Procaspase 8 overexpression in non-small-cell lung cancer promotes apoptosis induced by FLIP silencing

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We found that procaspase 8 was overexpressed in non-small-cell lung cancers (NSCLCs) compared with matched normal tissues. The caspase 8 inhibitor FLICE-inhibitory protein (FLIP) was also overexpressed in the majority of NSCLCs. Silencing FLIP induced caspase 8 activation and apoptosis in NSCLC cell lines, but not in normal lung cell lines. Apoptosis induced by FLIP silencing was mediated by the TRAIL death receptors DR4 and DR5, but was not dependent on ligation of the receptors by TRAIL. Furthermore, the apoptosis induced by FLIP silencing was dependent on the overexpression of procaspase 8 in NSCLC cells. Moreover, in NSCLC cells, but not in normal cells, FLIP silencing induced co-localization of DR5 and ceramide, and disruption of this co-localization abrogated apoptosis. FLIP silencing supra-additively increased TRAIL-induced apoptosis of NSCLC cells; however, normal lung cells were resistant to TRAIL, even when FLIP was silenced. Importantly, FLIP silencing sensitized NSCLC cells but not normal cells to chemotherapy *in vitro*, and silencing FLIP *in vivo* retarded NSCLC xenograft growth and enhanced the anti-tumour effects of cisplatin. Collectively, our results suggest that due to frequent procaspase 8 overexpression, NSCLCs may be particularly sensitive to FLIP-targeted therapies.
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Death receptors such as the TRAIL receptors DR4 and DR5 regulate the extrinsic apoptotic pathway.[1] Classically, activation of death receptors occurs through ligand binding, resulting in recruitment of Fas-associated death domain (FADD), which in turn recruits inactive procaspase 8 (FLICE).[2,3] The resulting complex is termed the death-inducing signaling complex (DISC). At the DISC, procaspase 8 is activated and subsequently activates executioner caspases 3 and 7, resulting in apoptosis. FLICE-inhibitory protein (FLIP) is a caspase 8 inhibitor and is predominantly expressed as long (FLIP$_L$) and short (FLIP$_S$) splice forms.[4] Both FLIP splice forms are recruited to the DISC, preventing the full activation of caspase 8, thereby inhibiting apoptosis.[5] FLIP has been found to be overexpressed in a number of cancers.[6,7] Although FLIP mRNA expression was found to be more highly expressed in squamous non-small-cell lung cancer (NSCLC) tissues compared with normal tissue,[8] no difference in expression of FLIP$_L$ protein was observed between matched tumor and normal tissues.[9]

Non-small-cell lung cancer accounts for ∼80% of lung cancer and is responsible for over one million mortalities worldwide each year.[10] Platinum-based therapies are used in early, locally advanced and metastatic disease; however median survival times are only 8–10 months in the latter setting, and the objective tumor response rate after second line therapy is below 10%. Clearly, new strategies are needed to treat this disease. DR5 has been reported to be over-

expressed in a high percentage of primary and metastatic NSCLC.[11] Unlike many SCLCs, in which the *caspase 8* gene is frequently silenced by methylation, NSCLCs typically retain caspase 8 expression.[12,13] In this report, we show that procaspase 8 and FLIP are frequently overexpressed in NSCLCs and that this preferentially sensitizes NSCLC cells to apoptosis induced by FLIP silencing.

Results

Differential expression of procaspase 8 and FLIP in NSCLC *versus* normal tissue. We analyzed procaspase 8 and FLIP expression in a panel of matched normal and tumor tissues from 20 patients with NSCLC (Figure 1a). Expression of the long form of FLIP (FLIP$_L$) was comparable in tumor and matched normal tissues for most patients, although 5/20 (25%) expressed higher FLIP$_L$ in the tumor tissue (Figures 1a and b). In 13/20 (65%) patients, expression of the short form of FLIP (FLIP$_S$) was significantly higher in the tumor than the matched normal tissues (Figures 1a and b). Most notably, 17/20 (85%) of the tumors had higher procaspase 8 expression in tumor compared with matched normal tissues (Figures 1a and b). There were no obvious correlations between procaspase 8 or FLIP expression and any clinicopathological details (Table 1). FLIP was over-expressed only in tumor tissue that also overexpressed

Figure 1 Expression of death receptor-signaling molecules in non-small-cell lung cancer (NSCLC) tumors and cell lines. (**a**) Western blot showing $FLIP_L$, $FLIP_S$ and procaspase 8 expression in matched NSCLC (T) and normal (N) tissues. Equal loading was assessed by analyzing β-actin expression. (**b**) Densitometry analysis of the relative expression of procaspase 8 in matched tumor and normal tissues. Tumors, which also overexpressed $FLIP_L$ and $FLIP_S$ are indicated by L and S, respectively. (**c**) Western blot showing $FLIP_L$, $FLIP_S$, caspase 8 and Fas-associated death domain (FADD) expression in NSCLC (H460, A549, H157 and H727) and normal lung (34LU and BEAS-2B) cell lines. (**d**) Cell surface expression of DR4 and DR5 in NSCLC and non-cancer lung cell lines. Expression was compared with an isotype control antibody

Table 1 Clinicopathological details of the NSCLC patients

Patient no.	M/F	Adeno/squamous	Age	Stage	Surgery date
1	Male	Squamous	66	IB	23.1.06
2	Male	Adenocarcinoma	73	IB	22.1.05
3	Female	Adenocarcinoma	56	IA	27.9.04
4	Male	Adenocarcinoma	79	IIIB	16.1.06
5[a]	Female	Adenocarcinoma	32	IIIA	7.11.05
6	Female	Squamous	57	IB	30.1.06
7	Male	Squamous	64	IIIB	16.3.06
8	Male	Adenocarcinoma	75	IIB	16.1.06
9	Male	Squamous	49	IB	19.12.05
10	Male	Squamous	68	IIIB	22.04.05
11[a]	Male	Squamous	79	IIIB	7.7.06
12	Female	Adenocarcinoma	54	IB	11.7.06
13	Male	Squamous	62	IB	14.7.06
14	Male	Squamous	73	IIB	20.6.06
15	Male	Adenocarcinoma	81	IA	4.7.06
16	Male	Adenocarcinoma	67	IA	5.5.06
17	Female	Adenocarcinoma	61	IIB	2.5.06
18[a]	Female	Adenocarcinoma	73	IIIA	14.7.06
19	Male	Adenocarcinoma	79	IIIB	9.1.06
20	Female	Adenocarcinoma	71	IIA	13.7.05

Samples from these patients were used to examine FLIP, procaspase 8, DR4 and DR5 expression in matched normal and tumour tissues (Figures 1a and b and Supplementary Figure 1a). [a]Patient nos. 5, 11 and 18 received neo-adjuvant treatment with Erbitux (Bristol-Myers Squibb, Princeton, NJ, USA)

procaspase 8, although not all procaspase 8-overexpressing tumors overexpressed FLIP (Figure 1b). We also examined DR4 and DR5 expression in the patient samples (Supplementary Figure 1a). Of note, DR4 and DR5 were expressed in every tumor sample. Moreover, DR4 and DR5 expression were similar in the majority of matched normal and tumor tissues, although 5/20 patients actually expressed higher levels of DR4 and/or DR5 in their tumors, and one patient (#12) had lower DR4 and DR5 in their tumor.

Procaspase 8 overexpression in NSCLCs suggested that these tumors may be sensitive to caspase 8-mediated apoptosis. To assess this *in vitro*, we used four NSCLC and two non-cancer 'normal' lung cell lines. The H460 large cell lung cancer cell line, the A549 adenocarcinoma cell line, the H157 squamous cell line and the H727 carcinoid cell line all expressed higher levels of procaspase 8 compared with the non-cancer 34LU lung fibroblast and BEAS-2B bronchial epithelial cell lines (Figure 1c). Apart from the H727 cell line,

the NSCLC cell lines expressed higher levels of $FLIP_S$ (Figure 1c). $FLIP_L$ expression was higher in A549 cells compared with the other cell lines, whereas FADD was expressed at similar levels in the panel. Overall, the patterns of FLIP and procaspase 8 expression in the tumor and normal cell line models were similar to those observed in the clinical samples. Of note, *caspase 8* gene expression in the cell line panel did not correlate with procaspase 8 protein expression (Supplementary Figure 1c), indicating that overexpression of procaspase 8 in the NSCLC cell lines is because of post-transcriptional regulation.

The cancer and normal cell line models expressed similar levels of cell surface DR5, with the exception of A549 cells, which expressed significantly higher levels (Figure 1d). More-over, western blot analyses indicated overall that, the relative expression of DR5 in the normal and tumor cell line models was similar to the pattern of expression observed in the majority of matched normal and tumor samples (Supplementary Figure 1a). H460 and A549 cells expressed high levels of DR4 on the cell surface; H157 cells expressed moderate levels and H727 cells expressed low levels. DR4 cell surface expression was barely detectable in the 34LU and BEAS-2B cell lines. By western blot analysis, DR4 was also expressed at a lower level in the BEAS-2B cells compared with the H460 cells (Supplementary Figure 1a). A549 and 34LU cells expressed relatively high levels of Fas, whereas the other cell lines expressed lower levels (Supplementary Figure 1b).

NSCLC cells are more sensitive to FLIP silencing than normal lung cells. The *FLIP* gene silencing induced cell death in the absence of death ligand co-treatment in NSCLC cells (Figure 2a and b). Western blot analyses indicated that FLIP downregulation correlated with increasing PARP cleavage and caspase 8 activation (Figure 2a). Similar results were obtained in H157 and H727 cells (data not shown). To assess the relative importance of the FLIP splice forms in NSCLC, we used $FLIP_L$-specific (FL) and $FLIP_S$-specific (FS) siRNAs. Compared with the dual targeted FLIP siRNA, little or no caspase activation or PARP cleavage was observed in cells that had been transfected with either of the splice form-specific siRNAs (Figure 2c). Despite successful knockdown of FLIP, no PARP cleavage, caspase 8 activation (Figure 2d and Supplementary Figure 1d) or apoptosis (Figure 2b) were observed in BEAS-2B or 34LU cells transfected with FLIP siRNA. Collectively, these results indicate that silencing both main FLIP splice forms induces caspase activation and cell death in NSCLC, but not in the normal lung cells.

Role of death receptors in regulating apoptosis after FLIP silencing. To analyze the roles of the TRAIL and Fas receptors in regulating FLIP siRNA-induced apoptosis, specific siRNAs were used to specifically decrease expression of each receptor (Supplementary Figure 2a and b). Downregulating DR4 or DR5 alone significantly reduced cell
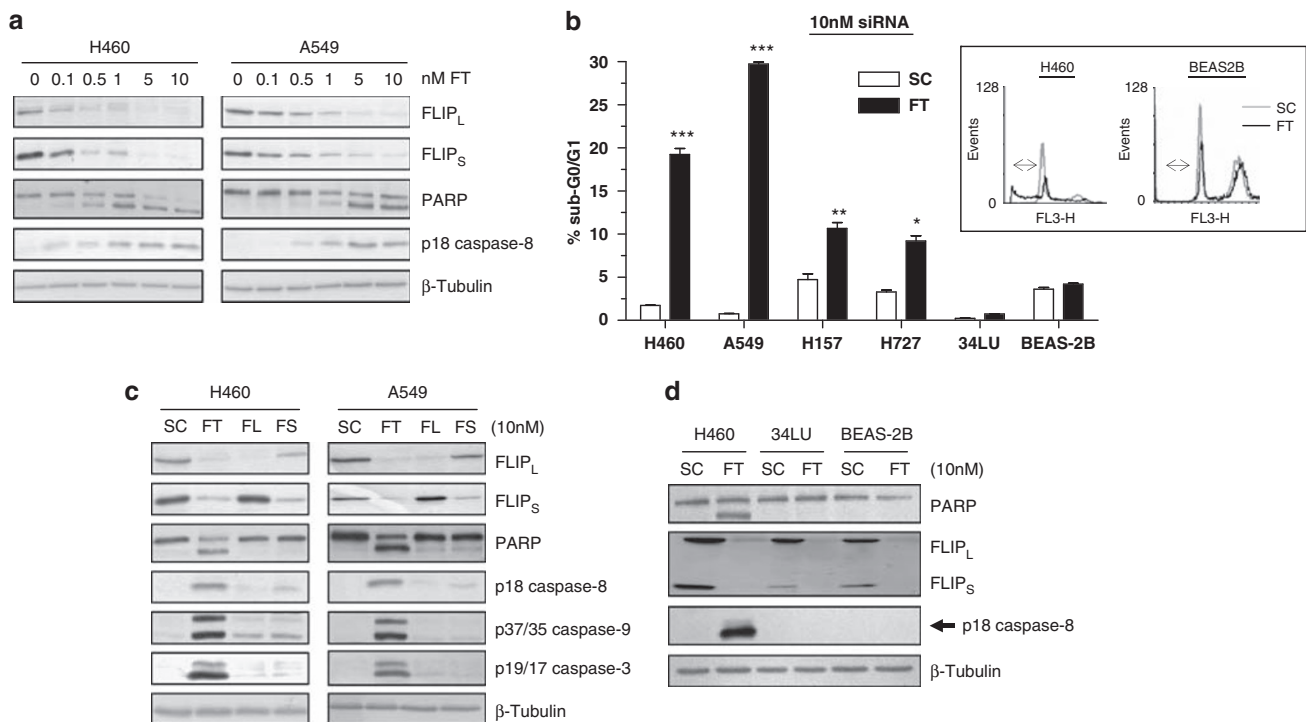
Figure 2 Effect of FLIP silencing on apoptosis. (**a**) $FLIP_L$ and $FLIP_S$ expression, caspase 8 activation (as shown by generation of the active p18-subunit) and PARP cleavage analysis in H460 and A549 cells transfected with FLIP-targeted (FT) siRNA for 24 h. (**b**) Flow cytometry analysis of apoptosis in non-small-cell lung cancer (NSCLC) and normal lung cell lines transfected with 10 nM SC or FT siRNA for 48 h. Apoptosis was assessed by analyzing the sub-G0/G1 fraction of cells. (*** denotes $P < 0.001$, ** denotes $P < 0.01$, * denotes $P < 0.05$; Student's *t*-test). Inset: representative histograms showing the relative effects of FT siRNA on the cell cycle profiles of H460 and BEAS-2B cells. The sub-G0/G1 apoptotic region is indicated by the arrows. (**c**) FLIP expression, caspase 8, 9 and 3 activation and PARP cleavage analysis in H460 and A549 cells transfected with 10 nM non-silencing control (SC), FT, $FLIP_L$-specific (FL) or $FLIP_S$-specific (FS) siRNA for 24 h. (**d**) FLIP expression, caspase 8 activation and PARP cleavage analysis in H460, 34LU and BEAS-2B cells transfected with 10 nM SC or FT siRNA for 24 h
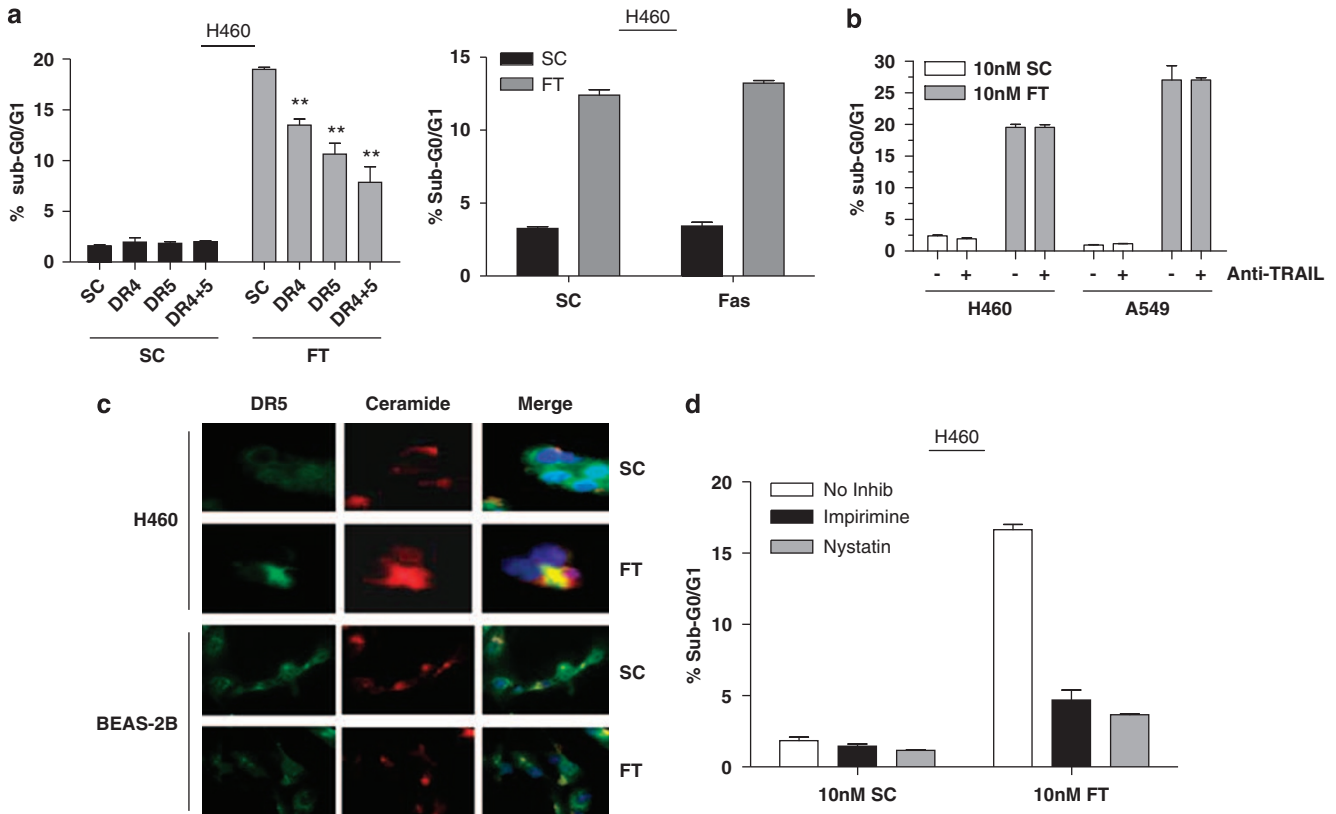
Figure 3 Death receptor involvement in apoptosis induced by FLIP silencing. (**a**) Flow cytometry analysis of apoptosis in H460 cells transfected with 10 nM non-silencing control (SC) or FLIP-targeted (FT) siRNA for 24 h. Cells were pre-transfected for 24 h with 10 nM DR4, DR5, DR4 + DR5, or Fas siRNAs as indicated (Student's *t*-test; *** denotes $P < 0.001$, ** denotes $P < 0.01$). (**b**) Flow cytometry analysis of apoptosis in H460 and A549 cells transfected with 10 nM SC or FT siRNAs for 24 h. Cells were co-treated with a TRAIL-neutralizing antibody (Anti-TRAIL; 100 μg/ml) as indicated. (**c**) Immunostaining of DR5 (green), ceramide (red) and nucleus (blue) in H460 and BEAS-2B cells transfected with 10 nM non-silencing control (SC) or FLIP-targeted (FT) siRNA for 16 h, as analyzed by fluorescent microscopy. (**d**) Flow cytometry analysis of apoptosis in H460 cells transfected with 10 nM non-silencing control (SC) or FLIP-targeted (FT) siRNA for 24 h and co-treated 20 μg/ml Nystatin or 50 μM Imipramine

death after FLIP silencing in H460 cells, and simultaneously downregulating both DR4 and DR5 had a further protective effect (Figure 3a). In contrast, silencing Fas had no effect on FLIP siRNA-induced apoptosis (Figure 3a). Similar results were observed in the A549 cell line (data not shown). Activation of DR4 and DR5 classically occurs after TRAIL binding; however, expression of membrane-bound TRAIL was very low ($\sim 1.5\%$ or less compared to isotype control, data not shown) in the NSCLC cell lines, and neither TRAIL siRNA (data not shown) nor TRAIL-neutralizing antibodies abrogated FLIP silencing-induced cell death (Figure 3b and Supplementary Figure 2c). Collectively, these results suggest that apoptosis induction after FLIP silencing is mediated through DR4 and DR5, but is not dependent on ligation of these receptors by TRAIL.

TRAIL can induce super clustering of TRAIL receptors into ceramide-containing lipid rafts.[14] Furthermore, it was shown that TRAIL-induced generation of ceramide and formation of DR5-containing ceramide-enriched membrane domains is dependent on acid sphingomyelinase (ASM) and is necessary for the induction of TRAIL-induced apoptosis. In H460 cells, FLIP silencing resulted in the formation of intense foci on the cell surface, at which ceramide and

DR5 were co-localized (Figure 3c). In contrast, the patterns of ceramide and DR5 expression were similar in control and FLIP siRNA-transfected BEAS-2B cells, indicating that DR5 and ceramide failed to cluster after FLIP silencing in this normal cell model. These results suggest that silencing FLIP preferentially induces TRAIL receptor clustering in cancer cells compared with normal cells. To examine whether the formation of DR5-containing lipid rafts was necessary for apoptosis induced by FLIP silencing, we used Nystatin, a cholesterol-depleting agent that has been shown to inhibit the redistribution of TRAIL receptors into lipid rafts.[15] We also investigated the role of ASM in FLIP siRNA-induced apoptosis using the ASM inhibitor imipramine. Notably, Nystatin and imipramine both inhibited FLIP siRNA-induced apoptosis in H460 cells (Figure 3d). Similar results were obtained in A549 cells (data not shown). These results suggest that after FLIP silencing in NSCLC cells, ceramide-enriched DR5-containing platforms are formed, and that the formation of these platforms is necessary for the induction of apoptosis. Furthermore, the failure to induce clustering of DR5 into ceramide-enriched lipid rafts may explain the resistance of BEAS-2B cells to apoptosis induced by FLIP siRNA.

Role of procaspase 8 and BID in regulating apoptosis after FLIP silencing. To further determine the mechanism of FLIP silencing-induced apoptosis, we examined the role of procaspase 8 expression. Downregulating procaspase 8 by ~threefold in H460 cells (to levels approaching the constitutive level of expression in BEAS-2Bs) was sufficient to abrogate apoptosis induced by both FLIP silencing (Figure 4a and b) and TRAIL (Supplementary Figure 2d), indicating that the expression level of procaspase 8 is a critical determinant of both FLIP siRNA- and TRAIL-induced apoptosis. In support of this, upregulating procaspase 8 expression by ~threefold in BEAS2B cells using interferon-γ (a known activator of *caspase 8* transcription[8]) sensitized BEAS-2B cells to apoptosis induced by FLIP silencing and TRAIL (Figure 4c). Dependence on caspase 8 for this sensitization was shown by its abolishment in the presence of caspase 8-targeted siRNA.

Cross-talk between the extrinsic and intrinsic apoptotic pathways is mediated by Bid, which, when cleaved by caspase 8, translocates to the mitochondria and promotes the release of pro-apoptotic factors.[16] Silencing Bid completely abrogated FLIP siRNA-induced apoptosis in the A549 cell line, but only partially attenuated apoptosis in the H460 cell line (Figure 4d). These results indicate that the A549 cells must amplify the apoptotic signal through the mitochondria and behave like type II cells after FLIP silencing, whereas the H460 cells behave more like type I cells.[17] Thus, FLIP silencing has the potential to activate apoptosis in both type I and II NSCLC cells.

FLIP siRNA sensitizes NSCLC but not normal lung cells to TRAIL. The H460 cell line was highly sensitive to TRAIL-induced cell death, whereas the A549, H727 and H159 cell lines were less sensitive (Figure 5a). The two normal lung cell lines were completely resistant to TRAIL-induced caspase 8 activation and apoptosis (Figure 5a). FLIP silencing synergistically increased TRAIL-induced apoptosis in all the NSCLC cell lines (Figure 5b); similar results were observed for the Fas agonistic antibody CH-11 (Figure 5c). Interestingly, the 34LU and BEAS-2B cell lines were not sensitized to TRAIL or CH-11 treatment after transfection with FLIP siRNA. Furthermore, TRAIL treatment alone and combined treatment with FLIP siRNA and TRAIL both failed to activate caspase 8 in the BEAS-2B cell line (Figures 5d and Supplementary Figure 1d). Thus, in contrast to the NSCLC cells, FLIP does not seem to be a critical determinant of TRAIL sensitivity in the normal lung cell line models. In addition, we found that TRAIL-induced apoptosis in the presence and absence of FLIP siRNA co-treatment could be equally mediated by either DR4 or DR5, as only simultaneous silencing of both receptors blocked apoptosis (Supplementary Figures 2d and e).
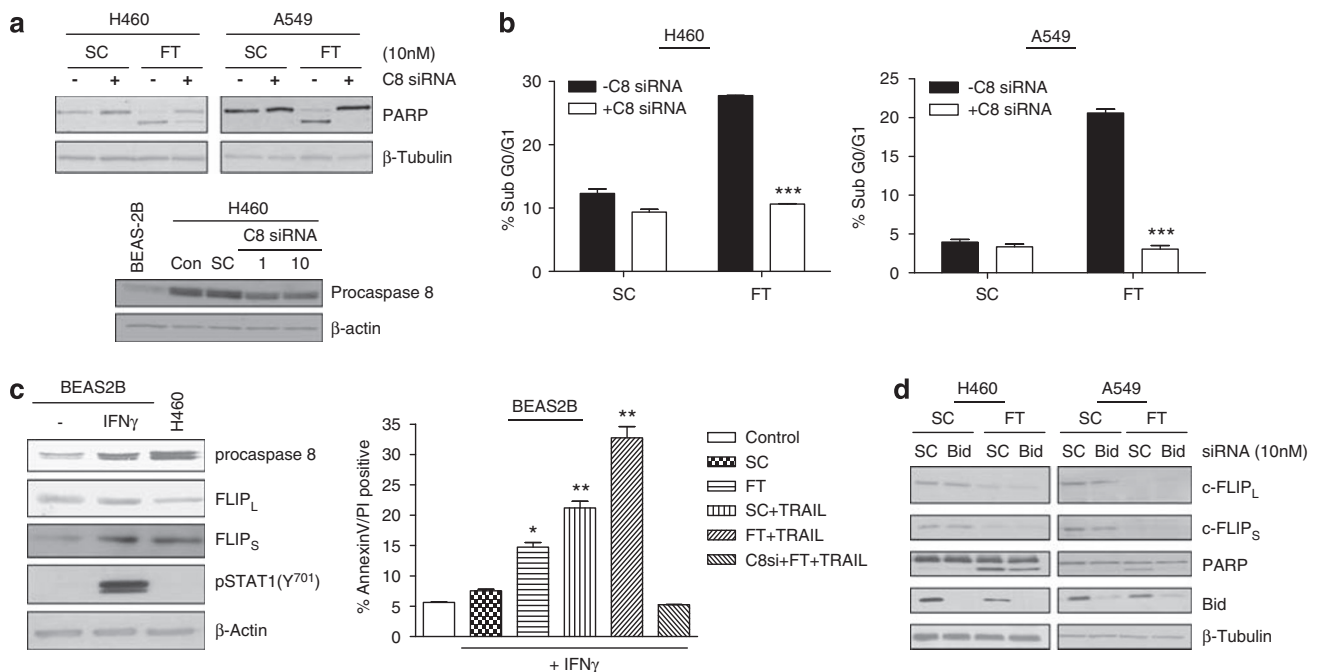
Figure 4 Roles of caspase 8 and BID in apoptosis induced by FLIP silencing. (**a**) Western blot analysis of PARP cleavage in H460 cells transfected with 10 nM SC or FT siRNAs for 24 h. Cells were pre-transfected for 24 h with 10 nM caspase 8 (C8)-targeted siRNA. (lower panel) Western blot analysis of procaspase 8 expression in H460 cells transfected with no siRNA (Con), 1 and 10 nM C8 siRNA and 10 nM control siRNA (SC); expression was compared with baseline expression in BEAS-2B cells. (**b**) Flow cytometry analysis of apoptosis in H460 cells transfected with 10 nM SC or FT siRNAs for 24 h. Cells were pre-transfected for 24 h with 10 nM caspase 8 (C8) targeted siRNA. (Student's *t*-test; *** denotes $P < 0.001$). (**c**) (Western blot) Procaspase 8 expression and STAT-1 activation in BEAS-2B cells treated with 1000 U/ml IFNγ; expression of procaspase 8 was compared with constitutive expression in H460 cells. (Graph) Flow cytometry analysis of apoptosis in BEAS-2B cells transfected with 10 nM SC or FT siRNAs and treated with 1000 U/ml IFNγ and 5 ng/ml TRAIL for 24 h. Cells were pre-transfected with 10 nM C8 siRNA as indicated (Student's *t*-test; * denotes $P < 0.05$ ($P = 0.011$), ** denotes $P < 0.01$). (**d**) Western blot analysis of PARP cleavage, FLIP and BID expression in H460 and A549 cells transfected with 10 nM SC or FT siRNAs for 24 h. Cells were pre-transfected with 10 nM SC or BID siRNAs for 24 h as indicated
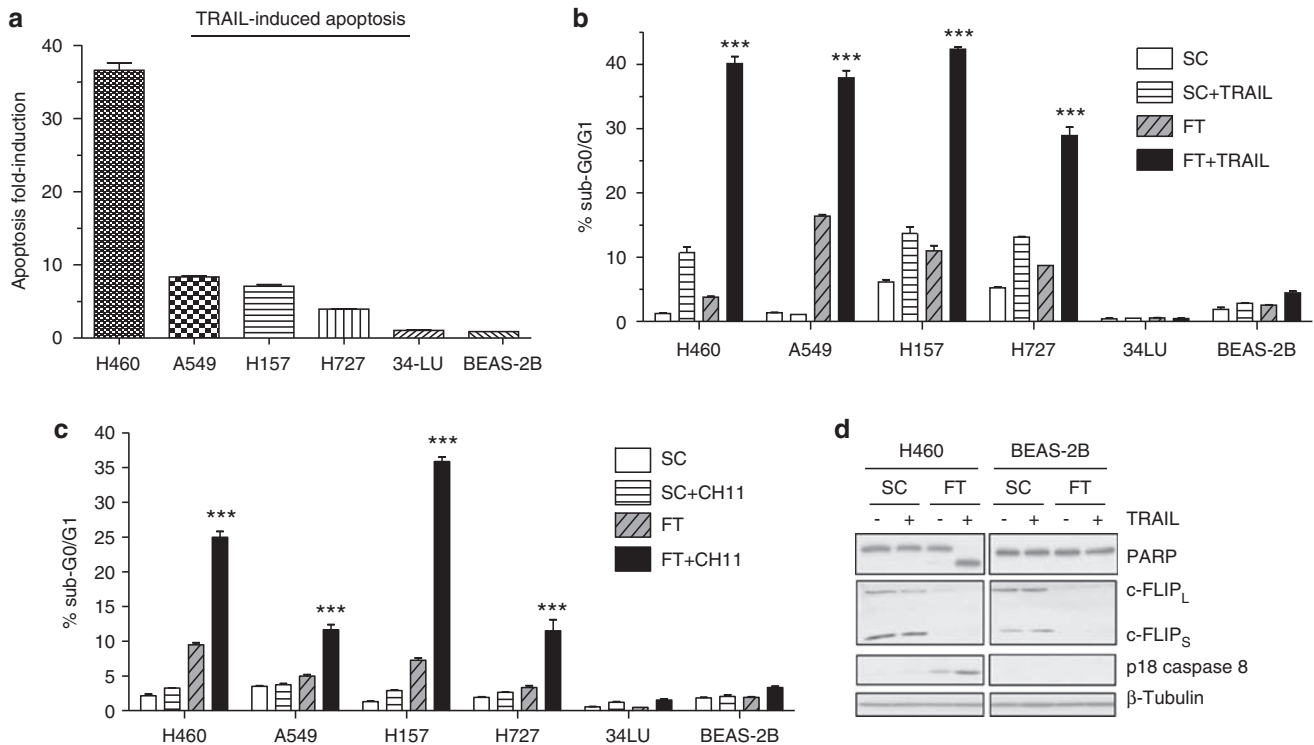
Figure 5 Effect of FLIP silencing on TRAIL and Fas-induced apoptosis *in vitro*. (**a**) Flow cytometry analysis showing the relative induction of apoptosis in non-small-cell lung cancer (NSCLC) and normal lung cell lines after TRAIL treatment (50 ng/ml). Apoptosis was assessed by analyzing the sub-G0/G1 population of cells, and the fold-induction of apoptosis was calculated compared with the control cells. (**b**) and (**c**) Flow cytometry analysis of apoptosis in NSCLC and non-cancer cells transfected with 1 nM non-silencing control (SC) or FLIP-targeted (FT) siRNA for 24 h. Transfected cells were co-treated with 5 ng/ml TRAIL (**b**) or 100 ng/ml of the Fas agonist CH11 (**c**). Apoptosis was assessed by analyzing the sub-G0/G1 fraction of cells (*** denotes $P < 0.001$, two-way ANOVA). (**d**) Western blot analysis showing FLIP expression, PARP cleavage and caspase-8 activation (active p18-subunit) in H460 and BEAS-2B cells transfected with 1 nM SC or FT siRNA and co-treated with 5 ng/ml TRAIL for 24 h. Equal loading was confirmed by analyzing β-tubulin expression

FLIP siRNA sensitizes NSCLC but not normal lung cells to chemotherapy. Notably, FLIP silencing significantly sensitized the four NSCLC cell lines to cisplatin-induced apoptosis (Figure 6a and data not shown). Furthermore, similar to the apoptosis induced by FLIP silencing alone, this supra-additive increase in apoptosis was mediated by DR4 and DR5 (Supplementary Figure 2f). However, FLIP siRNA failed to sensitize the 34LU or BEAS-2B cell lines to cisplatin-induced apoptosis (Figure 6b). Similar results were observed with Taxol (Paclitaxel, Mayne Pharma, Warwickshire, UK; Supplementary Figures 3a and b). We also found that silencing FLIP could overcome drug resistance in a cisplatin-resistant H460 daughter cell line (unpublished observations).

To assess the effects of targeting FLIP *in vivo*, we used an H460 xenograft model in Balb/c nude mice. Xenografts that were injected with FLIP siRNA expressed reduced levels of FLIP$_L$ and FLIP$_S$ compared with SC siRNA-injected xenografts (Figure 6c). Furthermore, caspase 3 activation was observed in FLIP siRNA injected xenografts indicative of enhanced cell death. Importantly, compared with untreated and siRNA controls, xenografts injected with FLIP siRNA grew significantly more slowly (Figure 6c). The *in vivo* effect of FLIP silencing in combination with cisplatin was also examined. Cisplatin treatment retarded xenograft growth to a very similar extent as FLIP siRNA (Figure 6d). Importantly, the combination of FLIP siRNA and cisplatin further inhibited xenograft growth compared with FLIP siRNA alone or cisplatin alone. Of note, there was almost no tumor growth in FLIP siRNA and cisplatin co-treated mice until after the last day of treatment. These results indicate that downregulating FLIP retarded xenograft growth and enhanced the growth inhibitory effect of cisplatin *in vivo*.

Discussion

FLIP protein overexpression has been reported in numerous epithelial cancers.[6,7] Despite overexpression of FLIP mRNA having been reported in squamous NSCLC, no overexpression of FLIP$_L$ protein was noted in NSCLC tissues.[9] In this study, we found that FLIP$_L$ protein expression was overexpressed in 5/20 of NSCLCs compared with normal tissues. Furthermore, FLIP$_S$ was expressed at a higher level in 65% of NSCLCs. Previous studies have shown that unlike SCLCs, NSCLCs express caspase 8 protein.[12,13] We found that 17/20 of NSCLCs actually had higher procaspase 8 expression than matched normal tissues, and the extent of overexpression was > two fold for 12/20 patients. Moreover, all but three of the NSCLCs that overexpressed procaspase 8 by > two fold also overexpressed FLIP. Expression of DR4 and DR5 in the NSCLCs were similar to those in the matched normal tissues for 15/20 patients and were actually higher in NSCLCs in the remaining five patients. Tissue microarrays are currently
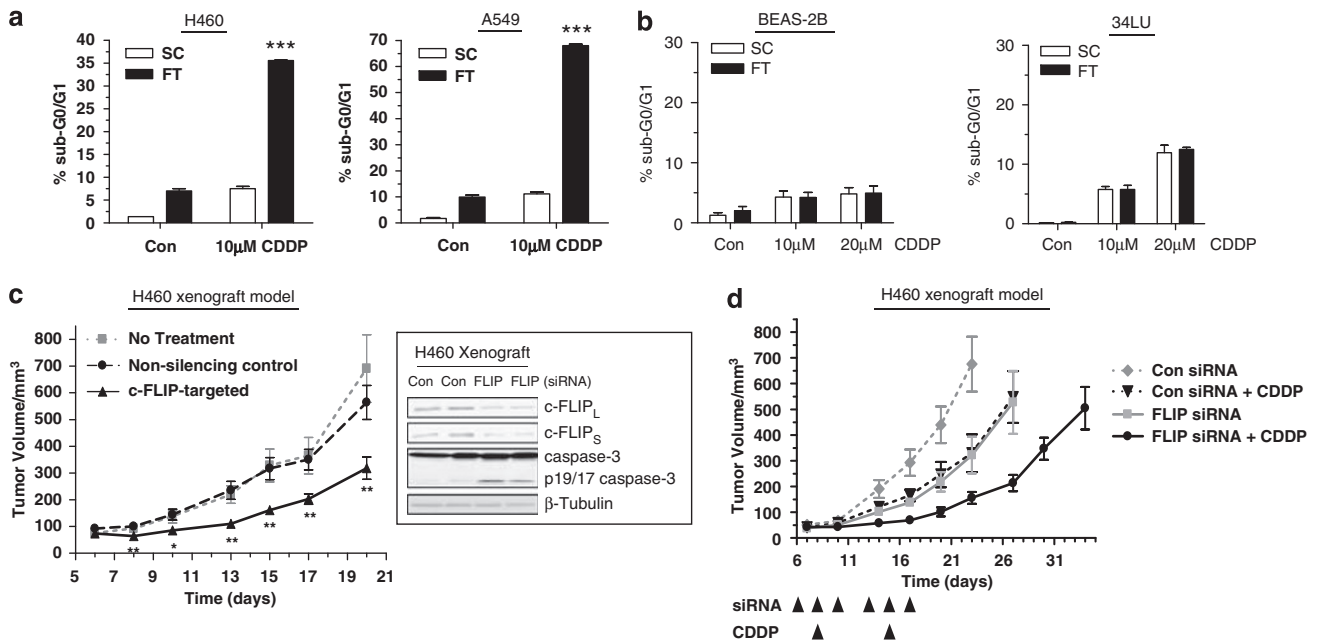
Figure 6 Effect of FLIP silencing on cispatin-induced apoptosis *in vitro* and *in vivo*. (**a**) Flow cytometry analysis of apoptosis in H460 and A549 cells transfected with 1 nM non-silencing control (SC) or FLIP-targeted (FT) siRNAs and treated with cisplatin (CDDP) for 48 h. Combined effects of FLIP siRNA and CDDP were synergistic (*** denotes $P < 0.001$; two-way ANOVA). (**b**) Flow cytometry analysis of apoptosis in BEAS-2B and 34LU cells transfected with 1 nM SC or FT siRNAs and treated with cisplatin (CDDP) for 48 h. (**c**) Growth of H460 xenografts injected with Stealth modified FLIP or control siRNAs. After implantation (day 0), the xenografts were injected with 1000 pmol siRNA on days 1, 3, 6, 8, 10, 13, 15 and 17. A no treatment group was included. (* denotes $P < 0.05$; **denotes $P < 0.01$; Student's *t*-test). Western blot analysis of FLIP expression and caspase 3 activation in H460 xenografts injected with 1000 pmol control (Con) or FLIP siRNAs for 24 h. (**d**) Growth of H460 xenografts injected with FLIP or control siRNAs. After tumour establishment (day 6), the xenografts were injected with 1000 pmol siRNA thrice a week for 2 weeks. Mice were treated with cisplatin (CDDP; 5 mg/kg; IP) once a week for 2 weeks as indicated

being constructed to assess caspase 8, FLIP, DR4 and DR5 expression and prognostic relevance in a larger cohort of NSCLC patients by immunohistochemistry. However, our initial western blot analyses prompted us to examine the roles of caspase 8 and FLIP in regulating apoptosis in NSCLC.

We have previously shown that *FLIP* gene silencing induced caspase 8 activation and apoptosis in colorectal cancer cells and that FLIP$_L$ was the more important splice form for this phenotype.[18] In the current study, we found that silencing both FLIP$_L$ and FLIP$_S$ was required for maximal caspase 8, 9 and 3 activation and apoptosis in NSCLC cells. Splice form-specific silencing had only a modest effect on apoptosis in NSCLC cells, suggesting that one splice form can efficiently compensate for loss of the other. Importantly, *FLIP* gene silencing failed to induce caspase 8 activation and apoptosis in normal lung cell line models. To our knowledge, these are the first studies to suggest that FLIP inhibition preferentially induces apoptosis in cancer cells compared with normal cells.

In agreement with previous studies,[19] we found that TRAIL treatment induced apoptosis in NSCLC cell lines (albeit to varying degrees), but not in normal lung models. Notably, we found that FLIP silencing potently sensitized each NSCLC cell line to TRAIL, but failed to sensitize the normal lung cells. This suggests that although FLIP clearly plays a key role in regulating the sensitivity of NSCLC cells to TRAIL, it may be less important in normal lung cells. These findings have clinical relevance, as a number of TRAIL receptor-targeted therapeutics are in early phase clinical development.[20]

Furthermore, Schimmer *et al.*[21] recently identified a small-molecule inhibitor of FLIP. Our observations suggest that targeting FLIP may preferentially sensitize NSCLC cells to TRAIL receptor-targeted therapeutics.

Downregulating FLIP has been shown to sensitize colon, ovarian and cervical cancer cells to chemotherapy-induced cell death.[22–24] We found that silencing FLIP sensitized NSCLC cells to cisplatin- and taxol-induced apoptosis, but failed to sensitize the normal lung cells. Thus, targeting FLIP not only preferentially sensitizes NSCLC cells to death receptor-targeted agents, but also to chemotherapeutic agents commonly used to treat this disease. Importantly, we were able to show that silencing FLIP *in vivo* retarded NSCLC xenograft growth. Moreover, we showed for the first time that targeting FLIP *in vivo* can significantly enhance the anti-tumour effects of chemotherapy. Collectively, these results strongly suggest that FLIP is a key regulator of chemoresistance in NSCLC. Of note, FLIP silencing was able to induce apoptosis in both type I (H460) and type II (A549) NSCLC cells. Moreover, we found that FLIP silencing sensitized both type I and type II NSCLC cells to TRAIL- and chemotherapy-induced apoptosis. Thus, targeting FLIP either alone or in combination with chemotherapy or TRAIL may be a viable therapeutic strategy, regardless of whether the apoptotic signal requires amplification through the intrinsic pathway.

It has been reported that even when both DR4 and DR5 are expressed, only one may function to induce apoptosis.[20] Using receptor-specific siRNAs, we found that induction of apoptosis after FLIP silencing was mediated by DR4 and

DR5, but not Fas. Consistent with this, the cell lines, which expressed the highest total levels of DR4 and DR5 were the most sensitive to FLIP siRNA-induced apoptosis. Although the two normal lung cell lines expressed low levels of DR4, it should be noted that FLIP siRNA-induced apoptosis was only partially blocked in NSCLC cells in which DR4 was downregulated, indicating that FLIP silencing can induce apoptosis in cells with low DR4 expression as long as DR5 is expressed. Therefore, lack of DR4 expression cannot explain the complete resistance of the normal lung cell lines to FLIP silencing. Of note, we found that blocking binding of TRAIL to its receptors failed to inhibit FLIP siRNA-induced apoptosis in NSCLC cells, indicating that downregulating FLIP triggers ligand-independent activation of DR4- and DR5-mediated apoptosis. The synergistic interactions between FLIP siRNA and both TRAIL and cisplatin were also found to be mediated through both DR4 and DR5.

Several studies have indicated that after ligation, death receptors cluster into lipid rafts, which are rich in cholesterol and sphingolipids.[25] Recently, Dumitru and Gulbins[14] showed that TRAIL treatment resulted in generation of ceramide at the cell membrane, which clustered with DR5 in a range of cancer cell lines. We found that FLIP silencing (in the absence of TRAIL co-treatment) induced clustering of DR5 in ceramide-enriched membrane platforms in NSCLC cells, but not normal lung cells. Furthermore, inhibiting co-localization of DR5 and ceramide or blocking ceramide production abrogated FLIP siRNA-induced apoptosis in NSCLC cells, suggesting that this co-localization is required to induce cell death. Caspase 8 activity has been found to be critical for ASM-regulated, ceramide-mediated FasL-induced Fas clustering and cell death.[25] We found that only a relatively modest ∼threefold downregulation of procaspase 8 was necessary to block FLIP siRNA-induced apoptosis. Moreover, a ∼threefold upregulation of procaspase 8 in BEAS-2B cells was sufficient to sensitize these otherwise resistant cells to FLIP siRNA and TRAIL. Thus, relatively small changes in the levels of procaspase 8 expression seem to profoundly affect sensitivity to FLIP siRNA and TRAIL, which may explain why the normal lung cell line models, which have lower procaspase 8 expression, are less sensitive to FLIP silencing or TRAIL. This finding may have important clinical relevance given the high percentage of NSCLC tumors that we observed to overexpress procaspase 8.

Numerous determinants of TRAIL sensitivity have been identified, including expression levels of FLIP, XIAP, Bcl-2 family members, TRAIL decoy receptors as well as TRAIL receptor glycosylation.[26–33] In addition, reduced or absent caspase 8 expression has been shown to result in lack of sensitivity to TRAIL-induced apoptosis in a restricted number of cancers, including neuroblastoma and small-cell lung cancer.[12,34] However, caspase 8 expression is retained in most cancers and a polymorphism in the *caspase 8* promoter that decreases caspase 8 expression has been associated with reduced susceptibility to multiple cancers, including lung cancer.[35] In this study, we found that procaspase 8 was actually overexpressed in a panel of 20 NSCLCs. The apparent selection for procaspase 8 overexpression in NSCLC is intriguing and suggests that in the presence of FLIP, procaspase 8 may activate proliferative or pro-survival

signaling pathways, for example through activation of NFκB.[36] Although the NSCLC cells overexpressed procaspase 8, we found that constitutive caspase 8 activity was similar in NSCLC and normal lung cells. However, when FLIP was silenced, caspase 8 was activated and apoptosis was induced in the NSCLC cells, but not in the normal lung cells. Collectively, our results suggest that FLIP actively prevents overexpressed procaspase 8 being activated by DR4 and DR5 in NSCLC cells, both constitutively and in the context of chemotherapy/TRAIL treatment. Thus, targeting FLIP may preferentially sensitize NSCLC cells to chemotherapy and TRAIL and may therefore represent a promising therapeutic strategy for NSCLC. Furthermore, tumor procaspase 8 expression may be a useful biomarker for the effectiveness of such strategies.

Materials and Methods

Reagents. Recombinant human TRAIL was purchased from Calbiochem (Gibbstown, NJ, USA), reconstituted in filter-sterilized PBS/0.1% bovine serum albumin (BSA) as a 20 ng/ml stock solution and stored at -70°C. Anti-TRAIL neutralizing antibody was obtained from R&D Systems (Minneapolis, MN, USA). Nystatin was obtained from Sigma, Dorset, UK. Interferon gamma (IFN-γ) was purchased from Calbiochem.

Cell culture. All the cell lines were maintained in 5% CO$_2$ at 37°C. H460, H157 and H727 cells were maintained in DMEM supplemented with 10% bovine calf serum, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine and 50 μg/ml penicillin/streptomycin (all from Invitrogen, Paisley, UK). A549 cells were maintained in DMEM supplemented with 10% bovine calf serum, 2 mmol/l L-glutamine and 50 μg/ml penicillin/streptomycin. 34LU cells were maintained in DMEM supplemented with 10% bovine calf serum, 2 mmol/l L-glutamine, 100 μmol/l non-essential amino acids and 50 μg/ml penicillin/streptomycin. BEAS-2B cells were maintained in F12 Ham Medium (Invitrogen) supplemented with 10% bovine calf serum, 2 mmol/l L-glutamine, 100 μmol/l non-essential amino acids (Invitrogen) and 50 μg/ml penicillin/streptomycin.

Western blotting. Western Blots were carried out as previously described.[18] FLIP (NF6; Alexis, San Diego, CA, USA), Caspase 8 (12F5; Alexis) and Poly-ADP-Ribose-Polymerase-1 (PARP; eBioscience, San Diego, CA, USA) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham, Buckinghamshire, UK). Caspase 9, caspase 3 and phospho-STAT1 (Tyr701) (Cell Signalling, Beverly, MA, USA) and DR4 (Calbiochem) rabbit polyclonal antibodies were used in conjunction with a horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Amersham). DR5 (N-19) goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used in conjunction with a horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma) or β-actin mouse monoclonal primary antibody (Sigma). For xenograft samples, FLIP was detected using the FLIP (Dave-2; Alexis) rat monoclonal antibody in conjunction with a horseradish peroxidase-conjugated goat anti-rat secondary antibody (Amersham).

Patient samples. The NSCLC patients were all part of an EU Biomarkers Study, and patient consent was received before the surgery. Patients' details are given in Table 1. The patients' tumors were resected at St. James' Hospital, Dublin. Immediately after resection, a consultant pathologist identified the normal and tumor tissues and cut a piece of each. The tissues were then immediately snap frozen in liquid nitrogen. Proteins were extracted using standard protein extraction procedures and western blot analysis carried out as described above.

Flow cytometry. Apoptosis was determined using propidium iodide (PI) staining to evaluate the percentage of cells with DNA content <2N as previously described.[18] For annexin V/PI analysis, cells were harvested and analyzed according to manufacturer's instructions (BD Biosciences, Oxford, UK). Cell surface expression of death receptors was performed as previously described.[18]

siRNA transfections. siRNA duplexes were designed to downregulate both splice variants of FLIP (FT), or to specifically downregulate either the long form (FL) or the short from (FS) of FLIP as previously described.[18] The non-silencing control (SC) siRNA, caspase 8-targeted (C8), Fas-targeted and DR5-targeted siRNAs were the same as those previously described.[18] The DR4-targeting siRNA sequence used was CAAACUUCAUGAUCAAUCAdTdT. siRNAs for *in vitro* transfection were obtained from Dharmacon (Chicago, IL, USA). Modified Stealth siRNAs based on the FT and SC sequences were obtained from Invitrogen. siRNA transfections were carried out on sub-confluent cells using OligofectAMINE reagent (Invitrogen) in the H460, A549, H157, 34LU and BEAS-2B cell lines, or using LipofectAMINE 2000 reagent (Invitrogen) in the H727 cell line, according to manufacturer's instructions.

Quantitative PCR. Total RNA was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, UK) according to the manufacturer's instructions. Reverse transcription of 2 μg of RNA was carried out using a Moloney murine leukemia virus-based reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantitative reverse transcription-PCR amplification was performed in a final volume of 10 μl containing 5 μl of 2 × SYBR green master mix (Qiagen, Crawley, UK), 4 μl of primers (2 μM) and 1 μl of cDNA using an Opticon DNA Engine Thermal Cycler (Bio-Rad Laboratories, Inc., Waltham, MA, USA). The caspase 8 primers used were: forward: CTGCTGGGGATGGCCACTGTG; reverse: TCGCCTCGAGGACACGCTCTC. The 18S rRNA primers used were: forward: CATTCGTATTGCGCCGCTA; reverse: CGACGGTATCTGATCGTCT. Reaction conditions were: activation at 95°C for 10 min; denaturation at 95°C for 15 s; annealing at 58.6°C for 30 s and extension at 72°C for 1 min. All PCR amplifications were carried out for 40 cycles. Melt curve analysis was used to examine the specificity of an amplified product. Standard curves were generated to quantify the absolute expression levels of each target gene.

Caspase 8 activity assay. The caspase-GLO® 8 assay system was used according to the manufacturer's instructions Promega (Southampton, UK).

Animal model experiments. Female BALB/c nude mice were maintained under sterile and controlled environmental conditions (22°C, 50 ± 10% relative humidity, 12-h light/12-h dark cycle and autoclaved bedding), with food and water *ad libitum*. After a minimum of 1 week of quarantine, mice were included in the protocols. All the experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986 under a Project Licence (PPL 2590b) approved by the Department of Health, Social Services and Public Safety, Northern Ireland. To calculate tumor measurement, two axes of the tumors were measured using digital Vernier calipers. Tumor volumes were determined by the following formula: shortest tumor diameter^2 × longest tumor diameter × 0.5. For analysis of the effects of FLIP siRNA on xenograft growth, mice were implanted sub-cutaneously on each flank with 2 × 10^6 H460 cells using Matrigel. The next day, 1000 pmol of Stealth modified (Invitrogen) non-silencing control (Con) or FLIP-targeted (FLIP) siRNA were injected intratumorally using a 29-gauge insulin syringe (Kendall, Tyco Healthcare, Princeton, NJ, USA). The siRNAs were complexed with Lipofectamine 2000 using Opti-MEM medium. siRNAs were injected on days 1, 3, 6, 8, 10, 13, 15 and 17. To analyze the effects of FLIP siRNA in combination with cisplatin, 2 × 10^6 H460 cells were implanted on each flank using Matrigel and the tumors were allowed to reach a volume of approximately 50 mm^3 (∼Day 6). After xenograft establishment, tumors were directly injected with 1000 pmol Con or FLIP siRNAs thrice weekly, with half the mice receiving cisplatin (5 mg/kg, intraperitoneal) once a week, for 2 weeks as indicated. Harvested tumors were snap frozen in liquid nitrogen. To extract protein, xenografts were homogenized in radio-immunoprecipitation assay buffer on ice.

Immunostaining of DR5 and ceramide. After treatment, adherent cells were washed with PBS and fixed for 10 min with 4% paraformaldehyde (Sigma). After three washes with PBS, cells were blocked for 60 min with PBS/3% BSA. Cells were then washed thrice with PBS/0.2% BSA and incubated with rabbit anti-DR5 (Calbiochem) and mouse anti-ceramide (MID15B4; Alexis) antibodies, diluted 1 : 100 overnight at 4°C. After three washes in PBS/0.1% triton-X (Roche Applied Science, Mannheim, Germany), cells were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgM and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) antibodies, diluted 1 : 1000 for 2 h at room temperature. After five washes with PBS/0.1% triton-X, slides were mounted using Vectashield containing Dapi (Vector Laboratories, Burlingame, CA, USA).

Images were obtained using a Zeiss Axiovert 200M inverted microscope (Thornwood, NY, USA) using a × 63 oil-immersion objective.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)