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E2F1 induces apoptosis and sensitizes human lung adenocarcinoma cells to death-receptor-mediated apoptosis through specific downregulation of c-FLIP_{short}

Running title: E2F1 targets c-FLIP_S to induce apoptosis.

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Abstract

E2F1 is a transcription factor which plays a well-documented role during S phase progression and apoptosis. We had previously postulated that the low level of E2F1 in primary lung adenocarcinoma contributes to their carcinogenesis. Here, we show that E2F1 triggers apoptosis in various lung adenocarcinoma cell lines by a mechanism involving the specific downregulation of the c-FLIP_S protein, leading to caspase-8 activation at the Death Inducing Signaling Complex. Importantly, we also provide evidence that E2F1 sensitizes tumor as well as primary cells to apoptosis mediated by FAS or TRAIL ligands, and enhances the cytotoxic effect of T lymphocytes against tumor cells. Finally, we describe the specific overexpression of c-FLIP_S in human lung adenocarcinomas with low level of E2F1. Overall, our data identify E2F1 as a critical determinant of the cellular response to death receptor-mediated apoptosis, and suggest that its downregulation contributes to the immune escape of lung adenocarcinoma tumor cells.

Keywords: Apoptosis, Death Receptor, E2F1, c-FLIP, Lung tumors.

Abbreviations: APAF-1, Apoptosis Protease-Activating Factor 1; FADD, Fas-Associated Death Domain-containing protein; FasL, Fas Ligand; FLICE, Fas-associated death domain-Like Interleukin 1 β -Converting Enzyme; DISC, Death-Inducing Signaling Complex; c-FLIP, FLICE Inhibitory Protein; RB, Retinoblastoma; TRAIL, Tumor necrosis Factor-Related Apoptosis-Inducing Ligand.

Introduction

The E2F transcription factors family plays a major role in regulating a diverse array of cellular functions including proliferation, differentiation and apoptosis (1). So far, seven distinct E2F proteins (E2F1 to 7) with different affinities for the pRB pocket family have been identified (2, 3). The interaction of E2Fs with the pRB members tightly controls their transcriptional as well as their repressive functions on target-genes mostly involved in G₁ to S phase transition (4). If individual E2F proteins demonstrate overlapping functions in the control of cell cycle progression, they also display distinct specificities (5). Actually, only E2F1, E2F2 and E2F3 are able to induce apoptosis in fibroblasts (5-9). In addition, E2F1-deficient mice exhibit a defect in thymocyte apoptosis as well as in negative selection demonstrating the role of E2F1 during the physiological apoptotic processes (10, 11). Importantly, as these mice also develop tumors (10, 12), it has been proposed that E2F1-mediated apoptosis could contribute to its tumor suppressive function. E2F1-induced apoptosis occurs via both p53-dependent and -independent pathways, and involves the transcriptional activation of numerous pro-apoptotic E2F1-target genes including *p14^{ARF}/p19^{ARF}*, *p73*, *apaf1* and *caspases* (8, 13, 14). Moreover, alternative models suggest that E2F1 also inhibits anti-apoptotic signals such as NF- κ B, or sensitizes cells to pro-apoptotic stimuli (for review, see (15)). Recently, a link between E2F1 and the extrinsic apoptotic pathway also called death receptor-related apoptosis has been established in murine primary T cells (16) and post-mitotic neurons (17).

FAS (also CD95, APO-1) is a 45-kDa cell surface protein which belongs to the tumor necrosis factor (TNF) / nerve growth factor (NGF) receptors family (18). Following stimulation by its cognate ligand (FasL) or by agonistic FAS antibodies, FAS oligomerizes and recruits several death-associated molecules, including the FADD adaptator (Fas-

Associated Death Domain-containing protein, also MORT1) (19, 20) and the caspase-8 (also FLICE /MACH/Mch5) (21-23), into a multimolecular signaling platform named the Death-Inducing Signaling Complex (DISC; (24)). Further activation of caspase-8 by autoproteolytic cleavage at the DISC level initiates the apoptotic signaling cascade (25, 26) which can either directly activate effector caspases or trigger the mitochondrial apoptosis loop amplification(27). FAS-induced cell death is tightly controlled by proximal regulators, among which is c-FLIP (FLICE Inhibitory Protein), a procaspase-8-like protease deficient protein (28-34). c-FLIP is mainly expressed as a long (c-FLIP_L) and a short (c-FLIP_S) spliced isoforms, which can be both recruited to the DISC. Similar to caspase-8, c-FLIP_L consists of two amino-terminal death effector domains (DED) followed by an unfunctional caspase homology domain (35). To date, the role of c-FLIP_L in apoptosis remains controversial. Indeed, pro-apoptotic, anti-apoptotic and proliferative functions have been reported (35-39). In contrast, only anti-apoptotic functions have been so far ascribed to c-FLIP_S (35).

We had previously shown that E2F1 expression is faintly detectable in human lung adenocarcinomas (40). As E2F1-deficient mice develop pulmonary adenocarcinoma with a high incidence (10), we had postulated that low levels of E2F1 contribute to the carcinogenesis of human lung adenocarcinomas by creating a defective response of tumor cells to apoptotic stimuli. Consistent with such hypothesis, we demonstrate that E2F1 induces apoptosis in various human lung adenocarcinoma cell lines by a mechanism involving a specific downregulation of the c-FLIPs protein, leading to the activation of caspase-8 at the DISC level. Importantly, we also provide evidence that downregulation of c-FLIPs by E2F1 restores tumor cells sensitivity to death-receptor-mediated apoptosis as well as to lymphocytes T-mediated cytotoxicity, indicating that E2F1 could regulate the cellular response to the immune system.

Results

E2F1-induced apoptosis in lung adenocarcinoma cell lines requires caspase-8 activation and an intact E2F1 DNA-binding domain

We first tested the ability of E2F1 to trigger apoptosis in A549 and H1299 human lung adenocarcinoma cell lines using transient transfection with a vector encoding wild-type E2F1. As compared to cells transfected with a control vector, enforced E2F1 expression strongly induced apoptosis in both cell lines (Figure 1a). In order to confirm the results of transient transfections, we then established stable E2F1 or E2F1(E132) DNA-binding defective mutant inducible clones in the H358 cell line derived from a human lung adenocarcinoma, using the doxycyclin-inducible expression system (Tet-On). Of note, E2F1 expression was undetectable in H358 cells (Figure 1b, upper panel). Several stable transfectant clones were obtained and were found to give similar results. Therefore, data presented with H358/Tet-On/E2F1 clone 6 and H358/Tet-On/E2F1(E132) clone 22 are representative of all clones tested. As shown in figure 1b (upper panel), expression of E2F1 and E2F1(E132) proteins was strongly induced after 48 hours of doxycyclin treatment and persisted even after 6 days of continuous induction. In H358/Tet-On/E2F1 cells, apoptosis was clearly detected after 72 hours of doxycyclin treatment and reached 20% of total cells after 6 days (Figure 1b, lower panel). In contrast, and consistent with previous studies (14, 41), apoptosis was never observed in H358/Tet-On/E2F1(E132) cells (Figure 1b, lower panel). Appearance of apoptotic cells upon E2F1 expression correlated with the cleavage of procaspases-8 and -3 in their p43/41 and p17 active fragments respectively, followed by the activation of procaspases-6 and -7 (Figure 1c). Interestingly and consistent with previous data (42), E2F1 also increased procaspase-7 expression level. Of note, activation of this proteolytic cascade was never detected following E2F1(E132) overexpression (data not shown). In order to confirm the involvement of the

caspase-8 initiator and the caspase-3 effector during E2F1-mediated apoptosis, we finally tested the effect of two specific caspases-8 and -3 inhibitors, namely z-IETD-fmk and z-VED-fmk respectively. As shown in Figure 1d, both inhibitors significantly reversed the ability of E2F1 to induce apoptosis. In contrast, a specific caspase-9 inhibitor (z-LEDH-fmk) had no significant effect and the cytochrome c was never released from mitochondria in response to E2F1 expression suggesting that the mitochondrial amplification loop was not involved in our model (data not shown). Overall, these results indicate that E2F1 requires an intact DNA-binding domain and caspases-8 and -3 activities to trigger apoptosis in adenocarcinoma cell lines.

E2F1 activates caspase-8 at the DISC level

Until now, two distinct cellular models of FAS signaling pathway activation have been described. In type I cells, the death signal is initiated by the accumulation of activated caspase-8 at the DISC level. In type II cells, procaspase-8 expression level is low and the caspase cascade requires further amplification through the mitochondrial loop (27). The inhibition of E2F1-dependent apoptosis by the z-IETD-fmk caspase-8 inhibitor prompted us to analyze the DISC components. We first observed that E2F1 did not affect FAS, FasL or FADD protein expression, nor increased the amount of the FAS receptor at the membrane (data not shown). Caspase-8 activation at the DISC results mainly from the binding of FasL to the FAS receptor but can also occur in the absence of FasL triggering (43-46). To determine whether E2F1-induced cell death involved an interaction between FAS and FasL, we used the antagonistic ZB4 anti-Fas antibody which inhibits FAS/FasL binding. Our data showed that treating cells with increasing concentrations of ZB4 did not modulate the ability of E2F1 to induce apoptosis as detected by Hoechst staining (Figure 2a, left panel) or caspase-3 activation (Figure 2a, right panel). These results suggested that a FAS/FasL interaction did not

play a central role in E2F1-induced apoptosis. In keeping with these data, we did not detect any FasL secretion into the culture medium, nor a modulation of FasL expression on the membrane in response to E2F1 (data not shown). In order to analyze the DISC, FAS co-immunoprecipitation experiments were performed in H358/Tet-On/E2F1 clones. E2F1 induction led to the recruitment of FADD, caspase-8 and caspase-10 at the FAS DISC (Figure 2b). Importantly, the procaspase-8 was activated as reflected by the appearance of its p43/41 active fragments in FAS co-immunoprecipitates (Figure 2b). In addition, FAS and FADD were found to co-immunoprecipitate with caspase-8 in E2F1-expressing cells when immunoprecipitation experiments were performed using an antibody against caspase-8 (data not shown). In this context, we were unable to detect FasL at the DISC that was consistent with FasL binding being not essential for FAS activation in response to E2F1. Finally, as DISC formation and caspase-8 activation stimulate the clustering of the FAS receptor at the cell surface (47), we used confocal laser scanning microscopy to further analyze the FAS receptor subcellular distribution in our cells. As expected from our co-immunoprecipitation experiments, E2F1 induced the clustering of FAS whereas E2F1(E132) did not (Figure 2c). Overall, these results demonstrate that E2F1 activates the DISC of the FAS receptor.

E2F1 downregulates c-FLIPs protein expression to induce apoptosis

As our results indicated that E2F1 triggered cell death by affecting downstream effectors of the FAS signaling pathway, we then focused on the proximal regulators of procaspase-8 activation at the DISC level. c-FLIP (cellular FLICE-inhibitory protein) is a specific endogenous caspase-8 inhibitor (32). Both c-FLIP long (c-FLIP_L) and c-FLIP short (c-FLIP_S) isoforms can be incorporated into the DISC and inhibit specific steps of caspase-8 activation (35). Consequently, elevated levels of c-FLIP protein protect cells from death receptor-mediated apoptosis. Interestingly, E2F1 but not E2F1(E132) induction was

associated with a modest increase and a strong decrease of c-FLIP_L and c-FLIP_S protein levels respectively (Figure 3a). These effects correlated with the appearance of apoptosis 72 hours post-induction and persisted even after 6 days of doxycyclin treatment. Of note, E2F1 expression also stimulated the cleavage of c-FLIP_L into its p43 fragment. In the context where E2F1-induced apoptosis requires the specific downregulation of c-FLIP_S as suggested by our results, we postulated that ectopic expression of c-FLIP_S could block E2F1-mediated programmed cell death. To test this hypothesis, H358/Tet-On/E2F1 cells were stably infected with a VSV-FLIPshort (FLIP_S) expression vector or a control mock vector. As compared to mock-infected cells, c-FLIP_S-overexpressing cells were significantly more resistant to apoptosis induced by E2F1 (Figure 3b). In contrast, silencing of c-FLIPs using RNA interference in H358/Tet-On/E2F1 cells enabled a 3-fold increase in the percentage of apoptotic cells upon E2F1 expression (Figure 3c). Interestingly, this was detected after 48 hours of E2F1 induction. Taken together, these results demonstrate that c-FLIP_S is a potent inhibitor of E2F1-induced apoptosis. Importantly, c-FLIPs protein expression was also specifically increased in E2F1-deficient MEFs as compared to wild-type MEFs while c-FLIP_L status was not affected (Figure 3d). These results indicate that c-FLIP_S is also an E2F1-target in primary cells.

Downregulation of *c-flip_s* mRNA by E2F1 requires protein synthesis

We further investigated the molecular mechanisms involved in E2F1-mediated c-FLIP_S downregulation. To this end, we used RT-PCR to analyze the effect of E2F1 on *c-flip_l* and *c-flip_s* mRNA levels. As shown in figure 4a, expression of E2F1 induced a specific decrease in *c-flip_s* mRNA level which was detected upon 72 hours of doxycyclin treatment, whereas *c-flip_l* mRNA level remained unaffected. In the same conditions, E2F1(E132) expression did not affect mRNA level of both *c-flip_s* and *c-flip_l*, confirming the requirement of

the E2F1 DNA-binding domain to regulate c-FLIPs. Importantly, E2F1 was not able anymore to downregulate *c-flip_s* mRNA level in the presence of cycloheximide, a protein synthesis inhibitor, indicating that *de-novo* protein synthesis is required for E2F1 to regulate *c-flip_s* (Figure 4b). Of note, E2F1 expression level was not affected by the cycloheximide treatment in our conditions (data not shown).

E2F1 sensitizes NSCLC cells to death receptor mediated apoptosis

Our data demonstrated that E2F1 overexpression was sufficient to activate the FAS signaling pathway by affecting c-FLIP_s expression, even in the absence of death receptor ligands. As the role of endogenous c-FLIP proteins, and more specifically of c-FLIPs, in resistance to FAS-related apoptosis had been established (32), we tested whether E2F1 could affect the response of lung adenocarcinoma cells to death receptor stimuli. Using transient transfections, we first demonstrated that E2F1 overexpression sensitized A549 and H1299 cells to apoptosis induced by the agonistic FAS CH11mAb as compared to control-transfected cells (Figure 5a). Same results were observed in H358/Tet-On/E2F1 clones which became sensitive to CH11 treatment in the presence of E2F1 (Figure 5b, upper). In contrast, E2F1(E132)-expressing cells remained highly resistant (Figure 5b, upper panel). Furthermore, E2F1-expressing cells were also more sensitive to FasL and TRAIL treatments as compared to uninduced or E2F1(E132)-expressing cells (Figure 5b, lower panel). Interestingly, E2F1 did not increase the cytotoxic effects of etoposide or paclitaxel (Figure 5c) indicating that E2F1 specifically sensitized H358 cells to death receptor stimuli. Importantly, these effects were not restricted to lung adenocarcinoma cell lines. Indeed, we demonstrated that E2F1^{-/-}MEF were more resistant to FasL or TRAIL treatment than wild-type MEF (Figure 5d), although they expressed the same level of FAS and DR5 at the membrane (data not shown).

These results supported the idea that endogenous E2F1 affects the FAS death pathway downstream of the receptor level.

Neutralization of c-FLIP_s restores the sensitivity of tumor cells and primary E2F1^{-/-} MEFs to death receptor-mediated apoptosis

Finally, we asked whether downregulation of c-FLIPs was sufficient to restore cells sensitivity to apoptosis triggered by death receptors stimuli. As shown in Figure 6a, *c-flip_s* transfected E2F1^{-/-} MEFs became sensitive to FasL- and TRAIL-mediated apoptosis as compared to *mismatch* transfected cells. These results indicated that overexpression of c-FLIPs in E2F1-deficient cells contributes to their resistance to death receptor stimuli. In the same way, neutralization of *c-flip_s* by siRNA highly sensitized H358/Tet-On/E2F1 clones to CH11 or TRAIL treatment when compared to cells transfected with *mismatch* siRNA (Figure 6b). Interestingly, concomittant E2F1 induction in the same cells did not significantly potentiate the effect of *c-flip_s* neutralization. These results indicate that E2F1 sensitizes these tumor cells to death receptor mediated apoptosis mainly through c-FLIP_s downregulation.

E2F1 enhances tumor cell sensitivity to lymphocytes T-mediated cytotoxicity

FAS/FasL interactions and perforin/granzyme B pathways are the two main lytic mechanisms used by cytotoxic T cells (48, 49). Recent data provided evidence that an immune response might occur in NSCLCs and especially that effector T cells might contribute to tumor regression (50). In addition, it was demonstrated that c-FLIP protein expression enabled tumor cells to escape from T cell immunity "in vivo" (51, 52). Since we established that E2F1 regulated the cellular response to death receptor stimuli by downregulating c-FLIP_s, we next tested its ability to sensitize H358 cancer cells to cytolytic T cells. To do so, a lymphocytes T-mediated cytotoxic assay was set up in the presence of EGTA which blocks

the perforin pathway. In this context, the cellular lysis is strictly dependent on a functional FAS/FasL interaction. The Jurkat T cells expressing high levels of the FAS receptor were used as positive control of effective lysis by activated T lymphocytes (Figure 7). When H358/Tet-On/E2F1 cells cultured for 72 hours in the presence or in the absence of doxycyclin were used as targets, the lysis mediated by cytotoxic T cells was significantly increased in E2F1-expressing cells as compared to uninduced cells (Figure 7). Therefore, we concluded from these data that E2F1 also enhances the response of tumor cells to the lymphocytes T-dependent cytotoxicity.

c-FLIPs is overexpressed in human lung adenocarcinomas

Finally, we investigated whether the c-FLIP_S isoform is deregulated in human lung adenocarcinomas which display undetectable E2F1 protein expression (40). To this end, we analyzed E2F1 and c-FLIP protein expression by western blotting on 12 tumor samples and 5 matched normal lung tissues. Representative results from 5 normal/tumor couples are presented in Figure 8 but same data were obtained in the 7 remaining tumor samples. Consistent with our previous study (40), E2F1 protein expression was hardly detectable in the tumor samples. Interestingly, c-FLIP_S protein expression was strongly enhanced in these samples as compared to normal lung tissues whereas c-FLIP_L protein expression was not altered (Figure 8). Overall, these results show that c-FLIP_S is specifically overexpressed in lung adenocarcinomas. Moreover, they provide evidence that both c-FLIP protein isoforms can be differentially expressed. Of note, as normal lung tissues do not overexpress c-FLIP_S despite a low level of E2F1 expression, it is likely that additional signals cooperate with E2F1 loss to induce c-FLIP_S overexpression in lung adenocarcinomas.

Discussion

A large body of evidence has established that apoptosis can occur through both intrinsic (caspase-9 mediated) and extrinsic (caspase-8 mediated) death pathways. The ability of E2F1 to promote apoptosis by activating the intrinsic pathway has been well documented, notably through transcriptional activation of *apaf-1* or *caspase-9* genes (for review, see (15)). In contrast, the relationships between E2F1 and the extrinsic pathway have been less studied. Here, we demonstrate that E2F1 triggers apoptosis in various human lung adenocarcinoma cell lines by a mechanism involving the transcriptional inhibition of *c-flip_s*, thus leading to the activation of caspase-8 at the DISC level. Importantly, we find that c-FLIP_S protein is specifically overexpressed in lung adenocarcinoma which display a low level of E2F1 expression, and provide evidence that E2F1 restores tumor cells sensitivity to death-receptor stimuli, by downregulating c-FLIP_S expression. Overall, our data identify a new mechanistic link between E2F1 and the death-receptor signaling pathway and suggest that downregulation of E2F1 expression level contributes to the immune escape of lung adenocarcinoma tumor cells.

E2F1 can induce apoptosis via several p53-dependent or -independent mechanisms, either through direct transactivation of proapoptotic genes such as *p73* (53, 54), *apaf-1* (apoptosis protease-activating factor 1) (55, 56) and *caspases* (42) or transcriptional repression (14, 41). Here, we show that E2F1 is able to induce a p53-independent apoptosis through the downregulation of the c-FLIP_S anti-apoptotic protein. So far, a few studies have reported a link between E2F1 and the death-receptor signaling machinery (16, 17, 57-59). In this work, we found that E2F1 activates the proteolytic cleavage of caspase-8 at the DISC and induces the clustering of the FAS receptor. We also found that ZB4, an antagonistic antibody which blocks FAS/FasL interaction, does not inhibit E2F1-induced apoptosis. In addition, we were

unable to detect FasL at the DISC in our co-immunoprecipitation experiments. These results suggest that FasL binding is not required for FAS activation in response to E2F1. Such model in which death receptors are activated without the need of their respective death ligand has been previously reported in a context of UVB exposure (44). However, as E2F1-induced apoptosis was a progressive process in our model with 20% of apoptotic cells at the time of immunoprecipitation, we cannot exclude that only a small fraction of FAS molecules are clustered and activated at each time making difficult FasL detection in co-immunoprecipitation studies. More importantly, we showed that caspase-8 activation by E2F1 is specifically associated with the downregulation of c-FLIP_S. Originally, c-FLIP proteins were solely considered as inhibitors of death receptor-induced apoptosis, functioning as procaspase-8 competitors for binding to FADD at the activated DISC (60-62). In this context, c-FLIP_S appeared to be more efficient than c-FLIP_L (31, 33, 63-65). Interestingly, recent works showed that recruitment of c-FLIP_L to the DISC could also promote caspase-8 activation (36-38). In our cells, we never detected the presence of c-FLIP_L at the DISC level. Thus, it is tempting to speculate that E2F1, by altering the stoichiometry of c-FLIP_S and c-FLIP_L molecules, enables activation of caspase-8 at the DISC and triggers programmed cell death. However, as c-FLIP_S neutralization does not induce apoptosis on its own in our cells, E2F1 probably activates other collateral signals which collaborate with the downregulation of c-FLIP_S to trigger caspase-8 activation and cell death. Interestingly, it was recently shown that loss of Rb-E2F repression resulted in caspase-8 mediated apoptosis through inactivation of focal adhesion kinase (66).

Both c-FLIP isoforms arise from alternative splicing and until now, the molecular mechanisms controlling their relative expression remain largely unknown. It has been demonstrated that the level of cellular FLIP is regulated both at transcriptional and post-translational levels, the latter involving the ubiquitin-proteasome pathway (67, 68). In this

study, we demonstrated that E2F1 differentially affects the expression of c-FLIP_S and c-FLIP_L proteins in human cell lines. Furthermore, we also showed the existence of a distinct expression pattern of the two isoforms in a few samples of human lung adenocarcinoma. Altogether, these results are consistent with the notion that c-FLIP_S and c-FLIP_L are differentially regulated and exhibit distinct functions. The *FLIP* gene was previously reported to be up-regulated by E2F1 (69). In contrast, we found that E2F1 induces a specific decrease of *c-flip_s* mRNA level by a mechanism requiring its DNA-binding domain indicating that E2F1 is able to regulate the expression of c-FLIP proteins in various way depending on the cellular context. As both c-FLIP_S and c-FLIP_L are expressed from the same promoter, our data are not consistent with a model in which E2F1 binds to the *FLIP* promoter and downregulates specifically *c-flip_s* mRNA without affecting *c-flip_L* mRNA. However, it remains possible that E2F1 binds to the *FLIP* promoter and recruits some specific co-factors to differentially regulate the alternative splicing of both isoforms, or affects the expression level of proteins controlling the alternative splicing of *c-flip_s*. Alternatively, E2F1 could also specifically decrease the stability of *c-flip_s* mRNA. We are currently testing these hypotheses. Of note, we observed that pre-treatment of cells with proteasome inhibitors did not prevent the ability of E2F1 to downregulate c-FLIP_S (data not shown), indicating that additional c-FLIP_S control at the protein level is not involved in our model.

We provided evidence that E2F1 sensitized primary and tumor cells to FasL- or TRAIL-mediated apoptosis. Such enhanced resistance to Fas-triggered apoptosis has been already described in E2F1^{-/-} cerebellar granule neurons (17), thymocytes (59) and lymphocytes (16). Altogether, these data are consistent with E2F1 being a critical determinant of the cellular response to death-receptor activation, at least in some cellular types. In addition, this is to our knowledge the first study demonstrating the involvement of E2F1 in the cellular response to TRAIL. High c-FLIP expression has been found in many tumor cells

and was correlated with resistance to CD95- and TRAIL-induced apoptosis (60). Recently, a genetic screen identified that the c-FLIP_S isoform conferred resistance to TRAIL in sensitive cells (70). Furthermore, c-FLIP_S was reported to be more efficient than c-FLIP_L in the inhibition of CD95-mediated apoptosis in T cells (71). Interestingly, E2F1-deficient mice accumulate mature T cells due to a defect in thymocyte apoptosis and negative selection (11, 12), a process functioning mainly through death-promoting ligands. Whether c-FLIP_S is overexpressed in E2F1^{-/-} thymocytes remains to be determined. In our model, selective neutralization of c-FLIP_S by siRNA was sufficient to restore tumor cells sensitivity to both Fas- and TRAIL-mediated apoptosis and concomitant induction of E2F1 in these cells did not lead to an additive effect. Therefore, these data are in favor of E2F1 sensitizing these tumor cells to death-receptor mediated apoptosis mostly through c-FLIP_S downregulation. In this setting, sensitization is likely to be mediated by a facilitated caspase-8 activation in the absence of c-FLIP_S. In addition, as we demonstrate that *c-flip_s* neutralization in E2F1^{-/-} MEF is sufficient to restore their sensitivity to FasL and TRAIL, our results extend to non transformed cells the role of E2F1 as a determinant of cellular response to death receptor-mediated apoptosis through c-FLIP_S regulation. The death receptor pathway represents one of the mechanisms by which immune cells kill target tumor cells. In this context, we also provided evidence that E2F1 enhanced tumor cell sensitivity to lymphocytes T-mediated cytotoxicity. Interestingly, c-FLIP expression was associated with tumor escape from T-cell immunity and enhanced tumor progression in "in vivo" experimental studies pointing to a role of FLIP as a tumor progression factor (51, 52, 72).

Many tumors are resistant to Fas- and TRAIL-induced cell death. Production of decoy receptors (73), expression of FasL to perform the so-called tumor counterattack (74), and upregulation of apoptosis inhibitors expression (72, 75) are among the molecular mechanisms involved in this process. We previously reported the frequent decrease of FAS expression in

NSCLC (76). Importantly, it has been shown that escape from T cell-mediated immunity "in vivo" by c-FLIP overexpression was not limited to tumors with high FAS surface expression (52). In the light of these data, the low level of E2F1 protein expression in NSCLC with reduced FAS expression could be another way to block FAS-triggered cell death, therefore contributing to the immune escape of tumor cells. In this setting, E2F1 restoration might represent a key step in therapeutic strategies to reconstitute the ability of tumor cells to undergo apoptosis.

Materials and methods

Cell lines, expression vectors and transfection

H358, A549 and H1299 human lung carcinoma cell lines as well as Jurkat T cells were cultured in RPMI-1640 medium (GibcoBRL, Life Technologies, Cergy Pontoise, France), supplemented with 10% (v/v) heat-inactivated fetal calf serum (GibcoBRL), 2mM L-glutamine and 100U/ml penicillin/streptomycin, in 5% CO₂ at 37°C. Primary murine embryonic fibroblasts (MEF) derived from wild-type and E2F1 ^{-/-} mice (kindly gift from Dr. Nevins), 293-FasL embryonic kidney cells stably expressing human Fas ligand and HT29 colon cancer cells were maintained in DMEM (GibcoBRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum and 2mM L-glutamine. To ensure exponential growth, cells were resuspended in fresh medium 24 hours before transfection or treatment. Stable inducible E2F1 (wild-type) or E2F1(132) (DNA-binding defective mutant) clones derived from H358 cells were obtained using a modified tetracyclin-regulated inducible expression system (Tet-On System, Clontech, Ozyme, Saint Quentin en Yvelines, France) as previously described (77). E2F1 expression was induced upon 1µg/ml doxycyclin treatment. Inducible clones stably infected with either VSV-FLIP_S expression vector or with a mock vector were provided by O. Micheau (INSERM U517, Dijon, France). Transient transfections of A549 and H1299

cells with either control pcDNA3.1/GFP or pcDNA3.1/E2F1 expression vectors were carried out using JetPEI™ (Polyplus Transfection, Illkirch, France) according to the manufacturer's protocol. In some experiments, genitacin (800 µg/ml) was added to the culture medium 48 hours after transfection and selection was performed during 3 additional days before analysis.

Tissue samples

Tissue samples taken at surgical resection of lung tumors were immediately frozen and kept at -80°C until use. They consisted of 12 adenocarcinoma according to the 1999 WHO international histological classification of lung tumors and 5 matched normal lung samples.

Reagents and antibodies

Agonistic anti-human Fas monoclonal antibody (clone CH-11) and caspase inhibitors z-IETD-fmk, z-DEVD-fmk and Z-LEDH-fmk were obtained from MBL (MBL, France Biochem, Meudon). For each experiment, caspase inhibitors (2 µM) were added to the culture medium 2 hours prior addition of doxycyclin (1µg/ml). Treatment with CH-11 mAb (250ng/ml) was carried out for 48 hours. Soluble Fas ligand was collected from 293-FasL supernatant. Recombinant soluble human FLAG-tagged FasL, Flag-tagged TRAIL and their enhancer were purchased from Alexis (San Diego, CA, USA). Etoposide (VP16), paclitaxel (Taxol™) and cycloheximide were obtained from Sigma (Saint Quentin Fallavier, France). Cycloheximide was used at 10µg/ml for 12 hours.

Monoclonal antibodies used included anti-procaspase-8 (5F7), anti-procaspase-10 (4C1) and anti-Fas (ZB4) (MBL), anti-FADD (1F7), anti-FasL (G247-4), anti-procaspase-6 and anti-E2F1(KH95) (Pharmingen BD, San Diego, CA, USA), anti-HSP 70 (Affinity Bioreagents, Golden, CO, USA), anti-actin (Santa Cruz, CA, USA), anti-E2F1(Ab4; Neomarkers, CA, USA), anti-Fas (APO-1 and 3D5) and anti-FLIP (NF6) (Alexis). To block

FasL/Fas interaction, anti-Fas (ZB4) was used at concentrations ranging from 2 to 30 µg/ml. Polyclonal antibodies used included anti-mouse TRAIL-R2 (R&D Systems), anti-procaspase-3 and anti-active caspase 3 (PharMingen BD), anti-procaspase-7 (Cell Signaling, Beverly, MA, USA), anti-procaspase-8 (C-20, Santa Cruz), anti-procaspase-10 (Sigma), anti-E2F1 (Santa Cruz Biotechnology) and anti-FLIP (H-202, Santa Cruz Biotechnology).

Cell viability, apoptotic and cytotoxic assays

Apoptosis was evaluated by scoring the percentage of apoptotic cells on 500 cells after Hoescht 33342 staining. Cytotoxic experiments were performed using the methylene blue colorimetric assay. Briefly, stable inducible clones were seeded at 10^4 cells/well into a 96-wells plate and were co-treated for 96 hours with increasing concentrations of cytotoxic drugs in the presence or absence of doxycyclin. Cells were then fixed in 100% ethanol, stained with 1% methylene blue in 0.01M borate buffer pH 8.5 for 15 min and optical density was measured at 630nm after elution in 0.1N HCl. Absorbance values were expressed as a percentage of untreated controls.

Allogenic T cells cytotoxic assays against tumor target cells were measured in standard 24 hours ^{51}Cr -release assays as previously described (78). Briefly, 10^4 ^{51}Cr -labeled target cells were mixed with the effector cells at different E/T ratios (25/1 to 1/1) with E=effector cells and T=target cells. The radioactivity in supernatants was counted after 24 hours incubation at 37°C in 5% CO₂. The percentage of specific lysis was calculated according to the following formula :% lysis = $100 \times (\text{ER} - \text{SR}) / (\text{MR} - \text{SR})$, where ER, SR and MR represent experimental, spontaneous, and maximum ^{51}Cr -release, respectively. All expressed values were derived from average quadruplicate determinations. Effector allogenic T cells were obtained from unidirectional mixed lymphocyte reaction (MLR) as previously described. To induce FasL expression at the cell surface, effector cells were preincubated for

2 hours with a mixture of 5ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1.5µg/ml Ca²⁺ ionophore ionomycin (Sigma). Cytotoxic tests were performed on E2F1 stable inducible clones which were pretreated or not with doxycyclin for 72 hours, then used as target cells in complete medium supplemented with 15% human A serum in the presence or absence of 3mM EGTA/4mM Mg²⁺. Jurkat T cells treated in the same conditions were used as positive control target cells.

Western Blot and immunoprecipitation analyses

For immunoblotting, cells washed three times in PBS or frozen tissue samples were lysed in RIPA buffer (150mM NaCl, 50mM Tris HCl pH 8, 0.1% SDS, 1% Nonidet P40, 0.5% Na deoxycholate, 0.1mM PMSF, 2.5µg/ml pepstatin, 10µg/ml aprotinin, 5µg/ml leupeptin, 0.2mM Na₃VO₄) for 30 min on ice and pelleted. Protein concentration was determined using the Biorad Dc protein assay. Proteins (40µg-80µg) were then separated in 10-12% SDS-PAGE gels and electroblotted onto PVDF membranes. Membranes were incubated overnight at +4°C with primary antibodies and proteins were detected using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). After washing, the blots were revealed using the ECL chemiluminescence method (Amersham, Les Ulis, France), according to the manufacturer's protocol.

For DISC immunoprecipitation, 50x10⁶ cells were lysed in lysis buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 2mM sodium vanadate, 1mM AEBSF and 10µg/ml aprotinin) for 15 min on ice and supernatants were collected by centrifugation at 12000 rpm for 15 min. Supernatants were precleared with Sepharose 6B (Sigma) for 1 hour at +4°C and immunoprecipitation of the FAS receptor was further performed overnight at +4°C on precleared lysates in presence of protein G sepharose and 2µg of FLAG-tagged-Fc-FasL

(provided by Olivier Micheau). Protein G sepharose beads were spun down, washed 4 times with lysis buffer, resuspended in SDS gel sample buffer and boiled at 95°C for 4 min. Immunoprecipitates were separated in 12% SDS/PAGE gel and immunoblotted with either anti-Fas (C-20, Santa-Cruz), anti-FADD (1F7, mouse IgG1, Immunotech), anti-caspase-8 (5F7, mouse IgG2b, Immunotech) or anti-caspase-10 (4C1, mouse IgG1, MBL) antibodies. Secondary antibodies used were anti-mouse HRP-IgG1 (Southern Biotechnology) to detect FADD and caspase-10, anti-mouse HRP-IgG2b (Southern Biotechnology) to detect caspase-8 or anti-rabbit HRP to detect Fas (C-20).

Indirect immunofluorescence and Fas clustering studies

Transfected A549 and H1299 cells were fixed in -20°C acetone for 5 min. After extended washes, non specific binding sites were saturated for 45 min at RT in 2% BSA, 10% normal goat serum, 1X PBS and incubation with anti-E2F1 mAb (Ab4, 1/2000) was carried out for 2 hours at RT in PBS, 2% BSA. After three PBS washes, Alexa TM⁵⁶⁸ goat anti-mouse IgG (H+L) conjugate (2mg/ml, 1/1000, Interchim Montluçon, France) was added and cells were further incubated for 30 min in dark at 37°C. Cells were then washed three times in PBS, counterstained with Hoescht 33342 and observed using an Olympus microscope. For Fas clustering analysis, 10⁴ H358 stable inducible cells were seeded onto immunofluorescence slides and cultured in the presence or in the absence of doxycyclin for 6 days. Cells were then fixed in 3% paraformaldehyde/PBS for 10 min, washed twice with PBS for 10 min, preincubated with 1% BSA for 15 min, and incubated for 2 hours at RT with anti-Fas mAb (Zb4) in 1% BSA, PBS. Samples were washed twice in PBS and stained using the Alexa TM⁵⁶⁸ goat anti-mouse IgG(H+L) conjugate. Cells were visualized using a confocal laser (Spectra Physics) microscope (Zeiss, LSM510). For all experiments, a nonrelevant isotype matching antibody was used as a negative control (data not shown). HT29 colon

cancer cells treated with 25 ng/ml soluble FasL (Alexis Biochemical) were used as a positive control of FAS clustering.

Transfection of siRNA oligonucleotides

The siRNA oligonucleotides were synthesized by Eurogentec and resuspended according to the manufacturer's protocol (Eurogentec, Seraing, Belgium). The sequence designed to target specifically human *flip short* mRNA was as follow: 5'-CACCCUAUGCCCAUUGUCCTT-3'. The sequence designed to target specifically the mouse *flip short* mRNA was as follow: 5-CCAGUGUAUGGAGUACCAGTT-3' The scrambled siRNA oligonucleotides used as control were 5'-ACAUACGCUUUCGCCCCUTT-3' for human cells and 5'-CCAAUGGGUUGACAGUCAGTT-3' for mouse cells. For siRNA transfection, stable E2F1-inducible clones or E2F1^{-/-} MEFs were seeded into a 6-well plate at 5x10⁵ or 2x10⁵ cells/well respectively. On the next day, the cells were transfected with siRNA oligonucleotides duplex using Oligofectamine™ (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Briefly, 10µl of siRNA duplex (at 20µM) were mixed with 185µl of serum-free RPMI/DMEM medium. 3µl of Oligofectamine™ was added to 12µl of RPMI/DMEM serum-free medium, vortexed, incubated at RT for 10 min and gently mixed with the siRNA duplex solution. Mixture was incubated for 20 min at RT and added to each well. 10% fetal calf serum was added 4 hours after transfection. Doxycyclin was added or not in culture medium 24 hours after transfection and cells were cultured additionally for 48 hours before analysis. In some experiments, stable inducible clones were transfected for 43 hours, then treated during 5 additional hours with either 500ng/ml anti-Fas agonistic CH11 mAb or 100ng/ml TRAIL before analysis. E2F1^{-/-} MEFs were transfected for 50 hours with *c-flip_s* or *mismatch* siRNA then treated for 20 additional hours with either

25ng/ml FasL in the presence of 1µg/ml enhancer or 40ng/ml KillerTRAIL™ (Alexis Biochemical).

Reverse transcription- polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated using Trizol reagent (Invitrogen). One µg of total RNA was subjected to RT in a 20 µl reaction volume using oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Three µl of the RT reaction was then amplified by PCR for 30 cycles using the following conditions: 94°C for 30 sec ; 58°C for 1 min and 72°C for 2 min. The primers used were: flip short forward (sense): 5'-CGAGGCAAGATAAGCAAGGA-3'; flip short reverse (antisense): 5'-CACATGGAACAATTTCCAAGAA-3'; flip long forward (sense): 5'-CTTGGCCAATTTGCCTGTAT-3'; flip long reverse (antisense): 5'-GGCAGAAACTCTGCTGTTCC-3'. Amplification of a fragment of the cDNA of G3PDH (Invitrogen) was performed in the same PCR reaction as internal control. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analyses

Quantitative experiments were analysed by the use of Student's paired *t* test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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Legends

Figure 1: E2F1 induces apoptosis in lung adenocarcinoma cells through a caspase-8- and DNA-binding domain-dependent mechanism.

(a) A549 and H1299 cell lines were transfected either with a control pcDNA3.1/GFP (open bars) or a pcDNA3.1/E2F1 (closed bars) vector. Apoptosis was monitored after 6 days of geniticin selection, in GFP- or E2F1-positive cells using Hoechst 33342 staining. Results are the mean \pm SD of three independent experiments. **(b)** Western blot analysis (upper panel) and percentage of apoptotic cells (lower panel) in H358/Tet-On/E2F1 (white and black bars) or H358/Tet-On/E2F1(E132) (hatched white and black bars) cells cultured in the presence (Dox +) or in the absence (Dox -) of doxycyclin for indicated times. Results are the mean \pm SD of

three independent experiments. **(c)** Western blot analysis of caspases in H358/Tet-On/E2F1 cells cultured in the presence (Dox +) or in the absence (Dox -) of doxycyclin for indicated times. HSP 70 was used as a loading control. **(d)** H358/Tet-On/E2F1 cells were pre-treated or not for two hours with 2 μ M of either z-VED-fmk or z-IETD-fmk, then 1 μ g/ml doxycyclin was added (Dox +) or not (Dox -) in the culture medium and cells were further cultured for 6 days. Percentage of apoptotic cells was scored on 500 cells after Hoescht staining. Results are the mean \pm SD. of three independent experiments. *: $p < 0.05$; *** $p < 0.001$ by Student's *t* test.

Figure 2: E2F1 activates caspase-8 at the DISC level

(a) H358/Tet-On/E2F1 cells were cultured for 6 days in the presence (+) or absence (-) of doxycyclin (Dox) and/or increasing concentrations (2, 5, 10, 30 μ g/ml) of antagonistic anti-FAS mAb (ZB4). *Left panel:* apoptosis was scored on 500 cells after Hoechst staining. *Right panel:* activation of caspase-3 was detected after immunoblotting. **(b)** H358/Tet-On/E2F1 cells were cultured in the presence (Dox +) or in the absence (Dox -) of doxycyclin for 6 days. FAS immunoprecipitates were analyzed for the presence of the components of the DISC by immunoblotting using specific antibodies. For all experiments, inputs are representative of 80 μ g of whole cell extracts. **(c)** Confocal laser microscopy analysis of FAS subcellular distribution was performed in H358/Tet-On/E2F1 and H358/Tet-On/E2F1(E132) cells cultured for 6 days in the presence (Dox +) or in the absence (Dox -) of doxycyclin. Magnification amplitude: x60. HT29 cells treated with FasL were used as a positive control.

Figure 3: The short isoform of c-FLIP is a downstream target of E2F1

(a) Western blot analysis of c-FLIP isoforms was performed in H358/Tet-On/E2F1 (left panels) and H358/Tet-On/E2F1(E132) (right panels) cells cultured for 6 days in the presence (Dox +) or in the absence (Dox -) of doxycyclin. Actin was used as a loading control. **(b)**

Apoptosis was analyzed in H358/Tet-On/E2F1 cells stably infected with either control (mock) or c-FLIP_S-expressing vectors (c-FLIPs) and cultured for 6 days in the presence or absence of doxycyclin. Fold induction of apoptosis represents the ratio: percentage of induced apoptotic cells/percentage of uninduced apoptotic cells. Results are the mean \pm SD of three independent experiments. Western blot analysis confirmed overexpression of c-FLIPs protein in VSV-FLIP_S-transfected cells as compared to mock-transfected cells. Hsp 70 was used as a loading control. **(c)** H358/Tet-On/E2F1 cells were transfected with either *mismatch* or *c-flip_s* siRNA and cultured in the presence (Dox +) or absence (Dox -) of doxycyclin. Apoptosis was scored on at least 500 cells using Hoechst staining. Results are the mean \pm SD of three independent experiments. Western blotting confirmed the neutralization of c-FLIP_S by siRNA. HSP 70 was used as a loading control. **(d)** Western blot analysis of c-FLIP_L, c-FLIPs and E2F1 expression in E2F1 deficient (E2F1^{-/-}) and wild-type control (E2F1^{+/+}) MEFs. Actin was used as a loading control.

Figure 4: E2F1 specifically downregulates *c-flip_s* mRNA

(a) RT-PCR analysis of *c-flip_s* or *c-flip_l* RNA expression level was performed in H358/Tet-On/E2F1 (left panel) or H358/Tet-On/E2F1(E132) (right panel) cells cultured in the presence (Dox +) or in the absence (Dox -) of doxycyclin for indicated times. Amplified G3PDH was used as an internal control. **(b)** H358/Tet-On/E2F1 cells were cultured in the presence (Dox +) or in the absence (Dox -) of doxycyclin for 72 hours and cycloheximide (10 μ g/ml) was added to the culture 12 hours prior cells harvesting. *c-flip_s* RNA expression level was analyzed as described in (a). Results shown are representative of three independent experiments.

Figure 5: E2F1 sensitizes NSCLC cells to death receptor-mediated apoptosis

(a) A549 and H1299 cell lines were transfected with either a control pcDNA3.1/GFP (open bars) or a pcDNA3.1/E2F1 (black bars) vector and treated with agonistic anti-Fas CH11 mAb (250 ng/ml). Apoptosis was assessed in GFP- or E2F1-positive cells using Hoechst 33342 staining after 6 days of genitacin selection. Results are the mean \pm SD of three independent experiments. **(b) Upper panel:** a 96-hours cell viability assay was performed in H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells co-treated with (Dox +; black symbols) or without (Dox -; white symbols) doxycyclin and increasing amounts of the FAS agonistic CH11 mAb. Results are expressed as the percentage of cell survival as compared to untreated cells. Mean value of three independent experiments \pm SD performed in triplicate are presented. **Lower panel:** H358/Tet-On/E2F1 and H358/Tet-On/E2F1(E132) cells were treated or not with 293FasL-supernatants or 40 ng/ml TRAIL, in the presence (Dox +) or in the absence (Dox-) of doxycyclin for 72 hours. Percentage of apoptotic cells was monitored after Hoescht staining. Results are the mean \pm SD of three independent experiments. **(c)** A 96-hours cell viability assay was performed in H358/Tet-On/E2F1 cells co-treated with (Dox +; black symbols) or without (Dox -; white symbols) doxycyclin and increasing amounts of either etoposide or paclitaxel as indicated. Results are expressed as the percentage of cell survival as compared to untreated cells. Mean value of three independent experiments \pm SD performed in triplicate are presented. **(d)** E2F1-deficient (E2F1^{-/-}, black bars) and wild-type control (E2F1^{+/+}, white bars) MEFs were treated for 24 hours with increasing amounts of FasL or TRAIL and apoptosis was evaluated using Hoescht staining. Fold induction of apoptosis represents the ratio: percentage of apoptotic treated cells/percentage of apoptotic untreated cells.

Figure 6: Downregulation of c-FLIP_S is sufficient to restore tumor cells and primary E2F1^{-/-} MEFs sensitivity to death receptor-mediated apoptosis.

(a) E2F1^{-/-} MEFs were transfected for 50 hours with *mismatch* (white bars) or *c-flip_s* (black bars) siRNA, then treated or not with 25ng/ml FasL or 40ng/ml Killer TRAILTM for 20 additional hours. Apoptosis was evaluated using Hoechst staining. Fold induction of apoptosis represents the ratio: percentage of apoptotic treated cells/percentage of apoptotic untreated cells in each condition. Upper blot: immunoblotting experiments showing the neutralization of c-FLIP_S in *c-flip_s* transfected cells as compared to *mismatch* transfected cells. Actin was used as a loading control. **(b)** H358/Tet-On/E2F1 cells were transfected either with *mismatch* or *c-flip_s* siRNA and treated or not with 500 ng/ml anti-FAS CH11 mAb or 100ng/ml TRAIL for 5 hours as indicated. In some experiments, E2F1 was induced by doxycyclin treatment. Percentage of apoptotic cells was assessed using Hoescht staining. Results shown are the mean ± SD of three independent experiments. Western blot analysis demonstrates the specific downregulation of c-FLIP_S protein expression. HSP70 was used as a loading control.

Figure 7: E2F1 enhances tumor cells sensitivity to lymphocyte T-dependent cytotoxicity

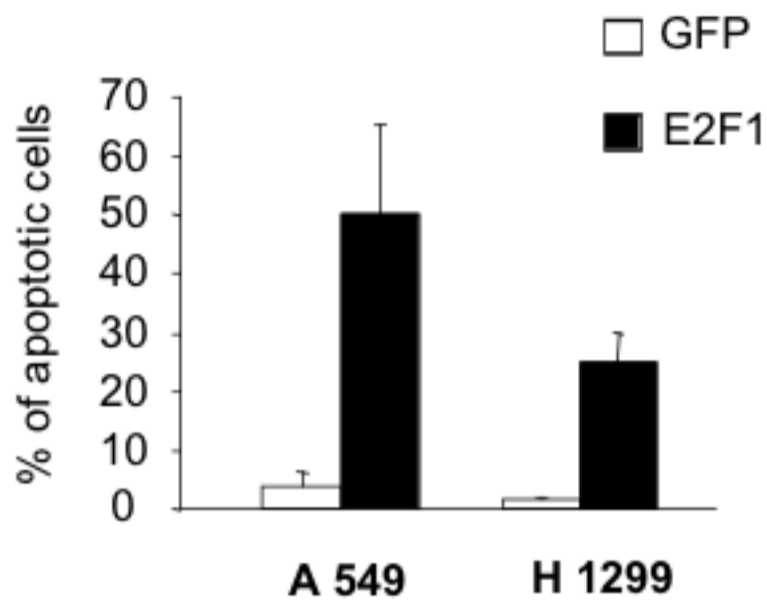
T cell-dependent cytotoxicity was measured in the presence of EGTA using a 24 hours ⁵¹Cr-release assay. Jurkat T cells were used as positive target control cells. H358/Tet-On/E2F1 cells were cultured in the absence or in the presence of doxycyclin for 72 hours and then used as target cells. Results represent the percentage of cell lysis in function of E/T ratio where E= effector T cells and T= target cells. Results are representative of two independent experiments.

Figure 8: Western blot analysis of E2F1 and c-FLIP expression in human lung adenocarcinoma and their matched normal lung tissues

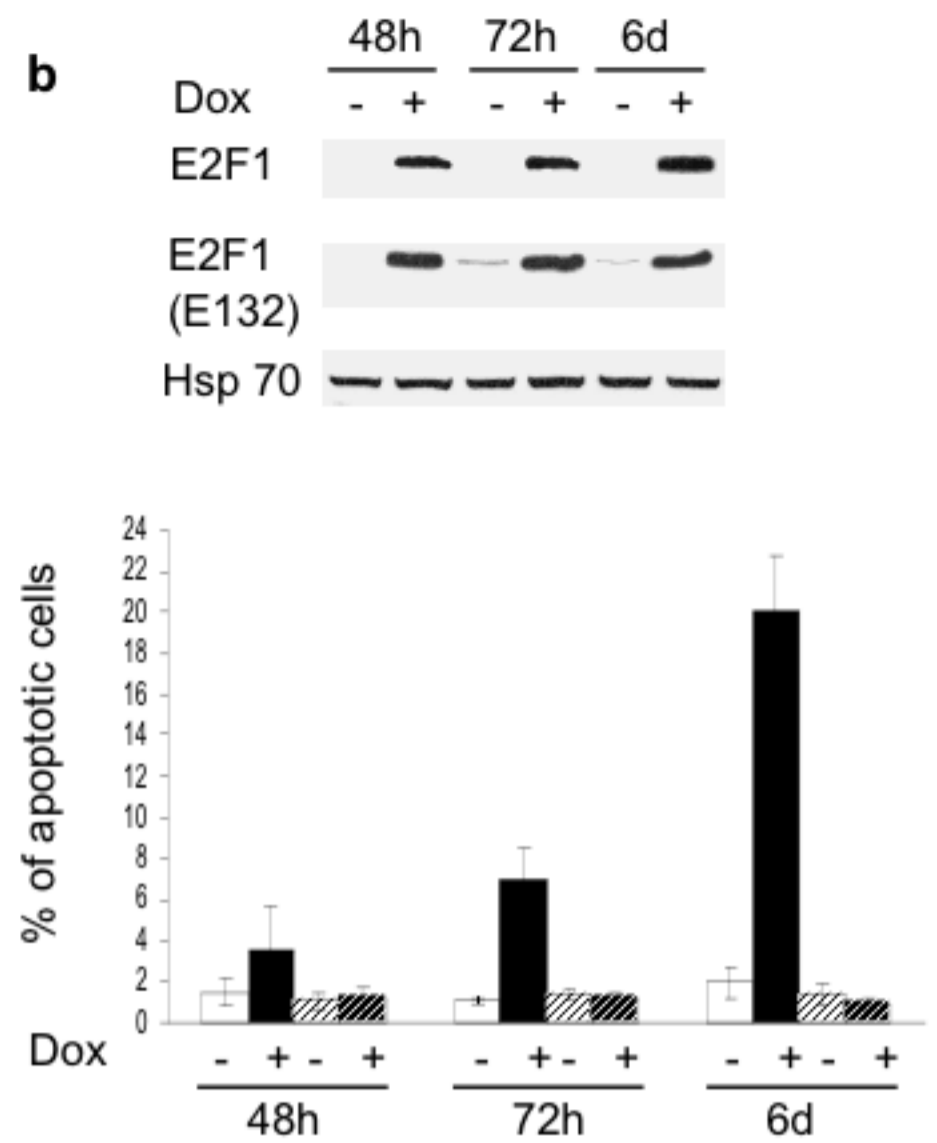
Western blot analysis of E2F1 and c-FLIP expression in 5 representative adenocarcinoma lung tumor (T) and their matched normal lung (N). Actin was used as a loading control.

Figure 1

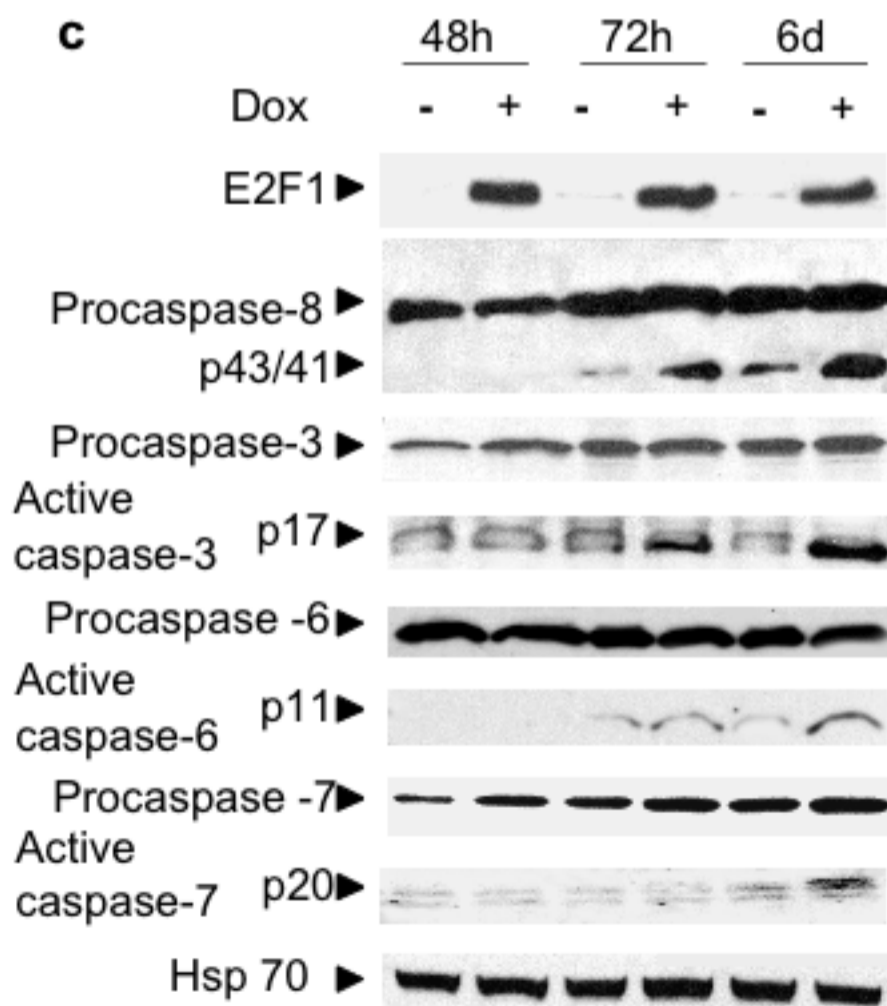
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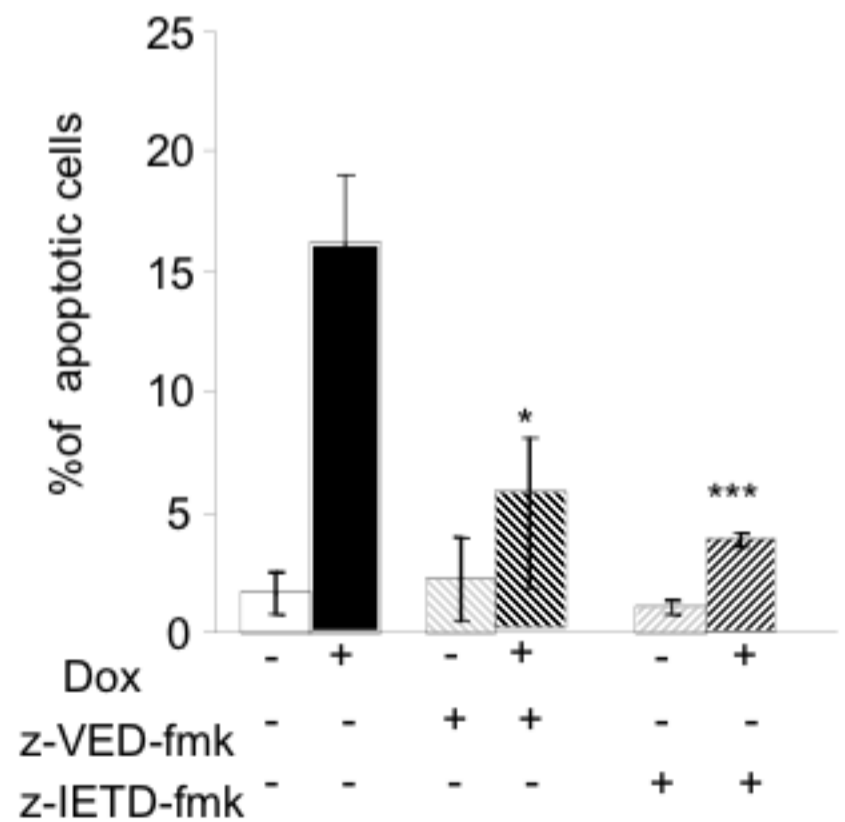
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c



d



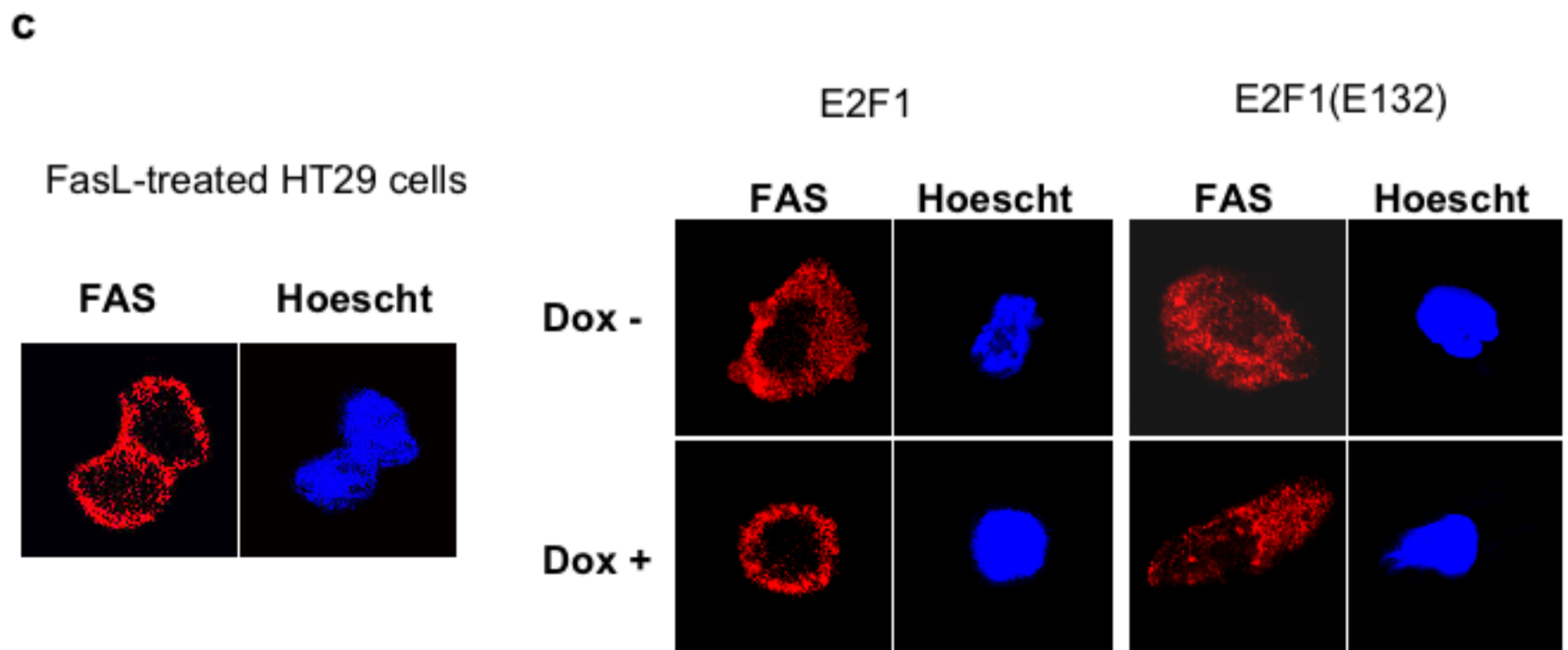
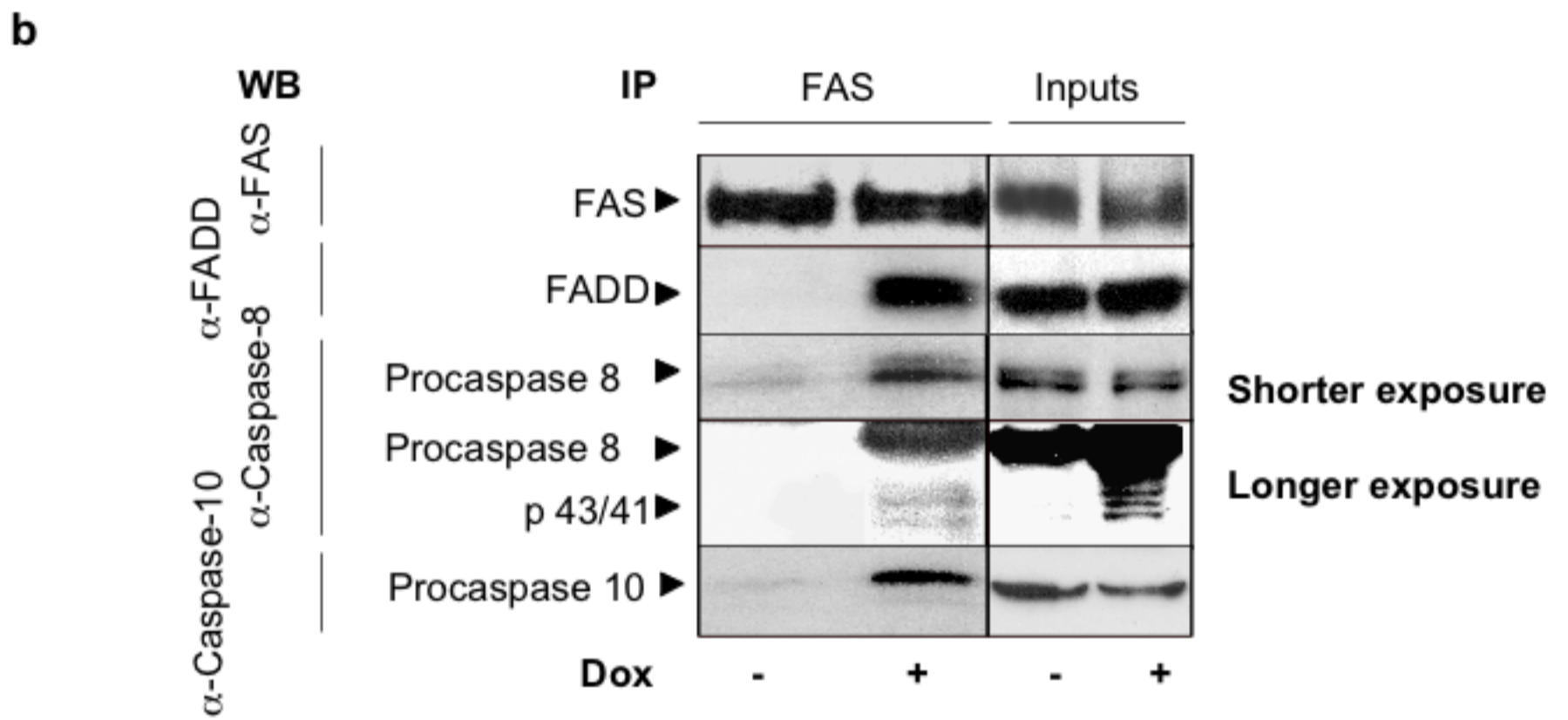
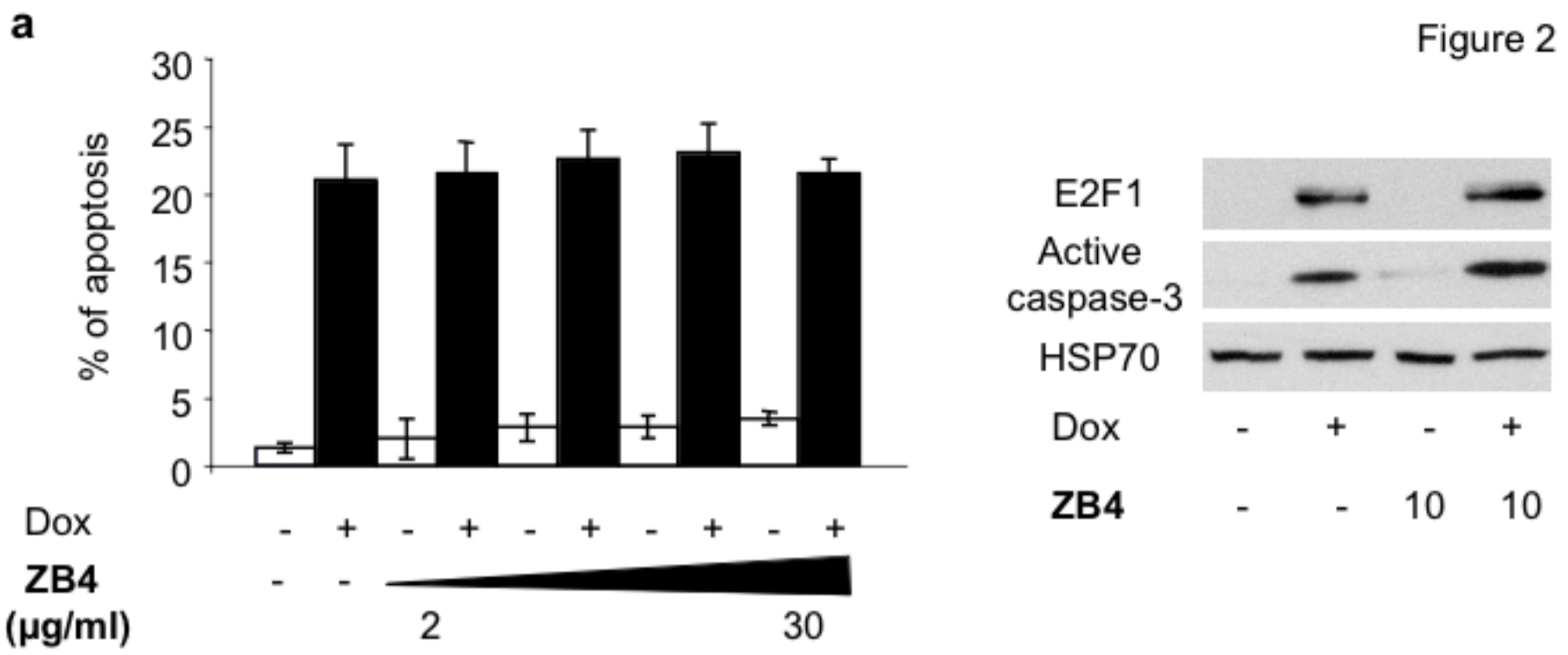


Figure 3

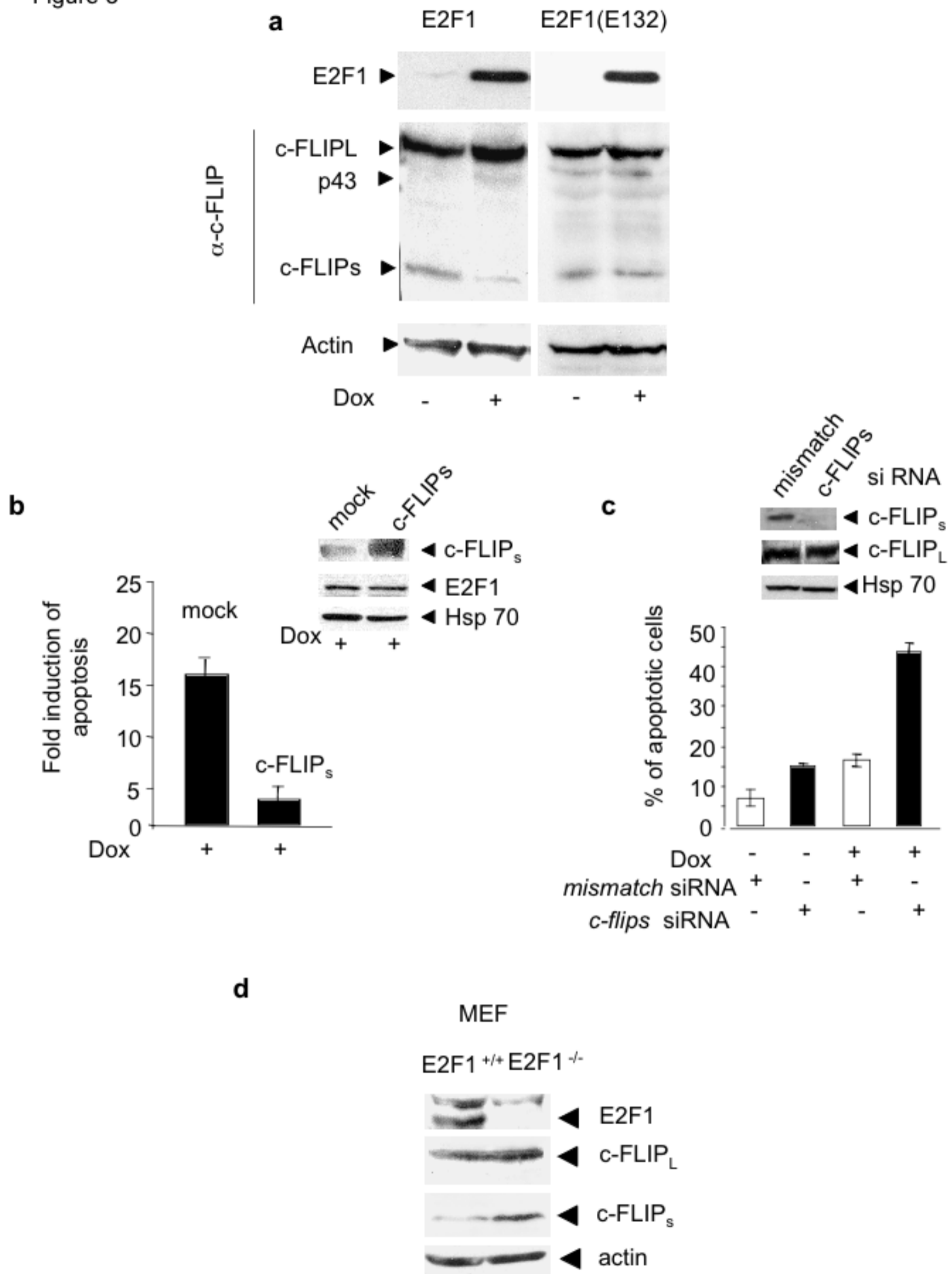
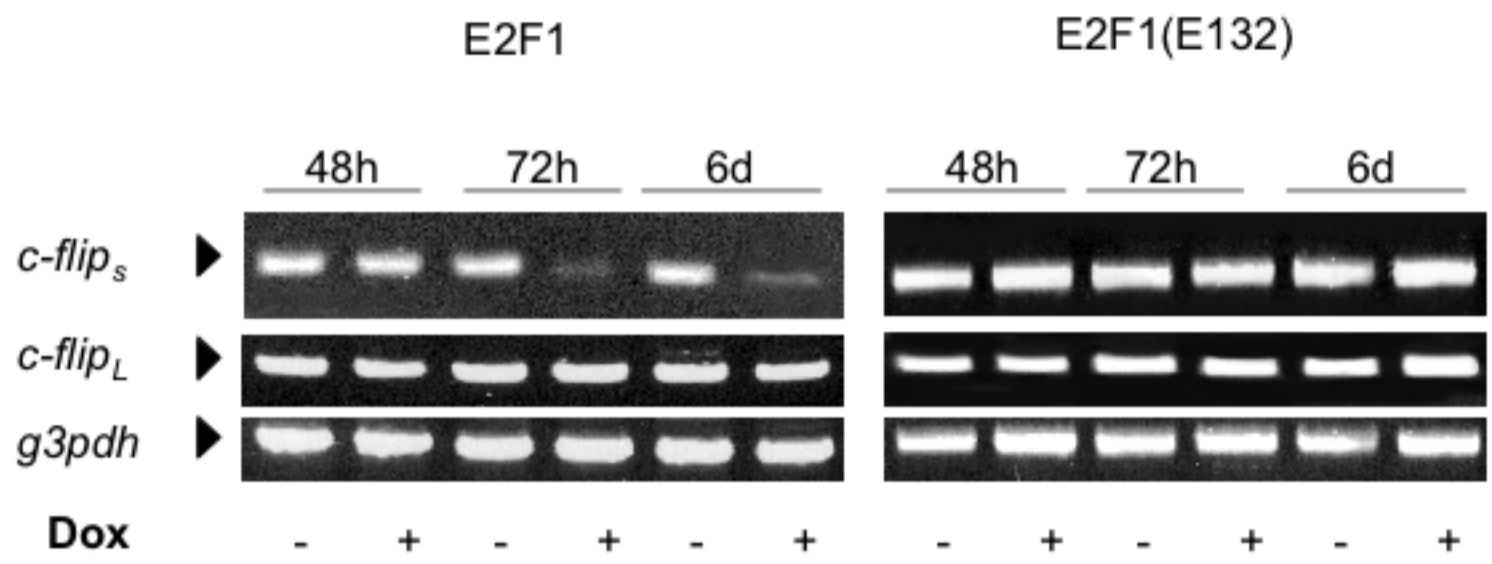


Figure 4

a



b

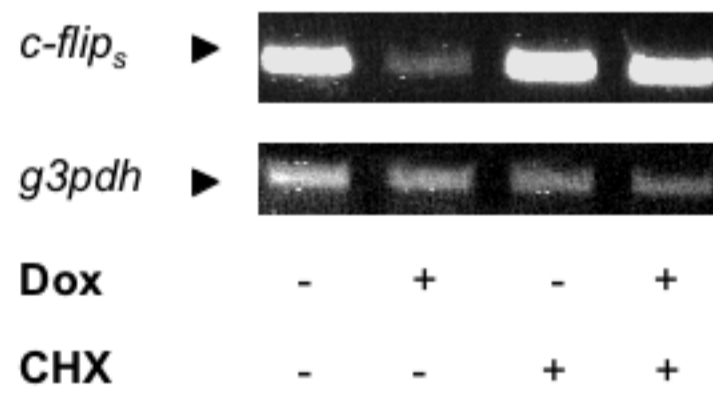


Figure 5

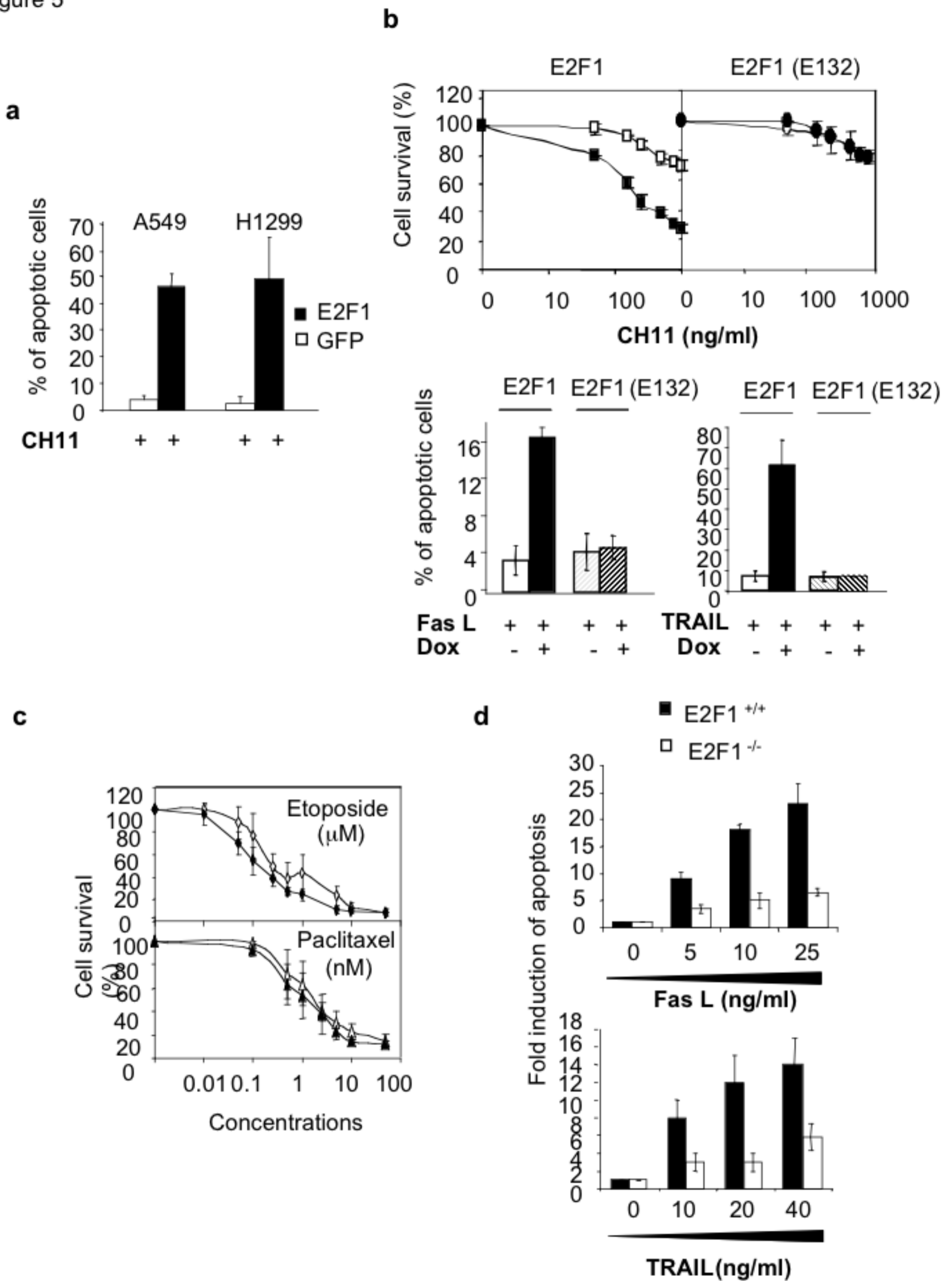
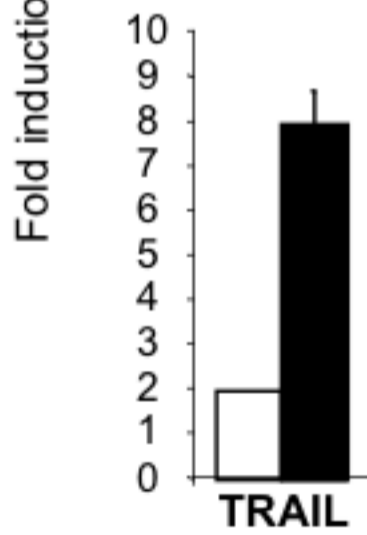
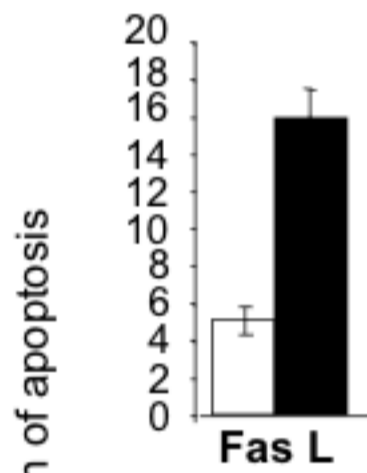
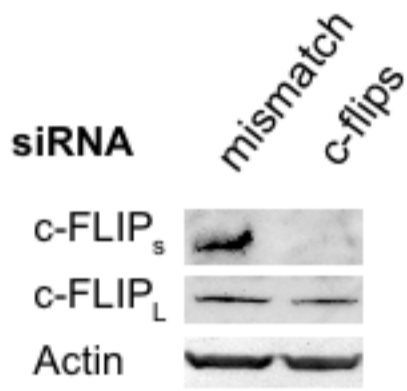
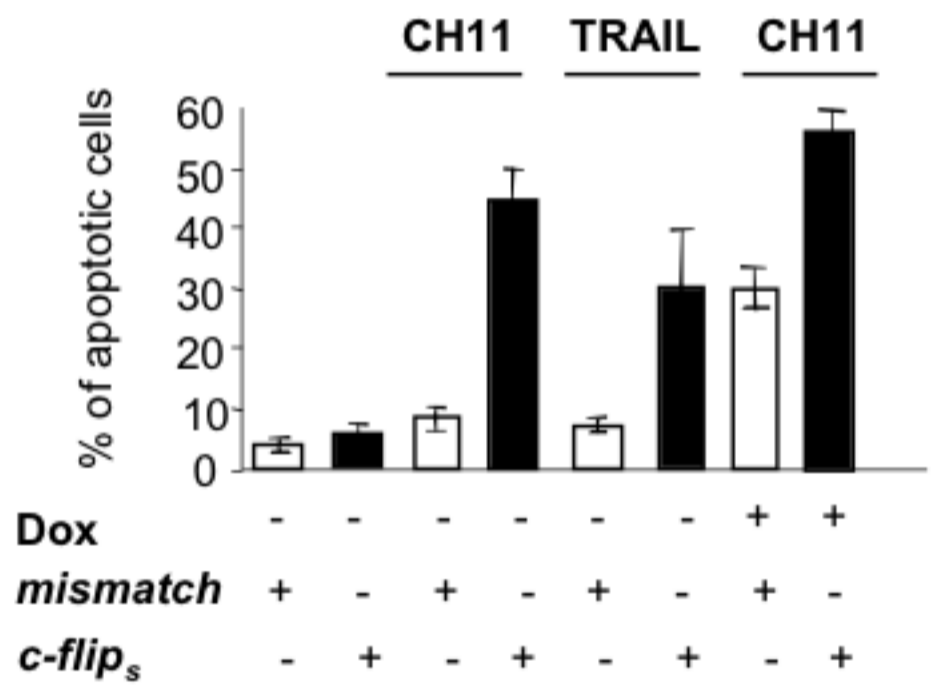
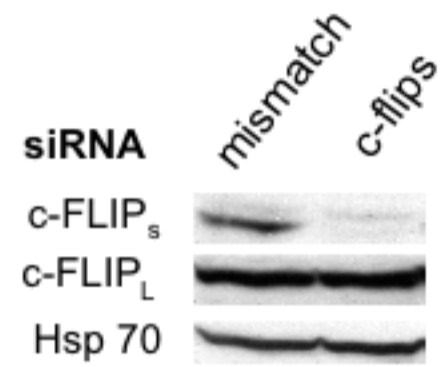


Figure 6

a



b



Condition	1	2	3	4	5	6	7	8
Dox	-	-	-	-	-	-	+	+
mismatch	+	-	+	-	+	-	+	-
c-flip _s	-	+	-	+	-	+	-	+

Figure 7

