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## Fatty Acid Synthase inhibition engages a novel caspase-2 regulatory mechanism to induce ovarian cancer cell death

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### Abstract

Blockade of fatty acid synthase (FASN), a key enzyme involved in *de novo* lipogenesis, results in robust death of ovarian cancer cells. However, known FASN inhibitors have proven to be poor therapeutic agents due to their ability to induce cachexia. Therefore, we sought to identify additional targets in the pathway linking FASN inhibition and cell death whose modulation might kill ovarian cancer cells without the attendant side effects. Here, we show that the initiator caspase-2 is required for robust death of ovarian cancer cells induced by FASN inhibitors. REDD1 (also known as Rtp801 or DDIT4), a known mTOR inhibitor previously implicated in the response to FASN inhibition, is a novel caspase-2 regulator in this pathway. REDD1 induction is compromised in ovarian cancer cells that do not respond to FASN inhibition. Inhibition of FASN induced an ATF4-dependent transcriptional induction of REDD1; downregulation of REDD1 prevented orlistat-induced activation of caspase-2, as monitored by its cleavage, proteolytic activity, and dimerization. Abrogation of REDD1-mediated suppression of mTOR by TSC2 RNAi protected FASN inhibitor-sensitive ovarian cancer cells (OVCA 420 cells) from orlistat-induced death. Conversely, suppression of mTOR with the chemical inhibitors PP242 or rapamycin sensitized DOV13, an ovarian cancer cell line incapable of inducing REDD1, to orlistat-induced cell death through caspase-2. These findings indicate that REDD1 positively controls caspase-2-dependent cell death of ovarian cancer cells by inhibiting mTOR, placing mTOR as a novel upstream regulator of caspase-2 and supporting the possibility of manipulating mTOR to enhance caspase-2 activation in ovarian cancer.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

C.-S.Y. and S.K. developed the concept and wrote the manuscript. C.-S. Y. and K.M. designed, performed experiments and analyzed data, with help from A.C.R. for the BiFC assays, from N.-J.H. for immunoprecipitation of active Bax/Bak, from B.H. for detection of caspase-2 phosphorylation and from L.Z. for qPCR.

## Keywords

lipogenesis; FASN; caspase-2; REDD1; ER stress; ovarian cancer; mTOR; ATF4

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## INTRODUCTION

Altered cellular metabolism is emerging as a hallmark of cancer and has been shown to support several steps along the pathway of cancer development.<sup>1-3</sup> In addition to aberrant glucose metabolism, as exemplified by the Warburg effect, accelerated *de novo* fatty acid synthesis is frequently observed in human malignancies. Elevated lipogenesis may provide one avenue for fulfilling the demand of cancers for increased genesis of membranes during unrestrained growth.<sup>4-6</sup> Indeed, inhibition of fatty acid synthase (FASN) has been shown to trigger ER stress in tumor cells,<sup>7</sup> while FASN inhibitors, such as orlistat and C75, have been found to produce antitumor effects in a variety of cancers, including ovarian cancers [reviewed in ref. <sup>5</sup>].

Interestingly, several recent studies suggest a “lipid addiction” phenotype for ovarian cancers. For example, FASN levels are upregulated in 80% of ovarian carcinoma samples and correlate with poor prognosis.<sup>8,9</sup> FABP4, a lipid chaperone, has been shown to be upregulated in ovarian-derived metastases to enable the uptake of exogenous lipids as an energy source.<sup>10</sup> Most importantly, blockade of *de novo* lipid synthesis with FASN inhibitors has been shown to be suppressive for ovarian cancer *in vitro* and *in vivo* by inhibiting cancer proliferation and stimulating apoptosis.<sup>11-13</sup>

Apoptosis is executed by caspases, a family of cysteine proteases. Although caspase-2 is the second member to be discovered, its biological role remains enigmatic, in part due to the lack of an obvious phenotype in caspase-2 knockout mice under unstressed conditions.<sup>14</sup> Interestingly, genetic deletion of caspase-2 has been recently found to shorten mouse life span, accelerate the development of age-related traits<sup>15</sup> and prompt tumorigenesis in mouse models of leukemia<sup>16,17</sup> and carcinoma,<sup>18</sup> suggesting a protective role for caspase-2 in ageing and tumorigenesis. Moreover, previous studies in our laboratory demonstrated that glucose-6-phosphate blocks caspase-2 activation and the subsequent *Xenopus* oocyte death induced by nutrient deprivation, revealing that caspase-2 is capable of coordinating glucose metabolism and cell death.<sup>19,20</sup>

A variety of physiological stresses have been shown to activate caspase-2 [reviewed in ref. <sup>21</sup>]. Of clinical interest, several common drugs used in chemotherapy, such as paclitaxel and cisplatin, appear to induce apoptosis, at least in part, through caspase-2.<sup>22,23</sup> Caspase-2 can engage the intrinsic apoptotic pathway by cleaving Bid, and thereby induces Bax/Bak-dependent mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, and subsequent cell death.<sup>24,25</sup> Caspase-2 has also been shown to mediate the activation of caspase-8 and the extrinsic apoptotic pathway in ceramide- and TRAIL-induced cell death.<sup>26,27</sup> Similar to other initiator caspases, the inert caspase-2 monomer is activated by dimerization, and subsequent intramolecular cleavage further stabilizes its proteolytic activities.<sup>28</sup> A p53 inducible protein, PIDD, with the aid of RAIDD, has been suggested to mediate caspase-2 dimerization by forming an activating platform, namely the

PIDDosome.<sup>29</sup> Nevertheless, several recent studies indicate that caspase-2 may be activated in a PIDD-independent manner,<sup>30, 31</sup> and a previously developed bimolecular fluorescence complementation (BiFC) assay that measures the activating dimerization of caspase-2 also provides the means to identify novel modulators that control caspase-2 dimerization, such as Hsp90 $\alpha$ .<sup>32</sup>

Here we report the identification of REDD1 as a novel caspase-2 regulator that facilitates caspase-2 dimerization/activation upon the suppression of *de novo* lipogenesis. REDD1 was transcriptionally upregulated through an ATF4-dependent pathway to permit caspase-2 dimerization following FASN inhibition. Interestingly, abrogation of REDD1-mediated mTOR inhibition by TSC2 RNAi prevented orlistat-induced cell death, whereas pretreatment with mTOR inhibitors reversed the resistance of REDD1-deficient ovarian cancer cells to orlistat by activating caspase-2. Collectively, these data demonstrated the involvement of caspase-2 in ovarian cancer cell death induced by FASN inhibition and identified REDD1 as a novel caspase-2 regulator that controls caspase-2 dimerization through the ATF4-REDD1-TSC2-mTOR axis.

## RESULTS

### Caspase-2 controls ovarian cancer cell death induced by inhibition of FASN

Our previous work linked caspase-2 to oocyte apoptosis upon nutrient deprivation;<sup>19, 20, 33</sup> here we sought to explore caspase-2's metabolic role in another paradigm. We hypothesized that caspase-2 might be involved in cancer cell death induced by the inhibition of *de novo* fatty acid synthesis. To test this hypothesis, we treated ovarian cancer cell lines OVCA420 (420) and DOV13 (DOV) with orlistat, an FDA approved drug capable of inhibiting FASN. Orlistat induced a dose-dependent increase in cell death (Fig. 1A) and effector caspase activities (Fig. 1B) in both ovarian cancer cell lines, consistent with its reported death-inducing effects for cancers of other origin. Notably, orlistat treatment caused significantly higher cell death and effector caspase activities in 420 cells, whereas its effects in DOV cells were relatively marginal (Fig. 1A & B, black bars). Similarly, orlistat-induced cleavage of caspases and PARP (an indicator for the activation of caspases) was much more pronounced in the 420 than in the DOV line (Fig. 1C, lane 3 vs. 7 and 4 vs. 8). The appearance of cleaved caspase-8 and -9 suggested that inhibition of FASN may activate both the intrinsic and extrinsic apoptotic pathways (Fig. 1C, lane 3 and 4). Note that these data are in contrast to a previous report in MDA-MB-435 melanoma cancer cells where caspase-8, but not caspase-9 cleavage was observed following FASN inhibition.<sup>34</sup>

Interestingly, the levels of full-length caspase-2 were concurrently decreased as other caspases became activated in 420 cells (Fig. 1C, lane 3 and 4), suggesting that caspase-2 might be involved in the death signaling. To examine this possibility, cleavage of caspase-2 was carefully examined with a polyclonal antibody capable of recognizing caspase-2 cleavage products. As shown in Fig. 1D, orlistat, but not DMSO (vehicle), readily induced the appearance of caspase-2 cleavage products (empty arrows), accompanied with a loss of its full-length form (solid arrow) in 420 cells. However, no clear loss of full-length caspase-2 or the appearance of its truncated species was detected in orlistat-treated DOV cells within the same time frame (Fig. 1C and data not shown). Concomitantly, orlistat

stimulated a dramatic increase in the proteolytic activity of caspase-2-like activity in 420 cells, but caused only a minor increase in activity against the caspase-2 preferred substrate VDVAD-pNA in DOV cells (Fig. 1E).

To evaluate a possible role for caspase-2 in orlistat-induced cell death, caspase-2 was downregulated by RNAi. The efficacies of different siRNAs were tested by immunoblotting and shown in Supplementary Fig. S1A. Knockdown of caspase-2 with an siRNA targeted against the 3'-UTR region of its transcript (siCaspase-2) greatly reduced the robust 420 cell death induced by orlistat (Fig. 1F, left panel). Similarly, 420 cells transfected with siCaspase-2 ORF, an independent siRNA directed against the coding region of the caspase-2 transcript, were resistant to orlistat-induced death as well (Supplementary Fig. S1B). By contrast, orlistat only caused limited cell death in DOV cells, and caspase-2 deficiency had no significant effect (Fig. 1F, right panel), likely due to the inability of orlistat to stimulate caspase-2 in this line (Fig. 1E). Together, these data indicated that inhibition of FASN was capable of activating caspase-2, and this activation was correlated with orlistat-induced cell death.

### **Caspase-2 acts as the dominant initiator caspase in cell death induced by suppression of FASN**

Caspase-2 has been implicated in both the intrinsic and extrinsic apoptotic pathways.<sup>24–27</sup> Consistent with its established role, caspase-2 was capable of cleaving Bid in response to inhibition of FASN. Suppression of caspase-2 with a chemical inhibitor zVDVAD-fmk blocked the orlistat-induced loss of full-length Bid (Fig. 2A, upper panel) and the appearance of truncated/activated Bid in 420 cells (Fig. 2A, middle panel). Cleaved Bid has been shown to cause MOMP and mitochondrial release of cytochrome c by activating Bax/Bak.<sup>24, 25</sup> Indeed, active Bax and Bak were detected in orlistat-treated 420 cells by immunoprecipitation using antibodies specific to their active forms (Fig. 2B). Bax/Bak-mediated MOMP appeared to be essential for orlistat-induced cell death, since knockdown of either Bax or Bak significantly impaired 420 cell death and the efflux of cytochrome c from mitochondria into the cytoplasm (Supplementary Fig. S2). Notably, orlistat-induced Bax activation was greatly suppressed in cells transfected with caspase-2 siRNA (Fig. 2C), suggesting a significant role for caspase-2 in triggering the intrinsic apoptotic pathway following the blockade of FASN. Moreover, activation of caspase-3 & -8 and cleavage of the effector caspase substrate, PARP, were almost completely suppressed by caspase-2 deficiency in orlistat-treated 420 cells (Fig. 2D, lane 2 vs. 4), whereas downregulation of caspase-8 partially prevented caspase-3 activation, but failed to inhibit loss of full-length caspase-2 (Fig. 2D, lane 2 vs. 6). These data suggest either that caspase-2 is upstream of both caspase-8 and Bax/Bak-mediated MOMP or that caspase-8 cleavage results from caspase 3 activation that occurs downstream of caspase-2-dependent MOMP. In either case, caspase-2 activation does not appear to depend on caspase-8, consistent with a dominant and apical role for caspase-2 in triggering the caspase cascade following the suppression of FASN.

### Induction of REDD1 by ATF4 is required to activate caspase-2 after FASN inhibition

To gain more mechanistic insight into how suppression of FASN activates caspase-2, we examined the involvement of several proteins that have been implicated in cell death triggered by metabolic stresses, such as REDD1, a 2-deoxyglucose-inducible protein involved in neuronal loss in Parkinson's disease and cigarette smoke-induced pulmonary pathology.<sup>35–37</sup> Basal expression levels of REDD1 were too scarce to detect in most ovarian cancer cell lines (Fig. 3A & B) and patient biopsies examined (Supplementary Fig. S3A); however, REDD1 was readily induced transcriptionally after orlistat treatment (Supplementary Fig. S3B). Among cell death regulators we examined, REDD1 showed the strongest transcriptional induction by orlistat in 420 cells (followed by PUMA and ATF4). Temporal examination of REDD1 induction after orlistat treatment showed that REDD1 levels were elevated prior to an increase in the cellular proteolytic activity against VDVAD-pNA (a caspase-2 preferred substrate) in 420 cells (Fig. 3A). This induction was not suppressed by the pan-caspase inhibitor zVAD-fmk (Fig. 3B), indicating that REDD1 induction was unlikely to be a consequence of caspase activation. In contrast, orlistat only induced limited levels of REDD1 in DOV cells (Fig. 3B). Given the fact that REDD1 rose prior to the increase in caspase-2-like activity and that the induction levels of REDD1 correlated with caspase-2 activation, we reasoned that REDD1 might be required to trigger caspase-2 after FASN inhibition. In support of this notion, orlistat-induced cleavage (Fig. 3C) and caspase-2-like activities (Fig. 3D, left panel) were largely suppressed in 420 cells transfected with REDD1 siRNA.

Since REDD1 induction appeared to be important for caspase-2 activation, we sought to elucidate how orlistat upregulates REDD1 levels. Several transcription factors, including HIF-1, ATF4 and C/EBP, have been shown to mediate REDD1 induction in response to various stimuli.<sup>36, 38, 39</sup> Among them, the transcription factor ATF4 has been implicated in the induction of REDD1 in response to glucose deprivation.<sup>39</sup> We found that downregulation of ATF4 drastically suppressed induction of REDD1 by orlistat and the subsequent cell death in 420 cells (Fig. 3E). As shown in Fig. 3F, REDD1 mRNA was increased by ~ 6-fold following orlistat treatment in control 420 cells (black bars, siControl, DMSO vs. Orlistat), whereas this induction was almost completely abrogated by ATF4 deficiency (black bars, orlistat-treated cells, siControl vs. siATF4). These data suggest that the increase in REDD1 protein following FASN inhibition most likely occurs via an ATF4-mediated transcriptional induction. Interestingly, DOV cells, which expressed much less REDD1 (Fig. 3B), were found to express significantly less ATF4 before and after orlistat treatment (Supplementary Fig. S3C), suggesting a plausible explanation for the differences in REDD1 induction levels and sensitivity to orlistat between 420 and DOV cells.

### REDD1 suppresses mTOR to enable caspase-2-dependent ovarian cancer cell death following FASN inhibition

REDD1 has been found to impinge on mTOR activity by activating TSC2 after hypoxia.<sup>40, 41</sup> Consistent with previous reports, REDD1 induction in orlistat-treated 420 cells was accompanied by a decrease in mTOR activity, as revealed by the dephosphorylation of 4EBP1, a well-characterized mTOR target (Fig. 4A, lane 1–4). Downregulation of REDD1 by RNAi prevented mTOR activities from falling after orlistat

treatment (Fig. 4A, lane 4 vs. 8). TSC2 deficiency rendered cells refractory to orlistat (Fig. 4B, C and Supplementary Fig. S4A), suggesting that the suppression of mTOR might be critical for orlistat to induce cell death in ovarian cancer cells. To test this hypothesis, we examined the role of mTOR suppression in cellular sensitivity to orlistat. In REDD1-deficient 420 cells, in which mTOR inhibition was compromised (Fig. 4A, lane 4 vs. 8), cell death was significantly reduced (Fig. 4D, orlistat-treated siControl vs. siREDD1, white bars). Importantly, pharmacological suppression of mTOR with synthetic inhibitors, PP242 or rapamycin, restored cell death in REDD1-deficient 420 cells (Fig. 4D, orlistat-treated siREDD1, white bar vs. grey and black bars), indicating that REDD1 facilitated orlistat-induced cell death at least in part by blocking mTOR.

To examine whether this finding could be applied to reverse the resistance of cells inherently lacking the REDD1 induction machinery, DOV cells were treated with orlistat in the presence and absence of mTOR inhibitors. As indicated by the phosphorylation at 4EBP1 S65, DOV cells exhibited a higher basal mTOR activity than 420 cells (Supplementary Fig. S4B, upper panel, lane 1 vs. 3), inversely correlated with basal REDD1 expression pattern in these two cell lines (Fig. 3B). Orlistat completely removed phosphorylation at 4EBP1 S65 in 420 cells, whereas residual phosphorylation remained in DOV 24 h post orlistat treatment (Supplementary Fig. S4B, upper panel, lane 2 vs. 4), indicating mTOR was still active. The degree of PARP cleavage following orlistat treatment correlated with mTOR suppression, and was more evident in 420 cells (Supplementary Fig. S4B, middle panel, lane 2 vs. 4). Although PP242 treatment did not directly result in PARP cleavage in DOV cells (Supplementary Fig. S4B, lane 6), cotreatment of PP242 with orlistat removed residual mTOR activity and enhanced the cleavage of PARP (Supplementary Fig. S4B, lane 4 vs. 5), suggesting that orlistat-induced caspase activities could be enhanced in DOV cells when mTOR was suppressed, whereas mTOR inhibition was not sufficient to trigger cell death. Accordingly, inhibition of mTOR with either PP242 or rapamycin sensitized DOV to orlistat-induced death (Fig. 4E, orlistat-treated siControl, white bar vs. grey and black bars). However, this sensitization was greatly reduced by caspase-2 deficiency (Fig. 4E, orlistat-treated siCaspase-2, white bar vs. grey and black bars). A similar trend was observed in DOV cells transfected with another caspase-2 siRNA (Supplementary Fig. S4C). These data demonstrate that REDD1 suppressed mTOR to stimulate orlistat-induced cell death via caspase-2 activation.

### REDD1 controls caspase-2 dimerization

Since our data supported a role for REDD1 in controlling caspase-2 activation following FASN inhibition, we wished to determine whether REDD1 could modulate caspase-2 dimerization, the first step in its activation. To this end, we adapted a recently developed bimolecular fluorescence complementation (BiFC) assay for caspase-2<sup>32</sup> using HeLa Tet-Off cells stably transfected with an inducible vector encoding a pair of caspase-2 BiFC constructs. Cells treated with either paclitaxel, a known caspase-2 activating drug, or orlistat emitted BiFC fluorescence, validating our experimental system and indicating that caspase-2 was activated following FASN inhibition (Fig. 5A, left panels). Notably, REDD1 was specifically induced by orlistat, but not by other caspase-2 activating drugs, even though they all caused similar levels of cell death (Fig. 5B). Importantly, orlistat-induced BiFC

signals were largely suppressed in REDD1 deficient cells (Fig. 5A, right panels). To quantify this effect, BiFC cells transfected with two different REDD1 siRNAs were treated with orlistat in the presence or absence of Doxycycline (Dox) to modulate the expression of BiFC constructs. As shown in Fig. 5C, orlistat increased the BiFC fluorescence positive population in control cells (white bars, DMSO vs. Orlistat). However, this induction was greatly dampened by two independent REDD1 siRNAs (Orlistat-treated, white bar vs. grey and black bars), and the suppressive effects of each siRNA correlated with its ability to reduce orlistat-induced REDD1 (Fig. 5C, right panel). Cotreatment with Dox blocked the induction of fluorescence, indicating that the signals were specifically from caspase-2 BiFC constructs (Fig. 5C, orlistat+Dox-treated group). In concert with our previous examination of the cleavage and activity of caspase-2 (Fig. 3C, D), these data demonstrated that REDD1 was essential for caspase-2 dimerization and activation following the inhibition of FASN.

## DISCUSSION

Accumulating evidence indicates that dysregulated lipid metabolism supports the progression of ovarian cancer, representing a potential vulnerability for the development of diagnostic or therapeutic interventions to combat this leading cause of cancer-related death in women.<sup>42, 43</sup> Experiments with inhibitors targeting FASN, a key enzyme mediating *de novo* lipogenesis, have encouragingly revealed their anticancer effects in cultured ovarian cancer cells or a xenograft mouse model.<sup>11–13</sup> However, how repression of FASN reduces the survival of ovarian cancer cells is still not fully elucidated. Here, we demonstrate that REDD1 is transcriptionally induced by ATF4 following FASN inhibition, permitting the dimerization/activation of caspase-2, which in turn functions as the apical caspase to trigger cell death through a pathway involving MOMP. It appears that caspase-2 may also act upstream of caspase-8 to promote caspase-8 activation as caspase-8 cleavage following orlistat treatment was reduced by caspase-2 deficiency.

In an attempt to explore caspase-2's role in cellular metabolism, we found that caspase-2 was activated in ovarian cancer cells following FASN inhibition, broadening caspase-2's role in coordinating cellular energy status and death. Downregulation of caspase-2 by RNAi significantly ameliorated ovarian cancer cell death induced by orlistat. Besides caspase-2, other apoptotic regulators have been suggested to mediate the tumoricidal effects of FASN inhibition. Downregulation of FASN in breast cancer cells stimulates the expression of pro-apoptotic proteins, such as BNIP3, TRAIL, and DAPK2,<sup>44</sup> whereas in melanoma, suppression of FASN reduces anti-apoptotic Bcl2 family proteins<sup>45</sup> and activates caspase-8.<sup>34</sup> It is not yet clear if there are cancer type/subtype specific signaling pathways or if particular pathways dominate in some cancer types. Our observations suggest that caspase-2 may act upstream of caspase-8 and induce MOMP through the Bid-Bax/Bak axis after FASN inhibition, at least in the case of ovarian cancer. Further investigation will help elucidate the functional hierarchy of caspase-2 and other apoptotic components in distinct cancer settings.

As we and others have previously shown that caspase-2 activation is suppressed by prodomain phosphorylations at S157 and S164,<sup>19, 27</sup> we monitored how FASN inhibition modulated phosphorylation of the caspase-2 prodomain. However, phosphorylations at S157

and S164 appeared unchanged (data not shown). Instead, we found that REDD1 was induced by orlistat prior to caspase-2 activation, and that downregulation of this stress-inducible protein prevented the activation of caspase-2, as assessed by its cleavage, proteolytic activities, and dimerization. Interestingly, although both orlistat and paclitaxel activated caspase-2, the intracellular distribution pattern of the induced BiFC signal (an indicator of caspase-2 dimerization) was distinct, suggesting that FASN inhibition might activate caspase-2 through a platform distinct from that engaged by paclitaxel. In support of this notion, REDD1 was induced following the inhibition of FASN, but not by other caspase-2 activating agents employed, including paclitaxel.

REDD1 was initially identified as a developmentally-regulated protein expressed under the control of p53.<sup>46</sup> Subsequent studies extended REDD1's role in stress sensing by showing its induction by various stimuli, such as hypoxia, glucose deprivation, and cigarette smoke-induced oxidative stress; several transcription factors, including HIF-1, ATF4 and C/EBP have been implicated in REDD1 induction in response to these stimuli.<sup>36, 38, 39</sup> Although the basal expression levels of REDD1 in ovarian cancer biopsies or cell lines that we examined were very limited, REDD1 protein levels were drastically upregulated in ovarian cancer cells that were sensitive to FASN inhibition, consistent with an increase in its mRNA observed by us and in a reported screen for genes upregulated in MDA-MB-435 cells following knockdown of FASN.<sup>34</sup> This induction appeared to be dependent on ATF4, as the induction levels of ATF4 correlated with the expression levels of REDD1, while deficiency of this ER stress-related transcription factor reduced both the mRNA and protein levels of REDD1, suppressing the subsequent cell death following orlistat treatment. Interestingly, inhibition of FASN has been shown to trigger ER stress in cancer cells, with unclear functional consequences.<sup>7</sup> Our findings, therefore, provide a potential molecular link between ER stress and cell death by showing that ATF4-induced REDD1 induction promotes caspase-2 activation. This suggests that the status of the ER functions may control the susceptibility of ovarian cancer cells to death following FASN inhibition.

Consistent with its reported function in hypoxia,<sup>40, 41</sup> we found that REDD1 induced by orlistat also caused a suppression of mTOR in ovarian cancer cells. Inhibition of mTOR with PP242 or rapamycin restored the sensitivity of REDD1 deficient 420 cells, and even overcame the lack of REDD1 induction machinery in refractory cells, such as DOV, by stimulating caspase-2-dependent cell death after orlistat treatment, indicating mTOR inhibition is a proximal determinant that controls cell death after FASN inhibition by permitting caspase-2 dimerization and activation. Therefore, the persistent mTOR activity and resistance to orlistat observed in DOV cells could be, at least in part, attributed to the lower steady-state levels of ATF4 and REDD1 and/or their weak induction after FASN inhibition.

Given the fact that mTOR is capable of stimulating FASN expression through SREBP,<sup>47</sup> in theory REDD1-mediated inhibition of mTOR could further inhibit lipogenesis by reducing the expression of FASN. However, FASN protein levels remained constant throughout the course of our experiments (data not shown). Meanwhile, since mTOR inhibitors did not directly trigger cell death, but sensitized DOV cells to orlistat-induced PARP cleavage and death, our data collectively indicate that mTOR inhibition is essential, though not sufficient,

to activate caspase-2 to initiate the death signaling in ovarian cancer cells after FASN inhibition. Precisely, how REDD1-mediated mTOR suppression facilitates caspase-2 activation is currently under investigation. Nonetheless, these data indicate, for the first time, that mTOR activity controls caspase-2 activation, and provide supportive rationale for the use of mTOR inhibitors to enhance caspase-2-mediated cell death in ovarian cancer treatment.

## MATERIALS AND METHODS

### Ovarian cancer biopsies and cell culture

Ovarian cancer biopsies and cell lines OVCA420 (420) and DOV13 (DOV) were kindly provided by Dr. Susan K. Murphy and Dr. Andrew Berchuck (Duke University, Durham, NC, USA), and grown at 37°C in RPMI 1640 media supplemented with 10% FBS. The purity of both lines was analyzed and confirmed using the PowerPlex 18D kit (Promega, Madison, WI, USA) for polymorphic short tandem repeat (STR) markers (The Duke University DNA Analysis Facility, NC, USA). Cells were treated with 100 or 200  $\mu$ M orlistat or DMSO (vehicle) and then collected for assays 18–24 h post treatment or at indicated time points. Caspase-2 specific inhibitor zVDVAD-fmk (Enzo, Farmingdale, NY, USA) was utilized at 50  $\mu$ M when indicated, while 50  $\mu$ M pan-caspase inhibitor zVAD-fmk (Enzo) was cotreated with orlistat to examine REDD1 expression, unless otherwise specified. To suppress mTOR, 4  $\mu$ M PP242 and 10  $\mu$ M (420) or 5  $\mu$ M (DOV) rapamycin were cotreated with orlistat as indicated. Cell death and caspase activity were measured as previously described by flow cytometry or using the substrates specific for different caspases [Ac-VDVAD-pNA (Caspase-2) or Ac-DEVD-pNA (caspase-3/7), Enzo]<sup>48</sup>. To monitor mitochondrial release of cytochrome c, treated cells were incubated in digitonin-based buffer (250 mM sucrose, 20 mM Tris, pH 7.4, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1  $\times$  Roche complete protease inhibitor, 250  $\mu$ g/mL digitonin) on ice for 10 min; cytosolic and mitochondrial fractions were then separated by centrifugation. Otherwise, cells were lysed as previously described for immunoblotting.<sup>48</sup>

### RNA interference

Cells were transfected with 50 nM siRNA twice on two consecutive days by Lipofectamine RNAiMAX reagents (Invitrogen, Grand Island, NY, USA), and assayed 48 h after the first transfection. The sources and sequences of siRNA could be found in the Supplementary information. To generate a stable TSC2 knockdown cell line, plasmids encoding scramble shRNA (Addgene, Cambridge, MA, USA, plasmid 1864) or TSC2 shRNA (Addgene, plasmid 15478) were used to generate lentivirus. 420 cells were infected with the virus and selected with 2  $\mu$ g/mL puromycin. Knockdown efficiency was examined by immunoblotting.

### Reagents and antibodies

Orlistat and cisplatin were from Sigma, St. Louis, MO, USA or Cayman Chemical, Ann Arbor, MI, USA; paclitaxel and rapamycin were from LC Laboratories, Woburn, MA, USA; PP242 was from Active Biochem, Maplewood, NJ, USA. Antibodies were purchased from Abcam, Cambridge, MA, USA (Polyclonal caspase-2 from rabbit, Actin, tubulin), Enzo

(Monoclonal caspase-2 from rat, 11B4), BD Pharmingen, San Jose, CA, USA (Active Bax 6A7, cytochrome c), Santa Cruz, Dallas, TX, USA (Actin), Proteintech (REDD1, ATF4), CalBiochem, Billerica, MA, USA (Active Bak Ab-1), Cell Signaling, Danvers, MA, USA (Caspase-8, Caspase-9, Caspase-3, cleaved PARP, Bid, Bax, Bak, COX IV, pS65 4EBP1, total 4EBP1, TSC2), and specified in the Supplementary information.

### Bimolecular fluorescence complementation (BiFC)

The BiFC assay was adapted from Bouchier-Hayes et al.,<sup>32</sup> and described in detail in the Supplementary information. The BiFC signals were recorded by Leica SP5 confocal microscopy or flow cytometry. To examine REDD1 induction, a similar experiment was performed, and cells were treated with DMSO (vehicle), 200  $\mu$ M orlistat, 10  $\mu$ M paclitaxel, or 10  $\mu$ M cisplatin for 24 h. Treated cells were assayed by flow cytometry for cell death, or lysed to examine REDD1 induction by immunoblotting. Pan-caspase inhibitor q-VD-OPh (10  $\mu$ M) was used in concert with pro-apoptotic drugs to suppress cell death.

### Immunoprecipitation of active Bax/Bak

Active Bax and Bak were detected as described previously.<sup>49, 50</sup> Immunoprecipitants collected with active Bax or Bak antibodies were resolved by a SDS-PAGE gel and detected by immunoblotting with antibodies against total Bax or Bak.

### Statistical analysis

Data from flow cytometry-based experiments and caspase assays were shown as mean $\pm$ sem; qPCR results were shown as mean $\pm$ sd. Data were analyzed by unpaired two-tailed Student's *t*-test.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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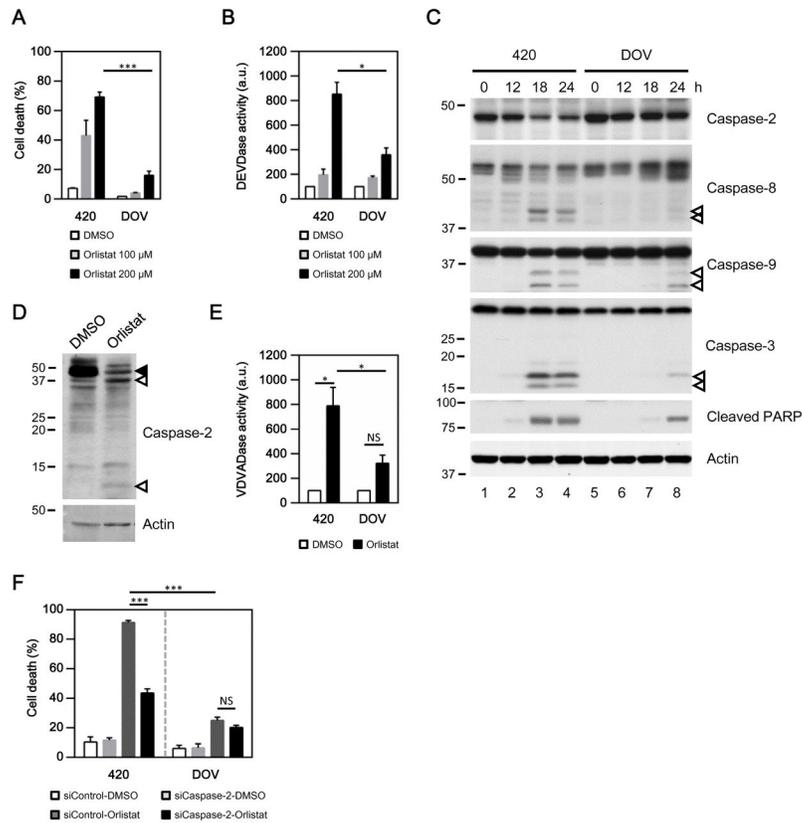
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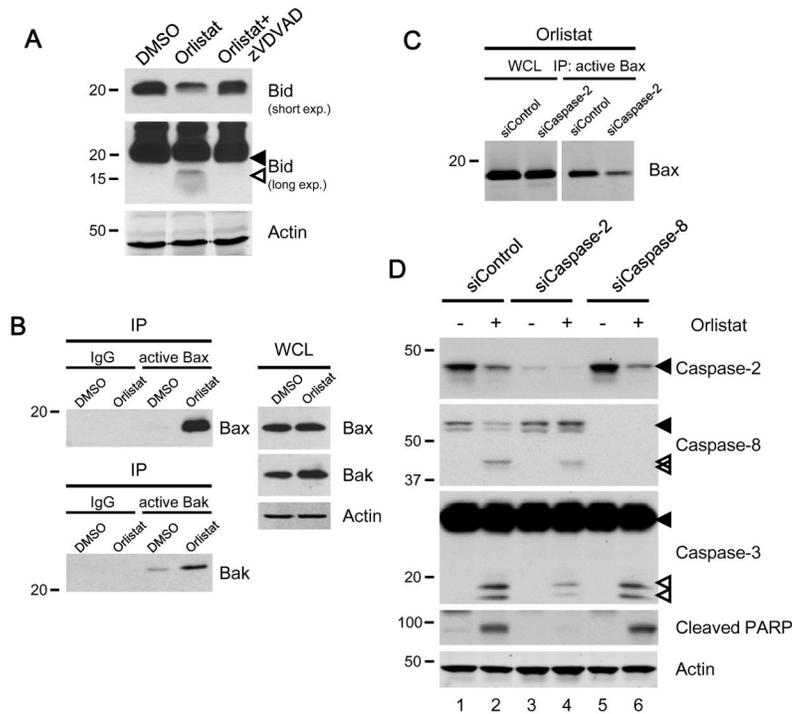
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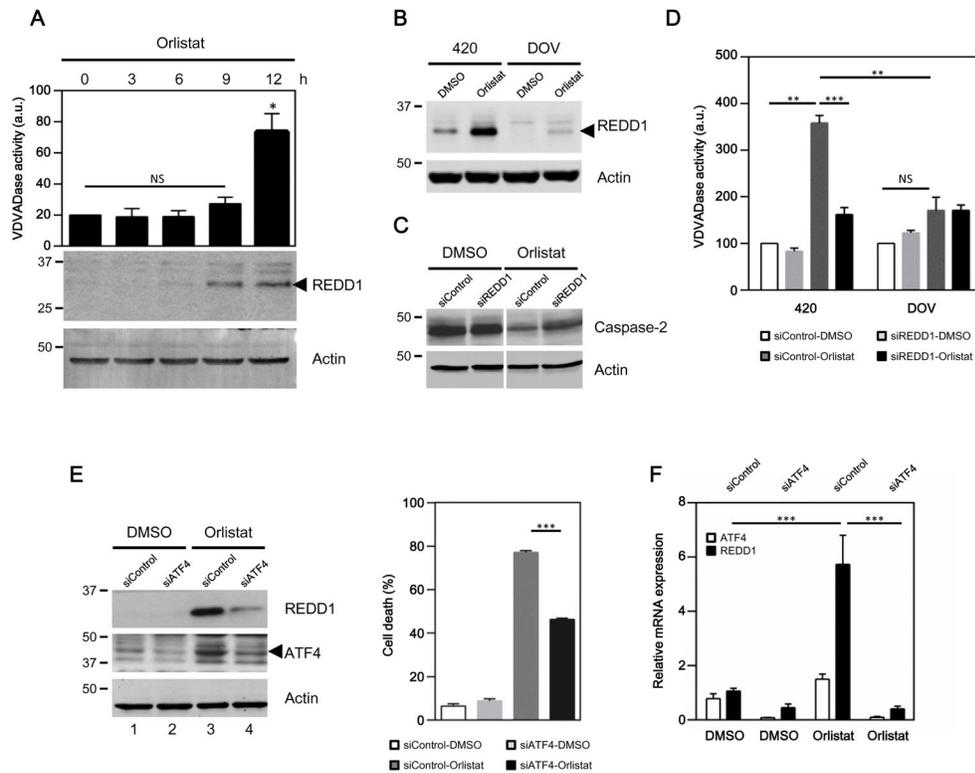


**Fig. 1. Blockade of *de novo* lipid synthesis activates caspase-2**

(A–B) FASN inhibitor orlistat induced strong cell death (A) and effector caspase (DEVDase) activities (B) in the ovarian cancer cell line OVCA420 (420), but only had marginal effects in DOV13 (DOV) cells ( $*p<0.05$ ;  $***p<0.001$ ; a.u., arbitrary units). (C) Activation of caspases induced by FASN inhibition was monitored at the indicated time points by the loss of full-length caspase-2, the appearance of cleaved caspase-8, -9, -3 (empty arrows), and caspase-mediated proteolysis of PARP. A rat monoclonal caspase-2 antibody (11B4) was used. (D) Orlistat induced the appearance of cleaved caspase-2 in 420 cells as monitored by a rabbit polyclonal caspase-2 antibody (Solid and empty arrows indicate full-length and cleaved caspase-2, respectively). (E) FASN inhibition activated the proteolytic cleavage of VDVAD-pNA (caspase-2 preferred substrate) in 420 ( $*p<0.05$ ), but not in DOV cells (NS, not significant;  $p=0.08$ ; a.u., arbitrary units). (F) Downregulation of caspase-2 effectively reduced cell death induced by orlistat in 420, but not in DOV cells ( $***p<0.001$ ; NS, not significant,  $p=0.137$ ).

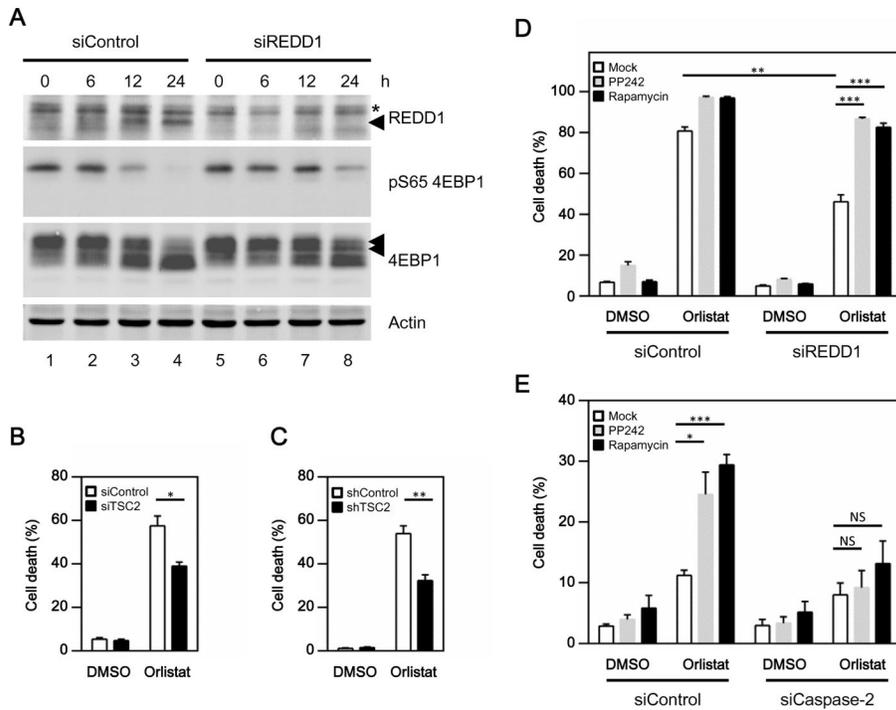


**Fig. 2. Orlistat-induced activation of the caspase cascade depends on caspase-2**  
 (A) Cotreatment with the caspase-2 inhibitor zVDVAD-fmk prevented orlistat from inducing a decrease in full-length Bid (short exposure) and the appearance of truncated Bid (long exposure; solid and empty arrows indicate full-length and cleaved Bid, respectively).  
 (B) Inhibition of FASN by orlistat activated Bax and Bak. Active Bax or Bak were immunoprecipitated (IP) from the whole cell lysate (WCL) and detected as described in the Materials and Methods. (C) Downregulation of caspase-2 by RNAi dampened orlistat-induced Bax activation. (D) Inhibition of caspase-2 suppressed the activation of the caspase cascade (Solid and empty arrows indicate full-length and cleaved caspases, respectively). The loss of caspase-2 was revealed by a monoclonal caspase-2 antibody 11B4. 420 cell lysates were collected 12 h (A–C) or 24 h (D) post treatment.

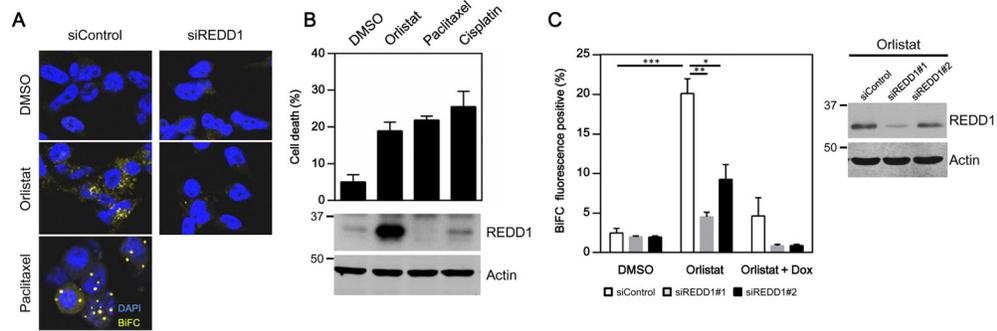


**Fig. 3. Inhibition of FASN activates caspase-2 through REDD1**

(A) REDD1 was upregulated prior to caspase-2 activation. 420 lysates were collected at the indicated time points and divided into two portions to examine cellular caspase-2-like (VDVADase) activities and REDD1 levels ( $*p < 0.05$ ; NS, not significant). (B) Caspase activities were dispensable for REDD1 induction by orlistat. REDD1 levels were monitored in 420 and DOV cells treated with DMSO or orlistat in the presence of zVAD-fmk. REDD1 is indicated by an arrow. (C) REDD1 siRNA prevented caspase-2 cleavage resulting from FASN inhibition in 420 cells. The loss of caspase-2 was revealed by a monoclonal caspase-2 antibody 11B4. (D) REDD1 deficiency suppressed caspase-2 activities induced after FASN inhibition. Orlistat induced strong caspase-2-like activities in 420 cells transfected with control siRNA, but this induction was largely suppressed in cells that received REDD1 siRNA (left panel;  $**p < 0.01$ ;  $***p < 0.001$ ). The same treatment with orlistat only caused negligible VDVADase activity in cells incapable of inducing REDD1 (right panel; NS, not significant,  $p = 0.98$ ). (E) REDD1 induction and the subsequent cell death induced by FASN inhibition was abrogated in 420 cells by siATF4 ( $***p < 0.001$ ). (F) 420 cells were treated as in (E), and indicated mRNA levels were analyzed by a semi-quantitative PCR. Orlistat significantly increased REDD1 mRNA levels in cells transfected with control siRNA (black bars, siControl, DMSO vs. Orlistat), but not in ATF4 deficient cells (black bars, orlistat-treated cells, siControl vs. siATF4) ( $***p < 0.001$ ).



**Fig. 4. REDD1 suppresses mTOR to control ovarian cell death induced by orlistat**  
 (A) Orlistat caused time-dependent induction of REDD1 (indicated by an arrow; an asterisk shows a non-specific signal), decreased phosphorylated 4EBP1 at S65, and induced a downshift of the 4EBP1 proteins in control 420 cells (lane 1–4). Knockdown of REDD1 by siRNA ameliorated the dephosphorylation of 4EBP1 at S65 (lane 3 vs.7 and 4 vs. 8) and preserved the slowly migrating/hyper-phosphorylated species (arrows) detected by the 4EBP1 antibody (lane 4 vs. 8). (B–C) Downregulation of TSC2 by siRNA (B) or shRNA (C) suppressed orlistat-induced 420 cell death (\* $p < 0.05$ ; \*\* $p < 0.01$ ). (D) Inhibition of mTOR by PP242 or rapamycin restored the sensitivity to orlistat in REDD1 deficient 420 cells (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). (E) Suppression of mTOR by PP242 or rapamycin greatly enhanced DOV cell death induced by orlistat through a caspase-2-dependent pathway (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ; NS, not significant).



**Fig. 5. Inhibition of FASN stimulates caspase-2 dimerization through REDD1**

(A) Caspase-2 dimerization was monitored by a BiFC-based assay and recorded by photomicrographs taken with a confocal microscope. Paclitaxel, a known caspase-2 activator, was used as a positive control, whereas solvent (DMSO) was used as a negative control. Orlistat induced caspase-2 BiFC signals (yellow) in control BiFC cells but not in those transfected with REDD1 siRNA. DAPI (blue) stained the nucleus. (B) REDD1 expression was specifically induced upon FASN inhibition. BiFC cells were analyzed after indicated treatments by flow cytometry for cell death, and then lysed to measure REDD1 levels by western blotting. Actin was used as a loading control. (C) Orlistat induced BiFC signals in control BiFC cells, whereas this induction was largely suppressed by two different REDD1 siRNAs (middle, orlistat-treated group) or by doxycycline (Dox) (right, orlistat+Dox-treated group) (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). The efficacy of REDD1 siRNA was examined by western blotting.