The combination of *TPL2* knockdown and TNFα causes synthetic lethality via caspase-8 activation in human carcinoma cell lines

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Most normal and tumor cells are protected from tumor necrosis factor α (TNFα)-induced apoptosis. Here, we identify the MAP3 kinase tumor progression locus-2 (TPL2) as a player contributing to the protection of a subset of tumor cell lines. The combination of *TPL2* knockdown and TNFα gives rise to a synthetic lethality phenotype via receptor-interacting serine/threonine-protein kinase 1 (RIPK1)-dependent and -independent mechanisms. Whereas wild-type TPL2 rescues the phenotype, its kinase-dead mutant does not. Comparison of the molecular events initiated by small interfering RNA for *TPL2* (si*TPL2*) ± TNFα in treatment-sensitive and -resistant lines revealed that the activation of caspase-8, downstream of miR-21-5p and cFLIP, is the dominant TPL2-dependent event. More important, comparison of the gene expression profiles of all of the tested cell lines results in the clustering of sensitive and resistant lines into distinct groups, providing proof of principle for the feasibility of generating a predictive tool for treatment sensitivity.

TPL2 | TNFα | synthetic lethality | caspase | apoptosis

Tumor necrosis factor α (TNFα), a member of the tumor necrosis factor cytokine superfamily, is a type II transmembrane protein that forms membrane-associated homotrimers. Proteolytic cleavage of the membrane-bound cytokine is mediated by the TNFα converting enzyme (TACE or ADAM17), which releases a soluble trimeric form of the cytokine. TNFα is produced primarily by activated macrophages, although it can also be produced by other types of cells, including neutrophils, eosinophils, mast cells, natural killer cells, and cluster of differentiation 4-positive ($CD4^+$) lymphocytes. Its expression is induced by Toll-like receptor (TLR), Nod-like receptor, and C-type lectin receptor signals, as well as by cytokine and chemokine receptor signals. In $CD4^+$ T cells, it can also be induced by T cell receptor signals (1, 2).

TNFα signaling can be initiated by both the membrane-bound and soluble forms of the cytokine, and it has an integral role in innate immunity and inflammation (1). Although in some normal and tumor cells, it induces apoptosis, most cells are protected. In some of them, protection is due to the expression of the caspase-8 inhibitor cFLIP and other antiapoptotic factors and is nuclear factor-κB (NF-κB)–dependent (3–5). In others, it depends on the ubiquitin ligases cIAP1 and cIAP2, which ubiquitinate receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and block its ability to activate caspase-8 (6). The role of these mechanisms has been confirmed with the use of NF-κB and protein synthesis inhibitors (cycloheximide [CHX]), which block the NF-κB–mediated protection (7), and with the use of Smac mimetics, which block cIAP1 and cIAP2 (6–8). TNFα and CHX-induced death is RIPK1-independent, while TNFα and Smac mimetic-induced death is RIPK1-dependent (7). The RIPK1-dependent cell death is inhibited by the IκB kinase complex and MK2, which inhibit RIPK1 (9–12). In this report, we identify the MAP3 kinase tumor progression locus-2 (TPL2) as a protective player in a subset of tumor cells and we delineate the relevant TPL2-regulated pathway(s).

TPL2 is an oncoprotein that is activated by provirus insertion in Moloney murine leukemia virus-induced rodent lymphomas and mammary tumor virus-induced mammary adenocarcinomas (13, 14). Expression of constitutively active TPL2 from a thymus-targeted transgene confirmed its oncogenic potential (15). Subsequently, it was shown that TPL2 is obligatory for the transduction of signals initiated by triggering TLRs and other receptors in different cell types and that it plays an important role in innate and adaptive immunity and inflammation (16, 17) (*SI Appendix*, *SI Introduction*). Although studies published over the last 5 y provide evidence that TPL2 has the potential to function as a tumor suppressor for several types of tumors (18, 19), TPL2 is primarily known to function as an oncogene. In human tumors, it is often expressed at high levels, and its expression promotes cell proliferation and drug resistance (20).

In the present study, we show that the combination of *TPL2* knockdown and TNFα induces synthetic lethality in TPL2-expressing human tumor cell lines. Using pharmacological and

Significance

Tumor necrosis factor α (TNFα) fails to induce apoptosis in most tumor cells. Defining the mechanism(s) responsible for the protection of these cells from TNFα-induced apoptosis may have significant therapeutic implications. Here, we show that tumor progression locus-2 (TPL2), a MAP3 kinase activated by TNFα, protects from TNFα-induced apoptosis by preventing the activation of caspase-8, and that the combination of *TPL2* knockdown and TNFα treatment induces synthetic lethality. More important, sensitivity to synthetic lethality can be predicted from the gene expression profiles of the tumor cells. In addition to their therapeutic potential, these results identify an important node in TNFα signaling.

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genetic tools, we showed that the dominant event responsible for TNFα-induced apoptosis in small interfering RNA for *TPL2* (si*TPL2*)/TNFα-treated cells is the activation of caspase-8, downstream of miR-21-5p and cFLIP. The mitochondrial pathway of apoptosis is also activated, but it has an auxiliary role. Most important, sensitive and resistant lines cluster in distinct groups when their gene expression profiles are compared, suggesting the feasibility of a tool that predicts treatment sensitivity.

Results

The Knockdown of *TPL2* Renders a Subset of Human Cancer Cell Lines Sensitive to the Apoptotic Action of TNFα. To determine the role of TPL2 in the response of different cell lines to TNFα, we examined the effects of TNFα stimulation in a set of cancer cell lines 48 h after treatment with si*TPL2* or siControl RNAs. Monitoring cell survival with light microscopy and the CellTiter-Glo assay 24 h later revealed that although TNFα and *TPL2* knockdown

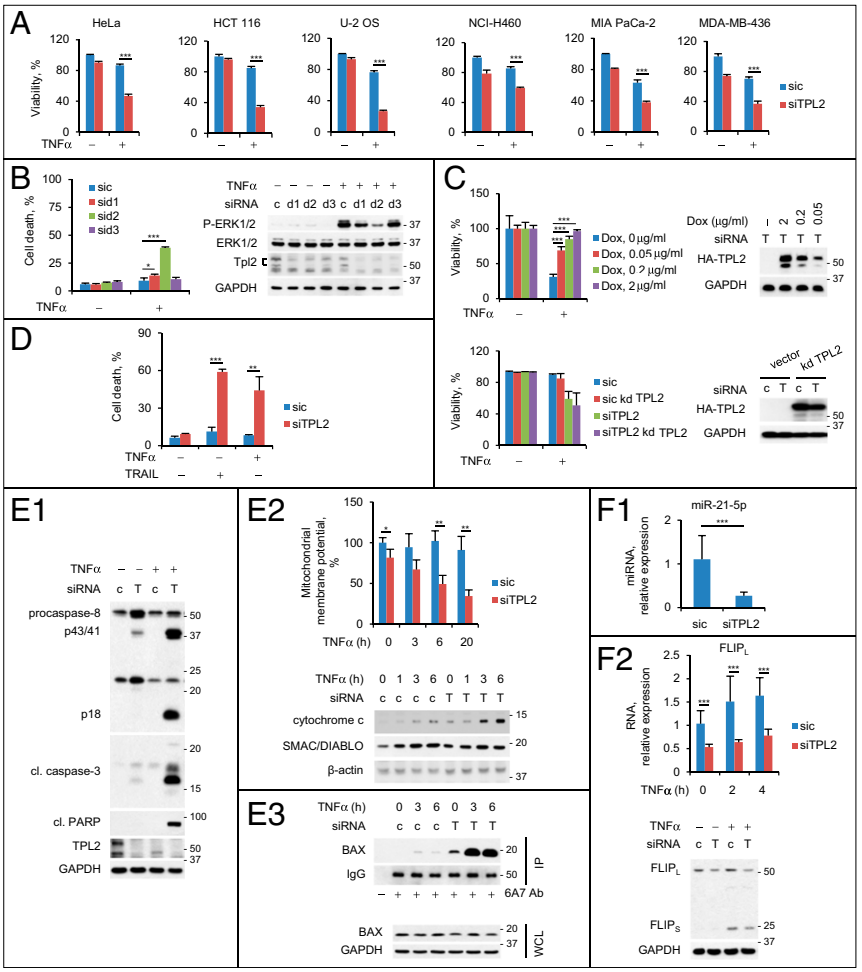

Fig. 1. si*TPL2* and TNFα induce synthetic lethality in human cancer cells by promoting the activation of caspase-8 and the mitochondrial pathway of apoptosis. (*A*) Viability of cancer cells transfected with control or *TPL2* siRNA was measured 24 h after the exposure to TNFα, using the CellTiter-Glo assay. sic, siControl. (*B*, *Left*) Synthetic lethality induced by the combination of TNFα and 3 different *TPL2* siRNAs (sid1, sid2, and sid3) was measured in HeLa cells by Annexin V/propidium iodide (PI) staining. (*B*, *Right*) Lysates of HeLa cells transfected with the same *TPL2* siRNAs (d1, d2, and d3) and treated with TNFα for 15 min were probed with an ERK1/2 phosphoantibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. (*C*, *Upper*) HeLa cells stably expressing si*TPL2*-resistant hemagglutinin (HA)-TPL2 from a doxycycline (Dox)-inducible promoter were transfected with si*TPL2*. Following the induction of HA-TPL2 with increasing concentrations of Dox, the cells were treated with TNFα. (*Left*) Viability was measured 24 h later, using the CellTiter-Glo assay. (*Right*) Dox-induced expression of HA-TPL2 was assessed in these cells by Western blotting with HA-tag antibody. (*C*, *Lower*) HeLa cells expressing the kinase-dead (kd) mutant TPL2K167M were transfected with sic or si*TPL2* and treated with TNFα. (*Left*) Cell viability was measured 24 h later by Annexin V/PI staining. (*Right*) Expression of kd TPL2 was measured in these cells by Western blotting with an anti-HA antibody. c, sic-transfected cells; T, si*TPL2*-transfected cells. (*D*) Synthetic lethality induced by TRAIL and si*TPL2* in HeLa cells was measured 24 h after exposure to TRAIL by Annexin V/PI staining. The si*TPL2*/TNFα treatment was the positive control. (*E*, *1*) Activation of caspase-8 and caspase-3 and cleavage of PARP were measured by immunoblotting 6 h after TNFα treatment. cl., cleaved. (*E*, *2*, *Upper*) Mitochondrial membrane potential was measured in cells treated with TNFα for 0, 3, 6, and 20 h. (*E*, *2*, *Lower*) The S100 fractions of cell lysates of c and T cells were harvested at the indicated time points after treatment with TNFα, and they were probed with anti-cytochrome *c* or anti-SMAC/DIABLO antibodies. Whereas the release of cytochrome *c* in the si*TPL2*/TNFα-treated cells was robust, the release of SMAC/DIABLO was weak. This may be caused by the ability of si*TPL2* to stimulate the expression of BCL2, BCL-XL and some of the IAPs (Survivin and XIAP) and to down-regulate the expression of BAX (*SI Appendix*, Fig. S1*G*), as these proteins have been shown to influence SMAC/DIABLO release from the mitochondria (37–39). (*E*, *3*) BAX activation was monitored by immunoprecipitation (IP) of cell lysates with the 6A7 antibody that recognizes the active monomeric form of BAX. Total BAX levels were monitored by immunoblotting of whole-cell lysates (WCL). (*F*, *1*) Levels of miR-21-5p were measured by qRT-PCR. miRNA, microRNA. (*F*, *2*) *FLIP$_L$* messenger RNA and FLIP$_L$ protein were monitored by qRT-PCR (*Upper*) and immunoblotting (*Lower*) before and after TNFα treatment (2 and 4 h for the mRNA and 6 h for the protein). Graphs show mean ± SD. Experiments in *A* were done in triplicate, and they were repeated more than 3 times. Experiments in *C*, *Upper*; *E*, *2*; *F*, *1*; and *F*, *2* were done in triplicate, and they were repeated 2 to 3 times. Experiments in *B*; *C*, *Lower*; and *D* were repeated 3 to 4 times. For *A* and *C*, *Upper*, error bars show the SD within the representative experiment shown in the figure. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

had minor effects on the viability of HeLa, U-2 OS, HCT 116, and NCI-H460 cells, their combination induced synthetic lethality. In this report, we will refer to these cell lines as si*TPL2*/TNFα (TST)-sensitive. MDA-MB-436 and MIA PaCa-2 cells were partially sensitive to both treatments, and their lethality was enhanced by the combination (Fig. 1*A*). Staining HeLa cells for Annexin V revealed that cell death was due to apoptosis (Fig. 1*B*, *Left*).

To confirm that the observed synthetic lethality was not due to off-target effects of si*TPL2*, we repeated the experiment with 3 *TPL2* siRNAs. Two of them induced synthetic lethality of a magnitude that correlated with their ability to inhibit the phosphorylation of extracellular signal-regulated kinase (ERK), an obligatory TPL2 phosphorylation target. The one that did not induce synthetic lethality also failed to inhibit ERK phosphorylation (Fig. 1*B*, *Right*). Additional experiments showed that whereas inducibly expressed wild-type (wt) TPL2 rescued HeLa cells from synthetic lethality, the kinase-dead TPL2K167M failed to do so (Fig. 1*C*). To determine whether other death receptor-targeting factors also synergize with the knockdown of *TPL2*, we repeated the experiment with TRAIL and confirmed that TRAIL also synergizes with si*TPL2* in HeLa cells (Fig. 1*D*).

Our initial attempts to determine the mechanism of synthetic lethality focused on the si*TPL2*-induced partial loss of the activating phosphorylation of ERK, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38MAPK) and the inhibitory phosphorylation of glycogen synthase kinase 3α/β (GSK3α/β) (*SI Appendix*, Fig. S1*A*). The inhibitory phosphorylation of GSK3α/β, both before and after TNFα stimulation, was shown to be under the control of AKR thymoma and the TPL2-dependent activation of mitogen-activated ERK kinase (MEK)/ERK (*SI Appendix*, Fig. S1*B*). The results of these studies showed that the pharmacological inhibition of MEK/ERK, JNK, and p38MAPK does not induce synthetic lethality in TNFα-treated cells (*SI Appendix*, Fig. S1*C*, *Upper*). Moreover, the pharmacological inhibition of GSK3 did not inhibit the si*TPL2*/TNFα-induced synthetic lethality (*SI Appendix*, Fig. S1*C*, *Lower*). We conclude that the si*TPL2*-induced inhibition of phosphorylation of these kinases is not responsible for the synthetic lethality phenotype.

si*TPL2* Promotes the TNFα-Induced Activation of Caspase-8 and the Mitochondrial Pathway of Apoptosis. TNFα stimulation induces apoptosis by activating caspase-8 and the mitochondrial pathway of apoptosis. We therefore examined whether the knockdown of *TPL2* sensitizes HeLa cells to the activation of these pathways by TNFα. The results confirmed that si*TPL2* enhances dramatically the activation of caspase-8 (Fig. 1 *E*, *1*). The mitochondrial pathway, which can be activated by caspase-8–dependent and –independent mechanisms, was also activated by TNFα in si*TPL2*-transfected cells, as evidenced by the fact that treatment with si*TPL2*/TNFα resulted in loss of mitochondrial membrane potential, release of mitochondrial cytochrome *c*, and activation of BAX (Fig. 1 *E*, *2* and *3*). Importantly, caspase-3, a target of this pathway, and PARP1, a caspase-3 target, were also cleaved in si*TPL2*/TNFα-treated cells (Fig. 1 *E*, *1*).

Two observations suggested mechanisms that may contribute to the si*TPL2*/TNFα-induced activation of caspase-8. First, si*TPL2* induced a substantial up-regulation of procaspase-8 (Fig. 1 *E*, *1*) and a substantial down-regulation of miR-21-5p, which is known to target procaspase-8 (21) (Fig. 1 *F*, *1*). Second, the catalytically inactive homolog of caspase-8 $FLIP_L$, an endogenous inhibitor of caspase-8 (22), was also down-regulated in both si*TPL2*- and si*TPL2*/TNFα-treated cells (Fig. 1 *F*, *2*). $FLIP_S$, a C-terminally truncated isoform arising via alternative splicing of cFLIP, was undetectable in HeLa cells, and its induction by TNFα was inhibited by si*TPL2* (Fig. 1 *F*, *2*, *Lower*).

si*TPL2* Down-Regulates *RIPK1* and *MCL1* and Induces BIM_L in both TNFα-Treated and -Untreated Cells, Blocks the Induction of *cIAP2*, and Promotes the Cleavage of BID and RIPK1 by TNFα. To determine whether additional regulators of apoptosis may contribute to the si*TPL2*/TNFα-induced synthetic lethality, we examined the expression of such regulators in siControl and si*TPL2*-transfected cells, with or without TNFα. We were searching primarily for changes induced by si*TPL2* only, because such changes may function as TNFα sensitizers. We observed that si*TPL2* down-regulates *RIPK1* and *MCL1* at both the RNA and protein levels, up-regulates BIM_L, and promotes the cleavage of BID and RIPK1, after treatment with TNFα (*SI Appendix*, Fig. S1 *D* and *E*). BIM_L, a strongly proapoptotic protein, is encoded by an alternatively spliced *BIM* RNA, and its up-regulation is due to si*TPL2*-induced changes in alternative splicing. Finally, si*TPL2* also blocked the induction of *cIAP2* by TNFα (*SI Appendix*, Fig. S1*F*).

Other apoptotic regulators include the inhibitors of apoptosis BCL2, BCL-XL, Survivin, XIAP, and cIAP1, whose expression was surprisingly up-regulated by si*TPL2*, and the inducers of apoptosis BAX and PUMA, which were surprisingly down-regulated (*SI Appendix*, Fig. S1*G*). These regulators of apoptosis therefore do not contribute to synthetic lethality, but they may be targets of feedback loops, activated via unknown mechanisms, by the activated caspase-8 and/or the activated mitochondrial pathway of apoptosis. The expression of BAK, another inducer of apoptosis, was not affected by si*TPL2* (*SI Appendix*, Fig. S1*G*). The down-regulation of PUMA, a target of p53 (23, 24), is p53-independent because p53 is inactive in HeLa cells and p53 levels were not affected in TST-sensitive HCT 116 and U-2 OS cells (*SI Appendix*, Fig. S1*H*).

It has been reported that inhibition of TAK1 (MAP3K7) also gives rise to synthetic lethality when combined with TNFα treatment, and that the TNFα/TAK1 inhibition-induced synthetic lethality is associated with the activation of caspase-8. This raised the question of whether TPL2 (MAP3K8) and TAK1 (MAP3K7) function in the same pathway. To address this question, we first examined the phosphorylation of TAK1 at Thr184/187 and Ser412 in HeLa cells transfected with siControl or si*TPL2*, before and at multiple time points after treatment with TNFα. Thr184/187 is an autophosphorylation site, and therefore depends on TAK1 activation (25). Ser412 phosphorylation is required for TAK1 activation by TNFα, interleukin-1β, and TLR ligands (26). The results showed that phosphorylation at Thr184/187 is delayed and phosphorylation at Ser412 is significantly impaired in TNFα-treated si*TPL2*-transfected cells. In addition, si*TPL2* transfection resulted in a significant down-regulation of the expression of TAK1 (*SI Appendix*, Fig. S1*I*). We conclude that TPL2 contributes to the regulation of TAK1 in HeLa cells and that si*TPL2*/TNFα-induced apoptosis in these cells may be due, at least in part, to the deregulation of TAK1.

Comparison of TST-Sensitive and -Resistant Tumor Cell Lines. To determine which of the events described above may contribute to the synthetic lethality phenotype, we compared 3 TST-sensitive (HeLa, U-2 OS, and HCT 116) cell lines with 3 TST-resistant (CACO-2, RKO, and SW480) cell lines, searching for events occurring selectively in TST-sensitive lines (Fig. 2). The same was done using MDA-MB-436 and MIA PaCa-2 cells, which were partially sensitive to both si*TPL2* and TNFα, and several additional TST-resistant lines (T-47D, PANC-1, NCI-H1299, and LS-174T) (*SI Appendix*, Fig. S2). These comparisons revealed that si*TPL2* up-regulates procaspase-8 in TST-sensitive, but not TST-resistant, lines, with the exemption of the TST-resistant SW480 and NCI-H1299 cells, in which procaspase-8 was also weakly induced (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). The up-regulated procaspase-8 was also activated selectively in si*TPL2*-treated, TST-sensitive lines (Fig. 2*B* and *SI Appendix*, Fig. S2*B*). The gene encoding procaspase-8 is a target of miR-21-5p (21), which was also down-regulated selectively

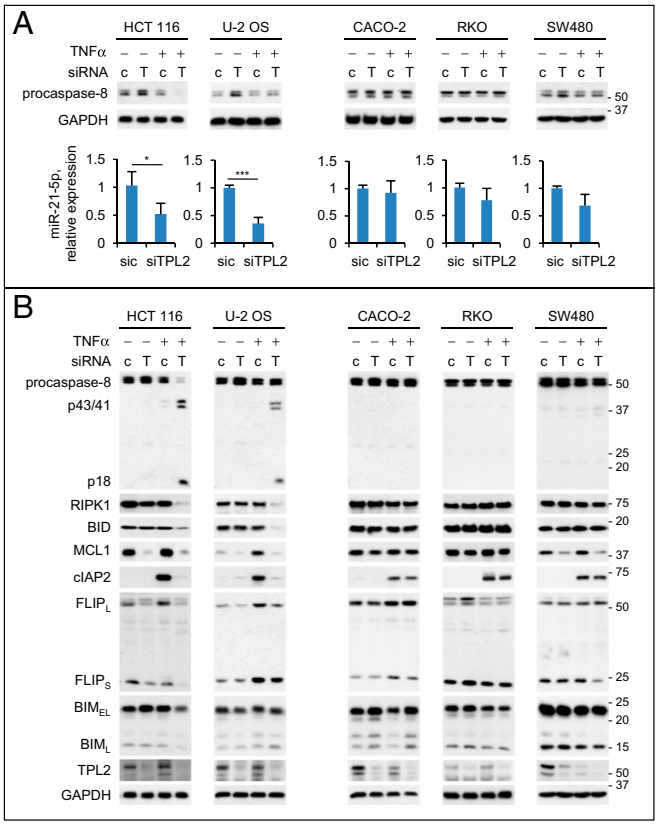

Fig. 2. Comparison of TST-sensitive (HCT 116 and U-2 OS) and TST-resistant (CACO-2, RKO, and SW480) tumor cell lines. The TST-sensitive and TST-resistant cell lines were transfected with control (c) or *TPL2* (T) siRNA. Transfected cells were harvested, following treatment with TNFα or vehicle for 6 h. (*A*) Expression of procaspase-8 (*Upper*) was monitored by immunoblotting, and expression of miR-21-5p was monitored by qRT-PCR (*Lower*). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sic, siControl. (*B*) Expression of the indicated proteins was monitored by immunoblotting. Graphs show mean ± SD. Experiments were done in triplicate and repeated 2 to 4 times. $*P < 0.05$, $***P < 0.001$.

by si*TPL2* (Fig. 2*A* and *SI Appendix*, Fig. S2*A*, *Lower*). Combined with the observation that miR-21-5p was also slightly down-regulated in the TST-resistant SW480 and NCI-H1299 cells in which procaspase-8 was weakly up-regulated, this finding suggests that si*TPL2* may up-regulate procaspase-8 by downregulating miR-21-5p.

The activation of caspase-8 is regulated by cFLIP (22), a catalytically inactive homolog of caspase-8. There are 2 main cFLIP isoforms, arising by alternative splicing: $FLIP_L$ and the C-terminally truncated $FLIP_S$. The latter ($FLIP_S$) binds FADD-associated procaspase-8 and strongly inhibits its activation. The former ($FLIP_L$) may also bind and inhibit FADD-associated procaspase-8. However, it may also bind procaspase-8 not associated with FADD, inducing conformational changes that result in caspase-8 activation in the absence of cleavage. Importantly, procaspase-8 activated via this mechanism does not induce apoptosis but has the potential to prevent necroptosis. Based on these studies and on structural considerations, it has been proposed that the regulation of cell survival by cFLIP may depend on the relative abundance of $FLIP_L$ and $FLIP_S$ and on the ratio of procaspase-8/$FLIP_L$-$FLIP_S$ (27).

Our experiments addressing the regulation of cFLIP revealed that si*TPL2* selectively down-regulates $FLIP_L$ in all of the sensitive lines (HeLa, HCT 116, MDA-MB-436, and MIA PaCa-2) and inhibits its up-regulation by TNFα in some (HeLa, U-2 OS, and MIA PaCa-2) (Figs. 1 *F*, *2* and 2*B* and *SI Appendix*, Fig. S2*B*). The expression of $FLIP_S$ in the sensitive lines was either undetectable (HeLa, MIA PaCa-2, and MDA-MB-436) or decreased, along with the expression of $FLIP_L$ by si*TPL2*, before, after, or both before and after treatment with TNFα (Figs. 1 *F*, *2* and 2*B* and *SI Appendix*, Fig. S2*B*). In the resistant lines expressing $FLIP_S$, si*TPL2* or si*TPL2*/TNFα down-regulated $FLIP_S$ weakly or not at all, and in 1 resistant line (PANC-1), they up-regulated it (*SI Appendix*, Fig. S2*B*). These data suggest that the sensitive cell lines may express only $FLIP_L$, but when they express both isoforms, si*TPL2* and si*TPL2*/TNFα alter the expression of *cFLIP* without altering the splicing; as a result, they do not substantially alter the ratio of the 2 isoforms. Therefore, the activation of procaspase-8 by si*TPL2*/TNFα in the sensitive lines is due to the combination of procaspase-8 up-regulation and cFLIP down-regulation. Combined, the preceding data suggest that both the expression and activity of caspase-8 in the TST-sensitive lines are regulated by TPL2, and that the ability of TPL2 to regulate caspase-8 in a given cell line may determine its sensitivity to the si*TPL2*/TNFα-induced synthetic lethality. RIPK1 and BID are cleaved by the activated caspase-8 (28, 29). Therefore, one would expect that RIPK1 and BID would also be selectively cleaved in the TST-sensitive lines. Probing Western blots of cell lysates from the experiment described above with antibodies against RIPK1 or BID confirmed this prediction (Fig. 2*B* and *SI Appendix*, Fig. S1*D*).

si*TPL2* dramatically inhibits the induction of *cIAP2* by TNFα in all of the TST-sensitive lines, but not in the TST-resistant lines (Fig. 2*B* and *SI Appendix*, Figs. S1*F* and S2*B*). However, since the induction of cIAP2 occurs 1 to 2 h after the start of the TNFα treatment, its inhibition by si*TPL2* may not contribute to the initiation of apoptosis. si*TPL2* also down-regulates MCL1 in all of the TST-sensitive lines (Fig. 2*B* and *SI Appendix*, Figs. S1*D* and S2*B*), as well as in some of the resistant lines (SW480, T-47D, and PANC-1) (Fig. 2*B* and *SI Appendix*, Fig. S2*B*). Therefore, MCL1 down-regulation by si*TPL2* may contribute to, but is not sufficient to induce, synthetic lethality.

Addressing the phosphorylation of TAK1 at Thr184/187 in the TST-sensitive cell line HCT 116 revealed that the phosphorylation was not inhibited but was slightly delayed by si*TPL2*, which is similar to what was observed in HeLa cells. In the TST-resistant lines CACO-2 and SW480, on the other hand, there was no delay in the phosphorylation of these sites. Regarding the phosphorylation of TAK1 at Ser412, there was a tendency for the phosphorylation to be lower in all si*TPL2*/TNFα-treated cells independent of their TST sensitivity. In fact, the most pronounced down-regulation was observed in SW480 cells, a TST-resistant line (*SI Appendix*, Fig. S2 *C*, *1*). Although the preceding data failed to show a clear pattern linking TPL2 and TAK1 regulation in TST-sensitive lines, the combination of TAK1 inhibition and TNFα treatment induced caspase-8 activation and cell death in the TST-sensitive lines, but not in the TST-resistant lines (*SI Appendix*, Fig. S2 *C*, *2*). These findings suggested that although TPL2 and TAK1 may functionally interact, their inhibition may induce synthetic lethality when combined with TNFα treatment via separate but interconnected pathways. To address this question, we treated HeLa cells with si*TPL2*/TNFα, in combination with increasing concentrations of the TAK1 inhibitor (5Z)-7-Oxozeaenol (*SI Appendix*, Fig. S2 *C*, *3*). The first observation we made based on this experiment was that TAK1 inhibition failed to up-regulate caspase-8. Given that caspase-8 up-regulation is one of the hallmarks of the treatment with si*TPL2*, this observation suggested that, indeed, TPL2 and TAK1 may regulate the response to TNFα via distinct pathways. Monitoring the activation of caspase-8 and caspase-3, the cleavage of RIPK1, and the viability of the cells after TNFα treatment supported the hypothesis of the parallel pathways by showing that the effects of si*TPL2* and the TAK1 inhibitor were additive.

Caspase-8 Activation Plays a Central Role in si*TPL2*/TNFα-Induced Synthetic Lethality. The preceding data show that the common theme in all of the TST-sensitive cell lines is the activation of caspase-8, along with the activation of the mitochondrial pathway of apoptosis. To determine the role of caspase-8 expression and activation in the synthetic lethality phenotype, we treated with TNFα HeLa cells transfected with si*TPL2* or siControl and pretreated with the caspase-8 inhibitor Z-IETD-FMK. This revealed that Z-IETD-FMK protects from TNFα-induced apoptosis and that the protection is dose-dependent (Fig. 3*A*). The protection correlated with inhibition of the TNFα-induced cleavage of BID, RIPK1, MCL1, caspase-9, and PARP. Caspase-3 was only partially processed, suggesting that full processing may depend on caspase-8. The si*TPL2*-induced block in the induction of cIAP2 by TNFα was not affected by the inhibitor (Fig. 3*B*).

The results of these experiments confirmed that the synthetic lethality phenotype depends on caspase-8 activation and identified BID, RIPK1, and perhaps MCL1 as potential effectors of caspase-8. BID is a BH3-only protein that is activated by cleavage. To determine whether BID cleavage contributes to apoptosis by si*TPL2*/TNFα, HeLa cells transduced with doxycycline-inducible constructs of wt BID or noncleavable BIDD60E were transfected with si*TPL2* and treated with TNFα or vehicle in the presence or absence of doxycycline. The results (Fig. 3*C*) showed that wt BID enhances apoptosis, while noncleavable BID does not.

RIPK1 kinase activity promotes the assembly of complex II (7, 8), which is required for caspase-8 activation. However, RIPK1 also undergoes ubiquitination by cIAP1 and cIAP2, and the ubiquitinated RIPK1 functions as a scaffold that contributes to the activation of NF-κB, which inhibits the assembly of complex II (6). Inhibition of RIPK1 with Nec1 inhibited si*TPL2*/TNFα-induced apoptosis by about 50% (Fig. 3*D*), suggesting that apoptosis is due to a combination of RIPK1-dependent and RIPK1-independent mechanisms. To further investigate the role of RIPK1, we knocked it out in HeLa cells via CRISPR/Cas9 (*SI Appendix*, Fig. S3 *A*, *1*). Treatment of 6 *RIPK1*$^{-/-}$ clones with si*TPL2* and TNFα revealed that all were TST-sensitive, although their degree of sensitivity varied (*SI Appendix*, Fig. S3 *A*, *2*). More important, their sensitivity correlated with the magnitude

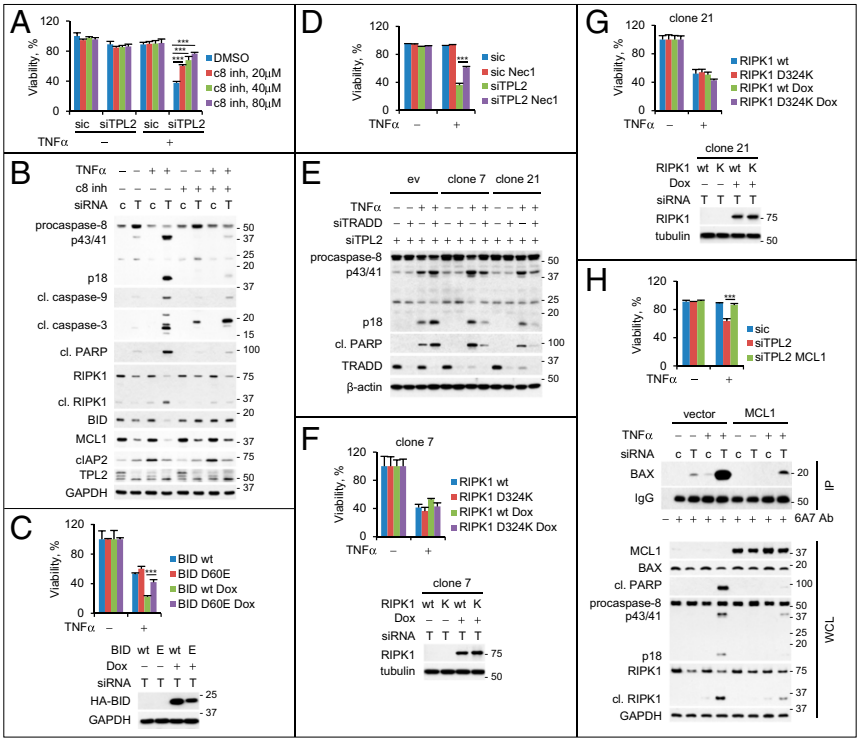

Fig. 3. Caspase-8 activation plays a central role in si*TPL2*/TNFα-induced synthetic lethality. (*A*) HeLa cells were transfected with control or *TPL2* siRNA and treated with the caspase-8 inhibitor Z-IETD-FMK (20 to 80 μM) for 1 h before TNFα treatment. Cell viability was monitored using the CellTiter-Glo assay 24 h later. c8 inh, caspase-8 inhibitor; sic, siControl. (*B*) Expression of the indicated proteins in the cells in *A* was monitored by immunoblotting 6 h after TNFα treatment. Cells were pretreated with 80 μM Z-IETD-FMK. c, control; cl., cleaved; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T, *TPL2*. (*C*) HeLa cells transduced with doxycycline (Dox)-inducible constructs of wt BID or the BIDD60E (E) mutant were transfected with si*TPL2* (T). Following induction of the BID constructs with Dox, the cells were treated with TNFα, and they were harvested 24 h later. (*Upper*) Cell viability was monitored, using the CellTiter-Glo assay. (*Lower*) Expression of wt and mutant BID was monitored by immunoblotting with the anti-hemagglutinin (HA) antibody. (*D*) HeLa cells were pretreated with the RIPK1 inhibitor Nec1 (30 μM) for 30 min before TNFα treatment. Cell viability was monitored by Annexin V/propidium iodide (PI) staining 24 h later. (*E*) Knockdown of *TRADD* protects *RIPK1*$^{-/-}$, but not *RIPK1*$^{+/+}$, HeLa cells from TNFα-induced caspase-8 activation. *RIPK1*$^{-/-}$ HeLa cell clones, clones 7 and 21, and *RIPK1*$^{+/+}$ HeLa cells (ev) were transfected with si*TRADD* or sic in combination with si*TPL2*. The transfected cells were treated with TNFα, and the expression and cleavage of the indicated proteins were monitored by immunoblotting 6 h later. *RIPK1*$^{-/-}$ HeLa cell clones 7 (*F*) and 21 (*G*) were transduced with Dox-inducible constructs of wt RIPK1 or RIPK1 D324K (K). Following treatment with Dox or vehicle, the transfected cells were exposed to TNFα, and their viability was measured 24 h later, using the CellTiter-Glo assay. Dox-induced expression of RIPK1 in these cells was monitored by immunoblotting. (*H*) HeLa cells stably transduced with an MCL1 construct or the ev were transfected with sic or si*TPL2*. (*Upper*) Cells were then treated with TNFα, and their viability was measured 24 h later by Annexin V/PI staining. (*Middle*) BAX activation in cell lysates harvested from similarly treated cells was examined at 6 h after treatment with TNFα by immunoprecipitation (IP) with the 6A7 antibody, which specifically recognizes the active form of BAX. (*Lower*) Expression and cleavage of other proteins involved in apoptosis were monitored in whole-cell lysates (WCL) from the same cells by Western blotting. Graphs show mean ± SD. Experiments in *A*, *C*, *D*, *F*, and *G* were done in triplicate, and they were repeated 3 times. Error bars show the SD within the representative experiment shown in the figure. For *H*, graphs show mean ± SD of 4 repeats. ***$P < 0.001$.

of si*TPL2*/TNFα-induced caspase-8 activation (*SI Appendix*, Fig. S3 *A*, *3*), which is consistent with other observations indicating that caspase-8 is central to this process. Phosphorylation/degradation of Iκ-Bα was impaired in *RIPK1*$^{-/-}$ cells, as expected (*SI Appendix*, Fig. S3 *A*, *4*). These data collectively show that the RIPK1 kinase activity contributes to si*TPL2*/TNFα-induced apoptosis. Given that *RIPK1* ablation did not fully block apoptosis, we hypothesized that si*TPL2*/TNFα treatment induces apoptosis in *RIPK1*$^{-/-}$ cells via TRADD, which can substitute for RIPK1 in the assembly of complex II (12). Support to this hypothesis was provided by experiments showing that whereas the knockdown of *TRADD* in *RIPK1*-expressing cells (empty vector [ev]) promotes si*TPL2*/TNFα-induced caspase-8 activation, it inhibits caspase-8 activation in *RIPK1*$^{-/-}$ clones (Fig. 3*E*).

It has been proposed that the functional interaction between caspase-8 and RIPK1 is bidirectional, with the cleavage of RIPK1 contributing to TNFα-induced caspase-8 activation and cell death (29, 30). To test this proposition, we reconstituted *RIPK1*$^{-/-}$ clones with wt RIPK1 or the uncleavable mutant RIPK1D324K. Testing the effects of the reconstitution revealed that neither construct alters the rate of si*TPL2*/TNFα-induced apoptosis (Fig. 3 *F* and *G*). We conclude that RIPK1 cleavage plays no major role in si*TPL2*/TNFα-induced apoptosis.

Expression of MCL1 from a lentiviral construct in HeLa cells inhibited the activation of BAX and the cleavage of PARP (Fig. 3 *H*, *Lower*) and rescued the synthetic lethality phenotype (Fig. 3 *H*, *Upper*). These data suggest that the down-regulation of MCL1 in si*TPL2*/TNFα-treated cells contributes to the apoptosis phenotype. In addition, MCL1 expression inhibited the cleavage of caspase-8 and its target RIPK1, suggesting that the functional relationship between caspase-8 and MCL1 is bidirectional.

Overexpression of cIAP2 did not affect the cleavage of the caspase-8 targets BID and RIPK1 and failed to rescue the synthetic lethality phenotype (*SI Appendix*, Fig. S3 *B*, *1*). Moreover, the knockdown of *cIAP2* did not induce apoptosis in TNFα-treated cells (*SI Appendix*, Fig. S3 *B*, *2*). Therefore, cIAP2 is dispensable for the synthetic lethality phenotype.

Although the inhibition of si*TPL2*/TNFα-induced BID and caspase-9 cleavage by Z-IETD-FMK indicates that caspase-8 stimulates the mitochondrial pathway of apoptosis, some mitochondrial pathway-dependent events were selectively promoted by caspase-8 inhibition. These include BAX activation and a decrease in the mitochondrial membrane potential, both of which were enhanced by Z-IETD-FMK (*SI Appendix*, Fig. S3 *C*, *1* and *2*). This unexpected observation suggests that caspase-8 protects the cells from si*TPL2*-induced BAX activation. We conclude that TPL2 and caspase-8 converge on a pathway that inhibits BAX and that robust activation of BAX occurs only when the signals transduced by both molecules are blocked (*SI Appendix*, Fig. S3, *C*, *1*).

miR-21 and FLIP$_L$ Inhibit the Activation of Caspase-8 in si*TPL2*/TNFα-Treated Cells and Rescue the Synthetic Lethality Phenotype. Data presented in Fig. 2 and *SI Appendix*, Fig. S2 show that miR-21 is selectively down-regulated by si*TPL2* in TST-sensitive cells and suggest that its down-regulation may be responsible for the up-regulation of caspase-8 and perhaps the synthetic lethality phenotype. Transfection of HeLa cells with pre–miR-21 prevented the induction of procaspase-8 by si*TPL2* and protected them from si*TPL2*/TNFα-induced apoptosis, providing support to this hypothesis (Fig. 4*A*). Parallel experiments in HeLa cells and 2 TST-resistant lines (CACO-2 and SW480) transduced with anti–miR-21 or anti-miR control lentiviral constructs revealed that anti–miR-21 promotes caspase-8 activation and PARP cleavage in both TST-sensitive and -resistant lines (Fig. 4*B*).

Whereas the abundance of procaspase-8 is regulated by miR-21, caspase-8 activity may be regulated by the abundance of both procaspase-8 and FLIP$_L$/FLIP$_S$. Given that FLIP$_L$/FLIP$_S$ is

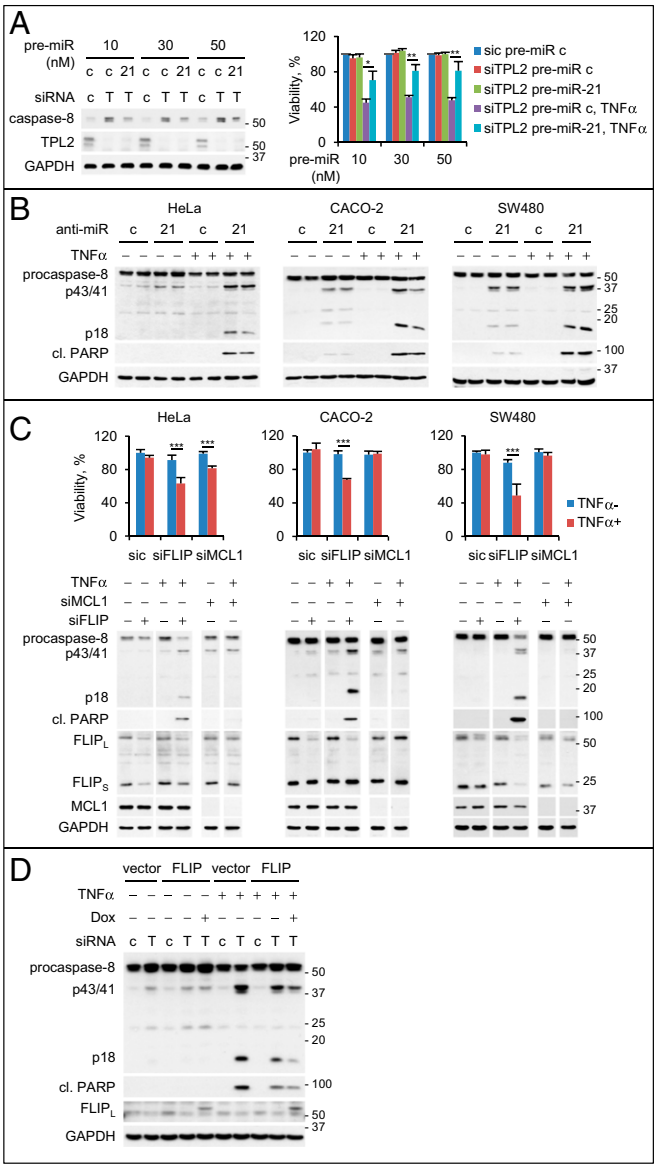

Fig. 4. miR-21 and FLIP$_L$ inhibit the activation of caspase-8 in si*TPL2*/TNFα-treated cells and rescue the synthetic lethality phenotype. (*A*) HeLa cells were transfected with increasing concentrations of the pre-miR precursor of miR-21-5p (10, 30, and 50 nM) or a negative control pre-miR, in combination with si*TPL2* (T) or siControl (c). (*Left*) Expression of caspase-8 in the transfected cells was monitored by immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (*Right*) Transfected cells were treated with TNFα, and their viability was measured 24 h later, using the CellTiter-Glo assay. sic, siControl. (*B*) Cleavage of caspase-8 and PARP was monitored by immunoblotting in HeLa, CACO-2, and SW480 cells expressing anti–miR-21 or anti-miR control (c) and treated with TNFα for 6 h. cl., cleaved. (*C*) HeLa, CACO-2, and SW480 cells were transfected with si*FLIP*, si*MCL1*, or siControl and treated with TNFα. (*Upper*) Cell viability was measured, using the CellTiter-Glo assay 24 h after TNFα treatment. (*Lower*) Expression of the indicated proteins was measured by Western blotting in similarly treated cells 6 h after the initiation of the TNFα treatment. (*D*) HeLa cells were stably transduced with a doxycycline (Dox)-inducible construct of FLIP$_L$ or the ev. The transduced cells were transfected with T or c, and 5.5 h later, they were treated with Dox or vehicle. Forty hours after the initial exposure to Dox, the cells were treated with TNFα. The expression of the indicated proteins was monitored by Western blotting in cell lysates harvested 6 h later. Graphs show mean ± SD. Experiments were done in triplicate, and they were repeated 2 (*C*) or 3 (*A*) times. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

down-regulated by si*TPL2* and/or fails to be induced by TNFα in TST-sensitive cells (Fig. 2*B* and *SI Appendix*, Figs. S1 *F*, *2* and S2*B*), we hypothesized that cFLIP down-regulation contributes to synthetic lethality. Treatment of HeLa cells in which *cFLIP* was knocked down with TNFα induced synthetic lethality (Fig. 4*C*), providing support to the hypothesis. In parallel experiments, HeLa cells transduced with a doxycycline-inducible lentiviral construct of $FLIP_L$ or the ev were transfected with si*TPL2* or siControl and treated with doxycycline or vehicle. Monitoring the cleavage of caspase-8 and PARP after TNFα treatment confirmed the hypothesis by showing that $FLIP_L$ partially rescues the cells from caspase-8 activation (Fig. 4*D*). We conclude that si*TPL2* sensitizes resistant cells to TNFα by deregulating caspase-8 downstream of miR-21, cFLIP, or both.

Whereas the regulation of caspase-8 cleavage clearly distinguishes the TST-sensitive from the TST-resistant lines, the regulation of MCL1 does not (Fig. 2*B* and *SI Appendix*, Fig. S2*B*). In addition, the knockdown of *MCL1* in the TST-resistant lines failed to sensitize them to TNFα-induced cell death (Fig. 4*C*), suggesting that MCL1 down-regulation by si*TPL2* may contribute to, but is not sufficient to induce, apoptosis by TNFα.

The Role of NF-κB in the Regulation of miR-21 and cFLIP by TPL2. TNFα activates NF-κB, which protects from apoptosis by regulating *cFLIP* (4, 5), *cIAP2* (3, 31), and other antiapoptotic genes. In addition, NF-κB activated by hypoxia, along with CREB, is required for the induction of miR-21 (32). Finally, NF-κB activated by TRAIL also induces miR-21 in TRAIL-resistant cell lines (21). Importantly, TPL2 is required for the activation of NF-κB by TNFα (33) and for the induction of cFLIP, cIAP2, and PUMA (34) in the TST-sensitive cell lines (Fig. 2*B* and *SI Appendix*, Figs. S1 *F* and *H* and S2*B*). Based on these observations, we examined whether the down-regulation of cFLIP and miR-21 in si*TPL2*-treated cells is due to the inhibition of NF-κB activation. The results showed that the knockdown of *TPL2* inhibits the activity of the NF-κB reporter in TST-sensitive (HeLa, U-2 OS, and HCT 116) lines, but not in TST-resistant (CACO-2 and SW480) lines (Fig. 5*A*). In addition, it blocks the activation of the NF-κB reporter by TNFα in HeLa cells (Fig. 5*B*). Overall, these findings agree with the delayed degradation of Iκ-Bα, observed selectively in TST-sensitive cells (*SI Appendix*, Fig. S4*A*).

To determine whether the inhibition of NF-κB activation in si*TPL2*-transfected TST-sensitive lines contributes to si*TPL2*/TNFα-induced synthetic lethality, we blocked the activation of NF-κB by transducing both sensitive and resistant lines with a tetracycline-inducible construct of the IKBA superrepressor (SR; IKBAS32A/S36A). Treatment with TNFα, combined with the induction of IKBA SR by doxycycline, induced caspase-8 activation and cell death in TST-sensitive HeLa cells and some of the resistant lines (Fig. 5 *C* and *D*). Therefore, interfering with the TNFα-induced NF-κB activation reproduces the si*TPL2* phenotype. The importance of NF-κB for si*TPL2*/TNFα-induced synthetic lethality was further supported by experiments showing that the coexpression of the NF-κB subunits p50 and p65 protects HeLa and U-2 OS cells from si*TPL2*/TNFα-induced apoptosis (Fig. 5*E*). The ability of the IKBA SR to induce cell death upon TNFα treatment in both sensitive and resistant cell lines suggests that the selective regulation of NF-κB activation by TPL2 in sensitive cells is a major determinant of the synthetic lethality phenotype. However, since IKBA SR renders only some of the resistant lines sensitive to TNFα-induced apoptosis, we conclude that some of the cell lines are intrinsically resistant to NF-κB inhibition.

Transfection of HeLa, U-2 OS, CACO-2, and SW480 cells with luciferase reporter constructs of a 4.6-kB miR-21 promoter (wt or mutant at the NF-κB and/or CREB binding sites) (32) revealed that the activity of the promoter depends on NF-κB selectively in the sensitive lines (*SI Appendix*, Fig. S4*B*). In agreement with this observation, the si*TPL2*-induced down-regulation of miR-21 promoter activity and miR-21 expression in TST-sensitive lines were both rescued by cotransfection of p50 and p65 (Fig. 5 *F* and *G*). Importantly, p50/p65 cotransfection also partially rescued the si*TPL2*/TNFα-induced down-regulation of $FLIP_L$, the activation of caspase-8, and the caspase-induced cleavage of PARP (Fig. 5 *H*–*J*).

A schematic diagram summarizing the functional interactions by which TPL2 protects cells from TNFα-induced apoptosis is presented in *SI Appendix*, Fig. S4*C*.

TST-Sensitive and -Resistant Tumor Cell Lines Are Characterized by Distinctly Different Gene Expression Profiles. The activation of caspase-8 downstream of cFLIP and miR-21 is common to all of the TST-sensitive lines, suggesting the feasibility of identifying biomarkers that distinguish the sensitive from the resistant lines. To address this question, we compared the gene expression profiles of the cell lines analyzed in this report, using the Cancer Cell Line Encyclopedia (CCLE) microarray data. The results revealed that sensitive and resistant lines cluster in 2 distinct groups that differ in the expression of a set of 1,126 genes (Fig. 6*A* and Dataset S1). Comparison of the sensitive and resistant lines for the expression of genes involved in TNFα signaling and apoptosis also showed that the sensitive and resistant lines cluster in distinct groups (*SI Appendix*, Fig. S5 and Dataset S2).

Given that the number of TST-sensitive lines in Fig. 6*A* was small, we continued our search, which led to the identification of 3 more sensitive lines (TCCSUP, BT-549, and 786-O) (Fig. 6*B*). The knockdown of *TPL2* in one of them (786-O) was sufficient to induce the cleavage of caspase-8, suggesting that this cell line may be producing TNFα, a hypothesis that was confirmed by qRT-PCR (Fig. 6*B*). Addition of these lines to the earlier cell line set gave rise to a new empowered set. Comparison of the gene expression profiles of the cell lines in the new set revealed that the sensitive and resistant lines also cluster in distinct groups, which differ in the expression of 1,427 genes (Fig. 6*C*, *SI Appendix*, Fig. S6*A*, and Dataset S3). The genes differentially expressed in sensitive and resistant lines in this set overlap with those differentially expressed in the original set. Functional analyses based on the differentially expressed genes in the empowered set, carried out using the GO_SLIM datasets and the Gene Set Enrichment Analysis (GSEA) platform, identified several functions that distinguish the sensitive from the resistant lines (*SI Appendix*, Table S1). Among the top functions enriched in the TST-sensitive lines were TNFα signaling and cell death (*SI Appendix*, Fig. S6 *B*–*D* and Datasets S4–S6).

One of the reasons we carried out these analyses was to determine the feasibility of a predictive tool that distinguishes TST-sensitive from TST-resistant tumor cell lines. To determine the potential predictive value of the data, we examined the clustering of a set of additional cell lines when compared for the expression of the top 200 genes differentially expressed in the empowered set (*SI Appendix*, Fig. S6*A*). This analysis identified 15 additional cell lines, of which 12 cluster with the sensitive lines and 3 with the resistant lines (Fig. 6*D*). Testing 2 of the putative TST-sensitive lines (A-498 and Caki-1) for the cleavage of caspase-8 upon treatment with si*TPL2* and TNFα revealed that caspase-8 was indeed activated in both (Fig. 6*E*, *Upper*). However, in Caki-1 cells, caspase-8 was activated by si*TPL2* even in the absence of TNFα because Caki-1 cells, similar to 786-O, also produce TNFα (Fig. 6*E*, *Lower*). The preceding data confirmed the feasibility of developing a tool that predicts sensitivity and resistance to the si*TPL2*/TNFα treatment.

Discussion

TNFα is a pleiotropic cytokine which initiates signals that may result in inflammation and/or cell death or cell survival. Here, we provide evidence that TPL2 protects cells from the cytotoxic

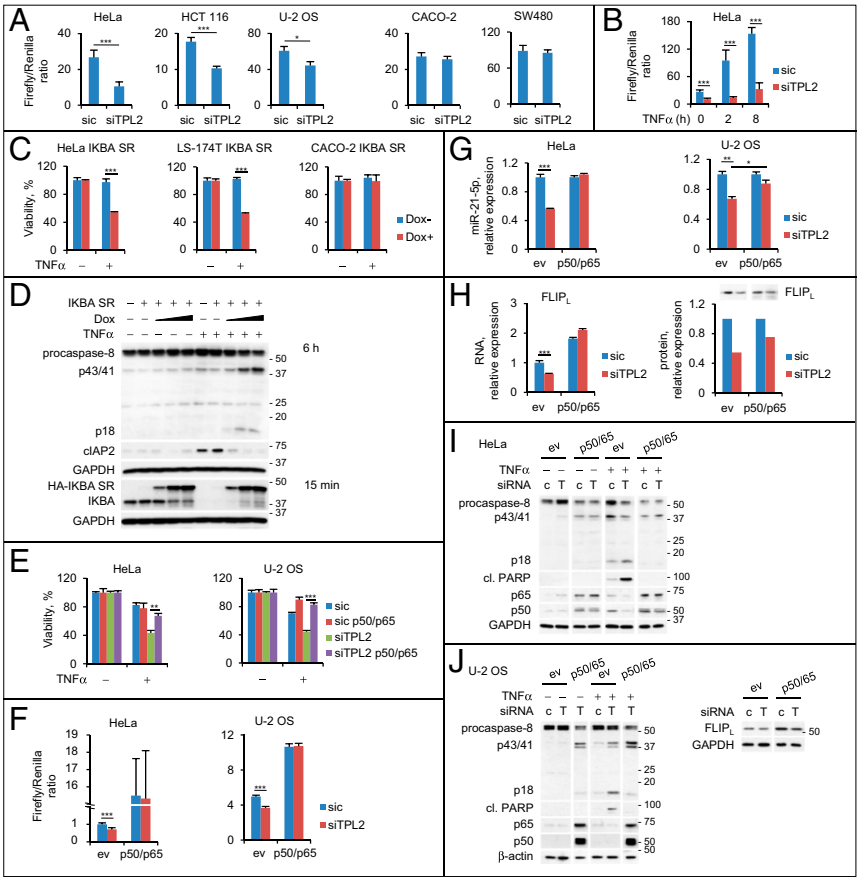

Fig. 5. NF-κB contributes to the regulation of miR-21 and $FLIP_L$ by TPL2. A dual-luciferase reporter assay was used to measure NF-κB reporter activity in multiple TST-sensitive and -resistant cell lines transfected with siControl (sic) or si*TPL2* (*A*) and in similarly treated HeLa cells, before and after (2 and 8 h) treatment with TNFα (*B*). (*C*) Indicated cell lines were transduced with doxycycline (Dox)-inducible constructs of the IKBA SR. After pretreatment with Dox for 16 h, the cells were treated with TNFα or vehicle. Cell viability was measured 24 h later, using the CellTiter-Glo assay. NCI-H1299 and SW480 cells were also sensitive to the combination of IKBA SR and TNFα, while PANC-1 and T-47D were resistant. (*D*) HeLa cells were transduced with a Dox-inducible construct of the IKBA SR or the ev and then pretreated with increasing doses of Dox. Forty hours later, the cells were treated with TNFα or vehicle. The expression of the indicated proteins in cell lysates harvested 15 min and 6 h later was monitored by immunoblotting. Inhibition of NF-κB signaling by the IKBA SR was confirmed by the inhibition of TNFα-induced cIAP2 expression in Dox-treated cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (*E*) HeLa and U-2 OS cells were transiently transfected with p50/p65 and si*TPL2* or sic. The transfected cells were treated with TNFα or vehicle, and their viability was monitored 24 h later, using the CellTiter-Glo assay. (*F*) HeLa and U-2 OS cells were transiently transfected with NF-κB p50/p65 constructs or the ev, in combination with si*TPL2* or sic. The activity of the wt miR-21 promoter was assessed in the transfected cells, using the dual-luciferase reporter assay. (*G*) miR-21 levels were measured by qRT-PCR in HeLa and U-2 OS cells transiently transfected with ev or p50/p65 and si*TPL2* or siControl. (*H*) $FLIP_L$ mRNA and protein levels were measured in HeLa cells transfected with ev or p50/p65 and si*TPL2* or sic. The intensity of the bands was quantified using ImageJ. Expression of indicated proteins was assessed by immunoblotting in HeLa (*I*) and U-2 OS (*J*) cells transfected with ev or p50/p65 and si*TPL2* (T) or siControl (c). cl., cleaved. Cells were treated with TNFα or vehicle, and they were harvested 6 h later. Graphs show mean ± SD. Experiments were done in triplicate, and they were repeated 2 to 3 times. Error bars show the SD within the representative experiment shown in the figure. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

effect of TNFα and contributes to the choice of survival over death. As a result, the combination of TNFα treatment and *TPL2* knockdown induces synthetic lethality in some, but not all, of the cancer cell lines we tested. The protective role of TPL2 against TNFα-induced cell death is due to a block in the activation of caspase-8 by TNFα downstream of miR-21-5p and $FLIP_L/FLIP_S$, which are down-regulated by si*TPL2*. The importance of miR-21-5p and $FLIP_L/FLIP_S$ was confirmed with experiments directly addressing their role in the activation of caspase-8 and the induction of apoptosis by TNFα in both TST-sensitive and TST-resistant lines. The knockdown of *TPL2* also promotes the activation of the mitochondrial pathway of apoptosis by TNFα (*Results* and *SI Appendix*, *SI Discussion*).

Of the 2 proapoptotic pathways activated by si*TPL2*, caspase-8 activation is the dominant one. This was confirmed by pretreating the cells with the caspase-8–specific inhibitor Z-IETD-FMK, which prevented the cleavage of caspase-8 targets and inhibited si*TPL2*/TNFα-induced apoptosis. It was also supported by experiments showing that expression of BIDD60E, a mutant of BID that cannot be cleaved by caspase-8, partially rescues TST-sensitive cells from apoptosis. The activation of the mitochondrial apoptotic pathway plays an auxiliary role in si*TPL2*/TNFα-induced apoptosis, as evidenced by the down-regulation of MCL1 in both TST-sensitive and -resistant si*TPL2*-transfected cells and by the observation that although MCL1 overexpression protects from si*TPL2*/TNFα-induced apoptosis, the *MCL1* knockdown fails to promote TNFα-induced apoptosis.

The activation of caspase-8 in TNFα-treated cells occurs in complex II, which is composed of procaspase-8, FADD, and RIPK1 (7). Experiments here showed that inhibiting the kinase activity of RIPK1 partially blocks apoptosis by si*TPL2*/TNFα and confirmed that apoptosis depends, in part, on RIPK1. However, knocking out *RIPK1* in HeLa cells failed to protect them from apoptosis because caspase-8 was activated via a TRADD-dependent, RIPK1-independent mechanism. Therefore, the knockdown of

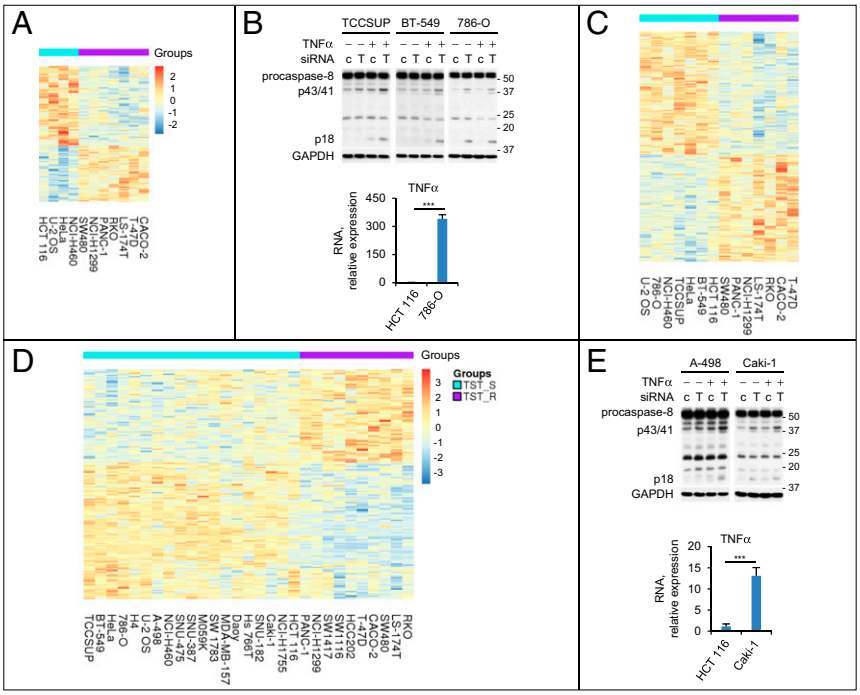

Fig. 6. TST-sensitive and -resistant tumor cell lines are characterized by distinctly different gene expression profiles. (*A*) Differential gene expression analysis of TST-sensitive and -resistant cell lines. Heatmap representation of the expression of 1,126 differentially expressed genes shows that TST-sensitive and -resistant lines cluster in distinct groups. (*B*, *Upper*) Search for additional TST-sensitive cell lines identified 3 more lines in which the combination of si*TPL2* and TNFα promotes the cleavage and activation of caspase-8. c, siControl; GADPH, glyceraldehyde-3-phosphate dehydrogenase; T, si*TPL2*. (*B*, *Lower*) In one of these cell lines (786-O), the knockdown of *TPL2* was sufficient to induce caspase-8 cleavage because these cells express substantial levels of TNFα, as determined by qRT-PCR. (*C*) Differential gene expression analysis of the expanded set of cell lines that includes the additional TST-sensitive and -resistant lines. Clustering of the TST-sensitive and -resistant lines was based on the relative abundance of 1,427 differentially expressed genes. (*D*) Cell lines that had not been tested earlier cluster with either the TST-sensitive or TST-resistant lines, based on the expression of the top 200 differentially expressed genes. (*E*) Clustering of cell lines in *D* identifies additional cell lines that may also be sensitive to the combination of si*TPL2* and TNFα. Two cell lines clustering with the TST-sensitive lines (A-498 and Caki-1) undergo caspase-8 activation when treated with si*TPL2* and TNFα, as expected. In one of them (Caki-1), caspase-8 is also activated by si*TPL2* only because these cells, similar to the 786-O cells, produce TNFα. Graphs show mean ± SD. Experiments were done in triplicate, and they were repeated 2 times. ***$P < 0.001$.

TPL2 promotes caspase-8 activation by TNFα via both RIPK1-dependent and -independent mechanisms.

Our studies on the role of TPL2 in the regulation of caspase-8 and the mitochondrial pathway of apoptosis by TNFα also revealed unexpected functional interactions between the 2 pathways. Treatment of si*TPL2*-transfected, but not siControl-transfected, HeLa cells with Z-IETD-FMK induced BAX activation both in the presence and absence of TNFα. Therefore, caspase-8 inhibition can activate the mitochondrial pathway of apoptosis, but activation is inhibited by TPL2. Alternatively, caspase-8 protects si*TPL2*-transfected cells from activation of the pathway. The molecular mechanism responsible for the protective role of caspase-8 is unknown. Our hypothesis regarding the mechanism of BAX activation by the combined inhibition of both molecules is based on earlier observations showing that caspase-8-(p55)/BID-(full-length) complexes are present in the mitochondria (35) and that full-length BID can induce apoptosis in a cleavage-independent manner (36). Activation of BAX may be due to the release of BID, which occurs only when *TPL2* is knocked down and caspase-8 is inhibited. As stated above, this model will be addressed in future studies. In other experiments, overexpression of MCL1 inhibited the cleavage of caspase-8 and its target, RIPK1, in si*TPL2*/TNFα-treated HeLa cells, suggesting that MCL1 controls a feedback loop that regulates caspase-8 activation by TNFα.

Another antiapoptotic molecule regulated by TPL2 in TNFα-treated cells is cIAP2, a known NF-κB target. *cIAP2* is induced by TNFα, and its induction is TPL2-dependent only in TST-sensitive cell lines. However, it is not affected by Z-IETD-FMK, and it is therefore caspase-8–independent. Its induction, starting at 1 to 2 h from the start of TNFα treatment, suggests that it may contribute to late events but not to the initiation of apoptosis by si*TPL2*/TNFα. This was confirmed by experiments showing that cIAP2 overexpression does not inhibit caspase-8 activation and does not rescue synthetic lethality.

Critically important in understanding the role of TPL2 in si*TPL2*/TNFα-induced synthetic lethality was the comparison of TST-sensitive and -resistant cell lines. This comparison provided evidence that caspase-8 activation was the dominant event giving rise to the vulnerability of certain tumor lines to the combination of si*TPL2* and TNFα, and that caspase-8 activation was due to the deregulation of miR-21-5p and $FLIP_L$/$FLIP_S$. These initial observations were subsequently validated with mechanistic rescue experiments. Inhibition of NF-κB activation contributes to the phenotype and is shared by all of the sensitive cell lines.

The data in this study lead to several conclusions that may have general implications. First, the knockdown of *TPL2* alters the activity of several signaling pathways, such as the MAPK pathways and the pathway leading to the phosphorylation of GSK3β at Ser9, which, although functionally important, are not responsible for the apoptotic response to TNFα. Another conclusion is that although si*TPL2* facilitates the activation of both caspase-8 and the mitochondrial pathway of apoptosis by TNFα, the dominant event is the activation of caspase-8. Finally, comparison of the gene expression profiles of TST-sensitive and -resistant lines revealed that despite the fact that sensitive lines

exhibit significant heterogeneity, they do cluster as a group, distinct from the group of the resistant lines. More important, the clustering of sensitive/resistant lines allowed us to predict the treatment sensitivity of cell lines that had not been tested earlier. These exercises provide proof of principle for the feasibility of generating tools that predict the response to targeted therapies in tumors with significant genetic heterogeneity.

Materials and Methods

Cells and Culture Conditions. HeLa, U-2 OS, HCT 116, CACO-2, RKO, SW480, MIA PaCa-2, MDA-MB-436, NCI-H1299, LS-174T, PANC-1, T-47D, TCCSUP, BT-549, 786-O, A-498, and Caki-1 cells were grown in Dulbecco's modified Eagle's medium, and NCI-H460 cells were grown in RPMI medium supplemented with penicillin/streptomycin, nonessential amino acids, glutamine, and 10% fetal bovine serum. Cells were used within 3 mo of resuscitation. Cell lines were periodically checked for mycoplasma.

Cells were transfected with siRNAs and microRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) and were serum-starved before TNFα or TRAIL stimulation. For lentiviral production, 293T cells were transfected with Lipofectamine 2000 and virus was collected 48 and 72 h after transfection. To generate stable cell lines, cells were selected with puromycin (average of 4 d) or G418 (average of 8 d).

Cell viability was measured using an FITC Annexin V Apoptosis Detection Kit (BD Biosciences) and CellTiter-Glo Luminescent Cell Viability Assay (Promega). Several proteins, such as RIPK1, BID, and p65, have the potential to induce cell death upon overexpression. In experiments in which these proteins were overexpressed, cell viability after TNFα treatment was expressed as the percentage of controls not treated with TNFα.

qRT-PCR and Western Blotting. Total RNA was extracted using a TriPure Isolation Reagent (Roche). RNA was reverse-transcribed using RETROscript (Thermo Fisher Scientific) and/or a miRCURY LNA Universal cDNA Synthesis Kit (Exiqon).

For Western blotting, cells were lysed directly in sample lysis buffer and protein concentration was measured using the RC DC Protein Assay (Bio-Rad). To detect activation of BAX, cells were lysed in 1% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) buffer and BAX was immunoprecipitated with mouse anti-BAX antibody (clone 6A7), which recognizes the active monomeric form of Bax.

Statistical Analysis. Data are expressed as mean ± SD. Error bars represent SD. Statistical significance was determined by one-way ANOVA. For all analyses, significance is shown by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Analysis of Gene Expression Profiling Datasets. High-throughput experiments were from the Gene Expression Omnibus repository and the CCLE database. Analysis was performed on microarray raw CEL files/data generated by Affymetrix. Differential expression analysis was realized at the probe level with the limma package. In all figures and supplementary Excel files, results were filtered with a $\log_2$-fold change cutoff of ±0.6 and P value significance of <0.05. Heatmaps were generated to present differentially expressed genes, along with Excel files describing the results (Datasets S1–S6). Row-wise scaling and Pearson correlation for row clustering were applied to all heatmap representations.

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