HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 May 18.

Published in final edited form as:

Oncogene. 2016 January 14; 35(2): 148–157. doi:10.1038/onc.2015.79.

mTOR inhibitors induce apoptosis in colon cancer cells via CHOP-dependent DR5 induction upon 4E-BP1 dephosphorylation

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Abstract

The mammalian target of rapamycin (mTOR) is commonly activated in colon cancer. mTOR complex 1 (mTORC1) is a major downstream target of the PI3K/ATK pathway and activates protein synthesis by phosphorylating key regulators of mRNA translation and ribosome synthesis. Rapamycin analogs Everolimus and Temsirolimus are non-ATP-competitive mTORC1 inhibitors, and suppress proliferation and tumor angiogenesis and invasion. We now show that apoptosis plays a key role in their anti-tumor activities in colon cancer cells and xenografts through the DR5, FADD and caspase-8 axis, and is strongly enhanced by TRAIL and 5-fluorouracil. The induction of DR5 by rapalogs is mediated by the ER stress regulator and transcription factor CHOP, but not the tumor suppressor p53, upon rapid and sustained inhibition of 4E-BP1 phosphorylation, and attenuated by eIF4E expression. ATP-competitive mTOR/PI3K inhibitors also promote DR5 induction and FADD-dependent apoptosis in colon cancer cells. These results establish activation of ER stress and the death receptor pathway as a novel anticancer mechanism of mTOR inhibitors.

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Disclosure of Potential Conflicts of Interest:
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J. Yu, K. He
Development of methodology: K. He, X. Zheng, J. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. He, X. Zheng, M. Li, L. Zhang, J. Yu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. He, X. Zheng, M. Li, J. Yu
Writing, review, and/or revision of the manuscript: K. He, J. Yu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. He, X. Zheng, L. Zhang, J. Yu
Study supervision: J. Yu

Keywords

mTOR inhibitors; DR5; FADD; ER stress; 4E-BP1; apoptosis

Introduction

Colorectal cancer (CRC) represents a major cancer burden worldwide, with over 1 million new cases and over 600, 000 deaths annually ([1]). CRC is the third leading cause of cancer death in the United States ([1]). The cancer genome project has greatly advanced our understanding of cancer biology in the last decade, and identified frequent somatic mutations in oncogenic kinases. For example, deregulation of the PI3K/AKT signaling pathway is estimated in 60-70% of human colon cancers, leading to enhanced cell survival and proliferation ([2]). Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell proliferation, motility, survival, protein synthesis, and transcription ([3]). mTORC1 is one of the two major mTOR complexes and a main downstream effector of PI3K/PTEN/AKT signaling in response to growth factors. mTORC1 phosphorylates downstream targets to increase protein synthesis, including S6K1 and its substrate ribosomal protein RPS6 and translation initiation inhibitor 4E-BP1 ([3-4]). Upon phosphorylation, 4E-BP1 frees the eukaryotic translation initiation factor 4E (eIF4E) for cap-dependent mRNA translation. Hyperactivation of mTOR is an early and common event in colon cancer ([5-7]), and generally caused by deregulation of upstream components ([3]).

Rapamycin is a natural product and non-ATP competitive inhibitor of mTORC1. Rapamycin binds to cytosolic FK binding protein (FKBP12) with high affinity, and the resulting FKBP12/rapamycin complex then interacts with mTOR1 and inhibits its function by interfering with mTOR kinase activity or complex assembly ([3]). Prolonged exposure to Rapamycin can inhibit mTORC2 in some cells ([3]). Phosphorylation of 4E- BP1 in cancer cells has been reported to be much less susceptible to inhibition by Rapamycin than that of RPS6, while the underlying mechanisms are not well understood ([8]). A number of Rapamycin analogs with improved pharmacologic properties, also referred as rapalogs, have been developed. Everolimus (RAD001), an orally available derivative of Rapamycin, and Temsirolimus (CCI-779) have been approved by the FDA for the treatment of patients with refractory renal cell cancer, and in clinical trials for other solid tumors including colon cancer ([9-10]). ATP-competitive mTOR or mTOR/PI3K dual inhibitors such as Torin-1 and NVP-BEZ235 have recently been developed. Antitumor activities of mTOR inhibitors are associated with suppression of tumor growth, angiogenesis and invasion ([3]).

Apoptosis is regulated by the intrinsic mitochondrial pathway ([11]), and extrinsic pathway ([12]). The mitochondrial pathway is activated through the Bcl-2 family members Bax and Bak ([11, 13]). The extrinsic pathway is activated upon binding of pro-apoptotic ligands to the extended TNF family receptors, and negatively regualted by decoy receptors (DcRs) ([12]). For example, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to death receptor 4 (DR4), and death receptor 5 (DR5), which further recruit the adaptor Fas-associated protein with death domain (FADD) and caspase-8, leading to caspase-8 activation ([12]). FADD is required for Fas- or TRAIL-induced apoptosis ([12]). Induction of apoptosis is

an important mechanisms of many anticancer drugs ([14]). Colon cancer cells deficient in *BAX* are highly resistant to anticancer agent-induced apoptosis ([15-18]), while the role of extrinsic pathway is much less understood.

mTOR inhibitors, particularly rapalogs, induce cancer cell apoptosis *in vivo*, but not in culture at concentrations that inhibit p70S6K or RPS6 phosphorylation, or cell proliferation ([3-4]). The significance of apoptosis induction in the anti-tumor activities of mTOR inhibitors remains undefined. In this study, we show that both rapalogs and ATP-competitive mTOR inhibitors can activate the extrinsic apoptotic pathway in CRC cells via the DR5/FADD/Caspase-8 axis upon rapid inhibition of 4E-BP1 phosphorylation. Everolimus strongly synergizes with TRAIL and chemotherapy to induce FADD and DR5-dependent apoptosis. FADD-dependent apoptosis is required for the antitumor activities of Everolimus in xenografts. These results demonstrate activation of the extrinsic apoptotic pathway as an antitumor mechanism of mTOR inhibitors.

Results

Rapalogs induce the activation of the death receptor pathway during apoptosis in colon cancer cells

Everolimus and Temsirolimus induced classical apoptosis in HCT116 colon cancer cells by 48 hours at high concentrations (18-20 uM), which was unaffected in *BAX* knockout (*BAX*-KO) or *BAX/BAK* double knockout (*BAX/BAK* DKO) HCT 116 cells (Fig. 1A, Fig. S1A-B). The apoptosis was preceeded by induction of DR5 as early as 8 hours followed by cleavage of caspase-3, −8 and −9, and Bid within 24 hours (Fig. 1B). RT-PCR analysis on a panel of extrinsic apoptotic regulators showed a strong induction of *DR4*, *DR5* and *TNFR1* (Fig. 1C). Significant apoptosis was induced in three other CRC lines including RKO, DLD1, and HT29 (Fig. 1D) by both agents, and the expression of extrinsic apoptotic regulators most notably DR5 (Fig. 1E, 1F and Fig. S2). Unexpectedly, the treatment of rapalogs inhibited 4E-BP1 phosphorylation much more rapidly and profoundly compared to RPS6 phosphorylation (Fig. 1B and 1F). These results indicate that rapalogs activate the death receptor pathway in CRC cells likely through inhibiting 4E-BP1 phosphorylation.

Rapid dephosphorylation of 4E-BP1 by rapalogs leads to induction of ER stress and DR5 in CRC cells

DR5 transcription is regulated by p53 following DNA damage ([19-20]) or CHOP after ER stress ([21]). We first ruled out p53, as *DR5* and apoptosis was induced irrespective of p53 status (Fig. 1, Figs. S2, S3A and S3 B), an p53 levels did not increase by either agent in p53 WT cells (Fig. S3). Interestingly, inhibition of 4E-BP1 phosphorylation and induction of CHOP were detected as early as 4 hours, followed by DR5 in 12 hours, only in cells treated with Everolimus at 20 μM (Fig. 2A). Overexpression of eIF4E attenuated CHOP, DR5 induction and caspase-3 cleavage (Fig. 2B). In contrast, inhibition of 4EBP1 phosphorylation, induction of CHOP or DR5, apoptosis was absent in cells treated with Everolimus at 50 nM or 1 μM, despite more effective inhibition of RPS6 phosphorylation (Fig. 2A) and reversible growth suppression (data not shown). However, knockdown of

raptor, rictor, or mTOR by siRNA did not cause apoptosis or loss of p4EBP1 (Fig. S3C-D), supporting mTOR-independent 4E-BP1 phosphorylation in CRC cells ([22]).

Furthermore, Everolimus treatment led to CHOP recruitment to the *DR5* promoter (Fig. 2C), and *CHOP* knockdown by siRNA attenuated the induction of *DR4* and *DR5*, as well as apoptosis and caspase activation by rapalogs (Fig. 2D and 2E). Interestingly, rapalogs-induced apoptosis was associated with strong activation of multiple ER stress markers besides CHOP induction, such as *XBP1* splicing, and upregulation of BIP, Ero1-Lα and IRE1α (Fig. 2F). These results demonstrate that CHOP is required for DR5 and apoptosis induction by rapalogs upon rapid inhibition of the 4E-BP1/eIF4 axis and activation of ER stress in HCT 116 cells.

DR5 and caspase-8 are required for rapalog-induced apoptosis in CRC cells

We further determined if DR5 is required for apoptosis induced by Everolimus and Temsirolimus. Knockdown of *DR5* by siRNA significantly suppressed apoptosis and cleavage of caspase-8 and −3 (Fig. 3A and 3B, and Fig. S4). Caspase-8 is an essential initiator caspase of the extrinsic apoptotic pathway downstream of the death receptors. *Caspase-8* stable knockdown significantly suppressed apoptosis induced by Everolimus and Temsirolimus (Fig. 3C and 3D), and largely rescued the long-term survival of HCT116 cells (Fig. 3E and 3F). These results demonstrate that DR5 and caspase-8 mediate mTOR inhibitor-induced apoptosis and long-term growth suppression in CRC cells.

FADD is required for rapalog-induced caspase-8 activation and apoptosis in CRC cells

FADD mediates caspase-8 activation downstream of Fas, TNF and death receptors. We further determined the role of extrinsic pathway by generating the *FADD* knockout (*FADD*-KO) HCT116 cells (Fig. 4A and 4B). Apoptosis and activation of caspase−3 and −8 induced by Everolimus and Temsirolimus was blocked in *FADD*-KO cells to near completion (Fig. 4C and 4D), and confirmed by flow cytometry (Fig. S5A and S5B). *FADD*-KO HCT116 cells were highly resistant to both agents in long-term clongenic assay (Fig. 4E and 4F). Stable expression a *FADD* allele lacking the caspass-8 recruiting domain (FADD-DN) also blocked apoptosis and activation of caspases-8 and -3 in HCT 116 and RKO cells (Fig. 4G and 4H, Fig. S5C-F). These data demonstrate that FADD-dependent caspase-8 activation is responsible for apoptosis and long-term growth suppression induced by rapalogs in CRC cells.

Induction of DR5 and FADD-dependent apoptosis by ATP-competitive mTOR inhibitors

ATP-competitive mTOR or mTOR/PI3K inhibitors block 4E-BP1 phosphorylation more effectively than rapalogs and induce apoptosis in cancer cells. Treatment of Torin 1 or NVP-BEZ235 induced expression of *DR4*, *DR5* or *TRAIL* in HCT116 cells, and *FADD*-dependent apoptosis (Fig. 5A, 5B and 5C, Figs. S1C-D and S6). Interestingly, Torin 1-induced apoptosis was dose-dependent and associated with induction of CHOP and complete inhibition of 4E-BP1 phosphorylation but incomplete inhibition of RPS6 phosphorylation (Fig. 5C). Similar to rapalogs, Torin 1 at 50 nM or 1 μM completely inhibited RPS6 phosphorylation by 24 hours, while induced no detectable apoptosis in HCT 116 (Fig. 5C). These results strongly suggest that mTOR inhibitors activate ER stress and

the extrinsic apoptotic pathway upon selective inhibition of 4E-BP1 phosphorylation (Fig. 5D).

Everolimus sensitizes CRCs to other anticancer agents via the extrinsic pathway

mTOR inhibitors activate apoptosis via p53-independent induction of DR5. We further hypothesized that they might sensitize CRCs to DR5 ligand TRAIL or chemotherapy that induces DR5 via p53 such as 5-FU (Fig. 5D). As expected, TRAIL or 5-FU in combination with Everolimus strongly induced activation of caspase-8 and caspase-3 compared to single agent (Fig. 6A). Apoptosis induced by the combinations was largely blocked in FADD-KO cells or by *DR5* siRNA (Fig. 6B and 6C). The combination of 5-FU and Everolimus enhanced *DR5* induction (Fig. 6D). Therefore, Everolimus sensitizes CRC cells to killing via the activation of DR5 and the extrinsic pathway.

Apoptosis mediates the antitumor effect of Everolimus in a xenograft model

To establish a potential role of apoptosis in the anti-tumor activities of mTOR inhibitors *in vivo*, we treated mice bearing WT and *FADD*-KO HCT116 xenografts with 5 mg/kg Everolimus or the vehicle by oral gavage daily for 10 days. Everolimus treatment caused 80% reduction in tumor growth in the parental tumors, but less than 30% in the *FADD*-KO tumors (Fig. 7A). Everolimus-treated tumors showed induction of DR5 associated with strong reduction in 4E-BP1 phosphorylation (Fig. 7B). Everolimus-treated tumors showed marked apoptosis in WT tumors as shown by TUNEL and active caspase-3 staining, which was significantly blocked in *FADD*-KO tumors (Fig. 7C and 7D). The inhibition of 4E-BP1 phosphorylation or angiogenesis was similar in WT and *FADD*-KO tumors in response to Everolimus (Fig. 7E and 7F). These data demonstrate an important role of the extrinsic apoptotic pathway and inhibition of 4E-BP1 phosphorylation in the antitumor activities of Everolimus *in vivo*.

DISCUSSION

The PI3K/PTEN/AKT/mTOR signaling pathway plays a key role in cell proliferation, survival and transformation ([14]). Recent studies suggest that activation of mTOR signaling is an early event in colon cancer, and its inhibition can lead to suppression of cell cycle progression, epithelial to mesenchymal transition (EMT), cell motility and metastasis ([5-7]). Our current study establishes that high dose mTOR inhibitors activate CHOP-dependent induction of DR5 and apoptosis via the extrinsic pathway in colon cancer cells, upon rapid and sustained inhibition of 4E-BP1 phosphorylation. To our knowledge, this is the first report to definitively show that apoptosis induction contributes significantly to the antitumor activities of mTOR inhibitors such as rapalogs *in vitro* and *in vivo*.

It is well established that, at sub micromolar concentrations, rapamycin or rapalogs do not inhibit phosphorylation of 4E-BP1 or cause apoptosis in cancer cells, despite effective inbibition of p70SK6 or RPS6 phosphorylation. These lower doses can promote formation of stress granules, reversible cell cycle arrest, and even apoptosis- and chemo-resistance ([23-24]). Interestingly, published work indicated that Temsirolimus (CCI-779) can reach high micromolars in the plasma of cancer patients ([25-27]). The highest weekly dose of CCI-779

of 250 mg/m^2 (whole blood exposure of 15.5 μM) was associated with improved medium and overall survival, including a complete response, in heavily pretreated renal cancer patients ([25]). The maximum drug concentaions in tumor tissues and underlying mechanmisms are however not known. We believe our findings therefore provide a potentially novel and clinically relevant antitumor mechanism of rapalogs, particularily in extrondinary responders whose tumors contain activating mTOR mutations are recently reported ([28_29]).

Emerging evidence supports targeting 4E-BP1 phosphorylation and translation in cancer. Our findings suggest that exposure to high doses of rapalogs causes a profound loss of 4E-BP1 phosphorylation and mTOR activity, both are likely required for the induction of ER stress, CHOP and DR5 and subsequent killing of cancer cells. This is consistent with that competitive eIF4E/eIF4G inhibitor 4EGI-1 induces apoptosis ([30_31]), mTOR-independent 4E-BP1 phosphorylation is associated with resistance to mTOR inhibitors ([22, 32_34]), and unresolved ER stress upon persistent CHOP induction leading to DR5-mediated apoptosis ([35]). However, the precise mechanism underlying inhibition of p4EBP1, translation and ER stress warrants further investigation, and we could not rule out mTOR-independent effects. It is tempting to speculate that intermittent high dosing of rapalogs promotes more robust antitumor responses via induction of ER stress and apoptosis in mTOR addicted cancers partiuclairy those with activating mTOR mutations ([28_29]).

The major challenge in the clinical use of mTOR inhibitors is the lack of biomarkers or clear resistance mechanisms ([10]). Induction of ER stress, death receptors and ligands, 4E-BP1 dephosphorylation and eIF4E/4E-BP ratio ([36]) might be potential biomarkers for selecting responders or monitoring responses. Rapalogs generally have a favorable safety profile and non-overlapping side effects with ATP-competitive inhibitors or DNA damaging agents. We showed that Rapalogs strongly synergize with TRAIL or 5-FU to induced cancer cell killing. Inhibitors of a variety of oncoproteins such as EGFR, MET, VEGFR, Raf, Hsp90, or Bcl-2/Bcl-xL, can activate mitochondria-dependent apoptosis in colon cancer cells ([17_18, 37_38]) Therefore, their rational combination with rapalogs might offer hopes to patients with chemotherapy-refractory metastatic colon cancers ([10]) by activating both the intrinsic and extrinsic pathways.

Apoptosis is part of the normal turn-over of intestinal epithelium and plays an important role in colon cancer biology, therapy and prevention ([39_40]), while its regulation can be different in normal and cancer cells and explored thearpuetically. For example, FADD or casaspe-8 is required for normal development and homeostasis of gut epithelium by suppressing RIP-3 dependent necrosis in mice ([41]), but dispensable for the survival of human CRC cells such as HCT116 and RKO. Rencent studies suggest functional links of ER stress and mTOR ([42]), and sensitization to ER stress upon abnormal activation of mTOR in normal but not cancer cells ([43_44]). Rapamycin or rapalogs suppress intestinal carcinogenesis in mouse models with little or no long-term side effects ([45_46]) It will be important to determine if and how ER stress selectively kills cells with aberrant Wnt signaling, the driver of virtually all CRC ([2]).

In conclusion, our work demonstrates an essential role of the death receptor-mediated apoptosis upon inhibition of 4E-BP1 phosphorylation in the antitumor activities of mTOR inhibitors in CRC cells and xenografts. These results provide a better understanding and potential biomarkers of the pro-apoptotic action of mTOR inhibitors.

Materials and methods

Cell culture and treatment

The human colorectal cancer cell lines, including HCT116, RKO, DLD1and HT29, were obtained from the American Type Culture Collection (Manassas, VA, USA). Isogenic HCT 116 knockout cell lines *p53*-KO, *BAX*-KO were from Bert Vogelstein, and *BAX/BAK* double KO were from Richard J. Youle. Stable *caspase-8* knockdown HCT 116 cells have been described ([47]). Loss of expression of targeted proteins was confirmed by western blotting and Mycoplasma testing was performed routinely by PCR. Details on cell culture and drug treatments are found in the supplemental materials.

Western blotting

Western blotting was performed as previously described ([48_49]). Details on antibodies are found in the supplemental materials.

Real-time Reverse Transcriptase (RT) PCR

Total RNA was isolated from cells using the Mini RNA Isolation II Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. One μg of total RNA was used to generate cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out as described ([18]). Details on primers are found in the supplemental materials (Table S1).

Analysis of apoptosis and cell death

Apoptosis was analyzed by nuclear staining with Hoechst 33258 (Invitrogen), and Annexin V/propidium iodide (PI) (Invitrogen) followed by flow cytometry as described ([50]). For colony formation assays, the same number of cells were treated and plated in 12-well plates at appropriate dilutions, and allowed to grow for 10–14 days before staining with crystal violet (Sigma, St. Louis, MO) ([16]).

Targeting FADD in HCT116 cells

Gene targeting vectors were constructed by using the recombinant adeno-associated virus (rAAV) system as previously described ([51]). HCT116 cells containing two copies of WT *FADD*. The FADD knockout cells with exon 2 deletion were generated following two rounds of gene targeting. Details for vector construction and identification knockout clone isolation are found in the supplemental materials.

Transfection

The human FADD dominant-negative (FADD-DN) and murine eIF4E expression constructs were kind gifts from Shiyong Sun (Emory University) and Nahum Sonenberg (McGill

University) respectively. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions. DR5, CHOP, Raptor, Rictor and mTOR small-interfering RNA (siRNA) duplexes were synthesized from Dharmacon (Lafayette, CO, USA). Details for transfection, drug selection and siRNA sequence are found in the supplemental materials.

Xenograft studies

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Female 5–6 week-old Nu/Nu mice (Charles River, Wilmington, MA) were housed in a sterile environment with micro isolator cages and allowed access to water and chow *ad libitum*. Mice were injected subcutaneously in both flanks with 4×10^6 WT or *FADD*-KO HCT116 cells. After implantation, tumors were allowed to grow 7 days before treatment was initiated. Mice were randomized into two groups (n = 6 per group) receiving either vehicle or Everolimus (5mg/kg/d) in saline on day 1-10 by oral gavage. Detailed methods on tumor measurements and analysis are found in the supplemental materials as described ([38], [52_53]).

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation (ChIP) was done using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology) according to manufacturer's instructions. The precipitates were analyzed by PCR using primers 5'-AGGTTAGTTCCGGTCCCTTC-3' (forward) and 5'-CAACTGCAAATTCCACCACA-3' (reverse).

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism IV software. P values were calculated by the student's t-test and were considered significant if $p < 0.05$. The means ± one standard deviation (s.d.) were displayed in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University) for *p53*- KO HCT116, Richard J. Youle (NIH) for *BAX/BAK*-DKO HCT 116 cells, Shi-Yong Sun (Emory University) for FADD-DN construct, and Nahum Sonenberg (McGill University) for eIF4E construct.

Grant Support

This work is supported by NIH grant CA129829, American Cancer Society grant RGS-10-124-01-CCE (Yu J), and NIH grants CA106348, CA172136 (Zhang L). This project used the UPCI shared facilities including flow cytometry, animal and histopathology were supported in part by award P30CA047904.

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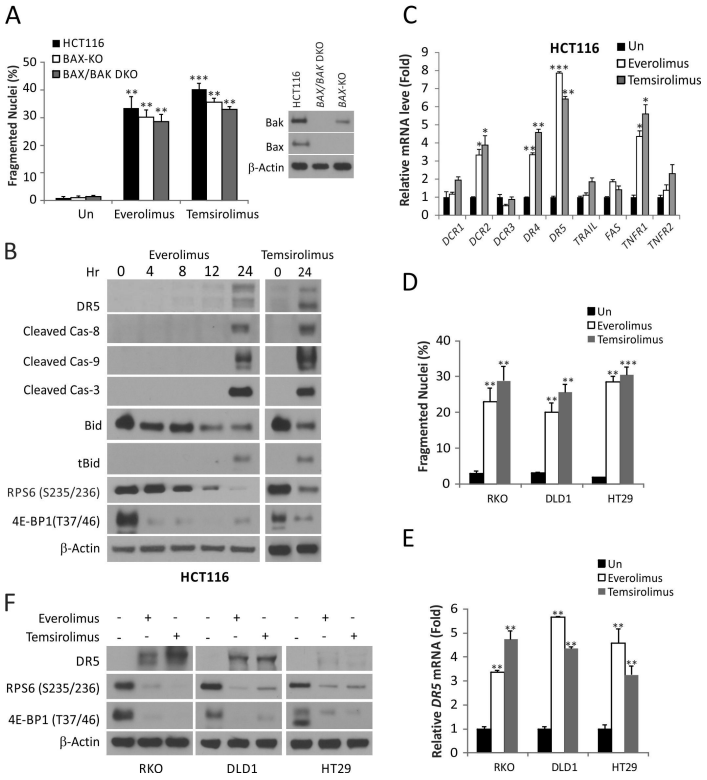

Figure 1. mTOR inhibitors activate apoptosis and expression of extrinsic apoptotic regulators. A-C

HCT 116 cells or derivatives were treated with vehicle (untreated, Un), 20 μmol/L Everolimus or Temsirolimus and analyzed at indicated times. **A**, apoptosis in the indicated HCT116 lines at 48 hours was analyzed by counting condensed and fragmented nuclei. Right, lack of protein expression in KO cells confirmed by western blotting. **B,** the indicated proteins were analyzed by western blotting. β-actin is a loading control. **C,** mRNA levels of the indicated genes at 24 hours were analyzed by real-time RT-PCR. The levels in vehicle (UN) treated cells were set at 1. **D,** RKO, DLD1 and HT29 cells were treated with 25 μmol/L Everolimus or 20 μmol/L Temsirolimus. Apoptosis was analyzed at 48 hours by counting condensed and fragmented nuclei. **E,** cells were treated as in D. mRNA levels of *DR5* at 24 hours were analyzed by RT-PCR. **F,** cells were treated as in D. The indicated proteins were analyzed by western blotting. β-actin is a loading control. **A**,**C**, **D and E**, values represent means + s.d. (n=3). *P < 0.05, **P < 0.01, ***P < 0.001 [Student's t-test, two-tailed]. Drugs *vs*. Un.

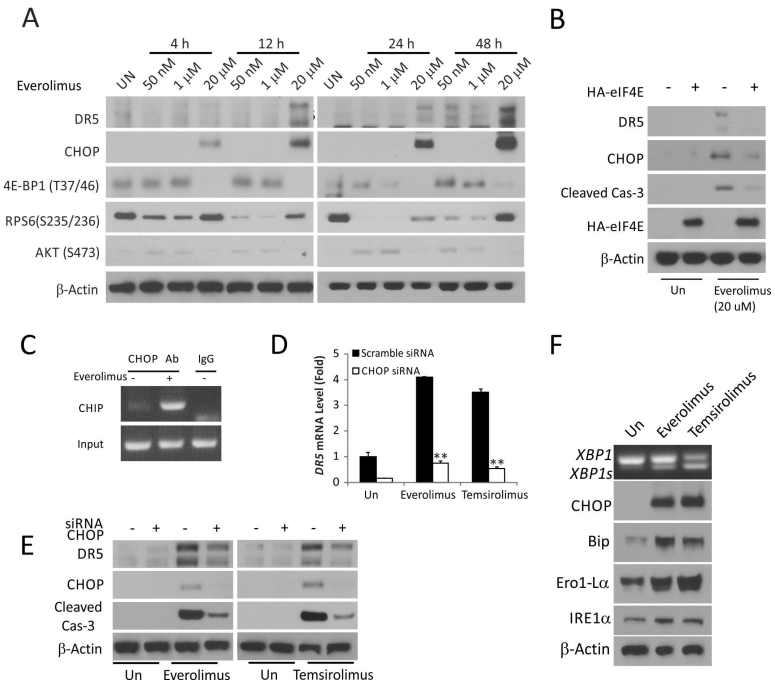

Figure 2. Induction of ER stress and CHOP-mediated DR5 and apoptosis by rapalogs

A, HCT116 cells were treated with various concentrations of Everolimus and analyzed for indicated proteins and times by western blotting. **B**, HCT116 cells were transfected with HA-eIF4E or vector for 24 hours, treated with 20 μmol/L Everolimus for 24 hours, and analyzed for indicated proteins by western blotting. **C,** chromatin immunoprecipitation (ChIP) was performed using a CHOP-specific antibody on cells treated with 20 μmol/L Everolimus for 24 hours. IgG was used to control for specificity. PCR was carried out using primers surrounding the CHOP binding sites in the *DR5* promoter. **D,** cells were transfected with either scramble or *CHOP* siRNA 24 hours prior to drug treatment. mRNA level of *DR5* at 24 hours were analyzed by RT-PCR. **V**alues represent means + s.d. (n=3). **P < 0.01 [Student's t-test, two-tailed]. Scramble *vs. CHOP* siRNA. **E,** cells treated as in **D** for 48 hours and analyzed for the indicated proteins by western blotting. **F,** ER stress markers were analyzed at 24 hours with 20 μmol/L Everolimus or Temsirolimus. Splicing of *XBP-1* was determined by PCR, and other markers by western blotting.

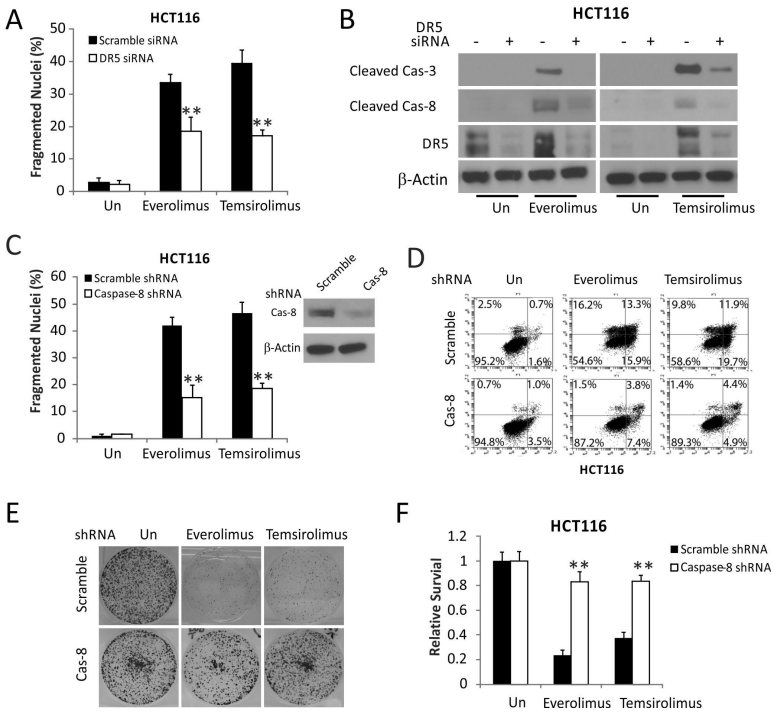

Figure 3. DR5 and Caspase-8 are required for mTOR inhibitors-induced apoptosis

HCT 116 cells or derivatives were treated with vehicle (Un), 20 μmol/L Everolimus or Temsirolimus and analyzed at indicated times. **A,** HCT116 cells were transfected with either a scramble siRNA or *DR5* siRNA for 24 hours prior to 48 hours of drug treatment. Apoptosis was analyzed by counting condensed and fragmented nuclei. **B,** active caspase-3 and −8, and DR5 were analyzed at 24 hours of drug treatment by western blotting. **C,** Apoptosis was analyzed in HCT116 cells stably expressed scramble shRNA or *caspase-8* shRNA at 48 hours of drug treatment by counting condensed and fragmented nuclei. Right, *caspase-8* knockdown confirmed by western blotting. **D.** Cells treated as in **C** were analyzed for apoptosis by flow cytometry following staining with Annexin V/propidium iodide. **E,** Colony formation assay of indicated cells treated for 24 hours, and the attached cells were stained with crystal violet after 14 days. Representative pictures are shown. **F**, quantification of colonies in **E**. **A**, **C** and **F**, values in represent means + s.d. (n=3). **P < 0.01, [Student's t-test, two-tailed]. WT *vs. DR5* siRNA *or Caspase-8* shRNA cells.

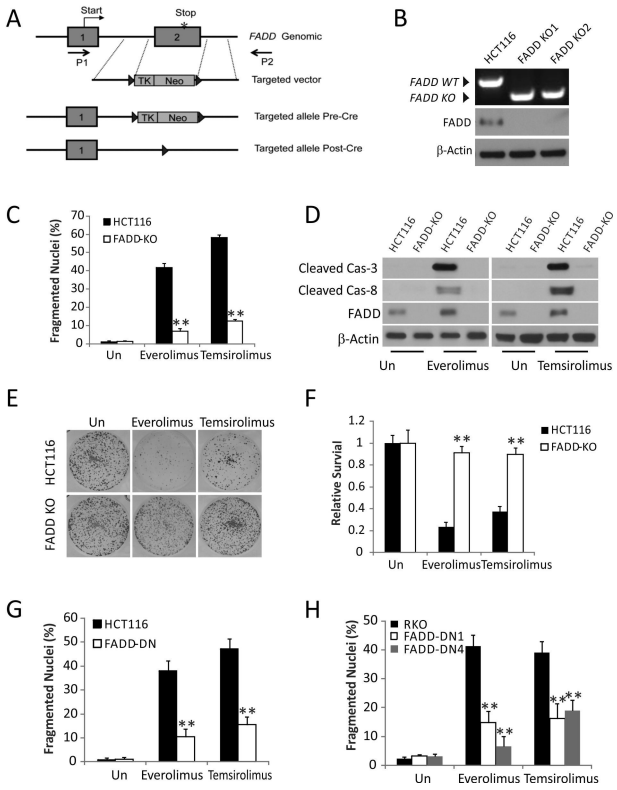

Figure 4. FADD is required for mTOR inhibitors-induced apoptosis

A, Schematic representation of the *FADD* genomic locus and the *FADD* targeting construct. P1 and P2 are PCR primers (sequences in Table S2) for identifying knockout clones. **B**, *FADD* knockout (KO) HCT116 clones were confirmed by genomic PCR and western blotting. **C,** Indicated cells were treated with 20 μmol/L Everolimus or Temsirolimus for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. **D**, active caspase-3 and −8 was analyzed in cells treated as in **C** by western blotting. **E,** colony formation of WT and *FADD*-KO HCT116 cells treated with 20 μmol/L Everolimus or Temsirolimus for 24 hours, and stained at14 days after treatment. Representative pictures are shown. **F**, quantification of colonies in **E**. **G,** WT and dominant negative FADD (FADD-DN) stable HCT116 cells were treated with 20 μmol/L Everolimus or Temsirolimus for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. **H**, WT, FADD-DN stable RKO cells (clone 1 and clone 4) were treated with 25 μmol/L Everolimus or 20 μmol/L Temsirolimus for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. β-actin is a loading control for all western blots. **C, F, G** and **H,** values represent means + s.d. (n=3). **P < 0.01 [Student's t-test, two-tailed]. WT *vs. FADD*-KO or FADD-DN cells.

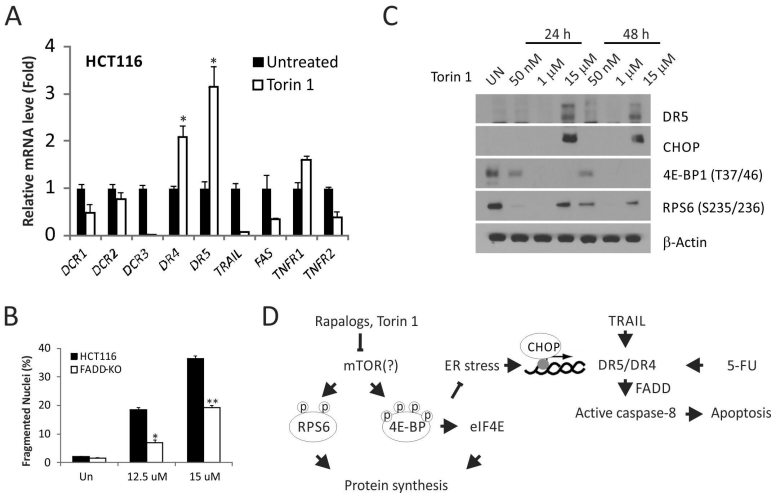

Figure 5. Induction of CHOP, DR4 and DR5, and FADD-dependent apoptosis by Torin 1

WT and *FADD*-KO HCT116 cells were treated with Torin 1 at indicated concentrations. **A,** mRNA levels of the indicated gene at 24 hours after 15 μmol/L treatment were analyzed by real-time RT-PCR. **B**, apoptosis in the indicated cells was analyzed at 48 hours by counting condensed and fragmented nuclei. **C,** the indicated proteins were analyzed by western blotting at 24 hours. β-actin is a loading control. **A** and **B,** values represent means + s.d. (n=3). *P < 0.05, **P < 0.01 [Student's t-test, two-tailed]. A, Torin 1 *vs.*Un, B, WT *vs. FADD* KO. **D**, A model. Selectively blocking 4EBP1 phosphorylation mediated by mTOR or an unidentified kinase inhibits eIF4E, and induces ER stress and CHOP-dependent DR5 upregulation and apoptosis. mTOR inhibitors are predicted to synergize with agents that active or induce DR5/DR4.

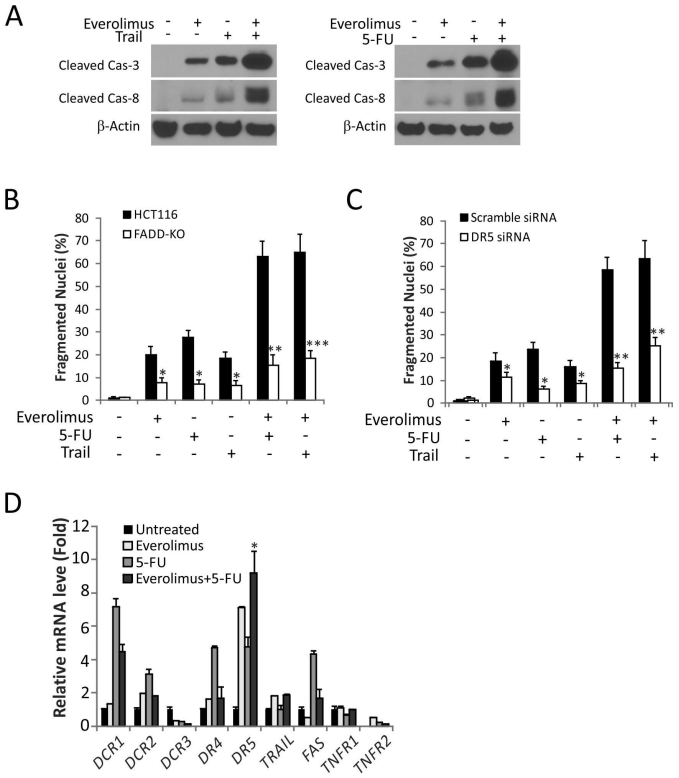

Figure 6. Everolimus induces drug sensitization via the extrinsic pathway

HCT116 cells and derivatives were treated with 18 μmol/L Everolimus, 10 ng/mL TRAIL, 50 μg/mL 5-FU, or indicated combination for 24 hours. **A,** active caspsae−3 and −8 was analyzed by western blotting. **B,** apoptosis was analyzed by counting condensed and fragmented nuclei in WT or FADD-KO cells. **C,** apoptosis was analyzed by counting condensed and fragmented nuclei in WT or *DR5* siRNA transfected cells as in **B**. **D,** mRNA levels of the indicated genes were analyzed by realtime RT- PCR. β-actin is a loading control for all western blots. **B**, **C**, and **D**, values represent means + s.d. (n=3). *P < 0.05, **P < 0.01, ***P < 0.001 [Student's t-test, two-tailed]. **B, C** WT *vs. FADD* KO, or scramble vs. *DR5* siRNA. **D** combination *vs.* single.

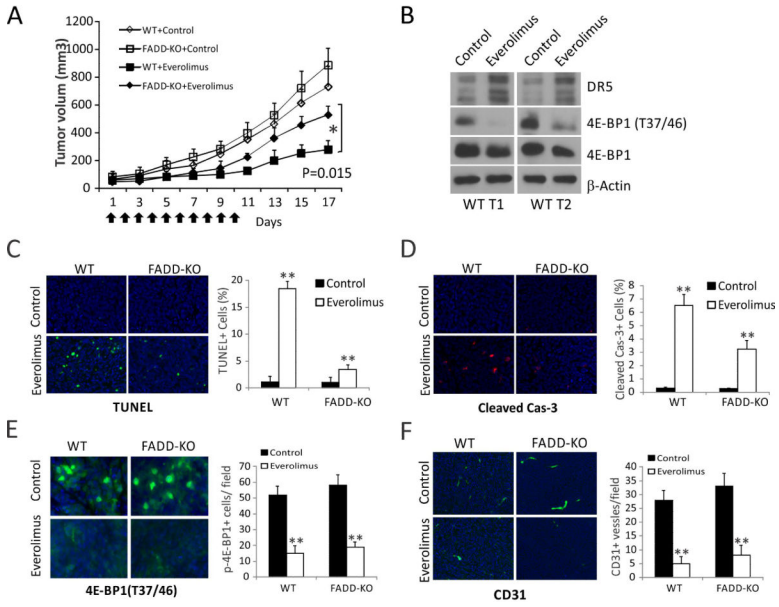

Figure 7. Apoptosis contributes to the antitumor effects of Everolimus in a xenograft model

A, nude mice after 1 week of implantation of 4×10^6 WT or *FADD*-KO HCT116 cells were treated with 5 mg/kg of Everolimus or the control buffer (vehicle) for 10 consecutive days. Tumor volume was plotted. N=6 mice/group. *P < 0.05 [Student's t-test, two-tailed]. WT *vs. FADD* KO. **B,** HCT116 WT tumors were harvested the day after the last treatment (day 11). The indicated proteins in randomly chosen tumors were analyzed by western blotting. β-actin is a loading control. **C** and **D,** WT and *FADD*-KO tumors were analyzed by TUNEL staining and active caspase-3 staining, respectively. Left, representative pictures; right, quantification. **E** and **F,** WT and *FADD*-KO tumors were analyzed by staining for phosphor-4E-BP1 and CD31 (endothelial cells), respectively. Left, representative pictures; right, quantification. **C -F**, values represent means + s.d, n=3. **P < 0.01 [Student's t-test, two-tailed]. WT *vs. FADD*-KO.