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1 **E2F1 controls alternative splicing pattern of genes involved in apoptosis**
2 **through upregulation of the splicing factor SC35.**

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23

1 **Abstract**

2

3 The transcription factor E2F1 plays a key role during S phase progression and apoptosis. It has
4 been well-demonstrated that the apoptotic function of E2F1 involves its ability to transactivate
5 pro-apoptotic target genes. Alternative splicing of pre-mRNAs also plays an important role in the
6 regulation of apoptosis. In this study, we identify the splicing factor SC35, a member of the Ser-
7 Rich Arg (SR) proteins family, as a new transcriptional target of E2F1. We demonstrate that
8 E2F1 requires SC35 to switch the alternative splicing profile of various apoptotic genes such as
9 *c-flip*, *caspases-8* and *-9* and *Bcl-x*, towards the expression of pro-apoptotic splice variants.
10 Finally, we provide evidence that E2F1 upregulates SC35 in response to DNA damaging agents
11 and show that SC35 is required for apoptosis in response to these drugs. Taken together, these
12 results demonstrate that E2F1 controls pre-mRNA processing events to induce apoptosis and
13 identify the SC35 SR protein as a key direct E2F1-target in this setting.

14

15 **Keywords:** Alternative splicing/Apoptosis/E2F1/SC35/SR proteins

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1 Introduction

2
3 Pre-mRNA splicing is an essential step for the expression of most genes in higher
4 eukaryotic cells. This process has emerged as an important mechanism of genetic diversity since
5 about 74% of human genes undergo alternative splicing, leading to the production of various
6 protein isoforms ¹. SC35 belongs to the serine/arginine-rich (SR) protein family, one of the most
7 important class of splicing regulators. Members of the SR family have a modular structure
8 consisting of one or two copies of an N-terminal RRM (RNA-recognition motif) followed by a C
9 terminus rich in serine and arginine residues known as the RS domain. They act at multiple steps
10 of spliceosome assembly and participate in both constitutive and alternative splicing ². Together
11 with most of the other splicing factors, SR proteins localize to nuclear subregions termed nuclear
12 speckles ³. Extensive serine phosphorylation of the RS domain plays an important role in the
13 regulation of both the localization and the activities of SR proteins ⁴. While the splicing functions
14 of SR proteins have been well documented "*in vitro*", their roles and physiological targets "*in*
15 *vivo*" are less well known. However, based on gene targeting experiments demonstrating that
16 they are required for cell viability and/or animal development, SR proteins undoubtedly control
17 essential biological functions.

18 Apoptosis is one of the cellular processes in which alternative splicing plays an important
19 regulatory role. Indeed, a remarkable number of transcripts that encode proteins involved in the
20 apoptotic pathway are subjected to alternative splicing. This usually drives the expression of
21 proteins with opposite functions, either pro- or anti-apoptotic ⁵. Interestingly, changes in SR
22 protein phosphorylation have been observed upon apoptotic stimulation following activation of
23 the Fas receptor ⁶. In addition, "*in vitro*" overexpression experiments have suggested a potential

1 role for SR proteins in the control of the splicing of pre-mRNAs encoding apoptotic regulators ⁷⁻
2 ⁸. Moreover, depletion of the ASF/SF2 SR protein has been reported to induce apoptosis ⁸⁻⁹.
3 Nevertheless, whether individual SR proteins are necessary to modulate alternative splicing of
4 mRNAs encoding apoptotic factors remains largely unknown, as well as the factors that control
5 expression and/or activity of SR proteins in this context.

6 The E2F1 transcription factor belongs to the E2F family encompassing eight members
7 involved in a diverse array of essential cellular functions ¹⁰. E2F1 is best-known for its role in
8 driving cell cycle progression in S phase. In addition, E2F1 can induce apoptosis by mechanisms
9 involving or not its transcriptional function. We previously demonstrated the ability of E2F1 to
10 trigger apoptosis through caspase-8 activation at the death-inducing signaling complex and
11 showed that E2F1 acts through specific downregulation of the cellular FLICE-inhibitory protein
12 short isoform, c-FLIP_{short} ¹¹. As *c-flip* predominantly encodes two isoforms arising from
13 alternative splicing, namely c-FLIP_{short} and c-FLIP_{long}, we postulated that E2F1 could control the
14 expression and/or activity of some splicing factors. In this study, we identify the SC35 splicing
15 factor as a direct transcriptional target of E2F1 and show that SC35 is involved in the ability of
16 E2F1 to trigger apoptosis through downregulation of c-FLIP_{short}. Importantly, we demonstrate
17 that E2F1 and SC35 also cooperate to switch the alternative splicing pattern of *caspases-8* and *-9*
18 as well as of *Bcl-x*, towards the expression of pro-apoptotic isoforms. Finally, we provide
19 evidence that E2F1 promotes the accumulation of SC35 in cells treated with DNA damaging
20 agents, and show that SC35 is required for induction of apoptosis in this setting. Taken together,
21 these results demonstrate that E2F1 and SC35 proteins control pre-mRNA processing events to
22 promote apoptosis.

23

1 **Results**

2

3 **E2F1 upregulates SC35 protein expression**

4 We previously established a model of stable E2F1-inducible clones in the H358 cell line
5 derived from a human lung adenocarcinoma ¹¹. Here we exploit this model to test whether E2F1
6 can control the expression pattern of three members of the SR protein family, one of the most
7 important class of splicing regulators, namely SC35, SRp20 and SF2/ASF. Immunoblotting
8 experiments demonstrated a 3-4 fold increased expression of SC35 in cells overexpressing E2F1,
9 whereas the total level of SRp20 and SF2/ASF was not affected (Figure 1A, left panel). This
10 effect required the DNA binding activity of E2F1 since overexpression of an E2F1(E132) DNA-
11 binding defective mutant did not affect SC35 protein level (Figure 1A, right panel).

12 To confirm these data, we knocked-down E2F1 expression by using small interfering
13 RNAs (siRNAs) in the H69 and H810 neuroendocrine lung carcinoma cell lines that
14 physiologically express high levels of E2F1, and analyzed SC35, SRp20 and SF2/ASF expression
15 by western blotting. As shown in figure 1B, the silencing of E2F1 was accompanied by marked
16 downregulation of the endogenous SC35 protein in both cell lines as compared to cells
17 transfected with *mismatch* siRNA. In contrast, the expression of SRp20 and SF2/ASF proteins
18 was not affected. In addition, we observed that SC35 protein levels were strongly reduced in
19 E2F1 knock-out MEF as compared to wild-type MEF, whereas those of SRp20 and SF2/ASF did
20 not change (Figure 1C). Taken together, these data demonstrate that SC35 expression is
21 positively regulated by E2F1.

22

23

1 **SC35 is a direct transcriptional target of E2F1**

2 To test whether E2F1 controls SC35 expression at the transcriptional level, we performed
3 RT-PCR experiments using primers localized at the 5'-end of the *sc35* ORF. The data showed
4 that expression of *sc35* mRNA was induced by E2F1 but not by the mutant E2F1(E132) (Figure
5 2A). To go further, we performed Chloramphenicol Acetyl Transferase (CAT) experiments using
6 a pR264-CAT plasmid that contains the 1kb human *sc35* promoter upstream of CAT cDNA ¹².
7 Co-transfection of H1299 (Figure 2B) or SAOS2 (data not shown) cells with pR264-CAT vector
8 and increasing amounts of an E2F1 expression vector resulted in a dose-dependent increase of
9 CAT activity. These results indicated that E2F1 can transactivate the promoter of *sc35*. To
10 confirm these data “*in vivo*”, we performed chromatin immunoprecipitation (ChIP) experiments
11 in the H358/Tet-On/E2F1 cells cultured in the presence of doxycylin (Figure 2C, upper panel).
12 As a positive control of ChIP assays, we used the proximal human *Skp2* promoter that was
13 recently reported as a target of E2F1 ¹³. In our conditions, binding of E2F1 to the proximal *Skp2*
14 gene promoter was clearly detected whereas no binding was observed with the *Gapdh* promoter
15 (Figure 2C, upper panel). Importantly, the *sc35* promoter fragment (-296/-79) that encompasses
16 two putative E2F1 binding sites was precipitated by an anti-E2F1 antibody (Figure 2C, upper
17 panel). In order to confirm that SC35 was a direct target of E2F1, endogenous E2F1 was
18 immunoprecipitated from H1299 cells and ChIP experiments were performed. As shown (Figure
19 2C, lower panel), endogenous E2F1 clearly bound to the *sc35* promoter in these cells. Taken
20 together, these results identify *sc35* as a direct transcriptional target of E2F1.

21

1 **E2F1 promotes SC35-dependent apoptosis through modulation of FLIP_{long}/FLIP_{short}**
2 **ratio**

3 As we previously demonstrated the ability of E2F1 to induce apoptosis through
4 downregulation of the c-FLIP_{short} protein isoform ¹¹, we next analyzed whether SC35 plays a role
5 in this setting. By the use of *sc35* siRNAs and Hoechst staining, we firstly observed that
6 neutralization of SC35 strongly reduced the number of apoptotic cells in response to E2F1
7 (Figure 3A). Therefore, these results indicated that SC35 is required for E2F1-induced apoptosis.
8 Importantly, RT-PCR (Figure 3B, left panel) and immunoblot analyses (Figure 3B, right panel)
9 revealed that downregulation of both *flip_{short}* mRNA and protein levels was also prevented in
10 these conditions. A high level of both c-FLIP protein isoforms has been found in many tumor
11 cells and was correlated with resistance to FAS- and TRAIL-induced apoptosis, two death
12 receptor ligands ¹⁴. Consistently, we previously reported that the downregulation of c-FLIP_{Short}
13 by E2F1 was sufficient to restore the sensitivity of tumor cells to these ligands ¹¹. In this study,
14 we showed that neutralization of *sc35* expression overrode the ability of E2F1 to sensitize H358
15 cells to FasL (Figure 3C) as well as to TRAIL (data not shown) treatments. Collectively, our data
16 demonstrate that E2F1 induces apoptosis through SC35-dependent modulation of the FLIP_{Long}
17 /FLIP_{Short} ratio, at the expense of the FLIP_{Short} protein isoform.

18

19 **E2F1 and SC35 co-regulate the splicing pattern of *caspase-8*, *caspase-9* and *Bcl-x***
20 **pre-mRNAs in favor of pro-apoptotic splice variants**

21 As the expression of numerous apoptotic genes is regulated by pre-mRNA alternative
22 splicing ⁵, we undertook a series of experiments to test whether E2F1 and SC35 can also affect
23 the ratio of other apoptotic splice variants. Various *caspases* are subjected to alternative splicing.

1 Alternative splicing of *casp-2* proceeds through selective insertion or removal of exon 9 giving
2 rise to anti-apoptotic caspase-2S and pro-apoptotic caspase-2L isoforms-respectively ⁷. The use
3 of a distant splice donor site at the 3'-end of exon 8 of human *caspase-8* pre-mRNA leads to the
4 synthesis of an alternative splice variant, *caspase-8L*, a competitive inhibitor of caspase-8 ¹⁵⁻¹⁶.
5 The inclusion or exclusion of an exon cassette in *caspase-9* causes the expression of two splice
6 variants, namely the pro-apoptotic *caspase-9a* and anti-apoptotic *caspase-9b* ¹⁷⁻¹⁸. To assess
7 whether E2F1 induces changes in the alternative splicing profile of these *caspases* pre-mRNAs,
8 RNAs recovered from non-induced or induced H358/Tet-On/E2F1 cells were analyzed by RT-
9 PCR using primers specific for each caspase splice variant (Figure 4A). The results showed that
10 E2F1 increased the expression of pro-apoptotic *caspases-2L*, *-8a* and *-9a* mRNA levels and
11 decreased those of anti-apoptotic *caspases-8L* and *-9b* (Figure 4B). In contrast, these effects were
12 not observed with mutant E2F1(E132). Interestingly, we were unable to detect the *caspase-2S*
13 transcript in our cells. Altogether, these data indicate that E2F1 can switch the splicing pattern of
14 *caspases-8* and *-9* in favor of pro-apoptotic isoforms. Therefore, besides its ability to
15 transactivate *caspases-8* and *-9* genes ¹⁹, E2F1 also controls their alternative splicing.

16 *Bcl-x* is a member of the *bcl-2* gene family that also plays a key role in apoptosis. Several
17 splice isoforms of *Bcl-x* have been reported. The use of a 5' proximal site generates the Bcl-x_L
18 long isoform which protects cells against apoptosis. In contrast, the use of a 5' distal site results
19 in the synthesis of a short pro-apoptotic Bcl-x_S isoform ²⁰. To analyze whether E2F1 regulates
20 the splicing pattern of Bcl-x, we performed RT-PCR analysis with specific primers in H358/Tet-
21 On/E2F1 cells (Figure 4A). The results showed that expression of E2F1 induced a concomitant
22 decrease of Bcl-x_L and increase of Bcl-x_S mRNA levels (Figure 4C, upper panel). As was
23 observed for *caspase* regulation, the mutant E2F1(E132) had no effect on *bcl-x* splicing. Western
24 blotting with Bcl-x antibodies specific for each isoform confirmed the RT-PCR results (Figure

1 4C, lower panel). Therefore, these data demonstrate that E2F1 also controls the alternative
2 splicing of Bcl-x.

3 Finally, we investigated whether SC35 was involved in these effects. siRNAs targeting
4 *sc35* were transfected in H358/Tet-On/E2F1 cells, and expression of *caspases* and *Bcl-x* splice
5 variants was analyzed by RT-PCR. In the absence of E2F1 induction, the knock-down of *sc35*
6 using two independent siRNAs (Figure 4D and Supplementary Figure 1) did not significantly
7 alter the level of *caspases-8L*, *-8a*, *-9a* or *-9b* mRNAs, nor that of *Bcl-x_L* or *Bcl-x_S*. Importantly,
8 the induction of E2F1 expression in these cells deprived of SC35 did not affect the splicing
9 pattern of these genes (Figure 4D and Supplementary Figure 1). Furthermore, when we
10 performed RT-PCR analyses in A549 cells transiently transfected with a vector encoding SC35,
11 we found that overexpression of SC35 affected the splicing profile of *caspase-8*, *-9* and *Bcl-x*
12 pre-mRNAs in a similar way than did E2F1 (compare Figure 4E to Figures 4B and C) and
13 induced apoptosis (data not shown). Altogether, these results demonstrate that E2F1 requires
14 SC35 to regulate the pre-mRNA alternative splicing of apoptotic genes.

15
16 **E2F1 and SC35 proteins are upregulated and required for apoptosis in response to**
17 **genotoxic stresses**

18 So far, our results demonstrate the ability of overexpressed E2F1 and SC35 to modify the
19 splicing pattern of various apoptotic genes. Thus, we next attempted to identify in which
20 physiological context both proteins could cooperate to induce apoptosis. It is now well-known
21 that DNA damaging agents stabilize E2F1 and induce its transcriptional activity towards
22 apoptotic genes thereby causing apoptosis²¹⁻²². In agreement with previous reports, treatment of
23 H358 cells with either methylmethanesulfonate (MMS) or cyclophosphamide, two alkylating

1 agents that create inter-strand DNA crosslinks, significantly increased E2F1 expression as
2 detected by immunoblotting (Figure 5A, upper panel). In these conditions, upregulation of E2F1
3 was accompanied by an increase of SC35 protein and mRNA expression (Figure 5A, upper
4 panels), as well as by the induction of apoptosis (Figure 5A, lower panel). In cyclophosphamide-
5 treated cells, the neutralization of E2F1 using siRNAs prevented the accumulation of SC35
6 (Figure 5B), indicating that E2F1 was involved in SC35 induction. In addition, as detected by
7 ChIP experiments, the binding of endogenous E2F1 to the *sc35* promoter was increased
8 following drug treatment, indicating that SC35 is a direct target of E2F1 in response to DNA
9 damaging agents (Figure 5C). Furthermore, inhibiting SC35 expression strongly repressed
10 apoptosis following cyclophosphamide treatment, as detected by immunoblotting of pro- or
11 cleaved caspase-3 and Hoechst 33342 staining (Figure 5D). Taken together, these results
12 demonstrate that E2F1 requires SC35 to induce apoptosis in response to genotoxic stresses.

13 Finally, we analyzed whether E2F1 and SC35 acted through regulation of the alternative
14 splicing of apoptotic genes and studied the expression pattern of *caspases -2, -8 and -9* as well as
15 *Bcl-x* splice variants by RT-PCR following cyclophosphamide treatment. As shown in Figure 6A
16 (left panel), an increase of pro-apoptotic *caspase-9a* and *Bcl-x_S* mRNA levels was observed in
17 cyclophosphamide-treated cells together with a decrease of anti-apoptotic *caspase-9b* and *Bcl-x_L*
18 splice variants. Again, immunoblotting experiments confirmed the downregulation and
19 upregulation of Bcl-x_L and Bcl-x_S proteins, respectively (Figure 6A, right panel). In contrast,
20 accumulation of *caspase-2L* and of both anti- and pro-apoptotic *caspase-8L* and *-8a* transcripts
21 was detected in cyclophosphamide-treated cells (Figure 6A, left panel). Therefore, these results
22 demonstrate that cyclophosphamide modifies the alternative splicing pattern of *Bcl-x* and
23 *caspase-9* pre-mRNAs in favor of pro-apoptotic splice variants. Importantly, the neutralization of
24 either *e2f1* or *sc35* before cyclophosphamide cell treatment prevented these modifications, as

1 detected by RT-PCR (Figure 6B, upper panel) and western blotting (Figure 6B, lower panel).
2 Overall, these data strongly suggest that E2F1 and SC35 proteins act together during apoptosis of
3 DNA-damaged cells by controlling *Bcl-x* and *caspase-9* pre-mRNAs alternative splicing.
4

5 **Discussion**

6

7 E2F1 is a transcription factor that plays a critical role in cell cycle progression by favoring
8 entry into S phase. Besides its role in cell cycle control, E2F1 is also widely accepted as an
9 inducer of apoptosis. It has been well-demonstrated that E2F1 promotes apoptosis through both
10 transcription-dependent and -independent mechanisms. So far, numerous apoptotic genes whose
11 transcription is enhanced by E2F1 have been identified ¹⁰. In this study, we show that E2F1
12 switches the alternative splicing pattern of key apoptotic genes in favor of their pro-apoptotic
13 splice variants, and identify the SC35 protein, a member of the SR family of splicing regulators,
14 as a key direct target of E2F1. Interestingly, two high throughput studies using ChIP on Chip
15 analysis ²³ or DNA microarrays ²⁴ previously suggested that SC35 is an E2F1-regulated gene. In
16 this study, we provide the first evidence of a direct functional link between E2F1 and SC35
17 proteins to regulate cellular apoptosis. Therefore, besides its ability to transactivate pro-apoptotic
18 target genes, E2F1 is also able to control pre-mRNA processing events to induce apoptosis.

19 It has now emerged from the literature that splicing not only depends on the interaction of
20 splicing factors with their target pre-mRNAs, but is also coupled to transcription ²⁵. Indeed,
21 variations of pol II promoter structure can lead to differences in alternative splicing of the
22 transcript ²⁶⁻²⁷ and components of the spliceosome such as the p54nrb (p54 nuclear RNA binding
23 protein) and PSF (polypyrimidine tract-binding protein-associated splicing factor) RNA binding

1 proteins are involved in both transcription and splicing processes ²⁸ (for review). In addition,
2 transcriptional coregulators of the nuclear receptor family recruited at the promoter level not only
3 enhance the transcriptional activity of this promoter, but also affect the nature of the splice
4 variants produced ²⁸⁻²⁹⁻³⁰. Moreover, some transcription factors have been reported to bind to
5 proteins of the spliceosome and/or display dual functions in splicing and transcription ³¹⁻³².
6 Taken together, these results indicate a role for proteins controlling transcription in splicing
7 regulation. In this study, we demonstrate that the transcription factor E2F1 transactivates the
8 expression of *sc35*, a component of the spliceosome and that both proteins regulate pre-mRNA
9 processing events. Altogether, these results strengthen the connection between the transcriptional
10 and splicing machineries. We show that E2F1 alters the splicing pattern of some of its
11 transcriptional targets such as *c-flip* (Figure 3B), *caspases-8* and *-9* (Figures 4B & 4D). As an
12 increased level of *caspases-8* and *-9* transcripts was also detected in this context, it is possible
13 that the transactivating functions of E2F1 cooperate with SC35 accumulation to regulate pre-
14 mRNA processing events. Interestingly, the expression of *c-flip* mRNA was apparently not
15 affected by E2F1 in the same conditions (Figure 3A). It was recently shown that the Spi-1/PU.1
16 transcription factor could modify alternative splicing of a transcriptional target gene, without
17 modulation of its mRNA transcription ³². Therefore, another but not exclusive possibility is that
18 E2F1 acts as a scaffold protein to drive SC35 to the nascent transcribed RNA of some of its target
19 genes, according to the cell-specific promoter occupation model ²⁵.

20 Apoptosis is one of the cellular processes in which alternative splicing plays important
21 regulatory roles ⁵. Several components of the splicing machinery have already been implicated in
22 apoptotic processes. For example, depletion of SF2/ASF ⁸ induces apoptosis. In addition,
23 overexpression of SC35 alters the splicing of *caspase-2* mRNA, in favor of the pro-apoptotic
24 isoform accumulation ⁷. Furthermore, phosphorylation of SR proteins, which is known to control

1 their sub-cellular localization as well as their activities, has been reported during apoptosis⁶⁻³³.
2 However, the upstream signaling molecules that regulate the expression and/or activity of SR
3 proteins during apoptosis, as well as the endogenous targets of SR proteins in this context remain
4 largely unknown. In this study, we provide evidence that E2F1 triggers apoptosis through SC35
5 accumulation and we demonstrate that both proteins cooperate to affect the splicing pattern of
6 *caspase-8*, *caspase-9*, *flip* and *bcl-x* genes, in favor of pro-apoptotic splice variants. Taken
7 together, our data identify SC35 as a new mediator of E2F1-induced apoptosis and identify some
8 of its endogenous targets in this setting.

9 Alteration of alternative splicing is believed to contribute to the resistance of tumor cells
10 to chemotherapy, notably through overexpression of anti-apoptotic splice variants³⁴⁻³⁵. However,
11 the molecular mechanisms involved in such processes remain largely unknown. Importantly, and
12 consistent with a role of SR proteins during the response to DNA damage, it has been previously
13 shown that SC35 accumulates following γ -irradiation³⁶. In addition, it was recently reported that
14 the expression of SRp55, another member of the SR protein family, is upregulated and required
15 for apoptosis of p53-deficient cells after mitomycin C treatment³⁷. In this study, we demonstrate
16 that SC35 is upregulated by an E2F1-dependent pathway in response to methylmethanesulfonate
17 or cyclophosphamide treatment. Furthermore, we show that the E2F1 and SC35 proteins are
18 required for apoptosis of DNA-damaged cells and control *Bcl-x* and *caspase-9* pre-mRNAs
19 alternative splicing at the expense of anti-apoptotic splice variants. Therefore, our data strongly
20 suggest that E2F1 and SC35 are key-determinants of the cellular response to chemotherapeutic
21 agents.

22 To conclude, we highlight the first functional connection between the transcription factor
23 E2F1 and a component of the splicing machinery, SC35, in the control of cellular apoptosis. It is
24 well-known that abnormalities in E2F signalling pathways contribute to tumorigenesis³⁸.

1 Accordingly, we previously described a differential pattern of E2F1 protein expression in human
2 lung tumors ³⁹. Interestingly, some SR proteins are overexpressed in ovarian cancer ⁴⁰ and
3 SF2/ASF was recently assessed as a proto-oncogene in human tumors ⁴¹. Therefore, it remains to
4 be determined whether alterations of both E2F1 and SC35 proteins could cooperate to promote
5 carcinogenesis.

6

7 **Materials and Methods**

8

9 **Cell lines, treatment, apoptotic assay, plasmids and transfection**

10 A549, H358, H1299 and H69 human lung carcinoma cell lines were cultured as
11 previously described ⁴². The H810 large cell neuroendocrine lung carcinoma cell line was
12 cultured in 5% CO₂ at 37°C in RPMI-1640 medium supplemented with 5% (v/v) FCS, 0.005
13 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-
14 estradiol and 10 mM HEPES. Murine embryonic fibroblasts (MEF) wild-type and E2F1 -/- were
15 cultured in DMEM (GIBCO) supplemented with 10%(v/v) heat-inactivated FCS. The human
16 lung adenocarcinoma H358/Tet-On/E2F1 and Tet-On/E2F1(E132) inducible clones were
17 obtained as previously described ¹¹. Apoptosis was evaluated by scoring the percentage of
18 apoptotic cells on 500 cells after Hoechst 33342 staining. Transient transfections were carried out
19 using Fugene 6 (Roche Diagnostic). Plasmids used in transient transfections were pcDNA3,
20 pCMV-E2F-1, pcDNA3-HA-SC35 and pR264-CAT. Recombinant soluble human FLAG-tagged
21 FasL was purchased from Alexis (San Diego, CA, USA). Methanesulfonic acid methyl ester
22 (MMS) and cyclophosphamide monohydrate were all purchased from Sigma (Saint Quentin
23 Fallavier, France).

1

2 **Antibodies**

3 The anti-E2F1 (C-20), anti-SC35 (H-55) and anti-Bcl-x_L (H5) antibodies were purchased
4 from Santa Cruz, the anti-Bcl-x_S (Ab-1) from Oncogene Research, the anti-E2F1 (KH95) and
5 anti-procaspase-3 from Pharmingen, the anti-FLIP (NF6) from Alexis, the anti-actin (20-33) from
6 Sigma, the anti-SC35 (4F-11) from Euromedex and the anti-SRp20 (7B4) and anti-SF2/ASF
7 from Zymed. Cleaved caspase-3 (Asp175) was from Cell Signaling.

8

9 **CAT assays**

10 For CAT assay measurements, 2×10^5 cells per well were seeded in duplicate in 6-well
11 plates, and transfected with the pR264CAT plasmid in the presence or absence of increasing
12 amounts of pCMV-E2F1 vector. pR364CAT vector contains the 1kb human *sc35* promoter¹²
13 and encompasses two putative E2F1 binding sites at -170 (TTTGGCCCG) and -236
14 (TTTCGCGGG) bp upstream of the transcription start site. Transfection was performed using
15 Fugene 6 according to the manufacturer's instructions, and CAT activity was measured 24-48
16 hours after transfection using CAT ELISA (Roche Diagnostic). CAT activity was then
17 normalized in each sample according to the protein amount.

18

19 **Chromatin immunoprecipitation experiments**

20 Chromatin immunoprecipitation experiments were performed in H358 cells, H1299 cells
21 or H358/Tet-On/E2F1 cells cultured in the presence of 1 µg/ml doxycylin for 24 hours. An equal
22 amount of chromatin (25 µg) was precleared and immunoprecipitated with a polyclonal antibody
23 specific for E2F1 (C-20, Santa Cruz) or unrelated rabbit IgG or no antibody, overnight at +4°C.

1 Co-immunoprecipitated chromatin was analyzed for the presence of *sc35* promoter DNA
2 between -296 and -79 bp, -upstream of the *sc35* transcription start site, -by semiquantitative
3 PCR. This fragment encompasses two putative E2F1 binding sites at -170 and -236 bp upstream
4 of the transcription start site. The primers used were as follow: forward 5'-
5 GAGCACCTCCTCTTCCTCCTG-3' and reverse 5'-CCGGAAATGAAACCTTCTGA-3'. PCR
6 conditions were 94°C for 3 min, (94°C 30 sec, 55°C 30 sec, 72°C 30 sec) for 36 cycles, and 72°C
7 for 10 min. For Skp2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter
8 analysis, the specific primers used were SKP2-95 5'-CTCCCCGCCTACCCCGTGG-3', SKP2-
9 +135 5'-CAGACCCGCTAAGCCTAGCAACG-3', GAPDH forward 5'-
10 AGCTCAGGCCTCAAGACCTT-3' and GAPDH reverse 5'-AAGAAGATGCGGCTGACTGT-
11 3' as previously described ¹³. PCR conditions were 94°C for 3 min, (94°C 30 sec, 57°C for
12 gapdh or 63°C for Skp2 30 sec, 72°C 30 sec) for 36 cycles, and 72°C for 10 min. Signals
13 obtained on ethidium bromide stained gels were quantified using ImageJ software and each CHIP
14 DNA sample was normalized according to the corresponding input DNA sample.

15

16 **Transfection of siRNA oligonucleotides**

17 The sequences designed to specifically target human *sc35* and *e2f1* RNAs were as follow:
18 *sc35*(1): 5'-GCGUCUUCGAGAAGUACGGTT-3'; *sc35*(2): 5'-
19 UCGUUCGCUUUCACGACAATT-3'; *e2f-1*(1): 5'-GUCACGCUAUGAGACCUCATT-3';
20 *e2f1*(2): 5'-ACAAGGCCCGAUCGAUGUUTT-3'. The second siRNA targeting *sc35* was from
21 Invitrogen (Stealth Select RNAi, SFRS2, Invitrogen). The scrambled siRNA oligonucleotides
22 used as control for all RNA interference experiments were as follow: 5'-
23 UCGGCUCUUACGCAUUCAATT-3' and 5'-CAAGAAAGGCCAGUCCAAGTT-3'. Cells

1 were transfected with siRNA oligonucleotides duplex using Oligofectamine reagent according to
2 the manufacturer's instruction (Invitrogen). Doxycyclin (1 µg/ml) was added or not in the culture
3 medium 4 hours after transfection. The cells were analyzed 48 or 72 hours post-transfection. For
4 experiments with cyclophosphamide, cells were transfected for 48 hours with *mismatch*, *sc35* or
5 *e2f-1* siRNAs, then cyclophosphamide (50 µM) was added in the culture medium for 24
6 additional hours.

7

8 **RT/PCR analyses of alternative splice transcripts**

9 Total cellular RNAs were isolated using Trizol reagent (Invitrogen). In all condition, 1 µg
10 of total RNA was reversed transcribed using oligo(dT) primer and MMLV reverse transcriptase
11 (Invitrogen) according to the manufacturer's protocol. The different primer sequences used in
12 this study as well as the PCR conditions are recapitulated in Table 1. Amplification of a cDNA
13 fragment of G3PDH (Invitrogen) was performed in the same PCR reaction as an internal control.
14 PCR products were run on a 1-2% agarose gel and visualized by ethidium bromide staining.

15

16 **Immunoblotting and immunoprecipitation experiments**

17 Immunoblotting experiments were performed as previously described ¹¹.

18

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11

12 Supplementary information is available at Cell Death and Differentiation's website.

13

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11
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13

14 **Titles and legends to figures**

15

16 **Figure 1: SC35 is upregulated following E2F1 induction.**

17 **(A)** H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells were incubated for indicated times in
18 the presence (+) or absence (-) of 1 µg/ml doxycyclin (Dox). Mutant E2F1(E132) is unable to
19 bind DNA. Expression of E2F1, SC35, SRp20 and SF2/ASF proteins was studied by western
20 blotting. Actin was used as a loading control. **(B)** H69 and H810 neuroendocrine lung carcinoma
21 cell lines were transfected for 72h with *mismatch* or *e2f1* siRNAs as indicated and subjected to
22 western blot analyses for the detection of E2F1, SC35, SRp20 and SF2/ASF proteins. Actin was
23 used as a loading control. **(C)** Western blot analyses of E2F1, SC35, SRp20 and SF2 protein

1 expression in E2F1-deficient (E2F1 $-/-$) and wild-type control Murine Embryonic Fibroblasts
2 (MEFs). Actin was used as a loading control.

3

4 **Figure 2: SC35 is a direct transcriptional target of E2F1.**

5 (A) RT-PCR analysis of *sc35* mRNA. H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells
6 were incubated for 48 or 72 hours in the presence (+) or absence (-) of 1 μ g/ml doxycyclin (Dox)
7 as indicated. Total RNAs were extracted as described in the material and methods section.
8 Amplified *g3pdh* was used as an internal control. (B) Chloramphenicol Acetyl Transferase
9 (CAT) experiments were performed in the H1299 cell line co-transfected for 48 hours with 1 μ g
10 pR264CAT plasmid, encoding CAT under the control of the *sc35* promoter, and increasing
11 amounts of pCMV-E2F1 as indicated. The CAT activity obtained in cells transfected with
12 pR264CAT alone was normalized to 1 and a relative CAT activity was then calculated for each
13 condition. Representative data of at least three independent experiments performed in duplicate
14 are shown. (C) *Upper panel*: H358/Tet-On/E2F1 cells cultured in the presence of doxycyclin for
15 48h were processed for ChIP analysis using C-20 antibody for E2F1. The coprecipitated
16 chromatin DNA was analyzed by semiquantitative PCR using pair of primers that amplify the
17 human *sc35*, *skp2* or *gapdh* promoter respectively, as described in the material and methods
18 section. IgG was used as an irrelevant antibody. No Ab means that no antibody was used in this
19 case. Input lane corresponds to PCR reactions containing 1% of total amount of chromatin used
20 in immunoprecipitation reactions. As compared to input amount, 0.13% and 0.3% of chromatin
21 was immunoprecipitated by E2F1 antibody at the *sc35* and *skp2* promoters respectively. *Lower*
22 *panel*: Similar ChIP analyses were performed in H1299 cells in order to detect the binding of
23 endogenous E2F1 to the *sc35* promoter. The input control corresponds to 0.7% of the amount of

1 chromatin used per immunoprecipitation. As compared to input amount, 0.035% of chromatin
2 was immunoprecipitated by E2F1 antibody.

3

4 **Figure 3: SC35 is required for E2F1-induced apoptosis.**

5 H358 Tet-On/E2F1 cells were cultured in the presence (+) or absence (-) of doxycyclin for 72h.

6 (A) H358 Tet-On/E2F1 cells were transfected for 72h either with *mismatch* or *sc35* siRNA.

7 Apoptosis was evaluated using Hoechst staining. Results shown are the mean \pm SD of three

8 independent experiments. (B) H358 Tet-On/E2F1 cells were transfected for 72h with either

9 *mismatch* or *sc35* siRNA and subjected to RT-PCR (left panel) and western blot (right panel)

10 analyses. (C) H358/Tet-On/E2F1 cells were transfected for 48h with either *mismatch* or *sc35*

11 siRNA, cultured with (+) or without (-) doxycyclin as indicated, and treated or not with 25 ng/ml

12 FasL for 20 additional hours. Apoptosis was evaluated as in (A).

13

14 **Figure 4: E2F1 and SC35 cooperate to affect the alternative splicing pattern of *caspase-8*,**
15 ***caspase-9* and *Bcl-x* pre-mRNAs.**

16 (A) Alternative splicing patterns of *caspsases-2*, *-8*, *-9* and *Bcl-x* primary transcripts. Coding and

17 alternative exons are indicated by \blacksquare and \square , respectively. The position of primers, the expected

18 sizes (in nucleotides) and the names of the RT-PCR products corresponding to the different

19 mRNA isoforms are indicated. The ability of each transcript to encode pro-apoptotic (+) or anti-

20 apoptotic (-) protein isoform is depicted on the right. (B, C) H358/Tet-On/E2F1 or H358/Tet-

21 On/E2F1(E132) cells were cultured for 48 hours in the presence (+) or absence (-) of doxycyclin,

22 as indicated. Total RNAs were extracted and subjected to RT-PCR analyses using the specific

23 primers depicted in (A). Representative agarose gels of RT-PCR products corresponding to *casps-*

24 *2*, *casps-8*, *casps-9* and *Bcl-x* splice variants are presented. The position of splice variants (in bp) is

1 shown on the right, and the various splicing isoforms are named on the left of each panel. *g3pdh*
2 was used as an internal control. (C) *Lower panel*: western blot analysis was performed using
3 specific anti-E2F1, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a loading control.
4 Results are representative of three independent experiments. (D) H358/Tet-On/E2F1 cells
5 cultured in the presence (+) or absence (-) of 1 µg/ml doxycyclin were transfected for 72 hours
6 with either *mismatch* or *sc35* siRNA and processed as in (B). (E) A549 human lung
7 adenocarcinoma cells were transiently transfected for 48 hours with 10 µg pcDNA3.1/SC35 or
8 control (Mock) vector and processed as in (B). *Upper panel*: immunoblot demonstrating the
9 accumulation of SC35 in cells transfected with pcDNA3.1/SC35 as compared to mock
10 transfected cells. Actin was used as a loading control.

11
12 **Figure 5: E2F1 and SC35 proteins are upregulated in cells undergoing apoptosis in**
13 **response to DNA damaging agents**

14 (A) H358 cells were treated or not for 24 hours with the indicated cytotoxic agents (50 µM, each)
15 and analyzed for E2F1 and SC35 protein levels by western blotting (upper panel). *sc35* mRNA
16 level was studied by RT-PCR in the same conditions (lower panel). Actin and *g3pdh* were used
17 as internal controls for immunoblotting and RT-PCR experiments, respectively. Apoptosis was
18 evaluated after Hoechst 33342 staining. Results shown are the mean ± SD of three independent
19 experiments. (B) H358 cells were transfected for 48 hours with either *mismatch* or *e2f1* siRNA
20 and treated (+) or not (-) for additional 24 hours with cyclophosphamide (50 µM). Western blot
21 analyses were performed for E2F1 and SC35 detection. (C) *Upper panel*: H358 cells were treated
22 or not for 24 hours with cyclophosphamide (50 µM) and ChIP analyses were performed using C-
23 20 antibody for E2F1 and primers specific for the human *sc35* or *gapdh* promoter respectively.
24 IgG was used as an irrelevant antibody. No Ab means that no antibody was used in this case. The

1 input reaction represents 0.5% of the total chromatin immunoprecipitated for each sample. As
2 compared to input amount, 0.04% and 0.12% of chromatin was immunoprecipitated by E2F1
3 antibody at the *sc35* promoter level in untreated and treated cells respectively. *Lower panel:*
4 Bands obtained with E2F1 ChIP DNA samples were quantified and normalized relative to the
5 corresponding input DNA sample. *Sc35* promoter occupancy in non-treated cells was arbitrarily
6 set at 1. **(D)** H358 cells were transfected for 48 hours with either *mismatch* or *sc35* siRNA and
7 treated (+) or not (-) for 24 additional hours with cyclophosphamide (50 μ M). Apoptosis was
8 evaluated after Hoechst 33342 staining and immunoblotting with anti-caspase-3 or cleaved
9 caspase-3 antibody. Results are the mean \pm SD of three independent experiments.

10

11 **Figure 6: E2F1 and SC35 regulate the alternative splicing pattern of *Bcl-x* and *caspase-9***
12 **pre-mRNAs in response to cyclophosphamide treatment.**

13 **(A)** H358 cells were treated (+) or not (-) for 24 hours with cyclophosphamide (50 μ M). *Left*
14 *panel:* RT-PCR analysis for detection of *caspases -2, -8, -9* and *Bcl-x* splice variants was
15 performed as previously described in Figures 4B and 4C. *Right panel:* western blot analysis was
16 performed using specific anti-SC35, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a
17 loading control. **(B)** H358 cells were transfected for 48 hours with either *mismatch* or *e2f1* or
18 *sc35* siRNA and treated (+) or not (-) for 24 additional hours with cyclophosphamide (50 μ M).
19 RT-PCR (upper panel) and western blot (lower panel) analyses of *Bcl-x* or *caspase-9* splice
20 variants were performed as previously described. *g3pdh* and actin were used as internal controls
21 for RT-PCR and immunoblotting experiments, respectively.

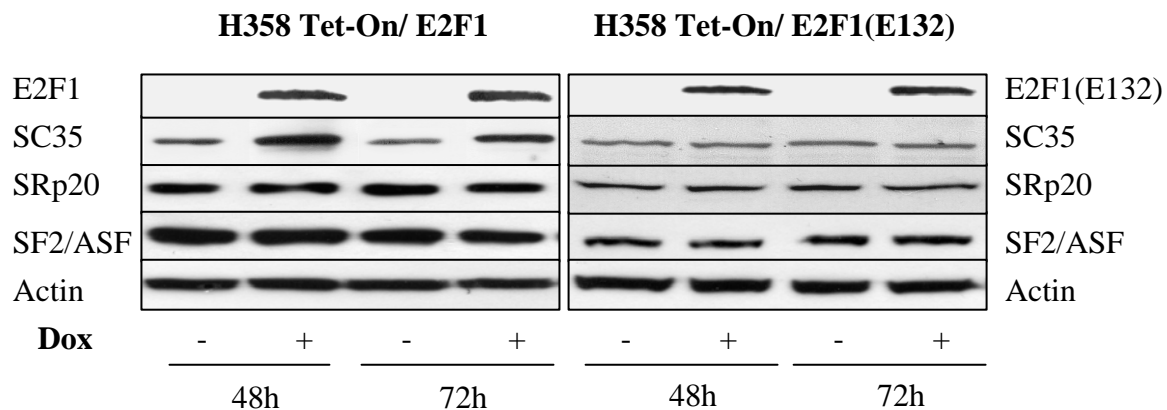
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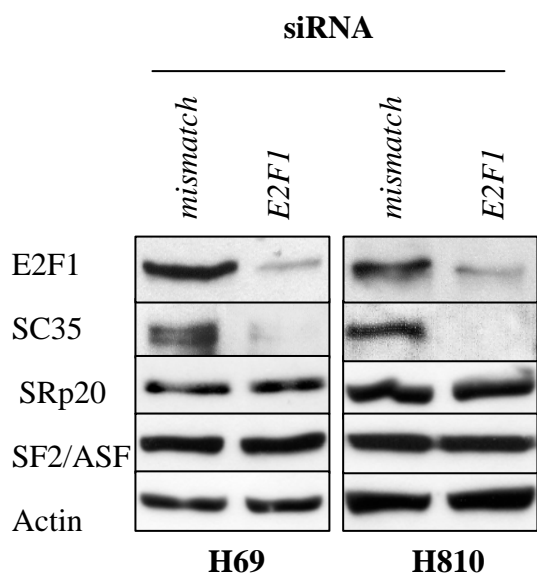
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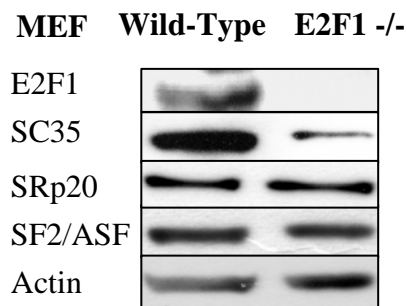
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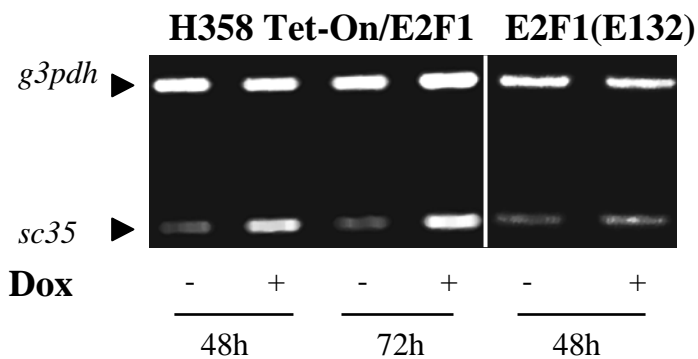
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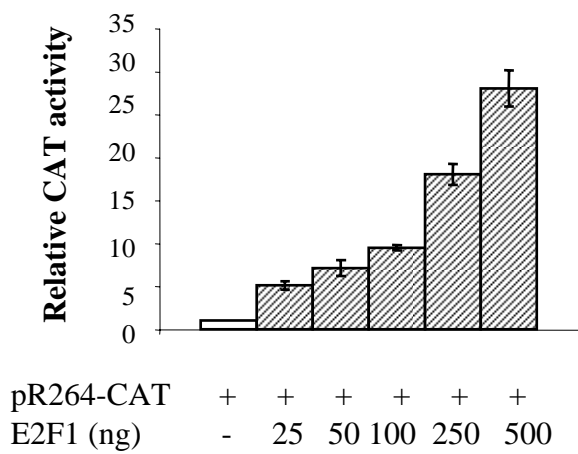
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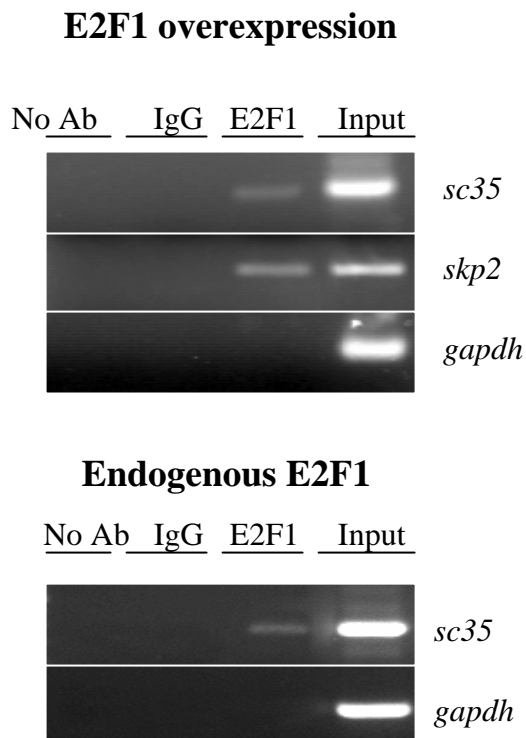
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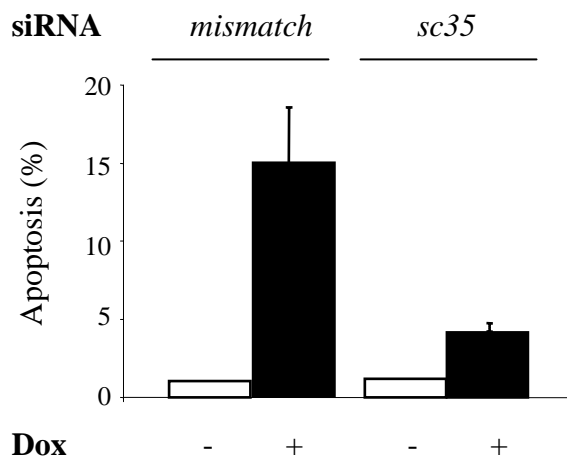
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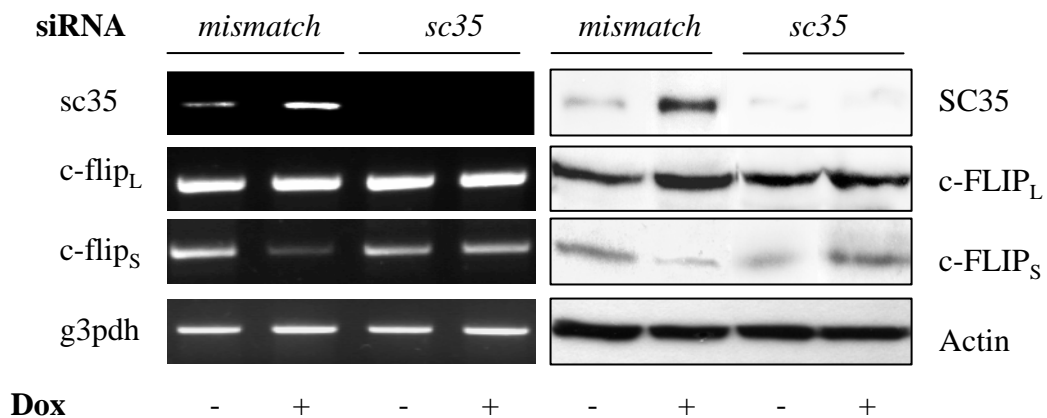
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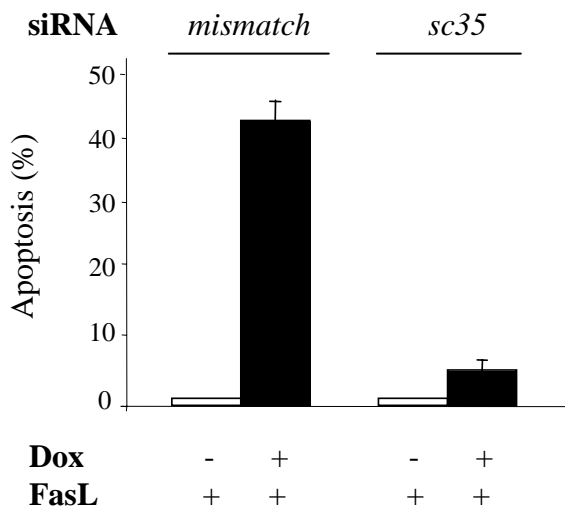
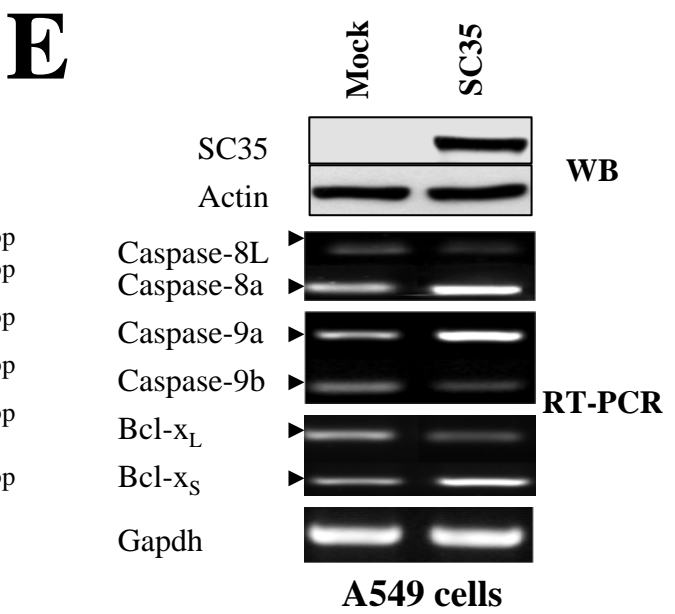
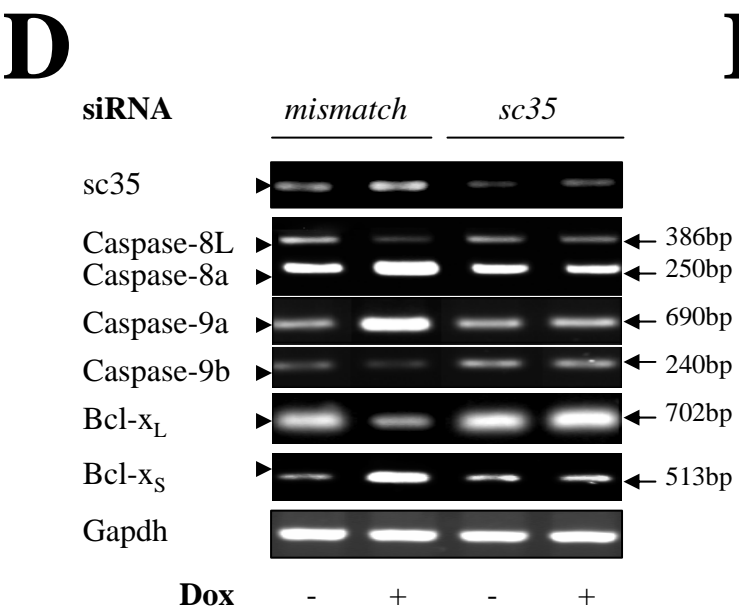
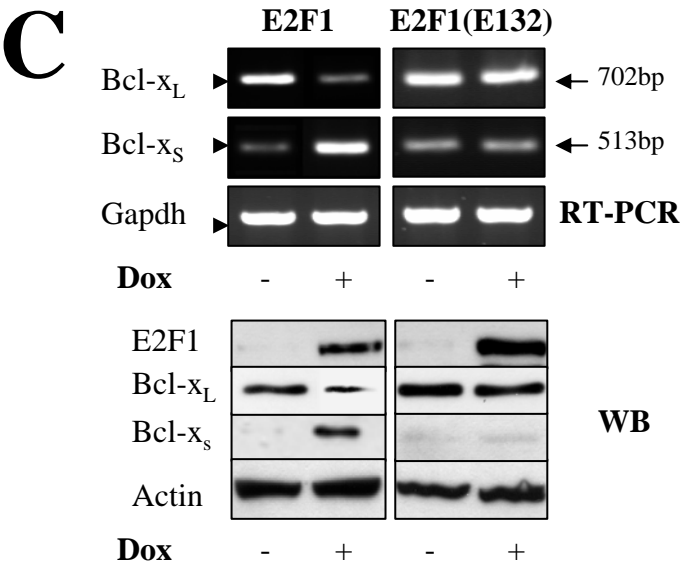
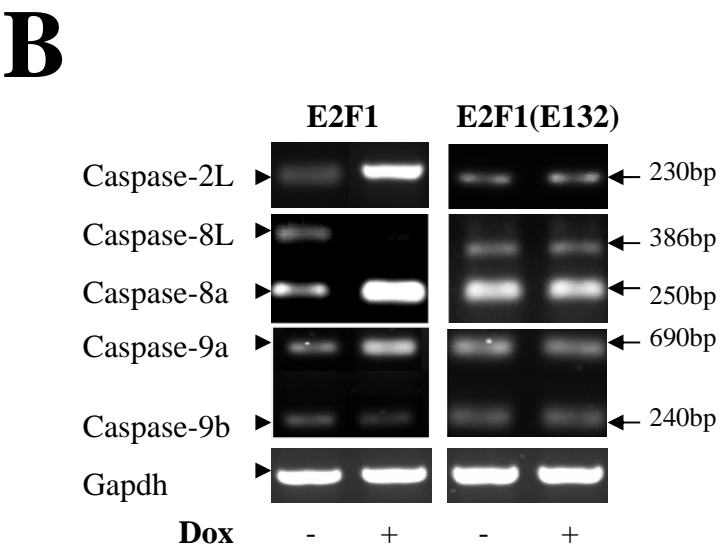
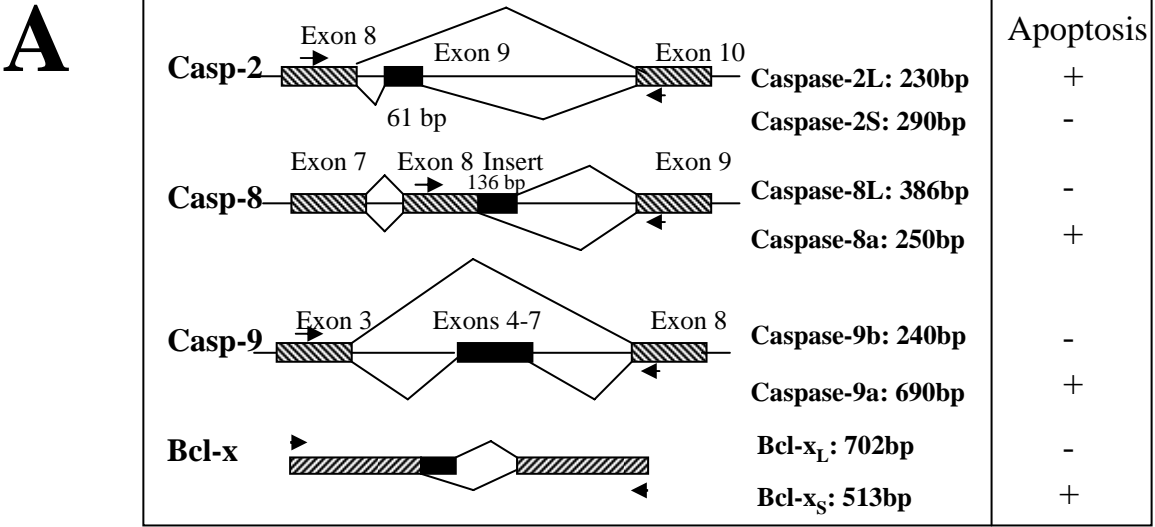
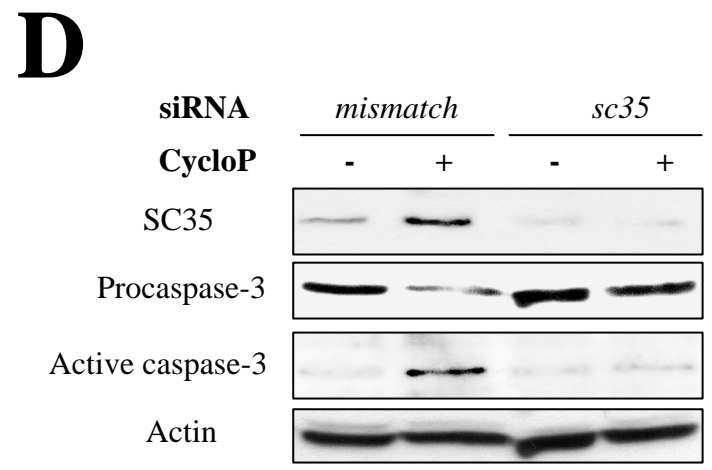
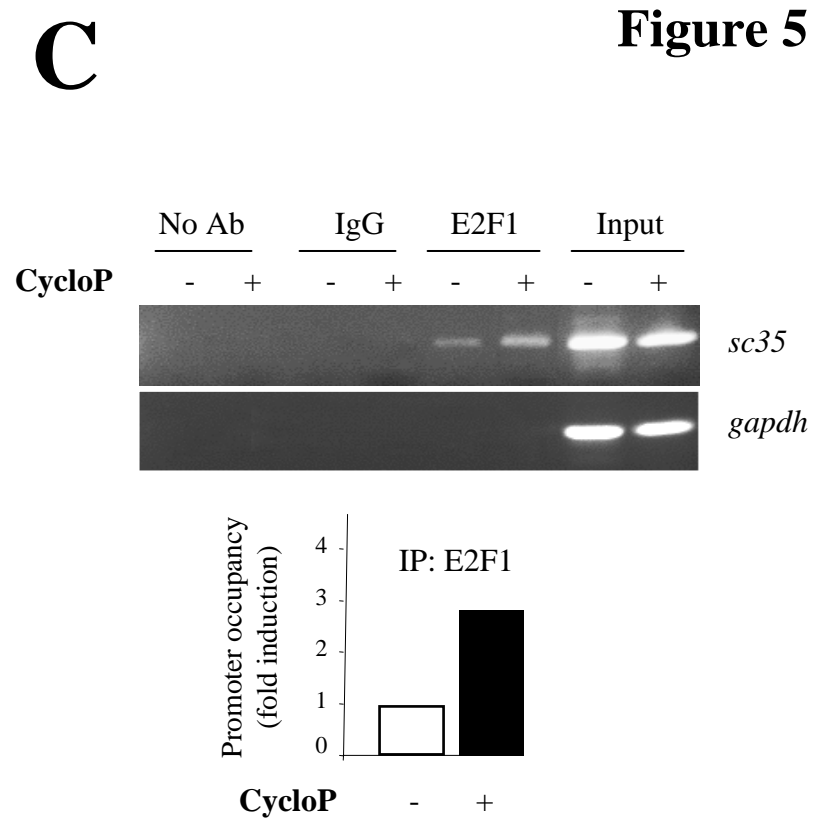
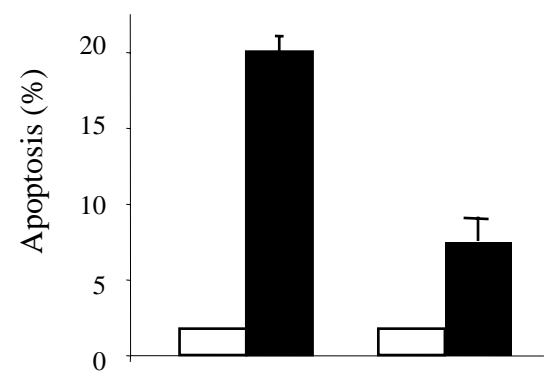
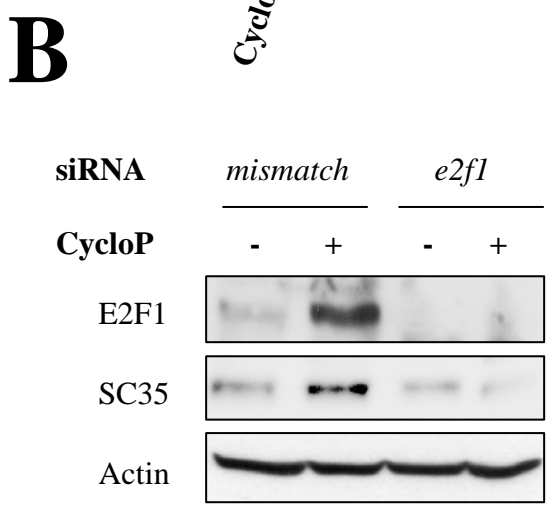
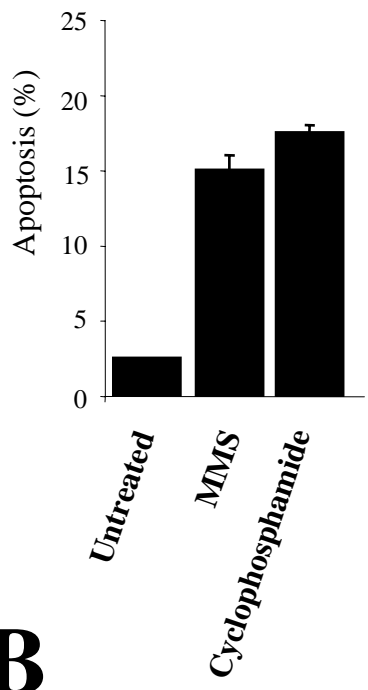
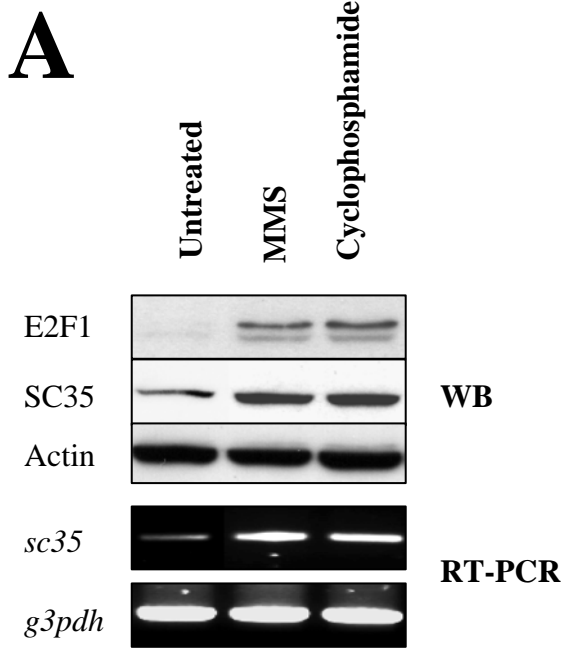
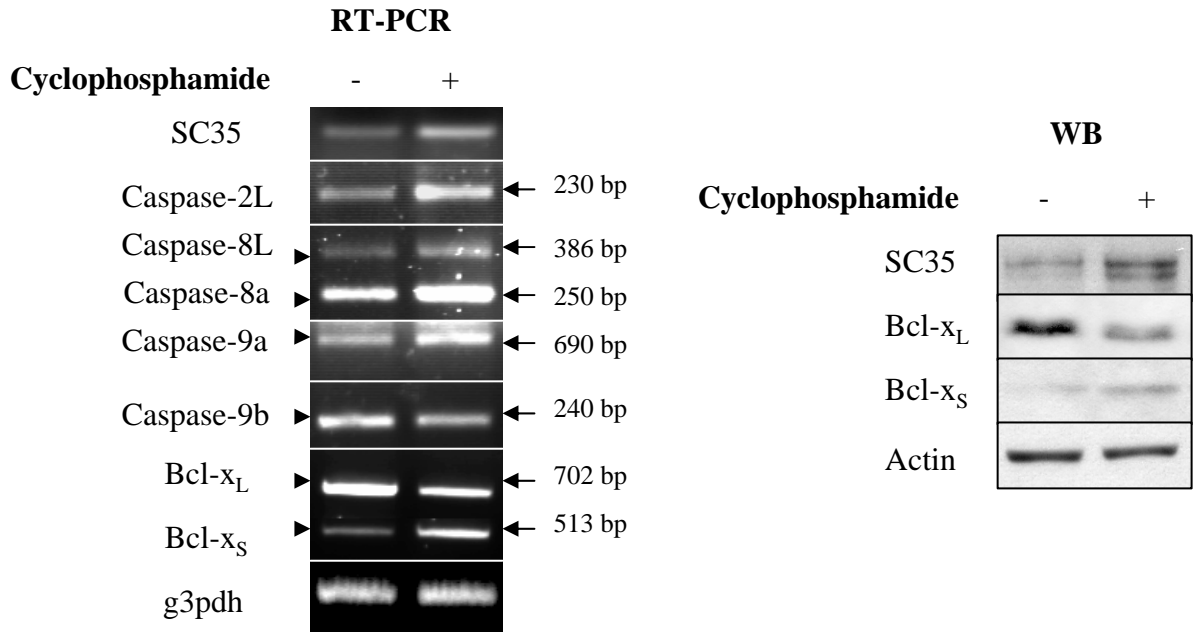


Figure 4





A



B

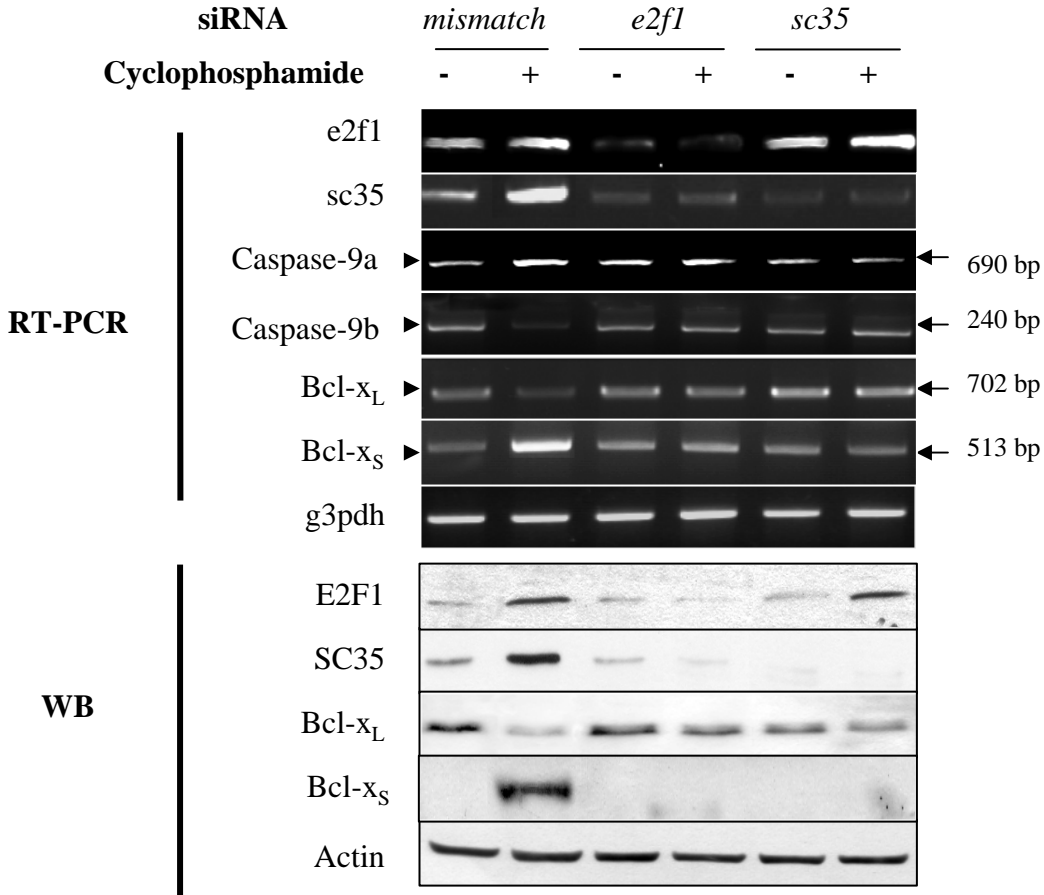


Table 1: Sequences of forward and reverse primers as well as PCR conditions used in this study.

Primer sequences	Forward (5'-3')	Reverse (5'-3')
sc35	CCACTCAGAGCTATGAGCTACG	ACTCCTTGGTGTAGCGATCC
94°C for 30s ; 57°C for 45s; 72° C for 1 min; 30cycles		
flip_{short}	CGAGGCAAGATAAGCAAGGA	CACATGGAACAATTTCCAAGAA
94°C for 30s ; 58°C for 1 min ; 72°C for 2 min ; 30 cycles		
flip_{long}	CTTGGCCAATTTGCCTGTAT	GGCAGAAACTCTGCTGTTCC
94°C for 30s ; 58°C for 1 min ; 72°C for 2 min ; 30 cycles		
caspase-2	TTACCTGCACACCGAGTCAC	TGGTTCTTTCCATCTTGTTGGTC
94°C for 30s; 64°C for 1 min; 72°C for 2 min; 28 cycles		
caspase-8	GGGATACTGTCTGATCATCAAC	GGAGAGGATACAGCAGATGAA
94°C for 30s; 55°C for 30s; 72°C for 1 min; 32 cycles		
caspase-9	AGACCAGTGGACATTGGTTC	GGTCCCTCCAGGAAACAAA
94°C for 30s; 59°C for 1 min; 72°C for 2 min; 30 cycles		
bcl-x	ATGTCTCAGAGCAACCGGGA	TCACTTCCGACTGAAGAGTG
94°C for 1 min; 54°C for 1 min; 72°C for 1 min; 25 cycles		
e2f1	TCTCCCCAGAACCGCTGTTT	CGAAGTGGTAGTCGAGGGC
94°C for 30s; 60°C for 1 min; 72°C for 1 min; 25 cycles		