Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade

Jianhui Ma,[1,2] Yan Meng,[1,3] David J. Kwiatkowski,[4] Xinxin Chen,[1] Haiyong Peng,[1] Qian Sun,[1] Xiaojun Zha,[1] Fang Wang,[1] Ying Wang,[1] Yanling Jing,[1] Shu Zhang,[1,5] Rongrong Chen,[1] Lianmei Wang,[1,6] Erxi Wu,[7] Guifang Cai,[8] Izabela Malinowska-Kolodziej,[4] Qi Liao,[9] Yuqin Liu,[10] Yi Zhao,[9] Qiang Sun,[5] Kaifeng Xu,[6] Jianwu Dai,[11] Jiahuai Han,[12] Lizi Wu,[13] Robert Chunhua Zhao,[3] Huangxuan Shen,[2] and Hongbing Zhang[1]

[1]Department of Physiology and Pathophysiology, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences and School of Basic Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China. [2]State Key Laboratory of Ophthalmology, Laboratory of Ocular Genetics, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, People's Republic of China. [3]Center of Excellence in Tissue Engineering, Institute of Basic Medical Sciences and School of Basic Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China. [4]Division of Translational Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA. [5]Division of Breast Surgery, Department of Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China. [6]Division of Respiratory Medicine, Department of Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China. [7]Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota, USA. [8]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA. [9]Center for Advanced Computing Research, Institute of Computing Technology, Chinese Academy of Sciences, Beijing, People's Republic of China. [10]Department of Pathology, Institute of Basic Medical Sciences and School of Basic Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China. [11]Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, People's Republic of China. [12]Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen, Fujian, People's Republic of China. [13]Department of Molecular Genetics and Microbiology, Shands Cancer Center, University of Florida, Gainesville, Florida, USA.

The receptor tyrosine kinase/PI3K/AKT/mammalian target of rapamycin (RTK/PI3K/AKT/mTOR) pathway is frequently altered in cancer, but the underlying mechanism leading to tumorigenesis by activated mTOR remains less clear. Here we show that mTOR is a positive regulator of Notch signaling in mouse and human cells, acting through induction of the STAT3/p63/Jagged signaling cascade. Furthermore, in response to differential cues from mTOR, we found that Notch served as a molecular switch to shift the balance between cell proliferation and differentiation. We determined that hyperactive mTOR signaling impaired cell differentiation of murine embryonic fibroblasts via potentiation of Notch signaling. Elevated mTOR signaling strongly correlated with enhanced Notch signaling in poorly differentiated but not in well-differentiated human breast cancers. Both human lung lymphangioleiomyomatosis (LAM) and mouse kidney tumors with hyperactive mTOR due to tumor suppressor TSC1 or TSC2 deficiency exhibited enhanced STAT3/p63/Notch signaling. Furthermore, tumorigenic potential of cells with uncontrolled mTOR signaling was suppressed by Notch inhibition. Our data therefore suggest that perturbation of cell differentiation by augmented Notch signaling might be responsible for the underdifferentiated phenotype displayed by certain tumors with an aberrantly activated RTK/PI3K/AKT/mTOR pathway. Additionally, the STAT3/p63/Notch axis may be a useful target for the treatment of cancers exhibiting hyperactive mTOR signaling.

Introduction

The receptor tyrosine kinase/PI3K/AKT/mammalian target of rapamycin (RTK/PI3K/AKT/mTOR) pathway, which plays multiple roles in cell growth, proliferation, and survival, is frequently deregulated in cancer (1, 2). mTOR, a serine/threonine protein kinase which exists as both rapamycin-sensitive (mTOR complex 1 [mTORC1]) and rapamycin-insensitive multimeric protein complexes (mTORC2) (3, 4), functions as a nutrient and energy sensor and regulates protein synthesis and autophagy to modulate cell growth and survival (1, 2, 5–9). It is frequently activated in human cancers by gain-of-function mutations in its activators, such as those encoding epidermal growth factor receptor, *PI3K*, or *AKT*, and by loss-of-function mutations in its suppressors, such as *PTEN*, *LKB1*, or the tuberous sclerosis complex (TSC) genes *TSC1* and *TSC2* (1, 2). However, the precise mechanisms of activation of the mTOR signaling pathway to enhance cancer development are less clear (1, 2, 10–12).

Aberrant cell differentiation occurs in nearly all cancers, and there is an association between poor differentiation and worsening clinical prognosis. Inactivating mutations of either *TSC1* or *TSC2*, major negative regulators of mTOR (10, 11, 13), cause TSC and LAM (12, 14) with tumor lesions featuring aberrant cellular differentiation and proliferation (15–17). Because physiological mTOR is required for cell differentiation

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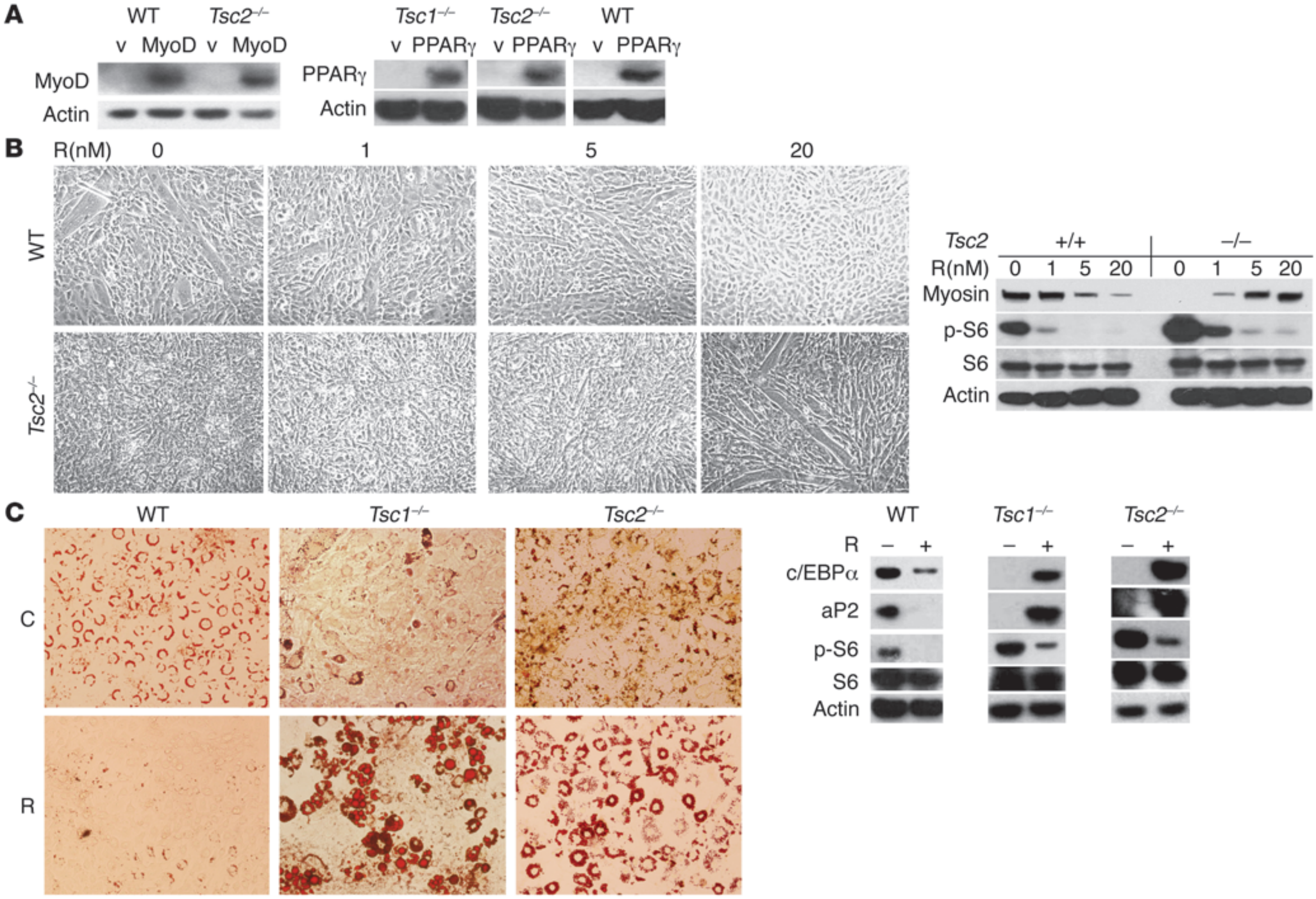

Figure 1

Hyperactive mTOR blocks cell differentiation. (**A**) Generation of MyoD- or PPARγ-expressing MEFs. Left: WT and *Tsc2*−/− MEFs were transduced with pMSCV (V) or pMSCV-MyoD (MyoD) retroviruses and subjected to immunoblotting for MyoD. Right: WT, *Tsc1*−/−, and *Tsc2*−/− MEFs were transduced with pMSCV (V) or pMSCV-PPARγ (PPARγ) retroviruses and subjected to immunoblotting for PPARγ. β-Actin served as a loading control. (**B**) WT and *Tsc2*−/− MEFs transduced with pMSCV-MyoD retroviruses were induced to differentiate into skeletal myocytes and form multicellular myotubes in 2% horse serum and 10 μg/ml insulin with or without varying amounts of rapamycin (R) for 5 days. Left: Myofibers (original magnification, ×200). Right: Immunoblotting for myosin (muscle marker) and p-S6 (Ser235/236, an mTOR activity marker). (**C**) WT, *Tsc1*−/−, and *Tsc2*−/− MEFs transduced with pMSCV-PPARγ retroviruses were induced to differentiate into adipocytes in differentiation medium with or without 1 nM rapamycin for 6 days. Left: Oil Red O staining for lipid droplets (original magnification, ×200). Right: Immunoblotting for c/EBPα and aP2 (adipogenic markers) and p-S6. C, no treatment.

(18–21), hyperactivation of mTOR may play a role in abnormal cell differentiation. We thus reasoned that malfunctioning of cell differentiation regulators might contribute to the pathology of TSC and LAM. Among them, Notch is a major regulator of cell differentiation (22, 23). Ligand engagement causes the intracellular domain of transmembrane receptor Notch (NICD) to be cleaved from the membrane and translocated to the nucleus, where it associates with the CSL family of DNA binding proteins to form a transcriptional active complex to activate transcription of Notch target genes such as hairy and enhancer-of-split 1 (*Hes1*) (22). All of these processes are subjected to multilevel regulation (24). Furthermore, Notch signaling regulates cell differentiation, proliferation, and survival as well as oncogenic transformation in a dose- and context-dependent manner (22, 25–30). Therefore, the potential functional interaction of mTOR and Notch might contribute greatly to tumor development.

To elucidate the putative relationship between mTOR and Notch signaling pathways, we analyzed various genetically defined mouse embryonic fibroblasts (MEFs) and kidney tumors, human cancer cell lines, and breast cancer and lung LAM tissues. We report here that the RTK/PI3K/AKT pathway activates the STAT3/p63/Jagged/Notch signaling cascade via mTORC1 and that mouse kidney tumors and human LAM resulting from TSC1 or TSC2 deficiency exhibited hyperactive mTOR/STAT3/p63/Notch signaling. Oncogenically activated mTOR impaired cell differentiation via this potentiated pathway. Uncontrolled mTOR signaling was strongly correlated with the enhanced Notch signaling in poorly differentiated but not in well-differentiated human breast cancers. Inhibition of Notch signaling blocked tumorigenesis of cells with activated mTOR signaling. We suggest that the newly identified mTOR effectors p63, Jagged1, Notch, and Hes1 are novel candidates for targeted therapy in diseases associated with deregulated RTK/PI3K/AKT/mTOR signaling.

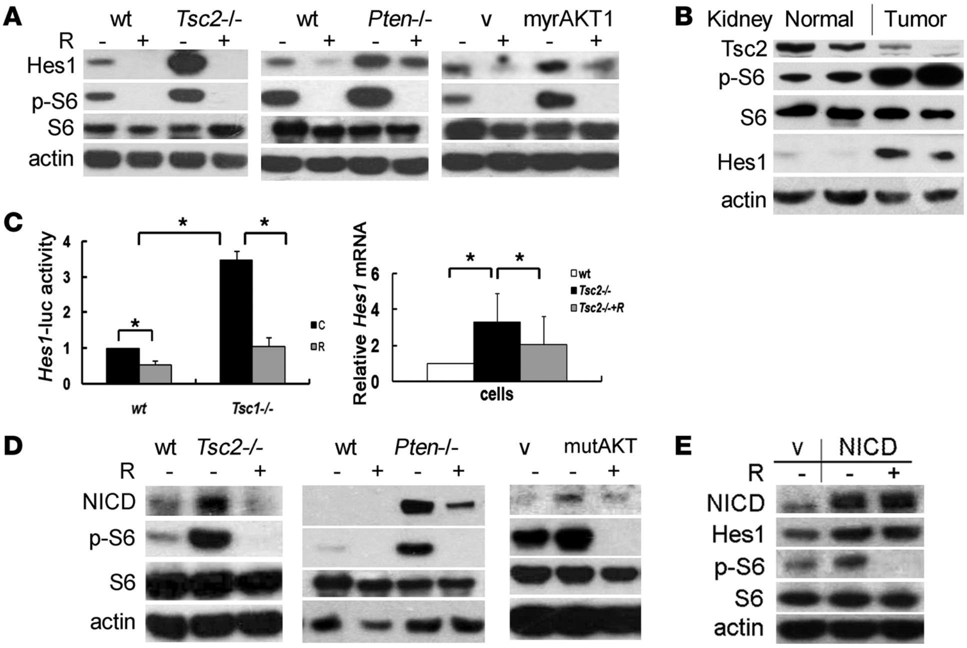

Figure 2

mTOR is a positive regulator of Notch signaling. (**A**) WT, *Tsc2*$^{-/-}$, and *Pten*$^{-/-}$ MEFs and WT MEFs transduced with the retroviruses for myristoylated AKT1 (myrAKT1) in pLXIN-hyg or its control vector pLXIN-hyg (V) were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for Hes1 and p-S6 (Ser235/236). (**B**) mTOR and Notch signaling were assessed by immunoblotting in age-matched normal kidneys and 2 kidney tumors from *Tsc2*$^{del3/+}$ mice. (**C**) Left: Hes1 promoter reporter luciferase assay for WT and *Tsc1*$^{-/-}$ MEFs treated with or without 10 nM rapamycin for 24 hours. The relative Hes1-luciferase activity (firefly luciferase activity/*Renilla* luciferase activity) is shown. Right: Quantitative real-time RT-PCR for relative *Hes1* mRNA from WT and *Tsc2*$^{-/-}$ MEFs. Values are the mean ± SD of triplicate samples. $^*P < 0.05$. (**D**) WT, *Tsc2*$^{-/-}$, and *Pten*$^{-/-}$ MEFs and WT MEFs transduced with the retroviruses for AKT1E17K (mutAKT) in pLXIN-hyg or its control vector pLXIN-hyg (V) were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for active Notch1 (NICD) and p-S6. (**E**) WT MEFs transduced with the retroviruses for NICD in pMig (pMig-ICN1) (NICD) or its control vector pMIG (V) in the presence or absence of 10 nM rapamycin for 24 hours and then subjected to immunoblotting for p-S6, NICD, and Hes1.

Results

Hyperactive mTOR blocks cell differentiation. Uncontrolled cell proliferation is usually coupled with blockage of cell differentiation, and we therefore postulated that hyperactive mTOR signaling might hinder cell differentiation, even though normal functioning of AKT-mTOR is required for cell differentiation (18–21). To test this idea, we utilized MEFs that were deficient in the mTOR suppressor genes *Tsc1* and *Tsc2* and consequently demonstrated hyperactive mTOR signaling (10, 31). MEFs have the ability to differentiate into various cell lineages, including myocytes and adipocytes (32, 33), and we therefore examined the effects of mTOR activation on this differentiation process (Figure 1). To induce myogenic differentiation, WT MEFs were transduced with retroviruses expressing MyoD, a master myogenic regulator (Figure 1A). The MEFs underwent myogenic differentiation, as demonstrated by the formation of myotubes and the expression of myosin, a muscle marker (Figure 1B). This differentiation was blocked by the mTOR inhibitor rapamycin (Figure 1B), indicating that normal mTOR signaling was required for myogenic differentiation. In contrast, *Tsc2*$^{-/-}$ MEFs with exogenous MyoD expression were unable to differentiate into myocytes until they were treated with rapamycin, at which point they differentiated in a dose-dependent manner (Figure 1B), suggesting that hyperactive mTOR signaling due to *Tsc2* deficiency inhibits myogenic differentiation.

Similarly, WT MEFs transduced with retroviruses expressing PPARγ (Figure 1A), a master adipogenic regulator, underwent adipogenic differentiation, as shown by the formation of lipid droplets and the expression of aP2 and c/EBPα, 2 adipocyte markers (Figure 1C). However, in parallel with the results of myogenic differentiation, both *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ MEFs expressing PPARγ failed to produce lipid droplets or express aP2 or c/EBPα (Figure 1C). In addition, adipogenic differentiation of the WT MEFs was inhibited by treatment with rapamcyin, while conversely, it was restored by rapamycin in *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ MEFs. Thus, these data on myogenic and adipogenic differentiation of MEFs suggest that a normal range of mTOR activity is critical for cell differentiation, but that cells with either too much or too little mTOR activity fail to differentiate normally.

mTOR is a positive regulator of Notch signaling. We next investigated any potential role of the Notch pathway in the mechanism underlying the impaired differentiation potential of mTOR-activated cells. We first investigated whether hyperactive mTOR could cause abnormal Notch signaling by monitoring the levels of Hes1, a direct target of Notch. We found that Hes1 protein expression was dramatically elevated in cells with constitutively active mTOR caused by loss of the *Tsc2* or *Pten* tumor suppressor gene or oncogenic myristoylation of AKT1 (myrAKT1) (34) (Figure 2A). The expression of Hes1 in all cell lines examined was reduced by rapamycin treatment, indicating that it was mTOR dependent (Figure

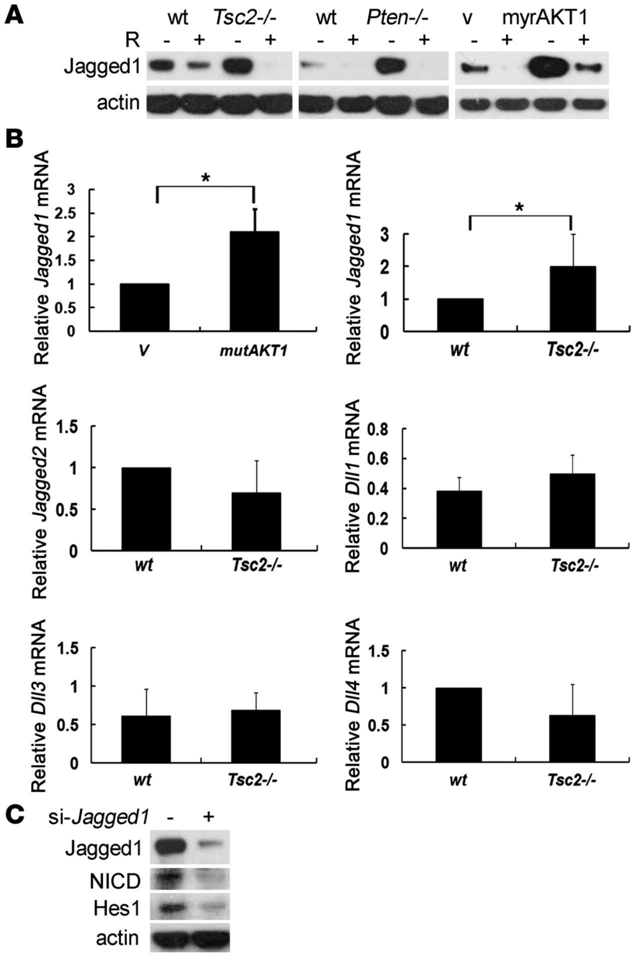

Figure 3

Expression of the Notch ligand Jagged1 is elevated in cells with active mTOR. (**A**) WT, *Tsc2*$^{-/-}$, and *Pten*$^{-/-}$ MEFs and WT MEFs transduced with the retroviruses for myristoylated AKT1 in pLXIN-hyg or its control vector pLXIN-hyg (V) were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for Jagged1. (**B**) Results of quantitative real-time RT-PCR for *Jagged1* mRNA in WT MEFs transduced with the retroviruses for AKT1E17K in pLXIN-hyg or its control vector pLXIN-hyg (V). Similar results are shown for *Jagged1, Jagged2, Dll1, Dll3*, and *Dll4* mRNA in *Tsc2*$^{-/-}$ and WT MEFs. Values are the mean ± SD of triplicate samples. $^{*}P < 0.05$. (**C**) *Jagged1* siRNA treatment of *Pten*$^{-/-}$ MEFs led to reduced levels of Jagged1, NICD, and Hes1, as assessed by immunoblotting. Non–target-directed random siRNA served as a control.

2A). The upregulation of Hes1 by mTOR was also observed in vivo, as mouse kidney tumors with hyperactive mTOR due to *Tsc2* exon 3 deletion (35) exhibited enhanced Hes1 expression (Figure 2B). Promoter reporter assays and quantitative real-time PCR analysis showed increased *Hes1* promoter activity and transcript levels, indicating that the upregulation of Hes1 expression likely occurs at the transcriptional level in an mTOR-dependent manner (Figure 2C). In addition, the Notch transactivator NICD domain was also elevated in cells devoid of either *Tsc2* or *Pten* or expressing oncogenic AKT1 E17K (AKT1-E17K) (36), and this elevation was attenuated by rapamycin treatment (Figure 2D). To provide further evidence that Notch-dependent Hes1 expression was downstream of mTOR, we examined the effects of ectopic expression of NICD on Hes1 expression and found that it was resistant to rapamycin treatment (Figure 2E). Taken together, these results show that activation of mTOR leads to upregulation of Notch signaling, generation of NICD, and expression of Notch targets both in vitro and in vivo.

mTOR positively regulates the expression of Notch ligand Jagged1. In light of this evidence for activated Notch signaling in cells with mTOR activation, we examined the expression of a Notch ligand, Jagged1, which was previously found to be elevated in some cancers with a more aggressive disease course (37). Jagged1 was expressed at higher levels in the cell lines with activated mTOR and was markedly reduced by rapamycin treatment (Figure 3A), which suggests that Jagged1 expression was also under the control of mTOR. This upregulation of Jagged1 by mTOR was due, at least in part, to higher levels of *Jagged1* mRNA in the *Tsc2*-null and mutant AKT-expressing cell lines (Figure 3B) and appeared to be directly responsible for Notch activation and Hes1 expression, as these were blocked by decreased Jagged1 expression using siRNA (Figure 3C). In contrast to *Jagged1*, levels of other Notch ligands such as *Jagged2*, *Dll1*, *Dll3, or Dll4* (38) were not significantly different in the WT versus *Tsc2*$^{-/-}$ MEFs we used here (Figure 3B). Since Notch also upregulates Jagged1 through a positive feedback mechanism (39) (Figure 4A), the enhanced expression of Jagged1 may be influenced by both upstream and downstream signaling events.

mTOR regulates cell differentiation through the Notch pathway. As mTOR is a positive regulator of the Jagged1/Notch/Hes1 pathway, we then investigated the effects of activated Notch signaling on the ability of cells with activated mTOR to differentiate. Inhibition of Notch signaling either by a dominant-negative form of the Notch transcriptional coactivator MAML1 (DN-MAML1) (32) (Figure 4A) or compound E, a γ-secretase inhibitor known to block Notch cleavage (Figure 4B), suppressed the adipogenic differentiation of WT MEFs expressing PPARγ. In contrast, treatment of *Tsc1*$^{-/-}$ or *Tsc2*$^{-/-}$ MEFs expressing PPARγ with either DN-MAML1 (Figure 4A) or compound E (Figure 4B) markedly restored their adipogenic differentiation capacity (Figure 4B). Moreover, reduction of Notch by siRNA potentiated the adipogenic differentiation of *Tsc2*$^{-/-}$ MEFs expressing PPARγ (Figure 4C). In addition, activation of Notch-Hes1 by overexpression of Jagged1 reduced the conversion of WT MEFs with PPARγ into adipocytes (Figure 4D). Therefore, Notch acts as an effector of mTOR signaling, while the deregulated mTOR activation inhibits cell differentiation through upregulation of Notch signaling. It is possible that mTOR exerts its dual effects on cell differentiation via the binary roles of Notch in cell differentiation.

Inhibition of Notch suppresses the tumorigenesis of cells with activated mTOR. Since the role of Notch in tumorigenesis is context dependent and Notch signaling was potentiated in cells with an oncogenically activated AKT/mTOR pathway, we reasoned that activation of Notch signaling might be critical for AKT/mTOR-mediated tumorigenesis and could therefore be targeted for the treatment of cancers with active mTOR signaling. We indeed found that suppression of the Notch pathway by the Notch inhibitor N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) compromised the proliferation of both MEFs and human cancer cells with activated mTOR signaling caused either by lack of the Tsc2 or PTEN (PC3 cells) tumor suppressors or by expression of AKT1-E17K (Figure 5A). Furthermore,

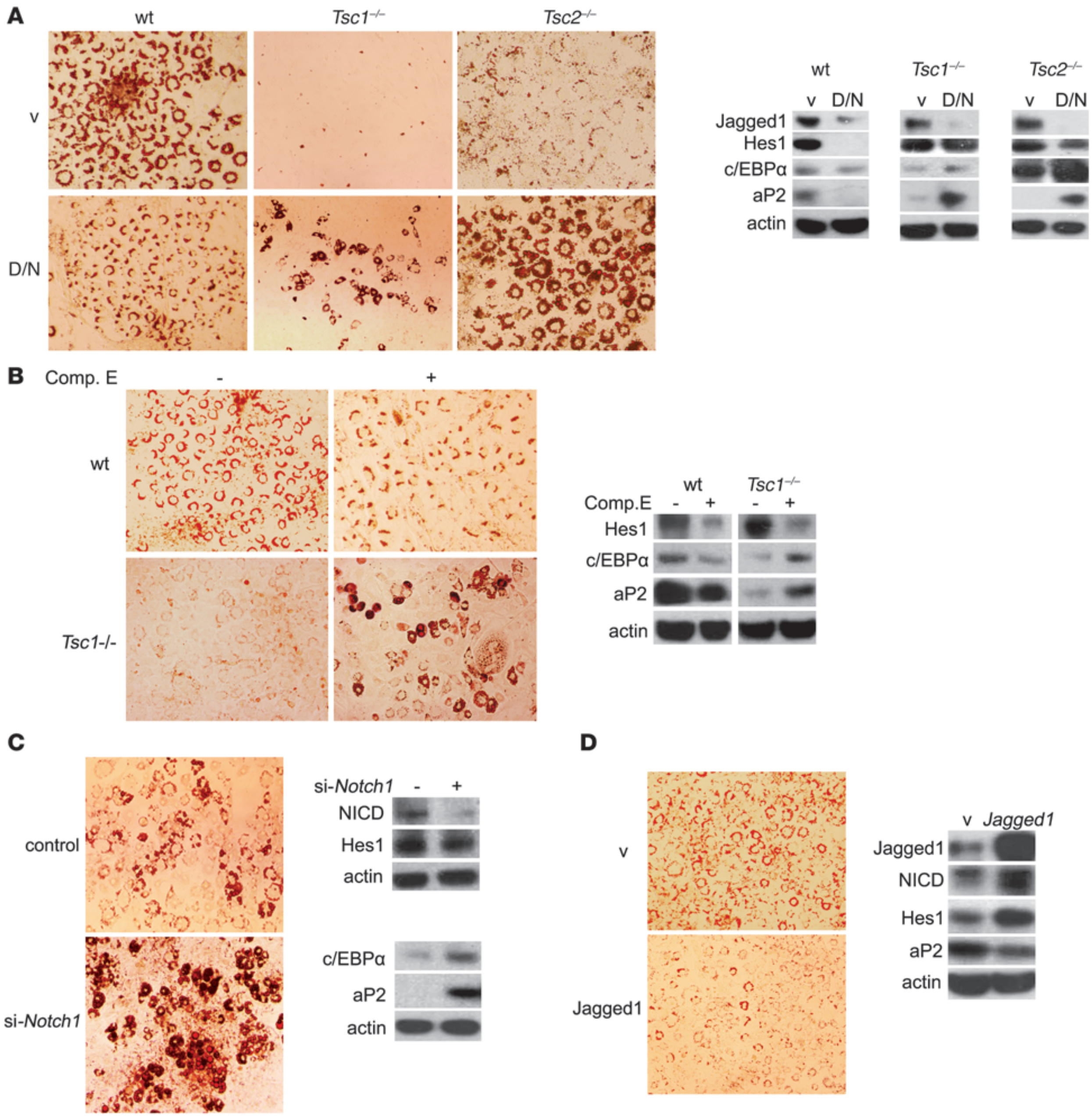

Figure 4

mTOR regulates cell differentiation through Notch. (**A**) WT, *Tsc1*−/−, or *Tsc2*−/− MEFs stably expressing exogenous PPARγ were transduced either with pMIG-DNL1 (D/N) or pMIG (V) retroviruses prior to undergoing adipogenic differentiation. (**B**) WT or *Tsc1*−/− MEFs stably expressing exogenous PPARγ were treated with or without 100 nM compound E (comp. E) during adipocyte differentiation. (**C**) *Notch1* was knocked down with siRNA in *Tsc2*−/− MEFs prior to undergoing adipocyte differentiation. Non–target-directed random siRNA served as a control. (**D**) WT MEFs were transduced with the retroviruses for Jagged1 in pLXIN-hyg or its control vector pLXIN-hyg (V) prior to undergoing adipocyte differentiation. Left panels: Oil Red O staining for lipid droplets (original magnification, ×200). Right panels: Immunoblotting.

the tumorigenicity of *Pten*−/− MEFs in nude mice was attenuated by inhibition of Notch signaling with dominant-negative Notch, DNL1 (DN-MAML1) (Figure 5B).

mTOR activates the Notch pathway through upregulation of p63. To explore the mechanism by which mTOR activation leads to the upregulation of Jagged1/Notch/Hes1 signaling, we examined the expression of p63, a TP53 family member, since p63 is a positive regulator of Jagged1 or Jagged2 expression and Notch activity (40–42) and PI3K is an inducer of p63 expression (43). The *p63* gene has 2 promoter regions generating *TA-p63* and *ΔN-p63* isoforms, each of which has 3 splicing variants on its C terminus as α/β/γ of *TA-p63* and *ΔN-p63* isoforms (41). The TA-p63 isoform has been observed to activate the expression of ΔN-p63 isoform (44). Both TA-p63 and ΔN-p63 were overexpressed in cells with mTOR activation because of either a lack of *Tsc2* or *Pten* or expression of AKT1-E17K, and were repressed by rapamycin (Figure

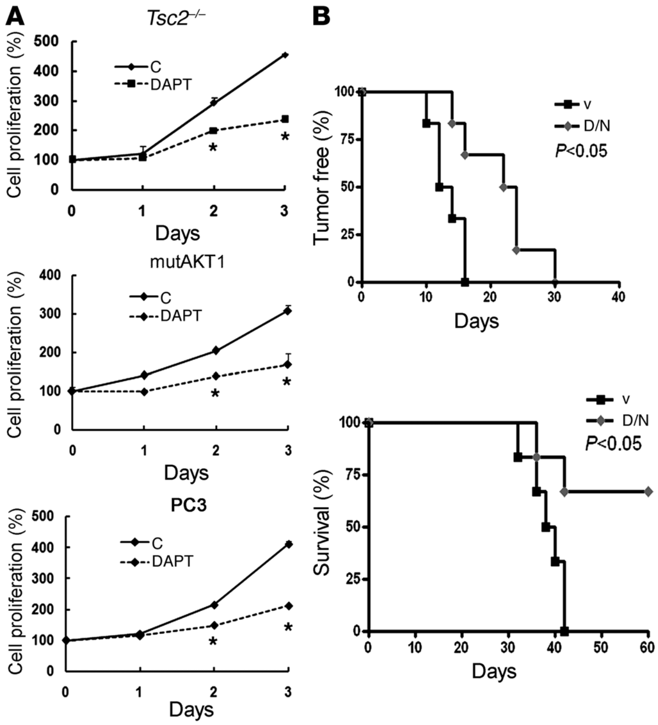

Figure 5

Inhibition of Notch suppresses the tumorigenesis of cells with activated mTOR. (**A**) The proliferation of *Tsc2*$^{-/-}$ MEFs, AKT1E17K MEFs, or PC3 cells treated with or without 50 μM DAPT was examined by MTT assay. Values represent the mean ± SD of triplicate samples. $^{*}P < 0.05$ (DAPT-treated cells compared with untreated cells on day 2 or 3). (**B**) *Pten*$^{-/-}$ MEFs transduced with either pMIG-DNL1-GFP (D/N) or pMIG (V) retroviruses were inoculated subcutaneously into nude mice and followed for tumor development and survival.

6A). Moreover, reduction of the expression of all the *p63* isoforms using pan siRNA for *p63* (39, 45, 46) decreased the expression of both Jagged1 and Hes1 in WT MEFs, MEFs without *Tsc1* or *Tsc2*, and WT MEFs expressing myrAKT1 (Figure 6B). Similar to the effects of mTOR and Notch signaling on cell differentiation, a normal range of p63 levels is critical for the adipogenic differentiation of MEFs expressing PPARγ (Figure 6C). These findings suggest that p63 expression is regulated by mTOR and that mTOR signaling is at least partially relayed to the Notch pathway through p63. Moreover, the mTOR/p63/Notch signaling cascade plays an important role in cell differentiation.

STAT3 transduces mTOR signaling to the p63/Notch axis. We next explored the mechanism of how activated mTOR influences p63 expression. STAT3, which has been reported to be a transcriptional activator of p63 (47), is a downstream target of mTOR (48) and is highly activated in *Tsc1*- or *Tsc2*-deficient cells (16, 49). Therefore we examined the possibility that STAT3 might be the link between mTOR activation and p63 expression. Similar to previous results with *Tsc1*- or *Tsc2*-deficient cells (49), total STAT3 and phosphorylated STAT3 (p-STAT3; Ser705) levels were increased in *Pten*-knockout or AKT1-E17K–expressing cells and the activation of STAT3 in these cells was also mTOR dependent (Figure 7A). Inactivation of STAT3 by either the STAT3 inhibitor AG490 (Figure 7B) or knockdown by siRNA (Figure 7C) led to reductions in p63, Jagged1, and Hes1 in all the mTOR-activated cells examined. In addition, kidney tumors from *Tsc2*$^{+/-}$ mice (31) demonstrated dramatic activation of STAT3, p63, Notch, and mTOR (Figure 7D). These data indicate that STAT3 connects mTOR to Notch signaling via p63.

mTOR regulates STAT3/p63/Notch signaling in human tumors. We next examined whether this newly discovered mTOR regulation of Notch signaling would also be seen in human cancer cells. p-S6, p-STAT3, p63, Jagged1, and Hes1 were all reduced in human breast (MCF7 and MDA-MB-468), prostate (PC3), lung (A549), pancreatic (PANC-1), and liver (HepG2) cancer cell lines treated with rapamycin (Figure 8A). In addition, restoration of PTEN in the PTEN-deficient prostate cancer cell line PC3 normalized AKT/mTOR/STAT3/p63/Notch signaling (Figure 8B). Therefore, the mTOR/STAT3/p63/Jagged1/Notch/Hes1 signaling cascade also presents in human cancer cell lines.

Since mTOR regulates STAT3/p63/Notch signaling network in human cancer cell lines in vitro, we predicted that this signaling regulation should also exist in human tumors in vivo. We first tested this hypothesis by analyzing mRNA microarray data of lung LAM tissues, which are known for mTOR hyperactivation due to inactive mutations of either the *TSC1* or the *TSC2* gene, in comparison with normal pulmonary artery smooth muscle cells from the Gene Expression Omnibus database (GEO accession number GSE12027, mRNA microarray data deposited by Y. Zhang and G. Pacheco-Rodriguez) (Supplemental Tables 1–3; supplemental material available online with this article; doi:10.1172/JCI37964DS1). As expected, *VEGF-D* (activated by mTOR and considered to be a diagnostic marker for LAM) (50), *Jagged2*, and *Hes1* were significantly increased in LAM tissues (Table 1), suggesting that mTOR regulates Notch in human LAM. This finding is also consistent with our in vitro observation that mTOR-regulated expression of either Jagged1 or Jagged2 was cell type specific, but in a mutually exclusive manner (our unpublished observations).

We then examined the correlation between p-S6 levels and Notch markers in breast cancer. Using regression analysis of immunoblots for breast cancer tissues, we found that p-S6 levels correlated well with Hes1 expression in poorly differentiated but not in well-differentiated human breast ductal carcinoma samples (Figure 8C, Supplemental Table 4, and Table 2). In addition, p-S6, p-STAT3, p63, and Jagged1 were all correlated with each of their downstream targets and the proteins further downstream in the mTOR/STAT3/p63/Jagged1/Notch signaling cascade, with the single exception of p63: Hes1 (Figure 8C and Supplemental Table 5). Taken together, these results suggest that mTOR activates STAT3/p63/Notch and inhibits cell differentiation in these human tumors.

STAT3/p63/Notch signaling is controlled under mTOR complex 1. All of the observations presented thus far indicate that the effects of mTOR on STAT3/p63/Notch signaling is rapamycin sensitive, suggesting that STAT3/p63/Notch is under the control of mTORC1. To confirm this model, *mTOR* or *rictor* (an important component in mTORC2) (3, 4) was knocked down in *Tsc2*$^{-/-}$ MEFs (Figure 9). While the reduction of mTOR markedly abolished STAT3, p63, and Notch signaling (Figure 9A), decreased expression of rictor did not affect the STAT3/p63/Notch signaling cascade (Figure 9B). These data thus confirm that mTORC1 regulates STAT3/p63/Notch signaling, and this effect is independent of mTORC2 (Figure 9C).

NF-κB and STAT3/p63 control Notch signaling downstream of mTORC1 in parallel. During the preparation of this manuscript, Bedogni et al. reported that hyperactivated AKT signaling led

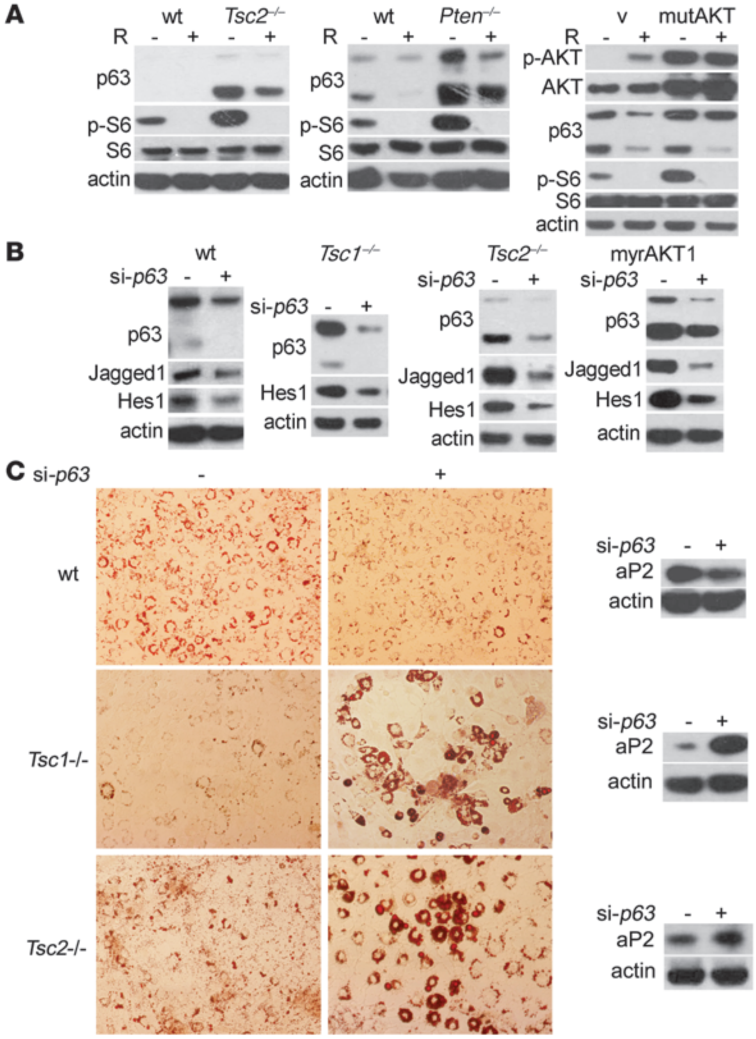

Figure 6

mTOR activates the Notch pathway through upregulation of p63. (**A**) WT, *Tsc2*$^{-/-}$, *Pten*$^{-/-}$, and WT MEFs transduced with the retroviruses for AKT1E17K in pLXIN-hyg or its control vector pLXIN-hyg (V) were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for both TA and ΔN p63 and p-S6 (Ser235/236). (**B**) WT, *Tsc1*-/, *Tsc2*$^{-/-}$, or WT MEFs transduced with the retroviruses for myristoylated AKT1 (myrAKT1) in pLXIN-hyg were transfected with *p63* siRNA (RNAi) to knock down *p63* expression for 48 hours and then subjected to immunoblotting. Non-target-directed random siRNA served as a control. (**C**) WT, *Tsc1*$^{-/-}$, or *Tsc2*$^{-/-}$ MEFs stably expressing exogenous PPARγ were transfected with either *p63* siRNA (RNAi) or non–target-directed random siRNA prior to undergoing adipogenic differentiation. Left: Oil Red O staining for lipid droplets (original magnification, ×200). Right: Immunoblotting.

to upregulation of Notch1 through NF-κB activity in melanoma (51). NF-κB is a key transcriptional factor family that plays important roles in multiple physiological and pathological processes, including cell proliferation/differentiation and tumorigenesis (51). Therefore, we examined the relationship of this newly identified AKT/NF-κB/Notch signaling cascade and the RTK/PI3K/PTEN/AKT/TSC1/2/mTORC1/STAT3/p63/Notch signaling pathway defined above. Because the enhanced NF-κB signaling in *Tsc2*$^{-/-}$ MEFs was abolished by rapamycin treatment, NF-κB signaling appeared to be downstream of mTORC1 (Figure 10A). However, knockdown of *Nfkb* did not change the state of STAT3 and p63, and knockdown of *STAT3* was unable to influence NF-κB, suggesting that NF-κB has no functional interaction with STAT3/p63 (Figure 10B). Since both NF-κB and p63 are inducers of Jagged1 expression (40, 52), NF-κB therefore, in parallel with STAT3/p63, modulates Notch signaling downstream of mTORC1 (Figure 10C).

Discussion

We provide evidence that mTORC1 positively regulates Notch signaling through upregulation of the STAT3/p63/Jagged cascade, and that Notch cascade activation impedes cell differentiation, in cells in which mTORC1 is activated due to loss of *Pten*, *Tsc1*, and *Tsc2* or acquisition of oncogenic AKT1 mutants. In addition, mouse and human tumors caused by hyperactive mTOR signaling present aberrant high STAT3/p63/Notch activity, while inhibition of Notch signaling extends survival in a *Pten*-null nude mouse tumor model.

Although the RTK/PI3K/AKT/mTOR pathway is one of the most frequently altered signaling networks in cancer, the mechanism by which it contributes to tumorigenesis remains uncertain. By studying various cell lines with activated mTOR signaling, we observed hyperactivation of STAT3/p63/Jagged1/Notch/Hes1 signaling in those cells and showed that these events were sensitive to rapamycin treatment and independent of mTORC2. We have therefore identified mTORC1 as a positive regulator of Notch signaling (Figure 9C). In support of this mTORC1-Notch connection, mouse kidney tumors and human LAM tissues driven by upregulated mTOR due to *TSC2* deficiency, as well as poorly differentiated breast cancers, exhibited hyperactive STAT3/p63/Notch signaling (Figure 2B, Figure 7D, Figure 8C, Tables 1 and 2, and Supplemental Table 5). Therefore, Notch effectors such as Hes1 may serve as surrogate markers for mTOR activation.

The p53 family consists of 3 transcription factors, p53, p63, and p73, which share overlapping and distinct functions as key regulators of cell cycle and cell death in the regulation of development, proliferation/differentiation, and response to cellular

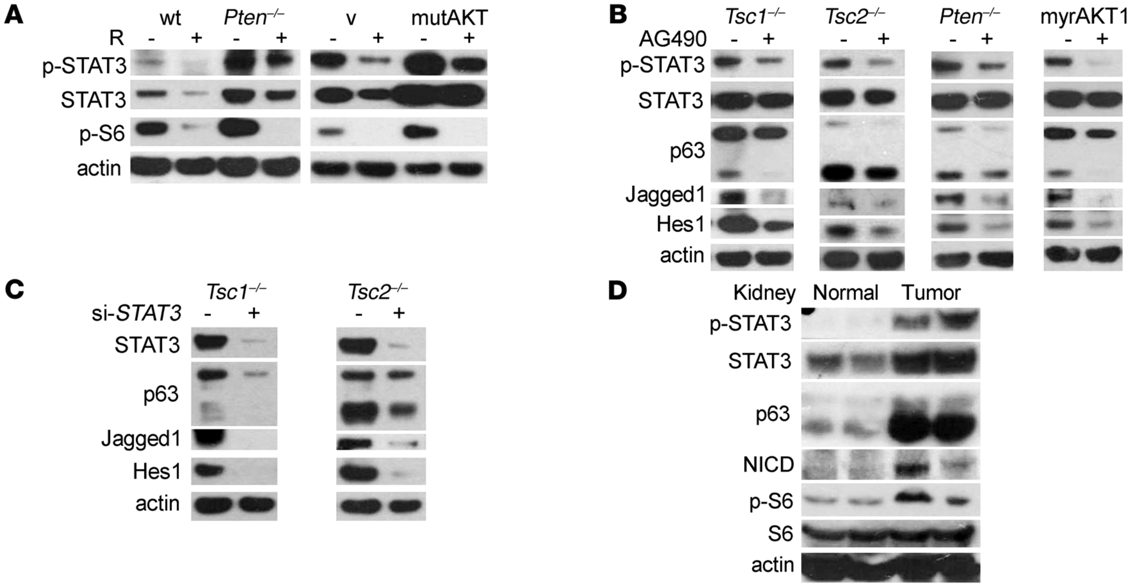

Figure 7

STAT3 transduces mTOR signaling to the p63/Notch axis. (**A**) WT and *Pten*$^{-/-}$ MEFs and WT MEFs transduced with the retroviruses for AKT1E17K in pLXIN-hyg or the control vector pLXIN-hyg (V) were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for p-S6 (Ser235/236) and p-STAT3 (Ser705). (**B**) *Tsc1*$^{-/-}$, *Tsc2*$^{-/-}$, and *Pten*$^{-/-}$ MEFs and WT MEFs transduced with the retroviruses for myristoylated AKT1 in pLXIN-hyg or the control vector pLXIN-hyg (V) were treated with or without 50 nM AG490 for 24 hours and then subjected to immunoblotting. (**C**) *Tsc1*$^{-/-}$ or *Tsc2*$^{-/-}$ MEFs were transfected with *STAT3* siRNA to knock down *STAT3* expression for 48 hours and then subjected to immunoblotting. Non–target-directed random siRNA served as a control. (**D**) Expression of STAT3, p63, NICD, and p-S6 were assessed in age-matched kidneys from 2 normal mice and kidney tumors from 2 *Tsc2*$^{+/-}$ mice by immunoblotting.

stress (53, 54). Dysfunction of this family has been implicated in the majority of human cancers (55). mTOR has been reported to be a positive modulator of p53 (31, 56) and a negative regulator of p73 (57). To our knowledge, we have now identified p63 as a novel effector of mTOR signaling downstream of the transcriptional activator STAT3. p63 in turn activates Notch signaling through stimulation of *Jagged1* gene expression, although we were unable to determine which of the 6 isoforms were responsible for Jagged1 activation at this time. Even if members of the p53 family are generally considered to be tumor suppressors, the function of p63 in tumorigenesis remains uncertain (55, 58). The mTOR/p63/Notch connection should cast some insights on the role of p63 and its regulation in tumor development. As another effector of mTOR (31, 56), p53 appears to play no role in mTOR-

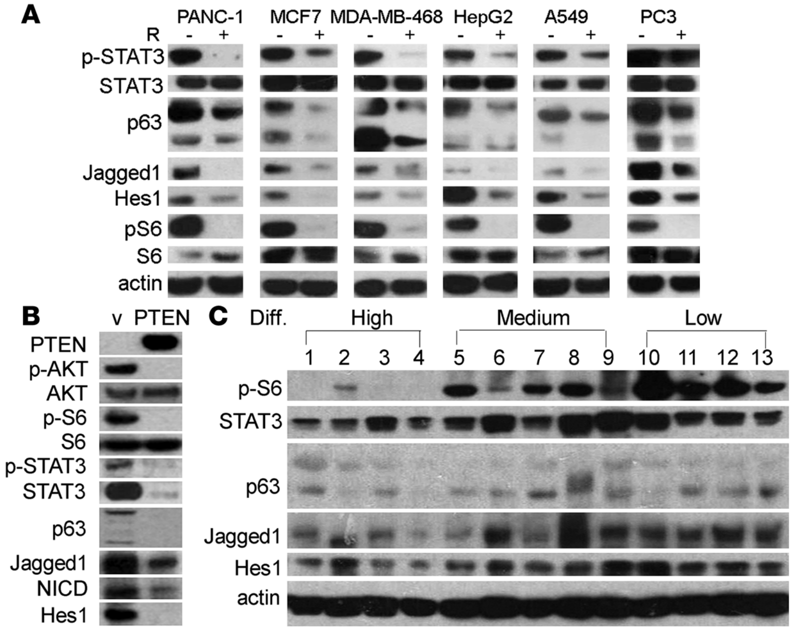

Figure 8

mTOR regulates STAT3/p63/Notch signaling in human cancers. (**A**) Human breast (MCF7 and MDA-MB-468), prostate (PC3), lung (A549), pancreatic (PANC-1), and liver (HepG2) cancer cell lines were treated with 10 nM rapamycin for 24 hours and then subjected to immunoblotting for components of the mTOR/STAT/p63/Notch signaling pathway. (**B**) PC3 cells were transduced with either pLXIN-hyg retroviruses (V) or pLXIN-hyg-PTEN retroviruses (PTEN) and then subjected to immunoblotting for PTEN and mTOR/STAT/p63/Notch signaling pathway components. (**C**) Human breast cancer tissues were immunoblotted for components of the mTOR/STAT/p63/Notch signaling cascade. A representative blot is shown. The differentiation states (Diff.) of cancer cells were as indicated.

Table 1
Human lung LAM with hyperactive mTOR/Notch signaling

	VEGF-D			Jagged2			Hes1		
Probe	**Fold change[A]**	***t*[B]**	***P***	**Fold change**	***t***	***P***	**Fold change**	***t***	***P***
1	30.2	–6.28	<0.001	2.8	–5.64	<0.001	2.2	–3.36	0.0034
2				1.8	–4.18	<0.001	3.4	–5.97	<0.001

mRNA microarray data of 14 lung LAM nodules and 7 pulmonary artery smooth muscle cell lines were derived from the Gene Expression Omnibus database. [A]Fold change is LAM versus pulmonary artery smooth muscle cells. [B]*t* statistic. $P < 0.01$ and a 1.5-fold increase were considered significant.

mediated Notch activation (our unpublished observations). It would be of interest to determine whether p73 participates in the regulation by mTOR on Notch.

The serine/threonine kinase AKT is a central regulator of cell proliferation, survival, and metabolism by phosphorylating multiple protein substrates including TSC2 and IκB kinase (IKK). AKT activates NF-κB via IKK and mTOR through TSC2 (1). Bedogni et al. recently reported that hyperactivated AKT signaling led to upregulation of Notch1 through NF-κB activity in melanoma (51). We have confirmed their findings here and further defined NF-κB, in parallel with the STAT3/p63 cascade, as a regulator of Notch signaling downstream of mTOR (Figure 10).

Furthermore, we found that this mTOR/STAT3/p63/Jagged1/Notch/Hes1 cascade plays an important role in the regulation of cell differentiation. Interestingly, while inhibition of mTOR/p63/Notch inhibits the differentiation of normal cells, sustained activation of mTOR impairs cell differentiation through overactivation of the p63/Jagged/Notch cascade. This observation is consistent with a recent report showing that differentiation of HepaRG cells into hepatocyte-like cells is attenuated by expression of an activated mutant mTOR (59). Given the dose-dependent binary effects of both mTOR and Notch on cell differentiation, the functioning of Notch appears to depend on the status of mTOR activity. This mechanism may be the molecular basis of the dose-dependent role of Notch in hematopoiesis and leukemogenesis (60). These observations are in accordance with the multifaceted roles of Notch signaling as both a repressor and an inducer of terminal differentiation in different settings, possessing both growth-promoting and tumor-suppressor functions in different contexts (26, 61).

The results of this study suggest that, in response to differential cues from mTOR, Notch serves as a molecular switch to couple 2 seemingly unrelated cellular processes: proliferation and differentiation. If the activity of RTK/PI3K/AKT/mTOR/p63/Notch deviates from the normal physiological range, cells may fail to differentiate or proliferate normally and may cause developmental defects or tumors. The inhibition of cell differentiation due to aberrant Notch signaling might be one of the underlying mechanisms responsible for tumorigenesis associated with aberrant RTK/PI3K/AKT/mTOR pathway activation, which is supported by the correlation between activation of mTOR and Notch signaling in poorly differentiated breast cancers (Figure 8C and Table 2). Moreover, uncontrolled RTK/PI3K/AKT/mTOR activation may underlie the ligand-mediated activation of Notch in tumors with overexpressed Jagged and unopposed Notch signaling. The Notch signaling pathway indeed appears to be critical for active mTOR-mediated tumorigenesis, since blunted Notch signaling compromised the proliferation and tumorigenic potential of cells with an activated mTOR pathway (Figure 5).

Even though the possibility of cancer treatment using Notch inhibitors is controversial due to the complex role of Notch in both embryonic and cancer development (26, 29, 62), our data suggest that components of the STAT3/p63/Jagged1/Notch axis may become novel targets for the treatment of cancers caused by deregulation of RTK/PI3K/AKT/mTOR signaling. Conversely, intervention in the PI3K/mTOR signaling pathway might be an option for cancers with activated Notch signaling. Furthermore, RTK/PI3K/AKT/mTOR/STAT3/p63/Jagged/Notch/Hes1 networks are likely the candidates for targeted combination cancer therapy. Because the Notch pathway has also been reported to be a positive regulator of the PI3K/AKT/mTOR pathway (63, 64), our current findings indicate that mTOR and Notch interact in a reciprocal regulatory loop. The interplay between mTOR and Notch may be of wider significance in development, physiology, and pathophysiology beyond its potential cancer connection.

Methods

Reagents and antibodies. Reagents were obtained from the following sources: rapamycin, AG490, DAPT, hygromycin B, and puromycin from Sigma-Aldrich; compound E from Axxora; DMEM, FBS, 4%–12% Bis-Tris Nu-PAGE gels, and Lipofectamine 2000 from Invitrogen.

Anti–p-S6 (Ser235/236) and anti-S6 antibodies have been described previously (34). The myosin antibody MF20 developed by Donald A. Fischman was obtained from the Developmental Studies Hybridoma Bank. TSC2, AKT, PPARγ, c/EBPα, MyoD, p63, and Jagged1 antibodies and all HRP-labeled secondary antibodies were from Santa Cruz Biotechnology Inc. aP2 and Hes1 antibodies were from CHEMICON. STAT3, p-STAT3 (Ser705), p65, p-p65 (Ser536), p-IκBα (Ser32), p-AKT (Ser473), and PTEN antibodies were from Cell Signaling Technology. Notch1 (NICD) antibody was from Epitomics, and β-actin antibody was from Sigma-Aldrich.

Plasmids pMSCV, pMSCV-MyoD, pMSCV-PPARγ, pMIG (pMSCV-IRES-GFP), pMIG-DNL1 (pMSCV plasmids expressing MAML13-74-GFP fusion protein as a dominant-negative mutant for blocking Notch signaling), the Hes1-luc reporter plasmid encoding firefly luciferase, and

Table 2
Correlation of mTOR and Notch signaling in human breast cancers is dependent on differentiation status

Differentiation	**Low** **(*n* = 12)**	**Medium to High** **(*n* = 17)**
p-S6 versus Hes1	0.333 ± 0.069[A]	0.196 ± 0.094[B]
r^2	0.70	0.22

Ordinary least squares regression analysis of the effect of mTOR on Notch signaling in human breast cancers. [A]Significance at 1%. [B]Significance at 10%.

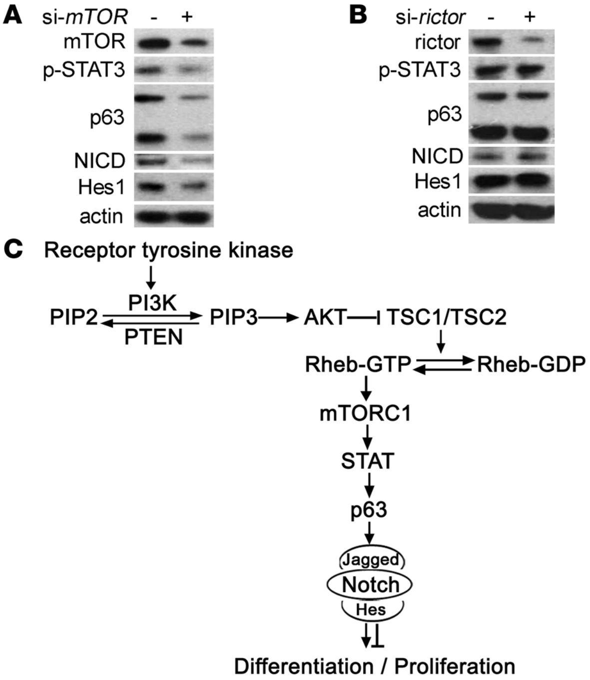

Figure 9

STAT3/p63/Notch signaling is controlled under mTORC1. (**A**) $Tsc2^{-/-}$ MEFs were transfected with siRNA to knock down *mTOR* expression for 48 hours and then subjected to immunoblotting for p-STAT3 (Ser705), p63, and Notch components. Non–target-directed random siRNA served as a control. (**B**) $Tsc2^{-/-}$ MEFs were transfected with siRNA to knock down *rictor* expression for 48 hours and then subjected to immunoblotting for p-STAT3, p63, and Notch components. Non–target-directed random siRNA served as a control. (**C**) Schematic illustration of how the RTK/PI3K/AKT/mTOR pathway regulates cell differentiation through the STAT/p63/Jag/Notch cascade. Upon stimulation of RTKs, PI3K activates AKT, which phosphorylates TSC2 and reduces the GAP activity of the TSC1/TSC2 complex toward Rheb-GTP, increasing Rheb-GTP levels. Rheb-GTP activates mTORC1, which in turn enhances Notch signaling through upregulation of the STAT3/p63 axis. mTORC1 regulates cell differentiation through Notch signaling in a dose-dependent manner.

the internal nonspecific control pRL-TK plasmid, pMIG-ICN1 plasmid expressing human NICD, and pLXIN-hyg-myrAKT1 have been reported previously (32, 34, 65, 66). pLNCX-HA-AKT1 was from Addgene. Generation of pLXIN-hyg-Jagged1, pLXIN-hyg-PTEN, and pLXIN-hyg-AKT1(E17K) is described in Supplemental Methods.

Cell culture. All MEFs were described previously (10, 31, 34). $Pten^{-/-}$ MEFs were provided by Ronald A. DePinho (Dana-Farber Cancer Institute). PT67 was from Clontech. MCF7, MDA-MB-468, PANC-1, A549, PC3, and HepG2 were from ATCC. Cells were cultured in DMEM with 10% FBS in 5% CO$_2$ at 37°C. Production of retroviruses and subsequent generation of stable gene expression cell lines have been described (34) and detailed in Supplemental Methods.

Quantitative real-time RT-PCR. Total RNA was extracted from cells using Trizol (Invitrogen) and reversely transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). cDNA was used as a template in a quantitative PCR reaction (Supplemental Methods).

siRNA knockdown. siRNAs were synthesized and transfected into cells for mRNA knockdown (Supplemental Methods).

Notch luciferase reporter assay. Hes1-luc reporter plasmid encoding firefly luciferase and an internal nonspecific control pRL-TK plasmid encoding renilla luciferase were used for the assay (Supplemental Methods).

Muscle differentiation. MEF cells were transduced with pMSCV-MyoD retroviruses or pMSCV control viruses for muscle differentiation (Supplemental Methods).

Adipocyte differentiation. MEF cells were transduced with pMSCV-PPARγ retroviruses or pMSCV control viruses for differentiation into adipocytes (Supplemental Methods).

Mouse kidney tumor assessment. For Figure 2B, the kidney tumors were from 2 heterozygous *Tsc2* exon 3 deletion ($Tsc2^{del3/+}$) mice (19 and 22 months old), and the kidneys were from 2 WT mice (22 and 23 months old) (35). For Figure 7D, the tumors from $Tsc2^{+/-}$ mice and kidneys from WT mice were previously described (31). Samples were sonicated and extracted for immunoblotting (10, 31). Animal protocols were approved by the Center for Animal Resources and Comparative Medicine of the Harvard Medical School and were compliant with federal, local, and institutional guidelines on the care of experimental animals.

Human breast cancer analysis. Invasive breast ductal carcinoma samples were freshly obtained from the patients undergoing surgery at the Peking Union Medical College Hospital. The institutional review board at Peking Union Medical College Hospital approved the study protocol, and all patients provided written informed consent. Detailed information for tumor samples is listed in Supplemental Table 4. A portion of the tissue specimens was assessed for differentiation stages by histology

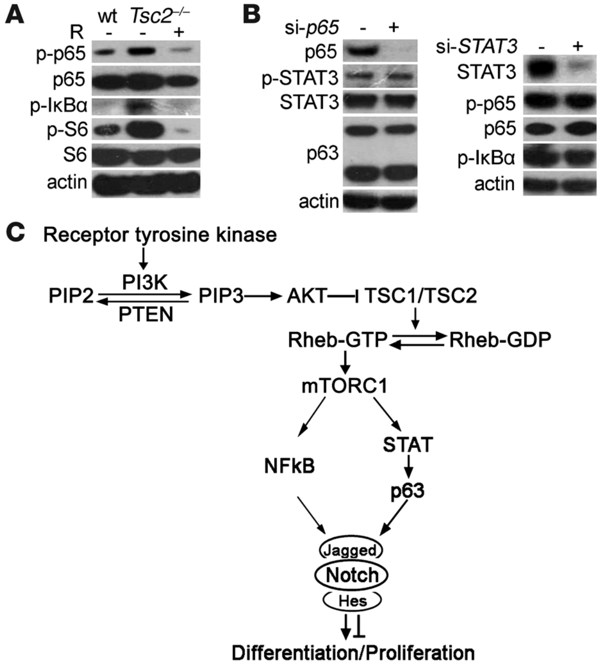

Figure 10

NF-κB and STAT3/p63 control Notch signaling downstream of mTORC1 in parallel. (**A**) WT and $Tsc2^{-/-}$ MEFs were treated with or without 10 nM rapamycin for 24 hours and then subjected to immunoblotting for NF-κB (p65), IκBα, and mTOR signaling. (**B**) $Tsc2^{-/-}$ MEFs were transfected with *p65* siRNA or *STAT3* siRNA to knock down *p65* or *STAT3* expression for 48 hours and were then subjected to immunoblotting for STAT3 and NF-κB signaling. Non–target-directed random siRNA served as a control. (**C**) Schematic illustration of how NF-κB and STAT3/p63 regulate Notch signaling downstream of mTORC1 in parallel.

analysis according to the modified Scarff-Bloom-Rochardson system (67). Additional tumor tissues were snap frozen and later were sonicated for immunoblotting (10, 31). The immunoblottings were quantified with the AlphaEaseFC Imaging Software (Alpha Innotech), and expression levels were normalized with β-actin.

Bioinformatic analysis of human tumor microarray data for mTOR regulation on Notch signaling. mRNA microarray data of 14 lung LAM nodules versus 7 pulmonary artery smooth muscle cell lines were derived from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE12027). Data analysis is described in the Supplemental Methods.

Cell proliferation assay. Cell proliferation was measured using an MTT Assay (Supplemental Methods).

Induction of subcutaneous tumors in nude mice. Subcutaneous tumors were established as described previously (34). Immunodeficient nude mice (BALB/c, 6–8 weeks old) were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS/PUMC). Six male mice were used in each cohort. Animal protocol was approved by the Animal Center of the Institute of Basic Medical Sciences of the CAMS/PUMC and was compliant with the regulations of the Beijing Administration Office of Laboratory Animals on the care of experimental animals.

Statistics. The Kaplan-Meier log-rank test was used to analyze mouse tumor development and survival data using GraphPad Prism software. Cell proliferation and quantitative real-time RT-PCR were analyzed using the 2-tailed Student's *t* test with Excel software. Quantified protein expressions of mTOR/STAT3/p63/Notch components in human breast cancer tissues were subjected to OLS regression analysis using the Stata 9.0 software (StataCorp). A *P* value less than 0.05 was considered significant.

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Address correspondence to: Hongbing Zhang, Department of Physiology, Institute of Basic Medical Sciences, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China. Phone: 01186-10-65296495; Fax: 01186-10-65296491; E-mail: hbzhang2006@gmail.com or hbzhang@ibms.pumc.edu.cn.

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