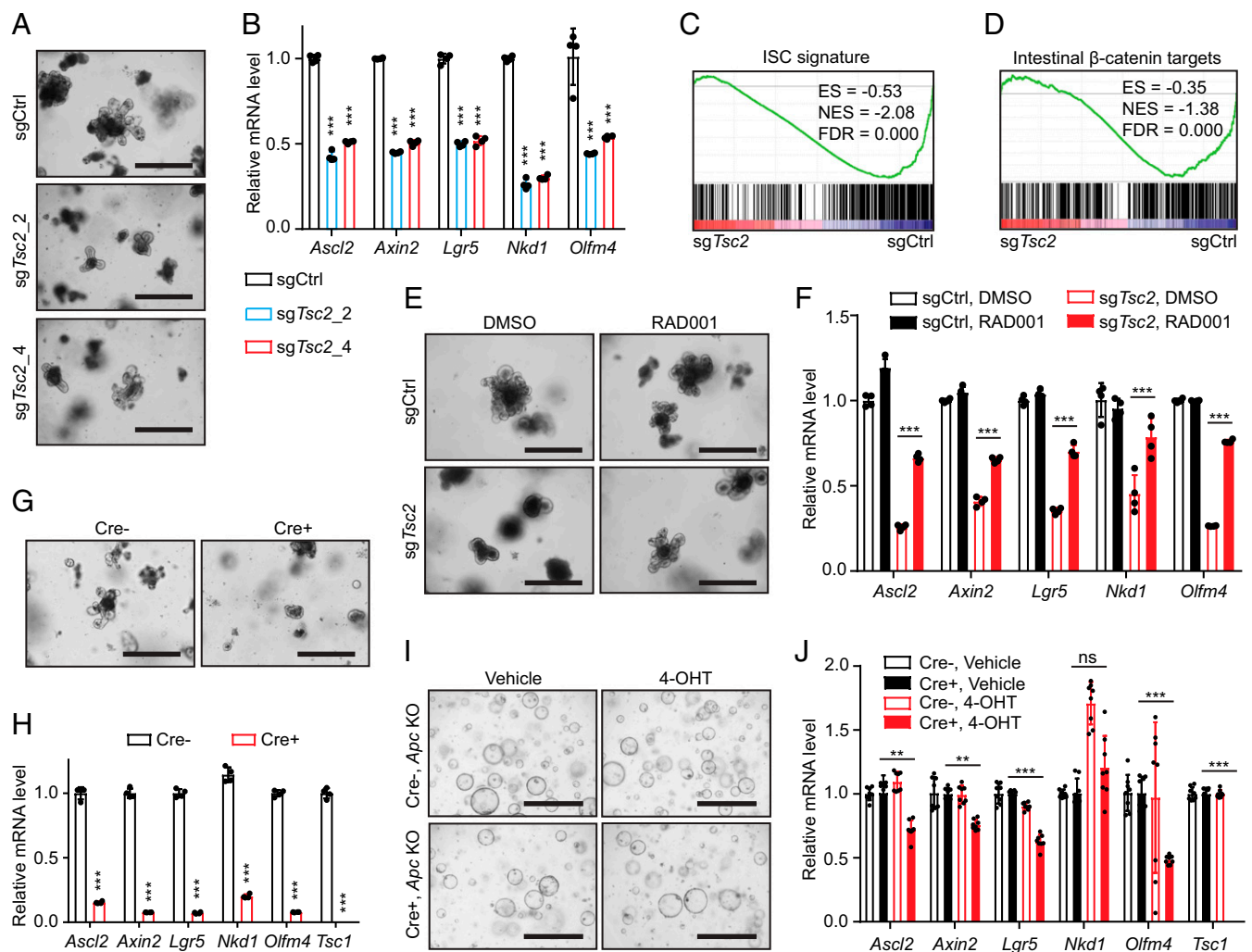
regulation is DVL dependent and ZNR/F3/RNF43 independent, it is possible that mTORC1 signaling regulates FZD turnover through affecting DVL-AP-2 interaction. To test this hypothesis, we assessed the interaction of endogenous DVL2 and AP2M1

proteins by coimmunoprecipitation assay. AP2M1 was coimmunoprecipitated with DVL2 antibody in control cells, but not DVL TKO cells, demonstrating the specificity of the coimmunoprecipitation assay (Fig. 2J). Knockout of *Tsc2* using two

independent sgRNAs increased DVL2–AP2M1 association (Fig. 2J), while RAD001 treatment decreased DVL2–AP2M1 association in both control cells and *Tsc2* knockout cells (Fig. 2K). Notably, effects of *Tsc2* deletion or RAD001 treatment on DVL2–AP2M1 interactions correlated perfectly with the effects of these treatments on cell surface FZD level (Fig. 2A). Collectively, these results suggest that mTORC1 signaling regulates cell surface FZD level by influencing the association of DVL with AP-2 adaptor.

**Activation of mTORC1 Signaling Impairs Wnt Signaling and Stem Cell Functions in Intestinal Organoids.** Wnt/ $\beta$ -catenin signaling is essential for intestinal stem cell (ISC) maintenance and organoid functions ex vivo (23, 24). Having established the crosstalk between mTORC1 and Wnt/ $\beta$ -catenin signaling in vitro, we wondered whether sustained mTORC1 activation could destroy Wnt/

$\beta$ -catenin–regulated ISC functions in organoids. To test this idea, we isolated small intestine (SI) crypts from a Cas9-expressing C57BL6/J mouse and cultured them as organoids ex vivo, followed by lentiviral infection of two independent sgRNAs targeting *Tsc2* to achieve genetic *Tsc2* deletion (*SI Appendix, Fig. S64*). *Tsc2* deletion markedly impaired crypt formation in organoids (Fig. 3A), indicating a decline in ISC functions typically associated with reduced Wnt/ $\beta$ -catenin signaling (25). Accordingly, gene expression analysis revealed that *Tsc2* deletion repressed the expression of Wnt/ $\beta$ -catenin targets and ISC markers (Fig. 3B). Gene set enrichment analysis (GSEA) further demonstrated significant down-regulation of both ISC gene signature (26) (Fig. 3C) and intestinal  $\beta$ -catenin targets (27) (Fig. 3D) in *Tsc2*-deficient organoids. Conversely, mTORC1 inhibition by RAD001 treatment largely rescued *Tsc2*-deletion–induced crypt-loss



**Fig. 3.** Activation of mTORC1 signaling impairs Wnt signaling and stem cell functions in intestinal organoids. (A) Morphology of control or TSC2-deleted intestinal organoids cultured for 4 d. (B) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/ $\beta$ -catenin target genes and intestinal stem cell marker genes in organoids described in A.  $n = 4$ . (C and D) GSEA of the ISC gene signature (C) and intestine-specific  $\beta$ -catenin target gene sets (D) in TSC2-deleted versus control organoids. (E) Morphology of control or TSC2-deleted organoids cultured in the presence of DMSO or 50 nM RAD001 for 5 d. (F) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/ $\beta$ -catenin target genes and intestinal stem cell marker genes in organoids described in E.  $n = 4$ . (G) Morphology of *Tsc1<sup>fl/fl</sup>* (Cre<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (Cre<sup>+</sup>) organoids cultured in the presence of 1  $\mu$ M 4-OHT for 4 d. (H) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/ $\beta$ -catenin target genes, intestinal stem cell marker genes, and *Tsc1* gene in organoids described in G.  $n = 4$ . (I) Morphology of APC-deleted *Tsc1<sup>fl/fl</sup>* (Cre<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (Cre<sup>+</sup>) organoids cultured in the presence of vehicle or 1  $\mu$ M 4-OHT for 4 d. (J) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/ $\beta$ -catenin target genes, intestinal stem cell marker genes, and *Tsc1* gene in APC-deleted *Tsc1<sup>fl/fl</sup>* (Cre<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (Cre<sup>+</sup>) organoids cultured in the presence of vehicle or 1  $\mu$ M 4-OHT for 4 d.  $n = 8$ . Data shown as mean  $\pm$  SD (B, F, H, and J); significance tested using two-tailed  $t$  test (B and H) and ANOVA (F and J); \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, not significant. [Scale bars: 200  $\mu$ m (A and E), 400  $\mu$ m (G), and 1,000  $\mu$ m (I.)] ES, enrichment score; FDR, false discovery rate; NES, normalized enrichment score.

phenotype (Fig. 3E) and gene expression defect (Fig. 3F) in organoids. Collectively, these data suggest that sustained mTORC1 activation suppresses Wnt/ $\beta$ -catenin signaling and impairs ISC functions in an organoid model.

To further strengthen the above findings, we isolated SI crypts from *Tsc1<sup>fl/fl</sup>* ( $\text{Cre}^-$ ) littermates or *Tsc1<sup>fl/fl</sup>; Rosa26-creERT2* ( $\text{Cre}^+$ ) mice and allow for organoid formation ex vivo. *Tsc1* deletion by 4-OH tamoxifen (4-OHT) treatment remarkably inhibited  $\text{Cre}^+$  organoid formation, whereas  $\text{Cre}^-$  organoids exhibited normal expansion upon 4-OHT treatment (Fig. 3G and *SI Appendix*, Fig. S6B). In line with the observations in MEF cells in vitro (Fig. 1F), mTORC1 activation in organoids significantly decreased the levels of pLRP6 and active  $\beta$ -catenin (*SI Appendix*, Fig. S6B). Additionally, *Tsc1* deletion resulted in severe repression of Wnt/ $\beta$ -catenin targets and ISC gene signature (26, 27) (Fig. 3H and *SI Appendix*, Fig. S6 C and D), confirming that persistent mTORC1 activity is detrimental to Wnt/ $\beta$ -catenin-mediated ISC maintenance. Compared with *Tsc2* CRISPR knockout, deletion of *Tsc1* using Cre-ERT2 showed stronger effects on organoids, likely due to more uniform gene inactivation. To evaluate whether mTORC1 activation-induced down-regulation of Wnt/ $\beta$ -catenin signaling is causative to impaired ISC functions, we activated Wnt/ $\beta$ -catenin signaling by lentiviral coinfection of Cas9 and *Apc* targeting sgRNA in  $\text{Cre}^-$  or  $\text{Cre}^+$  organoids to delete *Apc*, a core suppressive factor of the Wnt/ $\beta$ -catenin pathway. *Apc*-null organoids were established by R-spondin withdrawal from the organoid culture media. Activation of the Wnt/ $\beta$ -catenin pathway effectively prevented *Tsc1* deletion-induced organoid degeneration (Fig. 3I). The gene expression defect resulting from *Tsc1* deletion was also markedly ameliorated with Wnt/ $\beta$ -catenin pathway activation (Fig. 3J). The residual reduction in gene expression could be due to the effect of upstream Wnt signaling in APC KO cells (28) or the existence of a minor mechanism that affects Wnt/ $\beta$ -catenin signaling at the level of  $\beta$ -catenin. Taken together, these results demonstrate that persistent mTORC1 activity disrupts ISC functions by suppressing Wnt/ $\beta$ -catenin signaling.

**Activation of mTORC1 Signaling Decreases Wnt Signaling in Vivo.** We next determined the effect of mTORC1 activation on Wnt/ $\beta$ -catenin signaling and intestinal epithelium homeostasis in vivo. Histological evaluation of the small intestines of  $\text{Cre}^-$  or  $\text{Cre}^+$  mice after tamoxifen administration revealed a degenerative phenotype associated with loss of  $\text{Ki67}^+$  proliferating crypts upon *Tsc1* deletion (Fig. 4A). To examine whether *Tsc1* deletion reduced ISC numbers, we performed in situ hybridization (ISH) for *Olfm4*, a marker that is coexpressed by  $\text{Lgr5}^+$  ISCs (29, 30). *Olfm4*<sup>+</sup> primitive ISCs were remarkably reduced in *Tsc1*-deficient mice (Fig. 4A). Consistent with the degenerative phenotype, Wnt/ $\beta$ -catenin target gene expression was also significantly decreased in *Tsc1*-deficient small intestines (Fig. 4B). Together, these results suggest that sustained mTORC1 activation down-regulates Wnt/ $\beta$ -catenin signaling and inhibits the function of ISCs in vivo.

Emerging evidence have highlighted the Wnt/ $\beta$ -catenin pathway as a major regulator of metabolic liver zonation which dictates hepatocyte functions by its position along the portocentral axis of the liver lobule (31–33). Wnt/ $\beta$ -catenin activity is persistently high around the central vein of the liver lobule (34). To explore the impact of mTORC1 activation on Wnt/ $\beta$ -catenin-mediated metabolic zonation regulation, we collected livers from  $\text{Cre}^-$  or  $\text{Cre}^+$  mice after tamoxifen administration and observed significant down-regulation of pLRP6 upon *Tsc1* deletion (Fig. 4 C and D). In keeping with this notion, expression of classical Wnt/ $\beta$ -catenin targets *Axin2*, *Lect2*, and *Lgr5* was strongly inhibited (Fig. 4E), indicating the down-regulation of Wnt/ $\beta$ -catenin signaling upon *Tsc1* deletion in liver. Furthermore, expression of Wnt/ $\beta$ -catenin-dependent metabolic genes *Cyp1a2*, *Cyp2e1*, and *Glu1* was also markedly reduced in *Tsc1*-

deficient livers (Fig. 4E). Unbiased high-throughput RNA sequencing (RNA-seq) on total liver RNA samples isolated from  $\text{Cre}^-$  or  $\text{Cre}^+$  mice affirmed the down-regulation of Wnt/ $\beta$ -catenin target genes (Fig. 4F) and pericentrally expressed metabolic genes (33) (Fig. 4 F and G) in *Tsc1*-deficient livers. Collectively, these data support the idea that mTORC1 activation down-regulates the Wnt/ $\beta$ -catenin-mediated gene expression program involved in liver metabolic zonation. It should be noted that the in vivo study involved whole-body *Tsc1* deletion, so a paracrine effect from other tissues cannot be ruled out. Nevertheless, the effect of mTORC1 activation on Wnt/ $\beta$ -catenin signaling is highly consistent in in vitro cell culture, ex vivo organoid, and in vivo mouse models.

## Discussion

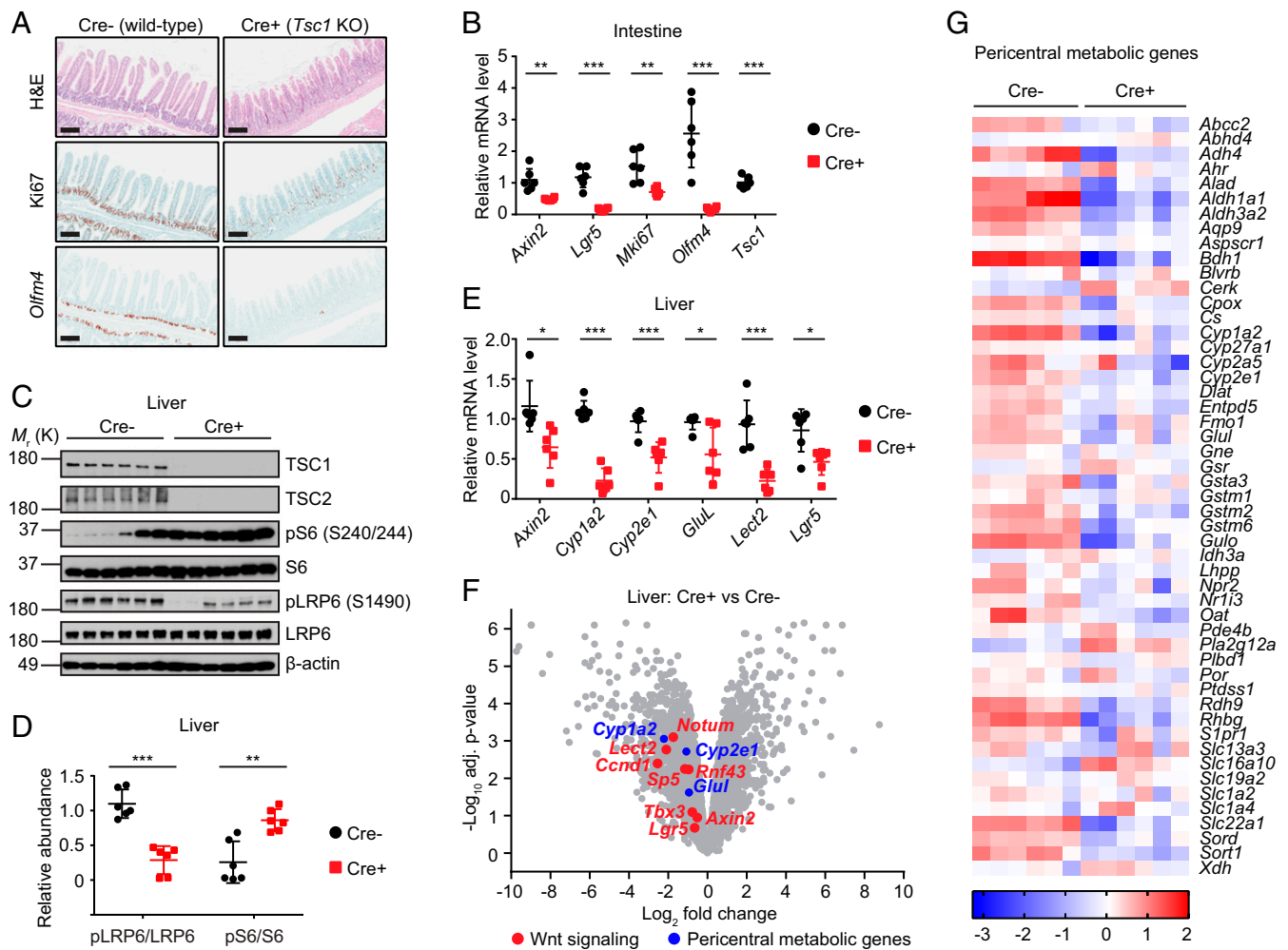
In this study, we discover an inhibitory role of mTORC1 activation in the Wnt/ $\beta$ -catenin signaling pathway through an unbiased genome-wide CRISPR screen and demonstrate its importance using ex vivo intestinal organoids and an in vivo mouse model. Although the regulatory outputs of mTORC1 signaling on Wnt/ $\beta$ -catenin targets could still be indirect, we favor a model in which mTORC1 signaling cell autonomously suppresses Wnt/ $\beta$ -catenin signaling through promoting DVL–AP-2 interaction and enhancing DVL-mediated FZD internalization. Thus, DVL-mediated FZD turnover represents a regulatory node of Wnt signaling for pathway crosstalk.

Although DVL–AP-2 interaction is required for FZD internalization (22), the exact binding mode between DVL and the AP-2 adaptor is not completely clear. The Kirchhausen laboratory initially identified that both a tyrosine motif (YHEL) and the DEP domain, particularly the K446 residue, of DVL contribute to AP-2 adaptor binding (22). Subsequently, the same group determined the crystal structure of a single-chain chimera with a variable linker between DEP and YHEL and a second variable linker between YHEL and the C-terminal domain of the  $\mu 2$  subunit of the AP-2 adaptor ( $\mu 2\text{C}$ ) (35). In this structure, both the DEP domain and YHEL motif are involved in  $\mu 2\text{C}$  interaction, but the K446 residue of the DEP domain is not (35). Although the YHEL motif makes a major contribution to DVL–AP-2 interaction in these two studies, the DVL2–AHEA mutant was fully capable of down-regulating the FZD level in both MEF DVL TKO cells (*SI Appendix*, Fig. S7) and HEK293 DVL TKO cells (10, 36). In contrast, the DVL2–K446M mutant failed to decrease the FZD level in both MEF DVL TKO cells (*SI Appendix*, Fig. S7) and HEK293 DVL TKO cells (10), consistent with the model that the K446 residue is directly involved in DVL–FZD interaction (37). Other regions of DVL, and even other proteins, might facilitate the physical interaction between DVL and AP-2.

Our data clearly demonstrated that mTORC1 signaling increases DVL–AP-2 interaction, though the exact mechanism is unclear. Given that DVL is a phosphoprotein, it is possible that mTORC1 regulates phosphorylation of DVL. Two previous mass spectrometry studies systematically identified mTOR-regulated phosphoproteome in immortalized MEF cells (38, 39). Neither DVL nor AP-2 adaptor was among the list of mTORC1 substrates which are sensitive to perturbation of mTORC1 activity. However, focused quantitative phosphoproteomic analysis on DVL is required to further delineate whether DVL phosphorylation is regulated by mTORC1. On the other hand, we noticed that RAD001 treatment appears to decrease the DVL2 protein level in MEF cells (Fig. 1F). Whether this observation is functionally relevant is still not clear. It is possible that a certain form of DVL promotes FZD degradation, and the expression of this form of DVL is decreased upon mTORC1 inhibition. Future studies are clearly needed to map out the mechanistic details of the mTORC1-regulated DVL–AP-2 interaction.

Accumulating studies have suggested an important regulatory role of the TSC/mTORC1 signaling in SC maintenance in multiple





**Fig. 4.** Activation of mTORC1 signaling by *Tsc1* deletion decreases Wnt signaling in vivo. (A) Hematoxylin and eosin (H&E) staining, Ki67 immunohistochemistry staining, and *Olfm4* in situ hybridization analysis in small intestines of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. (B) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/β-catenin target genes, intestinal stem cell marker genes, and *Tsc1* gene in small intestines of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. *n* = 6 mice per group. (C) Immunoblots of indicated proteins in livers of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. *n* = 6 mice per group. (D) Densitometry quantification of immunoblots in C showing the ratio of pLRP6/total LRP6 and pS6/total S6. (E) Quantitative RT-PCR analysis of relative mRNA levels of Wnt/β-catenin target genes and pericentral metabolic genes in livers of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. *n* = 6 mice per group. (F) Volcano plot showing selective pericentral genes (in blue) and Wnt/β-catenin target genes (in red) that are differentially expressed in livers of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. *n* = 6 mice per group. (G) Heatmap showing mRNA expression of pericentral metabolic genes in livers of *Tsc1<sup>fl/fl</sup>* (*Cre*<sup>-</sup>) or *Tsc1<sup>fl/fl</sup>; Rosa26-CreER* (*Cre*<sup>+</sup>) mice dosed with tamoxifen. *n* = 6 mice per group. Error bars denote SD (B, D, and E); significance tested using two-tailed *t* test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. [Scale bars: 200 μm (A).]

tissue types (11, 40). For instance, sustained activation of mTORC1 signaling leads to loss of stemness in hematopoietic SCs (17), hair follicle SCs (41), and tracheal epithelium SCs (42) in mice. Chronic mTORC1 activation also results in a decline of ISC numbers in flies (42), the phenotype of which is now extended to the murine model in the present study (Fig. 4A). Interestingly, reduction of mTORC1 activity by calorie restriction promotes ISC functions and increases the size of intestinal crypts in mice (43). Although a noncell-autonomous mechanism through Paneth cells is proposed (43), it is possible that cell-autonomous regulation of Wnt/β-catenin signaling by mTORC1 signaling in ISCs also contributes to this observation. The cell-autonomous effects of mTORC1 signaling on SC maintenance have been supported by another finding that dietary supplementation with rapamycin is sufficient to promote mouse tracheal SC maintenance and thus preserve regenerative capacity of airway epithelia (42), although whether Wnt/β-catenin signaling is accordingly involved remains to be resolved. Our data demonstrate cell-autonomous down-

regulation of Wnt/β-catenin signaling induced by mTORC1 activation, which is associated with the loss of ISCs as well as repression of the liver metabolic zonation-related gene expression program. Given that mTORC1 signaling coordinates various cellular events, including protein translation, autophagy, and metabolism, among others, we can thus not exclude the possibility that such downstream pathways are also involved in the degenerative phenotype resulting from mTORC1 activation.

In normal mouse intestines, mTORC1 signaling is active in normal intestinal crypts where Wnt/β-catenin signaling is also highly active (44, 45). Our data, together with the observation that calorie restriction increases the size of intestinal crypts, appear to be counterintuitive. Nevertheless, these findings suggest a potential self-check mechanism to precisely regulate mTORC1 signaling and Wnt/β-catenin signaling for tissue homeostasis. Interestingly, Inoki et al. (46) reported that Wnt activates mTOR signaling by inhibition of GSK3. Therefore, high mTORC1 signaling in ISCs could represent a negative feedback

mechanism to prevent overactivation of Wnt signaling. Hyperactivation of Wnt/ $\beta$ -catenin signaling, typically associated with loss-of-function mutations in the APC tumor suppressor, is the leading cause of development of colorectal cancer (1, 3). Numerous studies have demonstrated that mTORC1 inhibition blocks APC loss-driven colorectal tumorigenesis (45, 47–49), consistent with a critical role of mTORC1 signaling in tumorigenesis. Since mTORC1 inhibits the upstream part of the Wnt/ $\beta$ -catenin signaling pathway, mTORC1 inhibition is not expected to block  $\beta$ -catenin signaling in APC-deficient cells.

Lastly, emerging evidence has demonstrated that aging is accompanied by activation of mTORC1 signaling, and long-term inhibition of mTORC1 activity is capable of extending lifespan in various organisms (13). A recent study reported a decline of ISC functions upon aging due to decreased canonical Wnt/ $\beta$ -catenin signaling, and reactivating Wnt/ $\beta$ -catenin signaling in aged ISCs restores function in organoids (50). Considering that mTORC1 signaling inhibits Wnt/ $\beta$ -catenin signaling, enhanced Wnt/ $\beta$ -catenin signaling in stem cells might contribute to mTORC1 inhibition-associated increased function of stem cells and extension of life span. Our findings provide a

mechanistic connection of these two fundamental signaling pathways and may facilitate the deeper understanding of the interplay between mTORC1 and Wnt/ $\beta$ -catenin signaling pathways in stem cell maintenance and aging.

## Materials and Methods

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. Detailed procedures for the genome-wide CRISPR screen, generation of knockout cell lines, flow cytometry, immunoblotting, and immunoprecipitation, quantitative RT-PCR and RNA-seq, animal experiments, and intestinal organoid culture are described in *SI Appendix, Materials and Methods*.

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